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Artificial Intelligence Based Early Diagnosis of Sepsis

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Abstract—Sepsis is a major killer of those who are already in a serious condition. The morbidity and death rates in this field remain high, despite the fact that medical technology has been advancing steadily over the last several years. This is due mostly to people not beginning therapy quickly enough and doctors not following best practices. Medical decision support solutions have advanced greatly with the help of artificial intelligence (AI), a rapidly developing sector in the medical industry. Great promise has been shown in its ability to anticipate patients' clinical conditions and aid clinical decision-making. Early prediction, prognosis evaluation, mortality prediction, and optimum treatment are just few of the areas where algorithms developed using artificial intelligence may be put to use. This article summarizes the most recent research on AI based clinical decision support in sepsis as well as explains how this cutting-edge technology might aid in sepsis prediction, identification, sub phenotyping, prognostic evaluation, and clinical treatment. We also spoke about the difficulties of using this non-conventional approach in clinical practice.

Keywords—Sepsis, Artificial Intelligence, Machine Learning, Disease Prediction using AI.

I. INTRODUCTION

There has been a consensus definition of sepsis since 2015: "life-threatening organ failure due to a dysfunctional host response to infection." The prior definition, developed in 1992, relied on measures of systemic inflammation, but this one is more accurate. The most severe type of sepsis is septic shock, which occurs when the body's immune system and circulatory system fail simultaneously. It was also recommended that "adequate routine microbiologic cultures be collected before initiating antimicrobial treatment in patients with potential sepsis or septic shock if doing so yields in no considerable delay in the commencement of antimicrobials," which is in line with the new sepsis

classification. An analysis of the global mortality rate and occurrence of hospital-treated sepsis covering the years 2003-2015 discovered that sepsis and severe sepsis accounted for 16.9% and 25.76% of all in hospital mortalities, respectively, adding up to 5.28 million deaths annually utilizing the old description of sepsis and severe sepsis. Sepsis was responsible for 2% of all hospitalizations and 16.8% of all fatalities in hospitals in the United States in 2008 [1]. About 1.5 million individuals in the United States are affected with sepsis each year, and 250,000 of them die. In 2008, it was anticipated that treating these people would cost the United States \$15.1 billion. The revised criteria yielded an estimated yearly incidence of 838/100,000 for sepsis in Germany, three times greater than the rate of severe sepsis. Sepsis has a mortality rate of at least 9%, while septic shock has a mortality rate of at least 39%. This is a lot more than, say, the 5-6% mortality rate associated with acute myocardial stroke. Sepsis mortality is strongly correlated with age [2].

In hospitals, normally sepsis is one of the main reason of death. Patients at chance of heart failure, worsening, unanticipated ICU admittance, or death, as well as the necessity for hospital admission, were successfully identified by NEWS [3]. In cases of severe sickness when the warning signals are easily ignored, such as sepsis, scoring systems may be used as a tool to anticipate the need for operative admission and expected outcomes.

Earlier, more effective treatments may be possible if declining physiological indicators are detected sooner. Advance warning scores may assist shape a pre-alert strategy or identify particular pre-hospital therapy for sepsis patients, which may make a huge difference in their prognoses. Patients with sepsis have a better chance of survival if their condition is diagnosed and treated quickly. Patients' outcomes need to improve if they can be identified and treated sooner. Because effective treatment is

time-dependent, positive results are closely linked to good management in emergency medicine environments and emergency rooms (ED). Despite guidelines for patient treatment that emphasize the golden hour and the silver day, transfers from the ED to the ICU are often delayed. Similarities between the signs and symptoms of sepsis and those of other prevalent illnesses make it difficult for clinicians to reliably diagnose the condition [4].

II. LITERATURE REVIEW

The term "artificial intelligence" was coined during the 1955 Dartmouth Conference, when participants discussed the potential of then-new computing technology to let them build sophisticated robots with some of the same qualities as human intelligence [5]. Artificial intelligence progressed slowly, though, because of computing and memory power limitations. The most common use of AI based methods in sepsis is machine learning.

To make judgements and forecast future occurrences, the most fundamental kind of machine learning use algorithms to assess and acquire from data. Machine learning is a technique that allows computers to learn how to do a job without being explicitly programmed to do so (Figure 1). Even in the earliest phases of AI research, machine learning has made an appearance.

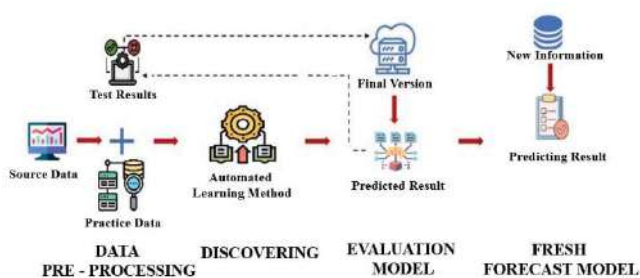


Fig. 1. Architecture of the Machine Learning Model [6]

There are many different ways to categorize machine learning. The machine learning algorithms are illustrated in Figure 2. Before the advent of deep learning, even small improvements in sepsis care using typical machine learning techniques were incredibly challenging [7].

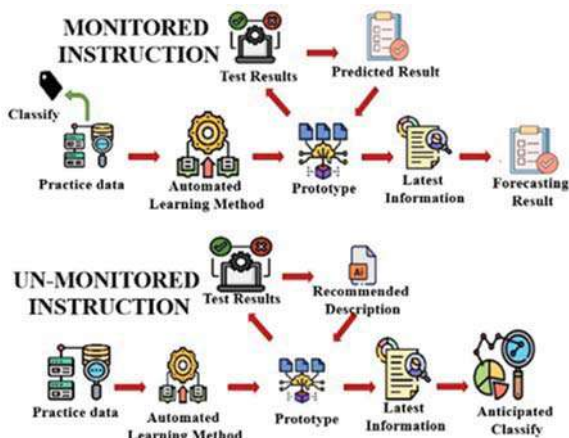


Fig. 2. Machine Learning Algorithms [8]

Initially, deep learning used deep neural networks to address the issue of feature expression. A DNN was not a novel idea in and of itself; rather, it was just a neural network

architecture with numerous hidden layers. If anyone wanted to boost the effectiveness of deep network training, they may modify the neuron connections and activation techniques. The success of deep learning in a number of healthcare applications, such as sepsis control, opens the door to its use in clinical settings [9]. The search for relevant literature was performed in (PubMed). We considered research articles, narrative reviews and systematic reviews published up through October 31, 2022. Full-text abstracts were not considered. ('machine learning' OR 'neural network' OR 'AI' OR 'sepsis') AND ('relevant literature') were used to discover the articles that were examined [10]. We were able to track down 400 separate publications. The articles included in the final batch were selected to offer the viewer the greatest possible broad overview of the issue, and the review is not intended to be exhaustive. Various research papers and reviews were chosen, and we also cited other narrative reviews. None of the writers have undertaken any original research with human or animal subjects for this article; rather, it is based solely on the results of other studies. The process of the method is shown in the figure 3. Existing medical warning scores for sepsis are not established on advanced mathematical models, but AI has the ability to give fast and accurate diagnosis. Using machine learning (ML) techniques trained on patient.

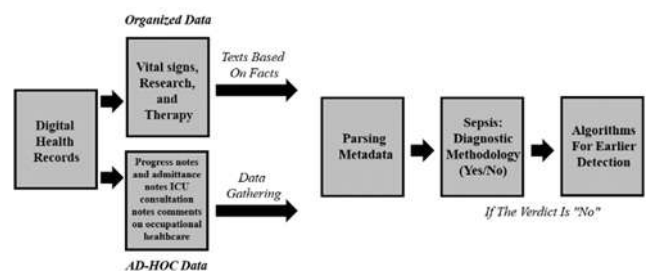


Fig. 3. Implementation of AI in Sepsis Early Prediction [11]

information, often drawn from electronic health records, biological signals, and laboratory findings, a decision support system might be built for earlier detection of sepsis. This scoping review will examine research on artificial intelligence techniques for detecting sepsis in its earliest stages. The phrases artificial intelligence (AI) and machine learning (ML) are not easily defined, and their use varies widely amongst writers. We have no strong opinions on the matter. Instead, we want to be comprehensive, thus any (mathematical) model that can be implemented digitally and has the potential to enhance sepsis detection accuracy will be considered for inclusion in our evaluation. When we talk about AI or ML here, we're really talking about a wide variety of mathematical models. Like the definitions of AI and ML, the language used to describe techniques that may determine whether or not a patient is at high risk for developing sepsis differs. It seems that in the literature, terms like "detect," "confirm," "predict," "recognize," and its derivatives (sensing, etc.), and "infer" are used consistently. We shall make a distinction between "detection" and related terminology and "diagnosis," the latter of which, in our opinion, should refer to a clinical benchmark for determining if a patient has sepsis. High-risk sepsis prediction will be referred to as "detect" or "detects" throughout.

III. AI IMPLEMENTATION IN ADVANCED SEPSIS DETECTION AND FORECASTING

The probability of death increases every hour sepsis patients continue without care. Knowing when sepsis may strike will enable us to start treating patients as soon as possible. The core of the first-generation sepsis forecasting system is experimental clinical decision rules (CDR), which are often applied to data received from bedside vitals. For instance, the bedside monitor collects five physiological indicators per minute. Only a few of the characteristics that make up the data streams that are subsequently classified by and blood pressure. With a mean detection rate of 82.8%, the model's Area Under Receiving Operational Characteristics (AUROC) of 0.779 indicates that it can accurately forecast the incidence of sepsis [12]. The basic artificial intelligence algorithm for sepsis early warning is shown here. A probabilistic model with an AUROC of 0.849 has been developed to assist in the identification of people at risk of sepsis. It measures six sepsis-related characteristics. The tool may be used to screen any hospitalized patient, and the results will be delivered to careers instantly [13].

There are more approaches to employ AI to treat sepsis now that deep learning has advanced. Bi-Directional Gated Recurrent Units (GRU) is a deep learning system that considers a broad variety of physiological, scientific, and demographic factors. With an AUROC of 0.968, our model is capable of predicting the beginning of sepsis six hours in advance. Our method performs far better than current artificial intelligence algorithms for sepsis diagnosis. A method for early sepsis identification that is based on deep learning is also available. Using EMR data, a unique algorithm was created that can predict the start of sepsis by hours and has an AUROC of 0.782. Yet another sepsis detection technique uses a CNN and an LSTM network separately. The model's quality is evaluated using conventional standards of accuracy and clinical applicability, and the effectiveness of the intervention is assessed using suppressive medication and blood cultures that are administered earlier than expected. In the three hours before to the onset of sepsis, there was a 0.849 AUROC [14].

An explainable AI framework for sepsis early detection has been developed by certain researchers as an alternative to the previously discussed deep learning. They created a model using publicly accessible data from ICUs and tested it on a covert sample to make sure it complied with the challenge's requirements. The interpretable AI model is trained for real-time sepsis prediction and is capable of retrieving 164 features per hour. To show the interpretability of the sepsis real-time predictions, we carefully evaluate the effects of each feature. In addition to being exceedingly effective at estimating sepsis risk in real time, the algorithm also provides insightful information about that risk. On the other hand, traditional supervised models often only perform well in a narrow range of tasks, while ensemble learning performs well in all domains. A kind of deep learning known as "ensemble learning" combines many "weakly supervised" models into a single "strongly supervised" model. If one weak classifier makes a prediction that is off, the other weak classifiers may make up for it, which is one intended advantage of ensemble learning. Recently, an ensemble learning framework for sepsis prediction and an autonomous feature extraction strategy employing long-term and short-term memory (LSTM) neural networks were developed. This

approach combines deep characteristics with synthetic features acquired from cutting-edge clinical data. With the use of ensemble learning, sepsis was detected 6 hours in advance. Results show how the model may help with an early sepsis diagnosis. The process of the method is shown in the figure 4.

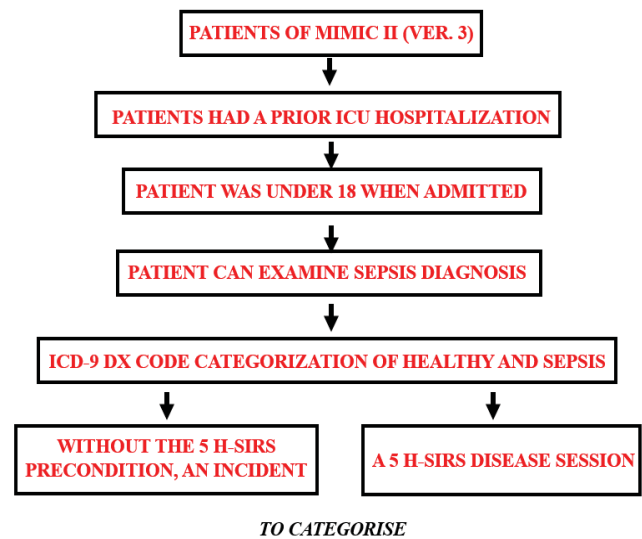


Fig. 4. Working Mechanism for Result Driven

Advance Forecasting of severe septic- One area of innovation in healthcare strategy is the creation of decision support systems that drew on improvements in machine learning. One of the active topics is predicting the onset of septic shock. To enhance clinical outcomes and advance real-time resource optimization in healthcare, several research have created intelligent decision support technologies for septic shock. With an AUROC of 0.9389 a sensitivity of 82.9%, and a specificity of 87.34 %, the model utilizing the Random Forest method fared finest. Investigations have also combined physiological parameters from the EHR with variables gleaned from natural language recognition in clinical health record data to create septic shock forecasting model, such as XG-Boost. The best approach among them has a median advanced warning of 6.5 hours, allowing for intervention several hours before the commencement of septic shock.

A recent branch of machine learning called transfer learning enables the spread of algorithms across multiple therapeutic settings. Transfer learning was included in order to evaluate how well AI Sepsis Expert predicted extended septic shock in the ED, and the viability of increasing external efficacy in the second site was confirmed. The effectiveness of this AI in forecasting deferred septic shock at 9 and 12 hours has the best AUROC of 0.79. Transfer learning significantly enhances the model's applicability and confirmability.

Enhancing the accuracy of Sepsis Detection- The presence of sepsis is often followed by the failure of several organ systems. However, the lack of characteristic clinical signs of multi-organ failure adds to the difficulty of sepsis identification and reduces diagnostic accuracy. Several research projects have created low-cost automated diagnostic technologies to address the difficulties associated with existing sepsis diagnosis. In [15], the authors build a temporal deep convolutional network architecture for sepsis

prediction and test it with three different types of validation data. The chosen model proved robust, and it may be used as a hospital-wide early diagnostic tool for sepsis. This diagnosing application's accuracy and precision were much better than those of competing methods. Another paper detailed the creation of an AI system for diagnosing sepsis and compared its efficacy to the SOFA score approach of diagnosis. To diagnose sepsis, the method relied on 102 clinical variables that were both previously chosen and chosen dynamically.

The AI was created with the help of the anonymized data. Several machine learning techniques' efficacies were evaluated using 4-fold cross-validation, and the highest-scoring approach, XGBoost based on a decision tree, was ultimately utilized to create the AI programme. The well-known AI algorithm has an AUROC of approximately 0.876 but a low SOFA score of 0.612. This artificial intelligence programme was created using data from actual EMRs of patients suffering from sepsis, together with hand-picked characteristics and XGBoost. A sepsis diagnosis made during the first few days of symptoms typically has an accuracy rate of above 80%. The health sector, medical personnel, and patients may all benefit from the prompt and correct reaction of this AI algorithm, which will allow physicians to deploy effective treatment approaches early, leading to decreased medical expenditures and better patient prognosis. According to the findings, the model outperforms human physicians utilizing the same characteristics by a significant margin. The AUROC is 93 percent, the sensitivity is 92.98 percent, and the specificity is 78.87 percent. Fertility, cervicovaginitis, newborn fever, dyspnea, hematocrit levels, gender, bradypnea, band cell, syringe usage, and infant weight are the 10 most important criteria in the assessment of neonatal sepsis.

IV. DIAGNOSIS AND RISK ASSESSMENT OF SEPSIS USING ARTIFICIAL INTELLIGENCE

Most individuals who are hospitalized due to an infection end up dying from sepsis. The current death rate is still excessive. Clinical decision-making and death rates may both benefit from the adoption of more accurate evaluation methods for the prediction of sepsis. The likelihood of a patient dying in the first 72 h after being admitted to the intensive care unit (ICU) may be predicted using a DNN model trained using LSTM. Multi-center study AUC was 0.867, whereas single-center study AUC was 0.847. This LSTM-based model might aid doctors in early identification of patients with poor prognosis, allowing them to "re-triage" and alter treatment strategies. Patients with sepsis-related AKI have varying clinical presentations and outcomes. To better treat patients with similar conditions, AI may classify them into different sub-phenotypes based on their level of risk. Deep learning was employed in a research to identify the sub phenotype of septicemia AKI and make predictions about the likelihood of death and the requirement for dialysis within 4 weeks. Type 1 AKI patients had the lowest dialysis needs (4%), and their 4-week death rate after AKI is likewise the lowest (22.77%). Subtype 3 has a mortality rate that is 1.87% higher than Type 1 after adjusting for risk factors.

In a similar manner, [16] employed AI to categorize the forms of organ failure seen in severe sepsis in the ICU, and they found clinically significant sepsis categories with diverse variations of organ dysfunction. Segmentation is

done via SVMs, SVM ensembles, and gradient boost trees. There are presently no reliable methods to predict the morbidity of sepsis-related coagulation abnormalities. The effectiveness of AI in predicting death in ED patients with probable disease or sepsis has also been assessed. Different AIs were tested to see how well they could classify ED patients with sepsis and predict whether or not they would die. RF, C4.5 decision tree, SVM, and artificial neural network were the four supervised learning techniques examined. As a consequence, the best discriminating effect is achieved by SVM and ANN when physiological variables are included. It has promising practical applications in evaluating sepsis categorization and prognosis. Additionally, the deep learning-based algorithm CNN with SoftMax may be used to forecast mortality in ED patients who are thought to have an infection. Early detection of patients in severe condition may be greatly aided by using deep learning.

Managing Sepsis using AI tools - Fluid sensitivity in sepsis may be predicted using the progressive leg rise (PLR), although this hydrodynamic challenge is often not used due to the patient's lack of mobility. Aiming towards anticipating fluid reaction. Transthoracic echocardiography data was used to train a machine learning model for the diagnosis of sepsis and septic shock. In comparison to PLR's hemodynamic reaction, the findings indicate that the most effective model is partial least regression (PLS), with an AUC of 0.965. Collapsibility of the defective vein artery, velocity-time summation, S-wave, E/Ea proportion, and E-wave are among the most important characteristics in echocardiography. Strategies for fluid management in sepsis patients were reported on in separate research. The "capped" setting for fluid volume delivery during the first 24hrs in the ICU is correlated with an increased risk of death, according to a study that employed causal inferred technologies to get that conclusion.

For over a decade, experts have advocated the use of "sepsis packs," a collection of treatments aimed at mitigating the severity of sepsis's negative effects. The fatality rate from sepsis remains high, and sepsis package compliance is subpar despite these advances. With the use of the Model Cell conceptual framework, researchers from other fields were able to compare actual death rates with those projected by using data from similar acute care institutions. According to the findings, the overall mortality rate dropped dramatically as bundle compliance rose. Nearly two-thirds of the variation may be explained by compliance alone in the linear model. The median fatality rate fell even lower, to 5.279%, when analyzing just the last 12 months of the experiment. When thinking about how to treat sepsis, knowing its root cause is crucial. The Inflammatrix bacterial-viral non-infected version classifier is a neural-network classification method that may be used to evaluate patients on admission who may be infected. Reduced healthcare expenses, better infection detection, and a halt to the development of sepsis are all possible outcomes. Because of their altered physiological state, patients in the intensive care unit (ICU) are at a higher risk of contracting an infection, and a number of environmental infections may further raise this risk. Antibacterial dosing in the intensive care unit (ICU) might become complicated for a number of reasons. These obstacles prevent the conventional antibiotic dosing regimen from accomplishing the aims associated to excellent patient outcomes. Innovative dosing algorithms using AI was created to aid in the modification of antibiotic medication in

intensive care unit (ICU) patients, and one of these techniques was via Bayesian forecasting. Antibiotic programmes may be tailored to individual patients based on their unique clinical features using data gathered through therapy monitoring.

The outcomes of using AI to quality enhancement activities were presented in research. The deep learning-based sepsis detection and treatment software Sepsis Watch was integrated into standard clinical practice. A better ability to diagnose and treat sepsis is the driving force behind Sepsis Watch. It was developed with input from statisticians, data analysts, data scientist, and physicians who collaborated on a performance improvement task report. Implementing Sepsis Watch in everyday clinical treatment is possible, as shown by the findings, and the process has also facilitated the development of locally based machine learning initiatives. The influence that using AI algorithms had on nursing staff's ability to detect sepsis early on was also discussed, as was the nurses' expertise using the technological tools created by AI algorithms. In the scenario presented, nurses actively engaged in the procedure of technological integration may make quick judgements in the early detection of sepsis.

As of the year 2020, COVID-19 may be found in every region of the globe. Patients infected with the virus have intense respiratory issues and are at risk for various consequences, including streptococcus pneumoniae, sepsis, septic shock, and multiple organ dysfunction. Finding efficient means of saving money and time is essential for reducing the impact of sickness. Research integrates systems biology and AI-based technologies to scour the whole medication market for new therapies for COVID-19. The effects of pixantrone and melatonin on COVID-19 were validated using the GUILDify v2.0 Web server as an alternate way. The therapeutic benefits of medication combinations for respiratory disorders are also predicted [17-18]. Sepsis is a complicated and variable disease with a wide variety of pathogenic causes and processes. The onset of systemic inflammation is the defining characteristic of this condition. Noninfectious forms of the acute inflammatory response syndrome (SIRS) can present with comparable symptoms. Given the same pharmacological and genetic features of sepsis and SIRS, clinical differentiation between the two is challenging. This may be because not enough attention has been paid to identifying the critical genes or pathways involved in the development of these illnesses. Differentiating sepsis from SIRS is feasible with the use of reasonably used genetic indicators that are suitable for diagnostic testing. Using two-tier gene scanning and ANN data mining technologies, researchers were able to find biomarkers that may be utilized to diagnose SIRS, sepsis, and septicemia in patients using publicly available gene expression data sets.

Creating and testing digital twin models that mimic critically sick patients may help doctors predict how sepsis patients will respond to therapy using causal AI. In this study, we used a combination of agent-based modelling, discrete event modeling, and Neural nets to simulate the treatment impacts and interactions of key organs at different phases, with the results shown by means of pertinent clinical indicators. While comparing the digital twin's anticipated reaction to the patient's actual response during advance treatment of a serious illness, the AI model was demonstrated to be surprisingly accurate. With a trustworthy

digital twin model at their disposal, doctors may simulate the outcomes of treatments before implementing them on actual patients.[19-23]

V. CONCLUSION

In some conditions, including sepsis, the possibility of developing AI-based medical services may equal or even surpass the competence of professionals. In spite of this, health care is a dynamic and intricate industry with stringent standards for patient protection. Patients might be harmed by a malfunction in the technology. Human physicians and safety engineers have little to no say in the decision-making process when an AI system is involved, and it may be difficult to grasp exactly how the AI system arrives at its conclusions. Artificial intelligence (AI)-based technologies have less safety restrictions and ethical restraints than traditional healthcare treatment. Because of the intricate nature of the sepsis clinical situation, numerous factors may influence the final outcome. Although the mathematical model displays the needs of many clinical situations, satisfying these needs during the technical design phase is challenging. In this way, the system's software behavior could not accurately represent the intended therapeutic outcomes. Unfortunately, the current solution to this issue—limiting the quantity of information input—may have unexpected repercussions. The loss of an insensible fluid would be one example. It can't be saved digitally, which may trigger an AI reminder to replenish the fluid supply. However, the doctor can see that the patient has been soaked in reality. Further, unlike human clinicians, machines are unable to reason on the most crucial material while interpreting data. For instance, doctors could decide to ignore very anomalous test findings that might have been caused by sample, screening, or documenting mistakes. There are also issues with the AI model itself, such as the fact that much research has only trained and verified the model using the same clinical cohort without considering the model's generalizability to different populations. Extra comprehensive testing is required to demonstrate the usefulness of these models in terms of clinical or other consequences. Many challenges will arise along the process of putting AI into clinical practice. Unfortunately, few businesses now have the resources to hire enough AI specialists and develop the necessary IT skills to put AI into clinical practice, which includes tasks like evaluating, combining, continuously monitoring, and recalibrating AI. There is also a pressing need to address concerns about the privacy and trustworthiness of digital data collecting and processing. In addition, most healthcare systems lack the resources necessary to effectively incorporate AI into the present process. There is still a long way to go before decision making and prediction models can be properly integrated into the world's existing healthcare systems. For the time being, there is a significant chasm between the development of AI techniques and their use in actual clinical settings. Experts in sepsis clinical treatment cannot be replaced by AI. Until they can verify that the findings can be updated prospectively in numerous situations, AI-based techniques should always be utilized as development tools.

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BHAI-MS-9

Exploring The Potential of mRNA - A Comprehensive ReviewNagendra Prasad S V¹ Krishna Kumar V² and Chakrapani I S³**Abstract**

The success of mRNA vaccine technology in combating COVID-19 has ignited exploration for broader applications against various diseases. This versatile method allows scientists to target any virus by inserting codes for specific viral proteins, accelerating vaccine development. Over the past two decades, RNA-based technologies, including mRNA vaccines, have demonstrated safety and enduring immune responses in trials for diseases such as influenza, ebola, and SARS. The existing mRNA vaccine framework opens new possibilities for swift and efficient development against various infectious threats. mRNA vaccines offer a promising alternative to traditional approaches due to their potency, rapid development capability, and potential for cost-effective production and safe administration. This review provides a comprehensive look at mRNA vaccines, exploring future directions and challenges in advancing this promising vaccine platform for widespread therapeutic use.

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Introduction:

In recent years, mRNA vaccines have emerged as groundbreaking technology for rapid and targeted vaccine development against infectious diseases (Pardi et.al., 2018; Hogan and Pardi 2022). First proposed in 1990 by Wolff et.al., 1990 (42) mRNA vaccines have undergone a 30-year developmental journey, utilizing the body's cellular machinery to produce specific antigens and trigger immune responses (Kariko et.al., 2015). In contrast to traditional vaccines, mRNA vaccines offer advantages such as rapid development and production, with the ability to be designed and synthesized in weeks. Notably, they have demonstrated success against SARS-CoV-2 (Pardi et al., 2020, Polack et.al., 2020; Baden et.al., 2021). The modular nature of mRNA vaccines allows easy adaptation to different pathogens by adjusting the mRNA sequence (Li et.al., 2016; Svitkin et.al., 2017; Wojtcjak et.al., 2018). However, challenges in efficient delivery to target cells, susceptibility to degradation, limited stability, and barriers to reaching desired action sites exist (Tsui et.al., 2002; Kaczmarek et.al., 2017). Researchers are expanding the use of mRNA vaccines beyond COVID-19 to combat other infectious diseases like influenza, Zika, rabies, dengue, and malaria, showing promise in early animal tests. This review thoroughly explores mRNA vaccines, highlighting their production, stability, synthesis, and delivery methods. It convincingly demonstrates how these vaccines outperform traditional ones, emphasizing their importance in modern medicine and

showcasing the technological strides in their development.

Background:

mRNA vaccines gained attention during the COVID-19 pandemic upon approval for public use, but their groundwork began in the early 1990s. Feasibility became evident in the 2000s, with the first mRNA vaccine for prostate cancer, 'Provenge', approved in 2013. mRNA's immune-boosting capability against cancer paved the way for applications to infectious diseases, exemplified by an MERS vaccine in 2013. The pivotal breakthrough for mRNA vaccines occurred in late 2019 during the COVID-19 pandemic, with the swift approval of Pfizer-BioNTech and Moderna vaccines in December 2020, highlighting their efficacy (Plotkin 2014). Years of dedicated research positioned mRNA as a formidable tool in disease confrontation.

The Excitement Around mRNA Technology:

mRNA vaccines stand out for their remarkable speed and adaptability, epitomized by Moderna's SARS-CoV-2 mRNA vaccine, which entered clinical trials in a mere 63 days after the virus genome's publication. In stark contrast, the HPV vaccine, developed using recombinant DNA technology, endured a lengthy 15-year journey. The key differentiator is mRNA's agility. Traditional vaccine methods involve time-consuming processes like growing and weakening viruses, while mRNA vaccines provide a swift alternative. Oxford's 100-day vaccine team further underscores this agility, aiming to create



vaccines within just 100 days of identifying a new pathogen. As per the NIH, this underscores the accelerated pace made possible by the groundbreaking technology of mRNA.

mRNA Vaccines: Advancements Over Traditional Vaccines:

Traditional vaccines rely on weakened or inactivated germs to stimulate an immune response. In contrast, mRNA vaccines utilize genetically engineered molecules to instruct cells in producing harmless fragments of viral proteins. This prompts the immune system to generate antibodies, effectively neutralizing the targeted viral protein. According to NHS study, the advantages of mRNA vaccines lie in their rapid development, safety, and efficacy. They are non-infectious, degrade naturally, and being fully synthetic, allow for cost-effective manufacturing. The mRNA platform proves crucial in developing variant-proof, safe, and effective vaccines, ensuring accessibility and safety for mass vaccination (Plotkin S and Plotkin S, 2011).

The Rise of mRNA Technology:

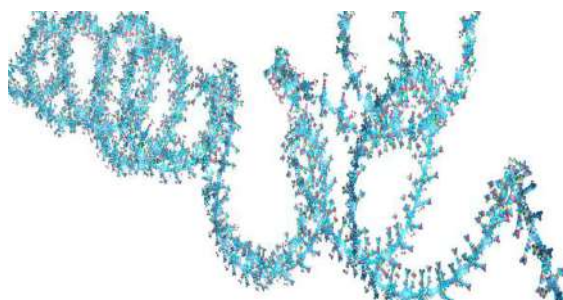
mRNA technology gained prominence during the Covid-19 pandemic, marking its first milestone with a candidate entering phase I clinical trials. Early experiments in 1990 showcased coding mRNAs potential to express proteins in mouse skeletal muscle. Over the subsequent decade, studies confirmed mRNA's ability to elicit immunologic responses across various mammalian cell types. What sets this technology apart is its precise triggering of the immune system to respond to specific threats, activating both arms of the immune

system for a robust defense. Unlike DNA vaccines, mRNA is more effective and safer, posing very low risk of causing genetic changes. The temporary effect of mRNA, coupled with its adaptable nature, allows for consistent manufacturing, irrespective of changes in the target virus. The use of in vitro cell-free transcription minimizes safety concerns compared to other vaccine methods (Schlake et.al., 2012). mRNA, acting as a messenger, provides instructions to cells for protein production, aiding in the fight against diseases like influenza, Ebola, and SARS. This flexible technology holds promise in combating viruses and advancing medical research (Pascolo S 2004).

Understanding mRNA: A Blueprint for Cellular Defense:

mRNA, or messenger RNA, serves as a molecular guide, furnishing instructions for cellular protein production. Encapsulated in a protective lipid nanoparticle, it smoothly enters cells. Inside, cells interpret mRNA instructions to construct proteins mimicking pathogen components, known as antigens. The immune system recognizes these foreign antigens as threats, mobilizing antibodies and T-cells for defense and training against potential future attacks. When faced with the actual virus, the body's recognition sounds the alarm, aiding in defense against infection and illness. The innovative use of mRNA as a blueprint for cellular defense signifies a paradigm shift in vaccine technology, offering enhanced safety, adaptability, and efficacy.





mRNA (image source: <https://www.pfizer.com/science/innovation/mrna-technology>)

Messenger RNA Vaccines: Transforming Medicine's Approach

mRNA vaccines revolutionize medicine by leveraging the body's natural processes for immune response, eliminating the need for live or inactivated viruses. This pioneering technology allows for a rapid and targeted reaction, exemplified by the successful response to the challenges of the COVID-19 pandemic.

How do the mRNA vaccines work?

The mRNA in vaccines provides cells with instructions to produce a viral protein fragment. The immune system responds by generating antibodies, which persist in the body, ready to identify and neutralize the virus if encountered. This process offers rapid protection without exposing individuals to the actual virus.

The Manufacturing Process of mRNA Vaccines: A Step-by-Step Overview

The production of mRNA vaccines involves a meticulous, multi-step process designed to generate an effective immune response. Scientists begin by identifying a

target protein from the pathogen, such as the spike (S) protein in the case of SARS-CoV-2 (1). This protein is selected based on its dissimilarity to human cells, ensuring the initiation of specific immune responses against the pathogen.

Once the target protein is identified, its gene sequence is inserted into a plasmid, a process executed in host bacteria, typically *Escherichia coli*, using a fermentor. The resulting plasmid DNA undergoes purification and linearization. The plasmids, adept at replicating gene sequences, facilitate efficient production.

In a cell-free in vitro transcription (IVT) process, the plasmid DNA serves as a template for mRNA synthesis. Enzymes and nucleotides are employed to produce mRNA, with additional steps involving specific enzymes to cap and stabilize the material. Once a sufficient amount of mRNA is generated, the plasmid DNA is broken down, ensuring that only mRNA is included in the vaccine.

Due to mRNA's susceptibility to degradation, a lipid nanoparticle (LNP) or another suitable delivery system is developed to encapsulate and transport the mRNA to cells. The purified and sterile-filtered mRNA is encapsulated, and final formulation activities result in the vaccine drug product. Upon administration, the encapsulated mRNA is taken up by recipient cells, directing them to produce the SARS-CoV-2 S protein, thereby eliciting a robust immune response, including the production of antibodies.



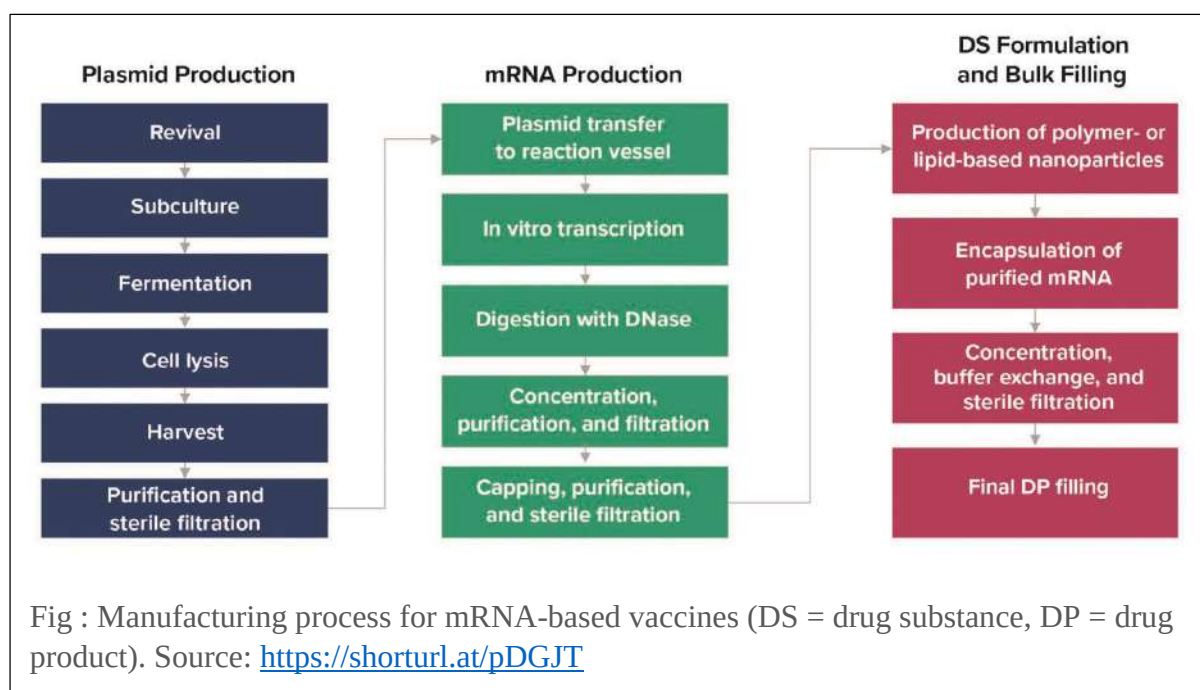
Revolutionizing Medicine: The Impact of mRNA Vaccines

mRNA vaccines spearhead a transformative era in medical technology, playing a pivotal role in the battle against COVID-19 and offering promising applications across various medical fields. Exemplified by leading COVID-19 mRNA vaccines from BioNTech-Pfizer and Moderna, these vaccines showcase remarkable efficacy against infection and a remarkable ability to adapt swiftly to new variants. Moderna's feat of developing a variant-targeted vaccine in just 30 days underscores the unparalleled flexibility of this technology (Hogan et.al., 2023).

establishment of regional mRNA vaccine production capacity not only contributes to the ongoing battle against COVID-19 but also positions communities to address a myriad of health challenges in the future.

Streamlining mRNA Manufacturing: A Swift and Scalable Process

The production of mRNA for medicines and vaccines is marked by its simplicity, scalability, and remarkable speed. The process involves key steps where considerations are made for quality and testing requirements. In a laboratory setting, mRNA is synthesized using the in vitro transcription (IVT) process. What sets



Beyond their success in countering COVID-19, mRNA vaccines demonstrate adaptability by easily tailoring to different pathogens. This inherent flexibility opens avenues for producing diverse vaccines and therapeutics on a unified platform. The

this method apart is its avoidance of dealing with cells or host cell proteins, a departure from older methods. Crucially, the mRNA manufacturing process can employ the same materials and equipment for different applications. This adaptability enables



facilities, especially those adhering to strict manufacturing rules like Good Manufacturing Practice (GMP) facilities, to swiftly transition to producing new medicines or vaccines with minimal adjustments to the process. It represents an intelligent and efficient approach to mRNA production.

mRNA Manufacturing Simplified:

1. Making the pDNA Template:

- Design and purity of the pDNA template are crucial for mRNA optimization.
- pDNA is amplified within *E. coli*, purified, and linearized for use as a template.
- Impurities are removed using methods like tangential flow filtration (TFF) and chromatography, crucial for GMP production.

2. In Vitro Transcription (IVT):

- Linearized pDNA is transcribed into mRNA using RNA polymerase and nucleotide triphosphates.
- Addition of a 5' cap structure is essential for stability and efficient cell transduction.
- Co-transcriptional capping is faster but less efficient; enzymatic capping offers higher efficiency.

3. Purifying mRNA:

- Purification eliminates unwanted materials like salts, cap analogs, proteins, and residual DNA.
- Process chromatography is commonly used for commercial-scale mRNA purification (Heartlein et.al., 2014).

4. Choosing mRNA Delivery System:

- Lipids or polymers are used for mRNA delivery, with some systems combining both.
- The delivery system significantly impacts the effectiveness of mRNA vaccines and therapeutics.

5. Quality Control and Release:

- Quality control testing includes capping efficiency, poly A tail length, and residual plasmid DNA.
- Testing requirements vary based on the final mRNA application and clinical usage.

Streamlining these steps ensures efficient and scalable mRNA manufacturing, a critical aspect in the development of mRNA-based therapeutics and vaccines.

How Do mRNA vaccines Work?

mRNA vaccines, such as those developed for COVID-19, work by leveraging the genetic material messenger RNA (mRNA) to instruct cells in the body to produce a viral protein—in this case, the spike protein of the SARS-CoV-2 virus. Here's an elaborate breakdown of the process:

mRNA in Lipid Nanoparticles:

The vaccine contains strands of mRNA that encode the genetic instructions for making a fragment of the virus, usually the spike protein.

These mRNA strands are encapsulated in tiny lipid nanoparticles to protect them and facilitate their entry into cells.

Cell Entrance and Translation:

Upon injection, the lipid nanoparticles deliver the mRNA into the cells of the body, typically muscle cells near the injection site.



Once inside the cell, the mRNA serves as a blueprint, providing instructions to the cellular machinery on how to build the viral spike protein.

Spike Protein Expression:

The cellular machinery reads the mRNA and begins synthesizing the viral spike protein based on the provided instructions. Importantly, only a fragment of the virus is produced, not the entire virus. This means there is no risk of causing the disease.

Immune Response Activation:

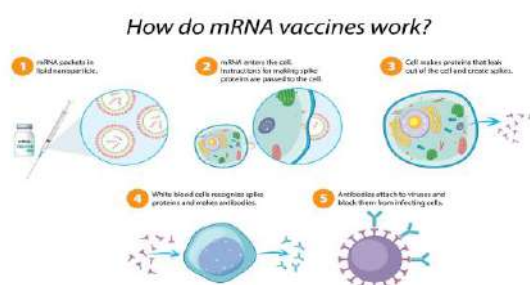
The newly synthesized spike protein is displayed on the surface of the host cell. The immune system recognizes this foreign protein as an invader and triggers an immune response involving T lymphocytes (cell-mediated immunity) and B lymphocytes (antibody-mediated immunity).

T and B Lymphocytes Activation:

T lymphocytes, specifically cytotoxic T cells, recognize and destroy cells displaying the viral protein. B lymphocytes produce antibodies against the spike protein.

Proprietary Antibodies:

The generated antibodies are specific to the viral spike protein. These antibodies are crucial for recognizing and neutralizing the actual virus if the vaccinated person is later exposed.



(Image source: <https://shorturl.at/aowJO>)

The triumph of mRNA vaccine technology in countering COVID-19 not only marks a significant milestone but also paves the way for its application across a wide spectrum of diseases. The intrinsic structure and delivery mechanism of mRNA provide a foundational blueprint for addressing various viruses. Scientists can seamlessly embed the code for precise viral proteins, instigating a prompt immune response. With the technology firmly established, the prospect of accelerated vaccine development becomes substantial. Human trials for mRNA vaccines, targeting diseases like HIV, Zika, and rabies, are already in progress, signaling a new era in medical advancement.

Applications of mRNA Vaccines:

1. Infectious Diseases and HIV:

RNA vaccines are at the forefront of research, with ongoing exploration in infectious diseases and cancer. While early-stage clinical trials investigate the potential of RNA vaccines for allergies, the broader applications extend to diseases beyond COVID-19. Trials are underway for different SARS viruses, MERS, hepatitis C, HIV, influenza, malaria, and tuberculosis. Moderna is even pioneering an adaptive mRNA vaccine for influenza. Notably, researchers from multiple institutions are progressing through early-stage clinical trials for mRNA-based HIV vaccine candidates, marking a transformative shift in vaccine development post-COVID-19(2,4,5,)

2. mRNA Vaccines in Cancer Treatment:



Beyond prevention, mRNA vaccines emerge as treatments for various cancers, conveying crucial information about tumors to kickstart the body's defense against cancer cells. Ongoing tests assess their efficacy and safety across different cancer types, including melanoma, brain, lung, ovarian, prostate, blood, digestive system, and breast cancers. Developing personalized mRNA vaccines poses challenges, but researchers are exploring combinations with anti-cancer drugs, like pembrolizumab, in ongoing tests, particularly for melanoma and glioblastoma.

Advantages of mRNA Vaccines:

1. Safety:

Non-infectious Particles: mRNA vaccines eliminate infection risks by excluding live or inactivated pathogens.

Limited Persistence: Swift degradation of mRNA after translation minimizes its presence within cells, reducing the risk of prolonged effects.

2. Efficacy:

Targeted Immune Response: Tailored for specific pathogen proteins, mRNA vaccines enhance efficacy by triggering a precise immune response.

3. Production:

Rapid, Inexpensive, and Scalable: mRNA vaccine production is swift, cost-effective, and scalable for large-scale manufacturing, thanks to high yields in in vitro transcription reactions.

Disadvantages:

1. Temperature Sensitivity:

Instability at High Temperatures: mRNA vaccines face challenges in packaging and distribution due to instability at high temperatures, impacting long-term storage and delivery, especially in remote areas.

2. Infrastructure Challenges:

Global Investments Required: Addressing temperature sensitivity demands substantial global investments in infrastructure, workforce training, and precise last-mile coordination.

3. Long-Term Uncertainty:

Extended Impact Uncertain: Despite positive outcomes in clinical trials and initial studies, the long-term effects of mRNA vaccines remain uncertain, necessitating ongoing monitoring and research for a comprehensive understanding.

Future Applications of mRNA Technology:

The triumph of mRNA vaccine technology in countering COVID-19 not only marks a significant milestone but also paves the way for its application across a wide spectrum of diseases. The intrinsic structure and delivery mechanism of mRNA provide a foundational blueprint for addressing various viruses. Scientists can seamlessly embed the code for precise viral proteins, instigating a prompt immune response. With the technology firmly established, the



prospect of accelerated vaccine development becomes substantial. Human trials for mRNA vaccines, targeting diseases like HIV, Zika, and rabies, are already in progress, signalling a new era in medical advancement.

Conclusion:

In summary, mRNA vaccines are a game-changer in medicine. They go beyond just preventing diseases; they have the potential to revolutionize global healthcare. Continuous improvements, like making them more affordable and efficient, are underway. Innovative methods, such as continuous production using microfluidics, show a commitment to making these vaccines more accessible. In the future, mRNA vaccines could make medical treatments not only better but also available to more people around the world.

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BHAI-MS-17

Biotechnological interventions in the treatment of Psoriasis - the promise of new 'biologicals'Chakrapani I S¹, Srinivas N² and Salini, K^{3*}**Abstract**

Psoriasis is a chronic, recurrent immune-mediated inflammatory dermatosis. Epidermal hyperplasia and infiltration of inflammatory cells into the dermis and neovascularization characterize this disease. The global prevalence rate is about 2-3%. It impairs the quality of life negatively, due to chronic complications and comorbidities like arthritis, cardiovascular issues, depression and metabolic disorders. The molecular mechanisms involved in psoriasis are complex, and deregulation of T-helper cells Th-1/Th-2/Th-17/Th-23 axis is implicated in the pathogenesis of the disease. Current treatment protocols for this disease have not been satisfactory. Biotechnological innovations have made possible the development of several new systemic therapies for psoriasis. 'Biologicals' are a new group of compounds including monoclonal antibodies, fusion proteins and recombinant proteins. Targets for the therapeutic strategies adopting the new 'biologicals' include inactivation of soluble mediators like tumor necrosis factor alpha, blockade of receptors for cytokines, adhesion molecules and interference with T-cell activation by antigen-presenting cells. About forty 'biologicals' are under examination for the management of psoriasis. Recombinant cytokines are in use to modulate immunological balance, as a therapy for psoriasis. This work reviews the recent developments in the treatment of psoriasis with new 'biologicals'.

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Introduction:

Psoriasis is an inflammatory disease. It is chronic, recurrent and immune-mediated. It affects about 2-3% of world population (Dogra and Mahajan 2016). The prevalence rate of Psoriasis is estimated to be varying between 0.44% and 2.8% in our country. Psoriasis affects the male population more than the female population (Dogra and Yadav 2010).

It is characterized by chronic inflammatory skin lesions of five types predominantly – plaque, guttate, inverse, pustular and erythrodermic. The most common form is plaque. Psoriatic plaques appear as raised and red patches covered with dead cells (Devrimci-Ozguven et. Al., 2000). Often itchy and painful, there is risk of cracks and bleeding affecting the quality of life. Patients suffering from this disease are often subjected to social stigma and humiliation, leading to anxiety and depression. It has a high impact on the motional and psychological aspects of the patients (Kim et.al., 2017), making it compulsory to adopt suitable management protocols (Dauden et.al., 2016).

The aetiology of this disease is unclear. At present, consensus about the manifestation is that it is T-Cell mediated and influenced largely by genetic and environmental factors (Kanwar et.al.,2010). Since therapeutic choice for this multifactorial disease is not easy, marketed formulations that encompass all the needs of a psoriasis patient are currently unavailable. The most common approach for clinical management of Psoriasis is use of a combination of topical and oral formulations, all of which

aim at reducing plaque formation. Corticosteroids, keratolytics, tazarotene, anthralin and calcipotriol are widely being used in the currently available therapies (Menter et.al., 2008). But, many side effects have been observed, resulting in therapeutic dropouts (Korman et.al., 2015; Sarma 2017). In addition, higher treatment cost is of concern amounting to 10 billion euros per annum and 40% work loss (Melnikova 2011). At present, routine clinical practice includes use of methotrexate (MTX), cyclosporine, acitretin, and small molecules, like apremilast (Armstrong et.al., 2013; Afra et.al., 2019), but many adverse effects have been reported from time to time (Fieldman et.al., 2016)

In this context, there arises an urgent need for development of new therapies for effective treatment regimes.

Biotechnological interventions

Biotechnological innovations made the development of new systemic therapies for Psoriasis. A new group of chemicals called biologicals seem to be promising. These include monoclonal antibodies, fusion proteins and recombinant proteins. These offer designer drugs to interact with specific targets and thus are safer (Graciliana Lopes et.al., 2020). Targets for these biologicals include blockade of cytokine receptors, inactivation of soluble mediators of the immune system, interference with T-cell activation etc. Currently, up to forty agents are under investigation for the treatment of psoriasis. Four of these agents, alefacept, efalizumab, etanercept and infliximab have already impacted on routine clinical practice. These



‘biologicals’ appear to be promising in combating Psoriasis. Current developments in the treatment of psoriasis with biologicals are briefly reviewed.

onwards, Second-generation biologics emerged. Their mechanism of action involves antibody targeting the IL-23/Th17-pathway. Examples include ustekinumab, secukinumab, ixekizumab, brodalumab, guselkumab, risankizumab, and tildrakizumab.

Table 1 – List of some of the biologicals currently used in clinical practice for treatment of Psoriasis

Sl.No	Name of the ‘biological’	Target/mechanism of action
1	Adalimumab (Humira)	TNF-alpha-blocking antibody
2	Adalimumab-adbm(Cyltezo)	biosimilar to Humira
3	Brodalumab (Siliq)	human antibody against interleukins
4	Certolizumab pegol (Cimzia)	TNF-alpha blocker
5	Etanercept (Enbrel)	TNF-alpha blocker
6	Etanercept-szsz (Erelzi)	A biosimilar like Enbrel
7	Guselkumab (Tremfya)	an antibody against interleukins
8	Infliximab (Remicade)	TNF-alpha blocker
9	Ixekizumab (Taltz)	An antibody that binds to inflammation-causing proteins/interleukins
10	Risankizumab-rzaa (SKYRIZI)	an antibody against interleukins
11	Secukinumab (Cosentyx)	A human antibody against interleukins
12	Ustekinumab (Stelara)	A human antibody against interleukins

These biologicals are used according to the international guideline recommendations and literature evidence (AAD 2008, AAD 2019, BAD 2017, European S3,) and protocol (Rajagopalan and Mital 2016).

Therapeutic approaches and challenges with biologicals

In the recent past, new biological therapies with are developed. Their mechanism of action involves more precise targets (porter et.al., 2017). First generation biologicals are developed targeting tumour necrosis factor (TNF- α). These include etanercept, infliximab, and adalimumab. These are used for patients who suffer from chronic moderate-to-severe psoriasis. From 2009

Secukinumab is the only second-generation biologic available in India at present. It is indicated as the first-line systemic in moderate-to-severe plaque psoriasis in adult patients (Rajagopalan et.al., 2021).

Clinical challenges in using biologicals

At present, only a limited evidence available is available about the practical issues faced with biologicals usage. Data available so far is inconsistent to arrive at a conclusion for primary failure or secondary failure and the management in such cases. Expert consensus are available for different countries, which can be used as a guide for clinicians.



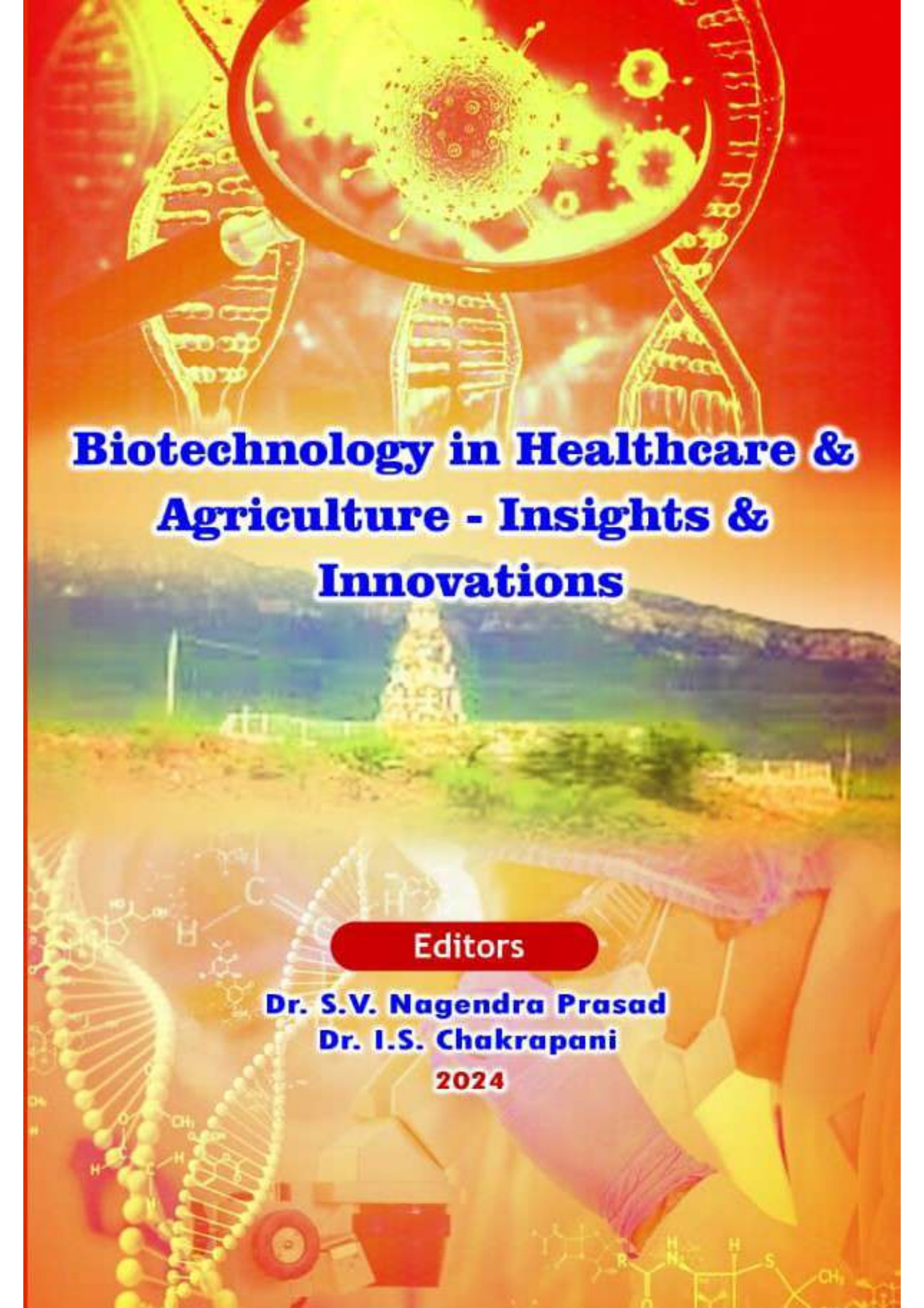
Conclusion

New biologicals are emerging as a hope for patients suffering from psoriasis. But, there is need to evolve country-specific recommendations.

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Biotechnology in Healthcare & Agriculture - Insights & Innovations

Editors

Dr. S.V. Nagendra Prasad

Dr. I.S. Chakrapani

2024

**BIOTECHNOLOGY IN HEALTHCARE &
AGRICULTURE – INSIGHTS AND INNOVATIONS**

Editors

Dr.I.S.Chakrapani

Dr.S.V.Nagendra Prasad

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FORE WORD

Biotechnology has made rapid strides into our daily life in the recent decades. Many biotechnological interventions in health care, agriculture, aquaculture, livestock management and other fields have made life easier. Biotechnology research is being funded and promoted by governments across the world, for the benefit of people. In this context, the Departments of Zoology, PRR & VS Govt. College, Vidavalur and MRR Govt. Arts & Science College, Udayagiri came forward to organize a national seminar on Biotechnology.

‘Biotechnology in Health care and Agriculture – Insights & Innovations’ is a collection of research and review articles contributed by different authors from across India. The national seminar held on 24.01.2024 on the same was fruitful with scientific deliberations about the latest trends in biotechnology research, the socio-ethical concerns, the daily life applications and future developments.

Researchers presented their work in the seminar and fruitful discussions were held about the local and global impact of such research. Many students participated in the event and were enlightened about the future developments and opportunities in the field of Biotechnology.

As editors, we are highly thankful to all the contributors for their efforts in bringing forth this edition. We profusely thank all the authorities who helped in conducting the event and bringing out this book. Every care is taken in publishing the content. Authors would be highly thankful to the readers for bringing to our notice, mistakes if any.

Reagrds..

Dr. S.V.Nagendra Prasad

Dr. J.S. Chakrapani

BIOTECHNOLOGY IN HEALTHCARE & AGRICULTURE

INDEX

Sl.No	Title of the Paper	Authors	Page No.
01	Bridging biology and technology: what is known and what is to be known?	S.B. Sainath	01
02	Xenobiotics and human health with special reference to pesticides	U. R. Sri Veena, P. Ramachandra Reddy*, S.B. Sainath*	09
03	Potential applications of <i>Corynebacterium glutamicum</i> to utilize harmful aromatic compounds into valuable products	B. Jaya Bhargavi*, M. Muralidhara Rao	17
04	Identification of putative drug targets in <i>Morexalla catarrhalis</i> using subtractive proteomics	M. Mounika, S.B. Sainath	52
05	Reverse vaccinology strategies to identify potential drug targets in <i>Candida albicans</i> sc5314	M. Ashok Kumar, S.B. Sainath	63
06	Protective action of <i>Moringa oleifera</i> on glyphosate-induced hepatotoxicity in albino rats	G. Anuradha , U. Kanchana Ganga , P. Annaiah , S.B. Sainath*, B. Kishori*	81
07	Advancements in Biopharmaceuticals: Revolutionizing Medicine	C Samson Raju, C Venkata Krishnaiah C, M Sharmila	88
08	Application of Biotechnology in Vaccine Development-A Review	P Sachi Devi*, D Veera Nagendra Kumar D, GLN Prasad	93
09	Exploring The Potential of mRNA - A Comprehensive Review	S V Nagendra Prasad, V Krishna Kumar, I S Chakrapani*	97
10	Monoclonal Antibodies: Revolutionizing Modern Therapies	K Nagaraju K* , N Anitha , Ch Lalitha Kumari	107
11	Aetiopathogenesis and Manifestations of Polycystic Ovary Syndrome	Shubhasmita Sahoo , L Suhasini Sahithi , Mohan Krishna Ghanta *	119
12	Probiotics - their possible mode of action and applications in Aquaculture	K R Shanmugam	127
13	Integrated Pest Management (IPM) Technology in Agriculture	N Anitha* , Ch Lalitha Kumari, K Nagaraju	139
14	Biotechnology Based Approaches to Disease Diagnosis	K Siva Prasad* , M Gurusekhar, I Anusha	145
15	Aquatic Immunity: Biotechnology's Contribution to Sustainable Aquaculture	Ch Lalitha Kumari , N Anitha, K Nagaraju	152
16	Applications of Biotechnology in Aquaculture	K Lavanya K*, Dayakar Y	160
17	Biotechnological interventions in the treatment of Psoriasis - the promise of new 'biologicals'	I S Chakrapani, N Srinivas, K Salini*	169
18	Vermi-biotechnology for Agricultural Development	G Amaravathi	173

MRR GOVT ARTS & SCIENCE DEGREE COLLEGE, UDAYAGIRI

Principal's Message

Dr. P. Subba Lakshumma
M.Com., M.Phil., Ph.D.
Professor & Principal
MRR Government
Arts & Science Degree College,
Udayagiri, SPSR Nellore dt



Dear Sir/ Madam,

I am very much pleased to express that the Department of Zoology and Internal Quality Assurance Cell of MRR Government Arts & Science Degree College, Udayagiri in Collaboration with Department of Zoology, PRR & VS Govt. Degree College, Vidavaluru is jointly organizing One day National Seminar on **“Biotechnology in Healthcare & Agriculture – Insights & Innovations”**, on 24th January, 2024 at MRR Government Arts & Science Degree College, Udayagiri, SPSR Nellore District.

I cordially invite all the eminent resource persons, delegates and participants for One day National Seminar on **“Biotechnology in Healthcare & Agriculture – Insights & Innovations”**, and the topic is well chosen to focus more on Health and Agriculture issues and finding solutions with innovative techniques of Biotechnology.

The seminar aims to bring different ideologies under one roof and provide opportunities to exchange ideas face to face, to establish research relations and to find research partners for future collaboration. I hope eminent speakers will cover the theme virtual reality from different perspectives with suitable solutions to health and agricultural issues and finding solutions with latest applications of Biotechnology. The themes and sub-themes for this seminar are indicative of relevant research areas to give scope for further research and ambit of discussion.

I extend my heartfelt thanks to eminent resource persons, participants, beloved philanthropists, staff, teaching fraternity, organisers and students for their contribution in successfully organising this event.

Thank you!


Principal
MRR Govt. Degree College
Udayagiri, SPSR Nellore Dt.





PRR & VS GOVT COLLEGE :: VIDAVALUR-524318, AP, INDIA
(NAAC 'B+' GRADE)

Dr. B.Sujatha, Ph.D
Principal
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Message



I am delighted that the Department of Zoology & IQAC, MRR Govt. Arts and Science Degree College, Udayagiri is organizing a **“One day Seminar on Biotechnology in health Care and Agriculture-Insights and Innovations”** on 24th January’ 2024, in collaboration with the Department of Zoology of our institution.

Biotechnology has revolutionized many a front of human life. Health care and agriculture have made rapid progress with the interventions of biotechnology, still there is a vast scope to utilize the inventions in biotechnology. With machine learning, blockchain technology and AI making rapid strides, the combination of IT and Biotechnology will definitely make human existence a miracle.

I expect that the deliberations of this conference will enlighten all the participants about the recent developments in the field of Biotech. Collaborative activities of this kind will definitely enhance and sustain the learning instincts of young research and also contribute to the growth of the institutions considerably. I congratulate all the stakeholders involved in this event.

I wish the organizers a grand success.

With Best Wishes


PRINCIPAL
PRR & VS GOVT. COLLEGE
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VIKRAMA SIMHAPURI UNIVERSITY :: NELLORE-524324, AP, INDIA

(NAAC 'A' GRADE)

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Message



I am extremely happy to know that the Department of Zoology & IQAC, MRR Govt. Arts and Science Degree College, Udayagiri is organizing an “**One day Seminar on Biotechnology in health Care and Agriculture-Insights and Innovations**” during 24th January’ 2024.

The first half of this century witnessed the technological developments in the field of biological sciences are revolutionizing different fields of life sciences to a point where the understanding of complex biological systems is possible. These technological advances in agriculture, medicine and food industry have seen unprecedented growth in global population and resulting in a rise in life expectancy and standards of living. Plant and Animal Biotechnology, as it is known today, did not simply appear on the scene with the discovery of DNA, but evolved over millennia in concert with the development of plant and animal agriculture and the biological sciences. Advances in science and biotechnology now occur at a rapid pace all over the world. Therefore, the impact of such advances on our lives and on posterity continues to be both exciting and sobering. The seminar aims to address all these aspects and their recent developments that revolutionize the areas such as animal physiology, toxicology, pharmacology, nanotechnology, and sericulture which are of high priority in the present scenario of scientific value.

I hope the deliberations at this conference will enlighten all the participants and provide a platform for young scholars and students to interact, exchange and collaborate with the community especially with the eminent scientists which will be useful for their future research endeavors. I am also confident that deliberations and discussions in the Seminar would be beneficial not only for the mankind but for all the living creatures and in dealing with some well-known environmental problems. I congratulate the organizers for conducting this National Seminar.

With Best Wishes



Dr. T. GOPI KRISHNA

Associate Dean (FAC)

Sri Mekapati Goutham Reddy

Agricultural College

ANGRAU, Udayagiri



PREFACE

It gives me immense pleasure to note that M. R. R. Government Arts & Science Degree College, Udayagiri taking initiative to organize One Day National Seminar on “Biotechnology in Healthcare & Agriculture - Insights & Innovations” under the Visionary and Dynamic Leadership of Principal of the College, Dr. P. Subbalakshumma.

Four Decades elapsed since the establishment of the College in 1983. The dream of the College to organise a Seminar and to bring the Souvenir as well as Seminar Proceedings in place like Udayagiri come True because of the Scintillating Efforts of Dr. S. V. Nagendra Prasad, In - Charge, Department of Zoology and Organizing Secretary of the Seminar & his Team in this Scenic Beauty Place of Historical Importance in the Foot Hills of famous Udayagiri Fort is Commendable.

I am highly appreciative of the Visionary Outlook with Scientific Tampere of the Organising Committee, Advisory Committee and Member in selecting the Themes of Interest such as Monoclonal Anti Bodies, Immune System Boosting, Bio Technology in Vaccine Development, Aquaculture, Genetic Modifications and Advances in Agricultural Biotechnology Research to Student Prodigies which will definitely be an Eyeopener to broaden their Vision and develop the Inquisitiveness in Higher Studies in General and Research in Specific.

Globally, India is among the top 12 Destinations for Biotechnology and 3rd Largest Destination for Biotechnology in Asia Pacific. India's Bio Economy has crossed estimated \$92 Billion by value in the year, 2022. Targeted to reach \$150 Billion by 2025 and \$300 Billion by 2030. Covid 19 given much needed push to Biotechnology. Indian Vaccines exported to 150 countries from Biopharmaceutical point of view. It is a leading destination for Manufacture



and Clinical Trials. India has 5th largest area of Organic Agriculture. To Promote Sustainable Agricultural Practices, Department of Biotechnology created 51 Bio – Tech Kisan Hubs (Biotech Krishi Innovation Science Application Networks) connecting farmers with best Scientists and Institutions empowering Farmers with information on Soil Health, Irrigation and new Agri Technologies. 44 Hubs have been created in 15 Agro - Climatic Zones conducting activities in 165 Districts. Global Agriculture Bio Tech Market Size valued at \$.106.62 Billion in 2022 and is projected to reach \$ 242. 17 Billion by 2032. There are 75 Bio Incubators, 800+ Core Bio -Tech Companies and more than 32, 000 jobs generated by this sector. Thus, the Importance of present Seminar on the said Themes.

I am sure that this is going to open a new chapter in the Educational Arena of Udayagiri, SPSR Nellore District, Andhra Pradesh with the Collective Efforts of everyone concerned in organising this event. I wish a grand Success and owe a lot for making me in the sense Sri Mekapati Goutham reddy Agricultural College, Udayagiri as the part of this event. I express my strong sense of Solidarity and Committed Support for this and such future Endeavours of the College.

Once again greeting for the best to happen

(T. GOPI KRISHNA)



MESSAGE

Dr. Krishna Kumar Vepakomma
Professor & Principal(Retd.)
Govt. Degree College
NAIDUPET



I'm delighted to share that the Department of Zoology and IQAC at the college is organizing a one-day National Seminar titled "Biotechnology in Healthcare & Agriculture: Insights and Innovations" on the 24th at M.R.R. Govt. Arts & Science Degree College, SPSR Nellore dt. The chosen topic genuinely captures the current trends, recent advances, and new approaches in biotechnology. I'm confident that this seminar will prove to be both engaging and productive. Personally, I hope you derive enjoyment and valuable insights from the event!

I extend my best wishes for a successful and enriching seminar and express my gratitude to all the organizers.

I look forward to seeing you at the Seminar.



MESSAGE

Dr. S. V. Nagendra Prasad
Organizing Secretary
Dept. of Zoology
MRR Govt. Arts & Science Degree College, Udayagiri
Nagzoo1966@gmail.com



I am happy to organize this seminar 'Biotechnology in Healthcare and Agriculture - Insights & Innovations (BHAI-2024).

The idea of conducting a seminar on 'Biotechnology in Healthcare & Agriculture-Insights & Innovations' was conceived during a chance discussion with the students at MRRGDC about treatment for metabolic syndrome. Enthusiastic students came forward to work for the seminar.

Then, we approached our Principal Dr. P. Subba Lakshumma with the idea, and she encouraged us a lot and provided the necessary academic and administrative support. The arrangements took a momentum.

I am thankful to De.V.Krishankumar, Rtd. Principal, GDC, Naidupeta for his suggestions about the theme and modalities of the seminar. I am thankful to Dr.V.Sainadh, Asst. professor, Dept. of biotechnology, VSU, Dr. T. Gopikrishna, Associate Dean, MGR Agricultural College, ANGRAU, Udayagiri for their support throughout.

I thank Dr.B.Sujata, Principal, PRRVS Govt. College, Vidavalur for her administrative support in collaborating with the Dept.of Zoology of their college in conducting this seminar.

I hope, this seminar will throw light on the recent developments in the field of Biotechnology that has impact on healthcare and agriculture.

With best wishes,



MESSAGE

Dr. I. S. Chakrapani
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I am delighted to collaborate with the Dept. of Zoology, MRRGASC, Udayagiri in organizing this seminar 'Biotechnology in Healthcare and Agriculture - Insights & Innovations (BHAI-2024).

The idea of conducting this seminar sprouted in discussion with students, and this is a testimony of the fact that the learning minds always ignite the teaching community to work more and more.

Biotechnology has made many path-breaking strides into all walks of life. The recent advancements in biotechnology that have an impact on healthcare and agriculture, contributing to the health security and food security of the nation, are highly commendable. This seminar will bring into the focus of UG students, the advanced research in biotechnology.

I sincerely thank all the authorities for providing the necessary administrative and academic support. I believe, this event will certainly pave way to engage students in pursuit of academic excellence.

With best wishes,



MESSAGE

K.C.VIKRAM
IQAC Co-ordinator
MRR.Govt.Arts & Science Degree College
Udayagiri.



Welcome to all delegates and participants,

It is with great pleasure that we convene for the National Seminar on Biotechnology in Healthcare and Agriculture, a forum dedicated to exploring cutting-edge insights and innovations that hold the potential to revolutionize these vital sectors. As we embark on this intellectual journey, let us collectively delve into the intricate realms of biotechnology, dissecting its applications in healthcare and agriculture.

This seminar serves as a platform for the exchange of ideas, fostering collaboration between experts, researchers, and industry professionals. May this discussions today not only broaden our understanding but also inspire us to contribute collectively towards a future where biotechnological innovations serve beacons of progress in healthcare and agriculture.

Wishing you a day filled with enlightening discussions and meaningful connections.

Sincerely,

A handwritten signature in green ink, appearing to read 'K.C. Vikram', with a stylized flourish at the end.

K.C.Vikram



Reviews & Research Articles

BHAI-MS-1

Bridging biology and technology: what is known and what is to be known?

S.B. Sainath

Department of Biotechnology, Vikrama Simhapuri University, Nellore-524324

Abstract

Biotechnology is a biology-based technology wherein organisms, cells or cellular components are exploited to develop new technologies that are useful in medicine, research, aquaculture, agriculture, industry and the clinic. With the advent of advanced molecular and analytical technologies, many ground breaking technologies happened and eventually, the perspective of biology. The present review provides a telescopic view of ground breaking bio-based technologies and their impact on humans and agriculture. One of the cogent examples of this trend are CRISPR technology used for gene editing applications not only in biomedicine but also in agriculture. The present review also highlighted different themes of biotechnology such as animal biotechnology, plant biotechnology, food biotechnology, energy biotechnology, environmental biotechnology, systems biology, nanobiotechnology, tissue, cell and pathway engineering, and pharmaceutical biotechnology. Finally, the gaps and challenges are discussed.

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Introduction

It is well accepted that the second half of this century was completely dominated by software revolution. Software revolution, known for building the electrons on a material known as silicon revolutionized technologies, companies and industries which at one point of time was unimaginable. On the other hand, we are going to witness that the second half of this century by approximately, 2050 will also be dominated by living software revolution known for its ability to program biochemistry and molecular networks on a material known as biology. Biotechnology comprises the study and manipulation of living organisms with the goal of creating new products and processes that can improve our health and/or standards of living. The biotechnology as we know today is the perfect amalgam of 'Biology' and its applications by utilizing advances in different fields such as engineering, physics, chemistry, and mathematics/information technology. The field of biotechnology includes emerging technologies that can address problems related to healthcare to food production to waste management.

Today, the field of biotechnology is considered as a broad discipline and popularly known as tinkering biology in which biological processes, cells or cellular components, organ or organ systems, organisms, are exploited to develop new technologies. Recent advances and developments in areas such as nanotechnology, molecular biology and OMICS, synthetic-biology, and genetic engineering tools further pave the way to achieve different selective criteria and development of 'tailor-made' microbe-metabolites that can be effectively applied for different applications. It is worth mentioning the impact of substantial academic and industrial research and developments in last decade on several attributes of different branches of biotechnology, especially in

nanotechnology for health and enzyme research, environmental applications, applications of extremophiles, proteomics, uses of toxins, and many more remarkable fields. The products developed through biotechnological tools by the scientists are useful in research, agriculture, industry and the clinic. There are two important areas, the medical and agriculture fields wherein the technological advancements are taking place in biology. Imagine plants that fix Nitrogen very efficiently or plants that resist against harmful pathogens or even development of plants in a perineal manner rather than annual through which crop yield increases twice a year, would certainly revolutionize agriculture. On the other hand, imagine devices that modulate your immune system and prevent against pathogens in an effective manner, would certainly revolutionize medicine. Thus, technological developments in these areas would certainly promote ever growing population fed and also aging population healthy. Some of the cogent examples are the production of transgenic animals, transgenic plants, gene therapy, tissue engineering, stem cell technology, monoclonal antibodies, somatic cell nuclear transfer technology, CRISPR, and omics technology. The Milestones of biotechnology in the area of health care and translational research were shown below (source: Liao et al., 2023)

1928: Discovery of transforming principle by Griffith experiments

1943: Discovery of transforming principle as DNA by McLoed, Mc

1952: Creation of continuous HeLa cell lines by George Otto Gey

1953: Discovery of helical model of DNA by Watson and Crick

1954: Kidney transplantation between identical twins by Joseph Murray

1957: An alteration in a single amino acid within hemoglobin cells induces sickle-cell anemia



- 1958: Creation of DNA in a test tube by Arthur Kornberg
- 1960: Discovery of mRNA
- 1961: Concept of operon by Francois Jacob and Jacques Monad
- 1962: green fluorescent protein in the jellyfish by Osamu Shimomura
- 1963: Samuel Katz and John F Enders developed the first measles vaccine
- 1963: Insulin production by USA, Germany, and China
- 1964: The prediction of the existence of reverse transcriptase
- 1967: Vaccine for mumps/rubella was developed
- 1968: The discovery of regulation of the cell's synthesis of proteins
- 1970: Discovery of restriction endonucleases
- 1972: Discovery of DNA ligases
- 1973: Southern blotting/ hybridization by Sir Edwin Mellor Southern
- 1974: Japan scholars developed the first vaccine for chicken pox
- 1975: Monoclonal antibodies BY Wholer and Milstein
- 1976: The NIH published the first guidelines for rDNA research
- 1977: development of the first pneumonia vaccine by the University of Pennsylvania
- 1978: The first test-tube baby, Louise Brown, was born in the UK
- 1980: Automatic gene machine was developed in California
- 1981: Cloning of mice by Switzerland research team
- 1982: First recombinant protein approved by FDA
- 1983: Kary Mullis discovered the polymerase chain reaction (PCR) technique
- 1984: Development of recombinant hepatitis vaccine
- 1985: Genentech launched its biopharmaceutical product
- 1986: DNA sequencing analysis using automated DNA sequencer
- 1987: FDA approved diagnostic kit (serum tumor marker test) development for ovarian cancer
- 1988: Human Genome Project
- 1989: First biologically derived human therapeutic developed by Amgen approved by FDA
- 1990: Hepatitis C antibody test approved by FDA
- 1992: Recombinant proteins in the treatment of renal cell cancer approved by FDA
- 1993: Recombinant proteins in the treatment of multiple sclerosis approved by FDA
- 1994: Recombinant proteins in the treatment of growth hormone deficiency approved by FDA
- 1995: The first baboon-to-human bone marrow transplant was carried out on an AIDS patient
- 1996: Gene Chips by Stanford University and Affymetrix
- 1997: Discovery of first human artificial chromosome
- 1998: Human skin was created in the laboratory
- 2000: Synthesis of DNA in a test tube by Har Gobind Khorana
- 2001: Human genome sequence
- 2002: The rapid shotgun sequencing of major genomes
- 2003: The sequencing of the human genome
- 2004: Monoclonal antibody and DNA microarray analysis system approved by FDA
- 2006: Recombinant vaccine for human papillomavirus approved by FDA
- 2007: Human skin cells were manipulated to produce embryonic stem cells
- 2008: Artificial DNA prepared entirely of artificial parts
- 2010: Development of prostate cancer medicine approved by FDA
- 2011: First cord blood therapy approved by FDA
- 2012: Draft regulations for biosimilar drugs approved by FDA



2013: CRISPR-Cas9 technology and successful gene editing in human cells
2015: First nasal spray version of naloxone hydrochloride approved by FDA
2016: Buprenorphine implant Probuphine approved by FDA
2018: CART Cell therapy (Novartis) for acute lymphoblastic leukemia approved by FDA
2019: Development of Agg-tag tagging system to identify misfolded and aggregating proteins
2020: AlphaFold2 technology based on AI algorithm to predict protein folding
2021: Development of COVID 19 diagnostic kits approved by FDA
2022: Development of COVID-19 vaccine approved by FDA

The present review provides a telescopic view of bio-based technologies and their impact on humans and agriculture. In the current review, outlines of different themes of biotechnology such as animal biotechnology, plant biotechnology, food biotechnology, energy biotechnology, environmental biotechnology, systems biology, nanobiotechnology, tissue, cell and pathway engineering, and pharmaceutical biotechnology was highlighted. For more information about the history, scope, basic concepts and development of biotechnology, the readers are informed to refer excellent reviews by Bhatia (2018), **Chekol and Gebreyohannes (2018)** and **Liao et al. (2023)**.

Animal biotechnology

Animal Biotechnology deals with the technological or genetic engineering principles applied to the animals which in turn have implications for human health and welfare. Animal biotechnology is a broad field encompassing the polarities of fundamental and applied research, including molecular modeling, gene manipulation, development of diagnostics and vaccines, and manipulation of tissue. Many areas of animal biotechnology

such as transgenic animal models, tissue engineering, nanobiotechnology, and proteomics are some of the examples. Further, in-depth examples of applications in human health and prospects for the future, including cytogenetics and molecular genetics, xenografts, and treatment of HIV and cancers.

Plant biotechnology

Plant biotechnology can be defined as the introduction of desirable traits into plants through genetic modification. It encompasses a multitude of scientific tools and techniques for screening and genetic manipulation of plants to develop beneficial or useful plant/plant products. An overview of literature on plant biotechnology innovations and the need to steer agriculture towards sustainability introduces a series of perspectives on how plant biotech can contribute to the major challenge of feeding our super population with enough nutritious food without further compromise of the environment. With all aspects of fundamental and applied research in the field of plant biotechnology, which includes molecular biology, genetics, biochemistry, cell and tissue culture, production of secondary metabolites, metabolic engineering, genomics, proteomics, and metabolomics.

Environmental biotechnology

Environmental biotechnology is the branch of biotechnology that addresses environmental problems, such as the removal of pollution, renewable energy generation or biomass production, by exploiting biological processes. The technological innovations introduced in this area could be effectively clean up many wastes than conventional methods and greatly reduce the dependence on methods for land-based disposal. For example, environmental biotechnology acts as a platform for researchers integrating apparently disjointed biological knowledge, and tailoring this knowledge to address specific engineering challenges related to the environmental pollution. This will be helpful to assess and



reduce the risk to human health using molecular based forensic tools in the identification of specific environmental problems including microbiological pollution.

Food biotechnology

The issue of food security and safety is a global issue that affects the whole food system. Questions arise on how to change food habits and ways to reduce waste in the food chain, from harvest to the moment of consumption. Although it is important to tackle these issues, it does not change the fact that we will need to change our dominant agricultural model in order to feed a growing global population in a way compatible with the sustainable use of global resources. Innovative agriculture and food systems must be tailored to a diverse global population whilst preserving the variety of its cultures i.e., fitting the characteristics and needs of various individuals, cultures, and social groups. Science alone will not solve food problems and hence, three major forces - science, the economy and society - shape our modern world needs to be harmonize. Food biotechnology deals with the current and emerging developments and applications of modern genetics, enzymatic, metabolic and systems-based biochemical processes in food and food-related biological systems which ultimately helps to produce and improve foods, food ingredients, and functional foods at the processing stage and beyond agricultural production. This area also includes microbial and fermentation based metabolic processing to improve foods, food microbiomes for health, metabolic basis for food ingredients with health benefits, and biochemical processes for food waste management. Food biotechnology also encompasses fundamental and applied educational and research aspects related to food quality and value-added processing challenges.

Systems biology

Systems biology is a branch of biotechnology that acts as an intellectual resource for integrative biology. It is a

comprehensive approach in biomedical research to decipher the complexity of biological systems to get a clear-cut picture, be it at the level of the organism, tissue, or cell. Systems biology is an integration of many scientific disciplines – biology, computer science, engineering, bioinformatics, physics and others to understand the complexity of biological systems. One of the major advantages of systems biology is to predict how these complex systems change over time and under varying conditions, and to develop solutions to the world's most pressing health and environmental issues. For example, systems biology wherein innovation in biology-based technology and computation is helpful to discover new biomarkers for disease, stratify patients on genetic profiles and development of targeted drugs. Another example of systems biology is to predict the drug targets in microbes that are resistance against antibiotics. This area is popularly known as reverse vaccinology.

Pharmaceutical biotechnology

It is well known that the designing stable and effective therapeutic proteins requires knowledge of protein structure and the interactions that stabilize the structure necessary for function. Pharmaceutical biotechnology is deals with the development of bioformulations like therapeutic molecules including antibodies, nucleic acid products and vaccines. Such bioformulations are developed through the principles of biotechnology and include several stages: understanding the principles underlying health and disease; the fundamental molecular mechanisms governing the function of related biomolecules; synthesis and purification of the molecules; determining the product shelf life, stability, toxicity and immunogenicity; drug delivery systems; patenting; and clinical trials. In the coming years, protein-based therapeutics is an emerging area of pharmaceutical biotechnology. Some examples of



pharmaceutical compounds are cytokines, interferons (eg Rebif, interferon beta-1a), interleukins (eg Ontak, denileukin difitox), tumour necrosis factors (eg Beromun, tasonermin), growth factors (eg Neupogen, filgrastim), hormones (eg Humalog, insulin lispro), enzymes (eg Benefix, nonacog alfa), antibodies (eg Avastin, bevacizumab) and vaccines (eg Engerix B, hepatitis B virus coat).

Nanobiotechnology

Nanobiotechnology is one of the revolutionary areas of biotechnology that encompasses physicochemical and biological properties including size (diameter), surface (porosity), pH, solubility, complexation (binding/ligand capacity), bioavailability, toxicity, and cellular and molecular effects of nanostuctures and their applications in various areas such as medicine and agriculture. It deals with the control, manipulation, synthesis, and biofunctionalization of structures/devices at the nanometer scale. It is an interdisciplinary science that utilize the use of biocompatible and biodegradable natural and synthetic polymers and their applications range from diagnosis (imaging), drug delivery, and targeting of extremely serious disorders, such as cancer, Parkinson and Alzheimer diseases, chronic inflammations, ocular dysfunctions, and microbial/viral infections. Currently, nanobiotechnology is progressing towards the development of modern nanosystems, including niosomes, polymeric nanoparticles, nanocomposite (colloidal) hydrogels, polymeric micelles, dendrimers, aptamers, capsosomes, nanoneedles, molecularly imprinted polymers, stimuli-responsive polymers, therapeutic polymers (polymer–drug conjugates), and polymeric artificial cells.

Tissue cell and pathway engineering

Tissue, cell and pathway engineering technology deals with regenerative medicine, wherein strategies aim at delivering solutions for those diseases and traumatic accidents that

occur in our life and impair the capacity of our tissues to repair and regenerate themselves, restoring their original functionality. Recent developments include clinic spanning from skin grafts to stem cell-based therapies for cornea regeneration and instructive biomaterials for bone regeneration. These models are instrumental to understand more fundamentally the cell-material interface and also future avenues of tissue regeneration combining developmental biology, organoids, and 3D bioprinting. With reference to the integrating developmental re-engineering with 3D bioprinting, regulation of several signaling pathways through the fabrication of mini-organ constructs for transplantation or *in vitro* screening of drugs using an organ-on-a-chip platform.

CRISPR technology

Thanks to the two women scientists Jennifer Doudna an American Biochemist, University of California, Berkeley and Emmanuelle Charpentier a French Microbiologist, Max Planck Institute for Infection Biology who make history for their discovery that an intrinsic immune system of bacteria and archea fend off viruses and plasmids which invade them using a molecular programming mechanism known as Clustered Regulatory Interspread Palindromic Repeats (CRISPR). Accordingly in 2020, the two female CRISPR scientists won Nobel Prize in chemistry for their discovery. It is worth to state that the microorganisms often surprise the scientists with plethora of molecular gadgets that have potential biotechnological applications and most notable example of this trend is the application of restriction enzymes as a molecular scissor in genetic engineering strategies. Currently, targeted gene editing is gaining popularity because of its simplicity in design and usage in biomedical and industrial applications.

Other ground breaking technologies include, a) AlphaFold, a protein folding



program developed by Google's DeepMind to understand the concepts like how proteins fold and better understanding of proteins may be helpful to human health; b) CRISPR-Based Gene Editing of DNA is offering potential cures for genetic disorders and also addressing inheritable diseases like cystic fibrosis to revolutionizing agriculture eg: grow drought-resistant crops; c) Epigenetics and digital therapeutics is another emerging areas of biotechnology wherein there is a scope for the development of precision medicine and tailoring treatments to individual genetic profiles. d) In order to get invaluable insights into drug effects and disease progression, a real time visualization of cellular processes in living organisms, Bioluminescent imaging is widely used; e) 3D bioprinting technology is a tissue crafting three dimensional technology that revolutionize the organ transplantation; f) Biosensors that track various biosignatures such as blood pressure, pulse, breathing, and body temperature will revolutionize precise monitoring system of our health; g) development of bioplastics is one of the profound technologies wherein the safety of humans, plants, wildlife and eventually environmental health against the toxicity of plastics and their metabolites is of prime concern.

Conclusion

Harmonization of biotechnological developments collated exactly what is known and what is to be known about the improvement of human health and also for sustainable agriculture and augment the researchers and clinicians to unlock the power of genome. Given the tools that are currently available and the translational potential for these studies, the power of technological developments in biosciences has become one of the most essential areas for those studying life sciences.

References

The present topic deals with the recent developments in the area of biotechnology. Due to limited space and also to provide a comprehensive data on the biotechnological developments, a broad outline on the selected topics were described in the current review. Hence, possible links to the articles are provided for further information on the aforementioned topics.

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3. <https://institut-curie.org/popin/spatial-omics>
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5. <https://www.tandfonline.com/doi/full/10.1080/02648725.2022.2116309>
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8. <https://www.nsf.gov/bio/pubs/reports/stctechn/stcmain.htm>
9. <https://www.pnas.org/doi/10.1073/pnas.191380098>
10. <https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3002135>
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Xenobiotics and human health with special reference to pesticides

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Abstract

There is a major concern towards the accumulation of xenobiotics such as pollutants, drugs, carcinogens, hydrocarbons, food additives and pesticides in the environment and their toxic effects on humans. They can able to interfere with the physiological aspects and eventually causes health hazards in humans and wildlife. Though the exact mechanism of xenobiotics is not clear, they exert their effects through oxidative stress and endocrine disruption. It is believed that these foreign substances after their entry, perturb anti-oxidant to pro-oxidant balance and interfere with endocrine system thereby target almost all compartments of humans including liver, kidney, brain, reproductive system, circulatory system and eventually affect metabolism, development, and reproduction. In the current review, we summarize the mechanisms underlying the toxic effects of xenobiotics on liver, kidney, development and reproduction.

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Introduction

The word xenobiotics is derived from the Greek word '*xenos*' meaning foreign. The xenobiotics are chemically synthesized and do not occur in nature. Most xenobiotics do not readily biodegrade and thus are persistent in environment (Fetzner, 2002). Such chemicals are known as persistent chemicals. It was estimated that humans are exposed to about one to three million xenobiotics in their lifetime. Most of these xenobiotics gain entry to the body via food, drinking water, air, drugs, inhalation, etc and they undergo a broad range of detoxication processes which render them more polar, less toxic, making them readily excretable. In the lower animals, detoxification reactions may have impact on human health. Incomplete detoxification in lower animals down the food chain may determine the extent of human exposure to xenobiotics (Patterson et al., 2010). This review article highlights the impact of xenobiotics and their toxic mechanisms underlying health hazards.

Endocrine disruption and its impacts

Hormones play a vital role in the regulation of physiological functions associated with growth, sexual development, reproduction, movement etc. in organisms. Endocrine disruptors (ED) are the agents that interfere with this endocrine system and can able to interfere with the synthesis, secretion, transport, binding or elimination of the natural hormones in the body (Diamanti-Kandarakis et al., 2009). They can act as agonists and/or antagonists to the endogenous hormones like estrogens, androgens, thyroid hormones and/or can able to bind their cognate receptors and

eventually lead to improper genomic actions that may induce harmful effects (Kabir et al., 2015).

For example, a number of chemicals that are structurally similar to thyroid hormones, have been shown to bind to the thyroid receptors and exert either agonism or antagonism effects on thyroid hormone signaling (Lyn, 2009) thereby perturb thyroid hormone mediated functions (Zoeller and Crofton, 2000). Studies have shown that the 4,4'-isopropylidenediphenol (BPA) mimic thyroid hormone and can able to bind thyroid hormone receptor (TR) and antagonizes T3 thereby inhibit(s) TR-mediated gene expression (Diamanti-Kandarakis et al., 2009). Thyroperoxidase (TPO) is a heme-containing enzyme that catalyzes the oxidation of iodine and thyroglobulin thereby thyroid hormone synthesis in the thyroid gland. Studies indicated that TPO was found to be blocked by 6-propyl-2-thiouracil (PTU, used therapeutically to treat Graves disease) (Kabir et al., 2015). It has been shown that the heavy metals, DDT, dioxins, PCBs, synthetic steroids, dithiocarbamates, phytoestrogens, some of the pesticides and herbicides (Kabir et al., 2015) directly target the nervous system thereby exert endocrine disrupting effects and modulate behavior, memory, learning, sensory function, attention, and even neuro-teratogenicity. Direct effect on steroid receptor activity is the most studied mechanism. Most of the xenoestrogens acts as ligands for estrogen receptor and may compete with estrogen for binding and are thought to exert their functions by activating estrogen receptor and regulates the target genes accordingly (Singleton and



Khan, 2003). Some of the disorders observed during the lifetime may be sexually dimorphic and related to endocrine disruption. Sexual differentiation in males is androgen-dependent and in females it is independent of estrogens and androgens. Therefore, different disorders seen in males and females are the result of endocrine disruptors that antagonize androgens and/or mimic estrogens (Diamanti-Kandarakis et al., 2009). In females, endocrine disruptors may cause polycystic ovary syndrome, precocious puberty, premature ovarian follicle, risk of breast cancer, and unwanted increase in the endometrium growth. In males, endocrine disruptors may cause hypospadias, ectopic testes, sperm anomalies and eventually decrease in fertility (Kabir et al., 2015).

Development and Reproduction

Prenatal period is believed to be one of the critical windows wherein crucial development of vital organs including reproductive tract takes place. Xenobiotics can able to cross placenta and can interfere and causes adverse effects on the developing fetus (Barr et al., 2007). The women who were exposed *in utero* to the estrogenic chemicals are at higher risk for the incidence of cancer when compared to the unexposed individuals (Colborn et al., 1993). Diethylstilbestrol (DES) is one of the drugs given to the pregnant women to prevent abortions. However, studies indicated that DES can able to cross placenta and acts as a development toxicant as evidenced by abnormality in pregnancies, reproductive organ dysfunction, reduced fertility, disorders of immune system in the children of women who were given DES during pregnancy

(Fraser and Adeoya-Osiguwa, 2006). Wide usage of PCBs causes environmental pollution. Many reprotoxic effects have been associated with spermatogram, testicular spermatogenesis and steroidogenesis and epididymal post testicular sperm maturation events. Interestingly, phthalates are a group of chemicals that affects Leydig cell function via inhibition of testosterone synthesis (Diamanti-Kandarakis et al., 2009).

Hepatotoxicity

Liver is one the metabolic organs in the body and is well appreciated for its detoxification mechanisms of chemical substances. It has been shown that xenobiotics lead to the accumulation of fat in the liver in humans. An example of this trend is the fat deposition in humans who consume alcohol and progressive liver injury can lead to fibrosis and finally to cirrhosis (Maher, 1997). A link between xenobiotics, fat accumulation in the liver and changes in nuclear receptor expression has been reported. A plastic raw material, phthalate ester Di (2-ethylhexyl) phthalate (DEHP) interact with peroxisome proliferator activator receptor (PPAR) α and causes fat accumulation, liver tumors and even changes the metabolism in liver (Klaunig et al., 2018). Liver is one of the important organs of detoxification systems. When xenobiotics enter the system, the phase I and phase II systems of liver degrade the xenobiotic and in most of the cases, the degraded metabolites are excreted, however some metabolites become toxic and such toxic products exert liver injury occurs via two types intrinsic and idiosyncratic mechanisms. Intrinsic injuries are generally predictable leading to



zonal hepatocyte necrosis which may be accompanied by little or no signs of inflammation and on the other hand, idiosyncratic liver injuries are mediated by immune mechanisms. The predominant pattern of injury is by diffuse necrosis and cholestasis (Sturgill and Lambert, 1997).

Apart from environmental pollutants, drugs such as acetaminophen are widely studied hepatotoxic. The cell death mechanisms are initiated by the formation of its metabolite N-acetyl-p-benzoquinone imine (NAPQI), cytochrome P450 enzymes (Jaeschke et al., 2014). Under normal conditions, NAPQI is converted into harmless reduced form by conjugation with glutathione, which is then excreted via the bile. However, NAPQI toxicity occurs when the glutathione is depleted due to the overdose of acetaminophen. The increasing amount of NAPQI binds to the mitochondrial protein and forms protein adducts which could be cytotoxic in nature. Glutathione levels can be replenished by N-acetyl cysteine which helps in the clearance of this toxic metabolite (Krenkel et al., 2014). Aflatoxins are the compounds produced by *Aspergillus* species. Aflatoxins specifically target liver and symptoms of hepatotoxicity caused by aflatoxins may include malaise, anorexia, fever, followed by abdominal pain, hepatitis and vomiting (Kumar et al., 2017).

Nephrotoxicity

Because of the active transport capability, high rate of perfusion, concentrating function, kidney is most often exposed to higher concentrations of xenobiotics than any other organ. Some xenobiotics may exert toxicity directly on renal cells. Examples of xenobiotics that

causes renal cytotoxicity are therapeutic agents (like analgesics, aminoglycoside antibiotics), heavy metals (like cadmium, mercury), or other chemicals (like carbon tetrachloride, chloroform) (Kluwe and Hook, 1980). The heavy metals in plasma exist as diffusible (complexed, ionized) and non-diffusible (protein bound) forms. It is well known that the tubular reabsorption is dependent on the form that is present in the glomerular filtrate and filtrate composition is in turn dependent on the dose and the mode of intoxication. Heavy metal ions readily bind to the most abundant protein, albumin, in plasma, however, a small amount of heavy metal may escape from this kind of binding. This eventually, lead to As a result, the plasma also contains the heavy metal in free form and glomerulus always filters a small amount of albumin. Hence, the luminal fluid delivered to the proximal tubule will contain both the albumin bound form of heavy metal and also the ionized form of heavy metal (Barbier, et al., 2005). The toxic form of heavy metal is the ionized form and the extent of severity of the renal damage depends on the mode of exposure. Cadmium causes the rupture of outer membrane, inhibition of electron transport chain resulting in the release of reactive oxygen species. Most of the heavy metals causes chronic intoxication like Fanconi syndrome which causes decrease in the glomerular filtration rate, proteinuria, increased urinary flow rate, glycosuria, loss of ions, and aminoaciduria (Barbier, et al., 2005). Chemicals such as chloroform is metabolized by cytochrome P450 to a reactive metabolite, phosgene, and this metabolite is responsible for renal toxicity



(Bailie, et al., 1984). Hypoxia and hypotension induced by phosgene may lead to anoxic injury to the kidneys. The abnormalities in the victims who are acutely exposed to phosgene include increase in blood urea nitrogen (BUN), abnormal urinalysis with numerous RBCs and WBCs (Borak and Diller, 2001). Drugs such as amphotericin B, an antifungal drug exhibits numerous side effects including kidney failure. During treatment with amphotericin B, 30% of the patients show renal malfunctions and as a result, serum manganese and potassium are reduced, and serum creatinine are elevated. The main reason of nephrotoxicity of amphotericin B is thought to be its high affinity for cholesterol and hence, to reduce these side-effects, monotherapy with amphotericin B is avoided whenever possible (Lemke, et al., 2005).

Natural toxins

Natural toxins are produced by plants as secondary metabolites and are not harmful to the organism itself but are toxic to other living forms such as, humans. Some plants produce a wide array of chemicals as a natural defense mechanism, to protect themselves against predators, microorganisms, or insects (WHO, 2018). Cycad plants were used as a source of food and medicine. These plants have also been recognized as hazards in tropical areas. Cycasin is the main toxin found in *cycas revoluta* and also in all genera of Cycadales. Cycasin is an azoxyglucoside and causes toxic effects on liver and gastrointestinal tract. In humans, there have been many cases of gastroenteritis caused by cycasin reported (Chang, et al., 2004).

Pyrrolizidine alkaloids are present geographically widespread distributed plants. More than 200 alkaloids were identified in 300 different types of plant species of up to 13 families. The commonly known pyrrolizidine alkaloids are lasiocarpine, heliotrine, senecionine, monocrotaline. The main sources of pyrrolizidine alkaloids are Compositae, Boraginaceae, Leguminosae (Prakash, et al., 1998). Many of these sources are weeds and can grow in fields and contaminate food crops. Pyrrolizidine alkaloids can cause adverse health effects. They have DNS-damaging potential and can lead to cancer (WHO, 2018). Mycotoxins are the toxic compounds produced by certain types of fungi. Fungi that produces mycotoxins can grow on many foodstuffs like nuts, dried fruits, spices, cereals (WHO, 2018). Fumonisin B1, aflatoxin B1, and ochratoxin A are toxic to mammals causing variety of adverse effects like mutagenicity, teratogenicity, hepatotoxicity, resulting in diseases like hepatic carcinoma, esophageal cancer, equine leukoencephalomalacia, toxic hepatitis, edema, hemorrhage, hepatic carcinoma, and kidney failure (Reddy, et al., 2010). Upon ingestion of mycotoxin contaminated food, the gastro-intestinal tract is affected. They can alter the normal functions of intestine like nutrient absorption and barrier function. Some of them can also affect the histomorphology of intestine (Liew and Mohd-Redzwan, 2018). Solanines and chaconine are the natural toxins found in all Solanaceae plants like potato, tomato, eggplant. They are found in potato sprouts, green parts, bitter tasting peel and also in green tomatoes (WHO, 2018). Solanines and chaconine can induce



gastro-intestinal effects by cell membrane disruption and systemic effects by acetylcholinesterase inhibition (Mensinga, et al., 2005).

To reduce the health risk caused from the natural toxins, WHO recommends people not to assume that when something is called as 'natural' it is automatically safe; do not consume and throw away damaged, bruised, or discolored food, particularly food contaminated with moulds; throw away the food item that does not taste or smell fresh or is having an unusual taste (WHO, 2018).

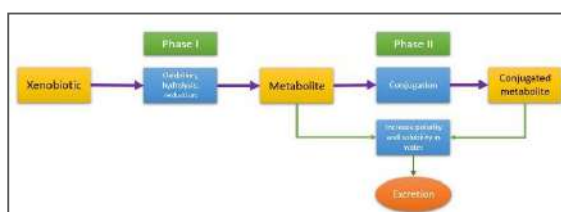


Figure 1: Biotransformation of xenobiotics

Mechanism of Action of Xenobiotics

The mechanism of action describes how a xenobiotic enters into the living organism, followed by its distribution, metabolism and its interaction with the cellular macromolecules. These events involve cellular, molecular and biochemical reactions (Park et al., 2014). In general, biotransformation of xenobiotics is believed to be one of the important underlying mechanisms of its toxicity. The mechanisms may broadly be classified into direct or indirect. Directly, xenobiotics may undergo hydrolysis and exhibit electrophilic property. This eventually targets the proteins thereby elicit toxicity. Such xenobiotics are classically known as direct acting xenobiotics. The xenobiotics

with the electrophilic property, interacts with the cellular macromolecules like DNA, lipids, proteins and results in the toxicity inside the organism (Park et al., 2014). On the other hand, xenobiotics undergo biotransformation in the liver wherein and phase I and phase II systems. Phase I system includes several cytochrome P450 enzymes and xenobiotics undergoes mainly oxidation reactions which in turn elevate the electrophilicity of the xenobiotics (Yagishita et al., 2016). Phase II system include enzymes that carry out the conjugation reactions include acetylation, glucuronidation, methylation, and glutathione mediated by transferases and convert the xenobiotics into more water-soluble forms (Park et al., 2014; Omiecinski et al., 2010). The metabolites of the parent xenobiotic compound, now elicit toxicity and hence, known as indirect acting xenobiotics (Patterson et al., 2010). The ultimate outcome of these events is toxicity to the living organism. Though the final goal of biotransformation is to convert the xenobiotics into harmless substances by converting lipophilic chemicals to more water-soluble and facilitate to excrete the same through urine. However, some substances, during their transformation become highly reactive and perturb redox-cycling system and eventually causes oxidative stress induced damage of the organs and prolonged presence of the reactive metabolites in the body may lead to adverse effects and disease conditions (Park et al., 2014). On the other hand, some substances, they interfere with the endocrine system and switch on or off genes thereby changes the molecular networks and physiological events of the



cells. Hence, xenobiotics induced toxicity relies on oxidative stress and endocrine disrupting properties. Figure 1 illustrates the biotransformation of xenobiotics through phase I and phase II systems.

Conclusion

To summate, the concept of xenobiotics and their toxic effects is of major concern. They exert their effects via endocrine disrupting properties and oxidative stress. Xenobiotics such as heavy metals, pesticides, natural toxins and chemicals/drugs interfere with the redox system and endocrine system. This eventually damage the organs. In addition, as prolonged exposure to xenobiotics at low concentrations may be mutagenic and teratogenic. To avoid the toxic impact of xenobiotics, some measures should be taken which include effective treatment of wastewater, strict laws and regulations must be adopted which are economically, environmentally, and socially acceptable with an emphasis on the removal of xenobiotics from the environment.

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BHAI-MS-3

Potential applications of *Corynebacterium glutamicum* to utilize harmful aromatic compounds into valuable productsB. Jaya Bhargavi*¹ and M. Muralidhara Rao¹**Abstract**

Corynebacterium glutamicum is a promising strain recognized for the production of various amino acids since its discovery in the year 1957, hence, popularly known as industrial work horse of white biotechnology. With the discovery of whole genome of *C. glutamicum*, the perspective of this organism revolutionized the industrial biotechnology through the development of strains that over-produce production of value-added products such as amino acids, L-glutamate family amino acids, non-amino acids, antioxidants. In the current review, the current perspectives of *C. glutamicum* with respect to the production of which have therapeutically and industrially potential value-added products via metabolically engineered strains from different substrates including substances other than glucose, lignin based bio-waste, and harmful substances. To accomplish this task, the review was broadly comprised of three aspects. The first aspect deals with a comprehensive treatise of basic biochemical and molecular aspects of *C. glutamicum*. The second aspect encompasses metabolically engineering approaches applied to *C. glutamicum* in the production of value-added products and the final aspect deals with the bioremediation pathways of *C. glutamicum* and metabolically engineered strains used in bioremediation. To conclude, the pleiotropic functions of *C. glutamicum* allowed this organism as one of the tools of microbial cell factory.

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1. INTRODUCTION

Microorganisms are versatile organisms as they are used for the fermentative production of various economically important compounds (Sanchez and Demain, 2008) and also play significant roles in biodegradation/bioremediation of aromatic compounds thereby sustain environmental pollution management. In recent times, a crosstalk between the utilization of aromatic compounds (that are harmful to the environment and humans) and their transformation into value-added products by microorganisms is one of the emerging areas of biotechnology. Interestingly, research is accelerated to apply *Corynebacterium glutamicum* to utilize harmful compounds into value added products (Shen et al., 2012).

C. glutamicum is a gram-positive, facultative anaerobic, non-spore-forming, non-pathogenic bacteria that has been used for decades to produce amino acids (Eggeling and Bott, 2015; Wendisch et al., 2016; Matanio et al., 2016; Hirasawa, 2016; Yang et al., 2020; Tsuge and Yamaguchi, 2021; Wolf et al., 2021). There are many advantages of *C. glutamicum* as illustrated in **Figure 1**.

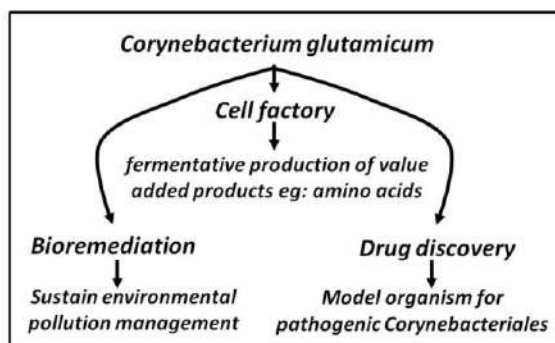


Figure 1: Broad applications of *C. glutamicum* in different areas of biotechnology

C. glutamicum is classically known as industrial workhorse as it can be used as a cell factory for the production of amino acids and value-added products (Wolf et al., 2021), b) it is preferred choice to develop therapeutic approaches against corynebacteriales pathogens (Si et al., 2014) and c) it is used in the bioremediation of toxic aromatic compounds phenol, resorcinol, benzyl alcohol, benzoate, 4-cresol and heavy metals including arsenic metalloids (Shen et al., 2012).

The use of *C. glutamicum* as a cell factory for the fermentative production of value-added products is well acknowledged. It is a major producer of L-glutamic acid and L-lysine (Wolf et al., 2021). As per the estimates, the annual production of L-glutamate and L-lysine from the *C. glutamicum* is 2,160,000 tons (Ajinomoto, 2010) and 1,480,000 tons (Ajinomoto, 2011), respectively. Due to industrial relevance and pharmacological benefits fermentative production of amino acids by *C. glutamicum* is well studied (**Table 1**).

The use of *C. glutamicum* as a choice to develop therapeutic approaches against corynebacteriales pathogens is quite interesting (Si et al., 2014). During recent past, few studies demonstrated that the knowledge obtained from the *C. glutamicum* might be used to develop therapeutic strategies. *C. glutamicum* belongs to the family Corynebacteriales wherein pathogenic species such as *C. diphtheria* (causes diphtheria) and *Mycobacterium tuberculosis* (causes tuberculosis) are also included. Because of



close relationship to pathogenic species, *C. glutamicum* is also used as model organism (Lawn and Zumla, 2011). Si et al. (2014) demonstrated that mycothiol (MSH) and mycothiol S-conjugate amidase (MCA) system similar to *M. smegmatis* also exists in *C. glutamicum* RES167 and experiments on this strain using MSH-MCA system might be to develop therapeutic strategies against pathogenic corynebacteriales.

There are other advantages of *C. glutamicum* such as its ability to grow quickly, easy propagation, no endotoxin production, its adaptability towards various carbon sources and to tolerate stress against various toxic substances. Because of no endotoxin production, *C. glutamicum* is generally recognized as safe (GRAS) and this status have been granted by United States Food and Drug Administration for the food and pharmaceutical industries. In addition, the properties such as secretion of functionally targeted proteins with proper folding into culture broth, and lack of extracellular enzymes with hydrolytic activity and low level production of endogenous proteins during production of targeted proteins, *C. glutamicum* is a very promising host system for the production of heterologous proteins, important enzymes and therapeutic proteins (Lee and Kim, 2018). With the advent of advanced molecular techniques, the complete genome picture of *C. glutamicum* became publically available in the year 2003 (Ikeda and Nagakawa, 2003). Kyowa Hakko provided the publicly available whole-genome sequence of *C. glutamicum* under the accession number in the DDBJ/GenBank/EMBL Database.

One of the important strains used in the production of various amino acids is *C. glutamicum* ATCC 13032 and its total genome size is 3,309,401 bp comprising of 3099 genes with a GC content of 53 %. Genome of *C. glutamicum* paved a way to develop methodologies for strain improvement such as metabolic engineering, and synthetic biology with desired phenotypes and also to stimulate production of value-added products (Zahoor et al., 2012). It is well accepted that the metabolism is a key aspect for the production of value-added compounds. Now it is possible to understand the ‘metabolic maps’ by using the genome of *C. glutamicum* thereby facilitated the researchers to design experiments to over produce biotechnologically relevant products which have significant applications in biopharma, cosmetic and food industries (Wolf et al., 2021).

Application of advanced molecular and analytical techniques revealed that, *C. glutamicum* is a source of range of compounds such as amino acids, non-protein products, organic acids, diamines, biofuels, and sugar based alcohols (Zahoor et al., 2012; Wolf et al., 2021). Additionally, an effort has been made to untangle the molecular network of *C. glutamicum* due to its strong relationship with pathogenic Corynebacteriales. According to studies by Brinkrolf et al. (2007) and Schröder et al., (2010), there are almost 140 transcriptional regulators, of which about 75 have been described. This improves strains relevant to industry and advances our understanding of the bio-molecular circuits in this organism.



Table 1: Synthesis of amino acids from their precursors and related pathways

Pathways	Intermediates and initial substrate or precursor	Amino acid related family Groups	Amino acids
Tri-carboxylic acid cycle (TCA)	α -ketoglutarate	Glutamate	Glutamate Glutamine Proline Arginine
	Oxaloacetate	Aspartate	Aspartate Lysine Asparagine Methionine Threonine Isoleucine
Embden-Meyerhof-Parnas (EMP)	Pyruvate	Pyruvate	Alanine Valine Leucine
	3-phosphoglycerate	Serine	Serine Glycine Cysteine
Hexose-monophosphate (HMP)	Erythrose-4-phosphate (HMP) and phosphoenol pyruvate (EMP)	Aromatic	Tryptophan Phenyl alanine Tyrosine

Thus, *C. glutamicum* is a versatile model system to design experiments related to over production of desired products or design eco-friendly strategies that are helpful in the degradation of aromatic compounds that cause environmental pollution.

1.1 Industrial Biotechnology and *C. glutamicum*

The fermentative production of useful products by microorganisms is known as industrial biotechnology. In modern times, the shape of industrial biotechnology has been changed and now it

acts as a platform to host other fields such as food industry, cosmetic industry and production of medicinal products, suggesting that the microorganisms are cell factories for the production of value-added products. Thus, their significance in white (industrial) biotechnology is well appreciated. This eventually enhanced industrial production of value-added products and also improved bio-based economy. It is believed that in near future industrial biotechnology might be recognized as an alternative to petro-based economy (Zahoor et al., 2012; Yang and Yang, 2017).



In 1950s, in search of natural L-glutamic acid producers, *C. glutamicum* was first isolated from the soil of Japan (Kinoshita et al., 1957; 1958). Since its discovery from the soil of Japan, several scientists did many experiments and concluded that the application of *C. glutamicum* for the fermentative production of amino acids as safe. The application of *C. glutamicum* for the production of various value-added products was shown in Table 2 and Figure 2.

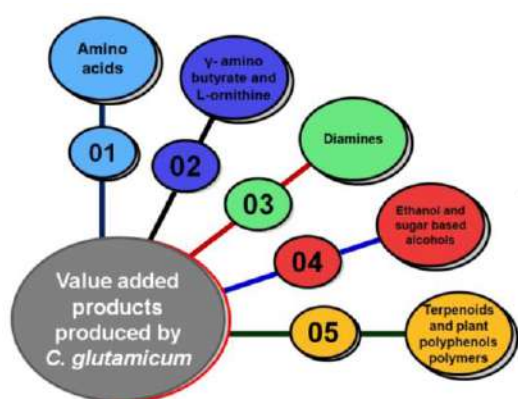


Figure 2: Value-added products produced by *C. glutamicum*

Amino acids are building blocks of proteins and thus they are essential for health. In addition, they are widely used in food commodities and preparation of medicines (Sanchez et al., 2017). According to the estimates, the production of amino acids in the year 2020 reached a volume of approximately 10 million tons and is equivalent to US\$ 21 Billion. Amino acids such as L-arginine (Park et al., 2014) and L-tryptophan (Bampidis et al., 2019), L-valine (Blombach et al., 2008), L-isoleucine (Kelle et al., 1996), L-serine (Stolz et al., 2007), L-methionine (Park et al., 2007) are produced from *C. glutamicum*. The other non-amino acid

compounds produced from *C. glutamicum* are a) non-proteinogenic derivatives including gamma amino butyrate (Shi and Li, 2011; Zhao et al., 2012), and L-ornithine (Schneider et al., 2011); b) diamines including putrescine (Schneider and Wendisch, 2010; Schneider et al., 2012), and cadaverine (Mimitsuka et al., 2007); c) organic acids such as succinic acid (Litsanov et al., 2012), pyruvic acid (Wieschalka et al., 2012), lactic acid (Okino et al., 2005), alpha ketoglutarate (Jo et al., 2012), 2-ketoisovaleric acid (Krause et al., 2010) and D-pantothenic acid (Chassagnole et al., 2003); d) alcohols such as ethanol (Inui et al., 2004; Sakai et al., 2007), isobutanol (Blombach et al., 2011) and 1,2-propanediol (Niimi et al., 2011); e) sugar based alcohols such as D-mannitol (Baumchen et al., 2009), xylitol (Sasaki et al., 2010), and cofactor regeneration (Siedler et al., 2013). In addition to the above, metabolic engineering of *C. glutamicum* led to the production of terpenoids (Henke et al., 2018; Henke and Wendisch, 2019), plant polyphenols (Kallscheuer et al., 2016), plant phenols (Milke et al., 2020), flavoring agents (Dikschat et al., 2010) and hyaluronic acid (Cheng et al., 2017; 2019). Glutamate family amino acids (GFAA; **Table 3 and 4**) comprises of the value-added products related to glutamate using metabolic engineering of *C. glutamicum* using genes related to the metabolic nodes: pyruvate-oxaloacetate and 2-ketoglutarate or alpha-ketoglutarate and many useful products like quercetin and resveratrol (antioxidants) are related to GFAA (Sheng et al., 2021). Based on the biotechnological applications of *C. glutamicum* in various food, pharma



and cosmetic industries, it is classically known as industrial workhorse (Baritugo et al., 2018).

1.2. L-glutamic acid and *C. glutamicum*

Amino acids are the simple organic compounds wherein α -carbon is connected to four functional groups: amino ($-\text{NH}_2$) terminal, carboxylic ($-\text{COOH}$) terminal, hydrogen ($-\text{H}$) and side chain ($-\text{R}$) and depending on the spatial arrangements of these four groups around the α -carbon, two stereoisomers such as L form known as levorotatory and D-form known as dextro-rotatory exist. Both L and D forms of amino acids are acknowledged for their roles. For example, L-amino acids are essential for the peptide bonds as they act as building blocks of proteins and play vital roles in the physiological activities in the form of enzymes, receptors and gene regulators etc in animal, plant and bacterial kingdoms. L-amino acids are major biological constituents which are used in food industries, feed supplements, agro-based chemicals, therapeutic compounds, infusion compounds and also used in the synthesis of peptides (Shyamkumar et al., 2014; Wei et al., 2019; Yang and Yang, 2017; Yang et al., 2020; Wolf et al., 2021).

L-glutamic acid is one of the aliphatic amino acids which are present in a wide array of foods. Among the amino acids, it is used in the form of sodium salts in various food products as additives and flavor enhancers. It was the first amino acid that was commercially produced in the year 1866 by the German Chemist, Karl Heinrich Leopold Ritt-hausen (Kurihara, 2009). There are two basic routes of L-glutamic acid production: chemical method (Brinbaum and Demain, 1969) and

biological method (Herman, 2003). The chemical synthesis of L-glutamic acid is often associated with the formation of racemic mixtures and hence, biological method using *C. glutamicum* is preferred (Yoshioka et al., 1999).

1.2.1. Production of L-glutamic acid

The first commercial production of glutamic acid was done in the year 1908 by Kikunae Ikeda. His experiments led to the compound with 'umami taste product' from the kelp, *Laminaria japonica* using wheat gluten (Kurihara, 2009). This seasoning product was patented and commercialized under the trade name, Aji-No-Moto. Almost forty seven years later, another scientist Kinoshita and his co-workers in the year 1957 made an attempt to identify novel bacterium that could convert non-protein raw materials into glutamic acid using fermentation technology. Fermentation is a process wherein selected microorganisms are used in the culture to produce large amounts of specific amino acid. The selected microbe when cultured in the presence of carbohydrates and ammonia, it releases L-amino acids into the culture medium. The production of L-amino acids is a consequence of reductive ammonia fixation wherein 2-oxoglutarate is catalyzed by glutamate dehydrogenase into glutamate. Kinoshita et al. (1959) found that the identified organism produced an amount of 10.3g/l glutamic acid into the medium using fermentation method. Based on the results obtained from the studies of Kinoshita (1959), several approaches have been made to identify glutamic acid producers such as *C. glutamicum*, *Brevibacterium lactofermentum*, and *B. flavum* (Sano,



2009). One of the major advantages of using *C. glutamicum* in the production of L-glutamic acid over chemical methods seems to be enantiopure form of L-glutamic acid, reduction in the production costs, and eco-friendly approach (Sano, 2009). Today, *C. glutamicum* is an important industrial amino acid producer of L-glutamate a flavor enhancer (Becker and Wittmann, 2020).

Scientists also performed experiments to investigate the conditions that can trigger glutamate over production and secretion. Studies of Shiio et al. (1962) suggested that the *C. glutamicum* over produce L-glutamate under biotin limitations. These studies have shown that the accumulation of glutamate occurs in the medium under biotin limitation only. This factor seems to be one of the obstacles for the usage of sugar molasses which contain biotin. To overcome this problem, several methods were discovered. Studies of Duperray et al. (1992) and Nunheimer et al. (1970) indicated that the addition of detergents like Tween-40 and Tween 60 and addition of beta lactum antibiotics such as Pencillin G also trigger L-glutamate production from *C. glutamicum*. The other compounds that influence the production of L-glutamate from *C. glutamicum* include ethambutol, (an inhibitor of cell wall synthesis), temperature, and glycerol or fatty acid auxotrophy (Kimura et al., 1997; Delaunay et al., 1999; Radmacher et al., 2005).

The biological production of L-glutamic acid occurs via sub-merged

fermentation of glucose, which is one of the major carbon sources. The other substrates utilized by *C. glutamicum* for the submerged fermentation of L-glutamic acid include palm waste hydrolysate (Das et al., 1995), cassava starch (Jyothi et al., 2005), sugarcane baggase (Amin et al., 2007) and date waste (Tavakkoli et al., 2012). With respect to the secretory system, studies of Nakamura et al. (2007) have shown that the transportation of glutamate occurs via a transporter known as YggB (Cg1434). One of the important points to be considered towards the over production of glutamate seems to be reduced activity of the TCA cycle enzyme, 2-oxoglutarate dehydrogenase complex (ODHC) (Shimizu et al., 2003; Shirai et al., 2007; Kataoka et al., 2006).

The available genome sequence led to the identification of metabolic maps. Therefore, now it is possible to manipulate genes associated with metabolism or transcriptional regulators. Such approach is known as metabolic engineering. This approach is now widely been used to develop *C. glutamicum* strains that can able to overproduce and secrete L-glutamate into the culture medium. For example, metabolic engineering of gene glutaminase (*Glsk*) in *C. glutamicum* led to faster growth and also overproduction of glutamate into the culture medium (Buerger et al., 2016).



Table 2: Fermentative production of value added products by *C. glutamicum* using metabolic engineering

Group	Substrate	Product	References
Polyphenols and related natural products	Glucose	6-methylsalicylate	Kallscheuer et al., 2019a
		Noreugenin	Milke et al., 2019
	<i>p</i> -coumaric acid	Naringenin	Kallscheuer et al., 2017
		Dihydrokaempferol	Kallscheuer et al., 2017
		Kaempferol	Kallscheuer et al., 2017
		Resveratrol	Kallscheuer et al., 2016; Kallscheuer et al., 2017
		Mono-O-methylated pinostillbene	Kallscheuer et al., 2017
		Di-O-methylated pinostillbene	Kallscheuer et al., 2017
		Raspberry ketone	Milke et al., 2020
	Cinnamic acid	Benzylacetone	Milke et al., 2020
		Pinosylvin	Kallscheuer et al., 2016
	Caffieic acid	Eriodictyol	Kallscheuer et al., 2017
		Dihydroquercetin	Kallscheuer et al., 2017
		Quercetin	Kallscheuer et al., 2017
		Piceatennol	Kallscheuer et al., 2016
	Ferulic acid	Zingerone	Milke et al., 2020
	Catechin	Cyanidin 3-O-glucoside	Zha et al., 2018
	Tyrosol	Salidroside	Kallscheuer et al., 2019b



Table 2: Fermentative production of value added products by *C. glutamicum* using metabolic engineering (continued)

Group	Substrate	Product	References
Terpenoids	Glucose	Astaxanthin	Henke et al., 2020
		Patchoulol	Henke et al., 2018
		CoQ10	Burgardt et al., 2021
Branched chain and non-proteinogenic amino acids	Glucose	L-valine	Hasegawa et al., 2012
		L-leucine	Huang et al., 2017
		L-isoleucine	Ma et al., 2016
		γ -aminobutyrate	Zhang et al., 2014
Extremolytes	Sugar and Molasses	Ectone	Gießelmann et al., 2019
	Glucose	Hydroxyectone	Becker et al., 2013
	Sucrose	α -glucosylglycerol	Ronneke et al., 2018
	Glucose and sucrose	L-pipicolic acid	Perez-Garcia et al., 2017

1.2.2. Biosynthesis of L-glutamate

The TCA cycle intermediate, 2-oxoglutarate is a common substrate for both oxoglutarate dehydrogenase complex (ODHC) and glutamate dehydrogenase, suggesting that 2-oxoglutarate marks an important branch point between amino acid production, L-glutamate and energy metabolism (TCA cycle). ODHC is a multimeric enzyme complex which comprises of three components: the first component is E1 (*odhA* gene, cg1280) which codes for OdhA protein, oxoglutarate dehydrogenase activity, the second component is E2 (*AceF*, cg2421), which codes for dihydrolipoamide acetyltransferase activity and the final component is E3 (*LpdA*, cg0790), which

codes for dihydrolipoamide dehydrogenase (Schwinde et al., 2001; Ikeda and Nakagawa, 2003; Kalinowski et al., 2003). This complex catalyzes the reaction wherein 2-oxoglutarate is converted into succinyl-CoA and NADH + H⁺ in the presence of NAD⁺ and coenzyme A, with the liberation of CO₂ (Usuda et al., 1996). In addition, reductive amination of 2-oxoglutarate to glutamate also occurs via glutamate dehydrogenase (*Gdh*, cg2280) activity, which takes place under nitrogen excess conditions (Börmann et al., 1992). Whereas under nitrogen limitation, glutamate is converted to glutamine via catalysis of glutamine synthetase (*GlnA*: cg2429) and glutamate-2-oxoglutarate aminotransferase (*gltBD*, cg0229, and



cg0230) for the assimilation of ammonium (Jakoby et al., 1997).

1.2.3. Regulation of L-glutamate levels

In general, the affinity of ODHC towards the 2-oxoglutarate is high as compared to the affinity between 2-oxoglutarate and glutamate dehydrogenase and thus, inhibition of ODHC seems to be an important step towards the increased metabolic flux of 2-oxoglutarate into glutamate (Bott, 2007). Though, the exact mechanism is unclear, studies have indicated that multilevel regulatory system promotes inhibition of ODHC activity and increased flux of 2-oxoglutarate to glutamate biosynthesis (Niebisch et al., 2006; Schultz et al., 2007; 2009; Krawczyk et al., 2010).

The regulatory mechanism of inhibition of ODHC complex underlies two aspects: phosphorylated and unphosphorylated form of a 15 KDa protein known as odhI protein (encoded by Cg1630). In phosphorylated form, OdhI is inactive and thus, it cannot able to bind ODHC and inhibits its activity. On the other hand, in unphosphorylated form OdhI becomes active and inhibits the activity of ODHC.

However, the switching of phosphorylated to unphosphorylated form of OdhI depends on multiple signaling cascades: 1) the first signal seems to be an interaction between L-glutamine and membrane bound lipoprotein (encoded by *GlnH*, Cg3045), followed by its transmission to a transmembrane protein known as GlnX (encoded by Cg3044) comprised of second signal. The signal from GlnX with its N- and C-terminal ends located in the cytoplasm, transmits signal

and activate a cytoplasmic membrane protein known as PknG, serine/threonine protein kinase (encoded by Cg3046), comprise of third step, 4) this activation led to its dissociation from the cytoplasmic membrane and with its kinase activity phosphorylate OdhI protein, 5) finally, OdhI protein in its phosphorylated form becomes inactive and cannot able to inhibit the E1 component of ODHC complex, thus, TCA cycle towards succinyl-CoA occurs. However, when signaling cascade of proteins are absent in the system, OdhI unphosphorylation takes place in the presence of an enzyme known as phosphoserine/threonine protein phosphatase (encoded by Cg0062) and thus, OdhI becomes active and can able to bind ODHC complex leading to its inhibition. Consequently, metabolic flux of 2-oxoglutarate towards glutamate occurs (Schultz et al., 2007; 2009; Krawczyk et al., 2010).

1.2.3. Metabolic engineering to overproduce L-glutamate

The production of glutamic acid from *C. glutamicum* depends on several factors such as external agents, and temperature. It has been shown that the culturing of *C. glutamicum* under conditions like biotin limitation, addition of penicillin, and Tween 40 showed increased channeling of L-glutamic acid into the culture media (Gopinath and Nampoothiri, 2014). It has also been shown that excessive biotin supplementation (eg: lignocelluloses biomass) did not show accumulation of glutamic acid (Jonsson et al., 2013). There appears to be two aspects in relation to excess biotin in the media, strengthen the cell wall which eventually



reduces the permeability for the secretion of glutamic acid and as the biotin favours TCA cycle, the metabolic flux i.e. 2-oxoglutarate for glutamic acid biosynthesis is reduced. To overcome biotin-induced lowered production of glutamic acid, scientists employed techniques like use of antibiotics, such as β -lactam antibiotics (pencillin), ethambutol (antibiotic used to treat tuberculosis) and surfactants like Tween 40 (Gopinath and Nampoothiri, 2014). Though promising, use of antibiotics negatively affects the cell growth also their excess usage leads to health and environmental concerns.

Interestingly, the method of temperature-induced glutamate secretion is preferred choice of over production of glutamate under biotin limitation (Kimura, 2005). The temperature induced production of glutamate includes two stages i.e. first cell growth is promoted at 32-33°C followed by production of glutamate at 39°C. However, the disadvantage associated with temperature triggered glutamate production depend the selection of mutants that resist higher temperatures and the mechanism is still unclear. Because of these factors, this application of this method is not progressed as expected (Gopinath and Nampoothiri, 2014). Based on the disadvantages associated with biotin limitation and temperature-triggered glutamic acid production, metabolic engineering approach is considered.

Metabolic engineering approach is well recognized strategy to produce desired products from microorganisms. Manipulation of genes i.e. introduction, deletion, or disruption is commonly used to get value added products from

microorganisms (Shi et al., 2019; Hirasawa et al., 2012). With the advent of available complete genome of *C. glutamicum*, this approach took a rapid pace in the production of value-added products including glutamic acid and its related amino acids (Sheng et al., 2021). Several genes such as *dtsR1* (Kimura et al., 1997), *odhA* (Nakamura et al., 2007), *murA*, *murB* (Shi et al., 2019), *itsA* (Hirasawa et al., 2001), *pyc* (Yao et al., 2009) and *pknG* (Schultz et al., 2007; Boulahya et al., 2010) were metabolically engineered in *C. glutamicum* to over produce glutamic acid. In recent times, an attempt to improve strains of *C. glutamicum* that utilize biomass for the over-production of L-glutamate using metabolic engineering approaches is gaining attention day-by-day (Hirasawa et al., 2012; Gopinath and Nampoothiri, 2014; Yokota et al., 2017; Wen et al., 2018; Sheng et al., 2021).

It was found that the reduction in the ODHC complex activity in order to over produce L-glutamic acid production, disruption (deletion of gene) or reduced (altering RBS sequence of gene) expression of *OdhA* gene in *C. glutamicum*. Though fruitful, this approach inhibits the TCA cycle thereby interferes with energy metabolism and cell growth. *DtsR1* is a homolog of beta subunit of biotin enzyme known as acetyl-CoA carboxylase enzyme. It is essential for fatty acid synthesis. Interestingly, biotin excess leads to over expression of *dtsR1* and stimulate ODHC activity thereby TCA cycle. This is further confirmed by the studies that the disruption of *dtsR1* gene leads to reduced ODHC activity and increased glutamate biosynthesis (Kimura et al., 1997; Yao et



al., 2009). This in turn reflects that the metabolic engineering of *ptsR1* leads to reduced activity of ODHC and thus metabolic flux reprogrammed towards glutamic acid production (Kimura et al., 2002; Chen et al., 2015).

Now-a-days, glutamate is used in the synthesis of glutamate family related amino acids (GFAA) compounds. It comprises of L-glutamate, L-proline L-citrulline, L-arginine, L-hydroxyproline, L-ornithine, 5-aminolevulinic acid and γ -aminobutyric acid. All these GFAs have wide array applications in food, animal feed, pharmaceutical and cosmetic industries. Currently, microbial fermentation of two *Corynebacterium* strains such as *C. glutamicum* and *C. crenatum* are used for the production of GFAA. In order to overproduce GFAA from these strains, metabolic engineering strategies are applied to these strains at two metabolic nodes such as pyruvate-oxaloacetate and 2-ketoglutarate. **Table 3** and **Table 4** show the production of different GFAA products using *C. glutamicum*. The information related to the GFAA products was reviewed by Sheng et al. (2021).

Metabolic engineering of GFAA production from *C. glutamicum* largely depends on the four steps: a) fast uptake, b) rapid transport, c) unobstructed metabolic flux and d) suitable configuration. Fast uptake is a process through which genes related to the uptake system are metabolically engineered so that the utilization of carbon sources and nitrogen sources which eventually metabolize into products that convert these sources into precursors for the production of value-

added products. Rapid uptake is to manipulate the genes that are not only useful for rapid transport of desired products from intracellular system to extracellular system but also useful to prevent degradation and/or re-absorption pathways that hinder the export of desired product. Unobstructed flux is a strategy through which the metabolic flux is redirected to biosynthesis of desired products via controlling genes at the metabolic nodes, and regulation of genes at the level of post-transcription (**Table 3 and 4**). Suitable configuration is an approach through which the *C. glutamicum* is metabolically engineered in such way that the GFAA biosynthesis pathways are introduced.

1.3. Bioremediation and *C. glutamicum*

Bioremediation is a process of removal of toxic compounds using microorganisms. This area can be studied at different levels such as ecological, biochemical and genetic levels using different tools of biology. Further, recent developments in rDNA technology, the boundaries of bioremediation are pushed wherein metabolic engineering techniques can be applied to develop microbial strains which can able to degrade diverse aromatic substances such as benzoate, phenol, 3-hydrobenzoate, protocatechuate, 4-hydroxybenzoate, 3,5-dihydroxytoluene, 4-cresol, resorcinol, benzyl alcohol, 2,4-dihydroxybenzoate, naphthalene, vanillin, ferulic acid (Shen et al., 2004; Shen et al., 2005a; 2005b; Shen and Liu, 2005; Merkens et al., 2005; Huang et al., 2006; Qi et al., 2007; Lee et al., 2010a, 2010b and 2010c). Interestingly, *C. glutamicum* utilize these diverse aromatic compounds as



energy sources via central carbon metabolism (**Table 5**; Shen et al., 2012). The ability of *C. glutamicum* to utilize the aromatic compounds aerobically mediate several peripheral pathways and converge to some common intermediate products such as catechol, protocatechuate, hydroxyquinol, genistin, and phenylacetyl-CoA (Diaz and Prieto, 2000; Díaz 2004; Cao et al. 2009; Fuchs et al. 2011). These compounds further enter into the energy cycle via central carbon metabolic pathways.

1.3.1. Catechol pathway

Catechol pathway includes the bioconversion of aromatic compounds into an intermediate catechol. The compounds such as phenol, benzyl alcohol and benzoate are utilized by *C. glutamicum* and metabolize them into catechol. Phenols and compounds related to phenol are waste by-products released as a consequence of manufacturing agricultural and industrial products. When they released into the environment, they become toxic to the environment and humans as well (Michałowicz and Duda, 2007).

Hence, there is a requirement to clean up from the environment. If untreated, it perturbs biogeochemical cycles thereby alter harmony of ecosystem equilibrium. Due to its potential harmful effects, phenol is placed on the list of priority contaminants. Biological approaches using microorganisms to remove phenol are eco-friendly in nature and also economically feasible and hence, well appreciated. *C. glutamicum* has been reported to utilize phenol as an energy source (Lee et al., 2010a; 2010b; 2010c; Shen et al., 2012). Studies of Lee et al.

(2010a) suggested that the bioconversion of phenol lead to the metabolites that are associated with TCA cycle and also amino acid production. Studies of Lee et al. (2010b) have shown that the conversion of phenol into glutamate by *C. glutamicum* ATCC 13032 comprised of transcriptional regulation of glutamate biosynthetic genes including glutamate dehydrogenase. Studies of Lee et al. (2010c) developed a nano-based system wherein phenol degrading *C. glutamicum* was immobilized on Fe₃O₄ nanoparticles. The results of their studies indicated the following: a) to enhance the capacity of Fe₃O₄ nanoparticles to adsorb the cell surface of *C. glutamicum*, the nanoparticles were encapsulated with silica and functionalized with amino groups; b) the functionalized and encapsulated nanoparticles showed strong adsorption capacity and did not affect the growth characteristics of bacterium; c) the immobilized bacterium exhibited a phenol degradation efficacy wherein it tolerated 50 ppm phenol in culture broth over a period of 8 days at 30 °C and d) this nano-based system coated bacterium can be easily separated using magnetic approaches and most strikingly, this nano-based system could be reused to degrade phenol.

Studies have shown that the bioconversion of benzoate to catechol occurs via a two-step mechanism (Shen et al., 2012): the first step catalyzes the oxidation of benzoate to benzoate diol by an enzyme benzoate dehydrogenase and the second step comprises of decarboxylation of benzoate diol to catechol catalyzed by 2-hydroxy-1,2-dihydroxybenzoate dehydrogenase. Genes coding for these conserved (Jiménez et al. 2002).



Table 3: Utilization of glucose for the production of various L-glutamate family amino acids (GFAA) by *C. glutamicum* using shake-flask, batch fermentation method

GFAA product	Gene manipulation	Reference
Ornithine	Manipulation at the level of <i>odhA</i> gene. Replaced start codon ATG; and natural RBS replaced with synthetic RBS with a strength of 837 au ^{\$} Manipulation at the level of <i>gdh2</i> gene ^{\$} Insertion of strong promoter upstream, <i>P_{eftu}</i> to the gene Manipulation of <i>gltA</i> gene ^{\$} Insertion of strong promoter upstream to the <i>gltA</i> gene for its over-expression*	Zhang et al., 2017; Zhang et al., 2019
GABA	Manipulation at the level of <i>odhA</i> gene. Replaced start codon GTG with TTG and removal of phosphorylation motif site of ODHC inhibitor protein, <i>OdhI</i> ^{\$}	Jorge et al., 2017
Proline	Manipulation at the level of <i>gdh</i> gene; Deletion of <i>avtA</i> gene*	Zhang et al., 2020
Glutamate	Deletion of <i>ldhA</i> gene*	Zhang et al., 2014
Arginine	Manipulation at the level of <i>lysC</i> gene; Replaced natural RBS with synthetic RBS sequence with a strength of 30 au*	Man et al., 2016
5-ALA	Manipulation at the level of <i>ppc</i> and <i>pyk</i> genes. Over-expression of <i>ppc</i> gene through insertion of a strong <i>P_{sod}</i> promoter in the upstream region and deletion of <i>pyk</i> gene*	Engels et al., 2008

*indicates the metabolic engineering of genes at the level of pyruvate-oxaloacetate metabolic node in *C. glutamicum*

^{\$} indicates the metabolic engineering genes at the level of alpha-ketoglutarate metabolic node in *C. glutamicum*

Table 4: Utilization of glucose for the production of various L-glutamate family amino acids (GFAA) by *C. glutamicum* using bioreactor, Fed batch fermentation method

GFAA product	Gene manipulation	Reference
Proline	Suppression of <i>odhA</i> gene by inducing mutation in the RBS sequence ^{\$}	Zhang et al., 2020
Arginine	Manipulation at the level of <i>pyc</i> gene (start codon GTG replaced with ATG) and insertion of copy of <i>gltA</i> gene on chromosome * co-expression of <i>glnA</i> , <i>aspA</i> and <i>gdh</i> genes ^{\$} overexpression of <i>icd</i> and <i>gdh</i> genes; Suppression of <i>odhA</i> by replacing natural RBS with synthetic RBS with a strength of 800 au ^{\$}	Man et al., 2016; Guo et al., 2017 Zhan et al., 2019

*indicates the metabolic engineering of genes at the level of pyruvate-oxaloacetate metabolic node in *C. glutamicum* ^{\$} indicates the metabolic engineering genes at the level of alpha-ketoglutarate metabolic node in *C. glutamicum*



Table 5: Pathways related to the utilization of various aromatic compounds by *C. glutamicum*

Pathway	Type of aromatic compound	Primary product of pathway	Reference
Catechol pathway	Phenol, benzylalcohol and benzoate	Catechol	Jimenez et al., 2002; Lee et al., 2010a;2010b; 2010c
Hydroxyquinol pathway	Polychloro- (chlorophenol, 2,4,6-trichlorophenol, dibenzo-p-dioxin, 4-aminophenol) and nitro-(4-nitrocatechol, 4-nitrophenol) aromatic compounds	Hydroxyquinol	Takenaka et al., 2003; Kitagawa et al., 2004; Nordin et al., 2005; Shen et al., 2005a; Huang et al., 2006
Ketoadipate pathway	Catechol and protocatechuate branch compounds	β ketoadipate	Shen and Liu, 2005 Davis and Sello, 2010
Protocatechuate pathway	Phenylprenoids (vanillin, vanillate, ferulate) 4-cresol	Protocatechuate	Shen and Liu, 2005; Qi et al., 2007
Gentisate pathway	3-hydroxybenzoate, naphthalene	Gentisate	Shen et al., 2005a; Lee et al., 2010b; Yang et al., 2010
Phenyl acetyl-CoA ring cleavage pathway	Phenylacetate	Phenyl acetyl-CoA	Ferrandez et al., 1998 Teufel et al., 2010

1.3.2. Protocatechuate pathway

Aromatic compounds such as phenyl propenoids mediate protocatechuate peripheral pathway wherein the intermediate compound is protocatechuate. The bioconversion of phenyl propenoids like vanillin, vanillate, and ferulate into protocatechuate was functionally demonstrated in *C. glutamicum*. The enzymes involved in the bioconversion of these aromatic compounds were identified in *C. glutamicum*. Genes encoding vanillin dehydrogenase (which is believed to convert vanillin to protocatechuate) was identified in the genome of *C. glutamicum* but not experimentally authenticated.

Vanillate demethylase catalyzes the conversion of vanillin to protocatechuate was functionally confirmed in *C. glutamicum* (Merkens et al., 2005). On the other hand, ferulate related enzymes such as feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase were identified in the genome sequence of *C. glutamicum* and it has been shown that these enzymes exhibit homology to that of enzymes related to metabolism of ferulate in *Pseudomonas* species (Jiménez et al. 2002).

4-cresol is another aromatic compound which mediate protocatechuate pathway. 4-cresol is a phenol related compound and contributes to the surface



and groundwater pollution (Said et al., 2021). It causes various adverse effects including respiratory problems, neurological problems, degeneration of cardiac muscle, liver and kidney problems (ATSDR, 2008). Studies of Peters et al. (2007), Johannes et al. (2008), and Carmona et al. (2009) indicated that the metabolism of 4-cresol to 4-hydroxybenzoate was catalyzed by 4-cresol methylhydroxylase and NAD-dependent 4-hydroxybenzylalcohol dehydrogenase in *Geobacter metallireducens*. Though, metabolism of 4-cresol into 4-hydroxybenzoate was observed in *C. glutamicum*, enzymes associated with this bioconversion was not identified (Shen and Liu 2005). Proteomic studies of Qi et al. (2007) indicated that two proteins related to enzymes viz., reductases and dehydrogenases were induced specifically when *C. glutamicum* was cultured on 4-cresol. Studies of Li et al. (2014) and Du et al. (2016) demonstrated the biodegradation ability of *C. glutamicum* against 4-cresol, a synthetic compound required for various aromatic compounds. These authors suggested that the biodegradation of cresol occurs via unique pathway mediating phosphorylated intermediates. The cascade of biodegradation of cresol includes phosphorylation of hydroxyl group of cresol catalyzed by 4-methylbenzyl phosphate synthase, followed by the recognition and oxidation of phosphorylated hydroxyl group of cresol, catalyzed by class I P450 system. These events lead to the formation of oxidation of aromatic methyl groups into –COOH groups comprising of alcohol and aldehyde intermediates and enzymes like alcohol

dehydrogenase and aldehyde dehydrogenase were also reported in *C. glutamicum* (Du et al., 2016).

Studies of Huang et al. (2008) have shown that the conversion of 4-hydroxybenzoate into protocatechuate in *C. glutamicum* was catalyzed by 4-hydroxybenzoate 3-hydroxylase (HBHase) using NADPH as a cofactor instead of NADH as cofactor used by other bacterial HBHase. It was shown that the enzymes (quininate/shikimate dehydrogenase, dehydroquininate dehydratase, dehydroshikimate dehydratase) were phylogenetically related to fungal enzymes but not to its respective family members (Elsemore and Ornston 1994; Teramoto et al. 2009).

1.3.3. Gentisate pathway

Gentisate pathway comprises of formation of gentisate from 3-hydroxybenzoate, naphthalene and catalyzed by an enzyme maleylpyruvate isomerase (MPI). It has been shown that in gram negative bacteria, MPI depends on glutathione (GSH) during degradation of aromatic compounds via gentisate pathway (Zhou et al., 2001). The assimilation of aromatic compounds through gentisate pathway was also discovered in *C. glutamicum* and gene related to MPI was also identified in the genome of *C. glutamicum* (Shen et al. 2005a; Lee et al. 2010b; Yang et al. 2010). Experiments revealed that MPI in gram positive bacteria (*Mycobacterium*, *Streptomyces*, *Rhodococcus*, and *Corynebacterium*) were different from that of MPI in gram negative bacteria in that the later depends on GSH, while the former MPI in gram positive depends on mycothiol (MSH) (Zhou et al.



2001; Newton et al. 2008; Jothivasan and Hamilton 2008). It has been indicated that *C. glutamicum* mutants lacking MPI did not grow on media containing 3-hydroxybenzoate but did grow on phenol suggesting the significant role of MPI (Shen et al., 2012). The crystal structure of mycothiol-dependent MPI was determined in *C. glutamicum* with a resolution of 1.75 Å. This structure revealed two factors: the C-terminal end contains a novel fold with alpha and beta sheets ($\alpha\beta\alpha\beta\alpha$ fold), while N-terminal end contains divalent Zinc-binding domain and suggested that the novel folding pattern at the C-terminal end is essential for the structural integrity of enzyme activity. Site directed mutagenesis also indicated that the amino acid residue Arg222 at the C-terminal domain was considered important for mycothiol-dependent MPI (Wang et al. 2007; Rawat and Av-Gay 2007).

Coupling metabolic engineering of microorganisms to enhance their degradation properties of toxic compounds is gaining popularity during the recent past (Shen et al., 2012). Gram negative and gram positive bacteria possess two important systems during detoxification mechanisms, glutathione mediated and mycothiol mediated systems. Mycothiol mediated detoxification systems include an enzyme known as mycothiol S-conjugate amidase, which catalyzes the compounds bound to mycothiol. This enzyme was first discovered in *Mycobacterium smegmatis*. Mycothiol mediated detoxification systems include two important steps: interaction of mycothiol to electrophilic compounds with its thiol moiety and recognition and cleavage of thiol-bound electrophilic

compounds by mycothiol S-conjugate amidase. Studies Si et al. (2014) demonstrated that mycothiol (MSH) and mycothiol S-conjugate amidase (MCA) system similar to *M. smegmatis* also exists in *C. glutamicum* RES167 using gene manipulation techniques. Using rDNA technology, two strains viz., strains which lack MCA system and strains complemented with MCA system were developed and assessed for their ability against alkylating agents (1-chloro-2,4-dinitrobenzene, N-ethylmaleimide, monobromobimane, iodoacetamide and methylglycol), antibiotics (lincomycin, spectinomycin, erythromycin and rifampin S), metals (cadmium, nickel, chromium and copper) and oxidative stress agents (hydrogen peroxide, dithiotretol, formaldehyde and diamide) using disk diffusion experiments. The results of Si et al. (2014) indicated that the strains of *C. glutamicum* mutants lacking MCA showed sensitivity against the selected alkylating agents, oxidative stress induced agents like hydrogen peroxide, formaldehyde and diamides, antibiotics such as rifamycin S and selected heavy metals as compared to wild strain, while strains complemented with MCA recovered against the tested compounds, suggesting that MCA-MSH system is crucial in detoxification mechanisms at least against the selected compounds.

1.3.4. Hydroxyquinol pathway

Some aromatic compounds such as polychloro- and nitroaromatic pollutants (chlorophenol, 2,4,6-trichlorophenol, dibenzo-p-dioxin, 4-aminophenol, 4-nitrocatechol, 4-nitrophenol (Takenaka et al. 2003; Kitagawa et al. 2004; Nordin et al.



2005) are assimilated by bacteria via hydroxyquinol pathway. Studies of Shen et al. (2005a) suggested that *C. glutamicum* assimilate aromatic compounds like 1,3-dihydroxybenzene/resorcinol, 2,4-dihydroxybenzoate; 3,5-dihydroxytoluene mediating hydroxyquinol pathway.

With the help of genome data mining, genes related to hydroxyquinol pathway were identified and biochemical studies have shown that hydroxyquinol 1,2-dioxygenases may be involved (Shen et al. 2005a; Huang et al. 2006).

1.3.5. β -ketoadipate pathway

β -ketoadipate pathway in *C. glutamicum* is believed to be one of the major pathways wherein lignin based aromatic compounds are assimilated (Davis and Sello, 2010). Most strikingly, the catechol branch and protocatechuate branch converge at the β -ketoadipate pathway in *C. glutamicum* through an intermediate, β -ketoadipate enol-lactone, followed by its conversion into Krebs cycle intermediates, acetyl-CoA and succinylCoA (Shen et al., 2012). Interestingly, *pca* genes (10 genes: *pcaHGB*, *pcaIJ*, and *pcaRFDO* with three independent transcriptional units) that encode enzymes related to the catalysis of protocatechuate to β -ketoadipate and single gene cluster (ncgl2317–ncgl2319) linked to assimilation of catechol β -ketoadipate pathway in *C. glutamicum* were identified (Shen and Liu 2005). Yet studies are going on to establish complete functional repertoire of genes related to β -ketoadipate pathway in *C. glutamicum*.

1.3.6. Phenylacetyl-CoA ring-cleavage pathway

The phenylacetyl-CoA ring-cleavage pathway is recognized as a unique pathway

wherein enzymes that are active only during anaerobic conditions are involved in the assimilation of aromatic compounds aerobically which is different from other reestablished pathways involved during assimilation of aromatic compounds (Teufel et al., 2010). It has been reported that phenylacetate-CoA ligase mediates the first step in the conversion of phenylacetate to phenylacetyl-CoA followed by the catalysis of its intermediates by CoA thioesters (which are actually active during anaerobic conditions rather than during aerobic conditions) (Ferrández et al. 1998; Teufel et al. 2010).

Interestingly, genes that encode amine oxidase and phenylacetaldehyde dehydrogenase related to the phenylacetate pathway were identified in the genome of *C. glutamicum* strain R (Shen et al., 2012), however, further experiments related to their functionality are awaited.

1.3.7. Arsenic and *C. glutamicum*

Apart from these pathways, the ability of *C. glutamicum* in the removal of arsenic is another interesting aspect of *C. glutamicum*-mediated bioremediation process. Arsenic is a metalloid and its accumulation causes deleterious effects in humans and wildlife. Arsenic contaminates soil, water and air and studies have shown that the contamination of drinking water by arsenic in West Bengal, India. The major routes for arsenic pollution include anthropogenic activities, smelting process, leaching process, burning of fossil fuels, mismanagement of medical and agricultural activities and gold mining industries. Thus, arsenic pollution is a serious ongoing problem all over the world (Sher and Rehman, 2019).



Interestingly, arsenic defense mechanisms have been developed by microbes due to ubiquitous nature of arsenic in the environment. At the molecular level, *C. glutamicum* genome comprises of two arsenic resistance operons which includes genes that code for metalloregulatory proteins, arsenite permeases and an enzyme involved in arsenate reduction i.e. arsenate reductases. These genes and their products are putatively involved in the biodegradation of arsenic.

To date, studies have shown that *C. glutamicum* resists 12 mM arsenite and 400 mM arsenate. Further, it has been shown that arsenate reductases are coupled to low molecular weight thiol mycothiol (MSH) and mycoredoxin-1 (Mrx-1) proteins. The combination of MSH and Mrx-1 acts as a MSH/Mrx-1 redox system and protect the cells against arsenic-oxidative stress induced damage (Mateos et al., 2017).

1.3.8. Biosorbent properties of *C. glutamicum*

Biosorbent properties of *C. glutamicum* were reported by (Vijayaraghavan and Yun, 2007). These authors suggested that *C. glutamicum* acts as an absorbent material to remove reactive black 5 (RB5) from aqueous medium. RB5 is most commonly used synthetic dye used in textile industry. It is water soluble dye and thus, there is a need to remove this dye during waste water treatments (Shah, 2014; Yaseen and Scholz, 2019). Studies of Vijayaraghavan and Yun, (2007) suggested that 0.1M HNO₃ enhances the biosorption capacity of *C. glutamicum* and enhances the RB5 uptake from the waste water. Interestingly, incorporation of 0.1M NaOH

solution promotes desorption of RB5 from the dye-loaded bacteria (Vijayaraghavan and Yun, 2007).

1.3.9. *In silico* analysis to identify genes in *C. glutamicum* linked to bioremediation of nitrile compounds

In silico analysis is one of the areas of interdisciplinary approach where there is a blend of computational strategies to address biological problems including the identification of genes with bioremediation properties. Nitrile compounds are cyanide-substituted organic compounds with wide array of applications in various industries (Ramteke et al., 2013). Benzonitrile and its derivatives are major constituents of herbicides such as 2, 6 dichlorobenzonitrile, 3, 5-dibromo- 4 hydroxybenzonitrile; 3, 5-dichloro-4-hydroxybenzonitrile and these compounds are extensively used in agriculture. The abnormal use of nitrile compounds in pharmaceutical, chemical, polymer and pesticide industries caused severe environmental pollution. For example, nitrilated herbicides pose serious threat to the environment and humans as well. Hence, there is a need to remove this pollutant from the environment (Kaul et al., 2007). Using *in silico* approach, studies of Amrutha and Nampoothiri (2022) have shown that the genome of *C. glutamicum* ATCC 13032 contains genes related to nitrilases i.e. carbon-nitrogen hydrolase (cg 3093) enzymes and further, using molecular docking studies they showed that these enzymes exhibit biodegradation.

To summate, genes related to the assimilation of aromatic compounds are arranged in several gene clusters in the genome of *C. glutamicum* and are tightly



regulated at the level of transcriptional regulation. It has been shown that at least DNA binding proteins like AraC (positive regulator of phenol degradation pathway i.e. catechol pathway), IclR (positively regulates gentisate pathway), LuxR (and IclR together regulate the utilization of benzoate, protocatechuate, 4-hydroxybenzoate via beta-ketoadipate pathway) and, PadR (regulation of bioconversion of vanillin, ferulate to protocatechuate branch of the beta-ketoadipate pathway), and TetR (negative regulator of hydroxyquinol pathway) which are involved in the transcriptional regulation of gene clusters related to aromatic compound assimilation. However, yet experiments are required to functionally establish the essential roles of specific enzymes in the biodegradation pathways (Brinkrolf et al., 2006; Shen et al., 2012).

1.4. *C. glutamicum* as a cell factory

C. glutamicum is used as a host system to produce value added compounds which are used in food, cosmetic and pharmaceutical industries (Huccetogullari et al., 2019). On the other hand, use of *C. glutamicum* also acts as a platform to utilize aromatic compounds like phenol, naphthalene, and 4-cresol that are harmful to the environment and humans into value added products (Shen et al., 2012). This aspect gained attention of scientific communities and the public. Thus, it seems apparent that *C. glutamicum* is a cell factory which is not only capable of assimilation of aromatic compounds and thus, offer a new platform for detoxification purposes, but can also utilize many aromatic compounds as sole carbon and energy sources.

1.4.1. Production of natural and non-natural aromatic compounds

Utilization of biological methods for the production of aromatic compounds minimizes the usage of petroleum-based compounds like benzene, toluene and xylene in various industries like food, cosmetic, pharmaceutical industries (Noda et al., 2017; Wu et al., 2018). With the rapid developments in the molecular and analytical techniques, microorganisms are metabolically engineered for the production of value added products, such as natural (eg: flavinoids) or non-natural (eg: cis-cis-muconic acid) aromatic compounds. The natural and non-natural aromatic compounds are broadly produced by engineered microorganisms through a) intermediates of shikimate pathway and b) aromatic amino acids like L-tryptophan, L-phenylalanine and L-tyrosine and their derivatives (Huccetogullari et al., 2019). With respect to the *C. glutamicum*, studies were designed to produce natural and non-natural aromatic compounds using metabolic engineering (Miyahisa et al., 2005; Hansen et al., 2009; Ikeda et al., 2011; Kallscheuer et al., 2016; Sun et al., 2016; Shin et al., 2018; Kitade et al., 2018; Kallscheuer and Marienhagen, 2018).

It is well known that phosphotransferase (PTS) is an enzyme which requires phosphoenolpyruvate (PEP) during the uptake of glucose in microorganisms. Studies of Ikeda et al. (2011) suggested that the uptake of glucose in *C. glutamicum* also occurs via myo-inositol transporter, a non-PTS system independent of PEP. However, non-PTS system is under the transcriptional regulation of *iolR* gene and it encodes a



repressor that inhibits the non-PTS system (Shin et al., 2018). Studies of Shin et al., (2018) suggested that the metabolic engineering of *C. glutamicum* ATCC 13032 wherein deletion of *iolR* gene led to the activation of non-PTS system thereby leading to the production of non-natural aromatic compound, cis-cis-muconic acid.

4-hydroxybenzoic acid (PHBA) a valuable aromatic compound used as a raw material for the production of liquid crystal polymers and paraben. It has been shown that *C. glutamicum* strain tolerant to chemical toxicity of 4-HBA was discovered, which is believed to be a major problem during microbial chemical production (Kitade et al., 2018). Studies of Kitade and co-workers (2018) have shown that over production of 4-HBA via shikimate pathway in *C. glutamicum* requires multiple steps: a) *aroG* which encode an enzyme 3-deoxy-d-arabinoheptulosonic acid 7-phosphate (DAHP) synthase from *Escherichia coli* was mutated and integrated into the chromosome of *C. glutamicum*, b) three genes which code for chorismate synthase, shikimate kinase, and 3-dehydroquinate synthase are involved in the over production of 4-HBA were expressed in *C. glutamicum* from wild type strains and c) expression of chorismate pyruvate-lyase (provides tolerance capacity against 4-HBA) from the intestinal bacterium *Providencia rustigianii* in *C. glutamicum* and d) to suppress the production of by-products like 1,3-dihydroxyacetone and pyruvate and to over produce 4-HBA, genes encoding haloacid dehalogenase superfamily phosphatase (*hdpA*) and pyruvate kinase (*pyk*) were also

deleted in *C. glutamicum*. Finally, these scientists successfully obtained a strain that was tolerant to high titres of 4-HBA and also over produce 4-HBA [(titre: 36.6 g/liter and yield: 41% (mol/mol)].

Studies of Kallscheuer and Marienhagen, (2018) demonstrated the over production of hydroxybenzoate through the deletion of aromatic catabolic pathways in metabolically engineered strain, *C. glutamicum* DelAro⁵ wherein, almost 27 genes which were organized in five gene clusters were deleted so as to abolish assimilation of aromatic compound via peripheral and central catabolic pathways. This engineered strain was used to produce aromatic compounds like protocatechuate (2.0 g/L), 2-hydroxybenzoate (0.01 g/L), 4-hydroxybenzoate (3.3 g/L) and 3-hydroxybenzoate (0.03 g/L) via shikimate pathway.

Studies of Kallscheuer et al. (2016) demonstrated that the *C. glutamicum* strains were metabolically engineered with codon-optimized and plant-derived genes such as chalcone synthase and chalcone isomerase, over production of naringenin (35 mg/L) and eridictyol (37 mg/mL) from polypropanoids like coumaric acid and caffeic acid, respectively. Further, these authors also suggested that the expression of genes coding for ligases (4-coumarate: CoA-ligases) and synthases (stilbene synthase) led to the production of pinosylvin, stilbens, and resveratrol from supplements like cinnamic acid, phenylpropanoids, and p-coumaric acid, respectively. Studies of Kubota et al. (2014) suggested that genetically engineered strain of *C. glutamicum* with *pab* genes (AB and C) produces para-aminobenzoate from



glucose in the culture medium and the genes *pabAB* was obtained from *C. callunae* and *pabC* gene from *Xenorhabdus bovienii*, respectively. *C. glutamicum* harboring plasmids with *pab* genes produced 314mM of PABA (titre: 43g/L and yield: 20%) via shikimate pathway under fermenter controlled conditions over a period of 48h. Violacein is well known for its efficacy as antimicrobial, anti-tumour and anti-oxidant activities and hence, grabbed attention in pharmaceutical industries. Engineered *C. glutamicum* strain expressing the *Chromobacterium violaceum* genes (operon: *vioABCDE*) related to the production of violacein, resulted in the production of violacein (5.436 g/L) from glucose in fed-batch process (Sun et al., 2016).

1.4.2. Degradation of harmful environmental aromatic compounds into value-added products

The major goal of this area is to manage environmental pollution of aromatic compounds at minimum level and also to convert them into useful products (Shen et al., 2012). Few efforts have been made in this area with respect to *C. glutamicum*. Studies of Lee et al. (2010b) reported that the assimilation of phenol and naphthalene by *C. glutamicum* yielded amino acids such as L-glutamate and proline suggesting that the reprogramming of metabolic flux could be a possible reason for the production of amino acids from toxic aromatic compounds. However, there are many gaps or challenges that need to be filled up in order to get a complete picture of bridging industrial biotechnology and bioremediation. This is because of the

following reasons: a) the first step is to identify microbial strains which can utilize aromatic compounds as energy sources, b) second step is to study the ability of microbe to exert dual activities such as fermentative production of value-added products and bioremediation properties, b) optimization of physico-chemical properties such as pH, temperature, time and concentration of substrate involves third step, c) fourth step addresses the need for understanding the metabolic fate in microbes i.e. assessing the biochemical events associated with the metabolism of selected substrate, d) fifth step comprises of understanding the molecular events i.e. regulation of different genes involved during fermentative production and bioremediation aspects, and e) final step deals with the reprogramming of metabolic flux linking aromatic compound degradation and production of value-added products. Deciphering these complex steps is of paramount in linking areas such as industrial biotechnology and bioremediation (Brinkrolf et al., 2006; Shen et al., 2012).

To summate, *C. glutamicum* ATCC 13032 is well appreciated strain for its production of L-glutamic acid and L-lysine. The added advantage of this organism is availability of its complete genome. It comprises of 3282708 base pairs with almost 3002 protein coding genes and based on the homology to other known proteins, the functions of 2489 genes out of 3002 protein coding genes were assigned. Several studies exploited the genome sequence for metabolic engineering approaches on *C. glutamicum* ATCC 13032 to produce desired value-added compounds



including glutamic acid. studies related to the production of aromatic compounds (Huccetogullari et al., 2019) and studies related to the degradation of aromatic compounds (Shen et al., 2012) are well studied. Thus, *C. glutamicum* is used as a cell factory not only to utilize toxic aromatic compounds but also to produce aromatic value-added products.

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BHAI-MS-4

Identification of putative drug targets in *Morexalla catarrhalis* using subtractive proteomicsM. Mounika¹ and S.B. Sainath^{1*}**Abstract**

Now a days, the emergence of drug resistance developed by bacteria is one of the major concerns. This eventually caused a high demand to prioritize an effective drug target. In these days, the reverse vaccinology strategies to identify a drug target is well acknowledged. Subtractive proteomics is one of the reverse vaccinology strategies to identify the drug targets in bacteria. In the current study, various *in silico* tools subjected to subtractive proteomics pipeline was used to identify the drug targets in *Moraxella catarrhalis*, a gram-negative bacterium that causes respiratory tract and ear infection in infants and adults. Almost, 52 potent drug targets were identified in this study and eventually, one protein was identified as a potential new drug target and forwarded to the structure-based studies i.e. nitrate reductase. Furthermore, virtual screening of 500 compounds from the ZINC database was performed against the nitrate reductase and based on the binding energies and ADMET analysis, three compounds were shortlisted. Nitrate reductase could be one of the potential drug targets identified in *M. catarrhalis*. However, further *in vivo* experiments were warranted.

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Introduction

Moraxella catarrhalis is a human-restricted commensal and has evolved from an emerging to a well-established pathogen and studies have shown that this organism has become one the major pathogens of upper and lower respiratory tract infections in humans. In children, it causes about 17% of acute otitis media infections in children (Verduin et al., 2002), sinusitis, bacteremia, otitis media, pneumonia, tracheitis, bronchitis, and, less commonly, ocular infections in children. Whereas in adults, it causes laryngitis, bronchitis, pneumonia, nosocomial infections and endocarditis. Furthermore, *M. catarrhalis* was found to cause other types of infections, such as bacteremia, endocarditis and meningitis, especially in immunocompromised patients (Tan et al., 2002; Smidt et al., 2013). Hence, this organism is considered to be one of the emerging pathogens associated with respiratory tract infections (Verhaegh et al., 2008).

In general, the classical antibiotic treatment reduces the clinical burden. However, improper regimen and unrestricted antibiotic use is one of the underlying mechanisms of the development of antibiotic-resistant bacteria (Ashraf et al., 2021). This eventually reduced the viable antibiotic options. One of the cogent examples of the development of antibiotic resistance by *M. catarrhalis* against the amoxycillin. On the other hand, studies have shown that the Chronic Obstructive Pulmonary Disease (COPD) and other respiratory diseases caused by *M. catarrhalis* are difficult to treat because of the rising MICs and antimicrobial drug resistance. Though

M. catarrhalis was thought to be a harmless commensal, the hospital outbreaks of this organism related respiratory diseases indicated that there may be shift from commensal to nosocomial pathogen. However, with the advanced molecular and analytical techniques, the pathogenic and virulence factors related to this shift (commensal to pathogenic) of *M. catarrhalis* came to limelight.

Published reports (de Vries et al., 2009; Parameswaran et al., 2009; Augustniak et al., 2011) have shown that the mechanisms of pathogenesis of *M. catarrhalis* may include a) evasion of immune surveillance such as complement system, residing in the lymphoid tissue and activation of non-specific B cells to produce polyclonal antibodies and redirecting adaptive immunity, b) biofilm formation; and c) interference at the level of protease-antiprotease balance. Other studies also suggested that the virulence factors such as periplasmic and outer membrane components released from the outer membrane vesicles to the host also favor colonization of this bacteria in the host system.

Since antibiotic resistance has been one of the major problems these days, development of antimicrobial drugs with bactericidal activities is one of the emerging areas of biomedicine. To accomplish this task, identification of suitable drug targets is a fundamental step. However, conventional studies to identify such potential drug targets is often associated with economic constraints and also not time bound. Hence, alternative strategies which can able to identify drug targets is urgently need. With the advancements in the



molecular and genetic engineering principles, genome architecture and proteome of many pathogenic organisms are known. Thus, comparative and reverse vaccinology strategies or subtractive proteomic analysis proved to be exigent job in the identification of drug targets and design of new drugs against the intended drug targets of pathogens. The major advantage of this approach is cost-effectiveness and opened avenues to unlock the new pathways for finding vaccine or drug candidates. The other advantage of this systems biology approach sped-up the drug discovery process and also useful to screen many targets at a time against the selected drug targets and also reduced the failure rate in clinical trial process. Using this comparative and subtractive genomic approach, so far, many drugs have been prioritized and identified therapeutic targets for harmful pathogens like *Clostridium botulinum*, *Mycoplasma pneumoniae*, *Rickettsia*, *Neisseria gonorrhoeae*, *Salmonella typhi*, and *Shigella dysenteriae* (Sudha et al., 2018; Vilela Rodrigues et al., 2019; Tanwer et al., 2020)

The major goal of this study was to identify the drug target(s) in *Moraxella catarrhalis* BBH18 and therapeutic candidates against this pathogen. To address this aspect, systems biology approach was used wherein the genome of *M. catarrhalis* was explored. In this study, comparative and subtractive genomics analysis approach, molecular docking analysis, Protein-Protein Interaction (PPI) network analysis, essentiality, drug ability of target proteins and ADMET properties

were used to identify drug targets and therapeutic candidates.

Materials and Methods

The approach of systems biology approach was employed for the drug target prioritization against the *M. catarrhalis* which holds clinical and biological importance. In this study, BBH18 strain of *M. catarrhalis* was selected and potent drug targets were identified. The flow chart represent the pipeline of in silico analysis performed in this study for the identification of drug targets and also selection of appropriate therapeutic candidate (Figure 1). The following methodology was performed to accomplish this task.

- a) The proteome of *M. catarrhalis* BBH18 strain (genome size: 1.9 Mb; protein coding genes of 1636) was retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000092265.1/) and subjected to CD-HIT analysis (60 % identity) to remove the paralog or duplicate sequences.
- b) The non duplicate protein sequences were subjected to BLASTp analysis and searched against the human proteome. This step was important to remove homolog or similar sequences.
- c) The non paralog and non-homologous protein sequences were now subjected to DEG database to identify essential sequences (> 50% identity; Bit score > 100).
- d) The subcellular localization of DEG proteins were analyzed using PSORTb database
- e) Cytoplasmic or membrane proteins were selected and its involvement in KEGG



pathways was evaluated using KAAS server available in KEGG database.

f) Efflux pump membrane transporter (D5VAP4) transporter was selected in the present study and its virulence factor efficacy was analyzed through VDFD database

g) D5VAP4 was searched against the drug data bank and also protein data bank to identify suitable ligands or drugs and also for template selection.

h) Further, if any drug was available in the drug data bank against the protein, the drug was selected as ligand and docked against the selected protein

i) SWISS-MODEL database was used for homology modelling

j) The predicted model was analyzed for its quality using ERRAT and Ramachandran plot analysis

k) Molecular docking was performed using EASY-DOCK pipeline

l) The protein was also screened against the zinc library for suitable ligands and redocking was performed against the step I (the coordinates of grid box used for docking analysis of zinc library compounds). Only compounds with molecular weights of 300 to 500, rotatable bonds with <10 and RMSD values at least 0.3 to 0.7 were considered as the cut-off values.

m) String database was used to analyze the protein-to-protein interactions

Results and Discussion

The protein coding genes as per the NCBI Ref Seq in NCBI database was found to be 1636 and CD-HIT analysis revealed that only six genes were paralogs (Table 1). The non paralogs were subjected to BLASTp and searched against the human

proteome to remove the homologs and accordingly, 519 homologs were identified and eliminated. The remaining 1121 non homologous protein sequences were analyzed for essential genes using DEG database (Table 1). Accordingly, 91 essential sequences were identified using DEG and were subjected to drug databank to investigate drugs if any. Out of 91 essential sequences, 38 sequences showed significant hits against drug databank. The subcellular localization of essential genes was shown in table 1.

Table 1. Non-homologous essential drug target identified out of 38 DEGs

S. No.	Uniprot Protein IDs	Protein's Name	PSORTb Results
1	D5VAP4	Efflux pump membrane transporter	Cytoplasmic Membrane

Molecular docking analysis

In the current study, efflux pump membrane transporter (Uniprot ID: D5VAP4) was selected as drug target. Efflux pumps are membrane proteins responsible for transporting substances across the cell membrane. In prokaryotes, these proteins are associated with intercellular signaling, processes associated with microbial virulence, and removal of unwanted metabolites and toxic substances from the cell. Thus, they contribute to the maintenance of cell homeostasis. The activity of efflux pumps is one of the reasons for bacterial resistance to certain antibiotics and bactericides. This occurs when the substance structurally resembles the pump's natural substrate or when the selectivity of the pump is modest



(multidrug resistance, MDR pumps). Chromosomal DNA usually encodes pumps with a broader spectrum of substrates. In contrast, mobile genetic elements, such as plasmids, typically contain genes encoding pumps with greater substrate selectivity.

Homology modelling

D5VAP4 was subjected to swiss model server to build model of this protein. Among the several templates, 7m4p.1 template (Figure 2; Zhang et al., 2021) was selected since it exhibited >60% identity with a GMSE and QSQE scores of > 80.

Homology model of D5VAP4

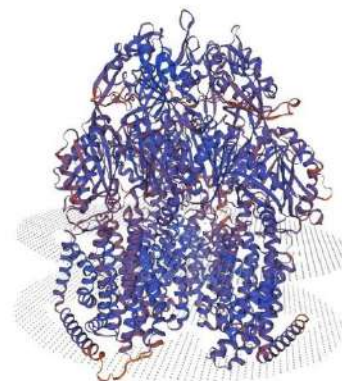
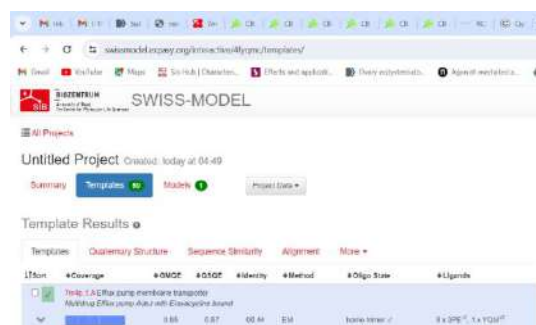
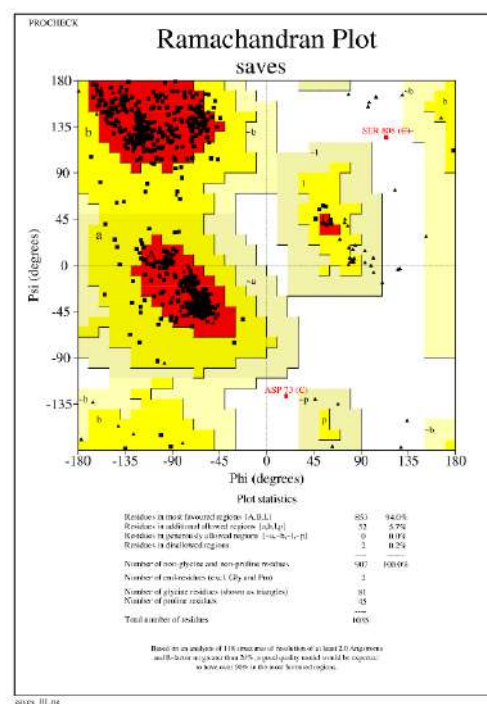


Fig 2:



Quality of predicted model

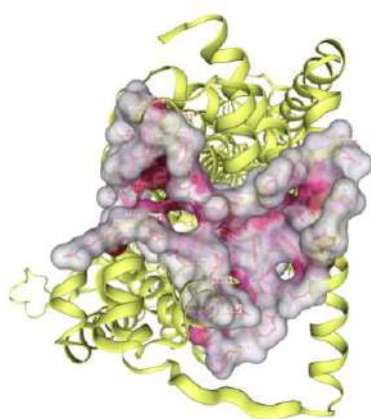
The pdb structure of predicted model was subjected to SAVES server to assess the quality. ERRAT values indicated that the predicted model was of good quality with a score > 96.277. Ramachandran plot analysis showed that the modelled protein exhibited >94 % amino acids in favoured regions, >5% in allowed regions. Only two amino acids, ASP 73 and SER 808 were present in disallowed regions.




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>tr|D5VAP4|Release 2023 02/2023 02|03-May-2023
```

Query
C

MSRFFIHPI FAWLITLIM LUGLIALNI PIEQPKTIAP PQVKVVRAP GAQRETVMS VTQIEQQK GIDGLYMSS NSSAAGSASV ILTFENGTP DTAQVQVKN LQASMSLPE TVQRITLNV
130 150 170 180 190 200 210 220 230 240
E KASDFLMVT AFVSEDSGQ QSDIAQVYAT SIVDPISRVE GVGGNVFGS SYAMRIIDP AKLRAYNPL SVDSASRIS NVQVSAGQL TPLNTDRVV INATISVQSY LQTFQEFQ
250 260 270 280 290 300 310 320 330 340 350 360
I LKXDTAGAQ VRKDVARVE LGSENVQRA QVNGQAASGL AIMPLAGANA LEVREAVGR LDELQNFPT GLKXNVPYDT TPFVRLSIQT WYTLLEAV LVFLWIFUL QVNRATIIIP
370 380 390 400 410 420 430 440 450 460 470 480
T LAVPVILGT FAVLSVFGYS INILTMFNV LAIGLVDDA INVLEVET LLENPSMIQ DATIQSMRE SVVYVICIAL LSVFVPMIF FGGSGVIYR QFAUTIMTS VLSAFTALV
490 500 510 520 530 540 550 560 570 580 590 600
F TPALCVIILK RQAHKDINIQ TGGFFVYRF YQTSRYEN FIGKTYASKL AYLAVYTGIV AMVALTFIRL PSSVFPEEQ GAVMTVLQPL AGSLDKTNA VIDKLANHYV DKETDNIES
610 620 630 640 650 660 670 680 690 700 710 720
V FTISGFGGFM SGQNSGHAFI KIKMDERAG SENTAQSIAR RAMPNVMIP EASLTPIIAP PPIQGFNTS GFDLQKDVQ GVGEALLDA RIQLMGMAQ NPATASIRPG QODEAPKL
730 740 750 760 770 780 790 800 810 820 830 840
V DIHQAAQAY GVLPTAINDT IAQMGSSYV NDFIDRGRK KWIQGEPSN RVPEIDINR VYRQSGEMV SFAGSFGSQY EYGSPLSAR NVGSSMYLGE SAALGVSTGD AMEAMQMA
850 860 870 880 890 900 910 920 930 940 950 960
S QLPAGIDFEN TGLSELOQKS GGOAPILYAL SILVFLCLLA QVENSVPF AVILVPLGV IGSLLTIKH GLANDVYLV ALLTVIGLSA KVALIIEFA KKLSSGGLT KASIMMAK
970 980 990 1000 1010 1020 1030
K RLRLPIINTSL AFGGVGVPLY IATGAGSGSQ NAVGTGVLGG VLTSTFLGIF FIPMYVYVWR ILTPY



CurPocket ID	Cavity volume ($\text{\AA}^3$)	Center (x, y, z)	Cavity size (x, y, z)
C1	3102	206, 205, 150	25, 27, 16
C2	2545	196, 204, 197	21, 20, 17
C3	2401	202, 219, 198	15, 22, 29
C4	1166	201, 205, 219	19, 16, 17
C5	743	206, 221, 218	14, 15, 21



Ligand selection

Based on the significant hits of selected protein (Table 2), dequalinium was selected as one of the ligands. Based on the literature survey, ciprofloxacin (Mikucka et al., 2000) and eravacycline (Sun et al., 2021) were also selected as ligands. Ciprofloxacin and eravacycline showed good efficacy against *M. catarrhalis*. Hence, they were selected and docked against the protein D5VAPR.

Molecular docking

Molecular docking was performed using Autodock Vina which was in built in EASY Dock tool. The x, y and z coordinates and active site prediction were performed using CB-dock server. The grid box coordinates were 206, 205, 150 from center and 25, 27 and 16 were the docking box size of x, y and z coordinates respectively.

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>tr|D5VAP4|Release 2023_02/2023_02|03-May-2023
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MSRFFIHRPIFAWVIAILIMLVGILAINNLPYQPKIAPPQVKVRAVYPGADAETVENSVTQIIQQMKGIDGL
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GTLPTNTDRVVINATISVQSYLQTPEQFEDILLKTDAGAQVRIKDVARELGSENYQFRAQYNGQAASGLAIM
LAPGANALEVREAVGVRLDELSQNFPTGLKMMVVPYDTPFVRLSITQVVYTLLEAVVLVFLVMFLFLQNWRTI
IPTLAVPVVILGTFAVLSVFGYSINILTMFAMVLAIGLLVDDAIIVVENVERILEENPSIQVMDATIQSMREISKVVI
GIALILSVVFPVPMIFFGGSSGVYRQFAVTLMTSMVLSAFIALVFTPALCVTILKRQAHKDINIQTGFFGWFNRFF
YQTSRRYENFIGKTYASKLAYLAVYTGIVAVMALIFMRLPSSFPPEEDQGAVMTLVQLPAGSTLDKTNVMDKL
ANYYHDKETDNIESVFTISGFGFMGSGQNSGMAFIKLDWDERAGSENTAQSIARRAMVMNMMIPEASLIF
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AAAYGVPLTAINDTIAQAWGGSYVNDIFDRGRIKKVYIQGEPNSRVVPEDINRWYVRNQSGEMVSFGAFSGS
QWEYGSPSLARYNGVSSMVLTSALGVSTGDAMEAMAQMASQLPAGIDFEWTGLSLEQQKSGGQAPILY
ALSILVFLCLAALYESWSVPFAVILVIPLGVIGSLLTKIHGLANDVYLQVALLTVIGLSAKNAILIEFAKEIQESGQT
LKASIMMAARMRLRPIIMTSLAFGVGVVPLYIATGAGSGSQNAVGTGVLGGVLTSTFLGIFPIMFYVWVRTLF
PYKPKAQPVTHAKSHEHV
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Interacting amino acids between dequalinium and D5VAP4

	Name	Visible	Color	Parent	Distance	Category	Types	From	From Chemistry	To	To Chemistry
1	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.64063	Hydrogen Bo...	Conventional Hydrogen Bond	d:UNK1:H40	H-Donor	C:GLN361:OE1	H-Acceptor
2	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.02373	Hydrogen Bo...	Conventional Hydrogen Bond	d:UNK1:H38	H-Donor	C:GLU415:OE2	H-Acceptor
3	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.79079	Hydrogen Bo...	Conventional Hydrogen Bond	d:UNK1:H37	H-Donor	C:MET970:O	H-Acceptor
4	C:ARG97...	☒ Yes		Ligand Non-bond Monitor	4.14516	Electrostatic	Pi-Cation	C:ARG973:NH2	Positive	d:UNK1	Pi-Orbitals
5	C:GLU42...	☒ Yes		Ligand Non-bond Monitor	3.29364	Electrostatic	Pi-Anion	C:GLU422:OE1	Negative	d:UNK1	Pi-Orbitals
6	C:ARG41...	☒ Yes		Ligand Non-bond Monitor	5.29481	Hydrophobic	Alkyl	C:ARG419	Alkyl	d:UNK1	Alkyl
7	C:ARG97...	☒ Yes		Ligand Non-bond Monitor	5.23071	Hydrophobic	Alkyl	C:ARG973	Alkyl	d:UNK1	Alkyl
8	C:TYR52...	☒ Yes		Ligand Non-bond Monitor	5.21656	Hydrophobic	Pi-Alkyl	C:TYR522	Pi-Orbitals	d:UNK1	Alkyl
9	d:UNK1 ~...	☒ Yes		Ligand Non-bond Monitor	5.4428	Hydrophobic	Pi-Alkyl	d:UNK1	Pi-Orbitals	C:ARG973	Alkyl

Interacting amino acids between Ciprofloxacin and D5VAP4

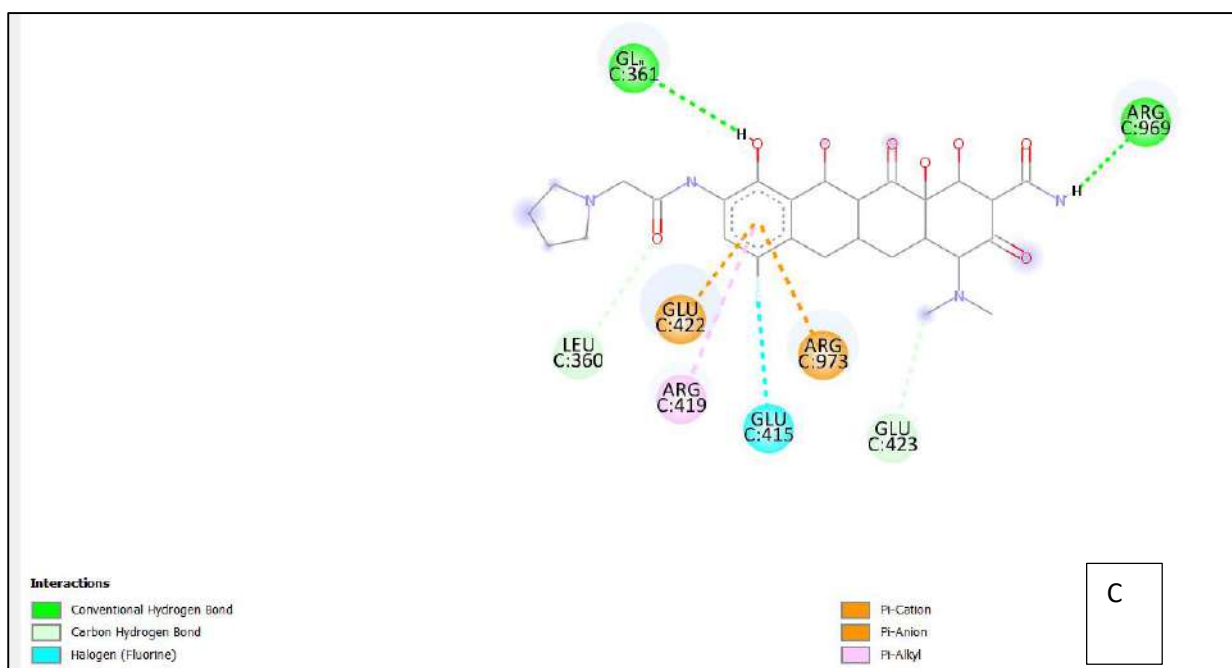
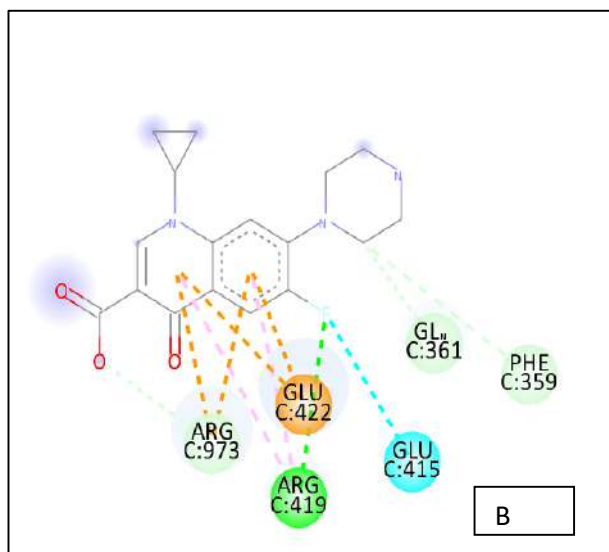
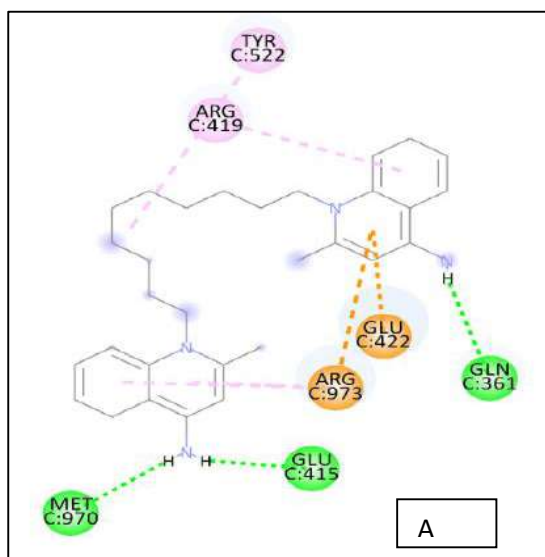
	Name	Visible	Color	Parent	Distance	Category	Types	From	From Chemistry	To	To Chemistry
1	C:ARG41...	☒ Yes		Ligand N...	3.53701	Hydrogen Bond;Halogen	Conventi...	C:ARG4...	H-Donor;Halogen Acceptor	d:UNK1:F1	H-Acceptor;Halogen
2	C:ARG97...	☒ Yes		Ligand N...	3.79598	Hydrogen Bond	Carbon ...	C:ARG9...	H-Donor	d:UNK1:O2	H-Acceptor
3	d:UNK1:...	☒ Yes		Ligand N...	3.70605	Hydrogen Bond	Carbon ...	d:UNK1:...	H-Donor	C:PHE359:O	H-Acceptor
4	d:UNK1:...	☒ Yes		Ligand N...	3.5637	Hydrogen Bond	Carbon ...	d:UNK1:...	H-Donor	C:GLN361:OE1	H-Acceptor
5	C:GLU41...	☒ Yes		Ligand N...	2.97091	Halogen	Halogen ...	C:GLU4...	Halogen Acceptor	d:UNK1:F1	Halogen
6	C:ARG97...	☒ Yes		Ligand N...	4.53572	Electrostatic	Pi-Cation	C:ARG9...	Positive	d:UNK1	Pi-Orbitals
7	C:ARG97...	☒ Yes		Ligand N...	3.71424	Electrostatic	Pi-Cation	C:ARG9...	Positive	d:UNK1	Pi-Orbitals
8	C:GLU42...	☒ Yes		Ligand N...	4.12743	Electrostatic	Pi-Anion	C:GLU4...	Negative	d:UNK1	Pi-Orbitals
9	C:GLU42...	☒ Yes		Ligand N...	3.36592	Electrostatic	Pi-Anion	C:GLU4...	Negative	d:UNK1	Pi-Orbitals
10	d:UNK1 ~...	☒ Yes		Ligand N...	5.26156	Hydrophobic	Pi-Alkyl	d:UNK1	Pi-Orbitals	C:ARG419	Alkyl
11	d:UNK1 ~...	☒ Yes		Ligand N...	5.00744	Hydrophobic	Pi-Alkyl	d:UNK1	Pi-Orbitals	C:ARG419	Alkyl

Interacting amino acids between Eravacycline and D5VAP4

	Name	Visible	Color	Parent	Distance	Category	Types	From	From Chemistry	To	To Chemistry
1	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.90586	Hydrogen Bond	Conventional Hydrogen Bond	d:UNK1:H31	H-Donor	C:GLN361:OE1	H-Acceptor
2	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.17265	Hydrogen Bond	Conventional Hydrogen Bond	d:UNK1:H28	H-Donor	d:UNK1:O1	H-Acceptor
3	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	2.0553	Hydrogen Bond	Conventional Hydrogen Bond	d:UNK1:H26	H-Donor	C:ARG969:O	H-Acceptor
4	C:LEU36...	☒ Yes		Ligand Non-bond Monitor	3.31537	Hydrogen Bond	Carbon Hydrogen Bond	C:LEU360:CA	H-Donor	d:UNK1:O8	H-Acceptor
5	d:UNK1:...	☒ Yes		Ligand Non-bond Monitor	3.52272	Hydrogen Bond	Carbon Hydrogen Bond	d:UNK1:C1	H-Donor	C:GLU423:OE2	H-Acceptor
6	C:GLU41...	☒ Yes		Ligand Non-bond Monitor	2.95201	Halogen	Halogen (Fluorine)	C:GLU415:O	Halogen Acceptor	d:UNK1:F1	Halogen
7	C:GLU41...	☒ Yes		Ligand Non-bond Monitor	3.02319	Halogen	Halogen (Fluorine)	C:GLU415:CD	Halogen Acceptor	d:UNK1:F1	Halogen
8	C:ARG97...	☒ Yes		Ligand Non-bond Monitor	3.9564	Electrostatic	Pi-Cation	C:ARG973:NH1	Positive	d:UNK1	Pi-Orbitals
9	C:GLU42...	☒ Yes		Ligand Non-bond Monitor	3.20464	Electrostatic	Pi-Anion	C:GLU422:OE1	Negative	d:UNK1	Pi-Orbitals
10	d:UNK1 ~...	☒ Yes		Ligand Non-bond Monitor	4.73275	Hydrophobic	Pi-Alkyl	d:UNK1	Pi-Orbitals	C:ARG419	Alkyl



Interacting amino acids between selected ligands (dequalinium: A; Ciprofloxacin: B; Eravacycline: C) and D5VAP4



The binding affinities were analyzed and as per the molecular docking analysis, dequalinium exhibited a binding affinity of -7 kcal/mol while ciprofloxacin and eravacycline against the D5VAP4 exhibited binding affinities of -7.6 kcal/mol and -7.5 kcal/mol respectively. The magnitude of binding affinities exhibited by the ligands against the D5VAP4 was in the order of eravacycline = ciprofloxacin > dequalinium. Ciprofloxacin (Pubchem ID: 2764) and the D5VAP4 of *M. catarrhalis* BBH18 were both used to screen the Zinc library with the defined parameters such as M.W: >300 to <350, RMSD: 0.7 Å³; rotational bonds: >2 and <8. This screening yielded almost 1200 significant hits against the zinc library. All the compounds were downloaded and molecular docking was performed using the zinc ligands and D5VAP4. Among the zinc ligands, the binding affinity between D5VAP4 and pipemidic acid (Drug databank id: DB13823; pubchem id: 4831; zinc ligand id: ZINC29345454) was equivalent to binding energy of ciprofloxacin and D5VAP4.

Compound	Binding affinity	Interacting amino acids	Hydrogen bond
Ciprofloxacin	-7.6 kcal/mol	Arg419, Arg973, Glu415, Phe359, Gln361, Glu422	Arg419, Arg973, Phe359, Gln361
Pipemidic acid	-7.2 kcal/mol	Gln361, Phe359, Glu418, Arg419, Glu422, Arg973	Phe359, Gln361

Conclusion:

In this study, new target i.e. efflux pump membrane transporter was identified from *M. catarrhalis* BBH18 strain. In addition, screening of zinc library and drug databank indicated two therapeutic targets such as pipemidic acid (quinole based compound) and dequalinium (bisquinoline) respectively. However, further in vivo and in vitro studies are warranted.

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BHAI-MS-5

Reverse vaccinology strategies to identify potential drug targets in *Candida albicans* sc5314M. Ashok Kumar¹ and S.B. Sainath^{1*}**Abstract**

Fungal infections are one of the serious threats to humans. Among the fungal infections, candidiasis caused by *Candida albicans* affect oral cavity, skin, gastro-urinary tract and mucosa- membranes of humans. *C. albicans* is a polymorphic and opportunistic fungus which escape the normal surveillance of immune system and causes candidiasis. The development of resistance by this pathogen against the antifungal drugs is of prime concern and should be immediately addressed. With the advent of advanced molecular and analytical technologies, the genome architecture of this pathogen is known which was explored in this study to identify potential targets using in silico analysis for the development of alternative drug design strategies. In this study, the proteome of *C. albicans* wild strain SC 5314 was retrieved followed by the removal of duplicate sequences, and human homologous sequences. A total of 357 sequences were found to be human non homologous and a total of 20 essential proteins involved in fungal unique metabolic pathways were identified. Among the pathways, genes related to isoprenoid pathway were selected as drug targets for this study. Both pharmaceutical and plant-based drugs were screened against ERG8 and based on molecular docking analysis. Among plant-based drugs, one of the plant-based antifungal agents that exhibited high binding affinity as compared to fluconazole and posaconazole was presented in this study. Lipinski 'Rule of 5' and ADME properties revealed that the selected antifungal agent was predicted to be safe and hence, could be used for *in vivo* experimental analysis.

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1. Introduction

The development of antibiotic resistance or multidrug resistance during fungal infections is one of the alarming factors worldwide and should be immediately addressed (Fisher et al., 2022). However, discovery of drugs is not an easy process because of economic constraints, and most strikingly development of drugs against specific fungal protein targets is time consuming (Ismail et al., 2018). Therefore, approaches that speed up the drug discovery process is of paramount importance. With the advent of advanced molecular techniques, the complete genome of several fungi is now known and offered several insights into the structural and functional organization which in turn led to the identification of proteins associated with fungal metabolic pathways (Basenko et al., 2018). Systems biology approach using various computational methods and tools allowed the biologists to isolate and identify proteins that are specific to fungal metabolism which consequently sped up and revolutionized the drug discovery process through a paradigm shift from ligand-based approach to target-based design of drugs (Mdluli and Spigelman, 2006; Karahalil, 2016). Systems-biology approach through ‘omics’ based technologies encompasses the exploration of comparative, subtractive, and functional methods to identify the proteins that are actively involved in pathogen metabolism (Bappy et al., 2023). This trend is particularly helpful to design antifungal drugs especially for drug resistant pathogens (Lin and Qian, 2007; Fischbach and Walsh, 2010).

Candida albicans is an archetypical opportunistic pathogen and published reports have shown that this fungus is carried on mucosal membranes of humans without causing any damage (Richardson and Rautemaa, 2009). In general, *C. albicans* is a diploid fungal pathogen and harmless in nearly 70% of human population, while in immunocompromised situations, the transition of *C. albicans* from a commensal to pathogen occurs and leads to candidiasis (Meiller et al., 2009; Schulze and Sonnenborn, 2009). Candidiasis is characterized by the formation of “thrush”, a spotty white infected membrane in gastrointestinal tract epithelial cells, vaginal region, and oropharyngeal mucosa and even causes mortality in severely ill patients (Niemeic et al., 2017). Estimates have shown that almost 50 % of mortality rate in US occurs due to cardiovascular and blood stream infections caused by *Candida* species (Kabir et al., 2012). Furthermore, the risk factors for invasive candidiasis which include antibiotic treatment, indwelling catheters and prolonged stay in ICU presents a persistent problem in patients and despite of the use of modern antifungal agents, the overall mortality is increasing day by day (Laupland et al., 2005; Eggimann et al., 2011). Studies in murine models have shown that the *C. albicans* negatively affect kidneys and causes sepsis (Lüttich et al., 2013).

Though, the exact pathogenesis of *C. albicans* induced mucosal and invasive infections is not clear, it has been shown that the adaptation of *C. albicans* in various niches of anatomical sites require versatile nutrient acquisition, metabolic status and stress resistance (Morgan et al., 2013;



Niemeic et al., 2017). To compliment these adaptation requirements, several changes such as phenotypic transition and genetic modifications play a pivotal role (Shin et al., 2004; Bougnoux et al., 2006; Yang et al., 2023). It has been shown that the phenotypic transition from yeast to hyphae could be one of the underlined mechanisms and the hyphal formation eventually establish biofilm network and ascribed to the colonization of fungi thereby candidiasis (Ramage et al., 2023; Do et al., 2023). On the other hand, genetic modifications such as gene expression, translation and post translation modifications, chromosomal rearrangements and genetic alterations like insertions, deletions and exchange of nucleotides also sustain the growth of fungi under stressful conditions (Selmecki et al., 2010; Nobile et al., 2012). Thus, the changes in the phenotype and genotype within the *C. albicans* populations promote their ability to adapt in host micro-environments which is at least in part defined by the properties of the local mucosa and also its ability to establish within the complex microbiota of that anatomical location (Morgan et al., 2013). Once established, they can able to produce various proteins including aspartic proteases which prevent opsonization process via cleaving a complement mediated pathway protein, a convertase C3b (which acts as a protein that activate a membrane attack complex proteins) and escape the immune surveillance (Reisbeck, 2013). Studies also indicated that the dissemination of increased risk of chronic mucocutaneous candidiasis is often associated with the impairment of T17 cells

due to genetic mutations of *C. albicans* (Okada et al., 2016). These events at least in part underlie antifungal drug resistance and/or tolerance. Antifungal drug resistance of *C. albicans* could be attributed to the mutations at the level of genes encoding drug targets (Fisher et al., 2018). For example, mutation of genes encoding lanosterol demethylase and beta-glucan synthase develop resistance against azole and echinocandids, respectively (Fisher et al., 2023). On the other hand, antifungal tolerance seems to be overexpression of genes encoding proteins in drug susceptible cells and sustain their growth at drug concentrations above the minimal inhibitory concentrations of fungistatic drugs like fluconazole (Berman and Krysan, 2020).

Considering the aforementioned data, the central objective of this study was twofold: identification of drug targets and screening of antifungal agents of plant origin against the selected drug targets of *C. albicans*. To accomplish this task, we took the advantage of genome of *C. albicans* and using systems biology approach, possible drug targets were identified. Further, molecular docking analysis was performed using plant based metabolites and modelled proteins of *C. albicans*. The current study might be helpful for scientists to develop *in vivo* and *in vitro* experiments and the computational strategy shown in this study may be useful for the scientists to screen the drug targets in various pathogenic strains of *Candida* species.

2. Materials and Methods

The pipeline of systems biology approach using several online bioinformatics tools in the identification



and prioritization of unique drug targets from the wild strain, *C. albicans* SC5314 was shown in Figure 1.

2.1. Retrieval of *Candida albicans* SC5314 proteome

The genome size of *C. albicans* SC5314 was about 14.3 Mb (Assembly: [ASM18296v3](#) ; NCBI RefSeq assembly: GCF_000182965.3) with a total number of 6263 genes (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000182965.3/). The representative *C. albicans* SC5314 proteome was retrieved from the Uniprot Universal Protein Knowledgebase (Uniprot: <https://www.uniprot.org/>; The Uniprot Consortium, 2021).

2.2. Elimination of paralogs and mini proteins

The reviewed proteins were submitted to CD-HIT tool (<http://cd-hit.org/>) with parameters such as “sequence identity cut-off” was set to 0.6 (60% identity) [Huang et al., 2010]. This resulted in the elimination of paralog sequences among the reviewed proteins. In general, protein sequences with <100 amino acids are actively involved in cellular biological and regulatory activities. However, they are less likely to be drug targets. Hence, non-paralog protein sequences with less than 100 amino acids were removed and proteins with >100 amino acids were further analyzed.

2.3. Identification of orthologs in host

The non-paralog protein sequences (with > 100 amino acids) were submitted to BLASTp (threshold e-value: 0.0001) in the NCBI database and searched against *Homo sapiens* (taxid: 9606) to separate the orthologs. The queries that resulted in no

significant hits were considered as non-homologous sequences which were specific to the pathogen and used for further investigation with the objective of developing a pathogen-specific drugs [Pourhajibagher and Bahador, 2016].

2.4. Identification of essential genes

The non-orthologous sequences, thus obtained from the previous step were submitted to BLASTp in the DEG database (www.essentialgene.org/) against eukaryotes to identify essential proteins (threshold e-value: 1e-100 and percent identity was set to >25 %).

2.5. Selection of unique metabolic pathways specific to fungus

KEGG pathway analysis (<http://www.genome.jp/kegg/pathway.html>) was used to identify metabolic pathways for both species i.e. *C. albicans* and *Homo sapiens* by entering the respective three-letter KEGG organism codes in the organism box -“cal” for *C. albicans* and “hsa” for *H. sapiens*. The fungus's unique pathways were then screened against the humans by manual comparison. The host non homologous sequences were then submitted to the KAAS server using BLASTp (https://www.genome.jp/kaas-bin/kaas_main) to identify KO numbers accordingly and the KO number was then used to search the KO server (KEGG ORTHOLOGY) (<https://www.genome.jp/kegg/ko.html>) for protein pathways that are unique to pathogen's metabolism. On the other hand, proteins which were found to be involved in common pathways were excluded from the study.

2.6. Identification of drug targets



The selected proteins that were unique to the fungal metabolic pathways were examined to know their distinctiveness as pharmacological targets by submitting the protein sequences to DrugBank [server database](https://www.drugbank.ca/structures/search/bonds/sequence) (<https://www.drugbank.ca/structures/search/bonds/sequence>) with default parameters. If significant results were obtained against the submitted proteins, it demonstrates the presence of druggability of targets and absence of significant hits indicates the distinctiveness of proteins as new targets.

2.7. Analysis of cellular localization

The unique protein drug targets obtained from the previous step were submitted to the CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>) server to forecast the subcellular localization.

2.8 Interactome analysis

Protein-protein interactions are key for the sustainability of the pathogen in the host system. To examine the probable interactions between the query protein and other interacting proteins, unique drug targets were submitted to the STRING 11.5 (<https://string-db.org/>) server with high confidence score of ≥ 0.700 .

2.8. Identification of plant-based antifungal drugs

Based on the available literature, twenty-nine plant based metabolites with antifungal activity were selected. The chemical structure of these plant based antifungal agents in SDF (3D) format were retrieved from the PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>) and the SDF format was converted into protein databank format using the Open Babel v2.3 program [44].

2.9. Molecular docking analysis

Homology modelling

In the current study, the molecular models of selected unique protein targets were modelled using a homology modelling tool Swiss model server (<https://swissmodel.expasy.org/>), an online resource for automated protein structure prediction based on the identification of structural templates, alignment of target sequence and template structure, model building and evaluation of model quality. To assess the quality of the modelled proteins were submitted to ERRAT and analyzed via Ramachandran plot available in the SAVES server (<https://saves.mbi.ucla.edu/>). The binding pockets of modelled proteins were determined using CASTp server (<http://sts.bioe.uic.edu/castp/>), which analyze the pockets based on recent theoretical and algorithmic results of computational geometry wherein all calculated parameters are rotationally invariant, and do not involve discretization and they make no use of dot surface or grid points.

Molecular docking

Molecular docking is one of the efficient procedures to understand the interaction between the ligand and protein and protein and protein interactions. This approach is widely used in drug development process and in reverse vaccinology. In the current study, the interactions between the ligand (antifungal agent) and receptor (target proteins) was performed by employing Server HDock (<http://hdock.phys.hust.edu.cn/>) as it allows for connection within therapeutic targets and potential treatments. Fluconazole, a



synthetic triazole is the currently employed antifungal drug and hence, selected as reference antifungal agent and docked against the selected modelled proteins. The binding affinity between the plant based antifungal agent and the target proteins were compared against the fluconazole and the target proteins. The docked complexes were visualized and analyzed the binding sites of the metabolites using discovery studio.

Results and Discussion

Candida albicans SC5314 is a pathogenic yeast that has developed as a multidrug-resistant organism. In many countries, community health experts have identified that *C. albicans* has affected hospitalized patients with serious illness. Diseases commenced by *C. albicans* frequently do not react to regularly consuming antifungal medications, rendering them tough to cure effectively. Hence, we investigated the proteome of this yeast to anticipate the prospective drug targets and drugs to deal with this pathogenic yeast.

Since most therapeutics tends to bind with essential gene products, essential proteins are considered to be among the most effective drug targets. A prospective drug target has to be an essential protein as it has a vital attribute for the existence of the pathogen and our target pathogen yeast contains such essential proteins specifying that drugs will act out in opposition to those proteins will lead the pathogen to death. The proteome of *C. albicans* SC5314 showed 6110 proteins and out of which 1111 proteins were reviewed proteins. The reviewed proteins (UniprotKB/Swiss-Prot) were used for further experimental studies.

Reviewed proteins represent the high quality manually annotated and non-redundant proteins based on experimental results and scientific conclusions and hence, they were selected and used in this study. After eliminating the paralogous sequences, 1063 non-duplicate large proteins were left since the CD-HIT service found at 60% identity. The non paralogous sequences were subjected to BLASTp analysis and searched against humans to eliminate homologous sequences. A total of 357 non-paralogous and non-homologous sequences were subjected to database of essential genes server under defined parameters i.e. > or equal to 25 % identity and bit score > 100 and also which exhibited significant hits against the fungal species (*Saccharomyces cerevisiae*: DEG2001; *Schizosaccharomyces pombe* 972hr: DEG2009; *Aspergillus fumigatus*: DEG2008 and *Komagataella phaffi* GS115: DEG2027). This step resulted in 102 essential sequences with defined parameters (Table 1).

Table 1: Retrieval of proteins from *Candida albicans* SC5314 and identification of non paralogs and non homologs

Number of reviewed proteins analyzed	1111
Number of non paralogs identified based on CD HIT	1063
Number of non-homologous sequences identified using BLASTp against humans	357
Number of essential proteins identified using DEG	102

The KEGG server had 127 *C. albicans* metabolic pathways (<https://www.kegg.jp/kegg->



[bin/search_pathway_text?map=cal&keyw ord=&mode=1&viewImage=true](https://www.kegg.jp/kegg-bin/search_pathway_text?map=cal&keyw ord=&mode=1&viewImage=true)) and 346 human metabolic pathways (https://www.kegg.jp/kegg-bin/search_pathway_text?map=hsa&keyw ord=&mode=1&viewImage=true), with 11 metabolic pathways being specific only for *C. albicans*. Proteins involved in these unique pathways can be used as therapeutic targets. Following a BLAST at the KAAS server, 20 out of 47 non-homolog essential proteins were discovered to have both KO orthology and metabolic pathway involvement. These 20 proteins (Table 2) are essential for the proper functioning of metabolism, and solely associated with *C. albicans* unique pathways (Table 4).

Table 2: KEGG pathway and KAAS analysis of non paralogous and non homologous sequences of *C. albicans*

Number of KEGG pathways identified from <i>C. albicans</i> SC5314	127
Number of KEGG pathways identified from <i>Homo sapiens</i>	356
Number of unique pathways identified from <i>C. albicans</i>	11
Number of essential genes related to the metabolic pathways of <i>C. albicans</i>	20

Among the 20 proteins of metabolic importance, a cytoplasmic enzyme phosphomevalonate kinase (sp|A0A1D8PLH0|ERG8_CANAL; [K00938](#) mvaK2; phosphomevalonate kinase [EC:2.7.4.2]) was selected for further experimental study because of its metabolic importance in terpenoid pathway.

Table 3: Unique metabolic pathways associated with *C. albicans* when compared against *Homo sapiens*.

KEGG Pathway	Metabolism
cal00261	Monobactam biosynthesis
cal00300	Lysine biosynthesis
cal00332	Carbapenem biosynthesis
cal00460	Cyanoamino acid metabolism
cal00660	C5-Branched dibasic acid metabolism
cal00680	Methane metabolism
cal00791	Atrazine degradation
cal00909	Sesquiterpenoid and triterpenoid biosynthesis
cal00999	Biosynthesis of various plant secondary metabolites
cal01110	Biosynthesis of secondary metabolites
cal01501	beta-Lactam resistance

The protein-protein interactions revealed that the selected enzyme ERG8 showed interactions with different metabolically active proteins involved in pathways like terpenoid backbone biosynthesis, biosynthesis of secondary metabolites, steroid biosynthesis, synthesis and biodegradation of ketone bodies, butanoate metabolism and valine, leucine and isoleucine degradation.

Molecular docking was performed between the ERG8 and selected antifungal plant-based candidates. Based on the SWISS-MODEL analysis, the structure of ERG8 was modelled. The modelled protein showed good quality as indicated by ERRAT value and Ramachandran plot analysis. Based on the literature survey, thirty-eight (38) plant based antifungal agents were collected from the NCBI database and docked against the ERG8.



Table 4: Unique proteins involved in metabolism of *C. albicans*

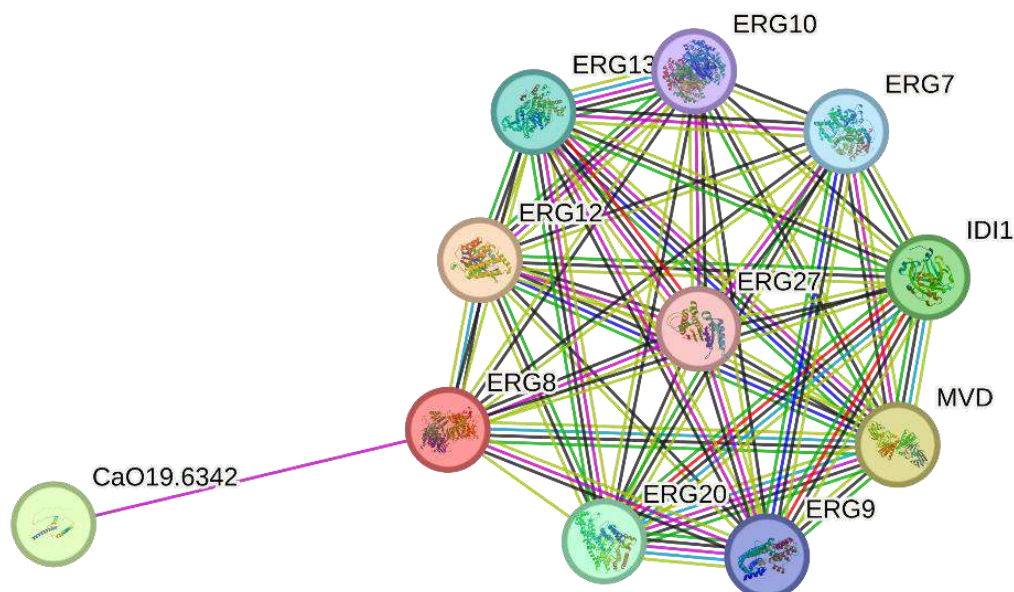
Polysaccharide
Storage polysaccharide
▼ Structural polysaccharide
sp O13318 PHR2_CANAL; K22832 GAS1; 1,3-beta-glucanosyltransferase GAS1 [EC:2.4.1.-]
sp P43076 PHR1_CANAL; K22832 GAS1; 1,3-beta-glucanosyltransferase GAS1 [EC:2.4.1.-]
sp Q59VW6 PGA5_CANAL; K22833 GAS2; 1,3-beta-glucanosyltransferase GAS2 [EC:2.4.1.-]
sp Q5AJY5 PGA4_CANAL; K22836 GAS5; 1,3-beta-glucanosyltransferase GAS5 [EC:2.4.1.-]
Histidine kinases
sp Q5A872 SLN1_CANAL; K11231 SLN1; osmolarity two-component system, sensor histidine kinase SLN1 [EC:2.7.13.3]
▼ 1.1.1.25 shikimate dehydrogenase (NADP+)
sp Q5AME2 ARO1_CANAL; K13830 ARO1; pentafunctional AROM polypeptide [EC:4.2.3.44.2.1.101.1.1.252.7.1.712.5.1.19]
▼ 1.1.1.270 3beta-hydroxysteroid 3-dehydrogenase
sp Q5A888 ERG27_CANAL; K09827 ERG27; 3-keto steroid reductase [EC:1.1.1.270]
▼ 2.5.1.19 3-phosphoshikimate 1-carboxyvinyltransferase
sp Q5AME2 ARO1_CANAL; K13830 ARO1; pentafunctional AROM polypeptide [EC:4.2.3.44.2.1.101.1.1.252.7.1.712.5.1.19]
▼ 2.7.1.71 shikimate kinase
sp Q5AME2 ARO1_CANAL; K13830 ARO1; pentafunctional AROM polypeptide [EC:4.2.3.44.2.1.101.1.1.252.7.1.712.5.1.19]
▼ 2.7.1.39 homoserine kinase
sp Q92209 KHSE_CANAL; K00872 thrB; homoserine kinase [EC:2.7.1.39]
▼ 2.7.4.2 phosphomevalonate kinase
sp A0A1D8PLH0 ERG8_CANAL; K00938 E2.7.4.2, mvaK2; phosphomevalonate kinase [EC:2.7.4.2]
▼ 2.7.13.3 histidine kinase
sp Q5A872 SLN1_CANAL; K11231 SLN1; osmolarity two-component system, sensor histidine kinase SLN1 [EC:2.7.13.3]
▼ 4.1.2.13 fructose-bisphosphate aldolase
sp Q9URB4 ALF_CANAL; K01624 FBA, fbaA; fructose-bisphosphate aldolase, class II [EC:4.1.2.13]
▼ 4.1.99.12 3,4-dihydroxy-2-butanone-4-phosphate synthase
sp Q5A3V6 RIB3_CANAL; K02858 ribB, RIB3; 3,4-dihydroxy 2-butanone 4-phosphate synthase [EC:4.1.99.12]
▼ 4.2.1.1 carbonic anhydrase
sp Q5AJ71 CAN_CANAL; K01673 cynT, can; carbonic anhydrase [EC:4.2.1.1]
▼ 4.2.1.10 3-dehydroquinate dehydratase
sp Q5AME2 ARO1_CANAL; K13830 ARO1; pentafunctional AROM polypeptide [EC:4.2.3.44.2.1.101.1.1.252.7.1.712.5.1.19]
▼ 4.2.3.4 3-dehydroquinate synthase
sp Q5AME2 ARO1_CANAL; K13830 ARO1; pentafunctional AROM polypeptide [EC:4.2.3.44.2.1.101.1.1.252.7.1.712.5.1.19]
▼ 5.3.1.24 phosphoribosylanthranilate isomerase
sp P43073 TRPF_CANAL; K01817 trpF; phosphoribosylanthranilate isomerase [EC:5.3.1.24]
▼ 3.6.1.74 mRNA 5'-phosphatase
sp O93803 CET1_CANAL; K01098 CET1; polynucleotide 5'-triphosphatase [EC:3.6.1.74]
▼ 6.5.1.3 RNA ligase (ATP)
sp P43075 TRNL_CANAL; K14679 TRL1; tRNA ligase [EC:6.5.1.3]



The interactions between the protopine and ERG8 and fluconazole and ERG8 was visualized using Discovery studio to predict the interactive bonds and type of amino acids involved. Inadequate ADME data is frequently linked with the malfunction of clinical trials in the course of several drug development projects. ADME analysis also revealed that the selected compound did not violate the Lipinski rule of five and also exhibited good gastrointestinal property.

[illegible]

Figure 2: PPI network of ERG8 with other proteins

Figure 3: Amino acid sequence of *C. albicans* ERG8

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.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120.....
MSKAFSAPGK AFLAGGYLV L EPIYDAYVTA LSSRMHAVIT PKGTSLKESR IKISSPOFAN GEWEYHISN TEKPEVQSR INPFLEATIF IVLAYIQPTE AFDLEIIIS DPGYHSQEDT ETKTSSNGE
130.....140.....150.....160.....170.....180.....190.....200.....210.....220.....230.....240.....
K TFLYHSRAIT EVEKTGLGSS AGLVSVWATS LLSHFIPNVI STNKDILHNV AQIAFCYACK KIGSGFDVAT AIYGSIVYRR FQALINDVF QVLESDEPKF PTELKKLIAS NWEFKHERC
250.....260.....270.....280.....290.....300.....310.....320.....330.....340.....350.....360.....
T LPHGIKLLYG DVKGGSETPK LVSRLQWKK EKPEESSVVY DQLNSANLQF MKELREMREK YDSPETIYIK ELDHSIEPLT VAIKNIRKGL QALTQKSEVP IEPDVQTQLL DRCQEIPGC
370.....380.....390.....400.....410.....420.....430..
V GGVPAGAGGY DAIAVLVLEN QVGNFKQKTL ENPDYFHNV WDLLEEQTEG VLEEKPEDYI GL

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Figure 4: Homology model of *C. albicans* ERG8 using SWISS-MODEL and Ramachandran plot analysis

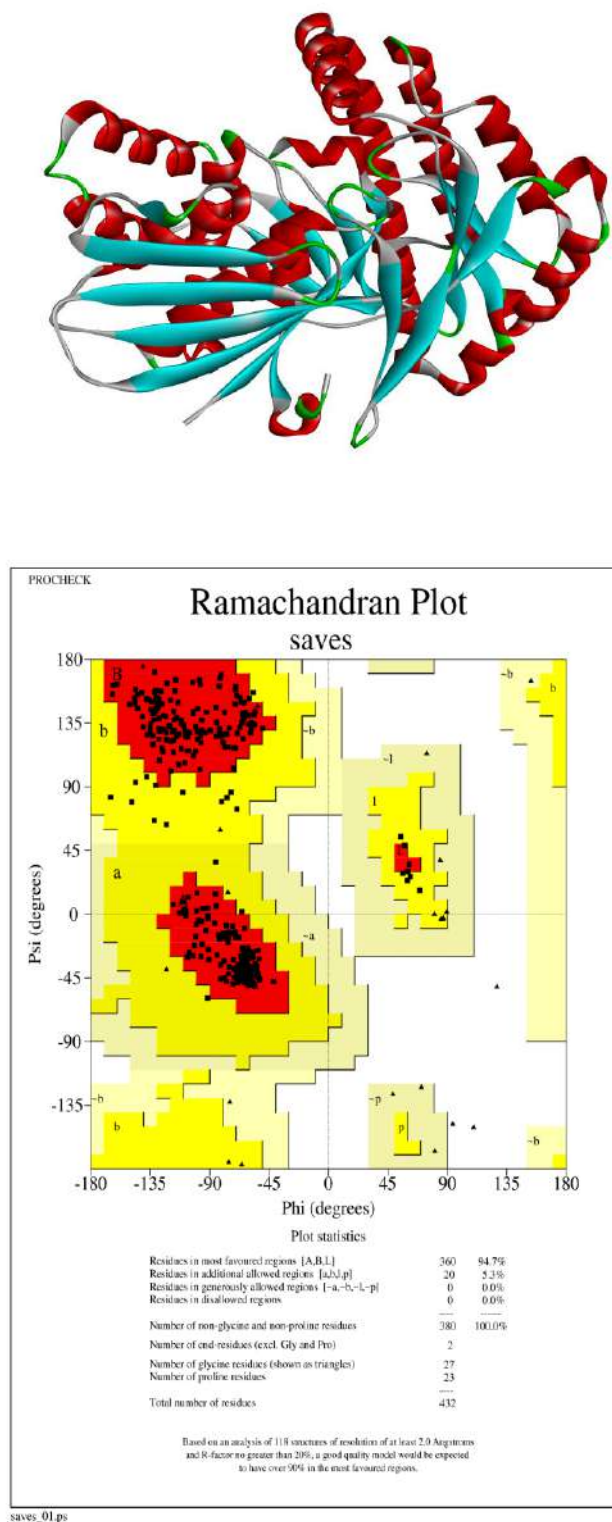


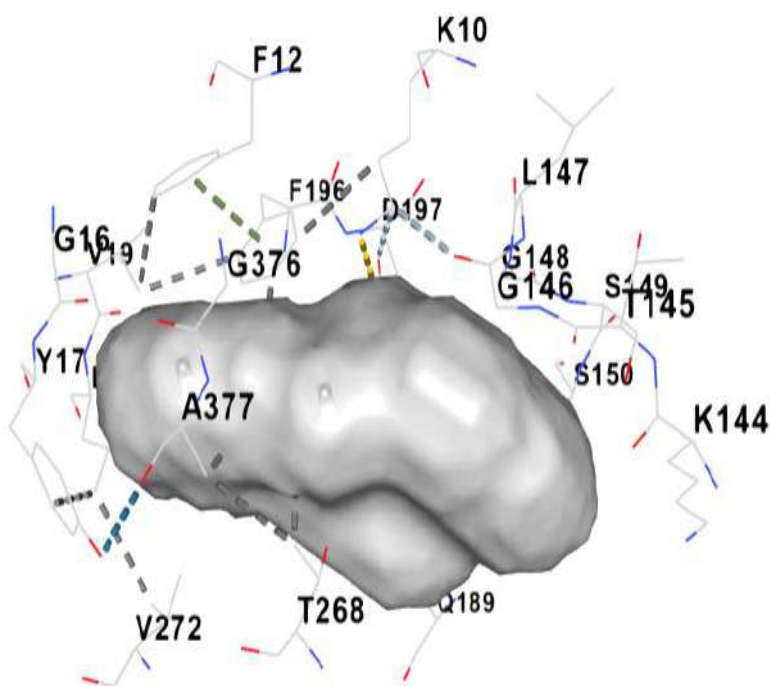
Table 6: Molecular docking analysis of ERG8 and different plant based antifungal agents using Autodock Vina in built in EASY-DOCK server

Compound	Pubchem	Kcal/mol
Protopine	4970	-8.9
Dicentrine	101300	-8.5
Caledonixanthone E	5464634	-8.4
Viniferin	5315232	-8.4
Chlorogenic acid	1794427	-8.2
Betulinic acid	64971	-8.2
Alpha-allocryptopin	98570	-8.1
Spirostanol	12304444	-8.1
Usnic acid	5646	-7.9
3methoxysampangine	122683	-7.8
Clausenidin	5315947	-7.8
Glaucine	16754	-7.8
Quercetin	5280343	-7.8
Jatrorrhizine	72323	-7.7
Eriobofuran	178939	-7.4
Flavones	10680	-7.4
Resveratrol	445154	-7.3
Fluconazole	3365	-7.2
Pterostilbene	5281727	-7
Acetoxychavicol acetate	<u>166872</u>	-6.9
Cyclocolorenone	160491	-6.4
abscisic acid	5375199	-6.3
Cerbinal	13786166	-6.2
4-Methyl-7-hydrox	5280567	-6.2
Agropyrene/ Capillen	3083613	-6
Carvacrol	10364	-5.9
Eugenol (Clove oil)	3314	-5.9
Gallic acid	370	-5.9
N-methyl-N-formyl-4-hydroxy-beta-phenylethylamine	11446673	-5.8
Caffeic acid	689043	-5.7
Thymol	6989	-5.7
Eucalyptol	2758	-5.5
Capric acid	2969	-5
Linalool	6549	-5
Ajoene	5386591	-4.6
Cycleanine	121313	-8.5



Figure 5: Molecular docking analysis of protopine and ERG8**Interactive amino acids**

LYS10 PHE12 GLY16 TYR17 VAL19 LEU20 LYS144 THR145 GLY146 LEU147 GLY148
SER149 SER150 GLN189 SER194 PHE196 ASP197 THR268 VAL272 GLY376 ALA377

**Conclusion**

This study of finding suitable drug targets and screening potential drugs will speed up the process to get remedies with fewer trials and error repeats of assays against *C. albicans*. Pathogen protein ERG8 were found as suitable drug target

and the metabolites protopine can be grater alternative to get rid of this deadly pathogen. Finally selected protein and metabolite to block those proteins may be greater foresteps of drug discovery against the pathogen. However, *in vivo* and clinical trials are highly recommended to validate the outcome of the study.



List of anti-fungal plant metabolites used for docking studies

Metabolites	Pub-chem ID	Source	References
Ajoene	5386591	<i>Allium sativum</i>	Meena et al.(2013); Ledezma & Apitz-Castro (2006)
Thymol	6989	<i>Thymus vulgaris</i>	Meena et al.(2013); Alagawany et al. (2021).
Carvacrol	10364	<i>Origanum vulgare</i>	Meena et al.(2013); Rathod et al.(2021).
Linalool	6549	<i>Ocimum basilicum</i>	Meena et al.(2013); An et al. (2021).
Caffeic acid	689043	<i>Eucalyptus globulus</i>	Meena et al.(2013); Zielińska et al. (2021).
Chlorogenic acid	1794427	<i>Malus pumila</i> ,	Meena et al.(2013); Chen et al. (2021); Navarro-Orcajada et al. (2021)
Pterostilbene	5281727	<i>Prunus amygdalus</i>	Meena et al.(2013); Wawarczyk et al. (2022).
Resveratrol	445154	<i>Vitis vinifera</i>	Meena et al.(2013); CAO, et al. (2021)
Viniferin	5315232	<i>Dryobalanopsaromata</i> .	Meena et al.(2013); Shang et al. (2021).
2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate	5277433	<i>Datura metel</i>	Meena et al.(2013); Raghuvanshi(2019).
N-methyl-N-formyl-4-hydroxy-beta-phenylethylamine	11446673	<i>Cyathobasisfruticulosa</i>	Meena et al.(2013); Thawabteh et al. (2019)
Acetoxychavicol acetate	166872	<i>Alpinia galanga</i>	Latha et al. (2009); Sok et al. (2021)
Eugenol(Clove oil)	3314	<i>Eugenia caryophyllus</i>	Latha et al. (2009); Rojas et al. (2021).
Spirostanol	129674828	<i>Smilax medica</i>	Sauton et al. (2006); Jastrzebska et al. (2021)
Coccoline	21579624	<i>Epineurum villosum</i>	Meena et al.(2013); Purwayantie et al. (2018)
Dicentrine	101300	<i>Glauciumoxylobum</i>	Meena et al.(2013); Perewalo et al. (2021).
Glaucine	16754	<i>Glauciumoxylobum</i>	Meena et al.(2013); Akaberi et al. (2021).
Clausenidin	5315947	<i>Clausena exca</i>	Sunthiktikawinsakul et al. (2003); Al-Abboodi et al. (2021)
Agropyrene/Capillen	3083613	<i>Agropyron repens Beauv</i>	Khare(2007); Grummer (1961).
Eriobofuran	178939	<i>Eriobotrya japonica Lindl</i>	Khare(2007); Li et al.(2016).
Cyclocolorenone	160491	<i>Magnolia grandiflora Linn</i>	Khare(2007); Jena et al. (2021).
Cerbinal	13786166	<i>Gardenia jasminoides Ellis</i>	Khare(2007); Li et al. (2019).
Flavones	10680	<i>Alpinia officinarum Hance</i>	Khare(2007); Zaragoza et al. (2020).
Capric acid	2969	<i>Arachis hypogaea Linn</i>	Khare(2007); Kim et al. (2020).
Usnicacid	5646	<i>Parmeliaperlata (Huds.) Ach</i>	Khare(2007); Araújo et al. (2015).
Protopine	4970	<i>Fumaria vaillantii</i>	Meena et al.(2013); Huang et al. (2022).
Alpha-allocryptopin	98570	<i>Glauciumoxylobum</i>	Meena et al.(2013)
3-methoxysampangine	122683	<i>Cleistopholis patens</i>	Meena et al.(2013); Liu et al. (1990)
Jatrorrhizine	72323	<i>Mahonia aquifol</i>	Jung et al. (2006); Li et al. (2014); Jiang et al. (2011); Wang et al. (2018); Wang et al. (2019)
Caledonixanthone E	5464634	<i>Calophyllum caledonicum</i>	Larcher et al. (2004)



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Protective action of *Moringa oleifera* on glyphosate-induced hepatotoxicity in albino ratsG. Anuradha¹, U. Kanchana Ganga¹, P. Annaiah², S.B. Sainath^{3*} and B. Kishori^{1*}**Abstract**

The present study was aimed to evaluate the protective efficacy of *Moringa oleifera* (MO), against glyphosate-induced hepatotoxicity in rats. Healthy male rats (n = 24) were randomly divided into four groups with six rats each. Rats in group 1 served as controls and did not receive any treatment. Animals in group II and III received glyphosate at a dose of 500 mg/Kg body weight and MO leaf extract at a dose of 500 mg/Kg body weight, respectively. Rats in group IV were maintained similar to that of group II but also received MO leaf extract at a dose of 500 mg/Kg body weight. The experimental duration was about 55 days. After completion of the experimental period, the rats were sacrificed and liver was isolated immediately and subjected to biochemical analysis. No significant changes in the body weights were observed between the control and experimental rats, however, the tissue somatic index of liver was reduced in glyphosate administered rats over controls. Significant reduction in the activity levels of superoxide dismutase, and catalase with a concomitant increase in the lipid peroxidation levels were observed in the liver tissue of glyphosate treated rats over controls. Whereas co-administration of glyphosate with MO significantly increased the levels of antioxidant enzymes and inhibited the levels of lipid peroxidation in liver tissue of rats over glyphosate treated rats. The results of present study indicating the protective role of *Moringa oleifera* to combat glyphosate-induced hepatotoxicity in rats at least in part mediated through antioxidant capacity.

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Introduction

Organophosphates are most commonly used insecticides in agriculture & pest control and studies indicated that the accumulated majority share in global insecticides usage (De Bleecker et al., 1994). Though promising, the wide usage of the OPs often causes side effects in humans which is of major concern (Robb et al., 2024). Published reports also suggest the deleterious effects in experimental animal models (Peter et al., 2014). Glyphosate [N-(phosphonomethyl)glycine] is one of the widely used OPs and a broad-spectrum herbicide to control weeds (Mahendrakar et al., 2014). Glyphosate also known as Roundup, and consists of isopropyl amine salt of N-(phosphonomethyl) glycine and surfactant. Roundup is a target specific herbicide, inhibits the shikimic acid pathway, which is important for plant protein synthesis (Schonbrunnet *al.*, 2001). It is a water-soluble and its half-life period >35 day's therefore it has been applied in the field 2 or 3 times, depending upon the growth of weeds. Glyphosate herbicides are among the world's most widely used herbicides (Monsanto, 2002) and the Glyphosate is the world's leading agrochemical (Baylis, 2000). Even though glyphosate is considered a low-toxic herbicide, recent studies have revealed toxic effects resulting from even low-dose commercial formulations. Roundup showed significant decrease in the Hb% and TLC and highly significant increase in alkaline phosphatase (Dhanarajamet *al.*, 2010). According to Adam (2002) effect of Round-up show respiratory effect, blood-stained weeping from noses and diarrhea. In case of chronic

exposure many reported carcinogenicity. Farmers in fields repeatedly use Roundup/Glyphosate with the recurrence of weeds. Since herbicides are intended to kill plants, accumulation of herbicides may take place in the soil and it may percolate in nearby water. They get fall on crops may not killing or destroying but accumulate inside the leaves, grains or fruits, by consuming such product by birds or mammals indirectly or accidentally herbicides enter in body. As farmers are not aware about the bad effects of herbicides therefore unusual and repeated use of herbicides may cause adverse effect on human beings and animals (EPA.1996). The liver plays vital functions in vertebrates, including nutrient metabolism and detoxification processes (Elufioye and Habtemariam, 2019). It is widely recognized that the liver is the major organ affected by xenobiotic exposure, and, experimental studies have demonstrated that exposure to glyphosate itself may have a hepatotoxic potential and may cause liver injury (Soudani et al. 2019; Milić et al. 2018; Mesnage et al. 2017; Tizhe et al. 2020).

Plant species that provide medicinal herbs have been scientifically evaluated for their possible medicinal applications. *Moringa oleifera* (Moringaceae family) is considered as a highly nutritious plant with its medicinal values (Talreja, 2011). The leaves are an excellent source of vitamins (A, B, C), calcium, proteins, potassium, carotenoids - leutin, alpha- and beta-carotene, xanthine, chlorophyll and other phytochemicals with known powerful antioxidant property (Fuglie et al., 1998; Kasolo et al., 2010; Pandey, A et al., 2012).



Hence extracts from different parts of this plant have been used for the treatment of fever, bronchitis, eye/ear infections, inflammation of mucous membrane, diarrhea, gastric ulcer including anti-cancer, anti-inflammatory and hepatoprotective agents (Pareek et al., 2023). Recently, novel derivatives of thiocarbamates and nitriles which stimulate insulin release in animals have been found in the plant, explaining its anti-diabetic actions also (Fahey, 2005). This study was designed to determine the leaf extract of *M. oleifera* could restore Glyphosate induced Hepato toxicity in rats.

Materials and Methods

Preparation of Moringa leaf extract

Plant materials

Samples of *M. oleifera* leaves were collected from plantations in Tirupati, Andhra Pradesh, India and were also identified and authenticated by a plant taxonomist.

Preparation of extract

The leaves of the plant were cleaned thoroughly, dried in room temperature and crushed into coarse powder, from which 20 g was taken and soaked separately in 100 ml of water and chloroform by keeping it in a Shaker for 3 days. It was filtered through cheese cloth and reduced to 10% of its original volume (organic solvent). The filtrate was concentrated in vacuum using a rotary evaporator while aqueous extract was dried using water bath.

Procurement and maintenance of experimental animals

Healthy male rats were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk, and provided

filtered tap water and rat food (purchased from HLL Animal Feed, Bangalore, India) ad libitum in an air-conditioned environment ($25 \pm 2^\circ\text{C}$) with a 12-hour light and 12-hour dark cycle. All the animals were maintained at the animal facility available at Department of Biotechnology, Sri Padmavati Mahila Visvavidyalam, and Tirupati.

Experimental Design

Rats were divided into 4 groups with 6 rats in each group. Animals in the group 1 served as control, and rats in groups II administered with glyphosate alone at a dose of 500 mg/kg body wt. Group III administered with MO leaf extract alone at a dose of 500mg/kg body wt and group IV treated with both glyphosate and MO leaf extract orally for 55 days.

Necropsy

After completion of the experimental period (55 days), the rats from control and experimental groups were fasted overnight, weighed and humanely euthanized via cervical dislocation. Blood was drawn immediately via cardiac puncture using a heparinized syringe and serum was separated by centrifugation at 2000g for 15 min after overnight storage at 4°C . Serum was stored at -20°C until further hormone analysis. Rats were dissected, liver was collected and weighed to the nearest milligram by using Shimadzu electronic balance after clearing off the adhering tissues. Organ indices were determined by using the formula:

$$\text{TSI} = [\text{weight of the tissue (g)} / \text{Body weight of the animal (g)}] \times 100$$

Estimation of lipid peroxidation in Liver



Lipid peroxidation in Liver was estimated spectrophotometrically by measuring a break down product of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) was determined by the method of Ohkawa et al. (1979). Briefly, the tissues were homogenized (10% W/V) in 1.15% KCL solution. 0.5 ml of saline (0.9% sodium chloride), 1.0 ml of (20% W/V) trichloro acetic acid (TCA) was added to 2.5 ml of homogenate. Samples were centrifuged for 20 minutes at 4000 x rpm. 0.25 ml of TBA reagent was added to 1.0 ml of supernatant, and samples were then incubated at 95°C for 1 hour. Equal volumes of n-butanol were added to the supernatant and mixed, the contents were centrifuged for 15 minutes at 4000 rpm. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. The rate of lipid peroxidation was expressed as μ moles of malondialdehyde formed/gram wet weight of Liver tissue.

Assay of antioxidant enzymes in Liver **Superoxide dismutase (SOD) (EC 1.15.1.1)**

The activity of SOD was determined by the method of Mishra and Fridovich, (1972). SOD was assayed in the microsomal fraction according to its ability to inhibit the autooxidation of epinephrine at alkaline medium. Briefly, the Liver were homogenized (10% W/V) in 50 mM ice-cold sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,500 rpm for 60 min. The supernatant (cytosol) fraction was used for the assay of the enzyme activity. The reaction mixture contained 0.05 M carbonate buffer (pH

10.2), 30 mM epinephrine (freshly prepared) and the enzyme source. Changes in absorbance were recorded at 480 nm, measured at 10 seconds intervals for 1 minute in a spectrophotometer. The enzyme activity was expressed as Units/mg protein/min.

Catalase (CAT) (EC 1.11.1.6)

The activity of CAT was determined by based on its ability to decompose H_2O_2 , from the method of Chance and Maehly (1955). The reaction mixture contained (2.5 ml, vol) mixer of 2.4 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of Liver tissue homogenate and 10 μ l of 19 mM H_2O_2 . Then the absorbance was read at 240 nm. CAT activity was expressed as μ M of H_2O_2 consumed/min/mg protein.

Statistical analysis

All data were expressed as means $\pm$ S.D. and analyzed by analysis of variance (ANOVA) and Duncan's multiple range test using SPSS unpaired student's t-test. All data were reported as mean $\pm$ standard deviation (SD). $p < 0.05$ was considered significantly different.

Results and Discussion

The body weights of rats did not show any significant change in all the groups (Table 1). The liver tissue indices of control and experimental rats were presented in Table 1. No significant changes were observed in the indices of liver tissue of control and MO alone administrated rats; significant decrease was observed in glyphosate administrated rats. Whereas in co-treatment of MO along with glyphosate, significant changes were not observed in the Liver tissue of rats over controls. The present findings i.e. no changes in the body weights and liver



Table1: Protective Action of MO on the body weights and liver TSI of glyphosate-treated rats

Parameter	Control	Glyphosate	MO	Glyphosate +MO
Body weights	223.65 ± 1.13	204.05 ^{ns} ± 3.68 (-8.78)	225.92 ^{ns} ± 3.46 (1.01)	214.38 ^{ns} ± 3.55 (-4.14)
Liver	7.54 ± 0.91	4.19 ^{ns} ± 0.63 (-44.4)	7.82 ^{ns} ± 0.7 (3.71)	7.23 ^{ns} ± 0.47 (-4.11)

Values are mean ± S.D. of 6 individuals for each group

Values in parenthesis are percent change from that of control.

Mean values with different superscripts in a row differ significantly at $p < 0.05$.

Table 2: Protective Action of MO on the Lipid peroxidation and SOD, CAT of glyphosate-treated rats

Parameters	Control	MO	Glyphosate	Glyphosate + MO
LPX	4.56 ± 0.20	2.65* ± 0.28 (-41.88)	6.10* ± 0.18 (33.77)	3.60* ± 0.12 (-21.05)
SOD	0.25 ± 0.04	0.56* ± 0.03 (49.6)	0.33* ± 0.02 (32.00)	0.45* ± 0.01 (80.00)
CAT	22.67 ± 0.28	27.44* ± 0.4 (21.04)	17.98* ± 0.43 (20.68)	23.39* ± 0.33 (3.17)

indices indicated that the general metabolism of the animals in glyphosate treated appears to be normal. The levels of malondialdehyde were significantly ($p < 0.001$) increased in the liver tissue of glyphosate treated rats when compared to control rats (Table 2). A significant decrease ($p < 0.001$) in the activity levels of SOD, and CAT were observed in liver tissue of glyphosate treated rats when compared with control rats (Table 2). Co-administration of MO and Glyphosate significantly ($p < 0.001$) reduced levels the malondialdehyde with a significant increase in the activity levels of enzymatic antioxidants ($p < 0.001$); however, no

significant increase was observed in MO alone received rats (Table 2).

The present findings suggest that the glyphosate intoxication perturb antioxidant to prooxidant system thereby accelerate oxidative stress in the liver of rats. These events eventually cause hepatotoxicity in glyphosate treated rats. Studies of Djaber et al. (2022) suggested that exposure to glyphosate deteriorated the hepatic antioxidant system as evidenced by an increase of lipid peroxidation (malondialdehyde) and advanced oxidation protein product (AOPP). Also, the antioxidant defense system such as reduced



glutathione (GSH), glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) and also raised the levels of serum hepatic marker enzymes as evidenced by an increase in aminotransferases (ALT, AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), and total bilirubin (TB), along with reduced total protein content and albumin in both the brain and liver of rats. The present study also supports the findings of Bali et al. (2019), Cattani et al. (2017), de Souza et al. (2019) and Qi et al. (2023). Interestingly, the present results also suggest that the co treatment of rats with glyphosate and MO leaf extract exhibited deteriorated lipid peroxidation levels with a concomitant increase in the antioxidant enzymes in the liver of rats suggesting negation of oxidative damage induced by glyphosate by MO. The results are in agreement with Reda et al. (2023). The antioxidant potential of MO leaf extract could be due to the desirable nutritional contents, including vitamins, amino acids, minerals, and fatty acids, besides various types of antioxidants including flavonoids, ascorbic acid, glucsinolates, carotenoids, and phenolics.

To summate, as MO leaf extract possess antioxidant potential and could be used as a tool to mitigate the OP induced oxidative stress in rats. The rat model system used in the current study might be used by researchers and toxicologists to authenticate the antioxidant potential of plants and their products.

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BHAI-MS-7

Advancements in Biopharmaceuticals: Revolutionizing Medicine

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Abstract

Biopharmaceuticals represent a revolutionary approach to therapeutic drugs, using living organisms for their production. In contrast to chemically synthesized traditional pharmaceuticals, biopharmaceuticals offer advantages such as high specificity, targeted action, and reduced side effects. These drugs which are derived from living organisms closely resemble human proteins, minimizing the risk of immune responses. Biopharmaceuticals play a pivotal role in transforming medical treatments by introducing innovative therapies like monoclonal antibodies, gene therapies and cultured cell-based treatments. The increasing number of USFDA-approved biologics from 2015 to 2022 reflects their growing significance in addressing diverse medical conditions.

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Introduction

Biopharmaceuticals is also known as biologics, which are therapeutic drugs produced using living organisms, cells, or biological systems. Traditional pharmaceuticals are chemically synthesized whereas Biopharmaceuticals are derived from living organisms. Conventional pharmaceuticals treat medical conditions, but they have drawbacks. These drawbacks include side effects, the risk of drug resistance, non-specific targeting affecting unintended areas, limited efficacy in addressing underlying causes, and complex, expensive manufacturing processes.

Biopharmaceuticals have revolutionized medical treatments by possessing the advantages like high specificity and targeted action, reduced side effects compared to traditional drugs and potential for personalized medicine. These are produced through biotechnological processes involving recombinant DNA technology, cell cultures, and other advanced techniques. Biopharmaceuticals target diseases precisely and minimize harm to healthy cells and reduce side effects compared to traditional drugs. They come from living organisms and closely resemble human proteins to decrease the risk of immune responses. Biopharmaceuticals use biological diversity to explore new treatment options, and their flexible design allows personalized medicine for better effectiveness. They also introduce new approaches to challenging diseases through innovative therapies like monoclonal antibodies, gene therapies, and cultured cell-based treatments. The common types of biopharmaceuticals include monoclonal

antibodies, vaccines, gene therapies, growth factors, and cytokines.

From 2015 to 2022, the U.S. Food and Drug Administration (USFDA) approved the following number of biologics each year: 2015 (13), 2016 (7), 2017 (13), 2018 (17), 2019 (12), 2020 (15), 2021 (14), and 2022 (15), totaling 106 approved biologics during this period. A few examples of USFDA-approved biopharmaceuticals are discussed here.

Vaccines:

During the ongoing global catastrophe of the COVID-19 pandemic, vaccines played a crucial role in mitigating the virus's impact and providing hope for control and prevention. In India, Covishield and Covaxin were widely administered, contributing to local efforts. Globally, vaccines from Pfizer-BioNTech, Moderna, Johnson & Johnson, and Sinovac combated the virus's spread. Beyond COVID-19, various vaccines, such as Recombivax HB for hepatitis B, Shingrix for shingles prevention, Prevnar 13 for pneumococcal disease, Fluzone for seasonal influenza, and Gardasil 9 for human papillomavirus (HPV), prevents a range of diseases, showcasing the broader impact and versatility of vaccines in public health efforts.

Interferons:

Interferons are proteins with antiviral properties which are used in treating viral infections and specific cancers. Interferon-A, targets hairy cell leukemia, AIDS-related Kaposi's sarcoma, and chronic hepatitis C by modulating the immune response. Interferon A is prescribed for melanoma, chronic hepatitis B and C, and certain leukemias. It activates the immune system for antiviral



and anti-proliferative effects. Pegasys, a PEGylated form of interferon alfa-2a, is widely used for chronic hepatitis C. It offers extended half-life, reduced dosing frequency, and maintained effectiveness against the hepatitis C virus.

Erythropoietin:

Erythropoietin (EPO) stimulates red blood cell production, and its applications include treating anemia, particularly in patients with chronic kidney disease. Epoetin alfa (Epogen, Procrit) addressed anemia related to chronic kidney disease, chemotherapy, and other conditions by stimulating red blood cell production in the bone marrow. Darbepoetin alfa (Aranesp) served a similar purpose with an extended half-life compared to Epoetin alfa, allowing less frequent dosing. Epoetin beta (Mircera) is approved for anemia associated with chronic kidney disease. It operates as a continuous erythropoietin receptor activator with a prolonged duration of action, reducing the necessity for frequent administration.

Cytokines:

Cytokines are crucial signaling proteins in immune response regulation. They are prescribed in treating certain cancers and autoimmune diseases. As Interferon alfa-2a (Roferon-A) has proven antiviral and antiproliferative effects, it is utilized for various cancers like hairy cell leukemia and melanoma, as well as certain viral infections. Interferon alfa-2b (Intron A) is prescribed for chronic hepatitis B, hepatitis C, melanoma, and specific leukemias. Interleukin-2 (Proleukin) stimulates the immune system to activate and proliferate T- Lymphocytes for targeting cancer cells

hence is employed for metastatic melanoma and renal cell carcinoma.

Growth Hormones:

Recombinant human growth hormones are vital for growth disorders in children and hormone deficiencies in adults. Somatropin (Genotropin, Humatrope, Norditropin, Saizen) is used for both pediatric and adult growth hormone deficiencies. These hormones stimulate growth by enhancing insulin-like growth factor-1 (IGF-1) production. Omnitrope, another somatropin product promote linear growth through cell growth and regeneration. For adults with growth hormone deficiency, Somapacitan (Sogroya) is administered a once-weekly. This growth hormone receptor agonist with sustained release, reduce the frequency of injections compared to daily growth hormone therapies.

Enzyme Replacement Therapies:

Biopharmaceuticals provide functional enzymes for individuals with enzyme deficiencies, such as in lysosomal storage disorders. Laronidase (Aldurazyme) is used to treat patients with Hurler syndrome, a form of mucopolysaccharidosis. Laronidase helps replace the deficient enzyme alpha-L-iduronidase. Alglucosidase alfa (Myozyme, Lumizyme) is approved for the treatment of Pompe disease, a lysosomal storage disorder. It replaces the deficient acid alpha-glucosidase enzyme, which decreases the accumulation of glycogen in muscles. Patients with Fabry disease, an X-linked genetic disorder is treated by Agalsidase beta (Fabrazyme) in enzyme replacement therapy. Agalsidase beta helps replace the deficient alpha-galactosidase A enzyme,



reducing the accumulation of globotriaosylceramide.

Fusion Proteins

Fusion Proteins are engineered by combining the properties of two different proteins, which are particularly used in cancer treatment. Etanercept (Enbrel) is a tumor necrosis factor (TNF) inhibitor which is used for autoimmune conditions like rheumatoid arthritis. This synthesized by combining the extracellular domain of the TNF receptor with the Fc portion of human IgG1. Abatacept (Orencia) is a fusion protein composed of the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the Fc portion of human IgG1 which is prescribed for the treatment of rheumatoid arthritis. Alefacept (Amevive) is a fusion protein for treating moderate to severe plaque psoriasis. It is prepared by combining the extracellular domain of lymphocyte function-associated antigen-3 (LFA-3) with the Fc portion of human IgG1.

Clotting Factors

Clotting Factors are vital in preventing excessive bleeding, are used to replace or supplement different deficient factors in individuals with hemophilia. Hemophilia A - is characterized by a deficiency of clotting factor VIII. Octocog alfa (Advate) is a recombinant factor VIII employed for treating hemophilia A. In a similar manner Hemophilia B is caused by a deficiency of clotting factor IX. It can be treated by Alprolix which has recombinant clotting factor IX with an extended half-life.

Conclusion

Though Biopharmaceuticals play a crucial role in modern medicine they face several

challenges like high development costs, complex manufacturing processes, and sometimes potential immunogenicity issues. These factors pose challenges of bringing biopharmaceutical products to market. Despite these challenges, the future of biopharmaceuticals is marked by promising trends. There is a growing emphasis on personalized medicine, tailoring treatments to individual patients based on their unique characteristics. Additionally, ongoing advancements in gene and cell therapies are expected to play a significant role in shaping the landscape of biopharmaceutical innovations, offering targeted and more effective approaches to disease treatment.

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BHAI-MS-8

Application of Biotechnology in Vaccine Development-A Review

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Abstract

Vaccine is an antigenic preparation that contains pathogenic substances which resemble disease causing microbe and is often made of inactivated microbes, live attenuated microbes, its toxins or a part of surface antigens. Vaccine administration is called vaccination that stimulates the immune system and makes the person resistant to a particular pathogen. Today six different types of vaccines are used. They are killed infectious organism called inactivated vaccines, live attenuated vaccines, naturally occurring infectious organisms which are closely related to pathogens, subunit vaccines, cell based and virus-based vaccines & DNA vaccines. Biotechnology has revolutionized the field of vaccination. The techniques of modern biotechnology such as genetic engineering and cell culture enable an effective, quick and economical development of vaccines. Recombinant DNA technology enables antigen of a certain pathogen to be produced in a host cell like E. coli or yeast which is relatively nonpathogenic. New vaccines employing biotechnological innovations are helping us to change the way for illness prevention. There is also an ongoing development on gene-based vaccines (DNA or RNA) from pathogens (including that of SARS-CoV-2) so that the production of antigen can occur in the bodies of vaccine receivers.

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Introduction

Vaccine is an antigenic preparation that contains pathogenic substances which resemble disease causing microbe and is often made of inactivated microbes, live attenuated microbes, its toxins or a part of surface antigens. Vaccination also called as immunization is the process of administration of vaccine. It is considered as the most effective method for disease prevention and control, saving millions of lives every year. Vaccines minimize the risk getting a disease by working with our body's natural defenses to build protection. We now have vaccines to prevent more than 20 life threatening diseases, helping people of all ages live longer and healthier lives. Vaccination prevents more than 3.5-5 million deaths every year from life threatening diseases like diphtheria, tetanus, pertussis, influenza and measles. In any country vaccination is a key component of primary healthcare and an indisputable human right. Vaccines are also vital to the prevention and control of infectious disease outbreaks. They safeguard global health security and will be a vital tool in the battle against infectious agents.

Types

Generally, six different types of vaccines are available today:

- Inactivated vaccines
- Live-attenuated vaccines
- Messenger RNA (mRNA) vaccines
- Subunit, recombinant, polysaccharide and conjugate vaccines.
- Toxoid vaccines
- Viral vector vaccines.

Method of action

Vaccination is carried out by various ways such as given by injection, oral, intranasal and subcutaneous administration. Once administered vaccine stimulates the immune system to detect a particular pathogen by producing antibodies and memory cells. As a result, the immune system is able to mount a defense if the person ever becomes infected.

Application of Biotechnology

Biotechnology is the technological usage of biological organisms, systems and processes to develop, make or modify products for specific applications like medicines, crops and livestock. It involves wide range of procedures, where living organisms are modified for human purposes. Modern biotechnology includes genetic engineering and cell culture techniques which enable an effective, quick and effective development of vaccines. Development of vaccines is closely related to biotechnology. Recombinant DNA technology enables the production of an antigen in a non- pathogenic host cell like E. coli or yeast, so that a direct harvest from the original pathogen is not required.

Recombinant sub-unit vaccines

A subunit vaccine contains purified pathogenic parts which are antigenic and can stimulate immune system. Recombinant subunit vaccines are made of protein or glycoprotein components of a pathogen and produced by Recombinant DNA technology. They are effective in inducing humoral and cell mediated immune response. Recombinant Hepatitis B surface antigen (HBsAg) was the first recombinant vaccine developed using cloning techniques. HPV vaccine is also



sub-unit vaccine composed of virus like particles made from the major L protein of Human Papilloma Virus responsible for cervical carcinoma in women. These vaccines can be used even on people with weakened immune system and long-term health problems. But these vaccines need booster doses to continue ongoing protection against the disease.

Recombinant protein vaccines

After infection, the pathogen produces proteins to elicit an immune response from the host body. The gene encoding the protein is isolated from the pathogen and used to construct a recombinant DNA which is injected to express in a heterologous cell such as bacteria, yeast. Cholera vaccine, diphtheria vaccine and tetanus vaccines are recombinant protein vaccines which are made of proteins or toxin antigens are produced in another host organism or purified from large number of pathogens.

mRNA vaccines

mRNA vaccines make proteins to initiate immune system. mRNA vaccines have several benefits compared to other types of vaccines like shorter manufacturing period and no risk of causing a disease in the vaccinated person. Recent covid vaccines are mRNA vaccines. There are three types of mRNA vaccines. They are

Non-Replicating mRNA: The mRNA is injected in body where it is taken up by cells and antigen is expressed.

In-vitro Dendritic Cell Non-Replicating mRNA Vaccine: Dendritic cells present antigen on their surface for immune cells which produce immune response. In this type of vaccine dendritic cells are extracted

and transfected invitro with antigen and reinjected into patient to stimulate immune system.

In-Vivo Self-Replicating mRNA: In this method pathogen mRNA is packed with additional mRNA to make sure it is copied inside cell.

Currently a great deal of research is going on in the field of mRNA vaccine for cancer as well as infectious diseases like AIDS.

DNA Vaccines

Deoxyribonucleic acid (DNA) vaccines are genetically engineered DNA, which when injected produce antigen and initiate a strong immune reaction. The gene responsible for immunogenesis is identified, cloned and then expressed in host cell by directly injecting it. DNA vaccines have tremendous potential than the other vaccines. They hold a very promising future in the immunization but till date no DNA vaccine for humans has been approved by FDA.

Benefits of Recombinant vaccines

Recombinant vaccines do not contain actual pathogens but only a part of it like DNA, RNA or protein, which are used in making vaccines. Thus, they are safer compared to conventional vaccines and people with weakened immune system can also be vaccinated. Moreover, recombinant vaccines induce both humoral and cell mediated immune response resulting in more effective vaccination.

Disadvantages

Complex vaccination schedule is the downside of recombinant vaccines, because they are produced by using parts of microbes such as DNA, RNA or protein. Therefore, it is necessary to have booster



doses for maximum immune responses or to boost response that fade over time. Another challenge is research and development of vaccines using biotechnologies is risky, costly and time taking. Most pharma companies and vaccine manufacturers have very minimal incentives to develop vaccines based on biotechnologies because of limited revenue.

Conclusion

The application of biotechnology in vaccination has revolutionized the field of medicine and public health, offering innovative solutions to prevent and control infectious diseases. In conclusion, the application of biotechnology in vaccination has transformed the landscape of preventive medicine. It has not only improved the efficiency and safety of vaccines but also opened up new possibilities for addressing global health challenges. Continued research and ethical considerations will be crucial in harnessing the full potential of biotechnology for the development and deployment of vaccines in the future.

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BHAI-MS-9

Exploring The Potential of mRNA - A Comprehensive ReviewNagendra Prasad S V¹, Krishna Kumar V², Srinivas N³ and Chakrapani I S⁴**Abstract**

The success of mRNA vaccine technology in combating COVID-19 has ignited exploration for broader applications against various diseases. This versatile method allows scientists to target any virus by inserting codes for specific viral proteins, accelerating vaccine development. Over the past two decades, RNA-based technologies, including mRNA vaccines, have demonstrated safety and enduring immune responses in trials for diseases such as influenza, ebola, and SARS. The existing mRNA vaccine framework opens new possibilities for swift and efficient development against various infectious threats. mRNA vaccines offer a promising alternative to traditional approaches due to their potency, rapid development capability, and potential for cost-effective production and safe administration. This review provides a comprehensive look at mRNA vaccines, exploring future directions and challenges in advancing this promising vaccine platform for widespread therapeutic use.

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Introduction:

In recent years, mRNA vaccines have emerged as groundbreaking technology for rapid and targeted vaccine development against infectious diseases (Pardi et.al., 2018; Hogan and Pardi 2022). First proposed in 1990 by Wolff et.al., 1990 (42) mRNA vaccines have undergone a 30-year developmental journey, utilizing the body's cellular machinery to produce specific antigens and trigger immune responses (Kariko et.al., 2015). In contrast to traditional vaccines, mRNA vaccines offer advantages such as rapid development and production, with the ability to be designed and synthesized in weeks. Notably, they have demonstrated success against SARS-CoV-2 (Pardi et al., 2020, Polack et.al., 2020; Baden et.al., 2021). The modular nature of mRNA vaccines allows easy adaptation to different pathogens by adjusting the mRNA sequence (Li et.al., 2016; Svitkin et.al., 2017; Wojtcjak et.al., 2018). However, challenges in efficient delivery to target cells, susceptibility to degradation, limited stability, and barriers to reaching desired action sites exist (Tsui et.al., 2002; Kaczmarek et.al., 2017). Researchers are expanding the use of mRNA vaccines beyond COVID-19 to combat other infectious diseases like influenza, Zika, rabies, dengue, and malaria, showing promise in early animal tests. This review thoroughly explores mRNA vaccines, highlighting their production, stability, synthesis, and delivery methods. It convincingly demonstrates how these vaccines outperform traditional ones, emphasizing their importance in modern medicine and

showcasing the technological strides in their development.

Background:

mRNA vaccines gained attention during the COVID-19 pandemic upon approval for public use, but their groundwork began in the early 1990s. Feasibility became evident in the 2000s, with the first mRNA vaccine for prostate cancer, 'Provenge', approved in 2013. mRNA's immune-boosting capability against cancer paved the way for applications to infectious diseases, exemplified by an MERS vaccine in 2013. The pivotal breakthrough for mRNA vaccines occurred in late 2019 during the COVID-19 pandemic, with the swift approval of Pfizer-BioNTech and Moderna vaccines in December 2020, highlighting their efficacy (Plotkin 2014). Years of dedicated research positioned mRNA as a formidable tool in disease confrontation.

The Excitement Around mRNA Technology:

mRNA vaccines stand out for their remarkable speed and adaptability, epitomized by Moderna's SARS-CoV-2 mRNA vaccine, which entered clinical trials in a mere 63 days after the virus genome's publication. In stark contrast, the HPV vaccine, developed using recombinant DNA technology, endured a lengthy 15-year journey. The key differentiator is mRNA's agility. Traditional vaccine methods involve time-consuming processes like growing and weakening viruses, while mRNA vaccines provide a swift alternative. Oxford's 100-day vaccine team further underscores this agility, aiming to create



vaccines within just 100 days of identifying a new pathogen. As per the NIH, this underscores the accelerated pace made possible by the groundbreaking technology of mRNA.

mRNA Vaccines: Advancements Over Traditional Vaccines:

Traditional vaccines rely on weakened or inactivated germs to stimulate an immune response. In contrast, mRNA vaccines utilize genetically engineered molecules to instruct cells in producing harmless fragments of viral proteins. This prompts the immune system to generate antibodies, effectively neutralizing the targeted viral protein. According to NHS study, the advantages of mRNA vaccines lie in their rapid development, safety, and efficacy. They are non-infectious, degrade naturally, and being fully synthetic, allow for cost-effective manufacturing. The mRNA platform proves crucial in developing variant-proof, safe, and effective vaccines, ensuring accessibility and safety for mass vaccination (Plotkin S and Plotkin S, 2011).

The Rise of mRNA Technology:

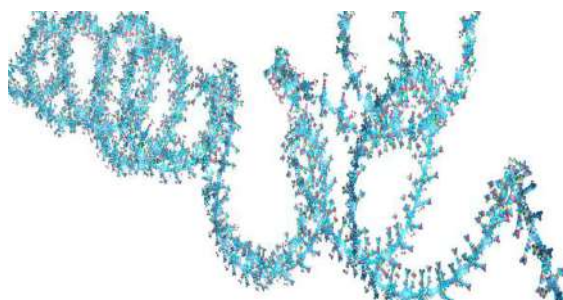
mRNA technology gained prominence during the Covid-19 pandemic, marking its first milestone with a candidate entering phase I clinical trials. Early experiments in 1990 showcased coding mRNAs potential to express proteins in mouse skeletal muscle. Over the subsequent decade, studies confirmed mRNA's ability to elicit immunologic responses across various mammalian cell types. What sets this technology apart is its precise triggering of the immune system to respond to specific threats, activating both arms of the immune

system for a robust defense. Unlike DNA vaccines, mRNA is more effective and safer, posing very low risk of causing genetic changes. The temporary effect of mRNA, coupled with its adaptable nature, allows for consistent manufacturing, irrespective of changes in the target virus. The use of in vitro cell-free transcription minimizes safety concerns compared to other vaccine methods (Schlake et.al., 2012). mRNA, acting as a messenger, provides instructions to cells for protein production, aiding in the fight against diseases like influenza, Ebola, and SARS. This flexible technology holds promise in combating viruses and advancing medical research (Pascolo S 2004).

Understanding mRNA: A Blueprint for Cellular Defense:

mRNA, or messenger RNA, serves as a molecular guide, furnishing instructions for cellular protein production. Encapsulated in a protective lipid nanoparticle, it smoothly enters cells. Inside, cells interpret mRNA instructions to construct proteins mimicking pathogen components, known as antigens. The immune system recognizes these foreign antigens as threats, mobilizing antibodies and T-cells for defense and training against potential future attacks. When faced with the actual virus, the body's recognition sounds the alarm, aiding in defense against infection and illness. The innovative use of mRNA as a blueprint for cellular defense signifies a paradigm shift in vaccine technology, offering enhanced safety, adaptability, and efficacy.





mRNA (image source: <https://www.pfizer.com/science/innovation/mrna-technology>)

Messenger RNA Vaccines: Transforming Medicine's Approach

mRNA vaccines revolutionize medicine by leveraging the body's natural processes for immune response, eliminating the need for live or inactivated viruses. This pioneering technology allows for a rapid and targeted reaction, exemplified by the successful response to the challenges of the COVID-19 pandemic.

How do the mRNA vaccines work?

The mRNA in vaccines provides cells with instructions to produce a viral protein fragment. The immune system responds by generating antibodies, which persist in the body, ready to identify and neutralize the virus if encountered. This process offers rapid protection without exposing individuals to the actual virus.

The Manufacturing Process of mRNA Vaccines: A Step-by-Step Overview

The production of mRNA vaccines involves a meticulous, multi-step process designed to generate an effective immune response. Scientists begin by identifying a

target protein from the pathogen, such as the spike (S) protein in the case of SARS-CoV-2 (1). This protein is selected based on its dissimilarity to human cells, ensuring the initiation of specific immune responses against the pathogen.

Once the target protein is identified, its gene sequence is inserted into a plasmid, a process executed in host bacteria, typically *Escherichia coli*, using a fermentor. The resulting plasmid DNA undergoes purification and linearization. The plasmids, adept at replicating gene sequences, facilitate efficient production.

In a cell-free in vitro transcription (IVT) process, the plasmid DNA serves as a template for mRNA synthesis. Enzymes and nucleotides are employed to produce mRNA, with additional steps involving specific enzymes to cap and stabilize the material. Once a sufficient amount of mRNA is generated, the plasmid DNA is broken down, ensuring that only mRNA is included in the vaccine.

Due to mRNA's susceptibility to degradation, a lipid nanoparticle (LNP) or another suitable delivery system is developed to encapsulate and transport the mRNA to cells. The purified and sterile-filtered mRNA is encapsulated, and final formulation activities result in the vaccine drug product. Upon administration, the encapsulated mRNA is taken up by recipient cells, directing them to produce the SARS-CoV-2 S protein, thereby eliciting a robust immune response, including the production of antibodies.



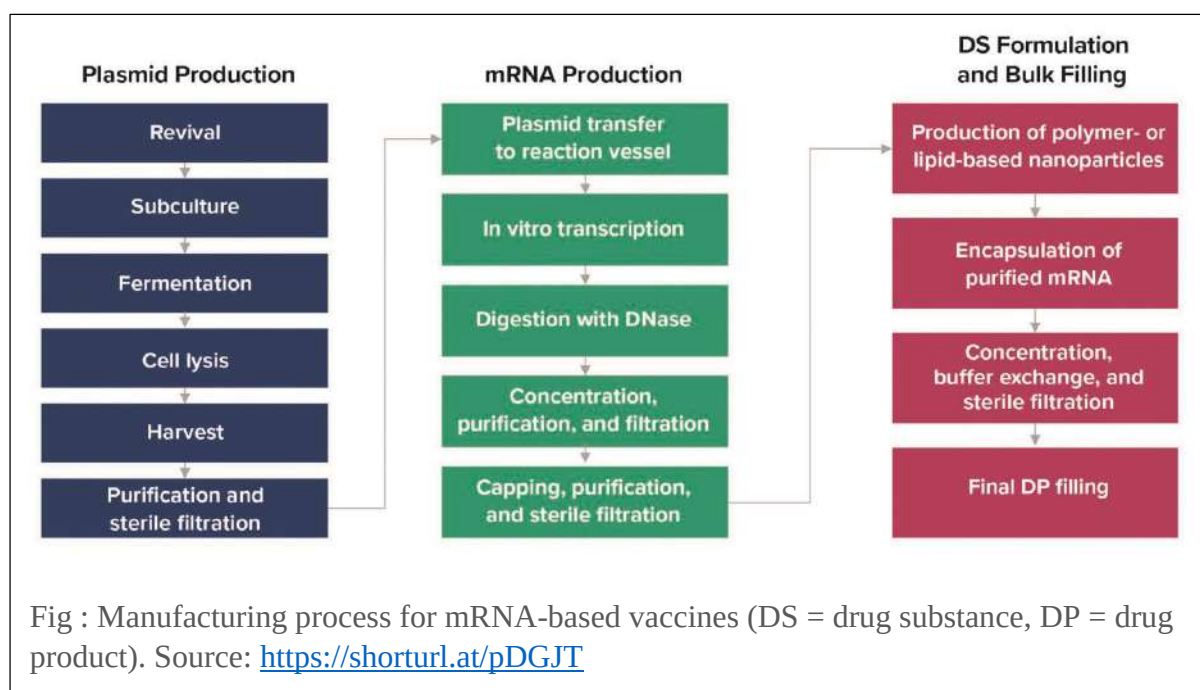
Revolutionizing Medicine: The Impact of mRNA Vaccines

mRNA vaccines spearhead a transformative era in medical technology, playing a pivotal role in the battle against COVID-19 and offering promising applications across various medical fields. Exemplified by leading COVID-19 mRNA vaccines from BioNTech-Pfizer and Moderna, these vaccines showcase remarkable efficacy against infection and a remarkable ability to adapt swiftly to new variants. Moderna's feat of developing a variant-targeted vaccine in just 30 days underscores the unparalleled flexibility of this technology (Hogan et.al., 2023).

establishment of regional mRNA vaccine production capacity not only contributes to the ongoing battle against COVID-19 but also positions communities to address a myriad of health challenges in the future.

Streamlining mRNA Manufacturing: A Swift and Scalable Process

The production of mRNA for medicines and vaccines is marked by its simplicity, scalability, and remarkable speed. The process involves key steps where considerations are made for quality and testing requirements. In a laboratory setting, mRNA is synthesized using the in vitro transcription (IVT) process. What sets



Beyond their success in countering COVID-19, mRNA vaccines demonstrate adaptability by easily tailoring to different pathogens. This inherent flexibility opens avenues for producing diverse vaccines and therapeutics on a unified platform. The

this method apart is its avoidance of dealing with cells or host cell proteins, a departure from older methods. Crucially, the mRNA manufacturing process can employ the same materials and equipment for different applications. This adaptability enables



facilities, especially those adhering to strict manufacturing rules like Good Manufacturing Practice (GMP) facilities, to swiftly transition to producing new medicines or vaccines with minimal adjustments to the process. It represents an intelligent and efficient approach to mRNA production.

mRNA Manufacturing Simplified:

1. Making the pDNA Template:

- Design and purity of the pDNA template are crucial for mRNA optimization.
- pDNA is amplified within *E. coli*, purified, and linearized for use as a template.
- Impurities are removed using methods like tangential flow filtration (TFF) and chromatography, crucial for GMP production.

2. In Vitro Transcription (IVT):

- Linearized pDNA is transcribed into mRNA using RNA polymerase and nucleotide triphosphates.
- Addition of a 5' cap structure is essential for stability and efficient cell transduction.
- Co-transcriptional capping is faster but less efficient; enzymatic capping offers higher efficiency.

3. Purifying mRNA:

- Purification eliminates unwanted materials like salts, cap analogs, proteins, and residual DNA.
- Process chromatography is commonly used for commercial-scale mRNA purification (Heartlein et.al., 2014).

4. Choosing mRNA Delivery System:

- Lipids or polymers are used for mRNA delivery, with some systems combining both.
- The delivery system significantly impacts the effectiveness of mRNA vaccines and therapeutics.

5. Quality Control and Release:

- Quality control testing includes capping efficiency, poly A tail length, and residual plasmid DNA.
- Testing requirements vary based on the final mRNA application and clinical usage.

Streamlining these steps ensures efficient and scalable mRNA manufacturing, a critical aspect in the development of mRNA-based therapeutics and vaccines.

How Do mRNA vaccines Work?

mRNA vaccines, such as those developed for COVID-19, work by leveraging the genetic material messenger RNA (mRNA) to instruct cells in the body to produce a viral protein—in this case, the spike protein of the SARS-CoV-2 virus. Here's an elaborate breakdown of the process:

mRNA in Lipid Nanoparticles:

The vaccine contains strands of mRNA that encode the genetic instructions for making a fragment of the virus, usually the spike protein.

These mRNA strands are encapsulated in tiny lipid nanoparticles to protect them and facilitate their entry into cells.

Cell Entrance and Translation:

Upon injection, the lipid nanoparticles deliver the mRNA into the cells of the body, typically muscle cells near the injection site.



Once inside the cell, the mRNA serves as a blueprint, providing instructions to the cellular machinery on how to build the viral spike protein.

Spike Protein Expression:

The cellular machinery reads the mRNA and begins synthesizing the viral spike protein based on the provided instructions. Importantly, only a fragment of the virus is produced, not the entire virus. This means there is no risk of causing the disease.

Immune Response Activation:

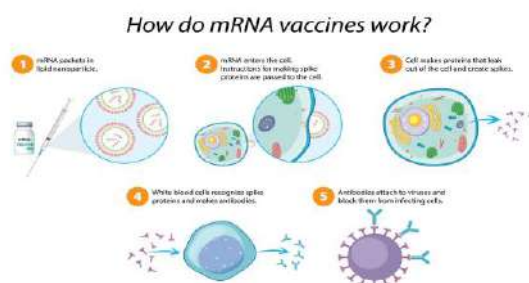
The newly synthesized spike protein is displayed on the surface of the host cell. The immune system recognizes this foreign protein as an invader and triggers an immune response involving T lymphocytes (cell-mediated immunity) and B lymphocytes (antibody-mediated immunity).

T and B Lymphocytes Activation:

T lymphocytes, specifically cytotoxic T cells, recognize and destroy cells displaying the viral protein. B lymphocytes produce antibodies against the spike protein.

Proprietary Antibodies:

The generated antibodies are specific to the viral spike protein. These antibodies are crucial for recognizing and neutralizing the actual virus if the vaccinated person is later exposed.



(Image source: <https://shorturl.at/aowJO>)

The triumph of mRNA vaccine technology in countering COVID-19 not only marks a significant milestone but also paves the way for its application across a wide spectrum of diseases. The intrinsic structure and delivery mechanism of mRNA provide a foundational blueprint for addressing various viruses. Scientists can seamlessly embed the code for precise viral proteins, instigating a prompt immune response. With the technology firmly established, the prospect of accelerated vaccine development becomes substantial. Human trials for mRNA vaccines, targeting diseases like HIV, Zika, and rabies, are already in progress, signaling a new era in medical advancement.

Applications of mRNA Vaccines:

1. Infectious Diseases and HIV:

RNA vaccines are at the forefront of research, with ongoing exploration in infectious diseases and cancer. While early-stage clinical trials investigate the potential of RNA vaccines for allergies, the broader applications extend to diseases beyond COVID-19. Trials are underway for different SARS viruses, MERS, hepatitis C, HIV, influenza, malaria, and tuberculosis. Moderna is even pioneering an adaptive mRNA vaccine for influenza. Notably, researchers from multiple institutions are progressing through early-stage clinical trials for mRNA-based HIV vaccine candidates, marking a transformative shift in vaccine development post-COVID-19(2,4,5,)

2. mRNA Vaccines in Cancer Treatment:



Beyond prevention, mRNA vaccines emerge as treatments for various cancers, conveying crucial information about tumors to kickstart the body's defense against cancer cells. Ongoing tests assess their efficacy and safety across different cancer types, including melanoma, brain, lung, ovarian, prostate, blood, digestive system, and breast cancers. Developing personalized mRNA vaccines poses challenges, but researchers are exploring combinations with anti-cancer drugs, like pembrolizumab, in ongoing tests, particularly for melanoma and glioblastoma.

Advantages of mRNA Vaccines:

1. Safety:

Non-infectious Particles: mRNA vaccines eliminate infection risks by excluding live or inactivated pathogens.

Limited Persistence: Swift degradation of mRNA after translation minimizes its presence within cells, reducing the risk of prolonged effects.

2. Efficacy:

Targeted Immune Response: Tailored for specific pathogen proteins, mRNA vaccines enhance efficacy by triggering a precise immune response.

3. Production:

Rapid, Inexpensive, and Scalable: mRNA vaccine production is swift, cost-effective, and scalable for large-scale manufacturing, thanks to high yields in in vitro transcription reactions.

Disadvantages:

1. Temperature Sensitivity:

Instability at High Temperatures: mRNA vaccines face challenges in packaging and distribution due to instability at high temperatures, impacting long-term storage and delivery, especially in remote areas.

2. Infrastructure Challenges:

Global Investments Required: Addressing temperature sensitivity demands substantial global investments in infrastructure, workforce training, and precise last-mile coordination.

3. Long-Term Uncertainty:

Extended Impact Uncertain: Despite positive outcomes in clinical trials and initial studies, the long-term effects of mRNA vaccines remain uncertain, necessitating ongoing monitoring and research for a comprehensive understanding.

Future Applications of mRNA Technology:

The triumph of mRNA vaccine technology in countering COVID-19 not only marks a significant milestone but also paves the way for its application across a wide spectrum of diseases. The intrinsic structure and delivery mechanism of mRNA provide a foundational blueprint for addressing various viruses. Scientists can seamlessly embed the code for precise viral proteins, instigating a prompt immune response. With the technology firmly established, the



prospect of accelerated vaccine development becomes substantial. Human trials for mRNA vaccines, targeting diseases like HIV, Zika, and rabies, are already in progress, signalling a new era in medical advancement.

Conclusion:

In summary, mRNA vaccines are a game-changer in medicine. They go beyond just preventing diseases; they have the potential to revolutionize global healthcare. Continuous improvements, like making them more affordable and efficient, are underway. Innovative methods, such as continuous production using microfluidics, show a commitment to making these vaccines more accessible. In the future, mRNA vaccines could make medical treatments not only better but also available to more people around the world.

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BHAI-MS-10

Monoclonal Antibodies: Revolutionizing Modern Therapies

Nagaraju K^{1*}, Anitha N², Lalitha Kumari Ch³

Abstract

Monoclonal antibodies (mAbs) have revolutionized the treatment landscape for various diseases, offering targeted and effective therapeutic interventions. In recent years more potent, versatile, and humanized antibodies have been produced and used for different purposes including in therapy. Scientists have engineered mAbs with human-like structures to minimize immunogenicity and improve compatibility with the human immune system. mAbs are combined with specific drugs {Antibody-Drug Conjugates (ADCs)} to deliver at specific sites in the body. Bispecific mAbs can simultaneously bind to two different antigens, enhancing their therapeutic potential and expanding their applications. Multi-specific mAbs can target multiple epitopes on a single antigen or engage multiple antigens simultaneously, leading to increased efficacy and broader therapeutic applications. The global market for monoclonal antibodies is experiencing remarkable growth, driven by their effectiveness, specificity, and expanding applications in the treatment of a wide variety of diseases. The present work reviews the latest developments in MAB technology.

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Introduction:

Monoclonal antibodies have emerged as a game-changing therapeutic option in the modern healthcare landscape. Their unique properties and versatile applications have significantly advanced treatment options for a wide range of diseases.

Monoclonal antibodies are laboratory-produced molecules engineered to mimic the immune system's ability to fight off harmful pathogens such as bacteria, viruses, and other foreign substances. These antibodies are designed to specifically target and bind to unique molecules known as antigens, which are typically found on the surface of cells. This targeted approach makes monoclonal antibodies valuable in various therapeutic and diagnostic applications.

The production of monoclonal antibodies involves several key steps:

1. **Antigen Selection:** The first step in monoclonal antibody production is selecting the specific antigen to target. This could be a protein associated with a particular disease or a marker present on abnormal cells.
2. **Immunization:** Animals, typically mice, are then immunized with the chosen antigen to stimulate an immune response. This prompts the production of a diverse range of antibodies against the antigen.
3. **Cell Fusion:** The next step involves isolating immune cells, typically B lymphocytes, from the immunized animal and fusing them with

myeloma cells, a type of cancer cell that can continuously divide.

4. **Screening and Cloning:** The resulting fused cells, called hybridomas, are screened to identify those producing antibodies that specifically bind to the target antigen. These selected hybridomas are then cloned to produce identical cells, ensuring a consistent supply of monoclonal antibodies.
5. **Harvesting and Purification:** The monoclonal antibodies produced by the cloned hybridoma cells are harvested and purified to remove any other cellular components, resulting in a highly concentrated and pure antibody solution.

The production process is carefully controlled to ensure the monoclonal antibodies are both effective and safe for use in various medical applications, including cancer therapy, autoimmune diseases, and infectious diseases. This precision in production has contributed to the significant advancements and widespread utilization of monoclonal antibodies in modern medicine.

Types of monoclonal antibodies**1. Murine Monoclonal Antibodies**

They are derived from mice. Murine mAbs are the earliest type of mAbs developed. They are produced by immunizing mice with a specific antigen and then fusing their B cells with myeloma cells to create hybridoma cells. These hybridoma cells can continuously produce mAbs against the target antigen.



2. Chimeric Monoclonal Antibodies

These are Combination of mouse and human components. Chimeric mAbs are engineered by fusing the variable regions of a mouse mAb, responsible for antigen binding, with the constant regions of a human antibody. This approach aims to retain the specificity of the mouse mAb while reducing its immunogenicity in humans.

3. Humanized Monoclonal Antibodies

These are predominantly human components, with minimal mouse sequences. Humanized mAbs are further refined by replacing most of the mouse sequences in chimeric mAbs with human sequences, except for the antigen-binding regions. This modification minimizes the risk of an immune response against the mAb in humans.

4. Fully Human Monoclonal Antibodies

This type of Antibodies are entirely derived from human sources. Fully human mAbs are generated using various techniques, such as phage display libraries, transgenic mice, or human B cell immortalization. These mAbs have the least immunogenicity and are preferred for therapeutic applications in humans.

5. Bispecific Monoclonal Antibodies

These are engineered from two different mAbs. Bispecific mAbs are designed to bind to two different antigens simultaneously. They are constructed by combining the variable regions of two mAbs, allowing them to target two distinct molecules or epitopes. Bispecific mAbs

have potential applications in cancer immunotherapy and targeted drug delivery.

6. Multispecific Monoclonal Antibodies

These Antibodies are engineered from three or more mAbs. Multispecific mAbs are similar to bispecific mAbs but can bind to three or more antigens simultaneously. They are more complex to engineer and have potential applications in targeting multiple disease pathways or cell types.

The selection of a particular type of mAb depends on factors such as the target antigen, desired specificity, immunogenicity concerns, and the intended therapeutic or diagnostic application.

Therapeutic applications of Monoclonal antibodies

1. Immunotherapy:

Monoclonal antibodies (MABs) play a crucial role in immunotherapy, targeting specific proteins on cancer cells to enhance the immune system's ability to combat cancer. Common monoclonal antibodies used in immunotherapy include Rituximab, Trastuzumab, and Pembrolizumab. Rituximab, for instance, is employed in the treatment of B-cell lymphomas by targeting the CD20 protein on cancer cells. Trastuzumab, used in breast cancer, targets the HER2 protein. Pembrolizumab is part of immunotherapies known as checkpoint inhibitors, helping the immune system recognize and attack cancer cells more effectively. These monoclonal antibodies can be utilized in various immunotherapy approaches, such as immune checkpoint blockade, antibody-drug conjugates, and radioimmunotherapy. In action, they either



stimulate the immune system by blocking inhibitory signals or directly deliver cytotoxic agents to cancer cells. The success of monoclonal antibodies in immunotherapy has significantly advanced cancer treatment, offering more targeted and less toxic alternatives for patients.

1. Immune Checkpoint Blockade:

Example: Pembrolizumab and Nivolumab.

Mechanism: These antibodies block immune checkpoints, such as PD-1 or CTLA-4, preventing cancer cells from evading immune detection. This allows the immune system to recognize and attack cancer cells effectively.

2. Antibody-Drug Conjugates (ADCs)

involve the coupling of monoclonal antibodies (mAbs) with cytotoxic drugs, creating a targeted therapy for various diseases. Several ADCs have been developed for the treatment of different conditions - Ado-Trastuzumab Emtansine (Kadcyla) in the treatment of HER2-positive breast cancer; Brentuximab Vedotin (Adcetris) in Hodgkin's Lymphoma & systemic anaplastic large cell lymphoma; Polatuzumab Vedotin (Polivy) in Diffuse large B-cell lymphoma; Trastuzumab Deruxtecan (Enhertu) in HER2-positive breast cancer; Enfortumab Vedotin (Padcev) in Urothelial cancer etc.

3. Radioimmunotherapy:

Radioimmunotherapy (RIT) integrates radiation therapy and immunotherapy, utilizing radiolabeled monoclonal antibodies to target specific cancer cells. This specific treatment protocol demonstrates promising applications, especially in cancer treatment, highlighting

the advancements in molecular-targeted therapy. Agents like Ibritumomab tiuxetan, Zevalin (Ibritumomab Tiuxetan), Bexxar (Tositumomab) have been developed for achieving effective treatment protocols.

Use of conjugated monoclonal antibodies with bacterial toxins in cancer therapy

Immunotoxins are a promising class of therapeutic agents in cancer therapy that combine the selectivity of monoclonal antibodies (mAbs) with the cell-killing properties of bacterial toxins. This innovative approach aims to specifically target cancer cells while avoiding harm to healthy tissues, offering a potential treatment option for several types of cancer. One intriguing strategy within this domain is the use of monoclonal antibodies conjugated with bacterial toxins in cancer therapy. Additionally, the development of recombinant immunotoxins further elevates the potential of this approach.

Bacterial toxins that are commonly conjugated with monoclonal antibodies for cancer treatment include Pseudomonas exotoxin A (PE), diphtheria toxin (DT), and ricin. These toxins are highly potent and can induce cell death by interfering with essential cellular processes. When conjugated with monoclonal antibodies, they can be selectively delivered to cancer cells, making them effective components of immunotoxins used in cancer therapy.

For example, the immunotoxin called moxetumomab pasudotox (Lumox) is used to treat hairy cell leukemia. Lumox is composed of an anti-CD22 antibody fragment fused to Pseudomonas exotoxin A



(PE). This immunotoxin targets cancer cells that express CD22, a molecule found on the surface of hairy cell leukemia cells, and delivers the cytotoxic payload specifically to these cancer cells.

Another example is inotuzumab ozogamicin (Besponsa), which is used in the treatment of acute lymphoblastic leukemia. This immunotoxin consists of a monoclonal antibody targeting CD22 conjugated to calicheamicin, a potent cytotoxic agent derived from bacteria. The specificity of the antibody component ensures the targeted delivery of the cytotoxic payload to cancer cells expressing the CD22 antigen.

In the context of cancer therapy, the conjugation of monoclonal antibodies with bacterial toxins facilitates the targeted delivery of these toxins to cancer cells. Once the monoclonal antibody portion of the immunotoxin binds to the specific antigen on the cancer cell surface, the toxin component gains entry into the cell, leading to its internalization. Once inside the cancer cell, the bacterial toxin component disrupts essential cellular processes, ultimately leading to cell death. This mechanism allows for the precise destruction of cancer cells while minimizing damage to healthy tissues, which is a significant advantage over traditional chemotherapy.

Use of recombinant immunotoxins in cancer therapy

Recombinant immunotoxins represent a further advancement in this field. These immunotoxins are created through genetic engineering techniques, wherein the gene encoding the antibody portion is fused with

the gene encoding the toxin component. The resulting recombinant protein retains the specificity of the monoclonal antibody and the potent cytotoxic properties of the bacterial toxin. This approach allows for precise targeting of cancer cells expressing the specific antigen recognized by the monoclonal antibody.

Several recombinant immunotoxins have been developed and are being investigated for their effectiveness in cancer treatment. For example, the immunotoxin moxetumomab pasudotox (Lumoxiti) targets CD22-positive cancer cells and has been approved for the treatment of hairy cell leukemia. Another notable example is inotuzumab ozogamicin (Besponsa), which targets CD22-positive B-cell precursor acute lymphoblastic leukemia. Additionally, clinical trials are underway to evaluate the potential of various recombinant immunotoxins, such as LMB-100 targeting mesothelin-expressing cancers and DT2219 targeting CD19-positive malignancies.

The working mechanism of recombinant immunotoxins involves several key steps. First, the monoclonal antibody component recognizes and binds to the specific antigen present on the surface of cancer cells. Upon binding, the immunotoxin is internalized via receptor-mediated endocytosis, allowing the bacterial toxin component to enter the cancer cell. Once inside, the toxin component exerts its cytotoxic effects by disrupting vital cellular processes, such as protein synthesis or DNA replication, ultimately leading to apoptotic cell death. Importantly, the selectivity of the monoclonal antibody ensures that the



cytotoxic effects are restricted to cancer cells expressing the target antigen, sparing healthy cells from harm.

Use of unconjugated monoclonal antibodies in cancer therapy

Unconjugated monoclonal antibodies, or naked antibodies, have proven to be valuable therapeutic agents in cancer treatment. These antibodies are designed to specifically recognize and bind to antigens expressed on the surface of cancer cells, eliciting various immune-mediated effects to combat the disease. Their mechanisms of action include stimulating the immune system, interfering with cancer cell signaling, and blocking pathways necessary for tumor survival and growth. Unconjugated monoclonal antibodies have been widely utilized in the treatment of various cancers, demonstrating efficacy as standalone therapies or in combination with other treatments.

One notable example is trastuzumab (Herceptin), a monoclonal antibody targeting the human epidermal growth factor receptor 2 (HER2). Herceptin is used in the treatment of HER2-positive breast cancer and gastric cancer. By binding to HER2, trastuzumab inhibits signaling pathways that promote cancer cell proliferation and survival, while also triggering immune-mediated mechanisms that contribute to cancer cell destruction.

Another example is rituximab (Rituxan), which targets the CD20 antigen found on B-cell lymphomas and leukemias. Rituximab induces antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), leading to

the destruction of CD20-expressing cancer cells.

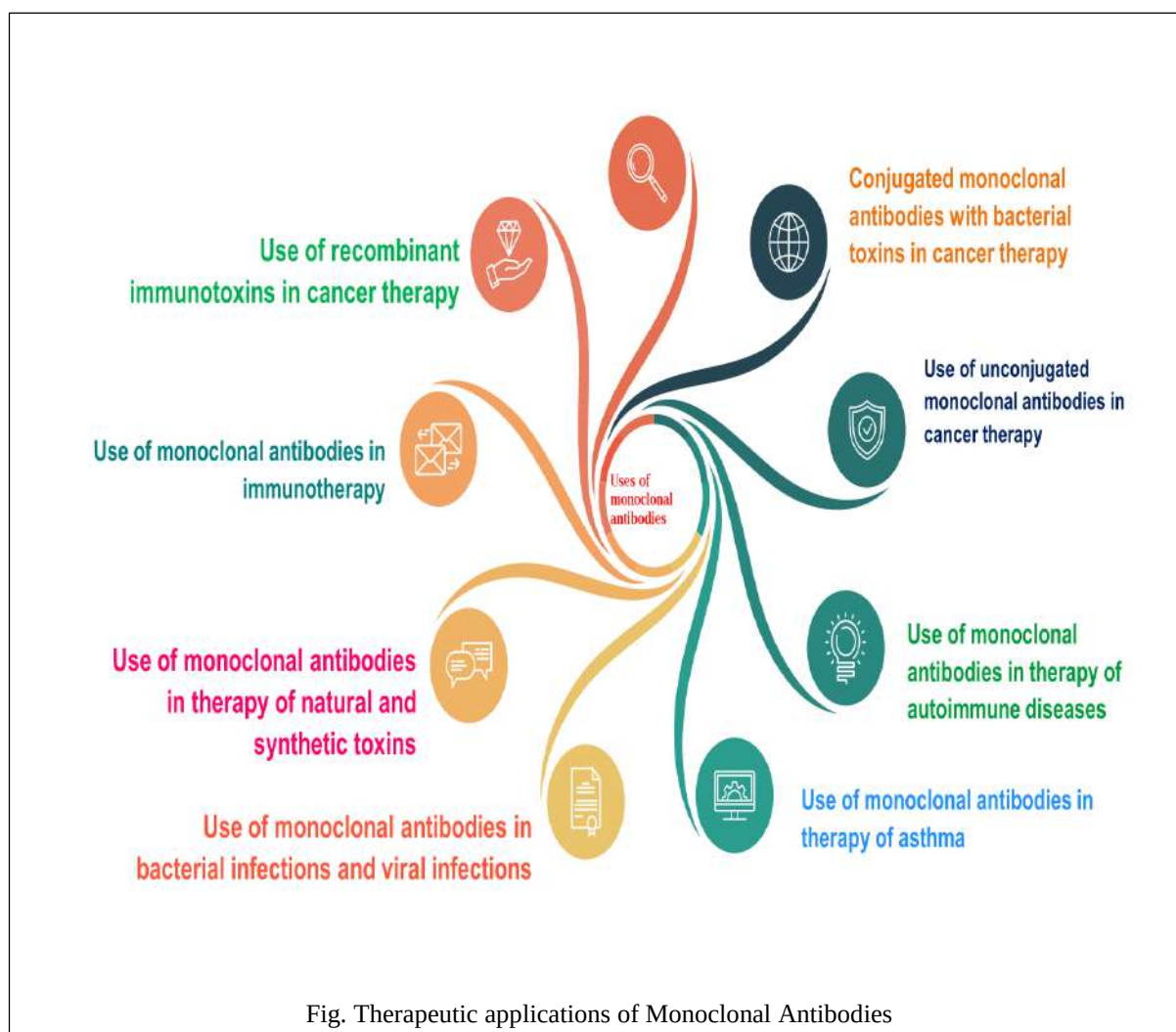
The working mechanism of unconjugated monoclonal antibodies involves their specific binding to cancer cell surface antigens, leading to various immune-mediated responses aimed at eliminating cancer cells. By targeting specific antigens, these antibodies can interfere with essential cancer cell functions and promote immune recognition and destruction of cancer cells.

In summary, unconjugated monoclonal antibodies have emerged as effective treatments for various types of cancer, including HER2-positive breast cancer, gastric cancer, and B-cell lymphomas and leukemias. Their ability to specifically target cancer cells and engage immune-mediated mechanisms makes them valuable therapeutic options, either as standalone treatments or in combination with other cancer therapies. Ongoing research and development in this field continue to expand the potential applications of unconjugated monoclonal antibodies in improving patient outcomes and quality of life.

Use of monoclonal antibodies in therapy of autoimmune diseases

Monoclonal antibodies are engineered proteins designed to target specific components of the immune system involved in autoimmune diseases. By blocking harmful immune reactions, these antibodies can alleviate symptoms and prevent tissue damage. Their precise targeting minimizes off-target effects, offering a promising avenue for therapeutic intervention in autoimmune diseases.





Examples include Secukinumab and Ixekizumab used for Interleukin-17A (IL-17A) Neutralization in Psoriasis treatment, Infliximab and Adalimumab used for Tumor Necrosis Factor Alpha (TNF- α) Inhibition in Rheumatoid Arthritis, Belimumab used for B-Lymphocyte Stimulation Inhibition in Systemic Lupus Erythematosus (SLE) etc.

Use of monoclonal antibodies in therapy of asthma

Monoclonal antibodies have emerged as a promising therapeutic option in the management of severe asthma, offering targeted interventions to modulate the underlying immune and inflammatory

pathways driving the disease. These antibodies are designed to specifically target key components of the immune system and inflammatory responses, thereby addressing the complex immunological mechanisms contributing to severe asthma.

The monoclonal antibody Omalizumab (Xolair) is used in the treatment of severe asthma. Omalizumab targets immunoglobulin E (IgE), an antibody involved in allergic responses. By binding to IgE, Omalizumab prevents its interaction with immune cells, thereby inhibiting



allergic and inflammatory processes that contribute to asthma exacerbations.

The MABs such as mepolizumab (Nucala) and reslizumab (Cinqair) target interleukin-5 (IL-5), a cytokine that plays a crucial role in promoting the survival and activation of eosinophils, a type of white blood cell involved in asthma-related inflammation. By neutralizing IL-5, these monoclonal antibodies help reduce eosinophilic inflammation in the airways, thereby providing beneficial effects in patients with severe eosinophilic asthma.

The MABs benralizumab (Fasenra) targets the interleukin-5 receptor α (IL-5R α), which is expressed on eosinophils. By binding to IL-5R α , benralizumab induces antibody-dependent cell-mediated cytotoxicity (ADCC), leading to eosinophil depletion. This helps to reduce the levels of eosinophils in the airways, thereby decreasing inflammation and improving asthma control in patients with severe eosinophilic asthma.

The working mechanism of monoclonal antibodies in controlling asthma involves targeting specific immune molecules or cells that drive the underlying inflammatory processes. For instance, Omalizumab prevents the binding of IgE to immune cells, thereby inhibiting the allergic response and associated airway inflammation. Similarly, mepolizumab, reslizumab, and benralizumab act by neutralizing IL-5 or its receptor, leading to reduced eosinophil-mediated inflammation in the airways.

Monoclonal antibodies employed in asthma therapy thus demonstrate diverse

mechanisms, including inhibition of allergic responses, reduction of eosinophilic inflammation, and modulation of specific immune cell populations. These targeted interventions have significantly expanded the treatment options for severe asthma, offering effective strategies to mitigate the underlying immunological processes and improve outcomes for patients with this chronic respiratory condition.

Use of Monoclonal Antibodies in the treatment of Bacterial and Viral Infections

Monoclonal antibodies (MABs) have emerged as promising therapeutic agents against both bacterial and viral infections. These antibodies are designed to target specific pathogens or their components, contributing to the development of targeted and effective treatments. Here's a concise overview of their use, examples, and working mechanisms in the context of bacterial and viral infections.

Monoclonal Antibodies in Bacterial Infections:

- 1. Clostridium difficile Infection (CDI):** Monoclonal antibodies such as bezlotoxumab target toxins produced by Clostridium difficile, aiding in the prevention of recurrent CDI.
- 2. Methicillin-resistant Staphylococcus aureus (MRSA):** Recent successes suggest that MABs could emerge as primary therapies against MRSA pathogens, with antibiotics serving as adjuvants in treatment plans.



3. **Anthrax:** Caused by the bacterium *Bacillus anthracis*, anthrax can be treated with Raxibacumab. Raxibacumab works by binding to the protective antigen of *B. anthracis*, thereby preventing the bacteria from entering and damaging human cells.

4. **Staphylococcus aureus infection:** MEDI4893 is a mAb that targets the surface protein of *S. aureus* bacteria, which is responsible for causing skin and soft tissue infections

5. **Neisseria meningitidis Infection:** Eculizumab is a mAb that targets the complement protein C5, which is involved in the immune response to *N. meningitidis* bacteria.

Monoclonal Antibodies in Viral Infections:

1. **COVID-19 (SARS-CoV-2):** Monoclonal antibodies like casirivimab and imdevimab have been authorized for emergency use in treating COVID-19, reducing the risk of severe disease progression.

2. **Influenza A and B:** Palivizumab is a monoclonal antibody used to prevent respiratory syncytial virus (RSV) infections, which can cause severe respiratory illnesses, particularly in infants.

3. **Human Immunodeficiency Virus (HIV):** Ibalizumab, a monoclonal antibody, is used in the treatment of multidrug-resistant HIV-1 infection, providing a novel approach to managing the virus.

4. **Respiratory Syncytial Virus (RSV):** Palivizumab, as mentioned earlier, is a key monoclonal antibody used to prevent RSV infections, particularly in high-risk infants.

5. **Hepatitis B Virus (HBV):** Monoclonal antibodies like HepaGam B and HyperHEP B are used for post-exposure prophylaxis against hepatitis B infections.

Use of monoclonal antibodies in therapy of natural under synthetic toxins

Monoclonal antibodies have emerged as a critical asset in treating both natural and synthetic toxin exposures, offering targeted interventions to neutralize toxic substances and mitigate their effects. These therapeutic antibodies work by specifically binding to toxins, preventing their interactions with target cells, and facilitating their elimination from the body.

Treatment of Natural Toxins:

1. **Botulism Toxin:** Botulism is a severe and potentially life-threatening condition caused by the neurotoxins produced by *Clostridium botulinum* bacteria. Monoclonal antibodies, such as BAT (Botulism Antitoxin Heptavalent), have been developed to neutralize the effects of botulinum neurotoxins, thereby preventing the progression of botulism and reducing its severity after exposure to contaminated food or wound contamination.

2. **Snake Venom:** CroFab is a polyvalent ovine-derived Fab antivenin used in the treatment of North American pit viper envenomation, including rattlesnake, cottonmouth, and copperhead bites. It



targets the venom components, preventing the progression of local tissue damage and systemic symptoms, providing essential support in managing snakebite envenomations.

3. Scorpion Venom: Anascorp, also known as Centruroides immune F(ab)2 (equine), is used to treat scorpion envenomation caused by Centruroides scorpions. It contains equine-derived F(ab)2 immunoglobulin fragments to neutralize the venom's effects, reducing systemic toxicity and improving clinical outcomes following scorpion stings.

4. Tetrodotoxin: Tetrodotoxin is a potent marine toxin found in various types of fish, such as pufferfish. A monoclonal antibody, Tectin, has been investigated for its potential to neutralize tetrodotoxin, offering a treatment option for tetrodotoxin poisoning following the ingestion of contaminated seafood.

Treatment of Synthetic Toxins:

1. Chemical Warfare Agents: Monoclonal antibodies, such as raxibacumab and obiltoxaximab, have been developed for the treatment of anthrax, which can be utilized in response to a deliberate release of anthrax spores, offering specific targeting and neutralization of the anthrax toxins to mitigate the effects of anthrax exposure.

2. Toxic Industrial Chemicals: Monoclonal antibodies targeting toxic industrial chemicals, such as organophosphates, serve as potential therapeutic agents for individuals exposed to these hazardous compounds, offering specific binding and neutralization of the

toxic effects to alleviate symptoms and prevent further harm.

3. Drug Overdose: In the context of drug overdose, monoclonal antibodies such as nalmeferene and naltrexone are used to neutralize the effects of opioids by binding and blocking opioid receptors, serving as a rescue therapy to reverse opioid toxicity and overdose.

4. Environmental Toxins: Monoclonal antibodies are also being explored as potential therapies for neutralizing the effects of environmental toxins such as polychlorinated biphenyls (PCBs) and dioxins, offering a targeted approach to counteracting the toxic effects of these substances in cases of accidental exposure or environmental contamination.

The working mechanism of monoclonal antibodies in toxin exposure involves their ability to specifically recognize and bind to the toxins, thereby preventing the toxins from exerting their deleterious effects. By neutralizing toxins and facilitating their clearance, monoclonal antibodies play a crucial role in mitigating the impact of toxin exposure and reducing associated morbidity and mortality. Whether in the context of natural venoms, biological toxins, or synthetic compounds, the targeted nature of monoclonal antibodies makes them valuable tools in managing toxin-related conditions.



Table1: MABs being used in cancer therapy

Brand Name	Antibody	Target (Full Form)	Diseases Treated
Avastin	Bevacizumab	Vascular endothelial growth factor A (VEGF-A)	Colorectal cancer, lung cancer, glioblastoma
Herceptin	Trastuzumab	Human epidermal growth factor receptor 2 (HER2)	Breast cancer, gastric cancer
Rituxan	Rituximab	Cluster of differentiation 20 (CD20)	Non-Hodgkin's lymphoma, chronic lymphocytic leukemia
Erbitux	Cetuximab	Epidermal growth factor receptor (EGFR)	Colorectal cancer, head and neck cancer
Keytruda	Pembrolizumab	Programmed cell death protein 1 (PD-1)	Melanoma, non-small cell lung cancer, head and neck cancer
Opdivo	Nivolumab	Programmed cell death protein 1 (PD-1)	Melanoma, non-small cell lung cancer, renal cell carcinoma
Tecentriq	Atezolizumab	Programmed death-ligand 1 (PD-L1)	Bladder cancer, non-small cell lung cancer
Kadcyla	Ado-trastuzumab emtansine	Human epidermal growth factor receptor 2 (HER2)	Breast cancer
Perjeta	Pertuzumab	Human epidermal growth factor receptor 2 (HER2)	Breast cancer
Zevalin	Ibritumomab tiuxetan	Cluster of differentiation 20 (CD20)	Non-Hodgkin's lymphoma
Blincyto	Blinatumomab	CD19 and CD3	Acute lymphoblastic leukemia
Gazyva	Obinutuzumab	Cluster of differentiation 20 (CD20)	Chronic lymphocytic leukemia
Polivy	Polatuzumab vedotin-piiq	Cluster of differentiation 79b (CD79b)	Diffuse large B-cell lymphoma
Empliciti	Elotuzumab	Signaling lymphocytic activation molecule family member 7 (SLAMF7)	Multiple myeloma
Darzalex	Daratumumab	Cluster of differentiation 38 (CD38)	Multiple myeloma
Lumoxiti	Moxetumomab pasudotox-tdfk	Cluster of differentiation 22 (CD22)	Hairy cell leukemia
Libtayo	Cemiplimab-rwlc	Programmed cell death protein 1 (PD-1)	Cutaneous squamous cell carcinoma
Bavencio	Avelumab	Programmed death-ligand 1 (PD-L1)	Merkel cell carcinoma
Imfinzi	Durvalumab	Programmed death-ligand 1 (PD-L1)	Non-small cell lung cancer
Jevtana	Cabazitaxel	Microtubule-associated protein tau (MAPT)	Prostate cancer
Yervoy	Ipilimumab	Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)	Melanoma
Libtayo	Cemiplimab-rwlc	Programmed cell death protein 1 (PD-1)	Cutaneous squamous cell carcinoma



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Aetiopathogenesis and Manifestations of Polycystic Ovary SyndromeShubhasmita Sahoo¹, L Suhasini Sahithi², Mohan Krishna Ghanta^{3*}**Abstract**

Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder affecting women of reproductive age, characterized by various symptoms that have a profound impact on overall well-being. The etiology of Polycystic Ovary Syndrome (PCOS) is multifactorial, involving genetic predisposition, hormonal imbalances (particularly elevated androgens), insulin resistance, chronic low-grade inflammation, and potential environmental influences. Symptoms include menstrual irregularities, weight gain, fatigue, hair loss, infertility, pelvic pain, mood swings, sleep problems, and headaches. Comprehensive management strategies involving healthcare professionals are crucial for addressing PCOS. The management of PCOS requires personalized treatment plans based on thorough evaluations. Lifestyle modifications, such as regular physical activity and healthy eating habits, play a vital role in managing PCOS. Pharmacological interventions may also be recommended to address hormonal imbalances, insulin resistance, and associated conditions like metabolic syndrome.

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Introduction:

Polycystic Ovary Syndrome (PCOS) is a highly prevalent and intricate endocrine disorder that frequently occurs in women of reproductive age, with a prevalence estimated at 4-8% (Aziz et.al., 2004). Stein and Leventhal first described the classic symptomatology in 1935, including amenorrhea, infertility, hirsutism, and enlarged polycystic ovaries (Stein and Leventhal 1935). Infertility is a common consequence of PCOS, primarily caused by ovulatory dysfunction, where the regular release of mature eggs from the ovaries is disrupted (Zehra and Khursheed 2018). Furthermore, a significant percentage (50-70%) of women with PCOS experience insulin resistance and hyperinsulinemia, which significantly elevate the risk of developing type 2 diabetes, dyslipidemia, cardiovascular disease, and endometrial carcinoma (Hardiman et.al., 2003). PCOS is categorized as a syndrome rather than a disease due to the lack of consistent symptoms and physical parameters. The disorder's complexity arises from its heterogeneity and unknown underlying causes, making it challenging to diagnose based on a single criterion.

Diagnostic criteria

During the 1990 NIH conference on PCOS, experts identified three essential characteristics: chronic anovulation, hyperandrogenism, and the lack of concurrent endocrine disorders (Lujan et.al., 2008). Subsequent studies revealed that genetic and environmental factors played a role in the variation of obesity and hirsutism. Both Insulin resistance and excess adrenal androgen were present

(Carmina et.al., 1992). PCOS is considered a heterogeneous disorder with varying degrees of gonadotropic and metabolic abnormalities, likely influenced by multiple inherited and environmental factors. The 2003 Rotterdam consensus conference criteria established that PCOS can be diagnosed when at least two out of three signs are present, such as, evidence of androgen excess, oligo-ovulation and multicystic ovaries (Azziz 2005). However, conflicts arose regarding the definition, with differing opinions on the inclusion of hyperandrogenism. The Androgen Excess Society presented a position statement emphasizing the importance of hyperandrogenism in PCOS (Azziz et.al., 2006).

Polycystic ovaries are present in around 20% of women and are associated with abnormal androgen and insulin levels (Michelmore et.al., 1999). They show histological characteristics like a thickened outer capsule, hypertrophied ovarian epithelium, and an excess of developing follicles (Jonard et.al., 2003). Despite being called "cysts," these follicles are healthy and hormone-producing (Takayama et.al., 1996). However, larger follicles may experience developmental arrest in anovulatory cycles. Transvaginal ultrasonography is the preferred method for diagnosis, using specific criteria such as the number and size of follicles or ovarian volume (Balen et.al., 2003). Polycystic ovaries can be distinguished by the string-like arrangement of follicles beneath the ovarian cortex, unlike the scattered follicles in normal or multi-follicular ovaries.



PCOS involves hormonal imbalances: elevated estrogen, LH, and insulin levels, insufficient progesterone production, and overproduction of androgens. This hormonal imbalances in PCOS disrupt the normal functioning of the ovaries, leading to irregular menstrual cycles, anovulation, and the characteristic symptoms associated with the condition.

Etiopathology

The etiology of PCOS is complex and multifactorial (Figure 1). The inheritance pattern of PCOS is difficult to determine, suggesting that it may involve multiple genes and/or environmental influences (Prodoehl et.al., 2009). PCOS exhibits wide variations in symptomatic characteristics and ethnic differences (Al-Fozan et.al., 2005). Therefore, the adoption of universal diagnostic criteria is considered crucial to unravel the specific cause of PCOS (Geisthövel and Rabe 2005). The observed heterogeneity in PCOS may indicate the presence of multiple distinct disorders with areas of symptomatic overlap between them (Welt et.al., 2006).

Inheritance

PCOS is a complex, polygenic disorder with autosomal dominant inheritance. and their interactions with the environment. Various genetic studies have identified specific genes and mutations associated with different symptoms of PCOS. These genes directly or indirectly affect the functioning of the ovaries. The development of PCOS is primarily driven by genes involved in processes such as steroid production and action, regulation of hormones related to reproductive function, insulin function and release, energy metabolism, and chronic inflammation (Singh et.al., 2023). In summary, PCOS is a polygenic condition with diverse genetic factors contributing to its pathophysiology, impacting various aspects of hormone regulation and metabolism. Polymorphic sequences, especially microsatellites, may play a role in PCOS by accounting for phenotypic variation and inconsistent results. Epigenetic modifications and transcriptional regulation require further investigation in PCOS.

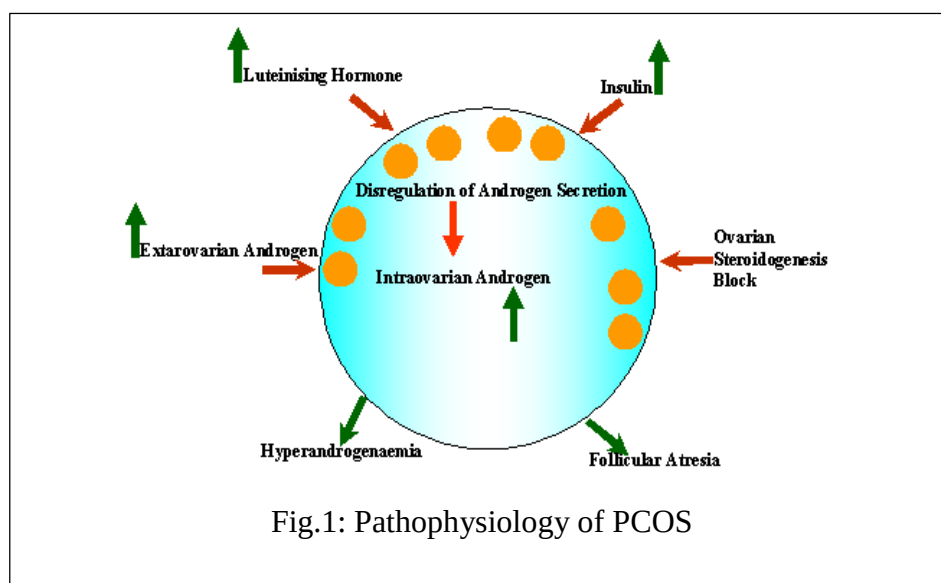


Fig.1: Pathophysiology of PCOS



Two methods commonly used to identify genetic loci for PCOS genes are association studies and linkage studies. Association studies compare the frequency of a specific allele associated with the disorder in affected individuals and the general population. Linkage studies investigate familial inheritance patterns to identify genomic markers linked to the phenotype. Aromatase, an enzyme complex, is involved in converting androgens to estrogens, which is relevant to the hormonal imbalances observed in PCOS (Zehra and Khursheed 2018).

Hyperandrogenism

PCOS is characterized by elevated androgen levels (hyperandrogenemia) that lead to clinical manifestations such as hirsutism, acne, and alopecia. Approximately 75-90% of PCOS patients with irregular menstrual cycles exhibit high levels of androgens. Genetic factors contribute to abnormal steroidogenesis and hyperandrogenism. In PCOS, the elevated androgens disrupt the normal growth of primordial and antral follicles, impacting folliculogenesis. Neuroendocrine dysregulation causes HPO axis imbalance, resulting in surplus amount of gonadotropin. Specifically, there is an excess of luteinizing hormone (LH) compared to follicle-stimulating hormone (FSH). In ovarian theca cells, LH triggers excessive androgen synthesis, while FSH aids in the conversion of androgens to estrogens in granulosa cells, supporting follicle growth. The excessive LH stimulation causes theca cell hyperplasia and the formation of cystic structures

resembling a string of pearls (Singh et.al., 2023).

Insulin resistance

Approximately 70% of PCOS-afflicted females have insulin resistance, indicating that their cells are unable to effectively utilize insulin. The significant contributors to the onset of PCOS are insulin resistance and hyperandrogenism, with insulin resistance affecting approximately 85 percent of cases (75 percent of lean individuals and 95 percent of those who are overweight). Studies have indicated that hyperinsulinemia in PCOS can result from primary insulin resistance or direct manifestation of pancreatic β -cell dysfunction. Studies have reported impairments of insulin action and function of pancreatic β -cell in PCOS. Furthermore, obesity is recognized as a primary factor leading to insulin resistance. Obesity and insulin resistance are both major risk factors for the development of type 2 diabetes. Insulin resistance significantly contributes to the pathophysiology of PCOS in 50% to 80% of women affected by the condition. An effective approach in reducing insulin resistance involves implementing the DASH eating plan, which was developed by the National Institutes of Health (NIH). When combined with weight loss and physical activity, the DASH eating plan has been demonstrated to effectively decrease insulin resistance (Zehra and Khursheed 2018).

Environment factor

Now a days, environmental factors are responsible for development of PCOS. Socioeconomic status, lifestyle choices,



and eating habits. Obesity, resulting from poor eating habits and a sedentary lifestyle, is a significant contributing factor. Research indicates that individuals with lower socioeconomic status often have unhealthy diets and limited physical activity, leading to obesity and increased insulin resistance. One distinctive hallmark of PCOS is insulin resistance, and individuals with lower socioeconomic status are more prone to developing cardiovascular diseases due to these combined factors (Ashraf et.al., 2022).

PCOS has been connected to environmental contaminants such as heavy metals, pesticides, and endocrine-disrupting chemicals (EDCs). Some study revealed that there is a positive correlation between elevated blood levels of BPA (bisphenol A) and increased serum testosterone levels in women with PCOS compared to women without the condition. PCOS-affected women also displayed lower urine concentrations of mono benzyl phthalate (mBzP), indicating impaired xenobiotic metabolism. These pollutants can influence various reproductive pathways, including orphan receptors, neurotransmitter receptors, nonnuclear hormone receptors, steroidogenesis, and hormonal metabolism. Overall, Environmental toxins may contribute to the onset and progression of PCOS (Ashraf et.al., 2022).

Role of life style in PCOS

A sedentary lifestyle is a significant contributor to PCOS in young girls and women. Lack of regular exercise and poor physical activity levels are common among women with PCOS. Factors such as physical inactivity, excessive use of TV and

mobile phones, high-stress levels, and unhealthy eating habits, including junk food consumption, contribute to obesity and overweight in PCOS women. Hormonal imbalances caused by factors like chicken intake and junk food further contribute to the development of PCOS. It is crucial for healthcare professionals, particularly pharmacists, to provide guidance and support to improve PCOS management and address the lack of knowledge among affected women (Ashraf et.al., 2022).

Clinical Manifestation

PCOS can present with various clinical manifestations, which can vary in severity and combination among affected individuals. The diagnosis of PCOS is typically based on the presence of specific criteria, including clinical manifestations, hormonal imbalances, and exclusion of other possible conditions. Proper diagnosis and management of PCOS involve a comprehensive evaluation by healthcare professionals. Some common clinical sign and symptoms of PCOS include:

Hirsutism

Hirsutism is the presence of coarse, dark, male-pattern hair growth in women. However, masculinization symptoms including increased muscular mass, a deeper voice, smaller breasts and clitoromegaly, are not typically associated with PCOS. If these virilization signs are present, it suggests elevated androgen levels, which may indicate the tumor in the ovary or adrenal gland synthesizes androgen. Further investigation and careful evaluation of symptoms are necessary to



determine the underlying cause of virilization (Zehra and Khurhseed 2018).

Acne

Hormonal changes, particularly related to androgens, can contribute to the development of acne issues. Additionally, dark patches of skin are often associated with PCOS. To assess potential signs of hyperandrogenism, experts suggest inquiring about the menstrual history of women experiencing acne and evaluating them for other relevant symptoms (Mukherjee 2020).

Metabolic syndrome

Both obesity and polycystic ovary syndrome (PCOS) are known to be associated with an elevated risk of getting metabolic syndrome, which encompasses high blood pressure (hypertension), high levels of LDL cholesterol (bad cholesterol) and low levels of HDL cholesterol (good cholesterol). These factors contribute to an elevated risk of developing conditions such as diabetes and angina pectoris (Mukherjee 2020).

Other symptoms

PCOS is associated with a range of symptoms that can impact women's well-being. These include menstrual irregularities, weight gain (especially around the waist), fatigue, hair loss, infertility, pelvic pain, mood swings (such as depression and anxiety), sleep problem, and headaches. These symptoms can have physical, emotional, and psychological implications for individuals with PCOS (Figure 2). Women who are experiencing these symptoms should visit a doctor and

explore appropriate management strategies to address the specific challenges associated with PCOS (Zehra and Khurhseed 2018). Women with PCOS often experience irregular periods, characterized by infrequent or absent ovulation and fewer than eight menstrual cycles per year. This can lead to heavier periods due to the prolonged buildup of the uterine lining. Another common symptom is the presence of dark patches of skin in body creases, such as the neck, groin, and under the breasts. In some cases, hormonal fluctuations associated with PCOS can also contribute to headaches (Mukherjee 2020). These symptoms are indicative of the manifestation of PCOS in affected individuals.

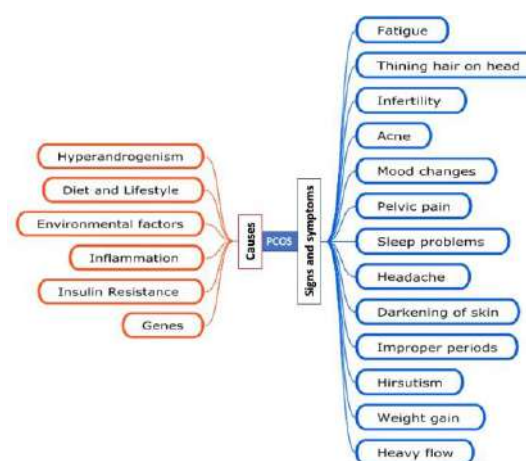


Figure 2: Sign and symptoms of PCOS.

CONCLUSION

In summary, PCOS is a complicated endocrine condition that affects women during their reproductive age. It presents with diverse symptoms such as irregular periods, infertility, hirsutism, and enlarged polycystic ovaries. PCOS is a heterogeneous disorder influenced by both genetic and environmental factors.



Diagnostic criteria include chronic anovulation, hyperandrogenism, and the exclusion of other endocrine disorders. Histologically, polycystic ovaries exhibit specific characteristics and are associated with abnormal androgen and insulin levels. The presence of insulin resistance and hyperandrogenism increases the risk of type 2 diabetes and cardiovascular disease. Additionally, environmental factors, sedentary lifestyle, and exposure to pollutants can contribute to the development and progression of PCOS. Proper diagnosis and management require the involvement of healthcare professionals, as women with PCOS may experience various clinical manifestations with physical and psychological implications. Further research is necessary to enhance our understanding of PCOS and develop improved approaches for its diagnosis and treatment.

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Probiotics - their possible mode of action and applications in Aquaculture

Shanmugam K R

Abstract

There was an elevated risk for diseases and substantial loss for aquaculture farmers. In view of several drawbacks caused by antibiotics, a strict vigilance have been established to ban and minimize their application in aquaculture. As an alternative to antibiotics, dietary administration of feed additives has received increasing attention during the past three decades. Probiotics, prebiotics, synbiotics and medicinal plants were among the most promising feed supplements for control and treatments of bacterial, viral and parasitic diseases of aquaculture animals. The possible mode of action of probiotics are strengthening immune response, competition for binding sites, production of antibacterial substances, and competition for nutrients in providing protection against diseases. Besides, we have classified different pathogens and separately described the effects of probiotics as protective strategy. Furthermore, we have addressed the gaps of existing knowledge as well as the topics that merit further investigations. Overall, the present abstract revealed potential of different probiont to be used as protective agent against various pathogens.

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Introduction

Today, aquaculture is the fastest growing food-producing sector in the world, with an average annual growth rate of 8.9% since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period.¹ World aquaculture has grown tremendously during the last fifty years from a production of less than a million tonne in the early 1950s to 59.4 million tonnes by 2004. This level of production had a value of US\$70.3 billion. The diseases and deterioration of environmental conditions often occur and result in serious economic losses. Fisheries and Aquaculture sector plays a vital role in meeting this growing demand for protein.

Antibiotics are used as traditional strategy for fish diseases management and also for the improvement of growth and efficiency of feed conversion. However, the development and spread of antimicrobial resistant pathogens were well documented. There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of non-pathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens.⁵ Considering these factors, there has been heightened research in developing new dietary supplementation strategies in which various health and growth promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated

2. Aquaculture

Aquaculture has a long history, originating at least in the year 475 B.C. in China, but became important in the late nineteen-forties, since the methods of aquaculture could be used to restock the waters as a complement to natural spawning. Nowadays, aquaculture is a lucrative industry. Aquaculture is the fastest growing food-producing sector in the world, with an average annual growth rate of 8.9% since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period. World aquaculture has grown tremendously during the last fifty years from a production of less than a million tonne in the early 1950s to 59.4 million tonnes by 2004. This level of production had a value of US\$70.3 billion. The diseases and deterioration of environmental conditions often occur and result in serious economic losses. (Boyd & Tucker 1998; Nomoto 2005).

Total fisheries and aquaculture production reached an all-time record of 214 million tonnes in 2020, comprising 178 million tonnes of aquatic animals and 36 million tonnes of algae, a slight increase (3 percent) from the previous 2018 record (213 million tonnes).

Global **aquaculture production** in 2020 reached a record 122.6 million tonnes, including 87.5 million tonnes of aquatic animals worth USD 264.8 billion and 35.1 million tonnes of algae worth USD 16.5 billion. Around 54.4 million tonnes were farmed in inland waters and 68.1 million tonnes came from marine and coastal aquaculture.



Global consumption of aquatic foods (excluding algae) increased at an average annual rate of 3.0 percent from 1961 to 2019, a rate almost twice that of annual world population growth (1.6 percent) for the same period, with annual per capita consumption reaching a record high of 20.5 kg in 2019.

India is the third largest fish producing country, contributing 8 percent to the global fish production and ranks second in aquaculture production. Globally, India stands 2nd in aquaculture production and is one of the top shrimp producing and exporting nations. The fish production in 2021-22 is 16.24 Million Tonnes comprising of marine fish production of 4.12 Million Tonnes and 12.12 Million Tonnes from Aquaculture.



Figure 1 : Fish Production in India from 1950- 2022

Over the past seventy-five years, the sector has gradually transformed into a commercial enterprise while maintaining its traditional and small scale character. Since 1950 and till the end of 2021-22, there has been a 22-fold increase in national fish production. During the last 9 years, the annual fish production of India has increased from 95.79 lakh tons (at the end of 2013-14) to an all-time record of 162.48 lakh tons (at the end of 2021-22) i.e. an

increase of 66.69 lakh tons. Further, national fish production for the year 2022-23 is also expected to touch or exceed 174 lakh tons (provisional figures), an 81% increase when compared to 2013-14.



Fig 2: Marine fish production in India (2021-22)

For aquatic animal production, this general trend masks significant variations between continents, regions, and countries. In 2020, Asian countries were the main producers accounting for 70 percent of the total, followed by the Americas, Europe, Africa and Oceania. China remained the first major producer with a share of 35 percent of the total. The expansion of aquaculture in recent decades has boosted the overall growth of aquatic animal production in inland waters, from 12 percent of total production in the late 1980s to 37 percent in 2020.

China remains the world's largest exporter of aquatic animal products, followed by Norway and Viet Nam, with the European Union the largest single importing market. The largest importing countries are the United States of America followed by China and Japan.



However, the intensification of aquaculture practices requires cultivation at high densities, which has caused significant damage to the environment due to discharges of concentrated organic wastes, that deplete dissolved oxygen in ponds, giving rise to toxic metabolites (such as hydrogen sulfide, methane, ammonia, and nitrites), that often are responsible for mortality. Additionally, aquaculture has appropriated of water bodies used for recreational purpose, and sometimes makes a water's waste because this natural resource is not reused in extensive aquaculture systems. Moreover, under these conditions of intensive production, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and causing a decrease in productivity (Balcázar et al., 2006).

The use of antibiotics in aquaculture as a preventive measure associated with the evolution and spread of several resistant pathogens like *Aeromonas* sp., *Escherichia tarda*, *E.coli*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, and many more (Allameh et al., 2016). The information regarding the resistance development in aquatic pathogens under long-term antibiotic pressure (Lakshmi et al., 2013). Thus, the uses of certain antibiotics in aquaculture industries have been restricted in several countries like the USA and Canada. So, the use of probiotics along with dietary supplementation is a very fruitful strategy to combat pathogenic agents through a variety of mechanisms as an alternative driving force of antibiotic treatment (Bandyopadhyay et al., 2015).

Probiotics:

The term 'probiotic' came from Greek words 'pro' (= favor) and 'bios' (= life) which are live organisms (usually bacteria or yeast or combination of both) and taken with food to confer beneficial effects to host in various ways (Fuller, 1989). The concept of probiotics, in the field of aquaculture, is fundamentally different from those which are used in terrestrial organisms depending upon certain critical influencing factors. It is now well established that probiotics play a vital role in maintaining the gut health by modulation of microbial community structure (Nayak, 2010).

The word "probiotic" was introduced by Parker, 1974. According to his original definition, probiotics are "organisms and substances which contribute to intestinal microbial balance". (Parker 1974). As per Fuller, (1989) the definition as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Therefore, several terms such as "friendly", "beneficial", or "healthy" bacteria are also commonly used to describe probiotics. Although application of probiotics in aquaculture seems to be relatively recent, the interest in such environment friendly treatments is increasing rapidly. A growing number of studies have dealt explicitly with probiotics, and it is now possible to survey its state of the art, from the empirical use to the scientific approach. (Wang & Xu 2006)

The first discovered probiotic was a certain strain of bacillus in Bulgarian yoghurt, called *Lactobacillus bulgaricus*.



The discovery was made in 1905 by Bulgarian physician and microbiologist Stamen Grigorov. The modern-day theory is generally attributed to Russian Nobel laureate Élie Metchnikoff, who postulated around 1907 that yoghurt-consuming Bulgarian peasants lived longer.

Three general modes of probiotics actions have been classified and presented by Oelschlaeger, (2010) as follow:

(1). Probiotics might be able to modulate the host's gut defences including the innate as well as the acquired immune system and this mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of inflammation of the digestive tract or parts thereof.

(2). Probiotics can also have a direct effect on other organisms, commensal and or pathogenic ones and this principle is in many cases is of great importance in the prevention, treatment and restoration of the microbial equilibrium in the gut.

(3). Finally, probiotic effects may be based on actions affecting microbial products, host products and food ingredients and such actions may result in inactivation of toxins and detoxification of host and food components in the gut. According to above summary, all three modes of probiotics actions are all likelihood associated with gut and/or gut microbiota. Therefore, it has become apparent that we are in fact dealing with another “organ”, the so called “microbiotic canal” with the increased knowledge of the specific activity of the gut microbiota.(Wolf 2006).

The microbes also proliferate independently of the host animal in response to diseases (Bondad-Reantaso et

al., 2005). The first experimental attempt of the probiotic application in aquaculture was made by Kozasa (1986), considering the beneficial effects of probiotics on humans and poultry (Kozasa, 1986). The rapid evolution of probiotics in aquaculture is well established due to the adverse effects of widely used antibiotics and broad spectrum chemicals which kill most of the beneficial bacteria along with the pathogenic bacteria to the aquatic species (Lakshmi et al., 2013). Additionally, probiotics also work through different mechanisms in aquaculture system to eliminate the organic wastes and pollutants, as a result of incorporation of ‘bioremediation’ and ‘biocontrol’ when dealing with the environmental problems. In this context, probiotics can play an effective role in aquaculture production by providing greater non-specific disease protection as well as pollution free water sources (Nandi et al., 2018).. The goal of this review is to summarize and evaluate the current information on the efficacy and mechanism of action of probiotics for the enumeration in a complex microbial community in aquaculture.

Outbreaks of viral, bacterial, and fungal infections have caused devastating economic losses worldwide. In recent decades, prevention and control of animal diseases has focused on the use of chemical additives and veterinary medicines, especially probiotics, which generate significant risks to public health by promoting the selection, propagation, and persistence of bacterial-resistant strains.



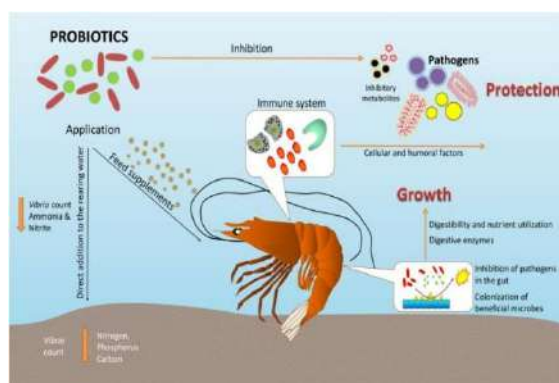


Fig 3: Effect of probiotics on pathogens

Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body. They can be found in yogurt and other fermented foods, dietary supplements, and beauty products. Prebiotics are nondigestible food components that selectively stimulate the growth or activity of desirable microorganisms. Probiotics may contain a variety of microorganisms. The most common are bacteria that belong to groups called *Lactobacillus* and *Bifidobacterium*. Other bacteria may also be used as probiotics, and so may yeasts such as *Saccharomyces boulardii*.

The extensive literature on probiotics revealed beneficial effects on host's gut defenses which has vital importance in diseases prevention as well digestive tract inflammation treatment (Modanloo et al., 2017). Apart from immunomodulation, probiotic microorganisms, such as lactic acid bacteria, *Brevibacillus brevis*, *Vagococcus fluvialis*, and *Vibrio harveyi*, stick with the mucosal epithelium of gastrointestinal tract and help to resist pathogens. In another way, probiotics increase feed digestibility through elevation of different digestive enzymes such as alginate lyases, amylases, and proteases.

They also produce organic acids, fatty acids, biotin and vitamin B12, hydrogen peroxide, antibiotics, bacteriocins, siderophores, lysozyme (Vine et al., 2006), which have positive effects on host health. Numerous studies have demonstrated that probiotics caused health benefits in aquatic organisms, such as Japanese flounder, black tiger prawns, *Penaeus monodon*, whiteleg prawns (Chiu et al., 2007), and western king prawns (Hai et al., 2010).

Different types of probiotics may have different effects. For example, if a specific kind of *Lactobacillus* helps prevent an illness, that doesn't necessarily mean that another kind of *Lactobacillus* or any of the *Bifidobacterium* probiotics would do the same thing.

The 2012 National Health Interview Survey (NHIS) showed that about 4 million (1.6 percent) U.S. adults had used probiotics or prebiotics in the past 30 days. Among adults, probiotics or prebiotics were the third most commonly used dietary supplement other than vitamins and minerals. The use of probiotics by adults quadrupled between 2007 and 2012. The 2012 NHIS also showed that 300,000 children age 4 to 17 (0.5 percent) had used probiotics or prebiotics in the 30 days before the survey.

Probiotics have shown promise for a variety of health purposes, including prevention of antibiotic-associated diarrhea (including diarrhea caused by *Clostridium difficile*), prevention of necrotizing enterocolitis and sepsis in premature infants, treatment of infant colic, treatment of periodontal disease, and induction or



maintenance of remission in ulcerative colitis.

Traditionally, probiotics used in food industry have been deemed safe, in fact, no human risks have been determined, remaining as the best proof of its safety. Theoretically, probiotics may be responsible for four types of side effects in susceptible individuals: systemic infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer. However, no hard evidence has been found.

Some of the suggested mechanisms of action of probiotics include (i) competitive exclusion through the production of inhibitory compounds, (ii) competition for nutrients, chemicals, or energy, (iii) adhesion site competition, (iv) contribution to digestion, (v) contribution to macro- and micronutrients, (vi) enhancement of immune response, and (vii) reduction of virulence through quorum sensing (QS) manipulation.

Table 1: Authorized probiotics in aqua feed

S. no.	Probiotic organism
1.	<i>Bacillus licheniformis</i>
2.	<i>Bacillus cereus</i> var. <i>toyoi</i>
3.	<i>Enterococcus faecium</i>
4.	<i>Bacillus subtilis</i>
5.	<i>Lactobacillus casei</i>
6.	<i>Lactobacillus farciminis</i>
7.	<i>Lactobacillus plantarum</i>
8.	<i>Pediococcus acidilactici</i>
9.	<i>Saccharomyces cerevisiae</i>
10.	<i>Streptococcus infantarius</i>

Applications of Probiotics in Aquaculture

The need for sustainable aquaculture has promoted research into the use of probiotics on aquatic organisms. The initial interest was focused on their use as growth promoters and to improve the health of animals; however, new areas have been found, such as their effect on reproduction or stress tolerance, although this requires a more scientific development.

1. As Growth Promoters

Probiotics have been used in aquaculture to increase the growth of cultivated species, in reality it is not known whether these products increase the appetite, or if, by their nature, improve digestibility. Some people are inclined to think that it could be both factors; furthermore, it would be important to determine whether probiotics actually taste good for aquaculture species. According to Balcázar et al. (2006) probiotic microorganisms are able to colonize gastrointestinal tract when administered over a long period of time because they have a higher multiplication rate than the rate of expulsion, so as probiotics constantly added to fish cultures, they adhere to the intestinal mucosa of them, developing and exercising their multiple benefits. This also depends on factors such as hydrobionts species, body temperature, enzyme levels, genetic resistance, and water quality. (Irianto & Austin 2002)

The use of probiotics as growth promoters of edible fishes has been reported. Diet of Nile tilapia (*Oreochromis niloticus*) was amended with a



probiotic *Streptococcus* strain, increasing significantly the content of crude protein and crude lipid in the fish, also weight has increased from 0.154 g to 6.164 g in 9 weeks of culture. Due to the commercial importance of this species, the effect of supplementing diet with probiotics produced an increase of 115.3% when commercial formulation was used at a concentration of 2%. (Lara et al., 2003).

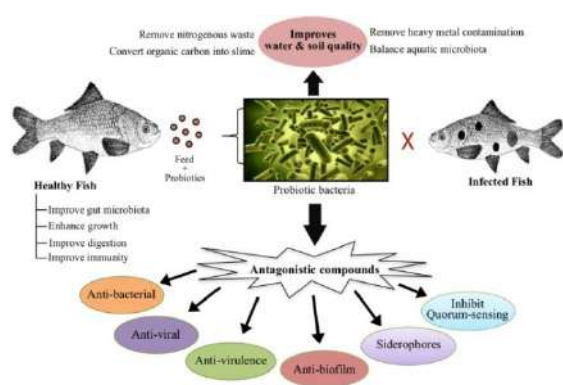


Fig 4: Probiotics applications

Examples of growth improvement of ornamental fishes include swordtail (*Xiphophorus helleri*, *X. maculatus*) and guppy, (*Poecilia reticulata*, *P. sphenops*), their feed was supplemented with *Bacillus subtilis* and *Streptomyces*, finding significant increases in growth and survival of *Xiphophorus* and *Poecilia* after 90 and 50 days of administration, respectively (Ghosh et al., 2008).

2. Inhibition of Pathogens

Antibiotics were used for a long time in aquaculture to prevent diseases in the crop. However, this caused various problems such as the presence of antibiotic residues in animal tissues, the generation of bacterial resistance mechanisms, as well as an imbalance in the gastrointestinal microbiota

of aquatic species, which affected their health. In fact, the European Union has regulated the use of antibiotics in organisms for human consumption. Today, consumers demand natural products, free of additives such as antibiotics; moreover, there is a tendency for preventing diseases rather than treating them. Thus, the use of probiotics is a viable alternative for the inhibition of pathogens and disease control in aquaculture species. (Nakano 2007).

Gómez et al. (2007) reported the use of *Vibrio alginolyticus* strains as probiotics to increase survival and growth of white shrimp (*Litopenaeus vannamei*), also by using probiotics in Ecuadorian shrimp hatcheries, production increased by 35%, while with the use of antimicrobials it decreased by 94%.

3. Improvement in Nutrient Digestion

A study has suggested that probiotics have a beneficial effect on the digestive processes of aquatic animals because probiotic strains synthesize extracellular enzymes such as proteases, amylases, and lipases as well as provide growth factors such as vitamins, fatty acids, and amino acids. Therefore, nutrients are absorbed more efficiently when the feed is supplemented with probiotics. (Balcázar 2006).

It has been reported that the probiotic yeast *Debaryomyces hansenii* HF1 has the ability to produce spermine and spermidine, two polyamines involved in the differentiation and maturation of the gastrointestinal tract in mammals. In addition this yeast secretes amylase and trypsin, enzymes that aid digestion in sea



bass larvae. Studies in juvenile common dentex *Dentex dentex* L. showed that when diet is supplemented with 0.5 g of *Bacillus cereus* strain E kg⁻¹ of food, increased fish growth due to more efficient use of the food. (Tovar et al., 2002; Hidalgo et al., 2006).

4.Improvement of Water Quality

In several studies, water quality was recorded during the addition of probiotic strains especially of the gram-positive genus *Bacillus*. Probably since this bacterial group is more efficient than gram-negative in transforming organic matter to CO₂. It is suggested that maintaining high levels of probiotics in production ponds, fish farmers can minimize the accumulation of dissolved and particulate organic carbon during the growing season. In addition, this can balance the production of phytoplankton. However, this hypothesis could not be confirmed on tests carried out during cultivation of shrimps or channel catfish, using one or more species of *Bacillus*, *Nitrobacter*, *Pseudomonas*, *Enterobacter*, *Cellulomonas*, and *Rhodopseudomonas*. Thus published evidence for improving water quality is limited, except for the nitrification. (Verschuere et al., 2000; Balcázar 2006).

5.Stress Tolerance

Aquaculture practices demand intensive productions in shorter times, causing stress in crop species. For example, it has been reported that chronic stress in zebra fish, *Danio rerio*, induces a general depression on the synthesis of muscle protein. As a result, it was sought to increase stress tolerance by using

probiotics. One of the firsts formal reports on this field studied the supplementation of *Lactobacillus*

delbrueckii ssp. *delbrueckii* in the diet of European sea bass (*Dicentrarchus labrax*). Another way to assess stress in fish involve subjecting them to heat shock, as in the case of Japanese flounder (*Paralichthys olivaceus*) grown in a recirculating system (Vianello et al., 2003; Taoka et al., 2006).

The potential health benefits of probiotics?

The beneficial microbes that live in different parts of our bodies assist us in a variety of ways. One of the most important ways is by fighting off the more harmful types of bacteria, fungi, viruses and parasites that might also want to live with us. Many probiotic products are formulated with beneficial bacteria and yeasts for the purpose of preventing or recovering from bacterial or yeast infections in your different body parts, including:

- Atopic dermatitis and acne.
- Cavities and gum disease.
- Vaginal and urinary tract infections (UTIs).
- Antibiotic-associated diarrhea.

Oral probiotics may have many additional benefits. The health benefits of probiotics:

- Helps break down and absorb certain nutrients and medications.
- Produces other important nutrients as byproducts.



- Helps break down and recycle bile after digestion.
- Helps train your immune system to recognize and eliminate harmful microbes.

information on the host/microbe interactions in vivo, and development of monitoring tools are still needed for better understanding of the composition and functions of the indigenous microbiota, as well as of microbial cultures of “probiotics”. The use of probiotics is an

Sl.No	Probiotic	Pathogen	Fish Species	Beneficial effects	Reference
1	<i>Carnobacterium divergens</i>	<i>V. anguillarum</i>	Atlantic cod	Reduced vibriosis	Gildberg et al., 1997
2	<i>Lactobacillus acidophilus</i>	<i>Pseudomonas fluorescens</i> , <i>Streptococcus iniae</i>	Nile tilapia	Improve immune function and disease resistance	Aly et al., 2008a
		<i>Staphylococcus xylosus</i> , <i>Aeromonas hydrophila</i> and <i>Streptococcus agalactiae</i>	African catfish	Reduced mortalities	Al-Dohail et al., 2011
3	<i>Lactobacillus rhamnosus</i>	<i>A. salmonicida</i>	Rainbow trout	Reduced mortalities	Nikoskelainen et al., 2001
		<i>Edwardsiella tarda</i>	Tilapia	Reduced mortalities	Pirarat et al., 2006
4	<i>Lactobacillus plantarum</i>	<i>Lactococcus (Lc.) garvieae</i>	Rainbow trout	Reduced mortalities	Vendrell et al., 2008
5	<i>Lactobacillus sakei</i>	<i>Edwardsiella tarda</i>	Rock bream	A non-significant decrease in the cumulative mortality	Harikrishnan et al., 2011
6	<i>Lactobacillus pentosus</i>	<i>Edwardsiella tarda</i> .	Japanese eel	Improved immune response and survival rate	Lee et al., 2013

Conclusion

The application of probiotics in aquaculture shows promise, but needs considerable efforts of research. It is essential to understand the mechanisms of action in order to define selection criteria for potential probiotics. Therefore, more

important management tool, but its efficiency depends on understanding the nature of competition between species or strains.



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BHAI-MS-13

Integrated Pest Management (IPM) Technology in Agriculture

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Abstract

Integrated Pest Management involves both chemical and non-chemical practices in the control of pests. It is used in various fields like agriculture, forestry, horticulture etc., to prevent and slow down the development of resistance. IPM is designed on the significance to control the intensity of pest and not eradication. It is a combination of all practices and methods to control the pest problems. Biological control is an important component of IPM programme. The strategy behind biological control of pests involve classical control method and conservation method. Integrated Pest Management is used for all other kinds of pests throughout the world like Livestock Operations, Greenhouses, National Parks, Food Services, Growth Chambers and many more. For the growing population farmers must produce more crops and to overcome the losses, more agricultural technologies has to be improved thereby protecting the environment. Regular training programmes related to IPM should be given to the farmers to get awareness about the management. India has adopted the integrated pest management as ‘National Policy’ for crop protection since 1985 and efforts are being made to encourage the farmers to adopt the sector. In the light of the above facts, the present paper reviews IPM usage.

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Introduction:

Integrated Pest Management (IPM) is an eco-friendly method of pest control. The term was first introduced by R. F. Smith and R. Van den Bosch and formalized by the US National Academy of Sciences in 1969. It involves the pest control programs by combining with several other methods in preventing and protecting the plants from pests. Presently, countries like Latin America, and Africa are using the integrated production and pest management together and concurrently.¹ The Government of India has adopted Integrated Pest Management to protect the crop since 1985. The crucial step in any pest management program is to identify the causative agent correctly and the failure in many cases is due to the lack of accurate information about the pest. For instance, plants may be damaged by factors like extreme weather conditions, inadequate soil fertilizers, water supply etc. that should not be confused or mistaken with the pest problem. Majority of the crop damage occurs during night by the pest like woodchucks and deer. The damage caused to the crop by the pest may be at any of its life-cycle stage and can be observed by the use of magnifying lens and the symptoms can be analyzed followed by the management processes.

The principles involved in IPM are to identify the pest before the action being taken monitoring guidelines for each pest species. The main objective of IPM is to minimize the usage of pesticides thereby conserving the energy. Throughout the world there are more than 600 species of pests that has developed resistance to pesticides and the resistance to the pests caused severe concern in agriculture leading to the poor yield. The more usage

of pesticides, the faster the pests become resistant. Insect pests cause severe crop loss and when insecticides fail, there are serious economic losses² and consequences for food security

The involvement of IPM in agricultural biotechnology involves the usage of scientific tools and techniques like molecular markers, molecular diagnostic vaccines and tissue culture to modify plants, animals and microorganisms. The desired characters are transferred from the known species to the desired species. According to UNs Food and Agricultural Organization IPM is defined as “the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest population and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment.”³

IPM is in practice throughout the world. It is used to solve pest problems without or at a very low level of risk to the mankind and the environment. Reducing the use of chemical insecticides for environmental and health concerns was an important motivator.⁴ The principle behind the IPM is to reduce the adverse impacts of pest control on human health, the environment and the non-target organisms by managing the pests effectively and reducing the exposure levels of pesticides to people, animals and environment.

Insecticides are widely used across the world to increase agricultural productivity and quality in vegetables and grains. In the practical agricultural aspect it can be defined as “A crop with resistant properties yield more than a susceptible crop in the



same insect pest attacking conditions”. In IPM, the uses of natural predators or parasites are used to control pests using selective pesticides when it is not considered by natural means. This method is used to manage pests across all the areas of the earth including natural. The strategy behind is to minimize the degradation of environment and to minimize the chemical inputs on humans and non-target organisms. *Bacillus thuringiensis* reduce pest damages more than insecticide treatments⁵. Through biological means control of insect pests and diseases is utmost component of IPM –

- The parasitoids are the organisms that lay eggs in or on the bodies of the host to complete their life-cycle on the host finally leading to the death of host.
- The natural enemy of a pest is introduced to control.
- By augmentation a large population of natural enemies are introduced for quick pest control.
- The predators are free living species that depend on other organisms for food. Ex: Spiders, dragonflies, lady bird beetles etc.

But the disadvantage of biological control is the non-target species may be attacked by the methods being followed. The effectiveness of using biological control using evolved extractions varies depending on how it is used; however, few programs are able to permanently suppress pest outbreaks⁶. In some cases, the presence of natural enemies such as parasitic wasps and predatory arthropods can significantly reduce pest numbers⁷ and some typical approaches for controlling endemic pests

include the regular release of commercially available natural enemies, the protection of natural enemy populations by providing refuges or avoiding acts that harm them⁸.

Cultural means of control method – The crop is to be selected based on the environmental factors that is suitable for it i.e., the right plant in the right place and fertility of the soil, water supply, fertilization are to be provided for the health of the crop. The healthy crop is less prone to the pest problem. The culture method disturbs the comfortable habitat of the pest. Sanitation and cultivation involves in eliminating the pest breeding sites and crop residues.

Mechanical and physical means of control method – In this the usage of machinery equipment like tilling machines, pest removal tractors, field ploughing tractors, traps with sticky devices and removing the pests manually by the labour involves in reducing the crops affected with pests.

Chemical means of control method – In this method the chemical are used in controlling the pests. Care should be taken then while using the chemicals the label instructions should be followed strictly or else they cause severe impact on the environment. Rotating pesticides with different modes helps in reducing the pest resistance.

The goal for agriculture is to keep pest population densities low and maintain a good health of population. By applying IPM, pest populations are kept below a point where they cannot harm the economy.¹¹





Fig.1 Five Step Management of Pests

Genetic means of control method – The genetically superior plants are selected that are pest resistant. Nematode-resistant plant, citrus greening resistance oranges are examples. These plants are better resistant to the pests. A complicated web of interactions is involved in the research on host plant resistance, and these interactivities are mediated by chemical and morphological characteristics that affect how much harm pests do.¹² The genetic changes result in modifications to the targets, so that the antimicrobial no longer interacts with its target site, or in the acquisition, induction or up-regulation of metabolic pathways that increase the degradation or removal of the pesticide from the organism¹³.

Further, IPM also plays an important role in sustainable agriculture like –

- Reducing the pesticide residues in food and water safety.
- Enhances in farm productivity and food availability by minimizing the pre and post-harvest crop losses.

- Helps in conserving the natural resources like soil, water and biodiversity.
- Aids in producing higher quality crops having less residues with high market prices that contributes to the farmer financially.

The pesticides are used in a wide range that exhibit their activity to control weeds, rodents, insects, molluscs and microorganisms like bacteria, fungi and virus. They are used during various stages of plant production, including at the time of planting, during plant growth and post-plant-based agricultural harvest for agronomic and horticulture purposes, as well as in floriculture, forestry, and by home gardeners¹⁴.

Prevention is the first and an important tool in pest management because it is eco-friendly, least expensive and more effective method. Selecting a healthy crop that can thrive adverse conditions and by taking precautionary measurements, a crop can be protected from pests and stress. The next step to prevent the crop from pest attack is to identify the early symptoms of affected crop, followed by record keeping. By maintaining the record, information regarding the planting of the crop, species variety, its habitat, onset of the disease to the crop can be determined and helps the farmer for further steps to take further steps.

Research suggests that tilling the soil is necessary and makes the developing stages in life-cycle of the pests to the surface. They get exposed to the sun light or get eaten away by the predators. Rotation of the crop should be strictly followed to minimize the pest problem. Insects pests can be controlled by planting attractive crops to



attract insects and then treating the trap crop with insecticide, like sunflowers attract *Lygus* plant bugs. Pheromone traps are used for detecting the presence of pests¹⁵. China clay or Kaolin clay can be used in the form of thin film on leaves and fruits to protect plants from Colorado potato beetle. The thin film irritates the insect body from eating the crop.

The strategy involved in the integrated pest management follows five steps and was developed by the Bio-Integral Resource Central in Berkeley, California.¹⁶

Identification of the pest: An important primary step is the identification of pest. Majority of the species are not pests hence, identifying the main causative agent should be done with an utmost care.

Observing the pest activity: The population of pest depends on the environmental factors where their number increases but causes no harm and vice-versa, Therefore, the treatment is depended on monitoring their activity.

Determine action thresholds: It is the point where further damage caused by the pests should to be stopped by involving pest control methods that shows lower pesticide effects.

Explore treatment options and make treatments: Giving less priority to the usage of pesticides, importance has to be given for prevention and it should be considered first. Involvement of Best Management Practices (BMPs) is another treatment option. If the crops are pest resistant varieties then they are less prone to be affected. Further, treatment of the crop depends on the fertility of the soil also as it

gives nutrients to the crop. Therefore, pesticide treatment should be given to the targeted species that are severely attacked and that should be less toxic to the mankind and also to the environment.

Evaluate Results: Monitoring after the treatment is an important factor to know how far the pest has been controlled and further the treatment is needed or not.

Conclusion:

Five step integrated pest management helps in reducing the usage of pesticides. Its goal is to identify the diseased or affected and to treat them without causing any impact on the environment. IPM helps in determining the correct treatment with less negative impact on the ecosystem. It encourages the healthy crops and minimizes the use of pesticides and improves the crop quality. Integrated pest management is a sustainable form of pest management – improve biodiversity.

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Biotechnology Based Approaches to Disease Diagnosis

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Abstract

DNA-based diagnostic tests determine the existence of specific nucleotide sequences, including human genetic mutations and sequences present in human pathogens. They are highly sensitive and specific and can detect single nucleotide mutations or copy number variations. The ability to diagnose diseases in humans at the genetic level makes it possible to determine the cause of an illness and to predict whether individuals or their offspring are predisposed to the disease. DNA analysis can be used for the identification of asymptomatic carriers of hereditary disorders, for prenatal diagnosis of serious genetic conditions, and for early diagnosis before the onset of symptoms. DNA sequence-specific diagnostic approaches include hybridization of a unique DNA probe to a complementary target sequence, target sequence amplification by PCR, microarray analysis to detect multiple sequences in a single sample, and mass spectrometry to identify single-nucleotide polymorphisms (SNPs). A diagnostic test involving DNA hybridization utilizes a DNA probe to detect a complementary target DNA sequence that is characteristic of the disease. The probe is labelled with a reporter molecule that indicates hybridization between the target and probe DNA. A hybridization probe used in a diagnostic assay must hybridize exclusively to the selected target nucleic acid sequence. Hybridization probes are often employed to detect the presence of microbial pathogens. This study is aimed at to investigate different Biotechnology tests to detect the human disease and pathogenesis.

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Introduction

The early 1970s were ripe for the development of recombinant DNA technology following the milestone discoveries of the structure of DNA by Watson and Crick (Watson and Crick, 1953) and the cracking of the genetic code by Nirenberg, Matthaei, and Jones (Nirenberg and Matthaei, 1961; Nirenberg, 1962). Building on this, rapid progress was made in understanding the structure of genes and the manner in which they are expressed. Advancing the discovery by Herbert Boyer and colleagues (Hedgpeth, 1972), who showed that the RI restriction endonuclease from *E. coli* (now known as EcoRI) made a staggered cut at a specific nucleotide sequence in each strand of double-stranded DNA, Mertz and Davis (Mertz and Davis, 1972) reported that the complementary ends produced by EcoRI could be rejoined by DNA ligase in vitro. In the meantime, Cohen and Chang (Cohen and Chang, 1973) had been experimenting with constructing plasmids by shearing large plasmids into smaller random pieces and introducing the mixture of pieces into the bacterium *E. coli*. One of the pieces was propagated. However, the randomness of plasmid fragmentation reduced the usefulness of the process. During a now-legendary lunchtime conversation at a scientific meeting in 1973, Cohen and Boyer reasoned that EcoRI could be used to splice a specific segment of DNA into a plasmid, and then the recombinant plasmid could be introduced into and maintained in *E. coli* (Cohen et al., 1973). Recombinant DNA technology was born. The potential of the technology was immediately evident to Cohen and others: “It may be possible to

introduce in *E. coli*, genes specifying metabolic or synthetic functions such as photosynthesis, or antibiotic production indigenous to other biological classes.” The first commercial product produced using this technology was human insulin. Mullis received the Nobel Prize in chemistry in 1993 for his invention of the PCR method. Replication of a specific DNA sequence by PCR requires three successive steps. Amplification is achieved by repeating the three-step cycle 25 to 40 times. All steps in a PCR cycle are carried out in an automated block heater that is programmed to change temperatures after a specified period of time.

1. Denaturation.

The first step in a PCR is the thermal denaturation of the double-stranded DNA template to separate the strands. This is achieved by raising the temperature of a reaction mixture to 95°C. The reaction mixture is comprised of the source DNA that contains the target DNA to be amplified, a vast molar excess of the two oligonucleotide primers, a thermostable DNA polymerase (e.g., Taq DNA polymerase, isolated from the bacterium *Thermus aquaticus*) and four deoxyribonucleotides.

2. Annealing.

The temperature of the mixture is slowly cooled. During this step, the primers base-pair, or anneal, with their complementary sequences in the DNA template. The temperature at which this step of the reaction is performed is determined by the nucleotide sequence of the primer that forms hydrogen bonds with



complementary nucleotides in the target DNA. Typical annealing temperatures are in the range of 45 to 68°C, although optimization is often required to achieve the desired outcome, that is, a product consisting of fragments of target DNA sequence only.

3. Extension.

Temperature is raised to ~70°C, which is optimum for the catalytic activity of Taq DNA polymerase. DNA synthesis is initiated at the 3' hydroxyl end of each annealed primer, and nucleotides are added to extend the complementary strand using the source DNA as a template.

The specificity, sensitivity, and simplicity of PCR have rendered it a powerful technique that is central to many applications in biotechnology. It is used to detect specific mutations that cause genetic disease, to confirm biological relatives, to identify individuals suspected of committing a crime, and to diagnose infectious diseases. Commonly, PCR is used to amplify target DNA for cloning into a vector. To facilitate the cloning process, restriction enzyme recognition sites are added to the 5' end of each of the primers that are complementary to sequences that flank the target sequence in a genome (Alberts B, Johnson A, Lewis J., 2002).

Review of Literature

Molecular diagnostic approaches detect molecular biomarkers of disease. In this context, a disease biomarker is a specific molecule that has been determined to be present, or present at higher or lower levels, in diseased tissues compared to normal tissue or to be an indicator of disease

prognosis or a response to therapy. It can be a protein, a specific sequence of DNA or RNA, or a small metabolite (Sarhadi VK, Armengol G, 2022). Specific molecules may be analyzed qualitatively or quantitatively in urine, blood serum, or tissue samples. Samples that contain a small amount of the target molecule, for example, small core needle biopsy samples or blood samples, may require some amplification of the target for accurate detection and quantification. The complex heterogeneity of clinical samples may also present a challenge for molecular diagnostics, and methods such as tissue micro dissection may be employed to increase sample purity. Molecular diagnostic tests are available to detect the etiological agents of infectious and genetic diseases (Datta S, Malhotra L, Dickerson R, Chaffee S, Sen CK, Roy S, 2015). The latter may be single-gene disorders such as cystic fibrosis or complex multigene disorders such as Alzheimer disease. Although many biomarkers have been identified, clinically relevant biomarkers are not well defined for most human diseases. Proteins play a critical role in all cellular processes, including metabolism, communication, defense, reproduction, transport, and motility. Regulatory proteins maintain tight control over protein production to ensure that cells function normally (Cooper GM, 2000). Dysregulation of these processes in diseased tissue is reflected in alterations in protein composition or levels of specific proteins. Characteristic changes in proteins have been used extensively to diagnose disease, either by detecting the presence or measuring levels of a specific protein biomarker or by determining protein



profiles in polygenic diseases (Neagu AN, Jayathirtha M, Whitham D, Mutsengi P, Sullivan I, Petre BA, Darie CC, 2022). Enzyme-linked immunosorbent assays (ELISAs) are widely used for diagnosing human diseases, including various cancers, autoimmune diseases, allergies, and infectious diseases. It is based on the specific and high-affinity interaction between an antibody and an antigen and is a sensitive assay that can be used for rapid detection on a large scale (Alhajj M, Zubair M, Farhana A, 2023). Detection relies on the activity of an enzyme that is covalently bound to an antibody employed in the assay. An indirect ELISA can detect the presence of specific antibodies in patient serum that indicates an immune response to the presence of a particular protein or pathogen. A sandwich ELISA detects the presence of a specific antigen in a patient's sample and hence is sometimes referred to as an antigen capture assay. In contrast to an indirect ELISA, a sandwich ELISA directly detects a particular antigen in a complex clinical sample. To capture the antigen, a monoclonal antibody that is specific for the target antigen is first bound to the surface of a microtiter plate. The patient's sample is then added to a well; if the specific antigen is present in the sample, it binds to the immobilized antibody. A labeled primary antibody is added to detect the presence of bound antigen. The labeled antibody may detect a different epitope on the same antigen. A sandwich ELISA is the basis for many diagnostic tests, including the home pregnancy test. In one pregnancy test, the human chorionic gonadotropin (hCG) protein produced during pregnancy by the developing placenta is detected in

urine. Specific for hCG are deposited on different regions of a membrane. Urine is applied to one end of the membrane and is Monoclonal antibodies that are not bound to hCG are carried away with the urine by capillary action. The monoclonal antibodies are conjugated to an enzyme that reacts with dye molecules contained in the region with the polyclonal antibodies. (Quinteros DA, Bermúdez JM, Ravetti S, Cid A, Allemandi DA, Palma SD, 2017). The U.S. Food and Drug Administration (FDA) has approved the use of a HE4 sandwich ELISA to monitor the recurrence or progression of ovarian cancer in women who are being treated for the disease. Several autoimmune diseases have been identified, including celiac disease, type 1 diabetes, lupus erythematosus, and rheumatoid arthritis (Frommer L, Kahaly GJ, 2020). Indirect ELISAs have been developed to diagnose some autoimmune diseases. In these assays, autoantibodies produced against a self-protein are detected in patient blood samples. Rheumatoid arthritis is an autoimmune disease that results in chronic, systemic inflammation of the joints, mainly the synovial joints (Song YW, Kang EH, 2010). Systemic inflammation may damage organs such as the heart and lungs. Rheumatoid factor is an autoantibody that targets the Fc region of immunoglobulin G (IgG) antibodies and contributes to rheumatoid arthritis. It is commonly used as a diagnostic biomarker to differentiate rheumatoid arthritis from other forms of arthritis and other inflammatory conditions (Fang Q, Ou J, Nandakumar KS, 2019). Viruses can be propagated only in host cells and cannot be identified using metabolic characteristics. Moreover, some clinical



isolates exhibit atypical metabolic profiles that confound accurate identification. Immunological detection methods such as agglutination assays and ELISAs eliminate the need to grow the pathogen in culture and can be used to detect specific viral, bacterial, fungal, or protozoan pathogens in body fluids and tissues. Because antibody-based approaches detect a target antigen with high specificity and sensitivity, they are well suited to distinguish a specific pathogen from the other hundreds of microbes that are normally present in some human tissues. Immunological assays for infectious disease may target proteins produced by a pathogen or may detect the presence of antibodies produced against the pathogen (Peruski AH, Peruski LF Jr, 2003). To prevent transmission of infectious disease, donated blood is screened for several pathogens, including human immunodeficiency virus, hepatitis virus, human T-lymphotropic virus, and *Treponema pallidum*, the bacterium that causes syphilis. Infection with hepatitis virus is relatively common in the United States (25 per 100,000 individuals) (Buerger CS, Jain H, 2023). Although five hepatitis viruses can infect the liver and cause liver inflammation, cell death, and even liver failure, most cases of chronic viral hepatitis are caused by hepatitis B and C viruses. Both viruses may be transmitted via blood transfusion. ELISAs have been developed for routine blood screening for hepatitis B surface antigen, a viral envelope lipoprotein, and for antibodies produced against both hepatitis B and C viruses (Kim SH, 2017). Viral surface antigens are the first markers to be detected in serum after infection, while antibodies appear up to

several months later. Detection of the viral surface antigen with a sandwich ELISA utilizes specific monoclonal antibodies to capture the hepatitis B surface antigens present in blood in wells of an assay plate. The monoclonal antibodies were generated using recombinant hepatitis B surface protein expressed in yeast. An anti-hepatitis B surface antigen detection antibody with a conjugated enzyme and the enzyme substrate are added to measure virus (Takeda K, Maruki M, Yamagaito T, Muramatsu M, Sakai Y, Tobimatsu H, Kobayashi H, Mizuno Y, Hamaguchi Y, 2013). A liquid bead array has been developed to quantify levels of these and other protein biomarkers in the serum samples. In this system, all of the different protein biomarkers are detected in a single well of a multiwell plate, which enables rapid analysis of many patient samples simultaneously (Parsa SF, Vafajoo A, Rostami A, Salarian R, Rabiee M, Rabiee N, Rabiee G, Tahriri M, Yadegari A, Vashae D, Tayebi L, Hamblin MR, 2018). Primary antibodies directed against each protein biomarker are attached to Patients with metastatic colorectal cancer were scored based on the phosphor proteome profiles of liver tumors analyzed using reverse-phase protein microarrays. (Wong GYM, Diakos C, Hugh TJ, Molloy MP, 2022). Many of the tumor cells had high levels of phosphorylated tyrosine kinases c-KIT, c-Abl, and platelet-derived growth factor receptor, which suggests that these patients may respond well to imatinib treatment (Iqbal N, Iqbal N, 2014). Diagnosis of Alzheimer disease using immunological methods may exploit the development of antibodies that differentiate



between the conformations of the disease-associated A β proteins and normal APP (Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M, 2019). To develop conformation-specific antibodies, researchers used an approach that mimics protein aggregation during the disease process, that is, the self-interaction of the A β proteins. (Ma B, Zhao J, Nussinov R, 2016).

CONCLUSION

Novel, biotechnology based approaches to disease diagnosis open a wide range of new possibilities and challenges in diagnostic various human diseases. Thus, it is strongly advised to consider powerful new methods and to introduce these new technologies at our diagnostic laboratories. However, it is important to diagnosis and conformation the exact diagnostic capacity of the new technologies.

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BHAI-MS-15

Aquatic Immunity: Biotechnology's Contribution to Sustainable Aquaculture

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Abstract

This study delves into the transformative impact of biotechnology on the sustainability of aquaculture health. Through genetic advancements, diagnostic precision, and targeted interventions, biotechnology emerges as a key player in fortifying aquatic ecosystems against disease threats. From genetically engineered resistance to tailored vaccines and advanced diagnostic tools, biotechnological innovations contribute to proactive disease management. The synergy of these approaches not only enhances the resilience of aquatic species but also promotes environmentally conscious practices. As we navigate the complexities of aquaculture, this abstract explores how biotechnology serves as a linchpin in fostering sustainable health for our aquatic environments.

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Introduction:

Aquaculture, as a pivotal contributor to global food security, faces the persistent challenge of disease outbreaks that can jeopardize both aquatic species health and the sustainability of the industry. In recent decades, biotechnology has emerged as a transformative force in the quest for effective disease management within aquaculture systems. This paper aims to provide a comprehensive exploration of the diverse roles played by biotechnological innovations in mitigating disease risks, enhancing resilience, and fostering sustainability in aquaculture.

The escalating demand for aquatic products has driven intensified aquaculture practices, creating an environment conducive to the emergence and spread of diseases. Traditional methods of disease control often fall short in addressing the dynamic nature of aquatic pathogens and the intricate ecosystems in which they thrive. Herein lies the significance of biotechnology, which offers a spectrum of tools and approaches ranging from genetic interventions and advanced diagnostics to targeted vaccines and eco-friendly disease management practices.

Disease resistance in aquaculture: refers to the ability of aquatic organisms, such as fish or shellfish, to withstand or exhibit reduced susceptibility to infections caused by various pathogens. This crucial aspect of aquaculture health is paramount for sustainable and productive practices. Disease resistance can be achieved through natural selection, selective breeding, or advanced biotechnological interventions.

1. Natural Selection: Over time, in response to environmental pressures, certain individuals within a population may develop inherent resistance to specific diseases. Natural selection favors these traits, and, given favorable conditions, the resistant individuals contribute more offspring to subsequent generations.

2. Selective Breeding: Aquaculturists employ selective breeding programs to enhance disease resistance in cultivated stocks. By identifying individuals with natural or acquired resistance, breeders can strategically mate them to pass on these desirable traits to the next generation. This traditional approach has been successful in developing more resilient strains of aquaculture species.

3. Genetic Engineering: Biotechnology plays a transformative role in disease resistance by enabling precise genetic modifications. Scientists can identify and introduce specific genes associated with resistance into the genome of target species. This approach allows for the creation of genetically modified organisms (GMOs) with enhanced resistance to prevalent diseases.

4. Immunization: Vaccination is another biotechnological method to bolster disease resistance. By exposing aquatic organisms to harmless components of pathogens or weakened forms of the pathogens themselves, the immune system can develop a memory response, providing a quicker and more effective defence against future infections.

5. Genomic Approaches: Advances in genomics have facilitated the identification



of genetic markers associated with disease resistance. This knowledge allows aquaculturists to selectively breed individuals with these markers, enhancing the overall disease resistance of the population.

Overall, disease resistance strategies in aquaculture represent a dynamic interplay between natural processes, selective breeding, and cutting-edge biotechnological interventions. These approaches are vital for maintaining the health and sustainability of aquaculture systems, reducing reliance on therapeutic treatments, and mitigating the impact of diseases on global food security.

Diagnostic tools in aquaculture: are essential for accurately identifying and monitoring diseases, enabling prompt intervention and effective management. These tools have evolved with advancements in biotechnology, providing more precise and rapid detection methods. Key diagnostic tools include:

1. **Polymerase Chain Reaction (PCR):** PCR is a molecular biology technique that amplifies and detects specific DNA sequences. In aquaculture, PCR is widely used to identify the presence of pathogens by amplifying their genetic material. This method allows for the early detection of diseases, even at low levels, facilitating timely responses.

2. **Loop-Mediated Isothermal Amplification (LAMP):** LAMP is an isothermal nucleic acid amplification technique that enables the rapid and cost-effective detection of pathogens. It is particularly useful in field settings where

sophisticated laboratory equipment might be limited.

3. **Enzyme-Linked Immunosorbent Assay (ELISA):** ELISA is an immunological method that detects the presence of antigens or antibodies. In aquaculture, it is commonly used to identify specific pathogens or antibodies in the blood or tissues of aquatic organisms, providing insights into the immune response.

4. **DNA Sequencing:** Advances in DNA sequencing technologies allow for the comprehensive analysis of the genetic material of pathogens. Whole-genome sequencing provides detailed information about the structure and characteristics of pathogens, aiding in their precise identification and understanding of their genetic makeup.

5. **Biosensors:** Biosensors are analytical devices that detect specific biological molecules. In aquaculture, biosensors can be designed to identify pathogenic substances or changes in the physiological parameters of aquatic organisms, offering real-time monitoring capabilities.

6. **Histopathology:** Although not strictly a biotechnological tool, histopathology involves the microscopic examination of tissues to identify pathological changes. Biopsy samples are analyzed to diagnose diseases and understand their impact on the health of aquatic organisms.

7. **Remote Sensing Technologies:** Remote sensing tools, such as imaging devices and underwater cameras, contribute to disease diagnosis by visually assessing the health and behavior of aquatic species. These technologies aid in the detection of external



signs of diseases, such as lesions or abnormal behaviour.

These diagnostic tools collectively empower aquaculturists and researchers to swiftly and accurately identify diseases, monitor their prevalence, and implement targeted management strategies. The integration of biotechnological advancements in diagnostics enhances the precision, speed, and efficiency of disease detection in aquaculture systems.

Vaccines : In aquaculture, vaccines represent a crucial biotechnological tool designed to bolster the immune response of aquatic organisms against specific pathogens. This proactive approach to disease management offers several key advantages:

Disease Prevention: Vaccines stimulate the immune system to recognize and mount a defense against specific pathogens, preventing infections before they can establish.

2. **Reduced Disease Impact:** Vaccinated aquatic species are better equipped to resist and fight off infections, leading to reduced morbidity and mortality rates in aquaculture systems.

3. **Environmental Sustainability:** Compared to traditional disease control methods such as antibiotics, vaccines contribute to environmentally sustainable practices by minimizing chemical inputs into aquatic ecosystems.

4. **Tailored Solutions:** Biotechnological advancements allow for the development of vaccines tailored to the specific needs of different aquatic species and the prevalent

diseases in a particular region or aquaculture system.

5. **Herd Immunity:** When a significant portion of the aquatic population is vaccinated, it creates a form of herd immunity, protecting even non-vaccinated individuals by reducing the overall prevalence of the targeted pathogen.

6. **Long-lasting Protection:** Vaccines often provide long-lasting protection, reducing the need for frequent treatments and interventions, and contributing to the overall health and resilience of the cultured species.

7. **Economic Benefits:** While there may be initial costs associated with vaccine development and administration, the long-term economic benefits include reduced losses from disease outbreaks and a more stable and productive aquaculture operation.

8. **Public Health Concerns:** In cases where aquaculture products are intended for human consumption, the use of vaccines aligns with public health considerations by minimizing the risk of antibiotic residues in seafood.

As the field of aquaculture vaccine development progresses, ongoing research focuses on optimizing formulations, delivery methods, and the identification of new target pathogens. The integration of vaccines into aquaculture practices represents a pivotal biotechnological strategy for ensuring the sustainability and health of global aquaculture systems.

Probiotics and Prebiotics: Probiotics and prebiotics play a crucial role in sustainable



aquaculture health, and biotechnology innovations have enhanced their application. Here's how:

1. Probiotics for Gut Health: - Biotechnology enables the isolation and cultivation of beneficial microorganisms (probiotics) that positively influence the gut microbiota of aquaculture species. These probiotics can improve nutrient absorption, enhance disease resistance, and contribute to overall digestive health.

2. Prebiotics to Support Probiotics: Biotechnological advancements allow for the development of prebiotics, compounds that stimulate the growth and activity of beneficial microorganisms. Combining prebiotics with probiotics promotes a synergistic effect, fostering a balanced and healthy microbial community in aquaculture systems.

3. Disease Prevention and Reduction of Antibiotic Use: - Probiotics contribute to disease prevention by outcompeting harmful pathogens for resources and producing substances that inhibit their growth. This can reduce the reliance on antibiotics, promoting more sustainable and environmentally friendly aquaculture practices.

4. Biotechnological Selection of Effective Strains: Biotechnology enables the identification and selection of specific probiotic strains with optimal characteristics for a particular aquaculture system. Tailoring probiotics to the specific needs of different species enhances their effectiveness in promoting health.

5. Application in Larviculture: Biotechnological innovations extend the use of probiotics to larviculture, supporting the health and development of juvenile aquatic organisms from the earliest stages.

6. Continuous Research and Development: - Ongoing biotechnological research allows for the discovery of new probiotic strains and the optimization of formulations for maximum efficacy.

Genetic Markers : Genetic markers play a crucial role in advancing sustainable aquaculture health through biotechnology innovations. By identifying and utilizing specific genetic markers, aquaculture practitioners can:

1. Disease Resistance: Selecting and breeding fish with genetic resistance to common diseases reduces the need for antibiotics and enhances overall health.

2. Optimized Growth: Genetic markers associated with rapid growth and efficient nutrient utilization help develop aquaculture species with improved feed conversion rates, promoting sustainability.

3. Environmental Adaptability: Identifying genetic traits that enhance tolerance to varying environmental conditions allows for the cultivation of species in diverse geographic locations, reducing the ecological impact.

4. Reproductive Performance: Selecting fish with favorable reproductive traits through genetic markers supports efficient and sustainable breeding programs.

5. Nutritional Content: Genetic manipulation can be used to enhance the nutritional content of fish, ensuring they



meet the dietary needs of both the species and consumers.

By incorporating these genetic markers, biotechnology innovations contribute to the development of robust and sustainable aquaculture practices, minimizing environmental impact and improving overall industry resilience

Water quality Management: Effective water quality management in aquaculture is crucial for sustainable and healthy operations. Biotechnology innovations play a key role in achieving this goal. Implementing advanced sensors and monitoring systems can provide real-time data on parameters like dissolved oxygen, pH, and nutrient levels, allowing for proactive adjustments.

Bioremediation techniques, such as using beneficial microbes or plants, can help in maintaining water quality by removing pollutants. Additionally, genetic improvements in aquaculture species can enhance their resistance to diseases, reducing the need for chemical treatments.

Innovations in recirculating aquaculture systems (RAS) with biofilters and water treatment technologies contribute to minimizing environmental impact. Sustainable practices, coupled with biotechnological advancements, ensure the long-term health of aquaculture ecosystems and support the industry's growth.

Nutritional Advancement : Biotechnology in aquaculture enhances nutritional advancement through genetic improvements for nutrient utilization, designer feeds, omega-3 production, biofortification, probiotics for gut health,

algae-based feeds, selective breeding for disease resistance, precision feeding with sensor technologies, bioremediation for water quality, and nutrigenomics for personalized nutrition. These innovations promote sustainable aquaculture by optimizing growth, reducing environmental impact, and ensuring species health.

Conclusion:

As we navigate the intersection of biotechnology and aquaculture, a deeper understanding of these innovations promises to reshape the landscape of disease management, ensuring the health and longevity of both aquatic species and the aquaculture industry at large.

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BHAI-MS-16

Applications of Biotechnology in Aquaculture

Lavanya K* and Dayakar Y

Abstract

Aquaculture and biotechnology are currently interacting in a positive way. Whereas aquaculture produces aquatic organisms under controlled systems with technological applications that allow managing population densities higher than the natural ones, aquaculture may be thought of as a collection of techniques used or applied to living organisms with the goal of modifying or manufacturing a product for use or consumption. While they are young, a large number of fish and shellfish species are comparatively resistant to chromosomal alteration. It has been investigated whether genetic modification, including polyploidy, can increase aquaculture productivity. The paper discusses the significant advancements in the aquaculture sector brought about by biotechnology, including the use of genetically modified stock, commercial feed infused with algae, vaccinations to prevent disease, and the management of water quality.

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Introduction

Aquaculture is the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies modifications and intervention in the production cycle, such as regular stocking, sorting, feeding and protection from predators in order to enhance production. It is important to note that aquaculture has a long tradition in the developing countries of the Asia-Pacific region, supplying most of the world's aquaculture production (over 90 percent), and making important contributions to the livelihoods and subsistence of small-scale farmers and coastal populations in many countries in the region.

1. Farming systems:

Aquaculture systems can range from an intensive indoor system monitored with high-tech equipment through to the simple release of fry and fingerling to the sea, but the aim remains the same: to improve production. Some of the simplest production systems are the small family ponds in tropical countries where carp are reared for domestic consumption. At the other end of the scale are high technology systems, such as the intensive indoor closed units used in North America for the rearing of striped bass or the sea cages used in Chile and Europe for growing salmon and bream.

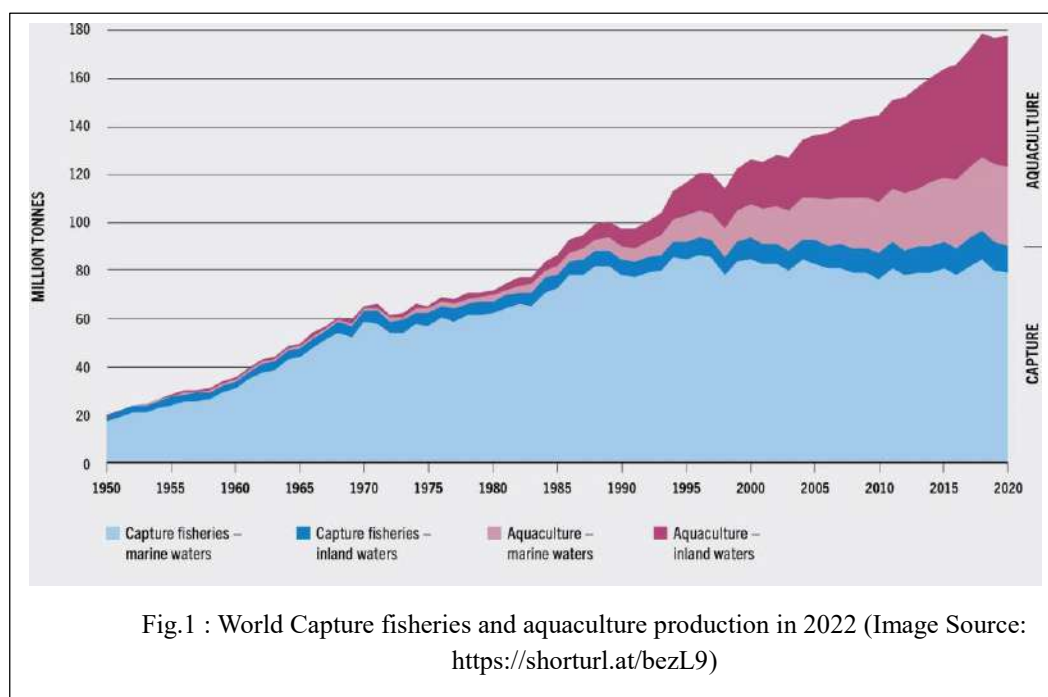
It is important to note that most of these small-scale aquaculture activities occur in developing countries, especially in regions or rural areas where food supply is at risk. For example, tilapia has become a globally important aquatic species that is produced in nearly 100 developing

countries worldwide. According to FAO, about 80 percent of the world's farmed tilapia comes from small-holders in developing countries, and this species is particularly prominent in production systems in the Asia-Pacific, the region that provides most of the world's aquaculture supply (De Silva, Subasinghe, Bartley, 2006).

Another good example of extensive aquaculture is the production of major carps in India. In this case, the majority of the production takes place in rural areas with relatively few impacts on the environment, particularly by using multitrophic culture of species such as catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*). It is true that some instances of uncontrolled aquaculture development have caused significant negative environmental and social impacts. However, except for a very few species, there are few negative environmental impacts associated with current production systems and practices. Moreover, most traditional and extensive systems produce fish with little or no negative environmental or social impact.

There has been a steady increase in the growth of aquaculture in developing countries, the rate of growth being twice that of developed nations. The most recent figures for global aquaculture production show that more than 90 percent of total fish production comes from developing countries, particularly China, which contributes about 70 percent of the total global fish and shellfish production (Subasinghe et al., 2009). Aquaculture is thus often one of the most important food





production sectors in developing countries, and in many cases, it is one of the most important sources of both food and income for rural populations.

2. Biotechnological Interventions in Aquaculture:

2.1 Genetic improvement and control of reproduction

Aquaculture is still the fastest growing food producing sector, compared to other food commodities. One of the reasons for this is the diversity of species in culture at present (over 230), and the genetic diversity that can be exploited through captive breeding and domestication, enabling the development of improved culture methods for a diverse array of species to expand commercial aquaculture (Subasinghe, 2009). A lack of knowledge of the biology of many of these species and the cost of technology development are constraints that explain in part why biotechnologies are only now emerging as useful tools for

increasing the productivity and sustainability of this sector. Aquaculture is a sector that is likely to benefit greatly from the application of appropriate genetic and reproduction biotechnologies to increase food production.

The use of hormones for the control of reproduction has been primarily developed for inducing the final phase of ova production, i.e. for synchronizing ovulation and for enabling bloodstock to produce fish in the first part of the season or when environmental conditions suppress the spawning timing of females. These procedures began by the pioneering work of Houssay (1930), who demonstrated that extracts of hypophysis (pituitary gland) can have an effect on sexual maturation of fish and reptiles (Zohar and Mylonas, 2001). These results allowed the development of a relatively simple procedure consisting of injecting hypophysial extracts purified by chromatography that contain products such as inductive hormones related to sexual



maturation. Human chorionic gonadotrophin and the gonadotrophin release hormone (GnRH) were also used to control the maturation of many fish species without limitations due to species-specific effects (Zohar and Mylonas, 2001). GnRHa, an analogous GnRH developed chemically, is more efficient in inducing maturation and is relatively inexpensive. It can be injected or administered by means of pellet implants, which facilitates its practical use. The use of hormones such as GnRHa has allowed advancement of the date of egg-laying in several species of fish, mainly salmonids, although for relatively short periods of time (Valdebenito, 2008). Several other molecules are currently under development for use in molluscs (e.g. scallops, oysters and mussels), where synchronous reproduction is required for the hatchery rearing of larvae for aquaculture production in developing countries, instead of using seed obtained from natural banks.

2.2 Biosecurity and disease control

Disease outbreaks are a serious constraint to the development of intensive aquaculture systems and can have a major impact on production due to mortality and decreased growth. It has been recognized that disease is the most significant factor impacting the intensive production of shrimp, salmon, carp and tilapia, with losses of 10 – 90 percent of total production (Peinado-Guevara, Melina and López-Meyer, 2006). Although many aquatic animal pathogens are well studied, unlike in terrestrial animals, the spread of pathogens is easy through water, and control is difficult due to high density culture in fluid

environment. Disease occurs in all systems, from extensive to intensive, although heavy losses are always possible in intensive production systems (Bondad-Reantaso, et al., 2005). There is a greater need for management intervention in intensive systems. Here biotechnological tools can be a valuable part of management approaches. Their scope of application is broad – they can be used as sensors in the production environment, for waste management (through controlled microbial technologies), and for disease detection and control (molecular methods). Traditionally, disease control is often carried out only after mortality has been observed. In the past, the diagnosis of fish diseases has been achieved primarily using histopathological methods, supported by parasitological, bacteriological and viral studies based on necropsy and in vitro cell culture.

2.3 Genetic modification

A genetically modified organism (GMO) is one whose genetic material has been altered through genetic engineering techniques with DNA molecules from different sources that are combined into one molecule to create a new set of genes. Typically, it involves introduction of a single gene from an unrelated species. After about two decades of very intensive research, the technology has reached the stage where it is possible to produce GM carp, tilapia and salmon. However, no aquatic GMOs have yet been approved for commercial release for food and agriculture purposes in any developed or developing country. There are potential concerns about the environmental impact of raising such fish (e.g. effects of possible interbreeding



with native populations) and the greater amount of feed required for sustaining the increased growth rates, as well as problems with consumer acceptance, which may be one of the most important reasons that transgenic technology has not developed beyond the experimental phase. Many developing countries have yet to develop a clear policy on the use of transgenic fish.

2.4 Marker systems

Molecular markers are identifiable DNA sequences, found at specific locations of the genome, transmitted by standard Mendelian laws of inheritance from one generation to the next. They rely on a DNA assay and a range of different kinds of molecular marker systems exist, such as restriction fragment length polymorphisms, random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and microsatellites. The different marker systems may vary in aspects such as their technical requirements, the amount of time, money and labour needed and the number of genetic markers that can be detected throughout the genome (reviewed in detail in FAO, 2007).

2.5 Pathogen screening and disease diagnostics:

The control of disease outbreaks relies heavily on having rapid and accurate diagnostic tools available in order to detect and identify the pathogen causing mortality. DNA and RNA methods have been used extensively for detecting a number of viral and bacterial pathogens in aquaculture worldwide. The techniques rely upon the fact that each pathogen species

carries a unique DNA or RNA sequence that can be used for identification. The techniques offer high sensitivity and specificity, and the commercial development of PCR primers and diagnostic kits allows rapid screening for a number of serious viral and bacterial infections and has direct application. Molecular-based techniques such as PCR also have an application in situations where the animal shows no antibody response after infection.

Considering the difficulties that developing countries may face in using advanced molecular diagnostics, and the importance of gradually improving national diagnostic capacities in developing countries, FAO recommended a three-level diagnostic process (FAO/NACA, 2000). This involves: field observations and necropsy (Level I); laboratory observations, bacteriology and histopathology (Level II); electron microscopy, molecular biology and immunology (Level III). There is a clear need to improve national diagnostic capacities to reach Level II and Level III diagnosis procedures, including molecular diagnostics.

These tools include both immunoassay and DNA-based diagnostic methods, e.g. fluorescent antibody tests, enzyme-linked immunosorbent assays (ELISA), radio-immunoassay, in situ hybridization (ISH), dot blot hybridization and PCR amplification techniques. They are currently used to screen and/or confirm the diagnosis of many significant pathogens of cultured finfish, such as channel catfish virus, infectious haematopoietic necrosis virus, IPNV, viral haemorrhagic



septicaemia virus, viral nervous necrosis virus and bacterial kidney disease, as well as shrimp diseases such as WSSV, yellow head virus (YHV), infectious hypodermic and haematopoeitic necrosis virus (IHHNV) and Taura syndrome virus (TSV) (Walker and Subasinghe, 2000).

2.5 Vaccines

Adams et al. (2008) reviewed the vaccine technologies in aquaculture. Vaccination is the action in which a host organism is exposed to organic (biological) molecules that allow the host to mount a specific immune reaction through which it has a better capability to fight subsequent infections of a specific pathogen compared with genetically similar non-vaccinated hosts. It has also been shown to be cost-effective and has led to the reduction in use of antibiotics. In Norway, for example, antibiotic use has decreased from 47 tons to approximately one ton annually (Markestad and Grave, 1997)

A wide range of commercial vaccines is available against bacterial and viral pathogens and many new vaccines are under development. Most target salmon and trout, and there are expanding opportunities for marine fish (Thompson and Adams, 2004). Traditionally, the organic molecules used for vaccination are directly derived from the pathogen in question. The most straightforward approach is to culture the pathogen after it has been inactivated and presented to the host. So far, vaccines containing more than ten bacterial pathogens and five viral pathogens have been produced based on such inactivated antigens (Sommerset et al., 2005).

One of the most important factors leading to reduced antibiotic use by the aquaculture sector is the availability of good prophylactic measures for diseases causing severe mortalities in cultured fish and shellfish. The use of vaccines provides good immunoprophylaxis for some of most important infectious diseases of finfish. In developed countries, their use has proved very effective at decreasing the unsustainable use of antibiotics. For example, in Norway antibiotic use in salmon farming has become almost negligible, at less than 1g per tonne of production, due mainly to the availability of vaccines for furunculosis and cold water vibriosis (Smith, 2008). As molecular-based vaccine production procedures rely heavily on biotechnological tools, vaccines are produced mainly in developed countries. A DNA vaccine is a circular DNA plasmid that contains a gene for a protective antigenic protein from a pathogen of interest (Kurath, 2008). Considerable industrial research has been conducted towards developing DNA vaccines for species such as salmonids for pathogens (generally, viruses) for which traditional methods have not been successful. Biosecurity and disease control

Disease outbreaks are a serious constraint to the development of intensive aquaculture systems and can have a major impact on production due to mortality and decreased growth. It has been recognized that disease is the most significant factor impacting the intensive production of shrimp, salmon, carp and tilapia, with losses of 10-90 percent of total production (Peinado-Guevara and López-Meyer, 2006). Although many aquatic animal



pathogens are well studied, unlike in terrestrial animals the spread of pathogens is easy through water and control is difficult due to high density culture in fluid environment. Disease occurs in all systems, from extensive to intensive, although heavy losses are always possible in intensive production systems (Bondad-Reantaso et al., 2005).

There is a greater need for management intervention in intensive systems. Here biotechnological tools can be a valuable part of management approaches. Their scope of application is broad – they can be used as sensors in the production environment, for waste management (through controlled microbial technologies), and for disease detection and control (molecular methods). Traditionally, disease control is often carried out only after mortality has been observed. In the past, the diagnosis of fish diseases has been achieved primarily using histopathological methods supported by parasitological, bacteriological, and viral studies based on necropsy and in vitro cell culture. These are well-proven techniques.

2.6 Environmental management and bioremediation

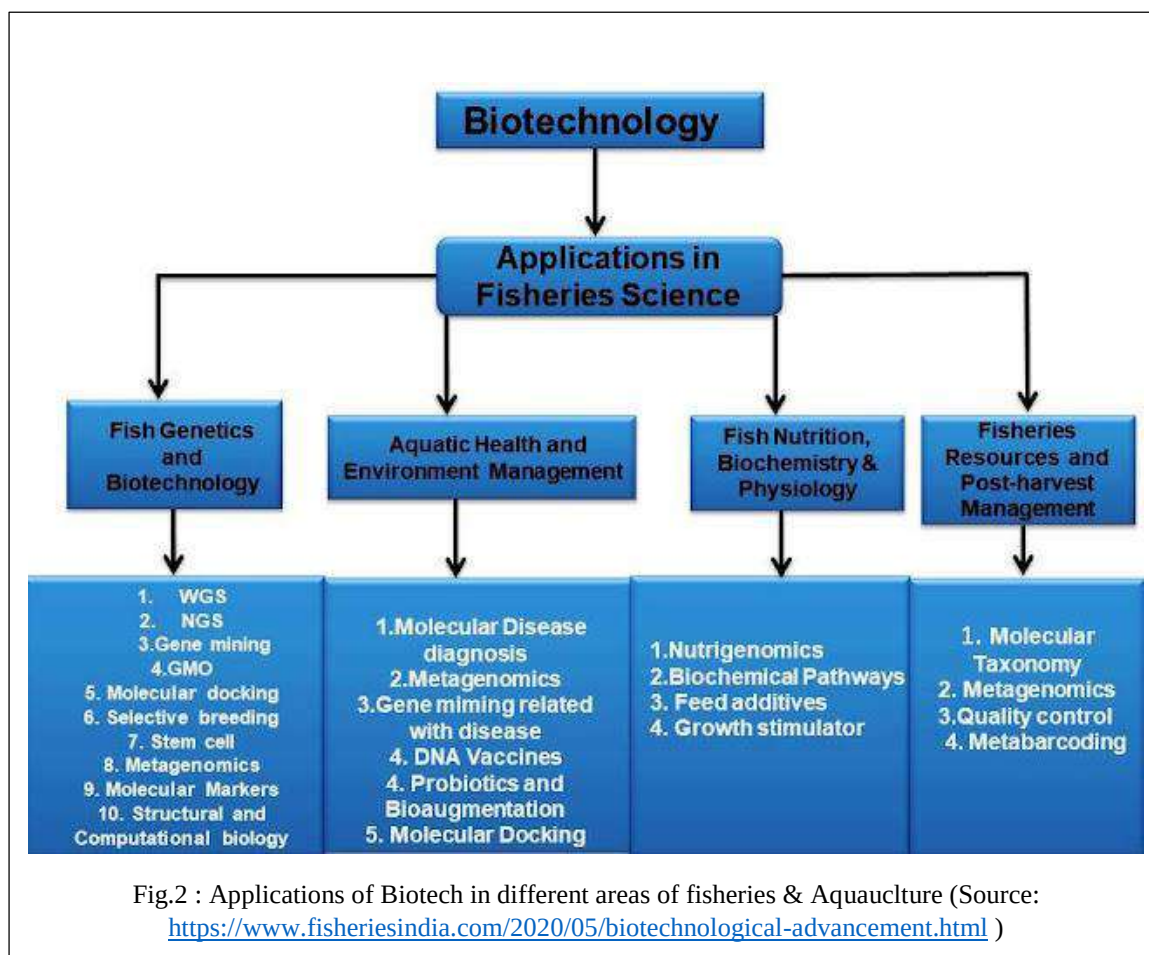
Aquaculture has often been accused of being unsustainable and not environmentally friendly. Although in some cases, where aquaculture development has failed to live up to the global expectations of sustainable development, these allegations are not entirely unfounded, the majority of aquaculture is practised sustainably and with a high degree of environmental conscientiousness. Reducing the impact of

effluent discharge, improving water quality and the responsible use of water are key areas to be considered during aquaculture development. Several biotechnologies are being used to address these areas: bioremediation for the degradation of hazardous wastes; the use of vaccination and probiotics to reduce antimicrobial use; and the use of DNA-based methodologies for the early detection of toxin-producing algae.

2.7 Biodiversity conservation and fisheries management

In fisheries management, conservation is an important concept. Good fisheries management requires effective conservation measures, which require better understanding of the population structure of the fishery. One of the most important population parameters for assessing the fate of a population is the effective population size (N_e), which determines the amount of genetic variation, genetic drift and linkage disequilibrium in populations and can be calculated as half the reciprocal of the rate of inbreeding (Tenesa et al., 2007). There is much concern in fisheries and aquaculture production about the potential loss of genetic variation that may result from the relatively high rates of inbreeding expected in these populations. Inadequate procedures for stock enhancement can yield a very small effective population size due to the high prolificacy of fish and shellfish species. Thus, a very small number of breeders could be used for restocking purposes, and bottlenecks can affect the fitness of the population in future generations. A range of biotechnology-





based approaches are being used to conserve wild fish populations such as the use of molecular markers: to estimate N_e in wild populations; to study gene flow between farmed and wild fish populations; and to monitor and understand changes in wild fish population sizes (FAO, 2006; Hansen, 2008).

CONCLUSION:

Identifying Options for Developing Countries:

Over the years, aquaculture biotechnologies and other technological innovations have had a positive impact on aquaculture diversification, investment potential, and international technology

exchange. The development of biotechnologies in aquaculture should therefore provide a means of producing healthy and fast-growing animals by environmentally friendly means. Improved exchange of information and discussion between scientists, researchers, and producers from different regions about their problems and achievements will undoubtedly help this important sector to develop with a view to increasing sustainable global aquatic animal production.

Based on the overview and analysis contained in this article, a number of specific options can be identified for developing countries to help them make



informed decisions regarding the adoption of biotechnologies in the future, such as when – and if – they should deploy one or more biotechnologies and, if they decide to do so, how they can ensure the successful application of the chosen biotechnologies to enhance food security in the future. Developing countries should therefore collect information on the aquatic animal biotechnologies that may be used and analyse their national-level adoption and socio-economic impacts.

The use of biotechnologies in aquaculture worldwide has increased incrementally over the past two decades. Several aquaculture biotechnologies have been used for improving aquatic food production in both developed and developing countries and have significant potential for future improvement. Since most aquaculture biotechnologies are still too technical and costly for small-scale farmers, efforts should be made to develop low-cost simple technologies that are easy to introduce to less advanced aquaculture farmers.

Major biotechnological achievements and advances in fisheries and aquaculture have been mainly restricted to aquaculture and to the fields of genetics, health and the environment. Genetic improvements using genetic manipulation (diploidy, triploidy) and hormonal therapy etc. have shown promise for producing fish and shellfish with improved and desirable production qualities. Disease prevention and health management in aquaculture have benefited significantly from advances in biotechnologies. Many reliable and accurate rapid diagnostic techniques have

been developed which can be used by small-scale farmers. There are several efficient vaccines now available for certain aquaculture species which have significantly reduced the use of antibacterials in their culture. However, more research is required to develop vaccines for tropical species, particularly the major species of global production. Some environmental remediation tools and technologies have been developed using several biotechnologies.

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BHAI-MS-17

Biotechnological interventions in the treatment of Psoriasis - the promise of new 'biologicals'Chakrapani I S¹, Srinivas N² and Salini K^{3*}**Abstract**

Psoriasis is a chronic, recurrent immune-mediated inflammatory dermatosis. Epidermal hyperplasia and infiltration of inflammatory cells into the dermis and neovascularization characterize this disease. The global prevalence rate is about 2-3%. It impairs the quality of life negatively, due to chronic complications and comorbidities like arthritis, cardiovascular issues, depression and metabolic disorders. The molecular mechanisms involved in psoriasis are complex, and deregulation of T-helper cells Th-1/Th-2/Th-17/Th-23 axis is implicated in the pathogenesis of the disease. Current treatment protocols for this disease have not been satisfactory. Biotechnological innovations have made possible the development of several new systemic therapies for psoriasis. 'Biologicals' are a new group of compounds including monoclonal antibodies, fusion proteins and recombinant proteins. Targets for the therapeutic strategies adopting the new 'biologicals' include inactivation of soluble mediators like tumor necrosis factor alpha, blockade of receptors for cytokines, adhesion molecules and interference with T-cell activation by antigen-presenting cells. About forty 'biologicals' are under examination for the management of psoriasis. Recombinant cytokines are in use to modulate immunological balance, as a therapy for psoriasis. This work reviews the recent developments in the treatment of psoriasis with new 'biologicals'.

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Introduction:

Psoriasis is an inflammatory disease. It is chronic, recurrent and immune-mediated. It affects about 2-3% of world population (Dogra and Mahajan 2016). The prevalence rate of Psoriasis is estimated to be varying between 0.44% and 2.8% in our country. Psoriasis affects the male population more than the female population (Dogra and Yadav 2010).

It is characterized by chronic inflammatory skin lesions of five types predominantly – plaque, guttate, inverse, pustular and erythrodermic. The most common form is plaque. Psoriatic plaques appear as raised and red patches covered with dead cells (Devrimci-Ozguven et. Al., 2000). Often itchy and painful, there is risk of cracks and bleeding affecting the quality of life. Patients suffering from this disease are often subjected to social stigma and humiliation, leading to anxiety and depression. It has a high impact on the motional and psychological aspects of the patients (Kim et.al., 2017), making it compulsory to adopt suitable management protocols (Dauden et.al., 2016).

The aetiology of this disease is unclear. At present, consensus about the manifestation is that it is T-Cell mediated and influenced largely by genetic and environmental factors (Kanwar et.al.,2010). Since therapeutic choice for this multifactorial disease is not easy, marketed formulations that encompass all the needs of a psoriasis patient are currently unavailable. The most common approach for clinical management of Psoriasis is use of a combination of topical and oral formulations, all of which

aim at reducing plaque formation. Corticosteroids, keratolytics, tazarotene, anthralin and calcipotriol are widely being used in the currently available therapies (Menter et.al., 2008). But, many side effects have been observed, resulting in therapeutic dropouts (Korman et.al., 2015; Sarma 2017). In addition, higher treatment cost is of concern amounting to 10 billion euros per annum and 40% work loss (Melnikova 2011). At present, routine clinical practice includes use of methotrexate (MTX), cyclosporine, acitretin, and small molecules, like apremilast (Armstrong et.al., 2013; Afra et.al., 2019), but many adverse effects have been reported from time to time (Fieldman et.al., 2016)

In this context, there arises an urgent need for development of new therapies for effective treatment regimes.

Biotechnological interventions

Biotechnological innovations made the development of new systemic therapies for Psoriasis. A new group of chemicals called biologicals seem to be promising. These include monoclonal antibodies, fusion proteins and recombinant proteins. These offer designer drugs to interact with specific targets and thus are safer (Graciliana Lopes et.al., 2020). Targets for these biologicals include blockade of cytokine receptors, inactivation of soluble mediators of the immune system, interference with T-cell activation etc. Currently, up to forty agents are under investigation for the treatment of psoriasis. Four of these agents, alefacept, efalizumab, etanercept and infliximab have already impacted on routine clinical practice. These



‘biologicals’ appear to be promising in combating Psoriasis. Current developments in the treatment of psoriasis with biologicals are briefly reviewed.

onwards, Second-generation biologics emerged. Their mechanism of action involves antibody targeting the IL-23/Th17-pathway. Examples include ustekinumab, secukinumab, ixekizumab, brodalumab, guselkumab, risankizumab, and tildrakizumab.

Table 1 – List of some of the biologicals currently used in clinical practice for treatment of Psoriasis

Sl.No	Name of the ‘biological’	Target/mechanism of action
1	Adalimumab (Humira)	TNF-alpha-blocking antibody
2	Adalimumab-adbm(Cyltezo)	biosimilar to Humira
3	Brodalumab (Siliq)	human antibody against interleukins
4	Certolizumab pegol (Cimzia)	TNF-alpha blocker
5	Etanercept (Enbrel)	TNF-alpha blocker
6	Etanercept-szsz (Erelzi)	A biosimilar like Enbrel
7	Guselkumab (Tremfya)	an antibody against interleukins
8	Infliximab (Remicade)	TNF-alpha blocker
9	Ixekizumab (Taltz)	An antibody that binds to inflammation-causing proteins/interleukins
10	Risankizumab-rzaa (SKYRIZI)	an antibody against interleukins
11	Secukinumab (Cosentyx)	A human antibody against interleukins
12	Ustekinumab (Stelara)	A human antibody against interleukins

These biologicals are used according to the international guideline recommendations and literature evidence (AAD 2008, AAD 2019, BAD 2017, European S3,) and protocol (Rajagopalan and Mital 2016).

Therapeutic approaches and challenges with biologicals

In the recent past, new biological therapies with are developed. Their mechanism of action involves more precise targets (porter et.al., 2017). First generation biologicals are developed targeting tumour necrosis factor (TNF- α). These include etanercept, infliximab, and adalimumab. These are used for patients who suffer from chronic moderate-to-severe psoriasis. From 2009

Secukinumab is the only second-generation biologic available in India at present. It is indicated as the first-line systemic in moderate-to-severe plaque psoriasis in adult patients (Rajagopalan et.al., 2021).

Clinical challenges in using biologicals

At present, only a limited evidence available is available about the practical issues faced with biologicals usage. Data available so far is inconsistent to arrive at a conclusion for primary failure or secondary failure and the management in such cases. Expert consensus are available for different countries, which can be used as a guide for clinicians.



Conclusion

New biologicals are emerging as a hope for patients suffering from psoriasis. But, there is need to evolve country-specific recommendations.

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BHAI-MS-18

Vermi-biotechnology for Agricultural Development

Amaravati G

Abstract

Agricultural yields could be improved with lowest cost using vermin-compost. The study focuses on the utilization of organic solid waste effectively using vermi-biotechnology. Municipal solid waste (MSW) is used as main substrate along with soil, dung etc. Out of the four substrates used, MSW with the addition of cow-dung appears to be best substrate for *Eisenia foetida* whereas MSW with cow-dung and soil are best substrates for *Perionyx excavatus*. Out of the two earthworm species used, the study indicates, that *Perionyx excavatus* is more suitable to convert the MSW mixed with soil and cow dung into nutrient rich vermin-compost. Further, among the different substrates used MSW mixed with soil and MSW mixed with cow- dung showed almost similar results in enhancing the soil nutrients. Since soil is easily available and less costly substance than cow-dung, it is advisable to use organic waste or MSW and soil in 3:1 ratio for efficient conversion of waste into valuable vermi-compost with *P.excavatus*. Therefore, it can be concluded that the vermicomposting of municipal solid waste alone (organic waste) will not yield good quality vermi-compost. The addition of cattle dung or soil or both to municipal solid waste in limited proportion will yield good quality vermi-compost.

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Introduction:

Vermi-biotechnology or vermi-composting of organic waste could be carried out with the use of different types of earthworms. At present vermin-composting of municipal solid waste or organic solid waste is accomplished by the use of the earthworm *Eisenia foetida*. This earthworm converts the organic matter into compost efficiently but they live in the top of the soil and they never go deep into the soil by making burrows when they are present in soil or agricultural fields. On the other hand, the earthworms naturally present in the soil (like *P. excavatus*) make burrows and bring the soil nutrients present in the deeper layers of the soil to the top layer thus making soil more fertile.⁶ Further, it is thought that the efficiency of *E. foetida* would be reduced if the organic waste contains significant amount of soil whereas the efficiency of indigenous variety of earthworms will not be reduced in the presence of soil (Dominguez J and Edwards, 2004; Viji J and Neelanarayanan, 2013). In this context, effort is made in this study to compare the ability of earthworm species in terms of growth and reproductive characters, environmental suitability, vermin-composting efficiency and quality of vermin-compost.

Materials and methods: Vegetable waste materials collected from the municipal vegetable market were used as the main substrate. Normal soil (garden soil), cow dung or farm yard manure are added in different proportions to municipal solid waste as supplements to the green waste. Five sets of plastic pots for each earthworm species are taken for this comparative study. Each set of pots is filled with five kg

of the substrate - municipal solid waste, cow dung and soil separately in the ratio given below. Set I Municipal solid waste (MSW) Set II MSW and soil (3:1 ratio) Set III MSW and Cow dung (3:1 ratio) Set IV MSW: Soil: Cow dung (2:1:1) Control Organic waste without earthworms Four sets are used for vermi- composting with each set using one species of earthworm and one set is used as control (without earthworms). Two species of earthworms, 16-20 weeks old individuals of each species, *Eisenia foetida* and *Perionyx excavates* (having individual live weight ranging from 250 to 300 mg) were collected from the stock culture and each of fifty adult individuals, are introduced on the top of the pre-composted substrate in each of the four sets of pots keeping aside another set for composting without earthworms. The process of vermin-composting is carried out for a period of 60 days and the samples are collected from each set of the substrate /substrate mixture at 0 day, 15, 30, 45 and 60th day and analyzed for various parameters such as temperature, moisture, weight, pH, EC etc., using standard protocols (Muragesan AG, Rajakumari C, 2005, Anderson and Ingram, 1993).

Results and discussion: The study is aimed at determining the optimal environmental factors required for successful vermicomposting of the waste. The environmental factors studied include temperature, moisture, pH, EC and weight reduction. The moisture content of the compost without earthworms is higher compared to vermi-composts of both the species of earthworms used. The moisture content of vermicompost (after 60 days) ranged from 50.3 to 57.8% in all the



samples kept for vermicomposting with *E.foetida* and for *P.excavatus* the moisture content ranged from 52.5 to 53.6%. It indicates that the vermicompost obtained using *P.excavatus* has lower level of moisture content compared to that of *E.foetida*. Lower level of moisture in the final product is desirable for longer storage and for its quality (Amaravathi and Mallikarjuna Reddy, 2015). The initial temperature of compost was 34.5⁰c and the temperature of vermicompost obtained after 60 days of vermicomposting was 27⁰c. For the better activity of microbes and earthworms, the pH of the substrate shall be neutral or around 7.0. The pH of the municipal solid waste is 6.3 and it is lower than the neutral pH. However, the pH of the municipal solid waste and other samples increased to 6.8-7.2 after vermicomposting especially with the addition of soil or cow dung or both (Table-1). The EC of the samples increased after vermicomposting particularly with the addition of cow dung or soil. Therefore, the addition of soil or cow dung appears to be essential in order to provide favorable medium for the growth of earthworms and for successful reclamation of municipal solid waste (Ndegwa, 2000).

Effect of substrate composition on the nutrients of vermi-compost prepared by different types of earthworms:

Soil nutrients or salts present in soil influence the fertility of the soil and plant growth. Soil nutrients such as nitrogen, phosphorus, potassium, sulphur etc. (N,P,K,S) are the required salts to be present in soil. The vermin-compost having considerably higher proportion of these nutrients is considered as very good fertilizer (Venkatesh RM. and

Eevera, 2008). The nitrogen% of msw is 0.56%, msw + soil is 0.45%, msw + cow dung is 0.64% and msw+cow dung + soil is 0.55% (Table-1). The results indicate that the addition of soil reduces the nitrogen 11% whereas the addition of cow dung increases the nitrogen percentage of substrate mixture. After 60 days of vermicomposting, the nitrogen% of all the samples increased gradually. After 60 days, the nitrogen% of msw + cow dung is higher compared to all the other samples in the case of both the earthworms. The nitrogen% of msw + cow dung sample is 0.92% for *E.foetida* and 1.05% for *P. excavatus*, (Bouche et al, 1997).

Thus, it is observed that the addition of cow dung increases the nitrogen% of vermi-compost irrespective of earthworm species. On the other hand, the addition of cow dung as well as soil increased the nitrogen% of vermicompost in the case of *P. excavatus*. Further, the nitrogen% of vermicompost of *P.excavatus* is higher than that of *E.foetida* (Table-1). The similar pattern to that of nitrogen could be observed in the case of phosphorus and potassium contents of vermicompost with regards to both substrate composition and earthworm type (Munnali and Bhosle, 2009). With regards to the effect of substrate on the organic carbon% (OC) of vermicompost a different pattern than that of nitrogen is observed. The organic carbon% of initial MSW is 20.4%, MSW + soil is 15.2%, msw + cow dung is 20.1% and msw+cow dung + soil is 18.02%. The results show that the addition of soil reduces the OC% of the substrate mixture whereas the addition of cow dung has no effect on the OC% of the substrate. (Table-1). Results show that the



vermicompost of msw + cow dung mixture has retained higher organic carbon percent in the case of *E.foetida*. In the case of *P.excavatus*, the vermicompost of msw+ cow dung mixture and msw + soil + cow dung has higher OC%. The C/N ratio of vermicopost of msw + cow dung, as well as msw + cow dung + soil is higher in the case of *E. foetida* compared to *P. excavatus*. On

the other hand, the C/N ratio of vermicopost of msw + soil, as well as msw + cow dung + soil is lower in the case of *P.excavatus*, indicating its efficiency in producing good quality vermicompost. The present study revealed that when the cow dung and soil are added to the organic waste, the resultant vermi-compost showed higher levels of plant nutrients like N, P, K, and lower C/N ratio.

<i>Eisenia foetida</i>								
Compost at 0 day					Vermicompost (after 60 days)			
Parameter	S1	S2	S3	S4	S1	S2	S3	S4
Moisture%	78	78	78	78	55.4	57.8	50.3	54.5
Temperature	34.5 ⁰ c	34.5 ⁰ c	34.5 ⁰ c	34.5 ⁰ c	27.4 ⁰ c	27.7 ⁰ c	27.2 ⁰ c	27.5 ⁰ c
pH	6.3	6.7	6.8	6.7	6.8	7.1	7.2	7.1
EC	2.87	2.40	4.85	3.86	3.25	4.90	5.17	4.80
Total Nitrogen%	0.56	0.45	0.64	0.55	0.82	0.65	0.92	0.72
Phospharous%	0.24	0.20	0.44	0.41	0.80	0.68	0.85	0.72
Potassium%	0.19	0.22	0.35	0.32	0.60	0.52	0.56	0.52
Organic carbon%	20.4	15.2	20.1	18.0	12.14	11.4	14.2	13.5
C/N ratio	36.42	33.77	31.40	32.76	15.95	17.53	16.52	20.13
<i>Perionyx excavates</i>								
Parameter	S1	S2	S3	S4	S1	S2	S3	S4
Moisture	78	78	78	78	53.2	53.6	52.5	52.6
Temperature	34.5 ⁰ c	34.5 ⁰ c	34.5 ⁰ c	34.5 ⁰ c	27.2 ⁰ c	27.3 ⁰ c	27.5 ⁰ c	27.4 ⁰ c
pH	6.3	6.7	6.8	6.7	6.8	7.2	7.2	7.2
EC	2.87	2.40	4.85	3.86	4.90	4.54	5.90	5.98
Total Nitrogen%	0.56	0.45	0.64	0.55	0.88	0.84	1.15	0.96
Phospharous%	0.24	0.20	0.44	0.41	1.02	0.70	1.09	1.01
Potassium%	0.19	0.22	0.35	0.32	0.64	0.68	0.78	0.75
Organic carbon%	20.4	15.2	20.1	18.0	12.5	12.10	14.50	13.8
C/N ratio	36.42	33.77	31.40	32.76	14.20	16.21	13.80	13.02

Table-1: Changes in various parameters of MSW before and after vermi-composting using different earthworms and with the addition of various substrates.



Conclusion: Among the different substrates used MSW mixed with soil and MSW mixed with cow- dung showed almost similar results in enhancing the soil nutrients. Since soil is easily available and less costly substance than cow-dung, it is advisable to use organic waste or MSW and soil in 3:1 ratio for efficient conversion of waste into valuable vermi-compost with *P.excavatus*. Therefore, it can be concluded that the vermicomposting of municipal solid waste alone (organic waste) will not yield good quality vermicompost. The addition of cattle dung or soil or both to municipal solid waste in limited proportion will yield good quality vermi-compost. Further, the study indicates that both the earthworm species, *Eisenia foetida*, and *Perionyx excavatus*, are equally efficient in converting municipal solid waste into good quality vermi-compost.

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Seminar Report



National Seminar

on

Biotechnology in Health Care & Agriculture – Insights & Innovations

24.01.2024

Jointly organized by

Departments of Zoology

PRR & VS Govt. College, Vidavalur & MRR Govt. Arts & Science College, Udayagiri

One Day National Seminar on “Biotechnology in Healthcare& Agriculture, Insights & Innovations” was jointly organized by the Department of Zoology, PRR & VS Govt. College, Vidavalur, Nellore dist. and the Dept. of Zoology, MRR. Govt. Arts & Science College, Udayagiri, SPSR. Nellore Dist. on 24th January 2024.

Dr.T.Gopikrishna, Associate Dean, MGR.Govt. Agriculture College, Udayagiri, attended as Chief Guest. Dr. V. Krishnakumar, Rtd. Principal, Govt. Degree College, Naidupet attended as Keynote speaker. Dr. P. Subbalakshumma, Principal, MRR. Govt. Arts & Science Degree College, Udayagiri and Dr. B. Sujatha, Principal, PRR & VS Govt. College, Vidavalur graced the occasion as guests of honour.

Dr. Sainadh, Asst.Professor, Dept. of Biotechnology, Vikramasimhapuri University, Nellore, Sri A.Narasmhulu, Vice-Principal of the College, Sri K.C.Vikram, IQAC Co-ordinator of the college, Dr.n.Madhu, IQAC coordinator from PRR & VS Govt. College involved actively in the conduct of this event.

Dr.I.S.Chakrapani, PRR&VSGC and Dr. S.V. Nagendra Prasad from MRRGASC were the organizing secretaries of the Seminar.

Nearly 150 students, 25 faculty members, research scholars, and industry personnel participated in this seminar. About 60 abstracts were received and 18 full-length papers were submitted by researchers from different institutions across India.

The inaugural session of the seminar was started with students prayer and lightening of the lamp.

Then Dr. P.Subbalakshumma,Principal addressed the participants and deligates. In her speech she said that it was a wonderful event organized by the Departments of Zoology of the two



rural colleges. She also said that these types of seminars are very much useful to the student community for their higher education as well as in research fields. She commented, “The present topic is very relevant to the recent trends and applications in the field of biotechnology as well as in healthcare.” Dr. S.V.Nagendra Prasad, Organising secretary gave inputs about the objectives of seminar, and the backdrop in which the event was conducted.

Chief guest of the seminar Dr. T.Gopkrishna in his message said that now-a-days biotechnology had been the most powerful branch of science. He gave a succinct idea of the applications of biotech in healthcare & agriculture. He gave an idea about various research programs and funding opportunities for research in biotech.

Dr.V.Krishnakumar, the Keynote speaker was received well by the audience. He gave good examples which are beneficial to the mankind and agriculture sector with good illustrations. He elaborated different applications of biotech in daily life, and gave an idea of the future research trends.

In the technical sessions Dr.P.Anil kumar, Professor, Dept.of Pathology, MGR.Govt. Agriculture College, Udayagiri gave an interesting talk on pathogens, pathogenecity in agriculture, remedies and applications of biotechnology in agriculture field.

Then, Dr.Sainadh, Asst.Professor, Dept.of Biotechnology, Vikramasimhapuri university, in his talk discussed about the recent trends in biotechnology, new research inventions, role of biotechnology in improving aquaculture yield in Nellore district.

22 oral presentations went on simultaneously in the auditorium. Mr. Srikanth from Vikrama Simhapuri University received the best oral presentation award. Nearly 50 Poster presentations were received. Dr.V.Krishnakumar & Dr.S. B. Sainath acted as experts in adjudicating the best Poster. Dr. Rajarajeswari received the best poster presentation award.

At the end of the event, valedictory session took place. The IQAC Coordinators and vice-principals of both the colleges lead the session.

Dr. I. S. Chakrapani, Organizing secretary presented the seminar report. Certificates and mementoes were distributed to the delegates. The program came to an end with national anthem.



Inaugural Session



Objectives of the seminar by Dr.S.V.Nagendra Prasad, Organizing Secretary. Speaking on the right is Dr.Vikram, IQAC co-ordinator



Release of Souvenir





Certificates distribution



Felicitation to the Resource Persons



Dr.I.S.Chakrapani, Organizing Secretary, presenting the seminar Report

సెమినార్లతో విద్యార్థులకు ఉపయోగం.. ఉదయగిరి: కళాశాలల్లో నిర్వహించే సెమినార్లు విద్యార్థులకు ఎంతగానో ఉపయోగపడతాయని మేకపాటి రాజమోహన్ రెడ్డి ప్రభుత్వ డిగ్రీ కళాశాల ప్రిన్సిపల్ డా. సుబ్బలక్ష్మమ్మ అన్నారు. బుధవారం కళాశాలలో జంతు శాస్త్ర విభాగ ఆధ్వర్యంలో బయోటెక్నాలజీ ఇన్ హెల్త్ కేర్ అగ్రికల్చర్, ఇన్సైట్స్ అండ్ ఇన్నోవేషన్ అంశంపై జరిగిన సెమినార్లో మాట్లాడారు.

సెమినార్లతో విద్యార్థులకు ఉపయోగం

ఉదయగిరిరూరల్, జనవరి 24: కళాశాలల్లో వివిధ విభాగాలకు సంబంధించి నిర్వహించే సెమినార్లు విద్యార్థులకు ఎంతో ఉపయోగకరంగా ఉంటాయని డిగ్రీ కళాశాల ప్రిన్సిపల్ సుబ్బలక్ష్మమ్మ అన్నారు. బుధవారం స్థానిక కళాశాలలో జంతుశాస్త్ర విభాగం ఆధ్వర్యంలో నిర్వహించిన సెమినార్లో ఆమె మాట్లాడుతూ ఇలాంటి కార్యక్రమాలతో విద్యార్థులు విజ్ఞానాన్ని పెంపొందించుకోవాలన్నారు. అనంతరం విద్యార్థులకు సర్టిఫికేట్లను అందజేశారు. కార్యక్రమంలో వైస్ చీఫ్ ప్రిన్సిపల్ నరసింహులు, కార్యనిర్వాహక కార్యదర్శులు నాగేంద్ర, ప్రసాద్, చక్రపాణి, కో ఆర్డినేటర్ విక్రమ్, గోపాలకృష్ణ, కృష్ణకుమార్, అనిల్ కుమార్, సాయి నాథ్ తదితరులు పాల్గొన్నారు.



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సంపాదకులు

డా. గోపవరం పద్మప్రియ

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Dr.I.S.Chakrapani - Dr. P. Anil Kumar

Unit – I Immunology – I (Overview of Immune system) 15 hrs

1.1 Introduction to basic concepts in Immunology -P 2-4

1.2 Innate and adaptive immunity, Vaccines, and Immunization programme – P 5- 8

1.3 Cells of immune system P 8-9

1.4 Organs of immune system P 10-13

Unit – II Immunology – II (Antigens, Antibodies, MHC and Hypersensitivity)

2.1 Antigens: Basic properties of antigens, B and T cell epitopes, haptens and adjuvants; Factors influencing immunogenicity P13-15

2.2 Antibodies: Structure of antibody, Classes and functions of antibodies. P-16-20

2.3 Structure and functions of major histocompatibility complexes P– 20-22

2.4 Exogenous and Endogenous pathways of antigen presentation and processing P 22-27

2.5 Hypersensitivity – Classification and Types P 28-34

UNIT III

3.1 Animal Cell, Tissue, and Organ culture media: Natural and Synthetic media P 35-37

3.2 Cell cultures: Establishment of cell culture (primary culture, secondary culture, types of cell lines; Protocols for Primary Cell Culture); Organ culture; Cryopreservation of cultures - P 37-43

3.3 Stem cells: Types of stem cells and applications- P 43-46

3.4 Hybridoma Technology: Production & applications of Monoclonal antibodies (mAb) P-47-19

UNIT IV

4.1 Genetic Engineering: Basic concept, Vectors, Restriction Endonucleases and Recombinant DNA technology P 49-55

4.2 Gene delivery: Microinjection, electroporation, biolistic method (gene gun), liposome and viral mediated gene delivery P- 55-59

4.3 Transgenic Animals: Strategies of Gene transfer; Transgenic – sheep, - fish; Applications P 59-61

4.4 PCR: Basics of PCR. -DNA Sequencing: Sanger's method of DNA sequencing- traditional and automated sequencing P-61-65

1.1 INTRODUCTION TO BAISC CONCEPTS OF IMMUNOLOGY

Immunology is a branch of biology and medicine that studies immune systems in all species. **Ilya Ilyich Mechnikov**, a Russian biologist who improved immunology research and was awarded the Nobel Prize for his efforts in 1908, invented the name. This was the body's active response to maintaining its integrity.

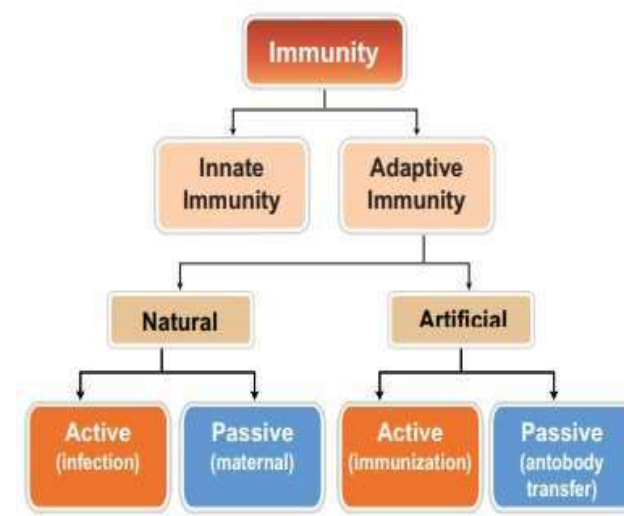
Immune System:

The immune system is a complex system of structures and functions that have evolved to keep us healthy over time. The components which make the immune system are **molecular and cellular components**. The study of the features that make up the innate and adaptive immune systems is known as **classical or fundamental immunology**.

Innate Immunity: Nonspecific
Innate immunity is the first line of defence. No matter how diverse the infections are, they all have the same reactions. **Physical barriers (such as skin, saliva, and cells) and cells make up innate immunity (e.g. neutrophils, macrophages, mast cells, basophils, etc.).** These components are pre-assembled and ready to use, ensuring that an organism is protected for the first few days after infection.

Adaptive Immunity: The second line of defence is adaptive immunity, which involves the creation of a memory of initial conditions to create a more tailored response to the pathogen or foreign material. **Antibodies are utilised in adaptive immunity** to target free foreign pathogens in the bloodstream. T cells are also involved since they are focused on

infections that have colonised cells and can either kill infected cells directly or help regulate the antibody response.



Immune Dysfunction and Clinical Immunology:

The immune system is a highly regulated and managed mechanism, and if the balance is broken, the disease can result. Immune system malfunctions cause two types of conditions:

Autoimmune Diseases: Autoimmune illnesses occur when the immune system attacks the body it is designed to protect. Autoimmune diseases are divided into **'primary' autoimmune diseases** that arise at birth or early in life, such as type-1 diabetes, and **secondary' autoimmune diseases** that appears later due to several factors. Rheumatoid arthritis and multiple sclerosis are regarded to be examples of autoimmunity.

Patients with autoimmune diseases have a deficit that makes it difficult to distinguish between 'self' and 'non self' or 'foreign' molecules. Furthermore, autoimmune disorders can be localised.

Immunodeficiency: Immunodeficiency disorders are problems with the immune system that make it harder for the body to fight against infections. As a result, these disorders are most often associated with significant conditions that persist, recur,

and induce complications, making them potentially fatal. The two forms of immunodeficiency disorders are **primary** and **secondary** immunodeficiency disorders.

Primary immunodeficiencies are congenital, mostly inherited, and relatively uncommon. One example is common variable immunodeficiency (CVID).

Secondary immunodeficiencies are more common later in life. They can develop due to an infection, such as HIV infection leading to AIDS.

Classical Immunology:

In classical immunology, epidemiology and medicine are tied together. It looks at how the human body's systems, infections, and immunity interact. Immunology is the scientific study of the molecular and cellular components of the immune system and their function and interaction. Invertebrates have an innate immune system that is more rudimentary than vertebrates', but vertebrates have an acquired or adaptive immune system.

The immune system can distinguish between self and nonself.

Antigens are molecules that cause the immune system to react. **Antibodies** are proteins that bind to and neutralise disease-causing germs. Antibodies do not directly eliminate infections; instead, they recognise antigens as targets for other immune cells like phagocytes and **natural killer cells** to destroy (NK cells). Antibodies are specific proteins produced by B lymphocytes, a type of immune cell, and antigens are anything that triggers the production of antibodies (antibody generators). On the basis of physicochemical and antigenic structure Igs are grouped into 5 classes or isotypes namely **IgG, IgA, IgM, IgD, IgE**.

Vaccination and immunization

- Introduction of the disease-causing agent (substance or the pathogen) into the body in attenuated or detoxified form that makes the body able to develop antibodies against it.
- Immunisation is the phenomenon of increasing specific antibody production and development of memory B- and T- cells against the potential attack of a pathogen.
- It is carried out through vaccination and injection of antiserum.
- When an immunized person is attacked by the pathogen, the existing antibodies immediately attack the antigen while the memory T and B cells give rise to a massive crop of lymphocytes and antibodies.

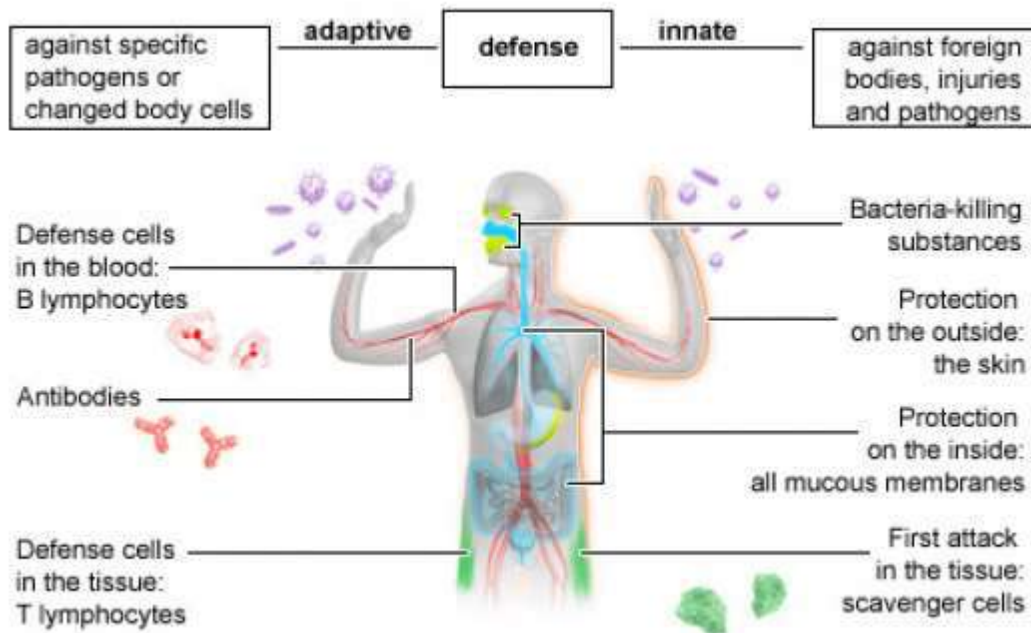


1.2 The innate and adaptive immune systems

The immune system fights germs and foreign substances on the skin, in the tissues of the body and in bodily fluids such as blood. The immune system is made up of two parts: the **innate, (general) immune system** and the **adaptive (specialized) immune system**. These two systems work closely together and take on different tasks.

Protection offered by the skin and mucous membranes

- All outer and inner surfaces of the human body a key part of the innate immune system.
- The closed surface of the skin and of all mucous membranes already forms a physical barrier against germs, which protects them from entering. Additionally, chemical substances like acid, enzymes or mucus prevent **bacteria** and viruses from gaining a foothold.



The innate immune system: Fast and general effectiveness

The innate immune system is the body's first line of defense against germs entering the body. It responds in the same way to all germs and foreign substances, which is why it is sometimes referred to as the "nonspecific" immune system. It acts very quickly: For instance, it makes sure that **bacteria** that have entered the skin through a small wound are detected and destroyed on the spot within a few hours. The innate immune system has only limited power to stop germs from spreading, though. The innate immune system consists of

- Protection offered by the skin and mucous membranes
- Protection offered by the immune system cells (defense cells) and proteins

- Movements created, for example, by hair-like structures in the bronchi (cilia) or bowel muscles stop germs from settling in the body.
- Tear fluid, sweat and urine (which flushes the organs of the urinary tract) have a similar effect.

Protection offered by the immune system cells (defense cells) and proteins

- The innate immune system activates special immune system cells and proteins if germs get past the skin and mucous membranes and enter the body.
- When a part of the skin is infected, immune system cells move to the area or immune system cells that are already there are activated. Specific immune system cells release

substances into the immediate area that make the blood vessels wider and more permeable. This causes the area around the infection to swell heat up and redden, and **inflammation results**. A fever may develop as well. Then the blood vessels expand further and even more immune system cells arrive. Certain proteins (enzymes) are also activated to help in the immune response (see below).

- Bacteria or viruses that enter the body can be stopped right away by phagocytes. Phagocytes special kinds of white blood cells (leukocytes). These cells enclose germs and "digest" them. The remains of these germs move to the surface of the cells to be detected by the adaptive immune system.
- There are also other types of immune system cells that release substances to kill bacteria and various germs. Both germs and body tissue and immune system cells die and decay during an immune system response. Their remains form pus, a yellowish fluid.

Several proteins (enzymes) help the cells of the innate immune system.

A total of **nine different enzymes activates one another in a process similar to a chain reaction**: One enzyme in the first stage alerts several enzymes of the second stage, each of which again activates several enzymes of the third stage, and so on. This allows immune system responses to escalate very quickly.

The tasks of these enzymes include:

- Marking germs as targets for immune cells,
- Attracting other immune system cells from the bloodstream,
- Destroying bacteria cell walls to kill them, and
- Fighting viruses by destroying the viral envelope (the outermost layer of a virus) or cells that have been infected with viruses.

Natural killer cells: Searching for changed body cells

- The natural killer cells are the third major part of the innate immune system. They specialize in identifying cells that are infected by a virus or that

have become tumorous. To do this, they search for cells that have changes in their surface, and then destroy the cell surface using cell toxins.

The adaptive immune system: Fighting the germs directly

The adaptive immune system takes over if the innate immune system is not able to destroy the germs.

- It specifically targets the type of germ that is causing the infection. But to do that it first needs to identify the germ. This means that it is slower to respond than the innate immune system, but when it does it is more accurate.
- It also has the advantage of being able to "remember" germs, so the next time a known germ is encountered, the adaptive immune system can respond faster.
- This memory is also the reason why there are some illnesses you can only get once in your life, because afterwards your body becomes "immune." It may take a few days for the adaptive immune system to respond the first time it comes into contact with the germ, but the next time the body can react immediately. The second infection is then usually not even noticed, or is at least milder.

The adaptive immune system is made up of:

- T lymphocytes in the tissue between the body's cells
- B lymphocytes, also found in the tissue between the body's cells
- Antibodies in the blood and other bodily fluids

1.2.1 VACCINATION AND IMMUNIZATION

Vaccination is an **administration used to give vaccines** i.e giving oral vaccines or injections to improve immune system development and fight against diseases.

Immunization is the **process in which the individual immune system is improved to fight against the infected agents**.

Difference between Vaccination and Immunization

Vaccines are used to artificially activate the immune system to protect against diseases.

Stimulating this immune response is known as Immunization. Immunization is the process after vaccination, to make someone immune to fight diseases. **Most people believe that taking a shot of the vaccine will make them immunized to kill microorganisms or treat infectious diseases. This belief is wrong, everyone's immune system reacts differently.**

Vaccine

- The vaccine is a substance used to produce antibodies and provide immunity against several diseases.
- It contains some amount of preservatives or some amount of antibiotics to preserve the vaccine for long.
- Some may contain aluminum salt to produce a better immune system.
- These vaccines are used to reduce the strength, kill bacteria, or remove toxins from the body.
- Vaccination is the most important and effective method to prevent infection and several diseases. It's responsible for the worldwide reduction of smallpox and diseases like polio and tetanus.
- Vaccines for more than 20 life-threatening diseases are available now and helping all age group people to live a healthy and longer life.

Types of Vaccines

- The development of vaccines is decided according to which method works the best for several diseases and is more effective.

Weakening Vaccines

- The theory behind the production of such vaccines is to educate the body and to improve the capability to fight the infection or virus.
- Some viruses multiply bacteria a thousand times and try to invade the body.

- Once these vaccines enter the body, it becomes difficult for the virus to reproduce. This type of vaccine introduces a live virus into the body. These vaccines are also known as the

Attenuated vaccine.

- **Advantages of Attenuated Vaccines are**

-
- They help to improve the strength of the immune system as they are very close to the virus.
- It shows results within one or two doses.
- These vaccines can be easily created and defend the virus.
- Weakening vaccines are used against the following viruses
- **Smallpox, Chickenpox, Yellow Fever, Mumps, rubella, and measles**

Inactivation Vaccines

- Inactive vaccines rely on a chemical or a pathogen and aims to completely stop the reproduction of the virus. The body will recognize the virus so it keeps producing the cells that protect against the virus.

Advantages of Inactive Vaccines

- Even a mild form of this vaccine is enough to stop the production of disease in the body.
- There is no requirement of a strong immune system for such vaccines.
- Types of diseases treated by inactive vaccines include
- **Polio, Hepatitis A, Rabies**

Subunit and Conjugate Vaccine

- Subunit vaccines use only the essential part of the antigen like protein on the surface of the virus to produce the vaccine and leave everything else. This type of vaccine is used when only a specific part of the virus needs to be treated to protect against the entire disease.

Some diseases treated by this type of vaccine are:

- **HPV, Hepatitis B, Whooping cough**

Immunization

According to WHO (World Health Organization) immunization saves millions of lives every year. Vaccines work with the body's natural defenses and reduce the risk

of getting diseases. Immunization protects more than 2-3 billion lives from tetanus, influenza, measles, and diphtheria diseases.

Immunization takes almost 2 weeks to show complete results. Most of these are needed to be used several times to keep the disease away and build long-lasting protection.

Immunization never gives lifelong protection. Tetanus vaccine lasts for 30 years whereas, whooping cough immunization protects only around 5 years after full course. Influenza is the disease that infects every year due to changes in the types of flu viruses. Thus immunization is needed every year to stay away from such infections.

Types of Immunization

- **Active Immunization**

The term active immunization defines how the body responds to a vaccine. Vaccine stimulates the production of antibodies which actively fight the virus or the bacteria. The individual should have a strong immunity to achieve the desired results.

- **Passive Immunization**

Passive immunity carries the antibodies from outside the body. The substance such as immune globulin is transfer to protect from a certain disease. This protection takes place immediately as compared to active immunization which takes time to produce antibodies.

1.3 Cells of Immune System

WBCs are the principle cells of immune system formed hematopoietic stem cell by the process of hematopoiesis. Hematopoiesis occurs in yolk sac during 1st week of gestation. After 3rd month of gestation, hematopoiesis occurs in liver and spleen of fetus and after birth, it occurs in bone marrow.

The cells of immune system are:

1. Lymphocytes	T-lymphocytes B- lymphocytes NK cell

2. Mononuclear Phagocytic cells	Monocytes Macrophages
3. Granulocytic cells	Neutrophils, Basophils, Eosinophils
4. Dendritic cells	

1. Lymphoid cells (Lymphocytes)

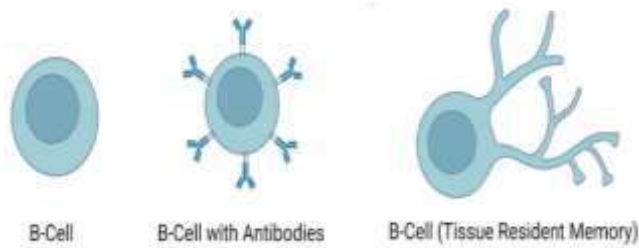
The lymphocytes are the central cells of the immune system which are responsible for adaptive immunity and immunological features of diversity, specificity, memory, and self/non-self recognition. Lymphocytes make up 20%–40% of the body's white blood cells and 99% of the cells in the lymph. There are about 10^{11} lymphocytes in the human body. These lymphocytes circulate continuously in the blood and the lymph hence they are able to migrate into the body tissue spaces and lymphoid organs, therefore integrating the immune system to a high degree.

Types of Lymphocytes

The lymphocytes are broadly divided into three populations based on their functions and cell-membrane components i.e:

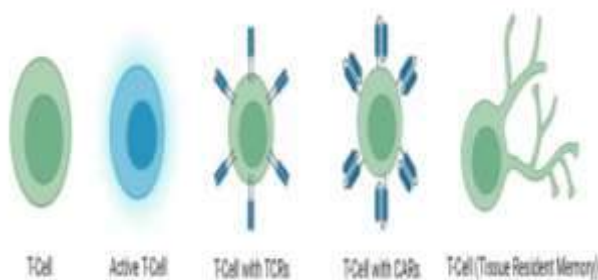
I. B-lymphocytes

- B-lymphocytes are also known as B-cells and on lab reports, they are known as **CD19 or CD20** cells.
- They are the specialized cells of the immune system whose major function is to produce antibodies also known as immunoglobulin's or gamma globulins.
- B-lymphocytes are synthesized and mature in the bone marrow from the hematopoietic stem cells, and after which they mature, migrate, and express themselves by forming unique antigen-binding receptors on their membranes, known as B-cell receptors or antibodies.



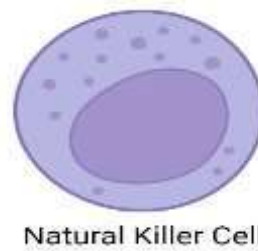
II. T-Lymphocytes

- T-lymphocytes are also known as T-cells, often named in lab reports as **CD3 cells**
- They also arise in the bone marrow but migrate to the **thymus gland** for maturation, where they express a unique antigen-binding molecule on its membrane known as the T-cell receptor.
- The name T originated from its site of maturation, the Thymus.
- Mature T-cells leave the thymus and populate other organs of the immune system, such as the spleen, lymph nodes, bone marrow, and blood.
- Unlike the B-cell receptors that can recognize antigens alone, T-cell receptors only recognize antigens that are bound to cell membrane proteins known as Major Histocompatibility Complex (MHC) molecules.



- The T-cells are classified into three categories: **T helper (Th)**, **T cytotoxic (Tc)**, and **T suppressor (Ts) cells**.

III. Natural killer cells (NK cells)



- These are large granular lymphocytes, that do not express surface markers like the B and T-cell

lineages

- These cells also indicated that they play key roles in host defense against tumor cells and cells infected with some, not all viruses.
- They constitute 5-10% of lymphocytes in the human peripheral blood.

2. Mononuclear Phagocytes

- These are immune cells i.e **Monocytes** that are freely circulating in blood and **Macrophages** that are found in the tissues.
- During hematopoiesis in the bone marrow, **granulocyte-monocyte progenitor** cells differentiate into **promonocytes**, which leave the bone marrow and enter the blood, where they differentiate further into **mature monocytes**.
- Monocytes circulate in the bloodstream for about 8 h, during which they enlarge and then migrate into the tissues and differentiate into specific tissue **macrophages** or into **dendritic** cells.
- **Macrophages** are dispersed throughout the body. Some take up residence in particular tissues, becoming fixed macrophages, whereas others remain motile and are called free, or wandering, macrophages.
 - Macrophage-like cells serve different functions in different tissues and are named according to their tissue location:
 - Alveolar macrophages in the lung
 - Histiocytes in connective tissues
 - Kupffer cells in the liver
 - Mesangial cells in the kidney
 - Microglial cells in the brain
 - Osteoclasts in bone

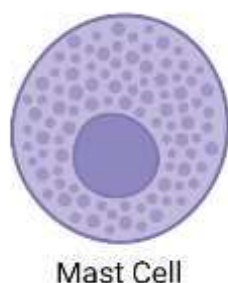
- Macrophages are normally in a resting phase but they can be activated by several immune responses.

3. Granulocytic Cells

- Granulocytes are white blood cells (leukocytes).
- They are classified based on their cellular morphologies and cytoplasmic staining characteristics and they include **neutrophils**, **eosinophils**, **basophils**, or **mast cells**.
- All granulocytes have multilobed nuclei that make them visually distinctive and easily distinguishable from lymphocytes, whose nuclei are round. The cytoplasm of all granulocytes is replete with granules that are released in response to contact with pathogens.
- These granules contain a variety of proteins with distinct functions: Some damage pathogens directly; some regulate trafficking and activity of other white blood cells, including lymphocytes; and some contribute to the remodeling of tissues at the site of infection.
 - Neutrophils have a multilobed nucleus and a granulated cytoplasm it is often called **a polymorphonuclear leukocyte (PMN) for its multilobed nucleus**.
 - The eosinophils have a bilobed nucleus and a granulated cytoplasm.
 - The basophil has a lobed nucleus and heavily granulated cytoplasm.
 - Both neutrophils and eosinophils are phagocytic, whereas basophils are not.

Mast Cells

- Mast cells are formed in the bone marrow
- They are released from the bone marrow into the blood as undifferentiated cells, and when they enter the tissues they then mature.
- Mast cells can be found in a wide variety of tissues, including the skin, connective



tissues of various organs, and mucosal epithelial tissue of the respiratory, genitourinary, and digestive tracts.

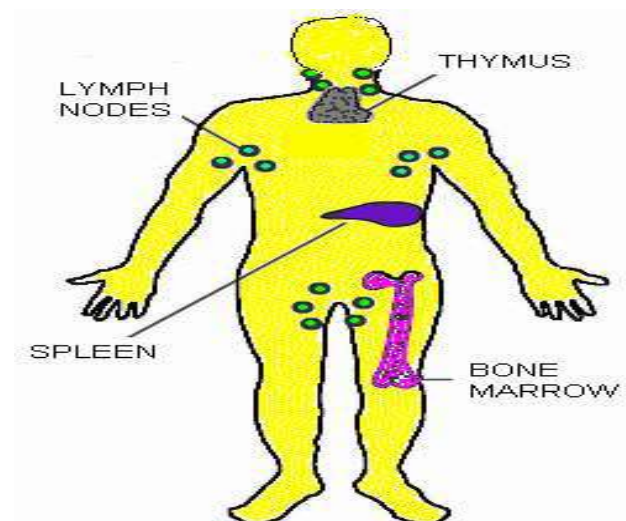
4. Dendritic Cells

- The dendritic cells acquire their name because they are covered with long membrane extensions resembling the dendrites of the nerve cells.
- Dendritic cells generally perform the distinct functions of antigen capture in one location and antigen presentation in another.
- Outside lymph nodes, immature dendritic cells monitor the body for signs of invasion by pathogens and capture intruding or foreign antigens.
- There are **four types of dendritic cells known**:
 - Langerhans cells
 - Interstitial dendritic cells
 - Myeloid cells
 - Lymphoid dendritic cells.

Each of these types arises from hematopoietic stem cells via different pathways and in different locations.

1.4 ORGANS OF THE IMMUNE SYSTEM

The organs of the immune system can be divided on the basis of their function into **primary lymphoid organs** and **secondary lymphoid organs**.



The location of primary and secondary lymphoid organs

The primary lymphoid organs include the thymus and bone marrow where maturation of lymphocytes takes place and thus these organs provide special microenvironment where antigen-independent differentiation of lymphocytes takes place. In these organs the lymphocytes develop antigenic specificity during maturation. The mature lymphocytes are then exported through the blood and the lymphatic system to the secondary lymphoid organs that include lymph nodes, spleen and MALT. These secondary lymphoid organs trap antigen and as a result undergo antigen-dependent differentiation.

Primary lymphoid organs

The lymphocytes only after maturation in the primary lymphoid organs become committed to a particular antigen and the cell becomes immunocompetent. In mammals, T cells mature in the thymus and B cells in the bone marrow.

Thymus

- It is a flat, bilobed organ situated above the heart and below the thyroid gland. At birth, it weighs around 15-20 g which grows more rapidly during the first two years and then more slowly reaching the maximum weight during puberty (40 g).
- Each lobe is surrounded by a capsule and is divided into lobules, which are separated from each other by strands of connective tissue called trabeculae.
- Each lobule is organized into two compartments: the cortex (outer compartment) the **medulla** (inner compartment). **Cortex** makes up 85 to 90% of the thymus.
- The progenitor T cells formed during hematopoiesis in bone marrow enter into the thymus where they multiply rapidly in the cortex. Cortical thymocytes are phenotypically and functionally immature but progressively differentiate into T cells within the thymic cortex.

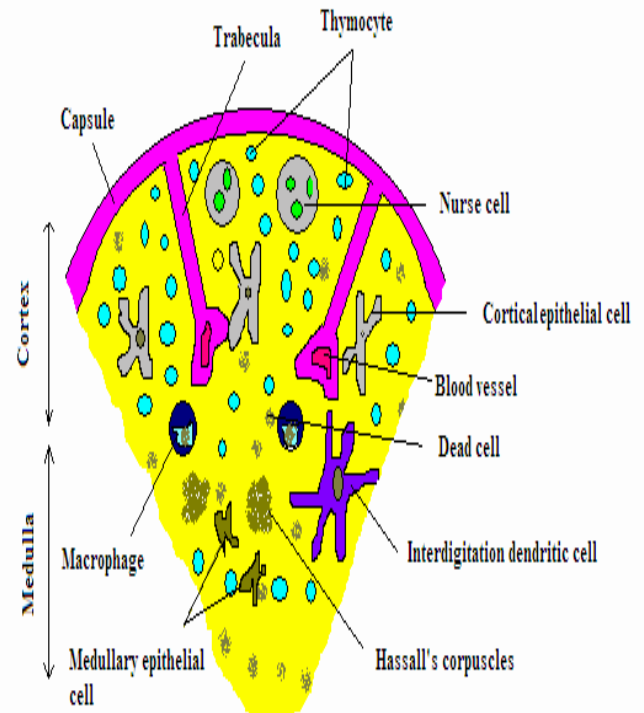


Fig: Diagrammatic cross-section of a portion of the thymus.

- For the differentiation and maturation of thymocytes into various types of mature T cells, several hormonal factors are required. The epithelial cells of the thymus serve as the source of these hormones namely, $\alpha 1$ -thymosin, $\beta 4$ -thymosin, thymopoietin and thymulin. In the course of thymocyte maturation within the thymus, the antigenic diversity of the T-cell receptor is generated by a series of random gene arrangements
- The absence of thymus exhibits dramatic decrease in the circulating lymphocytes of the T cell lineage and hence the absence of cell-mediated immunity, a congenital birth defect in humans called DiGeorge's syndrome.

Bone marrow

- Bone marrow is the source for all immune cells except during early fetal development.
- Bone marrow is the soft tissue in the hollow shafts of the flat bones (iliac bones, ribs, sternum, and vertebrae) and weighs about 3 kg in an averaged-size adult.

- The cellular composition of normal bone marrow is 5-15% lymphocytes, 1% plasma cells, and 4% monocytes and megakaryocytes.
- Immature B cells proliferate and differentiate within the bone marrow by interacting with the various cytokines secreted by the stromal cells.
- In birds, the bursa of Fabricius, a gut associated lymphoid tissue, is the primary site of B cell maturation.

Secondary lymphoid organs

Immune cells travel throughout the body by the circulatory and lymphatic systems. Lymphatic system serves to recover materials lost from the blood capillary network and antigens that enter the tissue spaces and return them to the blood. **Sites where lymphocytes encounter antigen and interact with other cells of the immune system are called the secondary lymphoid organs.**

Of all the secondary lymphoid tissue, **lymph nodes** and **spleen** are the most highly organized organs. Less organized lymphoid tissue, collectively called mucosal **associated lymphoid tissue (MALT)** occur in various body parts. The principal function of lymph nodes, spleen and MALT are to respond to antigens introduced into the tissues they drain, to respond to antigens in the blood and to protect against pathogens at major entry points respectively.

Lymph nodes

- Lymph nodes are encapsulated bean-shaped structures, normally <1 cm in diameter, packed with lymphocytes, macrophages, and dendritic cells
- Lymph nodes are present at the junctions of lymphatic vessels and serve as the first organized structures to encounter most antigens.
- The major function of the lymph nodes is to filter antigen from the lymph. The lymph, along with many cells and particles,

drain out of tissues and seep across the tiny single-cell-walled lymphatic vessels.

- The vessels transport lymph to the nodes where antigens are filtered out. As the lymph is filtered, it is enriched with antibodies, cytokines and mainly T lymphocytes.

Spleen

- The spleen is a large, ovoid filtering organ situated high in the left abdominal cavity. Unlike lymph nodes which are specialized to trap localized antigens from regional tissue spaces, the spleen filters blood and trap blood borne antigen and thus responds to systemic infections.
- The spleen is surrounded by a capsule that sends projections known as trabeculae into the compartments. The compartment is of two types-red pulp and white pulp separated by diffused marginal zone. The red pulp consists of erythrocyte rich blood intermingled with many dendritic cells, macrophages, a few lymphocytes and plasma cells. It is the site where old and defective red blood cells are removed.
- The white pulp surrounds the splenic arteries forming a periarteriolar lymphoid sheath (PALS) populated chiefly by T lymphocytes. The marginal zone, located peripheral to PALS, is rich in B cells and macrophages which is organized into primary lymphoid follicles.
- The spleen is the predominant organ in lymphocyte recirculation, moving more lymphocytes than all the lymph nodes combined.

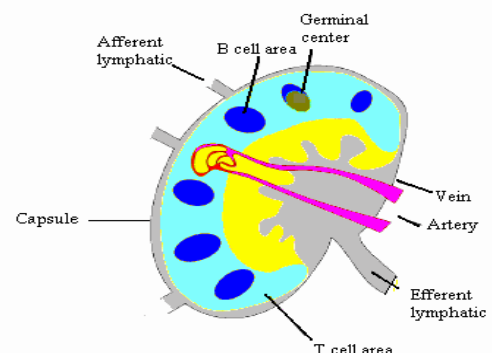
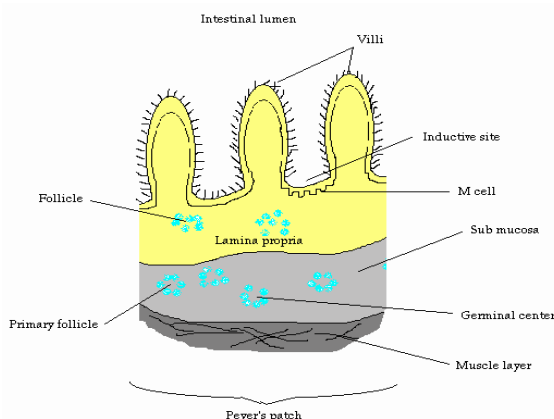


Fig. Structure of a lymph node

Mucosal-Associated Lymphoid Tissue (MALT)

- The non-encapsulated clusters organized lymphoid tissue with immune function is the mucosal associated lymphoid tissue.
- Approximately 400 m² surface area of the body are prone to attack by pathogens. These areas are the mucous membranes lining the digestive, respiratory and urinogenital tract. The defense of all these areas is provided by MALT.
- These tissue ranges from loose cluster or little organization of lymphoid cells in the lamina propria of intestinal villi to more organized structures such as the tonsils, appendix and Peyer's patches.
- The lamina propria contains large numbers of B cells, plasma cells, activated cells, and macrophages to impart better immunity than any other part of body.
- The epithelial cells of mucous membranes play an important role in promoting the immune response by delivering the antigen from the lumina of the respiratory, digestive and urinogenital tracts to the underlying MALT. This antigen transport is carried out by some specialized cells called M cells.



- **Fig. Cross-sectional diagram of the mucous membrane lining the intestine showing the M cell region.**

The M cells are flattened epithelial cells lacking the characteristic microvilli of the mucous epithelium and has a deep pocket or invagination in the basolateral plasma

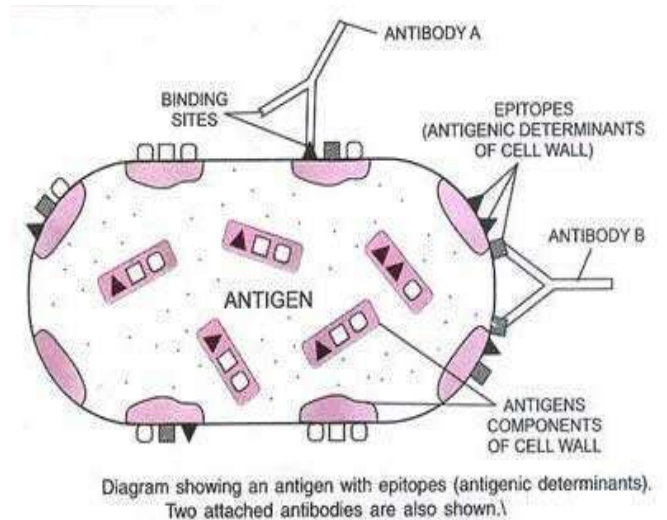
membrane filled with a cluster of B cells, T cells and macrophages

2.1 Antigen.

Any substance such as proteins, carbohydrates and lipid present in bacteria, fungi, parasites and viruses are considered foreign to Human or any vertebrate host and have the **capacity to induce immune response** is known as **Antigen**.

Immunogen:

Antigen which when introduced into vertebrate host, induces a specific immune response is called immunogen. They can mobilize immune system and provoke immune response



Antigenicity: the ability of compound or foreign molecules to combine specifically with antibody is known as antigenicity.

Immunogenicity: the ability of the molecule to induce immune response. Immunogenicity of an antigen is determined by four properties; Foreignness, molecular size, chemical composition and heterogeneity and ability to be processed and presented on the surface of Antigen presenting cells (APCs).

2.2.1 Properties of antigen/ factor determine immunogenicity

Foreignness: In order to induce immune response molecule must be foreign to the host. Immune response (antibody) is not

produced against self-antigen. Immunogenicity of an antigen increase with increase in degree of foreignness. Generally greater the phylogenetic gap between two species, greater will be the immune response.

Molecular size: There is correlation between molecular size of antigens and immunogenicity. Most active immunogen have molecular weight of 1 lakh (100,000 Da) or more. Generally, substances with molecular mass less than 5,000 Da are poorly immunogenic.

Chemical composition and heterogeneity: Proteins are the most potent immunogen with carbohydrate ranking the second. In contrast Lipid and nucleic acids generally do not act as antigen (immunogen) unless they are complexed with protein or carbohydrates. Homopolymers composed of polymer of single type less immunogenic. Heteropolymers are usually more immunogenic. In order to be immunogenic, molecules must be complexed and heterogeneous in composition.

Susceptibility to antigen processing and presentation: The development of both Antibody mediated immunity (AMI) and Cell mediated immunity (CMI) requires interaction of T-cells with antigen that has been processed and presented on the surface of Antigen presenting cells (APCs) along with MHC molecules. Larger and insoluble molecules are more immunogenic than small and soluble molecule because larger molecules are more easily phagocytosed and presented by APCs.

Dose and route of administration: Dose of antigen determines the amount of antibody produced in immunized host. An insufficient dose of antigen will not stimulate immune response either because it fails to activate enough T and B-lymphocytes or because of immunological tolerance in some cases. Conversely, excessive high dose can also induce tolerance.

Again, single dose of most immunogen will not induce strong immune response. Rather

repeated administration over a period of weeks is required. Such repeated administration is called boosters.

The antigen administered intravenously is carried to spleen where it activates T and B cells.

On the other hand, if antigen is injected subcutaneously, it enters into lymph node and activates T cells and B cells.

Genotype of recipient host: A major factor that determine immune response is genotype of recipient host. The genetic constitution of immunized host influences the type and degree of immune response.

The immune response of an animal to antigen is influenced and control by gene along with other environmental factors.

2.2. B and T cell epitopes: An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by an antibody, B-cell receptor or T-cell receptor.

- Generally, an antigen has several or many different epitopes and reacts with many different antibodies.
- Antigenic determinant is the region of antigen molecule that is bound by Fab region of Antibody or TCR of T-lymphocytes
- The antibody or TCR does not interact with antigenic macromolecules as a whole but only with distinct portion of the molecule called antigenic determinant or epitope.
- Antigenic determinant may include-sugars, amino acids and other organic molecules.
- A sequence of 4-6 amino acids is optimal size of an antigenic determinant of a protein antigen.

B Cell Epitopes:

- B cells recognize soluble antigens with the help of the antibody receptors present on their membrane, leading to a binary complex formation. It is the antigen binding site on the antibody molecules which decides/ selects the antigenic determinant to which it will bind to. B cells bind to free soluble antigens hence their epitopes are highly accessible sites on the exterior surfaces of the antigen and consist of hydrophilic amino acids.

- Mostly, the B cell epitopes are located in the most mobile regions of the antigen which helps in matching the complementarity with antigen binding site on the antibody molecule. Complex proteins contain multiple overlapping B cell epitopes, some of which are immunodominant.

T Cell Epitopes: T cells do not bind to soluble antigens but are capable of recognizing antigen only after it is processed and presented along with Major Histocompatibility Complex (MHC) molecules on APCs. Thus, it leads to formation of a trimolecular complex. The antigenic site which interacts with MHC molecules is called the **agretope** and is different from the epitope. The antigen binding cleft on an MHC molecule determines the size of the peptide(s) which can bind to the MHC molecule. T cell epitopes are often internal. Denaturation of proteins does not alter the T cell epitopes. MHC molecules expressed in an individual are one of the major deciding factors for the immunodominant epitopes.

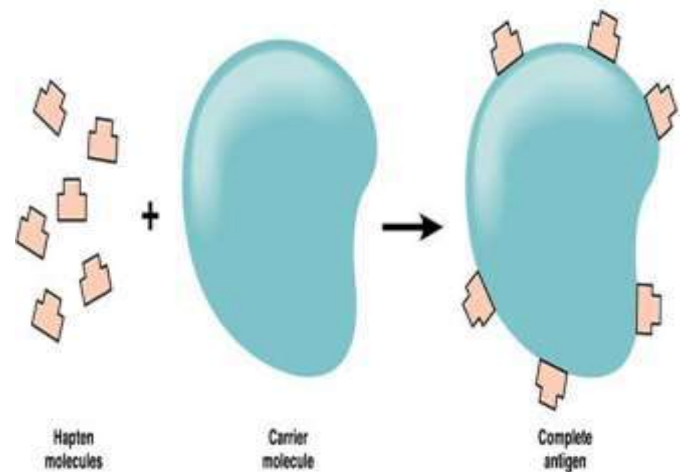
Paratopes: the corresponding binding site of antibody (Fab region) to epitopes of antigen is called paratopes.

2.1.3 Incomplete antigen hapten:

A substance which can bind with specific antibody but cannot induce immune response by itself is known as incomplete antigen or hapten. They have the property of antigenicity and not immunogenicity. They are also named as partial antigens

These small foreign molecules that is not antigenic and must be coupled with a carrier molecule to be antigenic. Simple haptens are non-precipitating and can inhibit precipitation of specific Ab by the corresponding Ag or complex Ag. They are univalent and hence non-precipitating as precipitation require two or more antibody binding sites on the antigen.

Complex haptens on the other hand can precipitate with specific antibodies and are multivalent/ polyvalent.



Hapten-Carrier Conjugate Chemical coupling of a hapten to a large protein called a carrier yields an immunogenic hapten carrier conjugate. These conjugates contain multiple copies of the hapten chemically linked to a large protein carrier. They are characterized by having native antigenic determinants of the carrier and hapten as well as new determinants formed by combined parts

2.1.4 Adjuvants:

- Adjuvants are substances that when mixed with antigen and injected with it, enhances the immunogenicity of that antigen.
- Adjuvants are used in research and clinical trials to increase the immunogenicity of antigen with low immunogenicity or available in low amount. They are mixed with antigen to prepare vaccine.
- For example; antibody response of mice toward BSA can be increased five folds or more if BSA is administered with adjuvants.
- Some of known adjuvants are synthetic polysaccharides and bacterial lipopolysaccharides.
- Adjuvants increase the immunogenicity by various ways. For examples-Alums
- Alums is an adjuvant that prolongs the persistence of antigen. It is the only adjuvant approved to human use.
- When antigen is mixed with alum, it causes precipitation of antigen. When this precipitate Ag-alum complex is injected, it results in slow release of antigen from injection site and thus activates immune system for long time.

- The alum precipitate also increases molecular size of antigen thus increase the susceptibility of antigen processing and presentation.

Effects of adjuvants:

- Antigen persistence is prolonged
- Co-stimulatory signals are enhanced
- Local inflammation is increased
- Non-specific proliferation of lymphocytes is stimulated

2.1.3. FACTORS INFLUENCING IMMUNOGENICITY

Contribution of the Immunogen

1. Foreignness: The immune system normally discriminates between self and non-self-such that only foreign molecules are immunogenic.

2. Size: There is not absolute size above which a substance will be immunogenic. However, in general, the larger the molecule the more immunogenic it is likely to be.

3. Chemical Composition: In general, the more complex the substance is chemically the more immunogenic it will be. The antigenic determinants are created by the primary sequence of residues in the polymer and/or by the secondary, tertiary or quaternary structure of the molecule.

4. Physical form: In general, particulate antigens are more immunogenic than soluble ones and denatured antigens more immunogenic than the native form.

5. Degradability: Antigens that are easily phagocytosed are generally more immunogenic. This is because for most antigens (T-dependant antigens, see below) the development of an immune response requires that the antigen be phagocytosed, processed and presented to helper T cells by an antigen presenting cell (APC).

B. Contribution of the Biological System

1. Genetic Factors: Some substances are immunogenic in one species but not in another. Similarly, some substances are immunogenic in one individual but not in others (i.e. responders and non-responders).

The species or individuals may lack or have altered genes that code for the receptors for antigen on B cells and T cells or they may not have the appropriate genes needed for the APC to present antigen to the helper T cells.

2. Age: Age can also influence immunogenicity. Usually, the very young and the very old have a diminished ability to mount an immune response in response to an immunogen.

C. Method of Administration

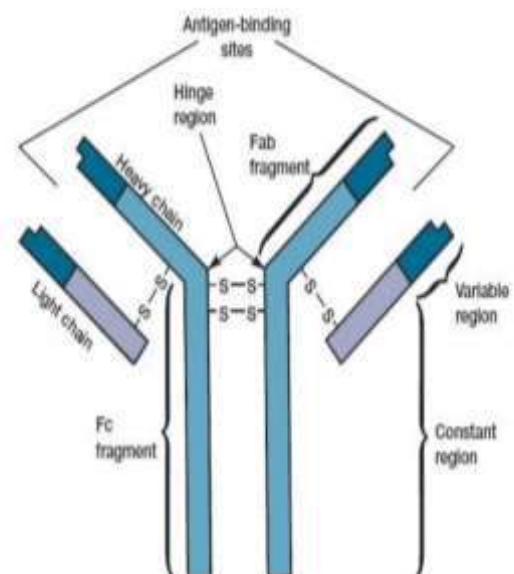
1. Dose: The dose of administration of an immunogen can influence its immunogenicity. There is a dose of antigen above or below which the immune response will not be optimal.

2.Route: Generally, the subcutaneous route is better than the intravenous or intragastric routes. The route of antigen administration can also alter the nature of the response

3. Adjuvants: Substances that can enhance the immune response to an immunogen are called adjuvants. The use of adjuvants, however, is often hampered by undesirable side effects such as fever and inflammation.

2.1. ANTIBODY OR IMMUNOGLOBULIN

The term antibody was first used by the scientists **Paul Ehrlich**. Membrane on antibodies refers antigenic specificity on B-cells. Secreted antibodies circulated, where they act as enhancer for stimulation of humoral mediated response, to neutralize antigens from the system.



The main categories of antibody action include:

- Neutralization
- Agglutination
- Precipitation
- Complement activation

All antibodies share structural features bind to antigen and participate in several functions.

Domain structure of antibody:

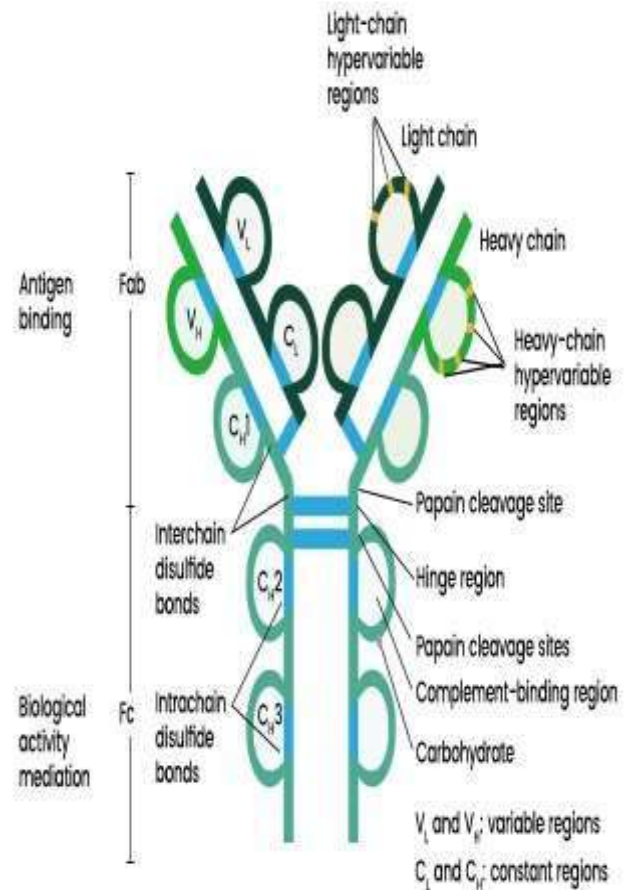
- Antibody molecules have common structure of four polypeptide chains. i.e., 2-light chains and 2 heavy chains respectively.
- Heavy chains consist of about 25000 molecular weight and light chain of 12000 molecular weight. Each Light chain is bound to heavy chain by non-covalent interaction.
- The light chain consists 220 amino acids while heavy chain consists of 440 amino acids.
- The Monomer has two kappa and two lambda
- Each antibody contains one or another type of light chains while consists of five types of heavy chains.
- Due to linkage of heavy chain and light chain antibody look like Y- shaped.
- The overall structure of immunoglobulin molecule is determined by primary, secondary, tertiary and quaternary organization of amino acid molecules.
- Two domains are found in L-chain i.e., one in variable region (VL) and other in constant region (CL).
- In H-chain, one domain is found in Variable region (VH). In IgA, IgG and IgD three domains are found in constant region (CH1, CH2 and CH3) whereas in IgE and IgM four domains are found in constant region of H-chain (CH1, CH2, CH3 and CH4).

- **Fab, Fc and Hinge region of antibody:**

1. Fab region

- Antigen binding is accomplished by amino-terminal (N-terminal) region and effector functions by carboxyl terminal (C-terminal) region of antibody.

- In an antibody molecule two Fab regions are found and they bind antigens.
- Hypervariable region on L-chain (VL domain) and H-chain (VH domain) form antigen binding site.



Detailed comparison of amino acid sequences of large number of VL and VH domain reveals that the sequence variation is concentrated in few discrete region of these domains. The variability plot of VH and VL domains shows maximum variation in certain region which is known as hypervariable region and this forms antigen binding site.

Antigen binding site is complementary to epitope of antigen, so it is also known as complementary determining regions (CDRs).

2.Fc region:

Fc region of immunoglobulin allows for interaction of immune complex with other phagocytic cells and complement. Take parts in various biological functions that are determined by amino acid sequences of

each domains of constant region. Many different form of Fc receptors exists.

3. Hinge region:

The γ , δ and α heavy chain contains an extended peptide sequence between CH1 and CH2 domain that has no homology with other domain, this region is known as hinge region. Hinge region is rich in proline residue and is flexible. Therefore IgG, IgD and IgA are flexible. The flexibility given by hinge region enable Fab region to assume various angle to bind antigen.

IgA- Alpha

IgE- Epsilon

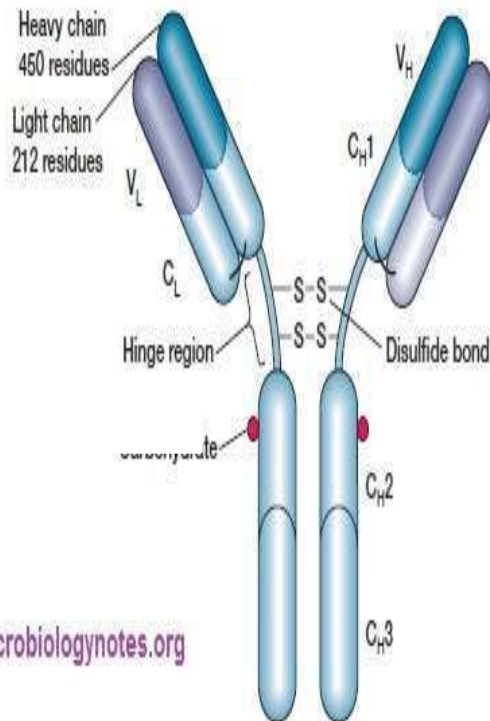
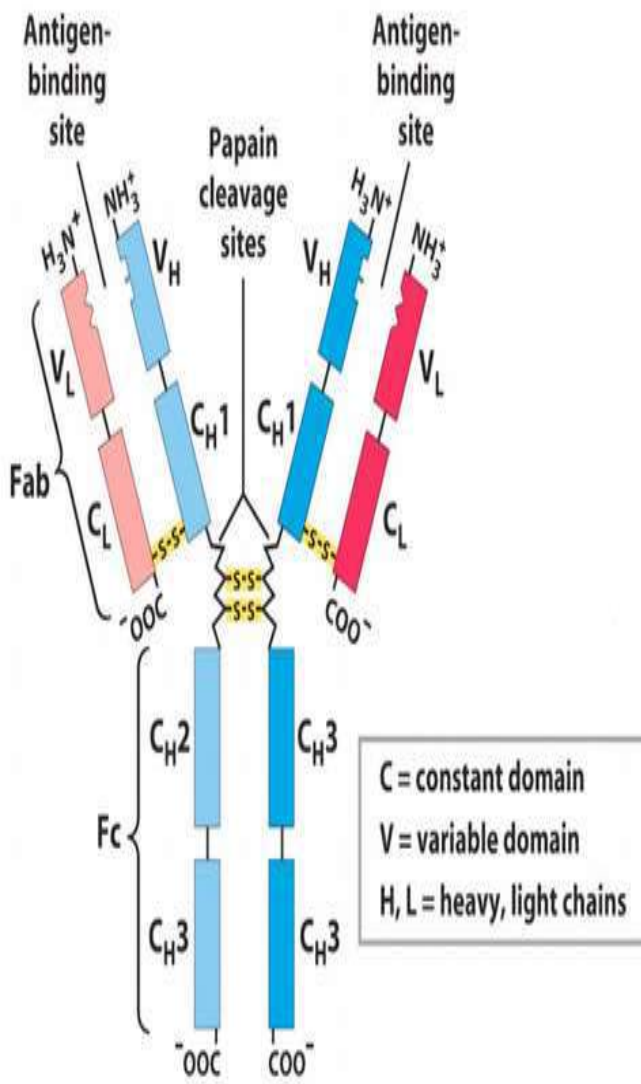
IgD- Delta

IgM- Mu

Heavy chains of antibodies had been named on Greek words each class of Ig plays important role in defence system.

IGG

IgG



- 50 to 80% antibodies contribute in the form of IgG in our serum.
- The maternal molecules can pass through placenta and provide passive immunity to fetus.
- These can also pass the cell walls of blood vessel.
- They are Monomer and molecular weight about 1,50,000 KD.
- They are responsible for neutralizing microbes and their toxins.
- They also enhance effectiveness of phagocytic cells to engulf and ingest them.

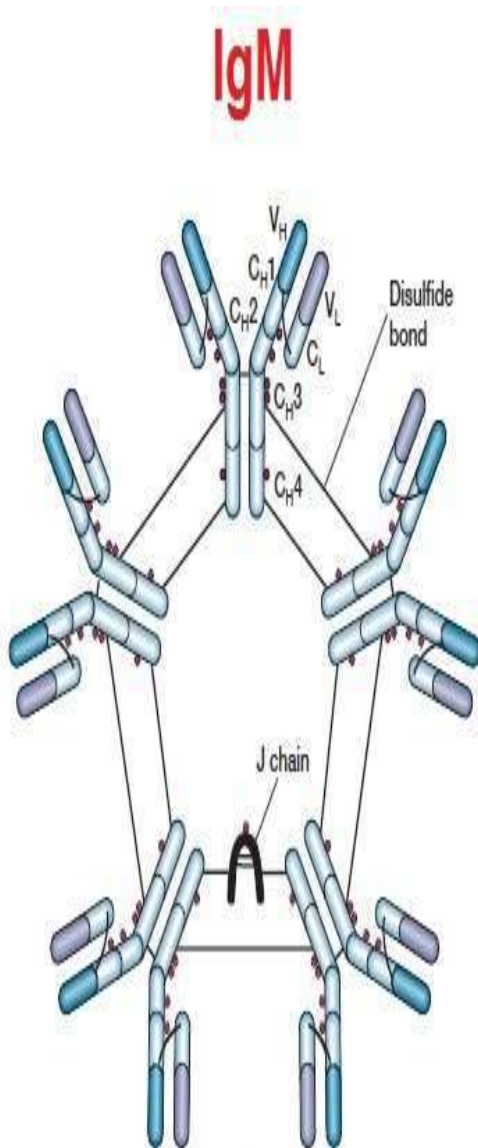
TYPES OF ANTIBODIES

There are five classes of immunoglobulin molecules such as:

IgG- Gamma

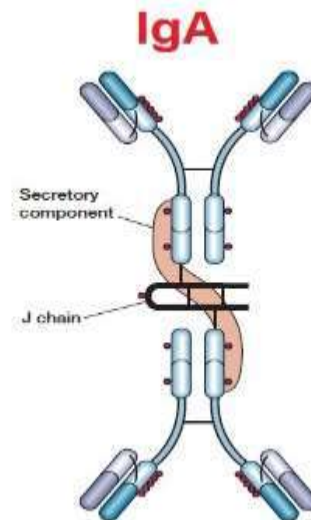
IGM

- The pentameric structure of human IgM. The disulfide bonds linking peptide chains are shown in black; carbohydrate side chains are in red. Note that 10 antigen-binding sites are present.
- They constitute about 5 to 10% in blood serum.
- It has pentamer structure.

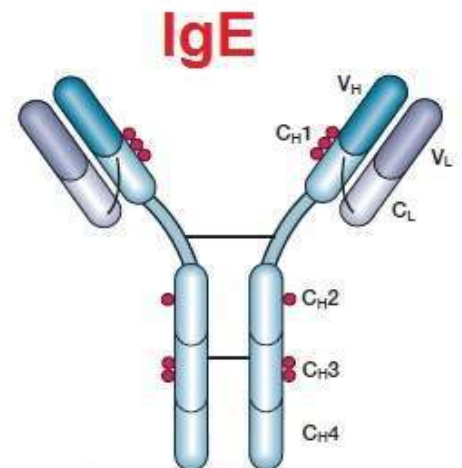


- When exposed to antigen, it is IgM that appears first.
- This specially cross-linked particulate antigen caused the aggregation due to numerous antigen binding sites.
- Molecular weight about 900,000 KD.
- It is very much effective as agglutination.

IGA



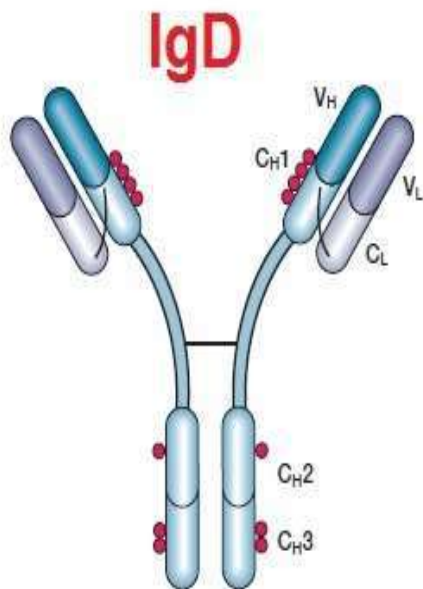
- The dimeric structure of human secretory IgA. Notice the secretory component (tan) wound around the IgA dimer and attached to the constant domain of each IgA monomer. Carbohydrate side chains are shown in red.



- Concentration of IgA in human blood remains about 15%.
- It consists J- chain with dimer.
- Molecular weight is about 3,20,000 KD.
- It is present in blood serum and surface of lymphocytes.
- It checks the attachment of pathogens to mucosal surfaces and protect the infants from infections.

IGD

- The disulfide bonds linking protein chains are shown in black; carbohydrate side chains are in red.
- It accounts only 0.2% of serum.
- It is also Monomer and consist of 1,85,000 KD molecular weight.



- These are present on the upper surface of T- cells and it cannot pass across placenta.
- They assist initiation of immune responses and their population remains very high on the surface of the B – cells.

IGE

- The concentration of IgE is 0.02% of the total antibodies.
- They are larger than IgG.
- It responds quickly to the receptor of the mast cells and basophils.
- Mast cell and basophils are spread cells that take part in allergic reaction.
- Molecular weight is about 2 lakh KD.
- They raise in infections and responsible for allergic symptoms.

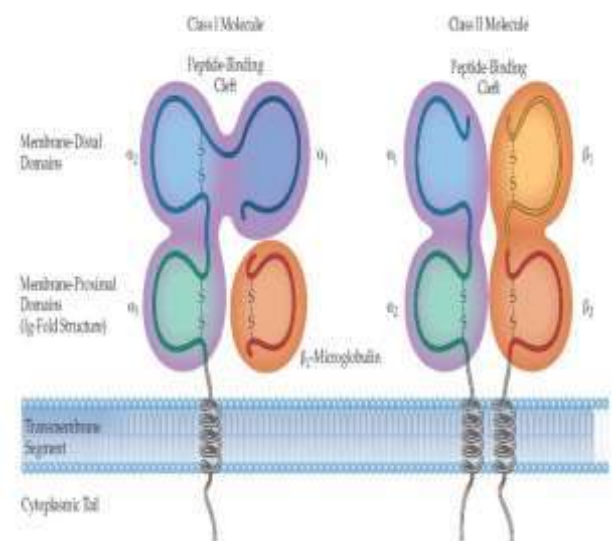
Applications

- Antibodies can be used to treat immune deficiencies as a means of passive immunity.
- The development of monoclonal antibodies has been used to treat several diseases like multiple sclerosis, rheumatoid arthritis, and different cancers.
- Antibodies can also be used in medical diagnostics as many biochemical assays allow the detection of antibodies for the diagnosis of diseases.
- Different classes of immunoglobulins can be used to analyse the antibody profile of patients.

- Antibodies can also be used as workhorse agents in biomedical research to study the workings of different antigens and their relationship with the host.

2.3 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC): STRUCTURE, TYPES AND FUNCTIONS

- Major histocompatibility complex (MHC) is the cluster of gene arranged within a long continuous stretch of DNA on chromosome number 6 in Human which encodes MHC molecules.
- MHC molecule is a cell surface glycoprotein receptor present in APCs and acts as antigen presenting structure. It plays vital role in immune recognition, including interaction between T cells and other cell types.
- In Human MHC is known as Human Leucocyte antigen (HLA) complex and the genes of MHC are recognized in three classes, consequently there are three types of MHC molecules.
- Types of MHC:
 - Class I MHC
 - Class II MHC
 - Class III MHC



MHC class-I:

- Class-I MHC gene encodes glycoprotein molecule which expressed on the surface of all nucleated cells and platelets.
- MHC-I molecule contains a 45KDa α -chain associated non-covalently with a 12KDa β_2 microglobulin molecule.
- Association of α -chain and β_2 microglobulin is required for expression of class-I MHC molecule on cell membrane.

α-chain of MHC-I:

- The α-chain is a transmembrane glycoprotein encoded by polymorphic gene within A, B and C region of Human HLA complex
- The α-chain is anchored in the plasma membrane by its hydrophobic transmembrane segment and hydrophilic cytoplasmic tail.
- α-chain is made up of 3 domains (α1, α2 and α3). Each domain containing approximately 90 amino acids, a transmembrane domain of about 25 hydrophobic amino acids followed by short stretch of charged (hydrophilic) amino acids of cytoplasmic tails of 30 amino acids.
- α1 and α2 domains interact to form a deep groove on the top which is a peptide binding cleft. It can bind antigen of 8-10 Amino acids long.
- α3 and β2 are organized into β-pleated sheets, each formed by antiparallel β-strand of amino acids, this structure is known as immunoglobulin fold. Because of this structure α-chain and β2 microglobulin are classified as member of immunoglobulin super-family receptor.

β2 microglobulin of MHC-I:

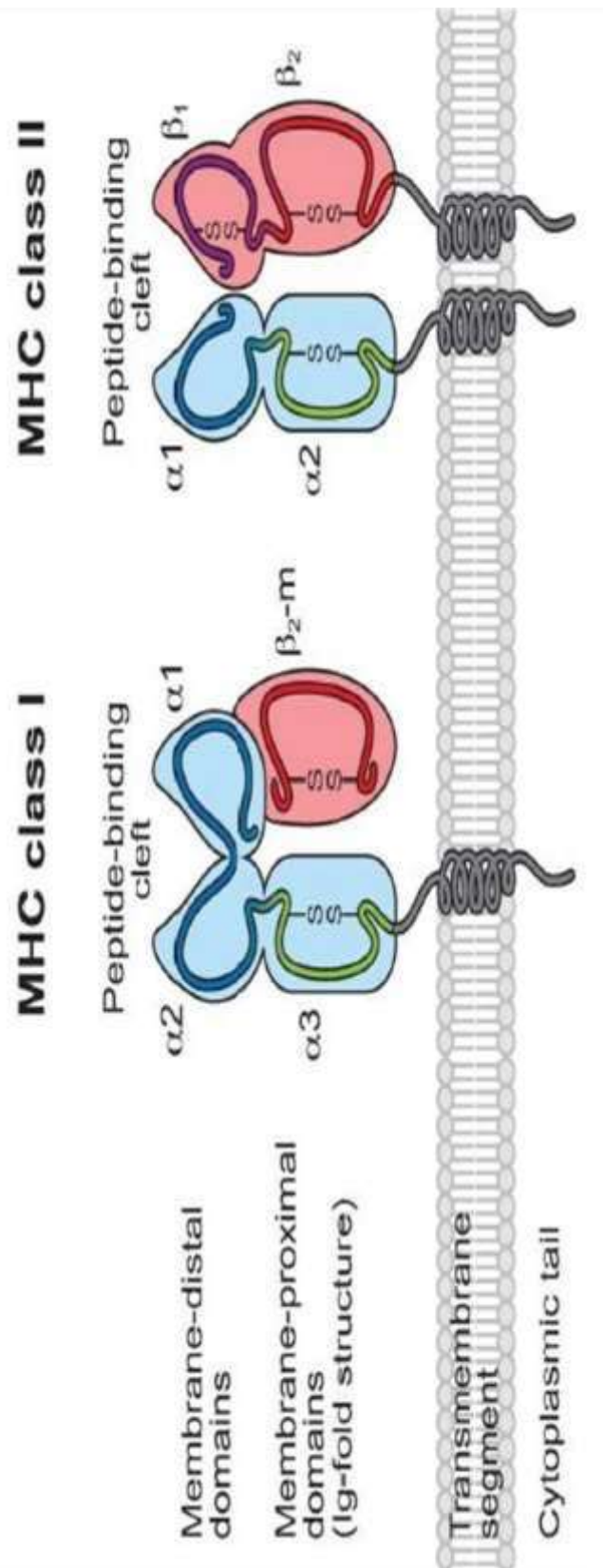
- β2 microglobulin is a protein encoded by a highly conserved gene located on different chromosome
- β2 microglobulin is similar in size and organization to α3 domain.
- β2 microglobulin does not contain transmembrane region and is non-covalently linked with α-chain.

Functions of MHC class I:

- Major function of MHC-I is to bind peptide antigens and present to CD8+ T cells (T helper cells)
- CD8 T cells are specific for MHC-I antigen
- MHC-I binds endogenous antigen and present to T helper cells.
- MHC-I molecules are found on surface of all nucleated cells.

MHC class-II:

- Class-II MHC is the glycoprotein molecule expressed primarily on antigen presenting cells such as macrophages, dendritic cells and B-cells.



- MHC-II molecules contain two different polypeptide chains, 1 33 KDa α-chain and 28KDa β-chain which are associated by non-covalent interactions.

α-chain and β-chain of MHC-II:

- α-chain and β-chain of MHC-II is a membrane bound glycoprotein that

contains external domains, a transmembrane segment and cytoplasmic tail.

- α -chain and β -chain are made up of two domains ($\alpha 1$ and $\alpha 2$) and ($\beta 1$ and $\beta 2$) respectively.
- The peptide binding cleft is an open ended groove formed between α -chain and β -chain at proximal end. The cleft can bind antigenic peptide of 13-18 amino acids long.

Functions of MHC class II:

- Major function of MHC-II is to bind peptide antigen and present to CD4 T cells.
- MHC-II are found on surface of Antigen presenting cells (APCs).
- CD4+T-cells are specific for MHC-II
- Activates B cells for antibody production
- MHC-II plays a significant role in graft versus host response and in mixed lymphocyte reaction (MLR) because the immune response gene is identical to MHC-II in human.

MHC class-III:

- MHC-III is a diverse group of molecules that serve a wide variety of functions in immune system.
- MHC-III is not a marker on cell surface.

Functions of MHC class-III:

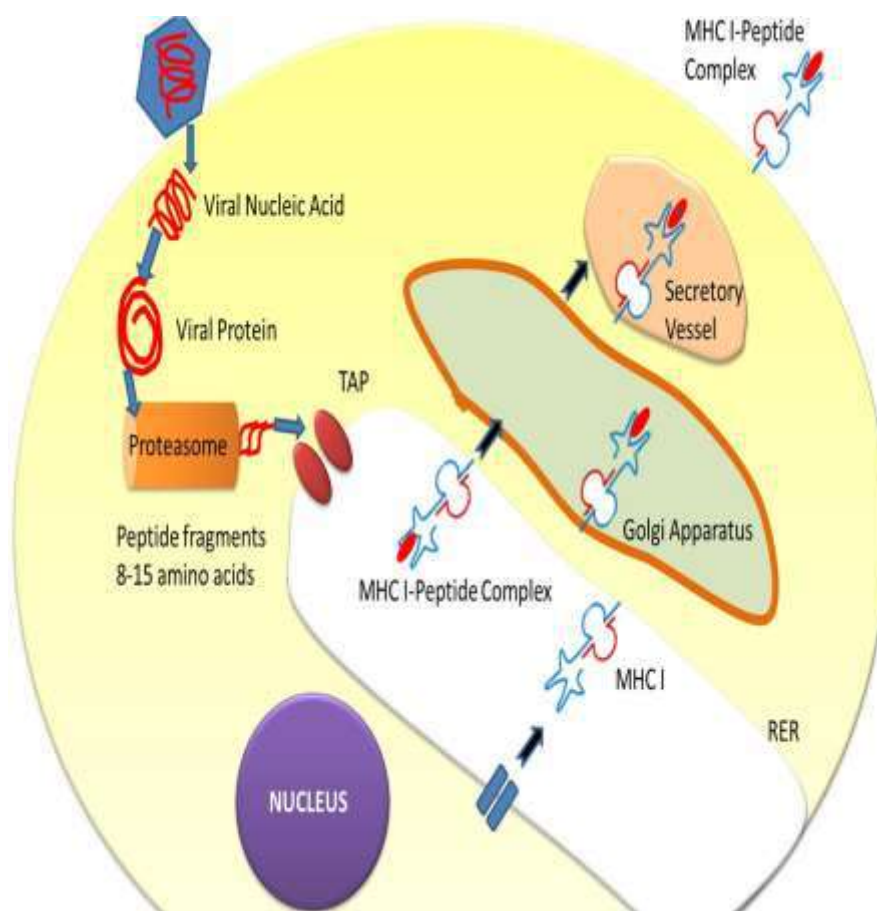
- Involved in complement activation
- Involved in inflammation caused by cytokines, tumour necrosis factors etc

2.3 Antigen processing and presentation by MHC molecules

Antigen processing and presentation are the means by which protein antigens become associated with self MHC molecules for presentation to T-cells with appropriate receptors. It can be broken down into two components;

Antigen processing: It converts large antigenic proteins into short peptides that can be displayed on the cell membrane together with an MHC molecule (either class I or class II) and recognized by T-cells.

Antigen presentation: In this process, certain cells in the body, especially antigen presenting cells (APCs) express processed antigen on their cell surface along with MHC molecules that are recognized by T-cells.



A. Endogenous Pathway or Cytosolic pathway:

- **Intracellular or endogenous antigens**, those generated within the cell, e.g., viral protein synthesized in an infected cell, tumour cells and intracellular pathogens (tuberculosis, Histoplasma capsulatum) are processed through this pathway.
- Thus, processed antigens are presented on the cell membrane with class I MHC molecules to CD8⁺ T_c cells for degradation.

Steps involved in Cytosolic pathway:

1. Proteolytic degradation of large antigenic protein into peptides:

- MHC molecules cannot bind large sized intracellular protein antigens. Therefore, they have to be degraded into short peptides of about 8-10 amino acids.

- Cytosolic proteolytic system or peptidase complex called proteasomes which are present in all the cells help in degradation of such proteins.
- The large (20S) proteasome is a barrel shaped complex composed of 14 sub-units' symmetrical rings. However, not all of these sub-units have protease activity.
- Large proteins enter the proteasome through narrow channels present at its two ends.
- A small protein called **ubiquitin** is attached to many proteins targeted for proteolysis.
- This **ubiquitin-protein complex** is then degraded by a **26S proteasome**, a **multifunctional protease complex** (20S proteasome + 19S regulatory component).
- The central hollow of the proteasome is the site where the **degradation of ubiquitin protein complex is thought to occur**.

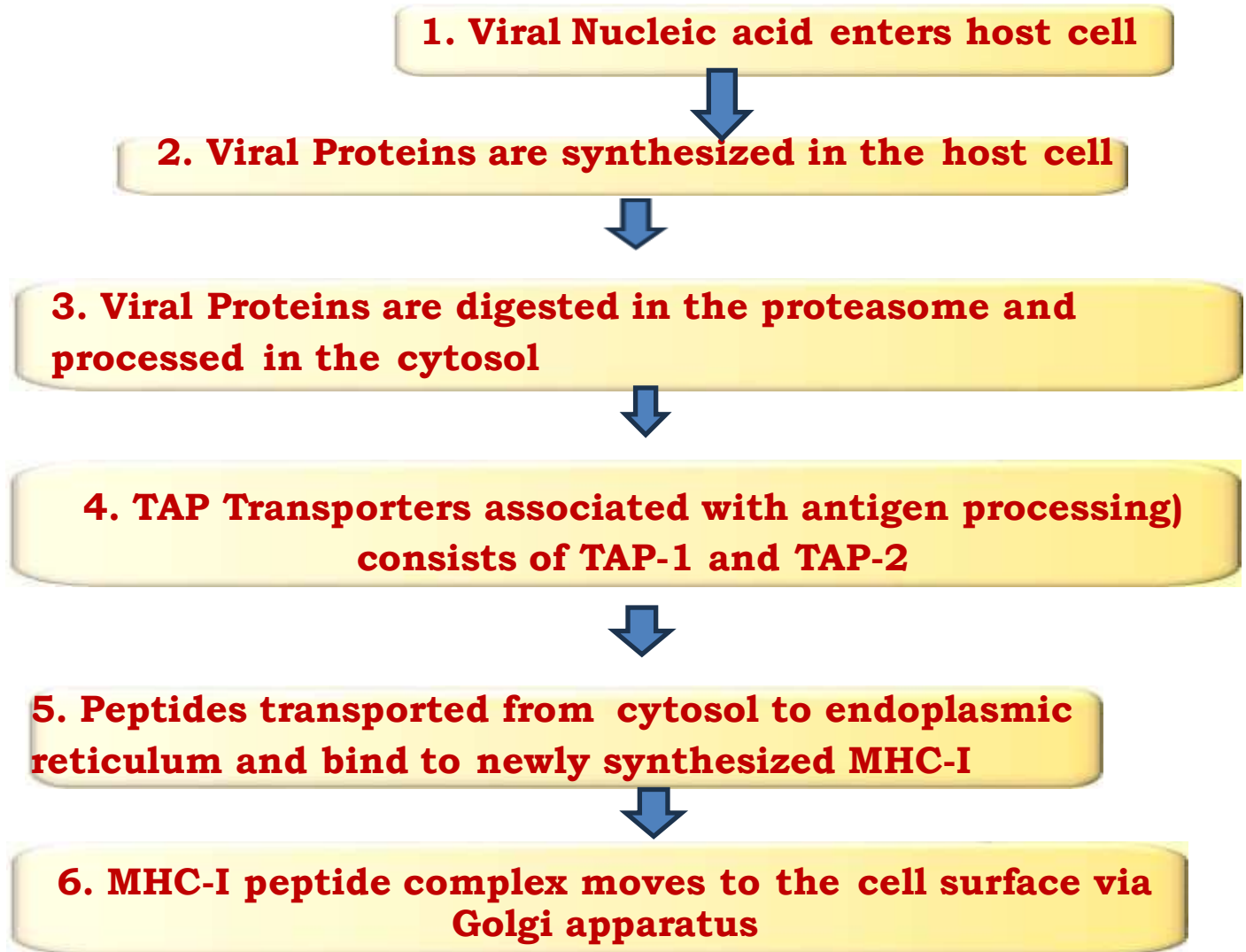
2. Translocation of peptides from cytosol to rough Endoplasmic Reticulum (RER)

- **TAP (transporter associated with antigen processing)** proteins transport small peptides (8-10 amino acids) formed in the cytosol by proteasome complex to rough endoplasmic reticulum (RER).
- This process requires the hydrolysis of ATP.
- TAP is a heterodimer of TAP1 and TAP2, which is anchored in the membrane of RER and has affinity for peptides having 8-16 amino acids.
- Class I MHC can most effectively bind peptides of nine amino acids that are formed by trimming the peptides with the help of endoplasmic reticulum amino-peptidase (ERAP).
- TAP also favours peptides having amino acids with hydrophobic or basic carboxyl terminal, also preferred by class I MHC molecules.

3. Assembly of peptides with class I MHC molecules:

- Class I MHC molecule has two components, the α -chain and β_2 macroglobulin which are synthesized on polysome along the rough endoplasmic reticulum (RER).
- Class I MHC molecule has a stable assembly of α -chain and β_2 macroglobulin and exit the RER only when its binding groove consists of a **peptide called molecular chaperones**.
- Molecular chaperones facilitate the **folding of polypeptides**.
- The first molecular chaperone involved in the assembly of class I MHC is **calnexin** which is a resident **membrane protein** of RER.
- It associates with free class I α -chain and promotes its folding.
- The binding of β_2 -microglobulin with α -chain, releases calnexin from class I MHC. Class I MHC then associates with other chaperones **calreticulin** and **tapasin** (TAP-associated protein).
- TAP transporter, carrying peptides is brought to the close proximity of class I MHC molecule by tapasin after which Class I MHC acquires the antigenic peptide.
- An additional protein with enzymatic activity, **ERp57**, allows the release of class I MHC after acquiring the antigenic peptide. This is possible because ERp57 form disulfide bond with tapasin and non-covalently associates with calreticulin to stabilize the interaction.
- As a consequence, peptide bound class I MHC is released from the complex of calreticulin, tapasin and ERp57, which then exits from RER and displays on the cell surface via Golgi complex.

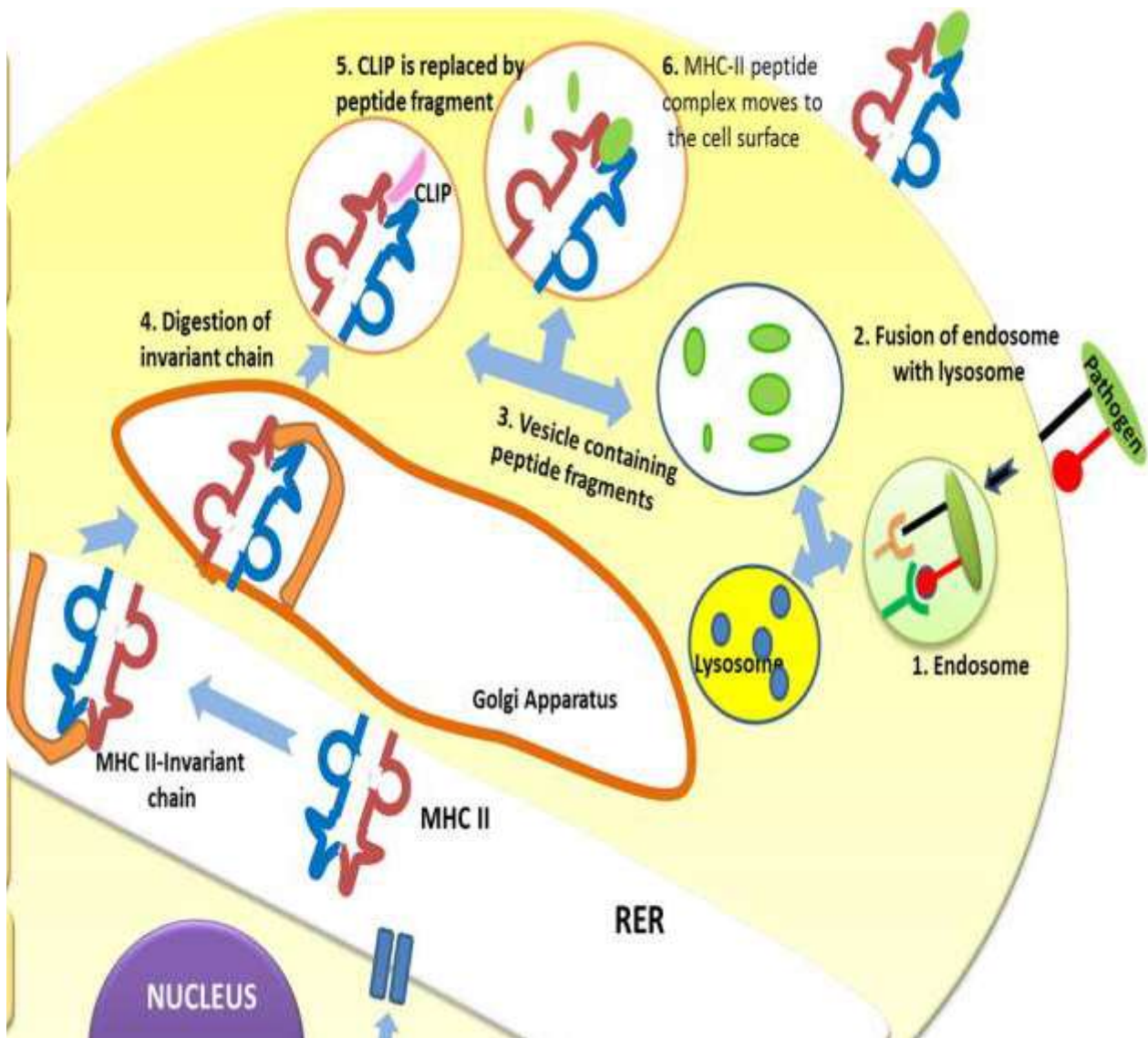
Endogenous Pathway or Cytosolic pathway: Flow chart



B. Exogenous or Endocytic pathway:

- Extracellular antigens like bacteria (those taken up by endocytosis) are processed in the endocytic pathway.
- At first, antigen presenting cells (APCs) **internalize** the extracellular antigen by phagocytosis or endocytosis or both.
- Dendritic cells and macrophages internalize antigens by both endocytosis and phagocytosis, whereas APCs which are non-phagocytic or poorly phagocytic, internalize the antigen by receptor mediated endocytosis or pinocytosis. e. g. B cells.

The processed antigens are presented on the cell membrane with class II MHC molecules which are recognized by CD4⁺ TH cells.



Steps involved in endocytic pathway:

1. Peptide generation from internalized antigen inside endocytic vesicles:

- Endocytic pathway involves the degradation of internalized antigens into peptides within acidic compartments.
- Three increasingly acidic compartments; **early endosomes** (pH 6-6.5), **late endosomes** or endo-lysosome (pH 5-6) and **lysosomes** (pH 4.5-5) are involved in this pathway.
- The internalized antigen gradually moves from early to late endosomes and finally to lysosomes, where it encounters with hydrolytic enzymes like **hydrolases**, **proteases**, **nucleases**, **lipases** etc. and the environment is increasingly acidic.
- Oligopeptides with 13-18 amino acids are generated from internalized antigen which then binds with class II MHC. This binding protects the oligopeptide from further proteolysis.
- The mechanism behind the movement of internalized antigen from less acidic to more acidic compartment has not been well explained.
- Small transport vesicles may carry antigen from one compartment to the next.

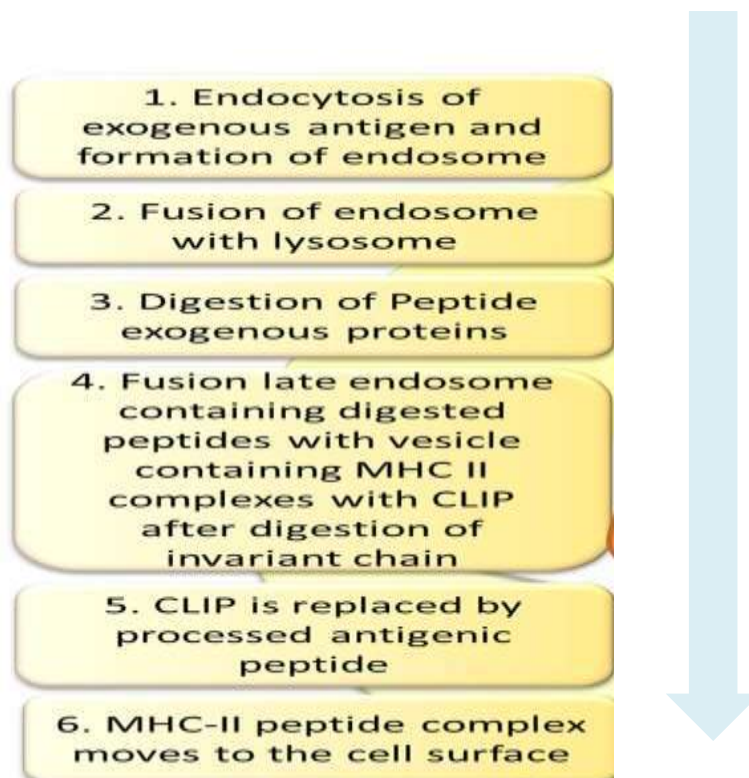
2. Transport of class II MHC molecule to endocytic vesicles:

- Since antigen-presenting cells express both class I and class II MHC molecules, something must prevent class II MHC molecules from binding to the same set of antigenic peptides as the class I molecules.
- A trimeric protein called **invariant chain (Li, CD74)** interacts with the peptide-binding cleft of class II MHC, preventing any endogenously derived antigen from binding to the cleft.

- Invariant chain, in its pre-assembled form is associated with three pairs of class-II α and β -chains when class II MHC molecules are synthesized within RER.
- The invariant chain also helps in the folding of the class II α and β -chains, their exit from the RER and the subsequent routing of class II MHC molecule to endocytic compartments from the trans-Golgi network.
- The invariant chain contains sorting signals in its cytoplasmic tail that direct the transport of the class II MHC complex from the trans-Golgi network to the endocytic compartments.

3. Assembly of peptides with Class II MHC molecules:

- Class II MHC-invariant chain complexes are transported from RER through trans-Golgi network to endocytic compartments; moving from early endosome to late endosome and finally to lysosome.
- The proteolytic activities inside each acidic compartment gradually degrade the invariant chain leaving a short fragment of **invariant chain called CLIP (Class-II associated invariant chain)**.
- CLIP physically occupies the peptide-binding cleft of class II MHC molecule and prevents any premature binding of antigenic peptides.
- HLA-DM (a non-classical class II MHC), is required to catalyse the exchange of CLIP with antigenic peptides.
- **HLA-DM** is a heterodimer of α and β -chains, which unlike other class II MHC is not expressed at the cell membrane.
- The reaction between HLA-DM and the class II-CLIP complex facilitating exchange of CLIP for another peptide is impaired in the presence of HLA-DO, which binds to HLA-DM and lessens the efficiency of the exchange reaction.
- This peptide-class II MHC complex is transported to the plasma membrane where neutral pH enables the complex to assume the compact and stable form with strongly bound peptide difficult to replace with another peptide at physiological conditions.



2.4 Hypersensitivity- Definition, Causes, Mechanism, Types, Examples

- Hypersensitivity is increased reactivity or increased sensitivity by the animal body to an antigen to which it has been previously exposed.
- The term is often used as a synonym for allergy, which describes a state of altered reactivity to an antigen.
- Hypersensitivity has been divided into categories based upon whether it can be passively transferred by antibodies or by specifically immune lymphoid cells.
- The most widely adopted current classification is that of Coombs and Gell which designates immunoglobulin-mediated (immediate) hypersensitivity reactions as types I, II, and III, and lymphoid cell-mediated (delayed-type) hypersensitivity/cell-mediated immunity as a type IV reaction.
- *“Hypersensitivity” generally represents the “dark side,” signifying the undesirable aspects of an immune reaction, whereas the term “immunity” implies a desirable effect.*

Causes of Hypersensitivity

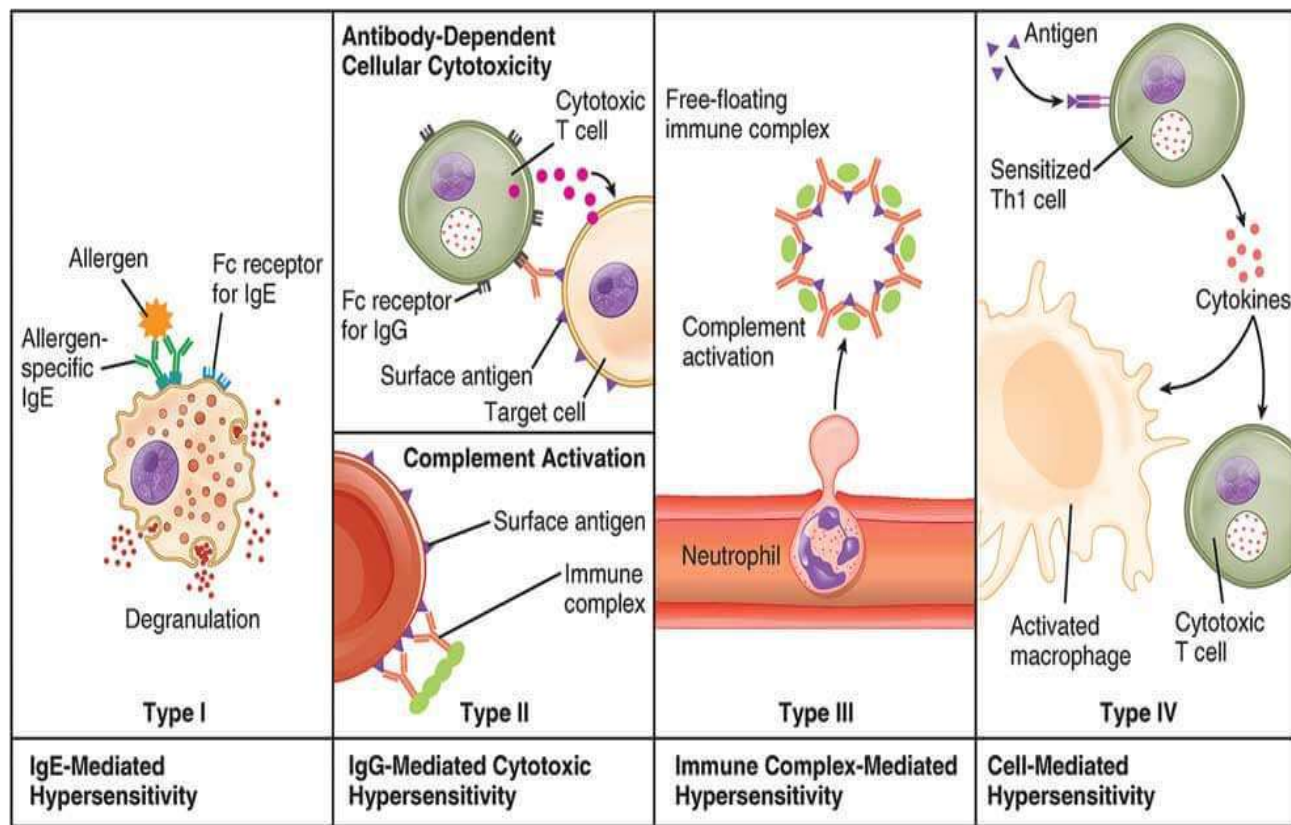
- Immune responses that are the cause of hypersensitivity diseases may be specific for antigens from different sources.
- Autoimmunity: reactions against self-antigens.
- Reactions against microbes.
- Reactions against non-microbial environmental antigens.

Mechanism of Hypersensitivity

Hypersensitivity diseases are commonly classified according to the type of immune response and the effector mechanism responsible for cell and tissue injury. These mechanisms include some that are predominantly dependent on antibodies and others predominantly dependent on T cells, although a role for both humoral and cell-mediated immunity is often found in many hypersensitivity diseases.

Types of Hypersensitivity Reactions

The Gell's and Coombs' classification of hypersensitivity reactions considers four types of reactions. **Type I, II, and III reactions are basically mediated by antibodies with or without participation of the complement system; type IV reactions are cell-mediated.**



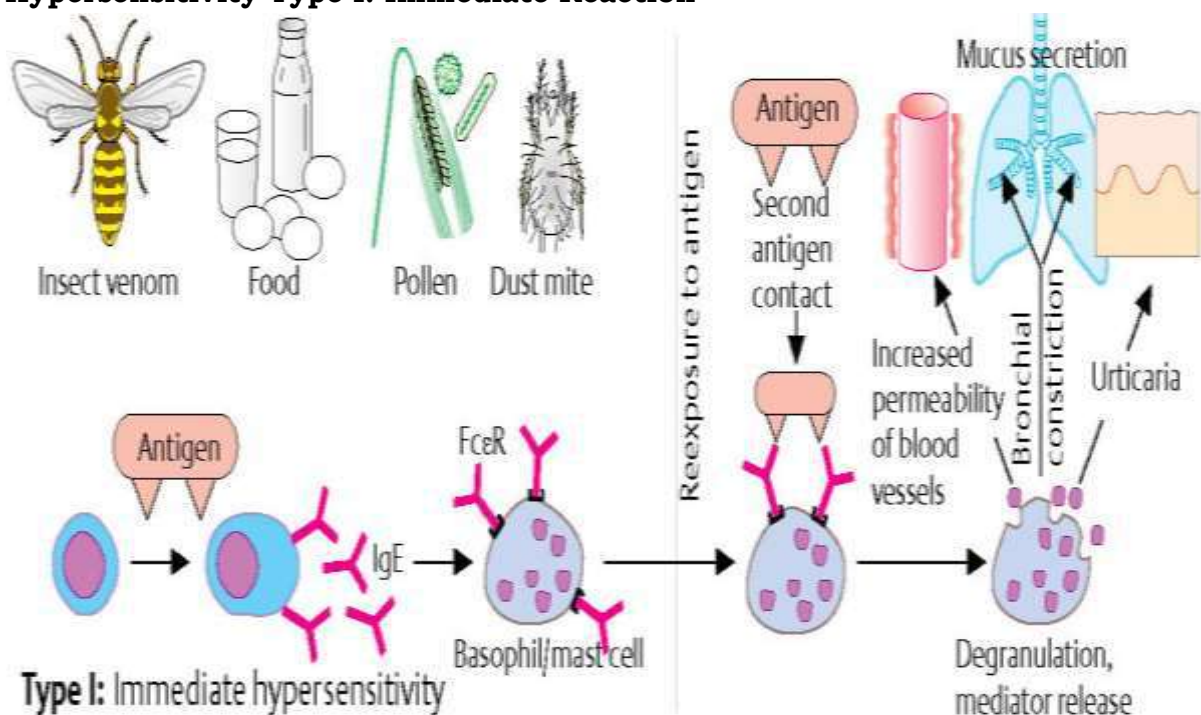
Hypersensitivity Type I, II, III and IV- Summary in table form

Character	Type I	Type II	Type III	Type IV
Alternative Name	Allergic hypersensitivity	Cytotoxic hypersensitivity	Immune complex hypersensitivity	Cell-mediated hypersensitivity/ Delayed type of hypersensitivity
Principle	Antibody-mediated degranulation of granulocytes leads to the destruction of cells.	Antibody-mediated destruction of healthy cells.	Antigen-antibody complex-mediated destruction of cells.	T lymphocytes mediated the destruction of cells.
Primary Mediator	IgE	IgG/IgM	IgG/IgM	Specific subsets of CD4+ helper T cells or CD8+ cytotoxic T cells.
Other components as mediators	Mast cells, Basophils, histamine & other pharmacological agents	Complement, Neutrophils	Complement, phagocytes and K cells	Dendritic cells, macrophages, and cytokines

Reaction time	Immediate or within a few hours	5-8 hours	2-8 hours	After 24 hours only, mostly 48-72 hours after contact
Antigen	Free in circulation (Soluble)	Fixed on cells	Free in circulation (Soluble)	Soluble or cell-bound
Antigen origin	Exogenous	Endogenous or exogenous	Exogenous or endogenous	Exogenous or endogenous
Antibody	Fixed on mast cells and basophils	Free in circulation	Free in circulation	Not applicable
Mechanism	Allergen-specific IgE antibodies bind to mast cells via their Fc receptor. When the specific allergen binds to the IgE, cross-linking of IgE induces degranulation of mast cells.	IgG or IgM antibody binds to a cellular antigen, leading to complement activation and cell lysis. IgG can also mediate ADCC with cytotoxic T cells, natural killer cells, macrophages, and neutrophils.	Antigen-antibody complexes are deposited in tissues. Complement activation provides inflammatory mediators and recruits neutrophils. Enzymes released from neutrophils damage tissue.	Th2 cells secrete cytokines, which activate macrophages and cytotoxic T cells.
Complement activation	No	Yes	Yes	No
Appearance	Weal & flare	Lysis & necrosis	Erythema & edema	Erythema & induration
Transfer with serum	Passive transfer possible with serum	Passive transfer	Passive transfer	Cannot be transferred with serum; but possible with T cells transfer
Desensitization	Easy but short-lived	Easy but short-lived	Easy but short-lived	Difficult but long-lived.
Examples	Asthma, Rhinitis, Atopic	Rhesus incompatibility	Glomerulonephritis, Systemic	The tuberculin reaction,

	eczema, Bee sting reaction	(Rh haemolytic disease), Transfusion Reactions, Cell Destruction due to autoantigens, Drug-Induced Haemolytic Anaemia	Lupus Erythematosus , Farmer's lung arthritis, Vasculitis	Granuloma formation, Allergic contact dermatitis, Type-1 diabetes

1. Hypersensitivity Type I: Immediate Reaction

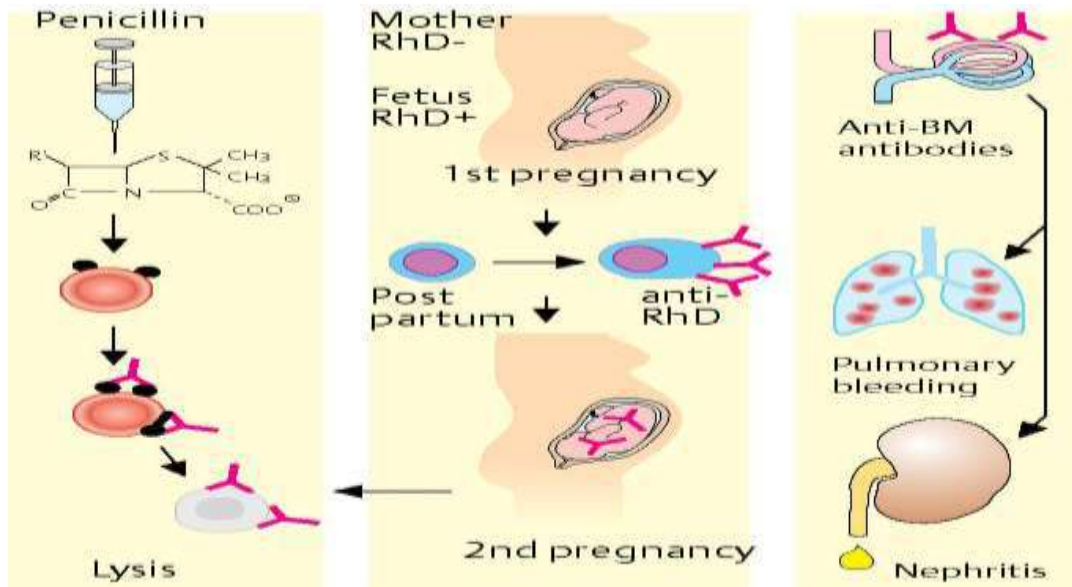


- Some antigens (allergens), such as **insect venom, foods, pollen, and dust mite, can induce the formation of IgE antibodies in individuals with a corresponding predisposition.**
- The IgE antibodies bind via Fc receptors to mast cells (sensitization). If the individual is re-exposed to the allergen, cross-linkage of the membrane-bound IgE occurs. This results in the immediate release of mediators (e.g., histamine, kininogen), which induce vasodilation, smooth muscle contraction, mucus secretion, edema, and/or skin blisters.
- Most allergens are small proteins that can easily diffuse through the skin or mucosa.
- They are frequently proteases and are active at very low doses. IL-4 favours the differentiation of TH2 cells.
- The allergen stimulates the induction of CD4⁺T cells.
- These T cells secrete cytokines that cause IgE production by plasma cells.
- The IgE molecule will bind to the Fc receptor on mast cells and basophils which in turn causes vasodilation increased vascular permeability and vascular spasm. this type may

occur as a systemic or local reaction: **systemic reactions:** skin erythema, followed by respiratory difficulty due to bronchial constriction.

local reactions: generally, on the skin or mucosal surface at the site of Ag exposure. Allergy to penicillin, Aspergillus spores, rupture of Echinococcus cyst.

2. Hypersensitivity Type II: Antibody-mediated cytotoxic reaction



- The immunization of individuals to erythrocyte antigens during pregnancy is a typical example of a type II reaction.
- Children who inherit the RhD erythrocyte antigen from their father can induce immunization against the RhD⁺ antigen in their RhD⁻ mother. Sensitization usually occurs at birth when foetal blood cells come into contact with the maternal immune system. In any subsequent pregnancies, maternal anti-RhD antibodies of the IgG type can pass into the placenta and cause severe hemolysis of fetal RhD⁺ erythrocytes.
- Other examples: Drugs (e.g., penicillin) can passively bind to erythrocytes. Antibodies directed against penicillin then lead to lysis of the erythrocytes.
- The formation of antibodies directed against the basement membrane (BM) of the glomerulus can develop during the course of kidney inflammation. Lung damage accompanied by pulmonary haemorrhage and renal inflammation (glomerulonephritis) may occur due to cross-reaction of these antibodies with the basement membrane of the lung.
- In this type Ab are formed against target Ag that are cell membrane components. Not really hypersensitivity, but cytotoxic reactions:

A) Complement-mediated

- Ab reacts with cell surface Ag leading to fixation of the complement system and then cell lysis. eg. red cells are the most common cells damaged by this mechanism

B) Many cells' types:

- (Macrophages, neutrophils, NK cells) cause lysis of target cell coated by IgG. Poststreptococcal rheumatic fever: molecular mimicry: Antibodies produced against *S. pyogenes* cross-react with various tissue eg. heart, joints – inflammation. *Oncocerca* worm infection may lead to blindness because of the cross-reaction of Ab produced against pathogens and proteins of the retina.

C) Antibody-mediated cellular dysfunction

- In some cases, Ab is directed against cell surface receptor impairing the function but not cause cell injury. In *MYASTHENIA GRAVIS*; Ab reacts with ACh receptors on the motor endplate.

3. Hypersensitivity Type III: Immune complex-mediated reaction

- Antibody-antigen complexes (immune complexes) can form during an immune response.
- Immune complexes can settle in vessel walls, the basement membrane of the lungs and/or kidneys, and in the joints (synovia). They can induce inflammatory processes in these structures by binding complement factors **C3a and C5a (anaphylatoxins)**.
- A particular type III reaction is the **Arthus reaction**: when an antigen has penetrated the skin of an individual who has performed IgG antibodies, the immune complexes can bind to Fc receptors of mast cells inducing degranulation inflammatory cells are recruited and complement is activated, leading to the release of C5a and local inflammation, platelet accumulation, and eventually to blood vessel occlusion (blocking) with necrosis.
- This type is mediated by ag-ab complexes which initiate an inflammatory reaction in the tissue. there are **2 patterns of immune-complex mediated injury**:

A) SYSTEMIC DISEASE (serum sickness, serum sickness type, SLE)

- This is because of a large excess of Ab and immune complexes are deposited at the site of injury especially within the vessel wall, the subsequent events will result in necrotizing vasculitides and accumulation of neutrophils-SLE (**Systemic lupus erythematosus -SLE**)

B) LOCAL DISEASE (Arthus reaction)

- Arthus reaction: intraductal injection of antigens to a personalized person may lead to local intradermal Ab – Ag complex formation and local vasculitis, redness, swelling.
- Example: Repeated (booster) vaccination with diphtheria or tetanus rarely leads to local vasculitis.
- Poststreptococcal acute glomerulonephritis: Ab-ag complexes deposit in glomeruli HBV infection: HBsAg-Ab complexes may HBV also cause acute glomerulonephritis

4. Hypersensitivity Type IV: Delayed-type hypersensitivity reaction

- Haptens are molecules of very small molecular weight (often < 1 k Da).
- They are too small to function as antigens, but they can penetrate the epidermis and bind to certain proteins in the skin (carrier proteins).
- Hapten-carrier complexes are bound by antigen-presenting cells of the skin (Langerhans cells), which then migrate to regional lymph nodes.
- T-cell stimulation then occurs at the lymph node.
- The so-called sensitization phase lasts ca. 10-14 days.
- If the individual is re exposed to the hapten, antigen-specific T cells migrate to the skin, where they accumulate and proliferate. They also cause edema formation and local inflammation with the help of cytokines.
- Compounds containing nickel or chrome and chemicals such as those found in rubber are typical triggers of type IV hypersensitivity reactions.
- This is mediated by T-cells. There are 2 types that involve CD4/8+T Cells.

Acute (within 2-3 days)

- Tuberculin test, contact dermatitis: mediated by CD4+ T helper cells cd4+ cells recognize ag (tuberculin), this leads to the formation of sensitized cd4+ cells.
- Upon cutaneous injection into previously sensitized individual sensitized cd4+cells become activated and secrete cytokines.
- Tuberculin/Mantoux Tuberculin/Mantoux test: intradermal injection of tuberculin = purified tuberculoprotein leads to swelling after 48- 72 h if the patient has been exposed to *Mycobacterium tuberculosis* previously.

Chronic (> 1 week)

- Granuloma formation, graft rejection: mediated by CD8+ cytotoxic T cell.
- Lymphocytes surrounding epitheloid cells lead to the formation of granuloma.

3.1 Introduction to cell culture:

- Cell culture is the technique where cells are allowed to grow under controlled conditions, usually outside of their natural environment.
- Likewise, animal cell culture is a technique in which the cells are removed and are allowed to grow in a favourable artificial environment.

Steps to culture animal cells:

- Harvest cells
- Isolation of the cells with the use of appropriate enzymes.
- In a culture dish with appropriate growth media, the cells are placed.
- The culture dish is now kept in incubator for the culture of cells.
- Cells can be sub-cultured in order to fix the problem or to get the pure culture.
- Now, cells are ready to be manipulated or modified for lab procedures.

Animal Culture media:

In animal tissue culture, 2 types of culture media are used:

1. **Natural media**
2. **Artificial media**

1. Natural media:

These media include the naturally occurring biological fluids and are of the following three types:

- Clots
- Biological fluids
- Tissue extract

i) Clots:

- Plasma clots are the most commonly used clots and has been employed for a long time.
- In the present time, plasma is commercially found in liquid state that can be prepared in the laboratory.

ii) Biological Fluids:

- Several biological fluids can be employed as culture media such as amniotic fluid, pleural and ascetic fluids, haemolymph of insects, aqueous humoral from eye, serum etc.
- Among them, serum is the mostly preferred.

iii) Tissue Extracts:

- The most commonly used tissue extract is chick embryo extract; however, bovine embryo extract is also used.
- In the culture media, the extracts from spleen, liver, bone marrow and leucocytes were also used.
- The substitution for tissue extract can be a mixture of amino acids and certain other organic compounds.

2. Artificial media:

For the following purposes, various artificial media have been employed:

- **Immediate survival** (a balanced salt solution with specified pH and adequate osmotic pressure)
- **Prolonged survival** (a balanced salt solution in addition with serum, or appropriate formulation of organic compounds).
- **Indefinite growth**
- **Specialized functions**
- **Artificial media may be classified into following types:**
- **Serum containing media**
- **Serum-free media**
- **Protein free media**
- **Chemically defined media**

Serum:

- Serum is the yellowish liquid and is a transparent content that remains left over after the removal of fibrin and cells from the blood.
- 2-10% of serum is often contained by normal growth media.
- The most commonly employed supplement in animal cell culture is foetal bovine serum (FBS).
- It supplies the basic nutrients for cells.
- It also contains several hormones and various growth factor.
- In addition to it, it also acts as a buffer.

Serum containing media:

- In animal cell culture media, foetal bovine serum is the most common supplement.
- In order to supply an optimal culture medium, it is employed as an economical supplement.
- Serum supplies carriers' water-insoluble nutrients, protease inhibitors, hormones and growth factors and binds and neutralizes the toxic moieties.

Serum free media:

- In case of immunological studies, presence of serum in media can result to serious misinterpretations.
- In general, these media are specifically designed to promote the culture of a single type of cell and incorporate specified amounts of purified growth factors, lipoproteins and other proteins normally supplied by the serum.
- As the components of these media are known, thus it is referred to as 'defined culture media

Chemically defined media:

- These media contain ultra-pure inorganic and organic ingredients free of contaminants and may also contain pure protein additives, such as growth factors.
- Their constituents are produced by genetic modification in bacteria or yeast with the addition of vitamins, cholesterol, particular amino acids, and fatty acids.

Protein free media:

- Protein-free media is devoid of any protein and only include non-protein constituents.
- Usage of protein-free media enhances superior cell growth and protein expression in contrast to serum-supplemented media and enables downstream purification of any expressed product.

Development of animal culture media:

- Eagle's Minimal Essential Medium (MEM) were broadly accepted, variously supplemented with human, horse or calf serum, protein hydrolysates, and embryo extract.
- In order to choose a medium, the minimum criteria required usually involve:
- The medium should supply the cells with all of the nutrients.
- Keep the physiological pH about 7.0 with ample buffering.
- The medium should be sterile, and isotonic to the cells.
- The balanced salt solution that was initially used to establish a physiological pH and osmolarity needed to sustain cells in vitro was the basis for the cell culture media.
- Various components (glucose, amino acids, vitamins, growth factors, antibiotics etc

have been added to promote cell growth and proliferation, and several media have been created.

Physicochemical properties of culture media

- In order to promote good growth and proliferation of the cultured cells, the culture medium is required to possess certain physicochemical properties (pH, O₂, CO₂, buffering, osmolarity, viscosity, temperature etc.).

pH:

- Most cells can develop in the 7.0-7.4 pH range, but there are minor differences depending on the cell type.
- Phenol red is usually used as an indicator.
- It becomes orange at pH 7.0, yellow at pH 6.5, lemon yellow below pH 6.5, pinker at pH 7.6, and purple at pH 7.8. It is red at pH 7.4.

CO₂, bicarbonate and buffering:

- The medium's carbon dioxide is in a dissolved state, the concentration of which depends on the atmospheric CO₂ tension and temperature.
- As seen below, CO₂ in the medium occurs as carbonic acid (H₂CO₃), and as bicarbonate (HCO₃⁻) and H⁺ ions.



- The CO₂, HCO₃⁻ and pH concentrations are interrelated, as is obvious from the above equation.
- The pH would be decreased by increasing atmospheric CO₂, rendering the medium acidic.
- Bicarbonate ions are neutralized by the addition of sodium bicarbonate (as a part of bicarbonate buffer).



- In summary, cultures in open vessels need to be incubated in the CO₂ atmosphere, the concentration of which is in equilibrium with that of sodium bicarbonate in the medium.

Oxygen:

- A large majority of in vivo cells rely on the availability of O₂ for aerobic respiration, which is made possible by haemoglobin's continuous supply of O₂ to the tissues.
- The cultured cells mainly depend on the dissolved O₂ in the medium that may be

toxic at high concentration due to the production of free radicals.

- The introduction into the medium of free radical scavengers such as glutathione, 2-mercaptoethanol (β -mercaptoethanol) or dithiothreitol erases the toxicity of O_2 .
- To decrease O_2 toxicity, the addition of selenium to the medium is often recommended.

Temperature:

- The optimum temperature for a provided cell culture is reliant on the body temperature of the organism, acting as the source of the cells.
- Therefore, the optimum temperature for cells obtained from humans and warm-blooded animals is $37^\circ C$.
- Higher temperatures cannot be handled by in vitro cells and most of them die if the temperature goes beyond $40^\circ C$.
- In addition to directly affecting cell growth, temperature also influences the solubility of CO_2 , i.e., higher temperatures increase solubility.

Osmolality:

- Once an osmolality is appointed for a culture medium, it should be sustained at that level (with an allowance of ± 10 mOsm/kg).
- Osmolality is typically determined by the depression of the medium's freezing point or vapor pressure elevation.
- Osmolality is influenced if acids, bases, medications, etc are applied to the medium.

Viscosity:

- A culture medium's viscosity is largely affected by the serum content and will have little impact on cell growth in most cases.
- Increasing the viscosity of the medium with carboxymethylcellulose (CMC) or polyvinylpyrrolidone (PVP) will minimize any cell damage that occurs under these conditions.

Surface Tension and Foaming:

- In suspension cultures in stirrer vessels or bioreactors, foaming can occur when 5 percent of CO_2 in air is bubbled via serum containing medium.
- The addition of 0.01 percent to 0.1 percent silicone antifoam (Dow Chemical) or Pluronic F68 (Sigma) helps avoid foaming in this situation by minimizing surface tension and can also protect cells from bubble shear stress.

3.1.2 Animal cell culture

- Animal cell culture is a type of biotechnological technique where animal cells are artificially grown in a favourable environment.
- The cells used in animal cell culture are usually obtained from multicellular eukaryotes and their established cell lines.
- Animal cell culture is a common and widely used technique for the isolation of cells and their culture under artificial conditions.
- In vitro culture of isolated cells from different animals has helped in the discovery of different functions and mechanisms of operations of different cells.
- Animal cell cultures can be performed from different complexities of cells as complex structures like organs can also be used to initiate organ culture in vitro.
- Depending on the purpose and application of the technique, cells, tissues, or organs can be used for the culture process.

Types of Animal cell culture

Animal cell cultures can be divided into two distinct groups depending on the number of cell divisions occurring during the process;

1. Primary cell culture: Primary cell culture is the first culture obtained directly from animal tissue via mechanical and chemical disintegration or enzymatic methods.

The cells of the primary cell culture are slow-growing cells that carry all the characteristics of the original tissue or cells.

✚ Since these cultures are obtained directly from the origin, they have the same number of chromosomes as the original cells.

✚ Primary cell cultures are performed in order to preserve and maintain the growth of cells on an artificial growth medium at a particular condition.

✚ Primary cell cultures can be subcultured to obtain other cultures that either continue to grow indefinitely or die after a few subcultures.

✚ The subsequent subculture of primary cell culture results in the introduction of

mutations into the cells, which might result in cell lines.

- ✚ The morphology of cells in the primary cell cultures might be different and varied, with the most commonly observed morphological structures being epithelium type, epithelioid type, fibroblast type, and connective tissue type.

- ✚ Primary cell cultures are difficult to obtain and usually have a shorter lifespan. Besides, these are prone to contamination by bacteria and viruses.

- ✚ The increase in cell numbers in the primary cell culture can result in exhaustion of the substrate and nutrients, which affects cellular activity.

- ✚ Usually, primary cell cultures need to be sub cultured in order to maintain continuous cell growth once they reach the confluence stage.

- ✚ Primary cell cultures can be further divided into two groups depending on the kind of cells present in the culture;

a. Anchorage-dependent/Adherent cells:

- ✚ The cells in the culture require a stable biologically inert surface for adherence and growth.

- ✚ The surface should be solid and nontoxic as these cells are difficult to grow as cell suspensions.

- ✚ These cells are usually obtained from the tissues of organs where the cells remain immobilized within the connective tissue.

- ✚ Examples of adherent cells include kidney cells and mouse fibroblast STO cells.

b. Anchorage-independent/ Suspension cells

- ✚ These cells can grow efficiently as cell suspensions and do not require a solid surface for attachment.

- ✚ These can be grown on liquid media continuously to obtain fresh subcultures.

- ✚ The ability of the cells to grow as suspension depends on the source of cells

as cells that remain as suspensions in the body are effective suspension cells.

- ✚ Examples of suspension cells include blood cells that are vascular and remain suspended in the plasma.

2. Secondary cell culture

- ✚ Secondary cell cultures are obtained after the primary cell cultures are subsequently sub cultured over a period of time in fresh culture media.

- ✚ The cells of the secondary cell cultures are long-lasting as these have a higher lifespan due to the availability of appropriate nutrients at regular intervals of time.

- ✚ Secondary cell cultures are favoured over primary cell cultures as these are more readily available and are easy to grow and preserve.

- ✚ These are formed from the enzymatic treatment of the adherent cells followed by washing and resuspension of cells in particular volumes of fresh media.

- ✚ Secondary cell cultures are prepared when the number of cells in the primary culture exceeds the capacity of the medium to support growth.

- ✚ Secondary cultures help to maintain an optimal cell density necessary for continued growth.

- ✚ The cells of the secondary cell culture might not resemble that on the parental tissue as mutations, and genetic alterations might be introduced during the subculture process.

- ✚ The cells can be transformed as, in some cases, the continuous subculture can lead to immortal cells.

- ✚ The risk of contamination by bacteria and viruses is less as the cells transform and become less susceptible to infections.

- ✚ An important disadvantage associated with secondary cell culture is that the cells might develop the tendency to differentiate over a long period of time and result in aberrant cells.

Cell Lines

✚ A cell line is a group of cells that are formed from the subculture of primary culture consisting of a pure culture of cells.

✚ Cell lines usually display functional features that are close to the primary cells, but the genotype and phenotype of the cells can be modified. A cell line consists of several cell lineages with similar or different phenotypes.

✚ Cell lines can be further divided into two groups based on the growth patterns of the cells;

a. Finite cell lines

✚ Finite cell lines are cell lines where the cells in the culture divide for a limited number of times, after which they eventually die.

✚ The cells in the finite cell lines can divide from 20 to 100 times before they eventually die and cannot divide anymore.

✚ The number of cell division and lifespan depends on a number of factors like cell lineage differences, species, culture conditions, and media.

✚ The cells of the finite cell lines grow as adherent cells on solid surfaces.

b. Continuous cell lines

✚ Continuous cell lines are cells that exhibit indefinite growth via subsequent subcultures.

✚ The cells in the continuous cell lines grow faster to form an independent culture. The cells are immortal and can divide indefinitely.

✚ The cells in the continuous cell lines can be transformed via genetic alterations and are also tumorigenic.

✚ The transformed cells are formed from the normal primary cell cultures after treatment with chemical carcinogens or by infection with oncogenic viruses.

✚ The cells are capable of growing to prepare higher cell density and can grow as suspensions on liquid media.

✚ These cells can even grow on top of each other to form multilayered structures on the culture vessels.

Examples of common Cell Lines

Some of the common examples of cell lines;

a. HeLa cell line

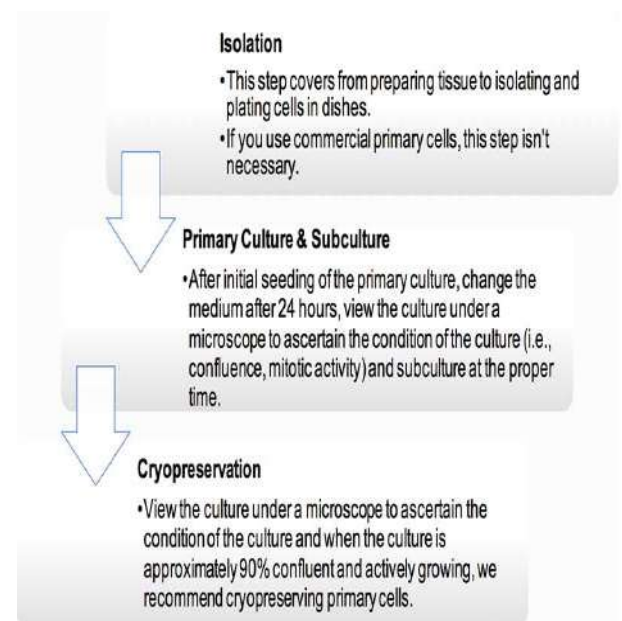
✚ HeLa cells are one of the first continuous culture human cell lines with the help of cells of the cervical carcinoma.

✚ These cells are used for processes like virus cultivation and preclinical drug evaluation.

b. HL 60 (Leukaemia)

c. MCF-7 (breast cancer cells)

Procedure or Protocol of Animal cell culture



1. Growth Conditions

✚ Animal cell culture requires the use of specific culture media that are more complex and specific than the basic culture media used for microbial growth.

✚ Some of the important basic components of the media are inorganic salts, nitrogen source, energy source, vitamins, fat and fat-soluble vitamins, growth factors, and hormones. Sometimes, pH buffering systems and antibiotics are also added.

✚ The temperature for the growth depends on the source of the cell as different organisms require different temperatures for cell growth and division.

✚ Warm-blooded animal cells can be cultured at 37°C as the optimal

temperature, whereas cold-blooded animals grow between 15°C-25°C.

2. Primary cell culture

Primary cell cultures are obtained from fresh tissues that are removed from the organs with the help of an aseptic razor.

In some cases, the cells are removed by the use of chemical disintegrators or proteolytic enzymes.

The cell suspension obtained is washed with buffering liquid in order to remove the proteolytic enzymes.

The cell suspension is poured onto a flat surface which can be a culture vessel or a sterile Petri plate.

The cells that can adhere to the base of the vessel are overlaid with an appropriate culture medium and incubated at room temperature.

3. Cell thawing

In the case of subsequent subcultures, the preserved cell culture might have to be used.

The water bath is heated to a temperature of 37°C, and the growth media where the cells are to be plated is warmed.

The warm medium is added to the culture vessel. The vial with the frozen cells is then placed in the water bath until thawed.

After thawing, the vial is washed with 70% alcohol on the outside. The cell suspension is pipetted into the cell culture vessel and swirled gently to mix everything.

The medium is then incubated overnight under the usual growth conditions. The growth medium is replaced the next day.

4. Trypsinizing Cells

Trypsinization is the method of separating adherent cells from the surface of the culture vessel with the help of proteolytic enzymes. It is done when the cells are to be used for passaging, counting, or other purposes.

The medium is removed, and the cells are recovered. The cells are then washed with phosphate buffer.

Warm trypsin-EDTA is added to the vessel so as to cover the monolayer. The vessel can be rocked to ensure that the monolayer is coated.

The vessel is incubated in a CO₂ incubator at 37°C for 1-3 minutes.

The vessel is removed from the incubator, and the flask is firmly tapped on the side with the palm of the hand to assist detachment.

Once the cells are dislodged, they are resuspended in an appropriate growth medium containing some amount of serum.

The cells are then separated with the help of syringe needles by disrupting the cell clumps and used accordingly.

Applications of Animal cell culture

The following are some of the applications of animal cell culture;

a. Production of vaccines

Animal cell culture is an important technique used for the development of viral vaccine production.

The technique has been used for the development of a recombinant vaccine against hepatitis B and poliovirus.

Immortalized cell lines are used for the large-scale or industrial production of viral vaccines.

b. Recombinant proteins

Animal cell cultures can also be used for the production of recombinant therapeutic proteins like cytokines, hematopoietic growth factors, growth factors, hormones, blood products, and enzymes.

Some of the common animal cell lines used for the production of these proteins are baby hamster kidney and CHO cells.

c. Gene Therapy

The development of animal cell culture is critical for the advances in gene therapy.

🚦 Cells with faulty genes can be replaced by a functional gene in order to remove such defects and diseases.

d. Model systems

🚦 Cells obtained from cell culture can be studied as a model system for studies related to cell biology, host-pathogen interactions, effects of drugs, and effects due to changes in the cell composition.

e. Cancer Research

🚦 Animal cell culture can be used to study the differences in cancer cells and normal cells as cancer cells can also be cultured.

🚦 The differences allow more detailed studies on the potential causes and effects of different carcinogenic substances.

🚦 Normal cells can be culture to form cancer cells by the use of certain chemicals, viruses, and radiation.

🚦 Cancer cells can also be used as test systems for studies related to the efficiencies of drugs and techniques used in cancer treatment.

f. Production of Biopesticides

🚦 Animal cell lines like Sf21 and Sf9 can be used for the production of biopesticides due to their faster growth rate and higher cell density.

🚦 Organisms like baculovirus can be produced through animal cell culture as well.

Advantages of Animal cell culture

🚦 Animal cell culture enables studies related to cell metabolism and understand the biochemistry of cells.

🚦 It also allows observation of the effects of various compounds like proteins and drugs on different cell types.

🚦 The results from animal cell cultures are consistent if a single cell type is used.

🚦 The technique also enables the identification of different cell types on the basis of the presence of markers like molecules or by karyotyping.

🚦 The use of animal cell culture for testing and other processes prevents the use of animals in experiments.

🚦 Animal cell culture can be used for the production of large quantities of proteins and antibodies, which would otherwise require a large investment.

3.1.3 CRYOGENIC PRESERVATION OF CELL CULTURES

Introduction

Maintaining healthy, growing cell cultures using cryogenic preservation to stop biological time for cell cultures, effectively putting them into true suspended animation

Advantages of cryofreezing Cell Cultures

Frozen cultures provide an important backup supply for replenishing occasional losses due to contamination or accidents and provide the assurance of a homogeneous culture supply. Cellular changes or alterations occur in all actively growing populations. These changes often result in the loss of important characteristics during evolution of the cultures there- by introducing unwanted variables into long-term experiments. Cryogenically preserved cultures apparently do not undergo any detectable changes once they are stored below -130°C

Therefore, the biological effects of in vitro cellular aging and evolution may be minimized by frequently returning to frozen stock cultures, allowing ongoing long-term culture experiments to be successfully completed without these unwanted variables. Frozen cultures also provide a valuable baseline against which future experimentally- induced changes may be compared or measured.

General Events During cryopreservation of cell ell Freezing:

To understand how freezing work, it is necessary to examine both the intracellular and extracellular events occurring in animal cell cultures during the freezing process.

- Initial cooling from room temperature to 0°C slows cellular metabolism, rapidly

disrupting active transport and ionic pumping. This does not result in cellular damage if the culture medium is osmotically balanced.

- As cooling continues (0° to -20°C) ice crystals begin to form in the extracellular environment which increases the solute concentration of the culture medium. As a result, water begins to move out of the cells and into the partially frozen extracellular medium, beginning the process of **cellular dehydration and shrinkage**.
- When the cooling process is rapid, intracellular ice crystals form before complete cellular dehydration has occurred. These ice crystals disrupt cellular organelles and membranes and lead to cell death during the recovery (thawing) process.
- When the cooling process is slow, free intracellular water is osmotically pulled from the cells resulting in complete cellular dehydration and shrinkage. This can also cause cellular death.
- The physical stresses of cellular shrinking may cause some damage resulting in irreparable membrane loss and cytoskeletal and organelle disruption.
- When the cooling rate is slow enough to prevent intracellular ice formation, but fast enough to avoid serious dehydration effects, cells may be able to survive the freezing and thawing process.
- Final storage temperature is also critical for successful cryopreservation. To completely stop biological time, storage temperatures must be maintained below 130°C, the glass transition point below which liquid water does not exist and diffusion is insignificant.
- Storage in liquid nitrogen at -196°C effectively prevents all thermally driven chemical reactions. Only photo-physical effects caused by background

ionizing radiation still operate at this temperature.

Practical Aspects of Cell cryopreservation Freezing

The following suggestions and recommendations are designed to augment the protocols referred to earlier.

I. Cell Selection

Select cultures near the end of log phase growth (approximately 90% confluent) and change their medium 24 hours prior to harvesting. Carefully examine the culture for signs of microbial contamination.

II. Cell Harvesting

Start with the standard harvesting procedure generally recommended for the culture and be as gentle as possible. Remove all dissociating agents by washing or inactivation (especially important when using serum-free medium).

III. Cryoprotection

Cryoprotective agents are necessary to minimize or prevent the damage associated with slow freezing, primarily by altering the physical conditions of both the ice and solutions immediately surrounding (external to) the cells.

IV. Storage Vessels

After the cryoprotective solution is mixed with the cell suspension, the resulting inoculum is added in small aliquots (usually 1 to 2 milliliters) to each storage vessel.

V. Labeling and Recordkeeping

Providing for long-term location and identification of frozen cultures is the most frequently overlooked area of cryogenic storage. Labels must contain enough information to locate the appropriate records; usually the culture's identity, date frozen, and initials of the person responsible are sufficient.

VI. Cooling Rate

The cooling rate used to freeze cultures must be just slow enough to allow the cells time to dehydrate, but fast enough to prevent excessive dehydration damage. A

cooling rate of -1°C to -3°C per minute is satisfactory for most animal cell cultures. The best way to control cooling rates is using electronic programmable freezing units.

VII. Cryogenic Storage

Only freezers capable of continually maintaining temperature below -130°C should be considered for long-term cryogenic storage. Although most liquid nitrogen-cooled freezers and some specially designed mechanical freezers meet this requirement, most cell culture laboratories prefer liquid nitrogen freezers

VIII. Thawing

Always use appropriate safety equipment when removing vials and ampules from liquid or vapor phase nitrogen freezers. A full-face shield, heavy gloves and lab coat are strongly recommended for protection against exploding vials or ampules.

IX. Recovery

Since some cryoprotective agents may damage cells upon prolonged exposure, remove the agents as quickly and gently as possible.

X. Problem Solving Suggestions

Viability problems associated with cryo- genic storage are usually noticed soon after cultures are thawed and plated. There are four major areas where problems occur:

1. During harvesting and processing of the cells. Problems may be caused by excessive exposure of the cells to dissociating agents; using a cryoprotective agent that is toxic; or allowing high density cell suspensions to remain too long at room temperature or at a pH that is too basic.

2. During the cooling (freezing) process. Excessive cell damage and reduced culture viability often result from using a cooling rate that is too fast or too slow, or when the cooling process is temporarily interrupted.

3. During cryogenic storage. Culture viability is often reduced when vials are allowed to warm up during transfer to the freezer, or if the repository temper ature is

not consistently maintained at appropriate cryogenic temperatures.

4. During thawing and recovery. Problems arise when the thawing process is too slow or the cryoprotectants are improperly removed.

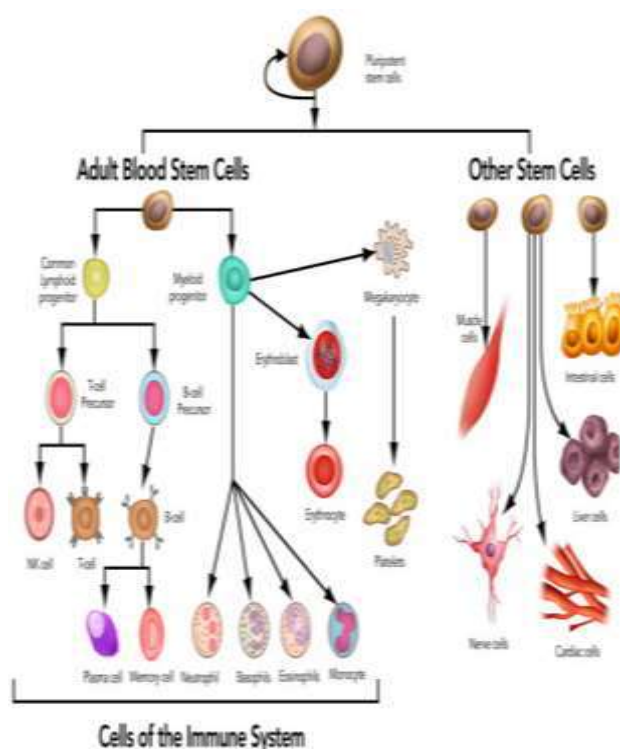
XI. Managing a Cell Repository

The effort and expenses of managing a repository should be kept in line with the value of the cultures stored within it.

3.3 Stem Cells Definition

Stem cells are unique cells present in the body that have the potential to differentiate into various cell types or divide indefinitely to produce other stem cells.

- ✚ Stem cells are essential cells that replace damaged cells or cells lost due to diseases.
- ✚ These cells are the earliest cells of the cell lineage in all tissues and are found in both embryonic and adult organisms.
- ✚ These cells provide a continuous supply of new cells that make up the tissues and organs of animals and plants. The stem cells in the embryonic organism are present in the inner cell mass of



the blastocyte, which then differentiates into all other cells in the body.

- ✚ The stem cells in adults, however, are localized to specific areas within the body like in the bone marrow and the gonads.

Properties of Stem Cells

All the stem cells found throughout all living systems have three important properties. These properties can be visualized in vitro by a process called clonogenic assays, where a single cell is assessed for its ability to differentiate.

The following are some properties of stem cells:

- ✚ Stem cells, of all origins, are capable of dividing and renewing themselves for long periods of time. These cells undergo a period of cell proliferation while preserving the undifferentiated state.
- ✚ All stem cells are unspecialized or undifferentiated. These are present as a mass of cells that differentiate later during their period of division.
- ✚ Another essential property of stem cells is their ability to differentiate into specialized cells that together make up different tissue types. These cells can be either pluripotent or multipotent.

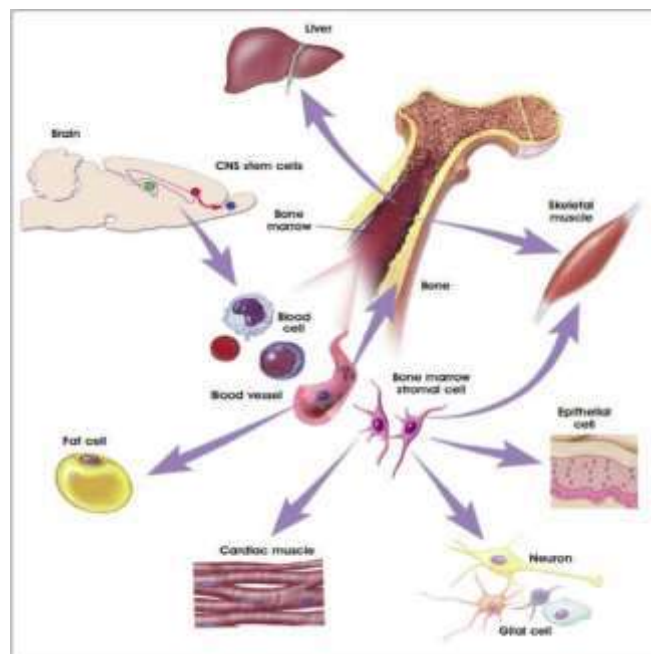
Types/ Sources of Stem Cells

Depending on the source of the stem cells or where they are present, stem cells are divided into various types;

1. Embryonic stem cells
2. Adult stem cells (Somatic or Tissue-specific stem cell)
3. Induced pluripotent stem cells (iPSCs)
4. Perinatal stem cells
5. Mesenchymal stem cells (MSCs)

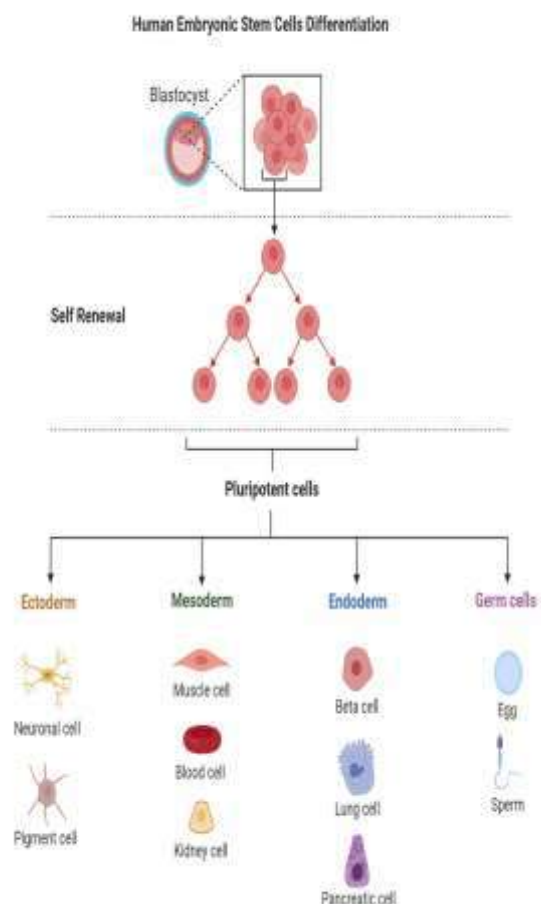
1. Embryonic stem cells

Embryonic stem cells are a group of cells that are present in the inner cell mass of the



embryo at a very early stage of development, called a blastocyst.

The blastocyst stage in embryonic development is reached within 4-5 days after



fertilization, and the number of cells at that point is about 50-150.

These cells are pluripotent, meaning they can develop and differentiate into various cell types (approx. 250 types) during their proliferation. These do not, however, contribute to the extraembryonic cells like the placenta.

Embryonic stem cells are present within the embryo, which divides and differentiates into germ layers as they become specialized.

These cells have been culture increasingly as they can be artificially cultured to produce cells of different types.

2. Adult stem cells (Somatic or Tissue-specific stem cell)

Adult stem cells, also called somatic stem cells, are the cells found in specific tissues that function to repair and form cells of only the tissues they are found on.

These cells are considered less potent than embryonic stem cells as they cannot differentiate to different cell types.

These cells are found in certain tissues that undergo continuous cellular turn over. Some tissue like the liver tissue, however, undergoes minimal division only when the tissue is damaged.

Adult stem cells are found in both children and adults and mostly localized in tissue like the epidermis, bone marrow, and lining of the intestine.

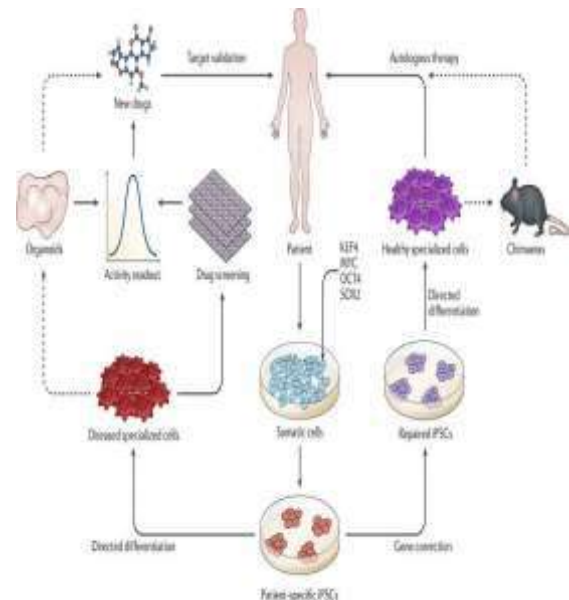
The cells in the epidermis layer divide continuously to form new cells as the keratinocytes are shed off.

Adult stem cells present in the bone marrow are the hematopoietic cells that differentiate to form three different types of blood cells and immune cells.

Stem cells are also found in the brain that differentiates to form very few nerve cells after birth.

3. Induced pluripotent stem cells (iPSCs)

- ✚ The limitations in adult stem cells led to the creation of novel pluripotent cells termed induced pluripotent cells from the adult cells by the process of reprogramming the genes.
- ✚ Induced pluripotent stem cells are formed when the adult cells are cultured with embryonic stem cells where a fusion of these two cells forms new cells with stem cell-like properties.
- ✚ Induced stem cells of the heart and the eyes can be used in the transplantation of



the cells during severe heart and eye-related diseases.

4. Perinatal stem cells

Perinatal stem cells are a type of intermediate cells carrying the characteristics of both embryonic stem cells and adult stem cells. They are derived from extra-embryonic cells of the fetal membrane, umbilical cord, and amniotic fluid.

Prenatal stem cells are known to possess immune-privileged characteristics, as well as broad multipotent plasticity.

Also, these cells, simply isolated from extraembryonic tissues that are typically discarded after birth, effectively avoid ethical issue involvement.

5. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells are a type of adult stem cell or somatic stem cell mostly found in the tissues of muscles, liver, and bone marrow.

Human MSCs (hMSCs) are the multipotent stem cells with the capacity to differentiate into mesodermal cell lines such as osteocytes, adipocytes, and chondrocytes as well ectodermal (neurocytes) and endodermal cell lines (hepatocytes).

Stem Cell Culture

There is increasing interest in optimizing stem cell culture, not only because cell culture is widely used in basic research for studying stem cell biology, but also owing to the potential therapeutic applications of cultured stem cells.

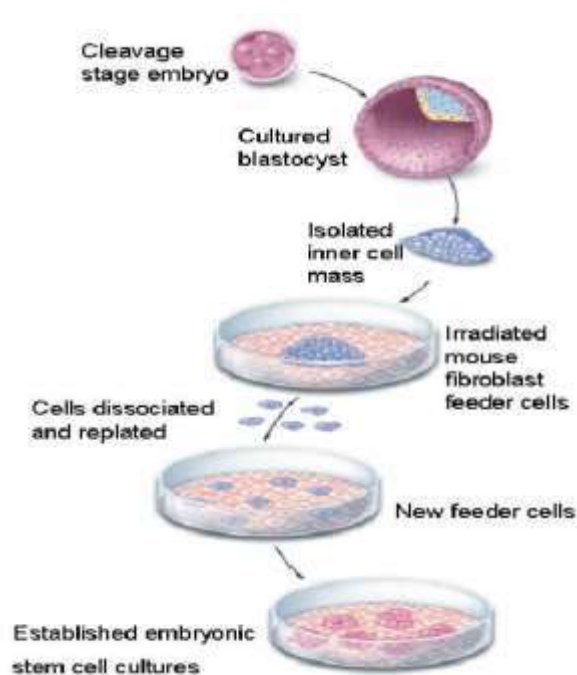
Because of their ability to replace damaged cells, these cells are cultured under artificial conditions to produce more of these cells.

Embryonic stem cells are more potent than adult stem cells as they are capable of differentiating into various cell types.

Adult stem cells, however, are less potent and more restricted as they can only differentiate into specific lineages.

Because of this reason, embryonic stem cells are mostly cultured to obtain more embryonic stem cells.

Stem cell culture conditions must be refined according to the stem cell type, e.g. cell culture requirements for embryonic stem cells (ES) or different kinds of adult stem cells may not be the same.



Applications of Stem cell

- ✚ Stem cell research has been used in the field of regenerative medicine, which deals with the restoration of tissues or organs in the patient suffering from severe injuries or some chronic disease.
- ✚ The progress made in the field of stem cell research has laid the foundation for other cell-based therapies of disease that cannot be cured with conventional medicines.
- ✚ Studies related to the human stem cell research has enormous potential for contributing to our understanding of fundamental human biology.
- ✚ Many years of research on stem cells has made it possible to transplant hematopoietic stem cells to the patients after the cancer treatments.
- ✚ Stem cell research has also been used for the testing of new drugs before they can be tested in animals or humans.
- ✚ Cultured stem cells are used for the transplant of cells in the case of various diseases like bone marrow for leukaemia, nerve cells for Parkinson's and Alzheimer's disease, heart muscle for heart disease, and pancreatic islets for diabetes.

Stem Cell Therapy

- Stem cell therapy, also known as regenerative medicine, is one of the applications of stem cells that promote the repair of dysfunctional and injured tissues and their derivatives.
- Pluripotent stem cells are not often used therapeutically in humans because some of these cells might result in the undesirable formation of unusual solid tumors, called teratomas.
- It has been used to treat spinal injuries and visual impairment.
- Multipotent stem cells, on the other hand, harvested from bone marrow have been used since the 1960s to treat leukaemia, myeloma, and lymphoma.
- The use of mesenchymal stem cells in the ability to form whole joints might be beneficial for other diseases as well.
- It is also better to use multipotent stem cells in the place of pluripotent cells as the multipotent stem cells would prevent the rejection of transplants by the body's immune system.
- Stem cell therapy thus is an excellent avenue for the improvement in the treatment facilities and methods of various chronic diseases.

3.4 Monoclonal Antibodies: Technology and Commercial Production

Hybridoma Technology:

- Two brilliant immunologists, German born **G.F. Koehler** and **C. Milstein** of Argentina developed a new technology, namely, hybridoma technology in 1975, which later (in year 1984) earned Nobel Prize for them in medicine.
- Koehler and Milstein demonstrated that somatic hybridization technique could be used to generate continuous **“hybridoma cell line (hybridomas)” or the “hybrid cell line”** secreting specific antibodies called **monoclonal antibodies (Mabs)**.
- Hybridoma cell is the fusion product of the B-lymphocyte and myeloma B-lymphocyte, it enjoyed the properties of both the partners; the property

inherited from normal B-lymphocyte to produce specific antibody, and the property inherited from myeloma (cancerous) B-lymphocyte to multiply uncontrollably and grow indefinitely in culture.

- Therefore, the hybridoma cells are capable of growing indefinitely in culture and of secreting specific antibody molecules continuously. All the antibodies secreted in culture by a particular hybridoma cell line remain identical.
- Since these antibodies are resulted in by a clone of cells developed from a single hybridoma in culture, **they are said to be monoclonal antibodies (Mabs)**.

Making a Hybridoma:

- Following main steps are involved in making a hybridoma and enabling it to successfully grow in culture medium to produce monoclonal antibodies:

(i) Immunization:

An animal, e.g., a mouse, is first immunized with the appropriate antigen. The animal is injected on several occasions and with such immunization, there is increased stimulation of B-lymphocytes which respond to the antigen.

(ii) Killing of Animal and Separation of Lymphocytes:

Three days after the final dose of antigen has been given intravenously to immunize the animal, the latter is killed. The spleen of the killed animal is aseptically removed and gently disrupted to release the spleen fluid containing lymphocytes and red blood cells. The lymphocytes are separated from the spleen fluid (and red blood cells) by density gradient centrifugation, and are washed.

(iii) Selection of Myeloma Cell Line:

Important point during the course of monoclonal antibody production is that just no myeloma lymphocyte be allowed to fuse with normal lymphocyte.

The myeloma cell line used must itself not be capable of synthesizing antibody otherwise hybridoma cell line will produce a mixture of antibodies.

(iv) Fusion of Separated Lymphocytes with Myeloma Lymphocytes:

After washing, the lymphocytes are mixed with myeloma lymphocytes of HPRT-negative cell line. The mixture of the two cell lines is

now exposed to polyethylene glycol, a fusion promoting agent.

(v) Selection of Hybridoma Cells:

Since the mixture of normal lymphocytes and myeloma lymphocytes contains hybridoma cells, un-fused normal lymphocytes, and un-fused myeloma lymphocytes, the hybridoma cells have to be selected from the mixture for further requirements. This is done by using HAT-medium for growth. HAT-medium represents a mixture of hypoxanthine, aminopterin and thymidine.

(vi) Isolation of a Monoclonal antibody Producing Hybridoma Cell:

If all the hybridoma cells that have been selected using HAT-medium are grown together, then a polyclonal antibody mixture would be obtained. Consequently, a single antibody (monoclonal antibody) producing hybridoma cells need to be isolated and grown individually.

This is done by diluting a suspension of hybridoma cells to such an extent that individual aliquots contain, on an average, only one cell. Such cells are transferred to separate fresh media for growth.

Each mass of hybridoma cells (clone) produced from a single parent hybridoma cell is now examined to determine whether it produces the desired monoclonal antibody.

Commercial Production of Monoclonal Antibodies:

Once the correct hybridoma has been selected and isolated, it can be stored frozen (cryopreserved) and cultured whenever required. Since the hybridoma is a transformed cell line, it grows readily in culture.

1. Microencapsulation Technology:

Microencapsulation technology is an interesting method of large-scale production of monoclonal antibodies and has been patented by the Damon Corporation. In this technology, the hybridoma cells/hybridomas are grown inside hollow microspheres which are surrounded by a porous membrane.

Protected inside the microsphere, the cells multiply and grow to higher cell densities than is possible with any other culture system.

This technology results in the production of 100 mg to one g of antibody per litre of culture medium and makes large scale

production of monoclonal antibodies (MABs) commercially feasible.

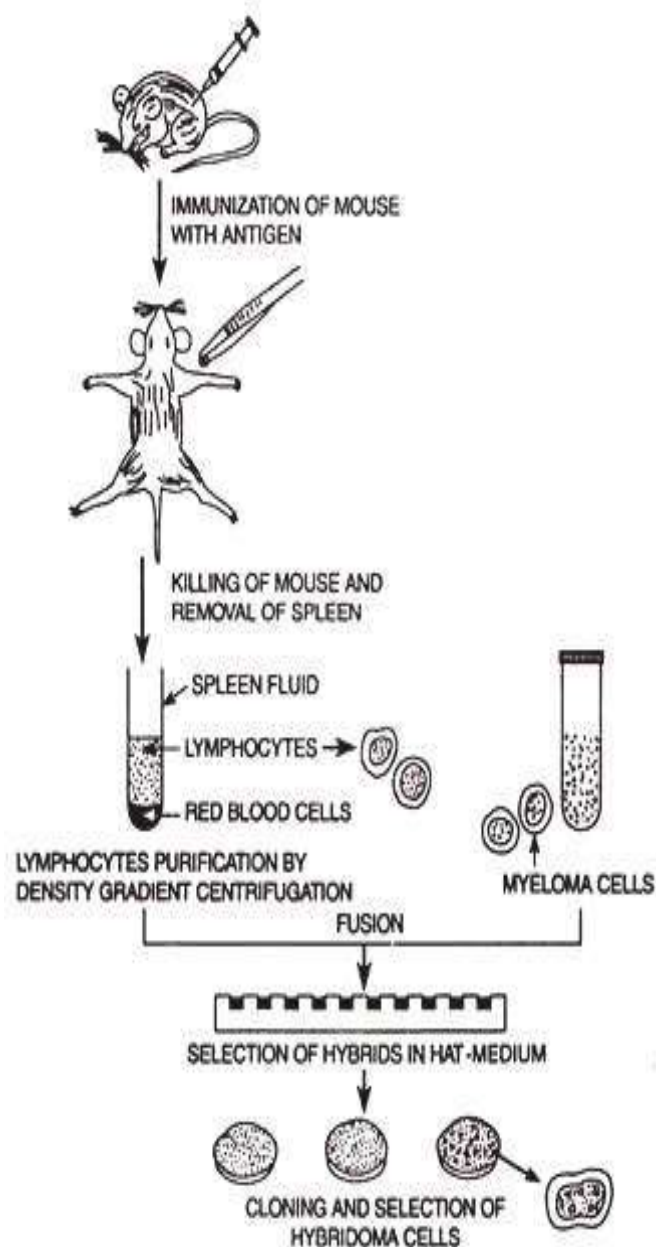


FIG. 41.10. Steps involved in the production of a hybridoma.

2. Ascites-route Technology:

Ascites-route technology provides an alternative for monoclonal antibody production commerciality. In this technique, the hybridoma is grown as ascites, i.e., as a suspension of tumour cells within the peritoneal cavity of an isogenic species.

3. Mass in Vitro Culture:

Large-scale culture of hybridoma cells has been done in stirred bioreactors, airlift fermenters and in vessels based on immobilized cells.

The medium used for in vitro culture of hybridomas must serve the following two purposes:

- (1) Support adequate cell proliferation and
- (2) Give high antibody yields.

Human Mabs

Human lymphocytes are usually collected from blood of volunteers who have been immunized with some antigen, e.g., tetanus toxoid.

The antibody producing lymphocytes are then immortalized in one of the following three ways:

(i) The B-lymphocytes are transfected with DNA from a tumour; this transforms the lymphocytes making them capable of indefinite proliferation.

(ii) Fusion of the B-lymphocytes with suitable lymphoid tumour cells to produce hybridoma clones. The tumour cell line must be antibody nonproducing (Ig-).

(iii) Transformation of B-lymphocytes following their infection with Epstein Barr virus to lymphoblastoid cell lines.

These lymphoblastoid cell lines can either themselves be used for Mab production or may be used to produce hybridoma clones (they are used in place of the B-lymphocytes).

At present, the second and third approaches of producing human-human hybridoma clones are being most commonly used.

Several human Mabs are available commercially, e.g., against lung carcinoma, breast carcinoma, tetanus toxoid (human-mouse hybridoma), against glioma, measles virus (human-human hybridoma), against RhD, hepatitis B surface antigen (Epstein-Barr virus transformed B-lymphocytes), and against tetanus toxoid (hybridoma involving fusion of Epstein-Barr induced transformed lymphocytes in place of normal B-lymphocytes).

It has been generally reported that Mabs exhibit lower affinities when compared with the effective affinities of their polyclonal counterparts. But Mabs recognizing peptide antigens with very high affinities have been produced, in practice it may be necessary to screen several different Mabs against the same antigen in order to identify a Mab suitable for the given purpose.

4.1.1 Concept of Genetic Engineering

By definition, genetic engineering is the direct alteration of an organism's genome which is achieved through manipulation of the DNA. This is achieved by using "recombinant DNA technology" which involves different techniques to insert, alter, or cut out pieces of DNA that contain one or more genes of interest.

This is also known as genetic modification, gene transfer or transgenesis. Doing this is possible because DNA is like a universal language; all DNA for all organisms is made up of the same nucleotide building blocks. Thus, it is possible for genes from one organism to be read by another organism. Main focus of genetic engineering is:

Gene isolation and Gene modification so that they can be transferred into and function within a new organism of a different species (trans genetics) or the same species (cis genetics),

Gene removal, and evaluating the success of resultant gene combinations. In practice, since DNA contains the genes to build certain proteins, by changing the DNA sequence, attempts are on to design a new gene for a cell/organism resulting in a different protein and also making a cell capable of performing the desired functions. The resultant organism is broadly referred as genetically modified organism (GMO).

The final aim of genetic engineering in higher eukaryotes results in two broad classes of GMOs which are:

Genetically Modified Plants / Animals are designed for expression of the cloned genes for basic research on gene expression or for the production of useful proteins in tissue culture.

Transgenic Plants / Animals are designed as a result of alteration of the genetic makeup of the organism in which all the cells will carry the genetic modification

4.1.2 Vectors

Vector- Definition, Features, Types, Examples, Applications, Limitations

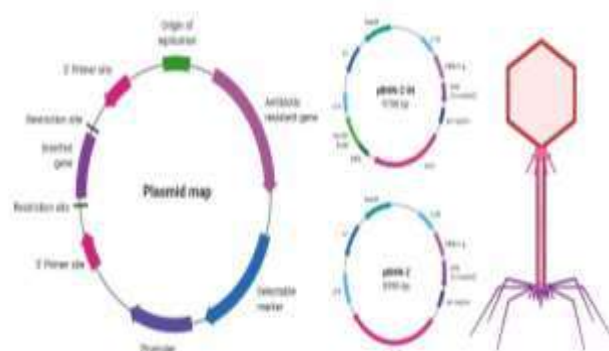
A vector is a substance, usually a piece of DNA that carries a sequence of DNA or other genetic material and introduces it into a new cell.

Vectors act as vehicles to transfer genetic material from one cell to the other for different purposes like multiplying, expressing, or isolation.

Vectors are used as a tool in molecular cloning procedures so as to introduce the desired DNA insert into a host cell.

The DNA insert that is transmitted by a vector is termed **recombinant DNA**, and the process is also known as **recombinant DNA technology**.

Usually, the vectors are DNA sequences that carry different parts involved in different functions. Vectors usually have an insert, also known as a **transgene**, that carries the recombinant DNA and a larger sequence called the backbone of the vector responsible for the structure of the vector.



Characteristics or features of vectors:

- ✚ Vectors should be capable of replicating autonomously, which, in turn, depends on the presence of particular sequences in the vector that enables them to initiate replication and propagation within the host cell.
- ✚ Some vectors might even have sequences that allow the production of proteins essential for the inserted DNA, regulation of the process, and further transfer of the insert between different vectors.

- ✚ The size of an ideal vector should also be small enough for it to be incorporated into the host genome.
- ✚ The small size of the vector also enables it to incorporate a large-sized insert for transfer.
- ✚ Vectors should be easy to isolate and purify as these need to be recovered and reused for multiple processes.
- ✚ In the case of gene transfer processes, it is important that the vector is capable of integrating itself or the recombinant DNA into the genome of the host cell.

Types of vectors

Vectors can be classified into different groups depending on the purpose of the process and the type of particles used in the process. The following are the commonly studied group of vectors that are used for different purposes;

1. Cloning vectors

Cloning vectors are vectors that are capable of replicating autonomously and thus are used for the replication of the recombinant DNA within the host cell.

Cloning vectors are of further different types that are defined by different features unique to each type of vector.

DNA Cloning Vectors

a. Plasmid vector

Plasmids are small extrachromosomal circular DNA molecules capable of replicating autonomously within the host cell.

These are also termed as the workhorse cloning vector in recombinant DNA technology.

Plasmids are widely used as vectors in all three domains of life; however, these are frequently used in bacteria and yeasts.

Some of the most widely used plasmids are pBR322, pUC, and vectors that use *E. coli* as the host.

b. Cosmid

Cosmid vectors are hybrid vectors composed of plasmid and phage λ vectors, capable of incorporating up to 42 kb of DNA.

Cosmid vectors are prepared by the insertion of the cos region of the phage vector into the plasmid vectors.

Cosmid vectors prepared and used in practice are cosmid pHC79 which is a cos-containing derivative of the vector pBR322.

c. Bacteriophage vector

Bacteriophage vectors are viruses that only infect bacteria and transform them efficiently while carrying large inserts.

Bacteriophages or phages have higher transformation efficiencies which increase the chances of recovering a clone containing the recombinant DNA segments.

Some of the common phages used as vectors include M13 phages, λ phages, and P1 phages.

d. Bacterial artificial chromosome

Bacterial artificial chromosomes are engineered DNA molecules that are used to clone DNA segments in bacteria cells (usually *E. coli*).

These consist of a bacteria-derived F-factor replication origin which enables the propagation of large DNA fragments in a supercoiled circular form.

However, BACs have been associated with the random insertion of DNA fragments into the host genome resulting in unpredicted expression.

e. Yeast artificial chromosome

Yeast artificial chromosomes are engineered DNA molecules that are used to clone DNA inserts within the yeast cells, particularly *Saccharomyces cerevisiae*.

One of the most commonly used examples of yeast artificial chromosomes includes pYAC4, which has been extensively used as a cloning vector.

f. Human artificial chromosome

Human artificial chromosomes are extrachromosomal DNA fragments that act as a new chromosome within the human cell.

The use of human artificial chromosomes has increased with advances in genetic engineering as it helps overcome problems commonly associated with traditional vector systems.

HACs can exist as single copy episomes without integration into the host

chromosomes allowing long-term stable maintenance.

2. Viral vectors

Viral vectors are one of the most effective means of gene transfer to modify host cells or tissues and manipulate them to express different types of genes.

The concept of using viruses as vectors arose from the fact that viruses are very effective in transducing their own genetic information into the host cell.

During viral transduction, the non-essential viral genes are replaced with foreign DNA sequences of therapeutic interest in order to produce recombinant viral vectors.

3. Expression vector

Expression vectors are vectors that enable the expression of cloned genes in order to determine the successful cloning process.

Usually, cloning vectors do not allow the expression of a cloned gene which is why the use of expression vectors is required.

The use of expression vectors facilitates the processing of introns in prokaryotes as these are designed with restriction sites next to the regulatory region.

Expression vectors can be plasmid-based or viral-based that are introduced into the host cells in order to code for particular mRNAs.

4. Shuttle vector

Shuttle vectors are that carry origins of replication from two different hosts, which enables them to 'shuttle' between the two hosts.

These vectors contain DNA plasmids that can usually replicate in both mammalian cells as well as bacterial cells.

Shuttle vectors function as hybrid vectors containing DNA sequences from bacterial plasmids and mammalian viruses.

The vectors contain three functional DNA sequences involved in the cloning process; a viral replication origin, a bacterial replication origin, and a drug resistance gene.

5. Secretion vector

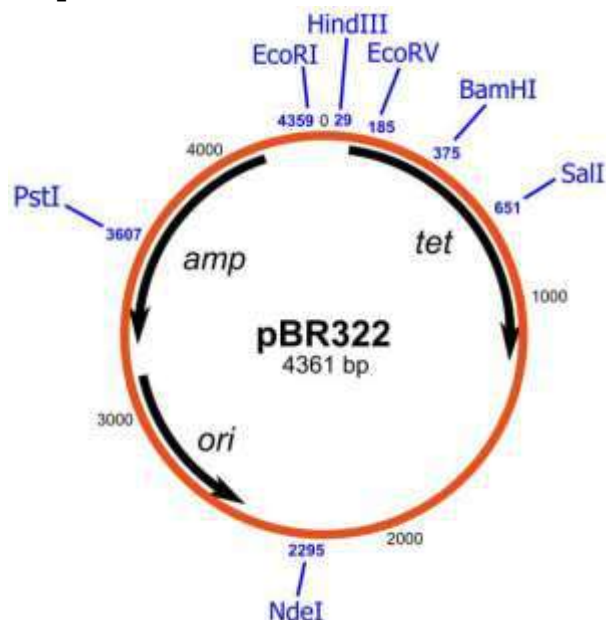
Secretion vectors are a type of specialized expression vector that expresses

the cloned genes in order to produce proteins at locations other than the cytoplasm.

The transport of protein product from the cell is achieved by the fusion of the inset DNA with a nucleotide sequence encoding the peptide of an easily secreted protein.

Some of the examples of vectors that are commonly used for different genetic engineering processes;

1. pBR322



The vector consists of an origin of replication from a ColE1-like plasmid, pMB1, an ApR gene (Ampicillin resistance gene) from the transposon, Tn3, and a TcR gene from pSC101.

pBR322 was designed to overcome the limitations with pBR312 and pBR313, both of which have extraneous DNA sequences and restriction enzyme cleavage sites that affected their function as vectors.

The structure of pBR322 was designed to maximize the number of restriction enzyme cleavage sites on the vector and to minimize its size.

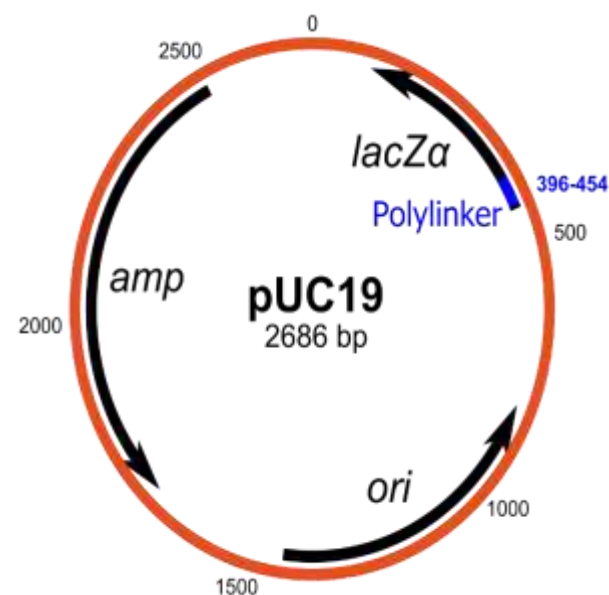
The vector contains twenty-one unique restriction enzyme cleavage sites, eleven of which are present in the TcR and ApR genes.

The structure also facilitates a unique EcoRI cleavage site within the plasmid in order to increase the efficiency of the vector.

The pBR322 family of vectors was initially created for general cloning purposes

in *E. coli* and other similar prokaryotes; however, over the years, derivatives of the vector have been designed for cloning purposes that specific to a particular organism or a particular function.

2. pUC19



pUC19 is also an example of a plasmid cloning vector that is used for the transfer of recombinant DNA fragments into a host cell.

The name 'pUC19' is given as the vector where the 'p' indicates plasmid and 'UC' indicates the University of California' where the vector was designed and constructed.

The vector has been extensively used for cloning purposes where the host cells containing the plasmid are distinguished from the ones that do not have it by the differences in the colour of the colonies on the growth medium.

The vector is a double-stranded DNA molecule with 2686 bp and a high copy number.

pUC19 consists of a 54 base-pair cloning site polylinker that further contains 13 different hexanucleotide-specific restriction endonucleases.

The colony screening after cloning with pUC19 is due to the presence of a selective marker that encodes for the N-terminal fragment of β -galactosidase.

3. λ phage

λ -phage is an example of a bacteriophage that infects the bacterial species, *Escherichia coli* (*E. coli*).

This vector is more effective than other plasmid vectors as it has a higher efficiency in entering bacterial cells so as to incorporate the recombinant DNA within the host genome.

It is a double-stranded DNA bacteriophage that contains an ori sequence requires for replication and a number of DNA sequences encoding regulatory and replicative proteins.

Applications of Vectors

- Cloning vectors are the most important group of vectors that are used for the transfer of foreign DNA into host cells for different purposes.
- One of the most important applications of vectors is to generate engineered organisms for a particular function, like engineering *E. coli* bacteria for insulin production.
- Vectors can be used to isolate a particular gene sequence within a genome and to determine its nucleotide sequence through DNA sequencing.
- It also helps determine control sequences and regulatory sequences in genomes for their study and analysis.
- Cloning vectors can be used for studying the structure, function, and production of protein in different organisms.
- Phage therapy is a form of therapy that uses bacteriophage vectors to treat different bacterial infections in humans and other animals.
- Vectors can also be used to identify mutations in different regions of DNA sequences as well as to diagnose gene defects related to certain diseases.
- Recombinant DNA technology has been used in clinical microbiology in different approaches like recombinant antigens, recombinant vaccines, and diagnostic probes.
- Recombinant antigens prepared by cloning techniques by using cloning vectors have

been used for the screening of diseases like HIV, HCV, and CMV.

- Vectors are one of the components in molecular biology which enable numerous studies related to cell structure, nucleic acid composition, and genetic engineering techniques.

The following are some of the limitations of vectors;

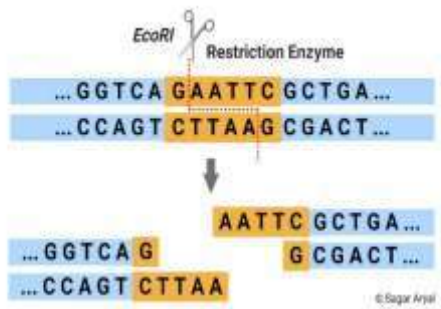
- Vectors are not very stable due to changes in metabolic energy and changing pH and temperature in different hosts. The stability of vectors depends largely on the type of vector and host genotypes.
- Over expression of a particular type of genes in the host cell is a common problem associated with the use of vectors.
- The use of a single type of vector might not be sufficient for a particular purpose. The use of multiple vectors is complex and results in difficulties during the process.
- Even though a large number of studies are done in the field of molecular biology for the production of more efficient vectors, it is a time-consuming and expensive process.

4.1.3 Restriction Enzyme (Restriction Endonuclease) Definition

Restriction enzyme, also called restriction endonuclease, is a protein produced by bacteria that cleaves DNA at specific sites along the molecule.

Restriction endonucleases cut the DNA double helix in very precise ways. It cleaves DNA into fragments at or near specific recognition sites within the molecule known as restriction sites.

They have the capacity to recognize specific base sequences on DNA and then to cut each strand at a given place. Hence, they are also called as **molecular scissors**.



Source of Restriction Enzymes

The natural source of restriction endonucleases are **bacterial cells**.

These enzymes are called restriction enzymes because they restrict infection of bacteria by certain viruses (i.e., bacteriophages), by degrading the viral DNA without affecting the bacterial DNA. Thus, their function in the bacterial cell is to destroy foreign DNA that might enter the cell.

The restriction enzyme recognizes the foreign DNA and cuts it at several sites along the molecule.

Each bacterium has its own unique restriction enzymes and each enzyme recognizes only one type of sequence.

Recognition Sites

The DNA sequences recognized by restriction enzymes are called **palindromes**. Palindromes are the base sequences that read the same on the two strands but in opposite directions.

For example, if the sequence on one strand is GAATTC read in 5'→3' direction, the sequence on the opposite strand is CTTAAG read in the 3'→5' direction, but when both strands are read in the 5'→3' direction the sequence is the same. The palindrome appears accordingly —

5' GAATTC 3'

3' CTTAAG 5'

In addition, there is a point of symmetry within the palindrome. In the example, this point is in the centre between the AT/AT.

The value of restriction enzymes is that they make cuts in the DNA molecule around this point of symmetry.

Some enzymes cut straight across the molecule at the symmetrical axis producing blunt ends.

Of more value, however, are the restriction enzymes that cut between the same two bases away from the point of symmetry on two strands, thus, producing a staggering break.

Mechanism of Cleavage of Restriction Enzymes

When a restriction endonuclease recognizes a particular sequence, it snips through the DNA molecule by catalysing the hydrolysis (splitting of a chemical bond by addition of a water molecule) of the bond between adjacent nucleotides. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

Types of Restriction Enzymes

Traditionally, four types of restriction enzymes are recognized, designated I, II, III, and IV, which differ primarily in structure, cleavage site, specificity, and cofactors.

Type I enzymes cleave at sites remote from a recognition site; require both ATP and S-adenosyl-L-methionine to function; multifunctional protein with both restriction and methylase activities.

Type II enzymes cleave within or at short specific distances from a recognition site; most require magnesium; single function (restriction) enzymes independent of methylase.

Type III enzymes cleave at sites a short distance from a recognition site; require ATP (but do not hydrolyze it); S-adenosyl-L-methionine stimulates the reaction but is not required; it exists as part of a complex with a modification methylase.

Type IV enzymes target modified DNA, e.g., methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA.

Nomenclature of Restriction Enzymes

Since their discovery in the 1970s, many restriction enzymes have been identified while Type II restriction enzymes have been characterized.

Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain.

For example, the name of the EcoRI restriction enzyme was derived as:

E – Escherichia: Genus

co- coli: specific species

R- RY13: strain

I- First identified: order of identification in the bacterium

Applications of Restriction Enzymes

Restriction enzymes can be isolated from bacterial cells and used in the laboratory to manipulate fragments of DNA, such as those that contain genes; for this reason, they are indispensable tools of recombinant DNA technology (genetic engineering).

The most useful aspect of restriction enzymes is that each enzyme recognizes the same unique base sequence regardless of the source of the DNA. It means that these enzymes establish fixed landmarks along an otherwise very regular DNA molecule. This allows dividing a long DNA molecule into fragments that can be separated from each

other by size with the technique of gel electrophoresis.

Each fragment, thus generated, are also available for further analysis, including the sequencing.

One value of cutting DNA molecule up into discrete fragments is being able to locate a particular gene on the fragment where it resides which is done by the general technique of Southern blotting.

One of the most popular restriction enzymes is called EcoRI from E. coli (bacterium).

Hundreds of other restriction enzymes with different sequence specificities have been isolated from several bacteria and are commercially available.

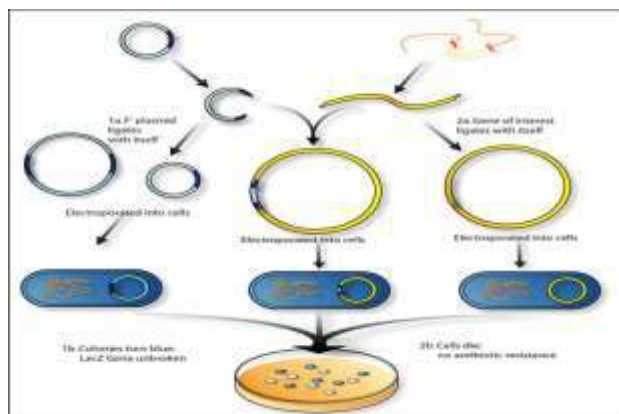
Enzyme	Obtained from	Recognition Sequence
EcoRI	Escherichia coli	5'GAATTC 3'CTTAAG
EcoRII	Escherichia coli	5'CCWGG 3'GGWCC
BamHI	Bacillus amyloliquefaciens	5'GGATCC 3'CCTAGG
HindIII	Haemophilus influenzae	5'AAGCTT 3'TTCGAA

4.1.4 Recombinant DNA technology

Recombinant DNA technology refers to the joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry.

Recombinant DNA (rDNA), on the other hand is the general name for a piece of DNA that has been created by the combination of at least two strands.

Recombinant DNA in a living organism was first achieved in 1973 by **Herbert Boyer**, of the University of California at San Francisco, and Stanley Cohen, at Stanford University, who used *E. coli* restriction enzymes to insert foreign DNA into plasmids.



Steps of Genetic Recombination Technology

1. Isolation of Genetic Material

The first step in rDNA technology is to isolate the desired DNA in its pure form i.e., free from other macromolecules.

Since DNA exists within the cell membrane along with other macromolecules such as RNA, polysaccharides, proteins, and lipids, it must be separated and purified which involves enzymes such as lysozymes, cellulase, chitinase, ribonuclease, proteases etc.

Addition of ethanol causes the DNA to precipitate out as fine threads. This is then spooled out to give purified DNA.

2. Restriction Enzyme Digestion

Restriction enzymes act as molecular scissors that cut DNA at specific locations. These reactions are called '**restriction enzyme digestions**'.

The technique 'Agarose Gel Electrophoresis' reveals the progress of the restriction enzyme digestion.

This technique involves running out the DNA on an agarose gel. On the application of current, the negatively charged DNA travels to the positive electrode and is separated out

based on size. This allows separating and cutting out the digested DNA fragments.

3. Amplification Using PCR

Polymerase Chain Reaction or PCR is a method of making multiple copies of a DNA sequence using the enzyme – DNA polymerase in vitro.

It helps to amplify a single copy or a few copies of DNA into thousands to millions of copies.

PCR reactions are run on 'thermal cyclers' using the following components:

- Template – DNA to be amplified
- Primers – small, chemically synthesized oligonucleotides that are complementary to a region of the DNA.
- Enzyme – DNA polymerase
- Nucleotides – needed to extend the primers by the enzyme.

4. Ligation of DNA Molecules

The purified DNA and the vector of interest are cut with the same restriction enzyme.

This gives us the cut fragment of DNA and the cut vector, that is now open.

The process of joining these two pieces together using the enzyme 'DNA ligase' is 'ligation'.

The resulting DNA molecule is a hybrid of two DNA molecules – the interest molecule and the vector. In the terminology of genetics this intermixing of different DNA strands is called **recombination**. Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the **recombinant DNA technology**.

5. Insertion of Recombinant DNA Into Host

In this step, the recombinant DNA is introduced into a recipient host cell mostly, a bacterial cell. This process is '**Transformation**'.

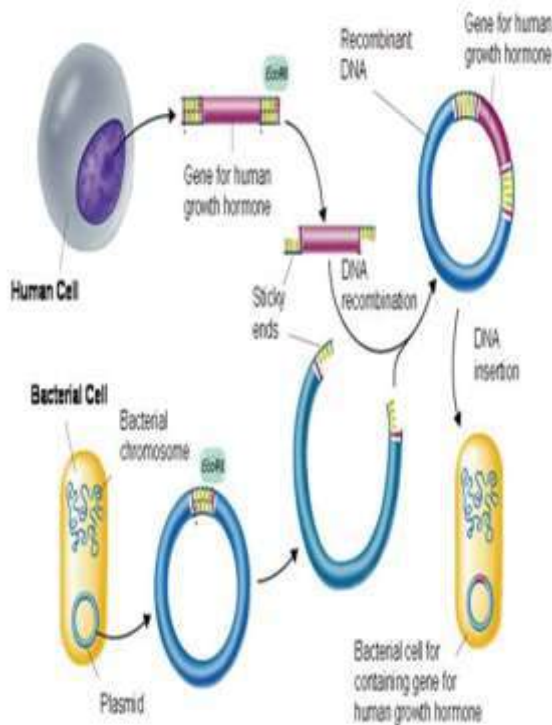
Bacterial cells do not accept foreign DNA easily. Therefore, they are treated to make them 'competent' to accept new DNA. The processes used may be thermal shock, Ca^{++} ion treatment, electroporation etc.

6. Isolation of Recombinant Cells

The transformation process generates a mixed population of transformed and non-transformed host cells.

The selection process involves filtering the transformed host cells only.

For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.



Application of Recombinant DNA technology

- Recombinant DNA is widely used in biotechnology, medicine and research.
- The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences.
- Recombinant DNA is used to identify, map and sequence genes, and to determine their function.
- Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.
- Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering.
- DNA technology is also used to detect the presence of HIV in a person.
- Application of recombinant DNA technology in Agriculture – For example, manufacture of Bt-Cotton to protect the plant against boll worms.

- Application of medicines – Insulin production by DNA recombinant technology is a classic example.
- Gene Therapy – It is used as an attempt to correct the gene defects which give rise to hereditary diseases.
- Clinical diagnosis – ELISA is an example where the application of recombinant DNA is possible.

Limitations of Recombinant DNA technology

- Destruction of native species in the environment the genetically modified species are introduced in.
- Resilient plants can theoretically give rise to resilient weeds which can be difficult to control.
- Cross contamination and migration of proprietary DNA between organisms.
- Recombinant organisms contaminating the natural environment.
- The recombinant organisms are population of clones, vulnerable in exact same ways. A single disease or pest can wipe out the entire population quickly.
- Creation of superbug is hypothesized.
- Ethical concern about humans trying to play God and mess with the nature's way of selection. Many people worry about the safety of modifying food and medicines using recombinant DNA technology.

4.2. Gene Delivery

Strategies for gene delivery can be broadly classified into three groups: **chemical, biological, and physical.**

Biological strategies are also called transduction technologies. Chemical and physical strategies are called transfection technologies. No one way can be applied to all cells and all experiments.

The method should be selected depending on your cell types and experimental needs, and should have high transfection efficiency, low cell toxicity, and minimal effects on normal physiology, and be easy to operate and repeat.

Biological methods:

Among the biological methods, some viruses have been used for gene delivery including adenovirus, adeno-associated virus, herpesvirus, and lentivirus. Non-viral gene delivery methods can be divided into

chemical and physical approaches. The chemical methods include certain polymers and lipids, while the physical methods utilize physical properties and forces to transport genetic material into cells.

Direct Inoculation of DNAs and RNAs

Biological ways of direct inoculation of DNAs and RNAs are based on genetically engineered viruses to transfer non-viral genes into cells via viral deliveries. This method provides an operation to reach hard-to-transfect cell types for protein overexpression or knockdown.

Physical Methods

The physical methods for gene delivery have revolutionized the efficiency of non-viral gene transfer, in some cases reaching the efficiencies of viral vectors.

- **Electroporation**

This technique includes the delivery of a voltage (current) across the surface of cells, which will result in the spontaneous creation of pores in the plasma membranes. While the voltage is removed, the pores will close. And then, the voltage is applied and the pores open up, current will travel through the cell.

- **Hydrodynamic intravascular injection**

The hydrodynamic gene delivery via tail vein injection is an effective technique to deliver nucleic acids to the liver within small animals. This way of gene delivery using a rapid injection of a relatively large volume of DNA solution has opened a new way for gene therapy studies in vivo.

- **Sonoporation**

For sonoporation, it is a non-viral gene delivery system for improving plasmid DNA transfer across the biological cell membranes. It works via transient permeabilization of the cell membrane and by transferring therapeutic DNA effectively across tissue and into cells by application of ultrasound energy.

- **Gene gun**

Microparticles on the order of ~0.5-1µm are used in gene gun, and they are typically made of gold. It can ionize to have a positive charge, and the positive charge will

interact well with DNA or RNA thanks to the negative charge on every phosphate group in the polynucleotide. This method is limited to exposed tissues, such as the epidermis, or cells in a plant leaf.

- **Jet injection**

Jet injection is to employ high pressure to deliver a liquid formulation of DNA. This technology has developed as an applicable alternative to viral or liposomal gene delivery systems. The major limitations to clinical use of this technique are the requirement for large amounts of DNA and the DNA degradation from the high-pressure delivery.

- **Chemical Methods**

Chemical-based methods of gene delivery can utilize natural or artificial compounds to form particles that improve the transfer of target genes into cells. Chemical vectors usually enter cells via endocytosis and can protect genetic material from degradation.

- **Polymers**

- Poly(L-lysine) (PLL)
- Poly(ethylene imine) (PEI)
- Chitosan
- Liposomes
- Dioctadecylamidoglycylspermine (DOGS)
- N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)
- Dioleoyl phosphatidyl ethanolamine (DOPE)

- **Viral Vectors**

Viruses, in theory, will yield up to 100% transduction efficiency in vitro. Practically, however, the range of 80% to 95% is more realistic. Some of the viruses used for gene delivery are adenovirus, adeno-associated virus, and certain retroviruses.

4.3 Transgenic animal

Transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology.

In addition to the gene itself, the DNA usually includes other sequences to enable it to be incorporated into the DNA of the host and to be expressed correctly by the cells of the host. Transgenic sheep and goats have been produced those express foreign proteins in their milk.

4.3.1 Two methods of producing transgenic mice are widely used:

- Transforming embryonic stem cells (ES cells) growing in tissue culture with the desired DNA
- Injecting the desired gene into the pronucleus of a fertilized mouse egg

The Embryonic Stem Cell Method - Method 1

Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, including its gametes.

1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing the gene you desire (e.g., the insulin gene) vector DNA to enable the molecules to be inserted into host DNA molecules promoter and enhancer sequences to enable the gene to be expressed by host cells

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

Prepare a pseudo pregnant mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive. Transfer the embryos into her uterus. Hope that they implant successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10–20% will have it, and they will be heterozygous for the gene.

Method 1

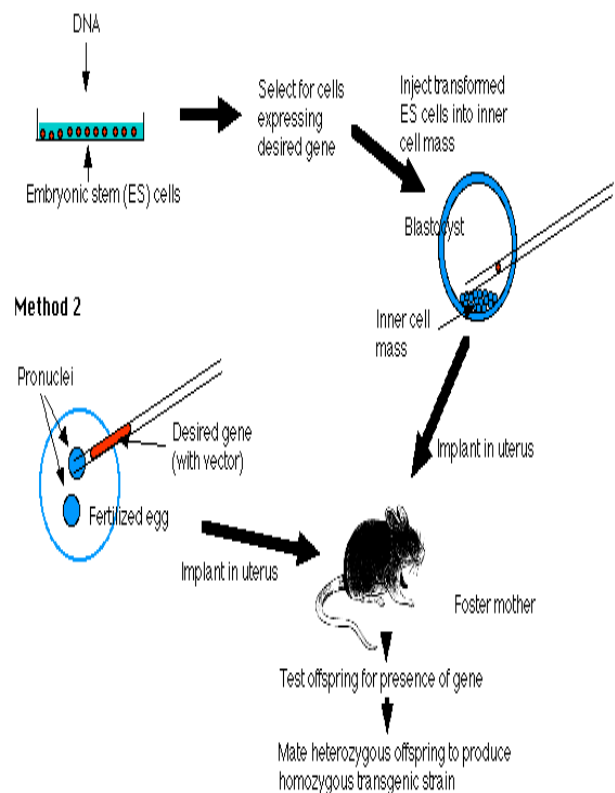


Fig. Methods to produce Transgenic mice

7. Establish a transgenic strain

Mate two heterozygous mice and screen their offspring for the 1 in 4 that will be homozygous for the transgene.

Mating these will find the transgenic strain.

The Pronucleus Method - Method 2

1. Prepare your DNA as in Method 1
2. Transform fertilized eggs
3. Harvest freshly fertilized eggs before the sperm head has become a pronucleus.
4. Inject the male pronucleus with your DNA. When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.
5. Implant the embryos in a pseudo pregnant foster mother and proceed as in Method 1.

4.3.2 Transgenic Sheep

Cloning Dolly:

In February 1996, Ian Wilmut and co-workers from the Roslin Institute and PPL Therapeutics, both in Edinburgh, Scotland, reported in Nature journal that they had successfully cloned a sheep from a cell taken from the udder of a six-year-old ewe.

The cloned lamb was named **Dolly**. In the past, genetically identical animal embryos had been created only with amphibian cells, and those created from adult nuclei had never successfully reached adulthood. Cloning in which the nuclei came from foetal cells or cells from cell lines had been successful before in mammals. The word clone means to create a genetically identical copy.

To clone an animal, it is necessary to begin with an egg, the only cell known to initiate and support development. In order to clone an individual, it is necessary to get an egg without a nucleus and then to transplant in it a nucleus of known origin.

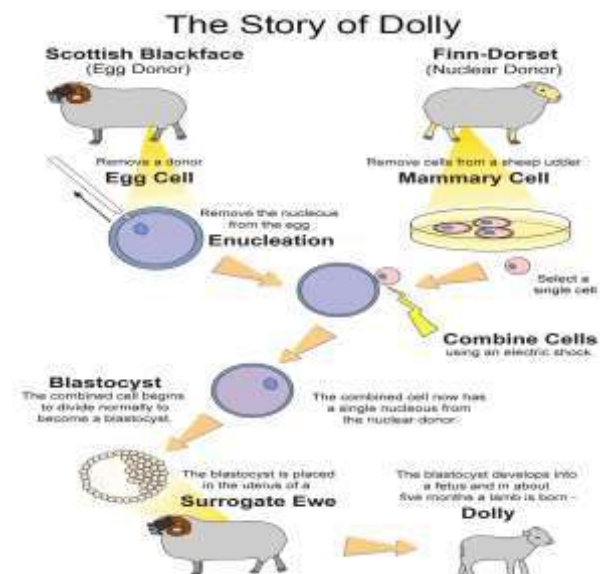
Techniques for nuclear transplantation had been worked out with frogs and toads in the 1950s. The Scottish scientists succeeded in obtaining sheep eggs, enucleating them (removing their nuclei) and then transferring in donor nuclei by fusing the donor cells and the enucleated eggs with an electric pulse.

The electric pulse also initiated development of the egg. Although only one pregnancy of the twenty-nine initiated was successful, the lamb that was born seemed normal in every respect; since it had produced offspring.

Others had tried this type of experiments with many types of animals, including mice. They were not successful for numerous reasons. The most likely explanation for the recent success, according to scientists, is that the donor cells were kept in a non-growth phase for several days, which may have synchronized them with the oocyte.

Thus, the nucleus and the oocyte were at the same stage of cell cycle and thus compatible. Other reorganization that had to

take place in the donor chromosomes are not really known for certain but one thing is clear: the nucleus of an adult cell in the sheep has all of the genetic material needed to support normal growth and development of the egg. The work has since been repeated with goats, cattle and mice.



4.3.3 Transgenic Fish

The genes that have been introduced by microinjection in fish include the following

1. Human or rat gene for growth hormone
2. Chicken gene for delta crystalline protein
3. E.Coli gene for beta galactosidase
4. E. coli gene for neomycin resistance
5. Winter flounder gene for antifreeze protein
6. Rainbow trout gene for growth hormone.

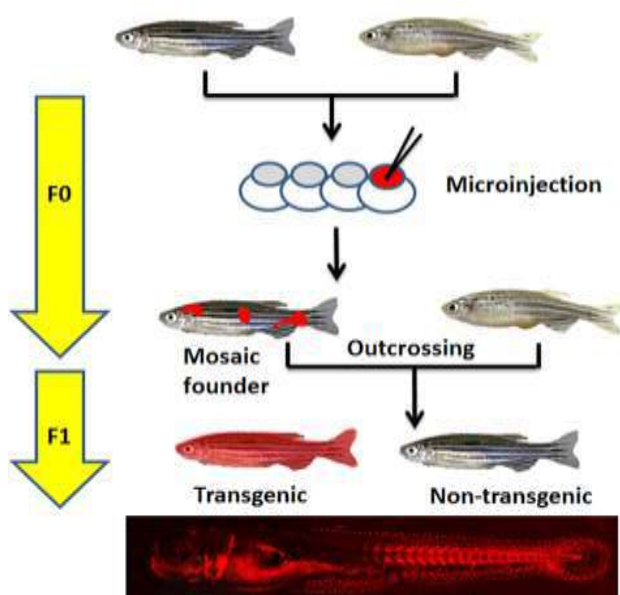
The technique of microinjection has been successfully used to generate transgenic fish in many species such as common carp, catfish, goldfish and salmon in other animals usually direct microinjection of cloned DNA into male pronuclei of fertilized eggs has proved very successful.

Fertilization is initiated by adding water and sperm to eggs, with gentle stirring to facilitate the fertilization process.

Egg shells are hardened in water. About 106 to 108 molecules of linearized DNA in a volume of 20 ml or less are microinjected into each egg within the first few hours after fertilization.

Following microinjection, eggs are incubated in appropriate hatching trays and dead embryos are removed daily.

Since in fish, fertilization is external, in vitro culturing of embryos and their subsequent transfer into foster mothers is not required.



Further, the injection into the cytoplasm is not as harmful as that into the nucleus, so that the survival rate in fish is much higher.

Human growth hormone gene transferred to transgenic fish allowed growth that was twice the size of their corresponding non-transgenic fish.

Similarly, antifreeze protein gene was transferred in several cases and its expression was studied in transgenic salmon.

It was shown that the level of AFP gene expression is still too low to provide protection against freeze. There is also a report of the production of transgenic zebrafish from an Indian laboratory.

In this attempt a plasmid containing rat growth hormone gene was microinjected into fertilized zebrafish eggs and its presence confirmed in adult fish.

4.3.4 Applications of Transgenic Animals

Biological Products: Biological products like some nutritional supplements and medicines are obtained from transgenic animals.

Safety of Vaccines: Some model organisms are used for the testing of the safety of vaccines before they can be injected to human beings.

Study of Diseases: They are used to study the role of genes in the development of diseases.

Other applications are:

- Transgenic animals have wider applications in the field of research, production of pharmaceuticals and therapeutics, development of disease resistant livestock, production of environment friendly animals and improving nutritional quality of the animal products.
- Transgenic mouse models have been developed for human genetic diseases, such as Alzheimer disease, Huntington disease and many others.
- Transgenic cattle can be used for production and secretion of pharmaceuticals and therapeutics in their milk.
- Xeno Mouse is transgenic mouse, produces fully human antibodies. First fully human monoclonal antibody, produced in the Xeno Mouse (Panitumumab), has received regulatory approval for use as a treatment for advanced colorectal cancer.
- Transgenic animals can produce organs for xenotransplantation to human beings. This may be achieved by inserting human complement inhibitor gene or deleting α -Gal epitope by knockdown the gene encoding 1,3- α -galactosyltransferase in the transgenic animals.
- Transgenic animals with improve nutritional quality have been produced. Increase casein quantity, secretion of the enzyme lactase in the milk and knockout of β -lactoglobulin gene are some of the desirable traits in the milk. The gene for enzyme desaturase from *C. elegans* is expressed in transgenic pigs. This gene can convert omega 6 fatty acid into unsaturated omega 3 fatty acid.
- Enviropig is environment friendly pig. It contains a transgene for the enzyme phytase. The enzyme phytase is secreted in the saliva and digest phytate present in the feed. This decreases phosphorus content in the excreta of the transgenic pig

4.4 “DNA sequencing is a process of determining or identifying the order of nucleotides present in a DNA sequence.”

DNA is a nucleic acid and genetic material of us. It is made up of nitrogenous bases, sugar and phosphate. Four nitrogenous bases in DNA are Adenine, Thymine, Cytosine and Guanine. Phosphodiester bonds join adjacent nucleotides while hydrogen bonds join nucleotides of opposite strands.

The whole DNA of a cell is known as a genome having two critical functions to encode proteins and regulate gene expression. These two goals are achieved by non-coding sequences and coding- genes, respectively.

What is DNA sequencing?

“Put simply, it is a process of determining the sequence of nucleotides.”

Sequencing is a combination of both wet and dry lab work. A machine performs a chemical synthesis while a computer program performs sequence analysis. There are many sequencing platforms available, each one uses different chemistry and relies on a different principle for sequencing.

Definitions:

“A laboratory technique employed to determine the sequence of a gene or DNA by a sequential chemical reaction is referred to as DNA sequencing.”

Principle of DNA sequencing:

In general, a broad principle of DNA sequencing is as stated.

- “DNA is denatured and converted into single-stranded. The sequence read by the sequencer machine gives signals to the detector which transfers them to the computer. A computer software inspects the signals and converts each read into a nucleotide sequence.”

Steps and Process in DNA sequencing:

- Sample preparation
- DNA extraction
- DNA fragmentation

- Adapter ligation and Library Preparation
- Library enrichment- amplification
- Library purification
- DNA sequencing
- Data collection and sequence analysis

Sample preparation:

Sample preparation starts with extracting pure and high-yield DNA. DNA of any organism, for example, animal, human, plant, bacteria or virus can be sequenced in the same process.

DNA extraction.

However, DNA extraction techniques vary between different organisms. Ideally, the concentration and purity of DNA for sequencing are ~50 to 500 ng and 1.80 (260/280 ratio), respectively. One should follow the instructions and suggestions provided by the manufacturers.

DNA fragmentation:

For a larger sequence or gene or whole genome, the DNA fragmentation step is so important. Here, using enzymatic catalytic reaction, chemical or physical treatment, the large chunks of DNA, which cannot be sequenced, are fragmented.

Adapter ligation and library preparation:

Sticky ends generated by the fragmentation are ligated with the known adaptor sequence and sometimes with a known barcode sequence too. After completion of ligation, the same-sized fragments are stored in the genomic library.

Adaptors and known oligonucleotide barcodes help in amplification and re-arrangement of sequence after accomplishment of the process.

Library enrichment- amplification:

To sequence the desired fragment, we need millions of copies of that particular fragment or a whole library. PCR amplification generates copies and facilitates library enrichment.

The amplification completes in three steps denaturation at 94°C, annealing at 55°C

to 65°C and extension at 72°C for 35 cycles. The enriched library is purified, later on.

Library purification:

If you check the purity of the PCR product at 260 and 280nm wavelengths, it will always be 1.80, exactly! because the amplicons are pure DNA fragments. If any contaminants are present in the DNA, amplification will not occur.

Unbound primers, primer-dimers, unused Taq DNA polymerase, unused DNA templates, and other unused PCR reaction buffer components can abort DNA sequencing. That is why amplicon purification is required.

Here, alcohol purification can't work. To achieve maximum purification and amplification yield, we need to use a ready-to-use amplicon purification kit. Such kits use spin-column technology which is handy, fast and accurate. The purified library is ready for sequencing.

DNA sequencing:

Put simply, in a sequencing machine, the machine reads each nucleotide of a target sequence, the detector detects signals and sends them to the computer. For example, let's take the example of sanger chain termination.

When a ddNTP is incorporated during amplification, the synthesis of the nucleotide chain is terminated, fluorescence is released, the detector detects the signal and sends it to the computer.

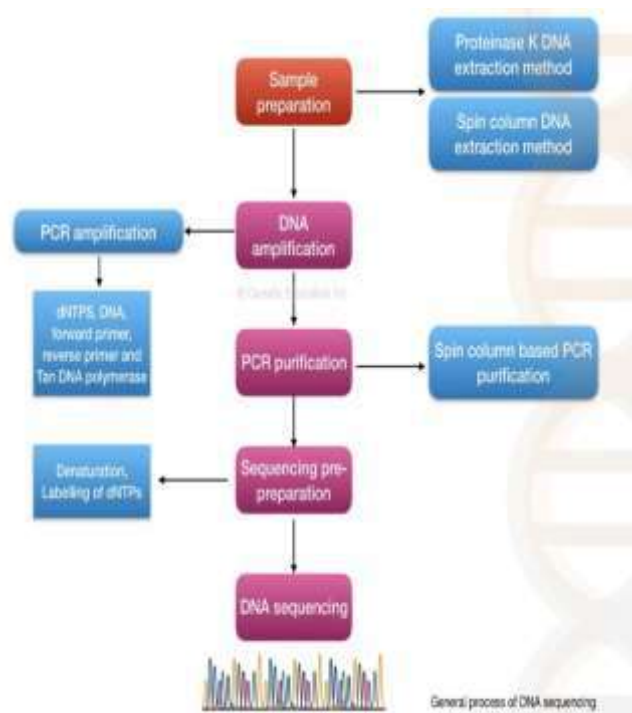
Data collection and sequence analysis:

In the final step, the sequencing file is processed in a computer program. The inbuilt software (provided by the manufacturer) processes the data and compares it with the available sequence information.

Through pairwise alignment, each nucleotide is compared with the reference, and any variations within the sequence are noted, reported and documented. The entire process of DNA sequencing is explained in the figure below.

Different methods of DNA sequencing:

- Chain termination (Sanger sequencing) method
- Semiautomated method
- Automated method
- Pyrosequencing
- Whole-genome shotgun sequencing method
- Clone by the clone sequencing method
- Next-generation sequencing method



Steps in DNA sequencing.

Sanger sequencing:

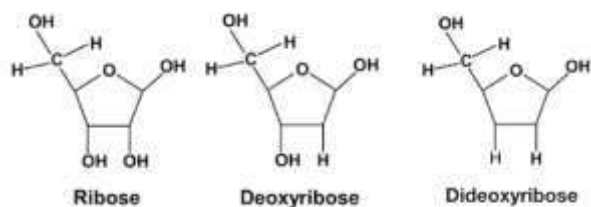
Year: 1977

Scientists: Sanger

Names: Sanger sequencing, chain termination sequencing, first-generation sequencing and dideoxynucleotide sequencing.

Automation: Possible.

Sanger and co-workers developed a chain termination method of DNA sequencing, after a few years of Maxam and Gilbert's method. It is also known as the first-generation DNA sequencing method. The present Technique is often referred to as dideoxynucleotide sequencing as it uses specially synthesized nucleotides- ddNTPs.



Difference between Ribose, Deoxyribose and Dideoxyribose

The image represents the difference between Ribose sugar, deoxyribose sugar, and dideoxyribose sugar.

Principle:

A denatured DNA is synthesized by DNA polymerase by adding dNTPs but when it encounters the ddNTPs, the presence of hydrogen (instead of hydroxyl group) terminates the chain synthesis. Each ddNTPs terminate the chain when encountering a complementary nucleotide every time. The results are analyzed on a PAGE gel which distinguishes each termination by length differences.

Sanger sequencing technique was utilized in the human genome project.

Process:

- The process of Sanger sequencing is broadly divided into 5 steps:
- DNA extraction- using any of the DNA extraction protocols.
- DNA denaturation- using heat or chemical treatment.
- DNA synthesis- using the flanking primers, dNTPs, Taq DNA polymerase and PCR buffer.
- Cycle sequencing- using all dNTPs, ddNTPs, Taq DNA polymerase and PCR buffer.
- Separation of fragments by size- using PAGE, or capillary gel electrophoresis.
- In the very first step, DNA isolation is achieved. DNA extraction is followed by DNA purification and DNA denaturation.
- Any DNA extraction technique can be used here; however, the goal is to isolate pure and good-quality DNA.
- DNA sample with a purity of 1.8 and quantity of 100 ng is selected and

processed further. In the next step, the PCR performs amplification to get copies of the desired fragment.

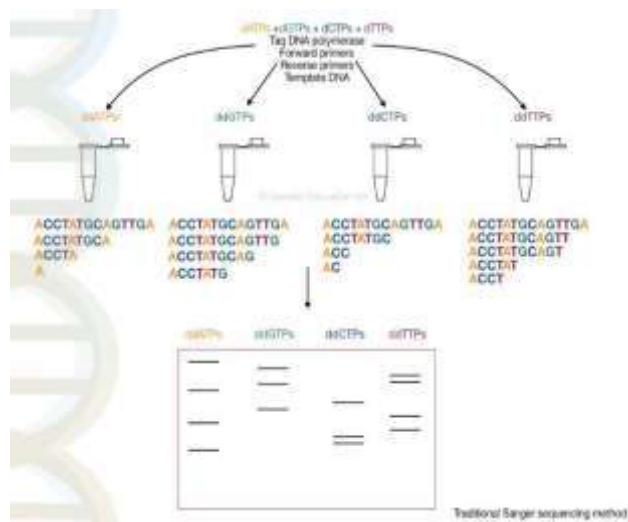
- In the next step, the cycle sequencing is performed by using the denatured PCR products. The reaction preparation is shown here.

- Each tube contains the same amount of PCR reagents but in each tube, extra ddNTPs are added as shown in the table. Here the flanking primers bind near the region of a sequence of our interest.

In the cyclic reaction, the Taq DNA polymerase incorporates nucleotides- dNTP on a denatured DNA. Here, high fidelity DNA polymerase is not needed. Normal Taq polymerase expands the growing DNA strand by the addition of the dNTPs.

Final amplicons from each tube run on a polyacrylamide gel which separates DNA fragments based on their size. Note that smaller fragments run faster than larger ones.

The final results are analyzed in a UV transilluminator, gel doc or X-ray film. We have hypothetically prepared the reaction, run it and analyzed the results. Take a look at it.



Automated Sanger sequencing:

The automated Sanger sequencing is a combination of amplification, capillary gel electrophoresis and detection by various techniques. The conventional Sanger

technique is a tedious process, but recent advancement makes it easy and rapid to use.

The semi-automated Sanger sequencing method is based on the principle of Sanger's method with some minor variations. Automation occurs in two ways, which also depend on the chemistry used by the manufacturer.

Fluorescent labelling of each ddNTPs with four different fluorochromes and run in a single capillary. And labelling of flanking primers with a single dye and running in four separate capillaries. However, for the second option, the detection technique used is an argon ion laser beam.

The samples are run in the automated CE setup and separate. By using the labelled primers or the ddNTPs, the machine reads the sequence accurately, on single-lane capillary electrophoresis. We can sequence more than 300 samples in a single run on an automated DNA sequencing platform.

The capillary electrophoresis is used to separate DNA molecules on the basis of size, It is powerful enough to separate a single base pair fragment. The chromatogram generated through the C.E sent the output as a fluorescent peak. What results it gives are known as electropherograms.

Applications

- Sanger and automated Sanger sequencing is the most anticipated, widely used and trusted technique to sequence DNA. Broadly, some of the important applications are enlisted here.
- Sequence both, prokaryotic and eukaryotic genomes.
- Sequence a gene.
- Microbial identification by 16s rRNA gene sequencing.
- Mutations and mutagenesis studies.
- Clinical and research studies.
- In recent times, robust and automated sanger sequencing helped to map the genome of SARS-CoV-2 COVID19.
- Used in identification, characterization and classification of microbes.



Welcome: **Dr. N. SREENIVAS** (Self Published Author)

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C-CONTENT



POSTHARVEST TECHNOLOGY OF FISH & FISHERIES

PAPER VII A-ZOOLOGY

SREENIVAS-CHAKRAPANI-ANILKUMAR

CONTENTS

Unit	Sub Topic	Topic Details	Page No
1	1. 1	Handling of fresh fish, storage and transport of fresh fish, post mortem changes (rigor mortis and spoilage), spoilage in marine fish and freshwater fish	1-5
1	1.2	Principles of preservation – cleaning, lowering of temperature, rising of temperature, denudation, use of salt, use of fish preservatives, exposure to low radiation of gamma rays.	5-7
2	2.1	Traditional methods - sun drying, salt curing, pickling and smoking.	7-10
2	2.2	Advanced methods – chilling or icing, refrigerated sea water, freezing, canning, irradiation and Accelerated Freeze drying (AFD).	11-16
3	3.1	Fish products – fish minced meat, fish meal, fish oil, fish liquid (ensilage), fish protein concentrate, fish chowder, fish cake, fish sauce, fish salads, fish powder, pet food from trash fish, fish manure.	16-21
3	3.2	Fish by-products – fish glue, Using glass, chitosan, pearl essence, shark fins, fish Leather and fish maws.	21-23
4	4.1	Sanitation in processing plants - Environmental hygiene and Personal hygiene in processing plants.	23-26
4	4.2	Quality Control of fish and fishery products – pre-processing control, control during processing and control after processing.	26-29
5	5.1	Seafood Quality Assurance and Systems: Good Manufacturing Practices (GMPs); Good Laboratory Practices (GLPs); Standard Operating Procedures (SOPs); Concept of Hazard Analysis and Critical Control Points (HACCP) in seafood safety	29-34
5	5.2	National and International standards – ISO 9000: 2000 Series of Quality assurance System, Codex Alimentarius	34-37

UNIT I: HANDLING AND PRINCIPLES OF FISH PRESERVATION

1. 1 Handling of fresh fish, storage and transport of fresh fish, post mortem changes (rigor mortis and spoilage), spoilage in marine fish and freshwater fish.

1.1 WET FISH HANDLING

Fish is a highly perishable food. If not handled properly the fish becomes inedible in a short time. Consumption of spoiled fish is injurious to human health. Spoiled fish contains harmful bacteria and toxic chemicals and these can cause large number of diseases like cholera, typhoid, gastroenteritis etc. to man.

Hence, it is ideal that fish should be preserved from the time it is caught till it is consumed. One of the best ways to arrest spoilage and associated deterioration of quality is to keep the fish at a lower temperature by keeping in ice or under frozen storage. Fish is often graded based on quality.

Ideal way to handle fish:

- 1) Keep the fish clean immediately after capture by washing in good quality water.
- 2) Arrest material contamination externally.
- 3) Chill the fish by keeping in ice.
- 4) Transport as quickly as possible to consumer sites (markets, pre-processing centres, houses etc.).

How to Reduce Spoilage

The fish growing in water is caught and kept in atmospheric temperature of 30°C it will spoil very rapidly.

If the rate of spoilage at this temperature is put at a scale of 100, it can be reduced to half, 50%, by cooling the fish to 25°C. If the fish is cooled to 20°C, the rate of spoilage becomes 25% and at 0°C temperature, rate of spoilage can be lower than 5%. This shows the importance of chilling fish.

Chilling thus enhances the shelf life of the fish. Shelf life means the number of days fish can be kept stored in ice in edible condition.

HANDLING THE FISH ON BOARD

Handling on board refers to handling the fresh fish on the deck of the fishing vessel after capture. Most fishing vessels having an Over All Length (OAL) up to 17 meters have insulated fish holds having capacity varying from 1 to 10 tonnes. They carry ice in fishing holds during fishing. The fish is kept in fish holds under ice until the vessels come to fish landing centres or harbours.

i) Refrigerated Sea Water Plants (RSW)

Large fishing vessels have fish holds containing brine (sea water) kept cooled by refrigeration plants installed on board fishing vessels.

ii) Chilled Sea Water Plants (CSW)

In this method, ice is carried on board fishing vessels in ice holds. Seawater is also pumped into the tanks.

iii) Freezing Plants

Large fishing vessels have freezing plants and frozen storage (-20°C) for storing fish. These types of vessels remain in sea for up to 2 -3 months or more and have huge storage capacity.

bags. Fish oil, a by-product, is often used as a fuel for boilers.

HANDLING FISH AT LANDING CENTRES

Ideal fish landing centre should have following facilities:

- Facilities for fishing vessels to be berthed,
- Fresh quality water,
- Ice Plants,
- Frozen Storage
- Fish Landing Platforms,
- Electricity,
- Connected by road or rail to transport the fish quickly to markets, and
- Should maintain high standards of sanitation and hygiene

TRANSPORT OF FISH

Since fish is a highly perishable item, handling procedures soon after capture determine the quality of the fishery products produced subsequently. Let us now look into the detailed procedures of handling of freshly caught fish.

Marine Fish

Marine fish arriving from distant seas are off-loaded into landing platforms. It should be immediately iced. These fishes are normally sold by auction. They are then transported to distant consumer markets by the following methods

Fresh Water Fish

Fresh water fish handling is totally different from marine fish. They can be caught and transported quickly to market or consumer centre. They can also be transported in live condition in tanks. Initial freshness being the highest priority, they can be maintained in high quality if they are kept immediately in ice or cold medium. The ideal way is to keep in small containers made of plastic or wood or bamboo. Fish is kept cold around 0 to 3°C in a fish to ice ratio of 1:1 and transported in ordinary trucks or insulated trucks to distant places. At arrival time, the temperature of the fish should be around 5°C.

Quality of raw fish or its freshness is very important for its utilization. Freshness of the fish depends on the method of capture and struggle it undergoes either in the net or after capture. Catching too much in one haul causes not only physical damage but also puts the fish under great stress. All these contribute to depletion of glycogen in the muscle, which affects its shelf life by rapid post-mortem changes. There is now a great preference to live fish. Often fish is brought alive and then partly processed by fish vendors. But quality and price are two factors that govern the market.

1. Kept packed in ice in small boxes (fish to ice ratio 1:1) in trucks for short distances.
2. In insulated trucks to processing factories, pre-processing plants and distant markets.

Live Fish Transport

Freshwater fish caught alive, without undergoing much struggle, is selected for live transport. They should be in good condition, healthy without any damage to skin.

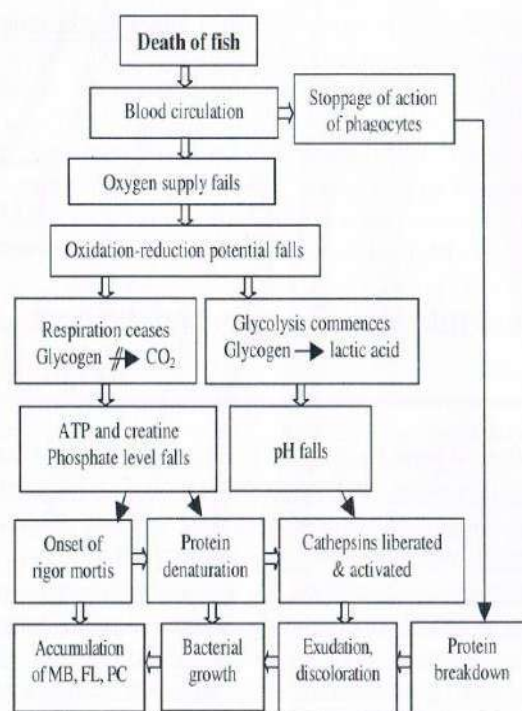
The fish then undergoes a process called **conditioning**. This is accomplished by

putting the fish in good quality oxygenated water. Conditioning reduces stress, inhibits metabolism and at the same time food remains are removed from the alimentary canals and oxygen demand of muscle is reduced. During the conditioning process, the fish is not fed, which further inhibits metabolism and also limits the excretion of ammonia and carbon dioxide. In short, for conditioning process, 1 m² of water is sufficient for 50-60 kg of carp, 30-40 kg of pike and 20-25 kg of trout.

Oxygen consumption or demand depends on the liveliness of fish. Sluggish species need only less quantity compared to fast moving ones. The amount of oxygen in water depends upon ambient temperature. For stenothermal species like carps, water temperature should not be less than 10-12°C in summer and 5-6°C in spring and autumn. Optimal temperature for conditioning and transportation of trout is 5-6°C in summer and 3-5°C in spring. During winter, fish tolerates temperatures of 1-2°C.

Now-a-days, special tanks are available with aeration facilities to keep fish alive. Water is cooled by ice.

RIGOR MORTIS IN FISH The catabolic



process taking place in a freshly dead animal body lead to stiffening of the muscles is known

as rigor mortis. There are 3 steps of rigor mortis progress in fish.

Immediately after death, the fish muscles are totally relaxed. The fish is soft and pliable and the texture is firm and elastic to the touch.

Changes in fish during rigor mortis

1 Physical changes

When rigor sets in., the muscle becomes hard and stiff and the whole body becomes inflexible. If the fish is filleted pre-rigor, the muscle can contract freely and the fillet will shorten and have a wrinkled surface. Dark muscle may shrink up to 50-52% while the white muscle up to 15-18% of the original length.

After rigor, the muscle tissue returns to a relaxed state. With some experience, it is possible to distinguish between the pre-rigor and post-rigor fish since pre-rigor fish is fully flexible and do not show pressure marks after gentle squeezing.

2. Chemical changes

The course of main biological process leading to rigor mortis is the same in muscles of great variety of species of fish and homeothermic animals. During the transition from living state to full rigor, fish muscle undergoes a series of biochemical changes. The most important reaction is the turnover of ATP, which proceeds for some hours in the dying muscle at about the same rate as in the resting living muscle. Living muscle cells obtain their biochemical energy (ATP) from respiring mitochondria.

This aerobic respiration ceases when blood circulation stops after death. Under these circumstances, the enzymatic machinery of the cell which is geared to maintain a constant level of ATP initially use creatine phosphate (CP), a reservoir of phosphate bound energy normally present in the muscle to generate ATP from ADP. ATP level starts to fall when 70% or more of the CP has been degraded.

3. Biochemical causes of rigor-mortis

Rigor mortis is caused by the bonding of myosin heads to the active centres of thin

filaments, the actin. This leads to the formation of a rather rigid structure of inter-connected myofilaments, the actomyosin

As the individual muscle fibres contain different quantities of ATP. they do not enter rigor at the same time post mortem. Thus, stiffness in muscle sets in gradually. During the later stages of post-mortem changes, the decomposition of nitrogenous compounds leads to an increase in pH in the flesh. The rate of pH changes depends on temperature.

In live or newly died fish, muscular contraction starts when a nervous impulse sets off a release of Ca from the sarcoplasmic reticulum to the myofibrils. When the Ca concentration increases at the active enzyme site on the myosin filament, the enzyme ATP-ase is activated.

SPOILAGE OF FISH

Introduction

Fish is one of the most consumed seafood and it is a highly perishable food product.

Fish and fish products are widely consumed as it is a good nutrition source due to their high protein content, unsaturated fatty acids, especially omega-3 fatty acids

Spoilage is caused by the action of enzymes, bacteria and chemicals present in the fish. In addition, the following factors contribute to spoilage of fish.

- High moisture content
- High fat content
- High protein content
- Weak muscle tissue
- Ambient temperature
- Unhygienic handling

Process of spoilage

Fish is highly nutritive. It is tasty because of its constituents. The main components of fish are water, protein and fat.

The biological and chemical nature of fish leads to its deterioration after it is caught. The spoilage process (Rigor mortis) will start within 12 h.

There are three modes of fish spoilage: Oxidation, Enzymatic and Microbial spoilage.

1. Oxidative spoilage

Lipid oxidation is a major cause of deterioration and spoilage of fish that contain high oil/fat content stored fat in their flesh.

Oxidation typically involves the reaction of oxygen with the double bonds of fatty acids.

In fish, lipid oxidation can occur enzymatically or non-enzymatically.

Lipid oxidation promotes protein denaturation, modification of the protein, electrophoretic profiles, nutritional losses, and endogenous antioxidant systems losses.

Lipid hydrolysis and oxidation cause “belly burst” in fish in which the enzymes and microorganisms of the digestive tract cause massive gas development.

2. Enzymatic spoilage

After capture, biological and chemical changes take place in dead fish due to the action of various enzymes found in fish.

The digestive enzymes cause extensive autolysis which results in fish muscle softening, rupture of the belly wall, and drain out of the blood.

Several proteolytic enzymes are found in fish that contribute to degradation in fish muscle and fish products during storage and processing.

Proteolysis is responsible for the degradation of proteins which leads to fish spoilage by microbial growth.

The changes in fish caused due to the various enzymes found in fish are:

Enzymes	Substrate	Effect
Glycolytic enzymes	Glycogen	Lactic acid production resulting in pH drop
Nucleotide breakdown enzymes	ATP, ADP, AMP, IMP	Gradual production of Hypoxanthine
Cathepsins	Proteins, peptides	Softening of tissue

Chymotrypsin, trypsin, carboxypeptidases	Proteins, peptides	Belly-bursting
Calpain	Myofibrillar proteins	Softening of tissue
Collagenases	Connective tissue	Softening and gaping of tissue
TMAO demethylase	Trimethylamine oxide (TMAO)	Formaldehyde production ATP

3. Microbial growth

Fish flesh is composed of protein, fats, carbohydrates, water, and amino acid compounds such as trimethylamine oxide (TMAO), urea, taurine, creatine, free amino acids, and trace glucose, etc.

The internal tissue of fish is generally considered sterile. Bacteria are present on the slime layer of the skin, gill surfaces, and the intestine.

The microbial growth in fish is the main cause of fish spoilage and produces amines, biogenic amines, organic acids, alcohols, aldehydes, and ketones with unpleasant and off-flavours.

The high-water activity, low acidity (pH > 6) of fish result in the fast growth of microorganisms that leads to undesirable changes in appearance, texture, flavor, and odour, reducing its quality.

At room temperature, *Bacillus*, *Clostridium*, *Escherichia*, *Micrococcus*, *Proteus*, *Sarcina*, and *Serratia* may predominate.

For unpreserved fish, spoilage is caused by Gram-negative, fermentative bacteria (such as *Vibrionaceae*), whereas psychrotolerant Gram-negative bacteria (such as *Pseudomonas spp.* and *Shewanella spp.*) tend to spoil chilled fish.

The fish spoilage is also caused by psychotropic, aerobic, or facultative anaerobic Gram-negative bacteria such as *Pseudomonas*, *Oraxella*, *Acinetobacter*, *Shewanella putrefaciens*, *Vibrio*,

Flavobacterium, *Photobacterium*, and *Aeromonas* Gram-positive bacteria such as *Staphylococcus* spp., *Micrococcus*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Brochothrix thermosphacta*, and *Streptococcus* are found in fish.

Lactic acid bacteria (LAB) can predominate in fish storage under vacuum or CO₂ storage. Some parasites can also be transmitted by fish, including tapeworm (*Diphyllbothrium latum*), nematodes (*Anisakis simplex* and *Capillaria philippinensis*), and trematodes (*Opisthorchis* and *Paragonimus*).

Spoilage compounds are produced by microorganisms during the storage of fresh fish.

Spoilage bacteria	Spoilage compounds produced
<i>Shewanella putrefaciens</i>	TMA, H ₂ S, CH ₃ SH, (CH ₃) ₂ S, Hypoxanthine, and acids
<i>Pseudomonas</i> spp., <i>Enterobacteriaceae</i>	CH ₃ SH, (CH ₃) ₂ S, ketones, esters, aldehydes, NH ₃ , and hypoxanthine
<i>Photobacterium phosphoreum</i>	TMA and hypoxanthine
<i>Vibrionaceae</i>	TMA and H ₂ S
Lactic acid bacteria	H ₂ S, ketones, esters, aldehydes, NH ₃ , and acids
Yeast, Anaerobic rods	Ketones, esters, aldehydes, NH ₃ , and acids
Aerobic spoilers	NH ₃ , acetic, butyric, and propionic acids

Prevention/Reduction of Spoilage.

The spoilage of fish is caused by enzymatic, bacterial and chemical action. The activity of organism can be controlled, reduced

or even retarded by proper handling and immediate lowering of the temperature.

The chilling of the fish immediately after catch and holding the fish at 0°C by proper icing will reduce the spoilage. In case of shrimps, removing head immediately after catch will reduce the rate of spoilage. In the case of big fishes, beheading and eviscerating will reduce the enzymatic actions which cause spoilage.

The spoilage is reduced or prevented in a number of ways like drying, salting, chilling, canning and freezing.

Chilling is a means of short-term preservation of seafoods achieved by the reduction in temperature using ice.

Freezing is the most satisfactory method currently available for a long-term preservation of seafood. It is, in fact by far the best way of preventing fish from spoilage, since fish continues to remain in almost the same natural conditions even after freezing.

Dehydration is another important reaction of a physical nature caused by the evaporation of ice due to differences in vapour pressure over the product surface and in the air of the store room. Loss of the moisture by evaporation of ice causes the product surface to dry resulting in dull appearance and even discolouration in some cases.

The evaporated water eventually condenses and freezes on the cooling surfaces of the store room and the transfer of moisture from the product will be continuous. Proper glazing and packaging eliminate this evaporation.

1.2 Principles of preservation – cleaning, lowering of temperature, rising of temperature, denudation, use of salt, use of fish preservatives, exposure to low radiation of gamma rays.

Fish preservation involves various principles and techniques to ensure the freshness, safety, and quality of fish products. The following are the principles associated with fish preservation:

- CLEANING,
- LOWERING OF TEMPERATURE
- RISING OF TEMPERATURE

- DENUATION
- USE OF SALT
- USE OF FISH PRESERVATIVES
- EXPOSURE TO LOW RADIATION OF GAMMA RAYS.

CLEANING

Removal of Dirt and Microorganisms: Proper cleaning involves removing dirt, scales, and gutting the fish to eliminate potential sources of contamination. This step helps reduce the microbial load on the fish.

During cleaning, the caught fish are first washed thoroughly in cold, clean water to remove bacteria, slime, blood, faeces, and mud, etc. from the body surface of the fish. It is being done under proper sanitary conditions. Large fishes are gutted (i.e. all the internal organs or viscera are removed) and the body cavity is washed.

LOWERING OF TEMPERATURE:

Low temperature storage is one of the most effective and principle for preserving fish and fish products.

In broad there are **three categories** of low temperature preservation: **chilling, super chilling** and **freezing**.

The important chilling methods of fish and fish products at non-freezing temperature are:

- ✚ Iced storage.
- ✚ Chilled seawater (CSW) storage.
- ✚ Mechanically Refrigerated seawater (RSW) storage.

Super chilling

- ✚ Super chilling has been defined as the lowering of the temperature of the flesh to within the range from -3° to -1°C . Brine is use as refrigerant in super chilling.

Freezing

- ✚ In the simplest terms refrigeration means removal of heat from the body or atmosphere, which is desired to be cooled and its transfer to another medium.

Lowering the temperature slows down the growth of spoilage-causing microorganisms and enzymes. Refrigeration and freezing are common principles to keep fish fresh. Rapid cooling after catching helps maintain the quality of the fish.

ISING OF TEMPERATURE:

Raising the temperature through cooking or canning is a preservation principle. Canned fish products, for example, are heat-processed to destroy harmful microorganisms and enzymes, extending the product's shelf life. The principles include

- Drying
- Wet curing
- Dry curing
- Smoking
- Canning

Sunlight is the cheapest and most abundant source of heat energy used for drying purposes.

Smoking is generally a combination of salting, and drying. Smoking is usually done in a kiln or a room, which is specially prepared for it. The fish that is salted and partially dried is used for smoking. Smoking can be cold, hot or liquid. If the temperature is below 35°C , it is cold and which is 70 to 80°C it is hot.



Canning is a common method of preserving fish in the fisheries industry. The process involves sealing fish in airtight containers, such as cans or jars, and subjecting them to heat to destroy microorganisms and enzymes that can cause spoilage.

Canning is particularly effective in extending the shelf life of fish products and ensuring their safety for consumption. Here is an overview of the canning process in fisheries

DENUATION:

Removal of Outer Layers: Denudation involves removing outer layers, such as scales and skin, which can accommodate microorganisms. This process can be part of the cleaning and processing steps in fish preservation.

USE OF SALT:

The use of salt in fish preservation has been a traditional and effective method for

centuries. Salt acts as a preservative by reducing water activity, inhibiting the growth of spoilage-causing microorganisms, and preventing enzymatic reactions that lead to the deterioration of fish.

Salt is used in curing and salting processes to draw out moisture from fish, reducing water activity and inhibiting bacterial growth.

In addition to preservation, salt is used to enhance the flavour of fish. It can bring out the natural flavours of the fish and contribute to the overall taste of the preserved product.

Salt creates an environment with lower water activity, making it inhospitable for many microorganisms that cause spoilage. This helps prevent the growth of bacteria, yeast, and molds that could otherwise lead to the deterioration of the fish.

USE OF FISH PRESERVATIVES:

Fish preservatives are substances or compounds used to extend the shelf life of fish and seafood products by inhibiting the growth of spoilage-causing microorganisms and delaying the oxidation of fats.

Various preservatives, such as antioxidants and antimicrobial agents, can be used to extend the shelf life of fish products.

These additives help prevent oxidation, inhibit bacterial growth, and maintain the



quality of the fish.

EXPOSURE TO LOW RADIATION OF GAMMA RAYS:

Exposure to low levels of gamma rays is a method of fish preservation known as **food irradiation**. Gamma radiation, which is a form

of ionizing radiation, is used to treat fish and other food products for various purposes, including extending shelf life, reducing spoilage, and ensuring food safety.

Gamma radiation can be used to irradiate fish as a method of preservation. This process helps eliminate or reduce bacteria and parasites, enhancing food safety and shelf life. The controlled application of gamma rays ensures the preservation without compromising the safety of the product.

These principles highlight the diverse methods employed in fish preservation, each serving specific purposes to ensure the fish remains safe, flavourful, and of high quality for consumption. The choice of preservation method often depends on the type of fish, intended use, and the desired shelf life.

UNIT II: METHODS OF FISH PRESERVATION

2.1 Traditional methods - sun drying, salt curing, pickling and smoking.

The traditional methods of processing fish by salting, drying, smoking pickling etc. are collectively known as 'Curing'.

Methods

- Drying
- Wet curing
- Dry curing
- Salt curing
- Pickling
- Smoking

DRYING

There are basically two methods of drying fish. The common one is by utilising the natural heat available from the sun. This is known as **sun drying**. The other is by using artificial means like **mechanical driers** for removal of moisture from the fish.

1. SUN DRYING

Sun drying Traditionally, simple sun drying was practiced widely. Here preservation was achieved by removing the water in the fish, thereby retarding the activity of bacteria and fungi.

The heat was able to destroy the bacteria to a certain extent. Later on, it was a combination of both salting and then drying or salting, smoking and drying. In the current

market situation both wet and dry cured



fishery products have commercial importance.

This is the simplest method of drying fish. The fishes dried in this way are small, lean ones, which are available in plenty during the glut season.

- They are usually spread out on the seashore as whole with little preprocessing.
- Sometimes they are given a washing in the seawater.
- Drying takes place usually by the removal of moisture from the surface and later from the interior of the fish.
- Depending on the relative humidity, temperature, air velocity, the removal moisture takes place continuously.

Factors, which affect the rate of drying, are:

- ✚ Size of the material, larger fish takes a longer time to dry whereas smaller ones lesser
- ✚ Surface area, large surface area will increase the rate of drying.
- ✚ Temperature, the higher the temperature the faster will be the rate of drying.
- ✚ Relative humidity, the lower the RH the faster will be the drying.
- ✚ Air velocity, the greater the speed of the air, the faster the drying
- ✚ Fat Content, fatty fishes will take a longer time to dry than lean fishes
- ✚ Water content, the higher the water

SOLAR DRYING

This is a method by which the sun's heat is converged and utilized for drying. Converging of solar heat can be affected by using a black surface which absorbs heat far more effectively than a light coloured one. Solar tent driers and cabinet driers work on this principle.

Mechanical driers

- ✚ In mechanical driers the removal of water from fish requires an external input of thermal energy.
- ✚ This is brought about by burning fuel. This is an expensive method since there is need for fuel for heating and maintenance of the temperature.
- ✚ The drying chamber consists of a long tunnel in which the product is placed on trays or racks.
- ✚ A blast of hot air is passed over the material to be dried. After the required degree of drying the product is removed from drier and packed.

2. WET CURING

- ✚ The initial stages of processing and salting are the same as for dry curing.
- ✚ Once the fish is put into the tank it is allowed to remain in the self-brine.
- ✚ The wet fish is then drained and packed in Palmyra leaf baskets or coconut leaf baskets and taken to the market.
- ✚ The fish is taken out only when there is demand. This method is particularly suitable for fatty fishes.
- ✚ When immersed in the tank there is no contact with outside air. These products have moisture content of 50-55% and the salt content around 25%.
- ✚ They are most susceptible to fungal attacks, bacterial degradation and general putrefaction.

They have a very short shelf life.

3. Dry Curing

- ✚ This is the most widely used method of fish curing.



- ✚ Here, the fish is gutted, beheaded or ventrally split open and the viscera removed.

- ✚ Scores are made along the thick flesh portion for better penetration of salt.
- ✚ Salt is then applied in the ratio 1:3 to 1: 10 (salt to fish) depending upon the size of the fish.
- ✚ The fish is then salted in cement tanks or containers. The bottom of the tank is covered with salt and a layer of fish is placed.
- ✚ The fish is kept in this condition for 24-48 hours. After this the fish is taken out, washed in brine solution to remove adhering salt and drained.
- ✚ It is then dried in the sun to required level of moisture. Yield of the product by this method is about 35-40%. This product has a shelf life of 6-10 weeks.

3. SALTING (SALT CURING):

Salting is a very old and common practice used in fish preservation in India and other parts of the world. Salting is nothing but the partial dehydration of fishes by osmosis with sodium chloride. Due to intense salting, microbes are killed and diastasis is stopped. In this process before salting, the heads of the fishes are removed, gutted and washed, and then salted as soon as possible. There are different methods of salting such as dry salting, cold salting, light, or strong salting.

✚ (a) Dry salting: In this method, alternate layers of fish and salt are placed. This is called dry salting.

✚ (b) Wet salting or brine: In this process, the fishes are dipped in salty water called brine. The brine or saltwater is used in two different concentrations. In light brine, the salt concentration is 16% and strong brine is used containing 25% salt. After this saltwater treatment, the fishes are dipped in salt.

Cold salting:

✚ In this process, salt and crushed ice are spread on the fish. This process is carried out in a cold room only where the temperature is maintained at 2 or 3°C. This is also called light cold salting.

A strong salting process is performed at normal room temperature. Salting is a very common practice adopted by fishermen near seashores. The small-sized fishes are sun-dried and preserved for a longer period.

5. PICKLING

Pickling is a preservation method that involves immersing food, in this case, fish, in an acidic solution called pickling brine.

The acid, typically vinegar, along with salt and various spices, acts as a preservative, creating an environment that inhibits the growth of spoilage-causing microorganisms. Pickling not only helps extend the shelf life of the fish but also imparts unique flavors to the product.:

Pickling Process for Fish:

- **Selecting Fish:**

Choose fresh and firm fish, preferably those with a mild flavour that can absorb the pickling flavors well. Common fish used for pickling include herring, mackerel, and salmon.

- **Cleaning and Preparing Fish:**

Clean the fish thoroughly, removing scales, guts, and any undesirable parts. Fillet the fish if desired.



- **Brining:**

Submerge the fish in a pickling brine, which typically consists of vinegar, salt, and sometimes sugar. The brine not only imparts flavor but also acts as a preservative.

- **Pickling Spices and Flavorings:**

Add spices and flavorings to the brine to enhance the taste. Common spices include peppercorns, mustard seeds, dill, bay leaves, and onions. The choice of spices can vary based on regional preferences and the desired flavor profile.

- **Marination:**

Allow the fish to marinate in the pickling brine for a specified period. The duration can vary depending on the size and type of fish, but it typically ranges from a few hours to a day or more.

- **Refrigeration or Fermentation:**

After marination, the pickled fish can be refrigerated to slow down the fermentation process. Some pickling methods involve allowing the fish to ferment at room temperature for a period, contributing to the development of complex flavors.

- **Packaging:**

Once pickled to the desired level, the fish can be packed in jars or other airtight containers. The pickling liquid (brine) is typically included in the packaging to maintain the fish's moisture and flavour.

- **Storage:**

Store the pickled fish in a cool place, such as a refrigerator, to prolong its shelf life. Properly sealed containers help prevent contamination and maintain freshness.

Types of Pickled Fish:

- ✚ Herring: Pickled herring is a popular dish in many cultures, often served with onions and a variety of sauces.
- ✚ Soused Mackerel: Mackerel is often pickled or "soused" with a vinegar-based solution and various spices.
- ✚ Pickled Salmon: Salmon can be pickled in a variety of ways, with ingredients such as dill, mustard seeds, and juniper berries.

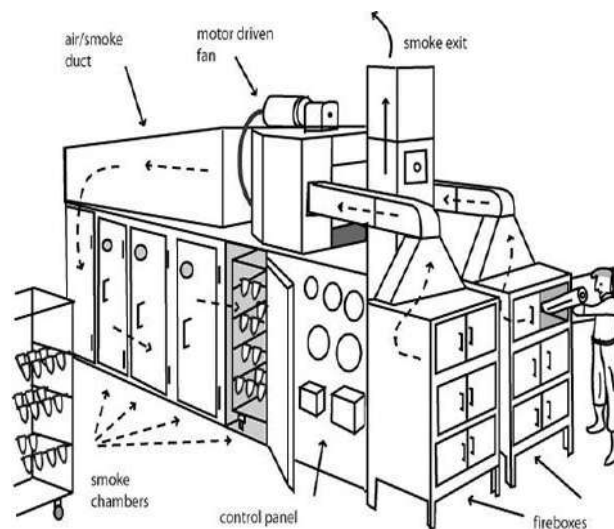
Pickled fish is enjoyed as a delicacy in many regions, and the variations in pickling methods and flavourings' contribute to diverse culinary traditions.

6.SMOKING OF FISH

- ✚ Smoking is generally a combination of salting, and drying.
- ✚ Smoking is usually done in a kiln or a room, which is specially prepared for it.



- ✚ The fish that is salted and partially dried is used for smoking.



- ✚ Smoking can be cold, hot or liquid. If the temperature is below 35 °C, it is cold and which is 70 to 80°C it is hot.
- ✚ The preserving effect of smoking on fishery products is attributed to a combination of surface drying, salting and deposition of phenolic and other anti-microbial constituents of smoke on the fish.
- ✚ Both salted and unsalted fishes are smoked.
- ✚ Smoked fish have the potential danger of containing certain carcinogenic compounds of smoke such as 3-4, bensopyrine harmful due to the presence of carcinogenic compounds

Types:

- ✚ Hot Smoking
- ✚ Cold Smoking
- ✚ Liquid Smoking
- ✚ Electrostatic smoking

Hot smoking

Hot smoking is typically not a single process. Several other steps such as brining, drying and smoking are also involved to produce a product of good quality

Cold smoking

Fish can also be subjected to cold smoking. Temperatures of cold smoking typically do not exceed 30 °C. Thus, cold smoked products are not cooked and typically heavily salted.

Liquid smoking

Liquid smoke is smoke condensate that is dissolved in a solvent, such as water or oil. Liquid smoke can be used directly on products by dipping or spraying. It is rapid and much easier to achieve a uniform smoke flavour than traditional cold and hot smoking processes, although the flavour and colour from the traditional smoking cannot be exactly duplicated.

Electrostatic smoking

Electrostatic smoking is another rapid way to smoke. In the electrostatic smoking, fish are sent into a tunnel where an electrostatic field is created. Smoke particles are given a positive charge and deposit onto the surface of the fish which are negatively charged.

2.2. Advanced methods – chilling or icing, refrigerated sea water, freezing, canning, irradiation and Accelerated Freeze drying (AFD).

Advanced methods of fish preservation include the following methods they are

1. Chilling or icing
2. Refrigerated seawater
3. Freezing
4. Canning
5. Irradiation
6. Accelerated Freeze drying

1. CHILLING

Chilling is an effective way of reducing spoilage in fish if it is done quickly and if the fish are kept chilled and handled carefully and hygienically.

The objective of chilling is to cool the fish as quickly as possible to as low a temperature as possible without freezing. Chilling cannot prevent the spoilage together but in general, the colder the fish, the greater the reduction in bacterial and enzyme activity.

The important chilling methods of fish and fish products at non-freezing temperature are:

- ✚ Iced storage.
- ✚ Chilled seawater (CSW) storage.
- ✚ Mechanically Refrigerated seawater (RSW) storage.

Iced Storage

The major advantage of using ice for chilling the fish is that it has a high latent heat of fusion so that it is capable of removing large amount of heat as it melts without changing

the temperature at 0 °C. Spoilage of the fish starts immediately after the death which is largely dependent on the temperature conditions, the sooner the fish can be cooled the better will be the quality and shelf life.

In ice storage it is not enough that there is sufficient quantity of ice to preserve the fish but the effectiveness of icing depends on how well the ice is dispersed among the fish.

Also, the smaller ice particles such as ice flakes do a more effective job of cooling than large pieces. This is because smaller particles give greater contact between fish and ice and the rate of heat removal depends on the size of the contact area.

Another advantage of small ice particles is that it avoids damaging fish. Large pieces can exert point forces and thereby damage the fish.

Types of Ice:

Block Ice

Ice is conventionally manufactured in the block form, which is crushed to smaller pieces for icing. Crushed ice has a large surface area, which will ensure rapid cooling than the large blocks.

Flake Ice

Ice with smooth contours and in the shape of very thin flakes is called flake ice.

Plate Ice

Plate ice is made by spraying water on to the surface of vertical hollow plates through which a refrigerant pass. A flat sheet of ice will be formed on the surface of the plate.

Tube Ice

The ice is formed on the inner surface of a series of hollow cylinders, the outer surface of which will be surrounded by a refrigerant.

Liquid ice or Flow ice

Liquid ice or flow ice looks like jelly. It actually consists of amorphous ice micro particles suspended in a non-corrosive, non-toxic solution. Liquid ice generator system yields a substance with ten times more cooling capacity than chilled water. **Soft Ice**

Soft ice is made by freezing a weak brine or seawater in a drum provided with refrigerated walls. The crystals of fresh water ice forms slurry in the brine as temperature falls and it is pumped into a storage tank.

Chilled Sea Water (CSW) storage

✚ In this case, the fish is surrounded with a mixture of ice and water.

✚ When enough ice is added to the system it will bring the temperature of the water near to 0 °C.

- ✚ The water will remove the heat from the fish. The transfer of heat from fish to water and from water to ice will continue until the system is brought to a state of equilibrium.
- ✚ Because of the maximum contact between the cooling medium and the fish the cooling rate of the fish in CSW is higher than that of fish in ice.
- ✚ CSW can be adopted in fishing vessels as it has many advantages over ice storage.
- ✚ CSW system is used for the containerized bulk storage of the catch onboard the vessels.
- ✚ The main advantages of the CSW system are listed below:
 - ✚ Faster cooling rate.
 - ✚ Maintenance of a uniform temperature.
 - ✚ Less damage to fish.
 - ✚ Easy unloading of fish using pumps.

Super chilling

- ✚ Super chilling has been defined as the lowering of the temperature of the flesh to within the range from -3°C to -1°C . Brine is used as refrigerant in super chilling. The storage time of fishes should not exceed 12 days. Above this day's ice formation is increased and makes the fish unsuitable for filleting or smoking.

2. REFRIGERATED SEA WATER (RSW) STORAGE

Refrigerated Sea Water (RSW) storage is a method commonly used in the fishing industry to maintain the freshness and quality of harvested fish. This technique involves using chilled sea water to cool and store fish immediately after they are caught.

RSW storage is particularly beneficial for preserving the quality of seafood products, especially those destined for the fresh market.

- ✚ In this system the seawater is cooled by mechanical refrigeration.
- ✚ Seawater has a salt content of around 3 to 3.5%. At 3.5% salt, seawater has a freezing point of about -2°C .
- ✚ Thus, if seawater is refrigerated, it is possible to reduce the temperature so that a storage temperature of -1°C can be achieved.
- ✚ The most important advantage of RSW over icing is the ease of handling and storage onboard, with a resultant saving of labour.
- ✚ Pumping systems have been developed for unloading fish as large as 10kg each at a rate of more than 25 tonnes/hour. In the most sophisticated systems, the vessel can be equipped with a pump which pumps the

fish out of the sea into holding tank and the same pumps can be used for unloading.

- ✚ RSW is popular on large vessels, while CSW can be applied even on small boats.
- ✚ For RSW, larger capital investment is required for the mechanical refrigeration system. In order to avoid inefficient, too slow chilling of the catch, not more than 800kg of fish should be packed per kilo litre of the brine.
- ✚ RSW may also be used to cool the catch by spraying over the top surface of ungutted fish or shrimp in the hold.
- ✚ Rapid chilling and holding of the catch in RSW is a popular practice on board large vessels which catch shrimps.
- ✚ Chilling of the catch in RSW before freezing on board freezer trawlers is a very effective method of preventing rapid quality loss of the fish on subtropical and tropical grounds.
- ✚ The shelf life and quality of seafoods chilled or stored in CSW or RSW depends upon the species characteristics and the condition of chilling.
- ✚ Generally, it is a few days longer than that of iced fish. Some species are vulnerable to loss of appearance due to bleaching or leaching. The uptake of salt may significantly decrease the market value of some seafoods, like shrimps, snappers.

The advantages of RSW are

Labour saving in stowage

- ✚ Rapid chilling, 2 to 3 times as fast as with ice
- ✚ Elimination of crushing and ice pitting
- ✚ Lower chill temperatures
- ✚ Washing effect
- ✚ Ease of unloading

3. FREEZING

In the simplest terms refrigeration means removal of heat from the body or atmosphere, which is desired to be cooled and its transfer to another medium.

The refrigeration cycle involves steps to remove the heat from the evaporating refrigerant by again converting it into the liquid state in order that it may be used repeatedly in a continuous process.

As more heat requires to be extracted during the second stage, in order to turn the bulk of the water to ice, the temperature changes by a few degrees and this stage is known as the period of "**thermal arrest**".

Quick freezing and slow freezing:

The freezing process in which the fish passes through the zone of maximum crystallization in 30 minutes or less is arbitrarily referred to as quick freezing.

Large ice crystals are formed during slow freezing which can damage the cell walls. This may cause textural changes and increased thaw drip.

Freezing methods and equipment
Direct and indirect systems

In the direct expansion system, the refrigerant absorbs heat directly from the material to be cooled.

In the indirect or brine system, the refrigerant absorbs the heat that the brine absorbs from the material to be cooled. Both these systems are commonly employed in commercial freezing operations. Freezing methods are broadly classified into

- ✚ Freezing in air
- ✚ Indirect contact freezing
- ✚ Immersion freezing
- ✚ Cryogenic freezing

Freezing in air

Air is the most common freezing medium. Air freezing systems are of two types- still air freezing and forced air freezing.

Freezing in still air:

The freezer consists of an insulated room or a cabinet maintained at -28 to -45 °C. Fish, packaged or otherwise, placed in aluminium trays, is kept on shelves made of pipes or coils through which the refrigerant is circulated. Time taken for freezing may be 12 hours or more. Freezing in still air is the least expensive method of freezing; however, is the slowest method as well.

Indirect Contact Freezing

Indirect contact freezing can be defined as freezing a product by keeping it in contact with a metal surface which is cooled by some refrigerant. They are of two types - The horizontal plate freezers and the vertical plate freezers.

Horizontal plate freezer:

These freezers in general have 15-20 plates. The product to be frozen contained in metal freezing trays are loaded between freezing plates and are maintained in close contact with top and bottom plates under slight hydraulic pressure to ensure maximum heat exchange. The freezing trays are also covered with tightly fitting lids which will help to

provide contact with the freezing plates at the top.

Vertical Plate Freezer:

These are most commonly used for freezing fish at the sea. They consist of number of vertical freezing plates that form partitions in a container called stations. Fish are loaded between the plates until each station is full and then the plates are closed together to form fish blocks. Temperature ranges between -30 to -40° C.

Contact plate freezer is very economical to operate. Dehydration of the product will be minimum and it remains in uniform blocks without bulging.

Rotary Drum Freezer:

This is a refrigerated stainless-steel drum revolving at a pre-set speed. The material to be frozen is fed on the external surface of the drum through a conveyor. It adheres immediately to the surface of the drum by freezing the water on the surface of the material. At the end of one revolution the frozen product is scraped off and is passed through an automatic glazer before packing. As there is no movement of the air and the freezing is rapid, there will be little or no loss of weight during freezing.

Immersion Freezing

In this method freezing is achieved by immersion in, or spraying with, a refrigerant that remains liquid throughout the process. Refrigerated aqueous solutions of propylene glycol, glycerol, sodium chloride, calcium chloride and mixtures of sugars and salt can be used as medium for freezing. Immersion freezing allows for intimate contact of every surface of the material with the freezing medium and thus ensures very efficient heat transfer.

i) Freezing in brine:

Brine freezing is rapid and is adaptable for continuous operation. However, the fish will absorb some salt, which will depend on several factors such as temperature of the brine, duration of immersion, fat content of the fish and the surface area. Contamination of the medium and subsequent cross contamination of batches is a major disadvantage of this process.

ii) Brine spray freezing:

Chilled brine is sprayed over the fish placed in trays. Heat is removed from the fish by the chilled brine in 1-2 hours.

Cryogenic Freezing:

In cryogenic freezing very rapid freezing is achieved by exposing the fish, unpacked or with a very thin package, to an extremely cool freezing undergoing a change of state.

The most common food grade cryogenic freezants are boiling nitrogen and boiling or subliming carbon dioxide.

Cryogenic freezing is much faster than air blast or contact plate freezing; but is only moderately faster than fluidized bed or liquid immersion freezing.

i. Freezing using liquid nitrogen:

In freezing using liquid nitrogen, liquefied nitrogen gas is sprayed over the product as it travels in a tunnel over a conveyor belt. The nitrogen gas passes counter current to the movement of the fish so that the fish gets pre-cooled before reaching the liquid nitrogen spray. After the spray the product is allowed to temper for a while being discharged from the tunnel.

ii. Freezing using liquid / solid carbon dioxide:

Liquid carbon dioxide is sprayed over the fish as it travels through a tunnel on a moving conveyor. The carbon dioxide is sprayed through the nozzles and while spraying the pressure is quickly reduced and about 50 % of it suddenly changes to tiny particles which absorb the heat from the surroundings and gets converted to gas resulting in rapid cooling of the fish. Freezing can be done by exposing the fish to powdered solid carbon dioxide.

Freezing with carbon dioxide has most of the advantages of freezing with liquid nitrogen. However, unpacked foods may absorb or entrap carbon dioxide to the extent of causing undesirable swelling.

iii. Double freezing:

It is a common practise to preserve the fish by freezing onboard vessels when the voyage lasts for few weeks. On reaching the shore the fish is thawed and reprocessed.

iv. Partial freezing:

Partial freezing or super chilling, involves lowering the temperature of the fish to about -2 to -3 °C. By cooling the fish to this temperature range, approximately half of the unbound water present in the fish will get converted to the solid phase. Storage life of partially frozen fish is almost double that of fish stored in ice. However, some loss of sensory quality is experienced when the

storage period extends to five days mainly due to the fluctuations in temperature.

4. CANNING:

Canning is a method of food preservation in which food is packed in metal or glass or plastic containers, sealed air tight and heated sufficiently to destroy the spoilage, pathogenic and fish food poisoning organisms making the food safe for consumption.

The steps involved in the canning process are as follows.

I. Pre-processing operations: Depending on the nature of the food, different preprocessing operations are carried out. Washing, separation of edible parts, cleaning, cutting into desired size and shape, brining, precooking, blanching, frying etc. are done prior to filling of the can. The difference in canning method for various foods stuffs lies in this stage.

II. Can filling: The preprocessed food is filled in to the cans along with suitable filling medium. The quantity of food to be filled varies with the size of the can. Sufficient space has to be provided between the lid and the top layer of the food.

III. Exhausting: The exhausting is the process to remove the air and gases inside the filled cans. This is achieved either by using steam or by creation of vacuum by a pump. The removal of air prior to can closing creates vacuum in the head space.

IV. Can closing/seaming: Can closing or seaming is a process wherein, the lid and the body of the can are tightly sealed. This is done by can seamer. Can closing should be airtight preventing the exchange of gases between atmosphere and inside of the can.

V. Heat processing (Retorting): Retorting is a process where the closed cans are subjected to heat processing at high temperature to destroy spoilage and pathogenic microorganisms. This is the most important step in canning. The temperature and duration of heat processing depend on the type of food, size and shape of can.

VI. Cooling of the cans: After heat processing, cans are cooled immediately either in air or more commonly in water to the

ambient temperature. Cooled cans are dried, labelled and stored.

Advantages of canning

- ✚ Canned products are safe and will not cause any ill health (or allergy etc)
- ✚ Canned product are ready to serve products and hence instantly available for consumption with little or no further preparation for the table.
- ✚ Canned product can be made available at any place any time.
- ✚ Canned products do not require special storage facility such as cold storage. Ordinary room temperature storage is sufficient.
- ✚ As inedible portions of foods stuffs are removed, it represents concentrated form of food
- ✚ Canned food has long shelf life measured in years not in weeks or months. The shelf life is about 2 years.
- ✚ The canning process is a sterilization technique that kills microorganisms already present on the fish, prevents further microbial contamination, and inactivates degradative enzymes.
- ✚ In this process fish are hermetically sealed in containers and then heated to high temperatures for a given amount of time.
- ✚ Canned fish can be stored for several years.
- ✚ In canning thermal conditions viz. exhausting and retorting at a temperature of 880C & 1210C respectively causes death of microorganisms integrated with prevention of oxidative changes.

5.IRRADIATION

Fish Irradiation is essentially non-thermal process of preserving fish because that treatment does not cause any significant raising temperature.

Temperature of the product being irradiated as an influence on the radiation induced changes.

Movement of free radicals increased with the temperature, affecting the overall rate of radiolysis lower temperature reduces the production of volatiles in food products

Fish Radurization

- ✚ Radurization is the application of irradiation to foods using a dose of

ionizing radiation sufficient to enhance its keeping quality.

- ✚ This causes a substantial decrease in numbers of viable specific spoilage microorganisms.
- ✚ The required dose is in the range of 0.4 – 10 kGy for a range of food product.
- ✚ Radurization processes have been developed for variety of fishery products including marine fresh water and shell fish.

Potential Application of Irradiations

- ✚ Under refrigeration conditions shelf-life extension of fresh fish and elimination of pathogens in fresh and frozen seafood's observed.
- ✚ Individually Quick Frozen (IQF) shrimp and reductions of pathogens including Hepatitis A virus, from oysters recorded with the application of irradiations.
- ✚ Development of shelf stable products from fish.
- ✚ Removal of off Odors from some species of lobsters and oysters.
- ✚ Reduction in fecal coli forms in live hard-shell clams.
- ✚ Hygienization of fishmeal

6. ACCELERATED FREEZE DRYING

Heat is used to dry foods or concentrates liquids by boiling to remove water and therefore preserves the food by reduction in water activity.

Heat causes a loss of sensory and nutritional qualities. In freeze drying a similar preservative effect is achieved by reduction in water activity without heating the food and sensory and nutritional qualities are consequently better retained.

Freeze drying has become an important commercial operation to dry expensive foods which have delicate aroma or texture (for example instant coffee, mushrooms, fruit juices and sea foods).

In freeze drying, foods are dried in two stages, first by sublimation to approximately 15% moisture content (on wet weight basis) and then by evaporative drying of unfrozen water to 2% of moisture content. (W.W.B).

Desorption is achieved by raising the temperature in the drier to near ambient temperature while retaining the low pressure.

The rate of drying depends mostly on the resistance of the food to heat transfer and on the resistance to vapour flow.

Equipment:

Freeze drier consists of a vacuum chamber containing trays to hold food during drying, source of heat to supply latent heat of sublimation, refrigeration coils to condense vapour with automatic devices to keep maximum area of the coil free of ice for vapour condensations and vacuum pump to remove non expensible vapour.

Accelerated freeze driers: Food is held between expanded metal mesh for heat transfer.

Freeze drying cycle consists of 3 stages.

- ✚ Freezing.
- ✚ Primary drying to remove water ice.
- ✚ Secondary drying to remove bound water molecules from the primary dried materials.

1) Freezing: Freezing the product temperature is reduced to -50°C at one atmospheric pressure to ensure that it is below the eutectic point.

2) Primary drying: This is a dynamic process involving transits of ice phase to vapour phase directly. This leaves the solid matrix formed in the freezing phase but without the ice crystals. During drying, an interface is set up which comes from the exposed surface to vacuum system to the surface where heat source is in contact or moves deeper in radiant heat. At this interface, actual drying takes place.

3. Secondary Drying: This is part of the process where remaining small proportion of "locked in" water is removed (Usually 2.5% by volume). This is not always considered necessary and depends on the product. The locked in or bound water is removed from the products by supplying additional heat energy to the system.

Effect of freeze drying on food:

- ✚ Freeze dried foods have high retention of sensory and nutritional qualities and shelf life of more than 12 months when correctly packed.

- ✚ Aroma retention is 80-100%. Shrinkage is very little and texture is well maintained. Allows rapid and full rehydration but the product is fragile and require protection from mechanical pressure.

- ✚ However, the open porous structure of the food may allow oxygen to enter and cause oxidative deterioration of lipids and subsequent browning.

Changes in thiamine and ascorbic acid content during freeze drying are moderate and there are negligible losses of another vitamin.

UNIT III: Processing and preservation of fish and fish by-products

3.1.1 FISH MINCE BASED PRODUCTS

MINCE AND MINCE-BASED PRODUCTS

Fish mince or minced fish is the flesh separated from the fish in a fragmented form free from scales, skin and bones.

Trimming from the fish filleting operations, whether hand or machine filleting, was the original source of fish mince.

Significant value addition will accrue to such fish if converted into fish mince because mince can be used to process a variety of value-added products having high commercial potential.

Another advantage of the process is that it conceals identity of the original fish from which it is made and consumers may not hesitate to accept mince or mince-based products even though the original fish would have been unacceptable as whole fish.

Production of fish mince

Mince can be prepared either from gutted fish or whole fish. Mince from gutted fish will be better in colour, appearance and flavour. For best quality minces it is desirable to use only a single species of fish so that mince of different quality and stability do not mix together.

The freshness of the raw material profoundly influences the quality of the mince. The mince will contain all the components like the enzymes, lipids, haem pigments etc. in the fish that affect its shelf stability. The mince is frozen and stored.

Mince-Based Products

The fish mince finds application in processing several convenience foods like fish finger, cutlet, burger and also in some low cost salted and dried products. In preparation of

fish finger, stick, sake etc., the mince stripped from the bone frame is incorporated to increase the yield.

Surimi

Surimi is a Japanese term for mechanically deboned fish flesh that has been washed with water and mixed with cryoprotectants for good frozen shelf life.

Kneaded products

Several kneaded products like kamaboko, chikuwa, hampen, fish ham and sausage are processed using surimi incorporating other ingredients.

The ingredients used in most of these preparations are identical; however, the classification is principally based on the manufacturing process involved.

The ingredients employed other than surimi include salt, monosodium glutamate, sugar, starch, egg white, polyphosphate and water.

The method of processing all these products involves grinding together of the various ingredients to a fine paste and some sort of heat treatment at some stage.

Fiberized products

Fiberized products are the greatest in demand among the surimi based imitation shellfish products.

Fish cutlets

Fish cutlets are prepared using cooked fish mince, which is mixed with cooked potato, fried onion and species etc. It is then formed into the desired shape, each weighing approx. 40 g. The formed cutlets are battered, breaded and flash fried for 20 seconds

Fish finger

Fish finger is a very popular product made out of fish mince. The mince is mixed with 1.0 per cent salt, made into rectangular slabs and frozen. The frozen mince is cut into suitable uniform sizes. These pieces are given a coating of batter followed by breading. The battered and breaded fish fingers are flash-fried in oil maintained at 180-200° C for about 20 seconds. After cooling the fingers are frozen and stored.

3.1.2. FISH MEAL & FISH OIL

Fish meal Fish meal is a dry product having brownish grey colour and milled to a fine to coarse powder.

It is produced by removal of 90 to 95% of water and fat present in the raw material. Generally oily pelagic fish is being used all over the world for fish meal manufacture.

It is a good source of major nutrients such as protein and contains fair amount of fat. It is rich in essential minerals, namely phosphorus, calcium and iron.

It is also a good source of micro minerals, oil soluble vitamins and water-soluble vitamins.

Fish oil is a by-product obtained during fish meal production and then subjected through various steps in order to yield the final product.

The oils contain mainly triglycerides of fatty acids (glycerol combined with three similar or different acid molecules) with variable amounts of phospholipids, glycerol ethers and wax esters.

It is characteristic of the oils that they contain a wide range of long-chain fatty acids with the number of carbon atoms ranging mainly from 14 to 22, and high degree of reactivity (unsaturation) ranging up to six double bonds per molecule.

Fish oil is high in unsaturated fats and also aids in reducing blood cholesterol level. They are imparting positive effect in powerful metabolic and physiological regulators, which also influence the excessive fat deposition in the arteries.

- ✚ Body oil of fish is more important as an industrial product besides its limited use in human consumption.
- ✚ Contains poly unsaturated fatty acids (PUFA), particularly n-3 PUFA
- ✚ n-3 PUFA used in the control of heart ailments in humans
- ✚ Known to have anticholesterolemic effects
- ✚ Highly unsaturated fish body oils can be used as drying oils in paints and varnishes
- ✚ Used as a medium in fish canning.
- ✚ finds use in margarine
- ✚ Used as carriers of fat-soluble vitamins A and D.
- ✚ Find use in the manufacture of linoleum, detergents, artificial rubber, lubricants, printing inks, soaps etc.

Process steps in reducing the fish waste to fish meal and oil

- ✚ Heating, which coagulates the protein, ruptures the fat depots and liberates oil and physio-chemically bound water
- ✚ Pressing (or occasional centrifugation), which removes a large fraction of the liquids from the mass

- ✚ Separation of the liquid into oil and water (stick water). This step may be omitted if the oil content of the fish is less than 3%
- ✚ Evaporation of the stick water into a concentrate (fish soluble)
- ✚ Drying of the solid material (press cake) plus added soluble, which removes sufficient water from the wet material to form a stable meal
- ✚ Grinding the dried material to the desired particle size

3.1.3 FISH LIQUID (ENSILAGE)

Fish ensilage

Fish silage can be defined as a product made from whole fish or parts of the fish to which no other material has been added other than an acid and in which liquefaction of the fish is brought about by enzymes already present in the fish.

The product is a stable liquid with a malty odour which has very good storage characteristics and contains all the water present in the original material.

It is a simple process and it requires little capital equipment particularly if nonoily fish are used. The use of oily fish usually requires oil separation. This involves expensive equipment and is only suited to a fairly large-scale operation.

Almost any species of fish can be used to make fish silage though cartilaginous species like shark and rays liquefy slowly.

The production of fish silage involves preferably organic acid like formic acid (35 kg/1 tonne of fish) to preserve the fish and then allow the enzymes already present in the fish to liquefy the protein.

Wooden or bitumen coated iron or concrete acid resistant tanks can be used for the process. Mineral acid like sulphuric acid can also be used for this purpose instead of formic acid. But in this case pH would be about 2.5 which requires neutralization before feeding.

When the minced fish is mixed with the acid the fish tends to stiffen initially and liquefaction will proceed and the rate at which liquefaction takes place depends upon the temperature of the mixture.

There are no problems in storage of fish silage if the correct acidity is maintained. There are Changes during storage the protein becomes more soluble and there is an increase in free fatty acids if any fish oil is present. If

silage is made from oily fish, it is desirable to separate the oil after liquefaction.

There are some advantages and disadvantages for silage over fishmeal. In the case of silage, the capital investment is significantly less and no skilled personnel is needed. But, in the case of silage the bulk is not reduced and so storage and transportation face problem. There is no considerable smell for silage in fact it is having a pleasant smell. Smell is a difficult problem in the manufacture of fishmeal. Marketing of fishmeal is well established, whereas silage is still not well known to the farmers. Recently silage production is considered to be the best method to address the problem of environmental production due to processing waste and underutilized by-catch.

There is an alternate method of production of silage by fermentation. The fish is mixed with a carbohydrate source like molasses and lactic acid is produced in the system to reduce the pH by introducing a lactic acid producing bacteria like *Lactobacillus plantarum*

3.1.4 FISH PROTEIN CONCENTRATE

Fish protein concentrate (FPC) is any stable fish preparation, intended for human consumption, in which the protein is more concentrated than in the original fish.

Fishmeal as produced throughout the world is very cheap potential FPC, but it is not intended for human consumption; it is used for making pig and poultry feeds for farming.

Ordinary fishmeal is unsuitable for human consumption for three main reasons:

- It is not normally made under sufficiently hygienic conditions to rule out the risk of occasional contamination by disease-causing bacteria.
- It usually contains rancid fat, which destroys certain vitamins and may lower the nutritive value of the protein;
- a fishmeal diet might precipitate vitamin deficiency in poorly nourished people.
- Moreover, the flavour of the rancid fat is unacceptable in many societies, though not in all.

- There is a slight risk that the rancid fat may have a cumulative toxic effect if consumed over a long period. The first of these reasons is the most important; thus, fishmeal made under hygienic conditions is called FPC type C.

The Food and Agriculture Organization of the United Nations defines three types:

- Type A: a virtually odourless and tasteless powder having a maximum total fat content of 0.75 per cent.
- Type B: a powder having no specific limits as to odour of flavour, but definitely having a fishy flavour and a maximum fat content of 3 per cent.
- Type C: normal fishmeal produced under satisfactorily hygienic conditions.

3.1.5 FISH CHOWDER

Fish chowder is a flavourful and hearty soup that typically features fish, vegetables, and sometimes bacon or other ingredients. It is a popular dish in many coastal regions and is valued for its rich taste and comforting qualities. It is basically a simple fish preparation, but is different from the other preparations because it is creamy and quite rich.

Ingredients like fish, potatoes and onions give this dish its heartiness, while butter, thyme and seasoning add a different dimension to this simple stew.

It is generally consumed as a soup.

Importance:

Nutrient-Rich: Fish is a good source of high-quality protein, omega-3 fatty acids, vitamins, and minerals. Including fish in chowder adds nutritional value to the dish.

Culinary Tradition: Fish chowder is often a traditional dish in coastal communities, reflecting the local culinary heritage and the availability of fresh seafood.

Versatility: Fish chowder can be adapted to include various types of fish, shellfish, and vegetables, allowing for regional and seasonal variations.

Comfort Food: Chowders, in general, are known for their comforting and satisfying qualities. The warmth and heartiness of fish

chowder make it a comforting choice, especially during colder seasons.

Sustainability: Choosing sustainable and responsibly sourced fish for chowder supports the health of marine ecosystems and ensures that seafood resources are managed responsibly.

3.1.6 FISH CAKE

A fish cake is a culinary dish made primarily from fish, often combined with other ingredients such as potatoes, breadcrumbs, and various seasonings. Fish cakes are typically formed into patties or balls and can be pan-fried, deep-fried, or baked. The exact ingredients and preparation methods can vary widely, and different cultures have their own versions of fish cakes.

Biologically, fish have significant importance in terms of nutrition and the ecosystem.

The importance of fish cakes lies in its culinary and cultural significance, offering a delicious and versatile way to enjoy fish.

Nutritional Value:

Fish are a rich source of high-quality protein, essential amino acids, vitamins (such as vitamin D and B vitamins), and minerals (such as iodine, selenium, and zinc).

Omega-3 fatty acids, found in fatty fish like salmon and mackerel, are crucial for brain health, cardiovascular health, and overall well-being.

Regular consumption of fish is associated with various health benefits, including reduced risk of heart disease, improved cognitive function, and support for healthy growth and development.

Fish is a valuable protein source that is easily digestible and contains all essential amino acids needed by the human body.

Fishing and the fishery industry contribute significantly to the global economy. It provides employment opportunities for millions of people worldwide.

Fish are an integral part of the diets and cuisines of many cultures around the world. Dishes like fish cakes showcase the versatility of fish in culinary creations.

Creation of dishes like fish cakes is just one way in which humans have harnessed the biological benefits of fish for culinary enjoyment and nutritional well-being. It's important to approach fish consumption with an awareness of sustainability to ensure the continued health of fish populations and their ecosystems.

3.1.7 FISH SAUCE

Fish sausages are prepared from minced fish flesh that are stuffed into a prepared intestine or similar other casings.

Fish sauce is a fermented product. Fish sauce is a very popular product in all south East Asian countries like Thailand, Malaysia, China, Vietnam and Philippines. Fish sauce is not a popular item in India.

Spices include salt, sugar, chillies, onion, coriander, glutamate, egg- white, hydrogenated vegetable oil, etc. Additives comprise antiseptics and antioxidants (ascorbic acid) to prevent rancidity. Colouring agents also may be added. These products are commercially manufactured.

The CODEX Committee on Fish and Fishery Products has defined fish sauce as “a clear liquid product with salty taste and mild fish flavour obtained from natural fermentation of a mixture of fish and salt”. (CX/FFP 08/29/ 9). The product is prepared by mixing fish, water and salt and kept in covered containers or tanks. The fermentation of the mixture is left at ambient temperature for a period not less than 6 months and the liquid is obtained as a result of protein hydrolysis. Optional ingredients (e.g. sucrose or caramel colour) may be added to the final product.

One advantage of sauce production is that fish of all types and slightly spoiled ones can also be used for sauce making. The production is popularly done in glut season when large quantity is landed. It is one of the best ways of preserving fish. Ageing is an important step in sauce production. This aroma differs from species to species.

3.1.8 FISH SALADS

Fishes are cleaned and pieces are boiled with steam. The boiled fish or prawns are mixed with tomatoes, salt, garlic, maida, pepper and oil to prepare fish salad. This can be used in fresh condition or can be stored.

Steps:

- ✚ Heat the oil in a large skillet, over medium-high heat.
- ✚ Add the fennel, shallots, salt and pepper and cook, stirring often, until the fennel begins to soften, about 6-8 minutes.
- ✚ Add the garlic and cook for an additional 2 minutes.
- ✚ Transfer to a large bowl and refrigerate until cool.
- ✚ Meanwhile, bring a large stockpot, filled with salted water, to a rolling boil, and prepare an ice bath.
- ✚ Add the shrimp to the boiling water and poach until cooked through, about 1 minute to 90 seconds. Quickly transfer to the ice bath.
- ✚ Once the water returns to a rolling boil, add the calamari and cook until tender, about 30-45 seconds. Quickly transfer to the ice bath.
- ✚ Once the water returns to a rolling boil, add the scallops and cook until opaque throughout, about 45 seconds to 1 minute. Quickly transfer to the ice bath.
- ✚ Line a sheet pans with paper towels. Using a spider, transfer the seafood to the sheet pan to drain as much excess water as possible.
- ✚ Add the drained seafood to the bowl with the fennel mixture.
- ✚ Add the parsley, vinaigrette and olives and toss to combine.
- ✚ Refrigerate until ready to serve.

3.1.9 FISH POWDER/ FISH FLOUR (HYDROLYSED PROTEIN):

Fish flour is a superior quality fish meal, produced under strict control and care, and forms an ideal protein supplement for human diet, even for infants of 3-4 months old. On commercial scale, it is produced by a sophisticated solvent extraction process.

The process is complicated and is not expensive. Fish is chopped and washed. It is then boiled with dilute acetic acid at 80°C. The mass is then washed thoroughly and the water is pressed out.

This is then treated with petroleum to remove fat and also to increase its keeping quality. The mass, thus, obtained is then hydrolysed with an alkali, preferably with caustic soda (10% at 80°C). The entire mass is then liquefied when it is neutralised with acetic acid (85%). The liquid is then dried

with a spray, which subsequently forms a dry, cream coloured powder.

Use of fish flour:

- ✚ It is considered as an ideal protein source to supplement diet for both adults and infants.
- ✚ It is used to enrich bakery products such as bread, biscuits, cakes and soup.
- ✚ As the product contains high protein (35%), it is very suitable for convalescing patients struck with malnutrition, anaemia, etc.

3.1.10 PET FOOD FROM TRASH FISH

The portion of the catch that is unsuitable for human consumption and includes diverse species originally trashed or discarded. Trash fish includes juveniles, slightly spoiled catch, species that we don't want to eat and endangered species.

Nowadays trash fish is sold to fish meal and fish oil (FMFO) industries to add nutrition to feed. India's bottom trawl fisheries now rely on trash fish (instead of fish we eat) for income. By feeding FMFO to our pets and farmed fish, we are encouraging unsustainable fishing practices.

We are also threatening the livelihoods of traditional and small-scale fishers.

3.1.11. FISH MANURE

Fish manure and guano are inferior quality of fish meal. It is not fit for animal consumption. Fish manure is a by-product of the curing yards, fish glue industries and oil extraction plants, where trash or spoilt fishes have been employed. Mackerel, horse mackerel, sardine, etc., which are spoilt and unfit for use as food is used for preparation of fish manure.

This manure has a high content of nitrogen (5-7%), phosphates about 4-6% and lime (Ca O) 1-5%. For such nutrient content, fish manure is useful in raising coffee, tea and tobacco crops.

Fish guano is the by-product of the body oil extraction plant which is the dried refuse left after the oil is pressed out. Here oil-bearing species such as oil sardines are used as raw material. Fish guano contains high concentration of nitrogen (8-10%) and appreciable quantities of phosphoric acid. For

this, guano is several times more effective than any animal manure.

3.2 FISH BY-PRODUCTS

3.2.1 FISH GLUE

Fish glue is a good adhesive obtained from trimmings, bones and skin of fishes belonging to order — Gadiformes (Cods, Pollack, Hakes, etc.). The raw materials are washed, chopped and steam-heated in steam-jacketed cookers.

The mass is then covered with water and to it is added small quantity of acetic acid. It is then cooked for 6-10 hours. The liquor is extracted and concentrated to form glue. The residue is dried and is used as manure.

Fish glue uses

- ✚ Smearing the backs of glued stamps and labels.
- ✚ Glue obtained from cod is of better quality and is used in photo-engraving.
- ✚ Low quality fish glue is used as adhesive for paper boxes, shoes and other things like furniture where joining is required.

3.2.2 ISINGLASS

Isinglass is a gelatine-like material obtained from the swim bladder or air-bladder of certain fishes. When put in water, it swells up but does not dissolve in it. At high temperature it hydrolyses in water to produce strongly adhesive gelatine.

Swim bladder is a hollow sac, the outer layer is thick and fibrous, while the inner one is thin, often with a silvery lustre. The thin, inner silvery shiny layer of the air-bladder of some fishes, such as sturgeons, carps and catfishes, is used to derive isinglass.

For preparing isinglass the air-bladders are collected, washed thoroughly to remove blood and other extraneous matters. Then the outer thick and fibrous layer of the wall is separated from the inner layer, which is exclusively isinglass raw material. This is then sun-dried and marketed.

Isinglass uses:

- ✚ Isinglass is used mainly for clarification of wine, beer and vinegar.
- ✚ It is used for the preparation of special grade cement and plaster.
- ✚ Formerly it was used as a substitute for gelatine in confectionery.

3.2.3 CHITOSAN

Chitin meant for deacetylation to chitosan is to be either dried or centrifuged or pressed well to remove as much water as possible.

The deacetylation is done by heating at 90-95°C with 40% (w/w) caustic soda for 90 – 120 mts. The water present in the chitin cake should also be taken into account while preparing caustic soda solution. To achieve this 50% caustic soda is prepared and calculated quantity of it is added to the chitin cake. The reaction is followed by testing the solubility of the residue in 1% acetic acid. As soon as complete desolution is reached the caustic soda is removed from the reaction mixture. The drained caustic soda can be reused for the next batch of deacetylation by fortification if necessary. The residue is washed with water free of alkali.

It is then centrifuged and dried in the sun or an artificial dryer at a temperature not exceeding 80°C and pulverized to coarse particles.

Chitosan is almost colourless, light in weight and soluble in dil. Organic acids but insoluble in water, alkali and organic solvents. It gives viscous solution when dissolved in dil. Organic acids such as formic acid, acetic acid etc.

Chitosan applications:

- ✚ Food industries
- ✚ Pharmaceutical applications
- ✚ Chemical industries
- ✚ Dental and surgical uses as a heamo-static agent
- ✚ Wound healing
- ✚ Biodegradable films as a substitute for artificial skins
- ✚ For removal of toxic heavy metals like lead, mercury, cadmium etc. from industrial effluents.

- ✚ Wine clarification
- ✚ Industrial effluent flocculation
- ✚ Agriculture
- ✚ Photography
- ✚ Cosmetic applications
- ✚ Textiles
- ✚ As a human health diet etc.

The above listed are only some broad areas of developed uses of chitin and chitosan. There are more than 300 products containing chitosan in the international markets and more applications are under investigation. It is likely that this waste from shrimp may fetch more money than the shrimp meat in future.

3.2.4 PEARL ESSENCE

A lustrous substance is present in the epidermal layer and on the scales of most pelagic fishes, such as sardine and mackerel. The lustrous effect is due to the presence of an organic compound, guanine, (2-amaino, 6-oxypurin) a constituent of cell membrane.

The crystalline guanine can be extracted from the fish scales. The suspension of guanine crystals in a suitable solvent is called “Pearl essence”. When the particles are deposited on the inside of hollow beads or outside of solid one, they produce an optical effect very similar to genuine pearls. There is good demand for the product in countries like Japan.

3.2.5 SHARK FINS

Shark fins are in great demand particularly among the Chinese, for making the ceremonial dish called shark fin soup. Dried shark fin is an item of export from India mostly to Singapore, Hongkong and the United Kingdom.

The commercial value of the fins depends on their colour, size, variety and quality. Depending on the quality and quantity of rays present in the fins they are broadly classified into two varieties, generally known as black and white. The black fins usually fetch a lower price than the white fins. Fins are generally marketed in dried form.

The preparation of shark fin does not require any elaborate treatment, but care is needed in cutting, trimming and drying operations.

Fins are cut from sharks of about 125 cm or more in length as soon as they are landed avoiding as much flesh as possible and are washed thoroughly in water after removing the adhering flesh. They are then dusted with salt in the ratio 1:10 (salt to fin), the cut portion being sprinkled liberally with salt. A little lime also is often sprinkled at the cut portion and the fins are set aside for 24 hours. They are then dried in sun after gently rinsing with clean water to remove solid salt and excess lime to a moisture content not more than 10%. The dry fins are graded according to the size and type of the fin.

3.2.6 FISH LEATHER

The skin of larger fishes is collected, soaked in brine and left for a day. On the next day it is salted and again put in brine containing 10% hydrochloric acid. The skin is then taken out, drained and scraped on the surface, particularly to remove fine denticles present in the skin of sharks. They are then limed and tanned by the usual process.

Fish leather has various uses, such as:

- ✚ Natives of old age used the dried and spiny skins of Globe fishes or Porcupine fishes for making war helmets.
- ✚ In Japan the dried skin of Puffer fishes is inflated and used to make lanterns.
- ✚ Now-a-days the skins of larger fishes, such as cod, salmon, halibut, toadfish, sharks and rays are tanned and marketed as ornamental leather and can be dyed in different colours.
- ✚ Fish leather, particularly of sharks, is used in making shoes, wallets, bags and tobacco pouches.
- ✚ The skin of sharks, ray, skates are very hard and is used as an abrasive for polishing wood or metal.
- ✚ Suitably prepared and dyed skin of sharks providing 'Shagreen' issued for covering card cases, jewellery boxes, sword scabbards, etc.

3.2.7 FISH MAWS.

These are also known as the Air-Bladder or Swim-Bladder in Fish. The primary function of this Air-Bladder is to act as a float to maintain its position in the water. In some fishes, the air bladder is an accessory to the gills, and in others it acts as a true lung, enabling them to breath. In some, the air bladder has some connection with the organ of hearing and in yet others, it has considerable vocal & sound producing properties. In the western world, it is also known as ISINGLASS, which is used in clarifying beverages, as an adhesive base in confectionary products, currencies, in Indian ink and for glass, pottery & leather.

UNIT IV: SANITATION AND QUALITY CONTROL

4.1 Sanitation in processing plants - Environmental hygiene and Personal hygiene in processing plants.

4.1.1 SANITATION IN PROCESSING PLANTS - ENVIRONMENTAL HYGIENE

Fish processing involves facilities along with other processes which includes receiving, washing & chilled storage, pre-processing-dusting, battering, breading, freezing cooking, curing, freezing, or making fish products.

Fish processing uses large quantities of water and generates wastewater which includes significant amounts of organic matter such as fat, blood, manure, hair, feathers, and bones. This wastewater can also contain disease-causing organisms, bacteria, parasite eggs, oil, grease, salt, nitrogen and ammonia compounds, phosphorus, and chlorine.

Air pollution generated by fish processing can include particulate matter, volatile organic compounds, and hazardous air pollutants. Other by-products of processing include odours, noise, and solid waste for treatment or disposal.

"No Objection Certificate" to be obtained from Municipality or Panchayat or applicable local bodies before grant of license. "No Objection Certificate" from the pollution control board of the State has to be obtained.

License/Registration under FSS Act' 2006 as per the Food Safety and Standards (Licensing and Registration of Food Businesses) Regulations, 2011.

Selection of Location & Surrounding Area

Fish and Fish Processing Industry shall be located away from:

- ✚ Environmentally polluted areas and industrial activities which produce disagreeable obnoxious odour, fumes, excessive soot, dust, smoke, chemical or biological emissions and pollutants, and which pose a serious threat of contaminating food.
- ✚ Areas subject to flooding, otherwise measures should be taken to locate the premises at elevated level in a sanitary place
- ✚ Areas prone to infestations of pests;
- ✚ Areas where wastes, either solid or liquid, cannot be removed effectively;

Fish and Fish Product Processing Units

- ✚ It shall permit adequate maintenance, cleaning and/or disinfection, prevent any potential contamination, and provide adequate working space to allow good manufacturing and hygienic practices for all the operations.
- ✚ It should have adequate drainage and easy provision for cleaning.
- ✚ All drains and gutters shall be properly and permanently installed.
- ✚ Drain or Storm water shall be prohibited to enter the premises.
- ✚ Access should be controlled, and site boundaries shall be clearly identified.
- ✚ Access to animals other than intended for slaughter shall be restricted.
- ✚ The external area of the site shall be maintained in good order to avoid any potential contamination like pest harbourage and infestation.
- ✚ Roadways and areas serving the establishment which are within its boundaries or in its immediate vicinity shall have all weather surfaces suitable for wheeled surface to allow ready transport of animals.
- ✚ There should be adequate facilities for the ETP and location of ETP shall be at such a distance as to avoid the possibility of contamination.
- ✚ The facility shall be used for the purpose that it is meant for.
 - ✚ The location should be preferably near to fish landing site, or accessible by road very easily.
 - ✚ There shall be continuous provision of sufficient electrical power supply with proper back up to maintain the cold chain and to operate freezers and cold stores

General Requirements

- ✚ The establishment shall be such so that there is sufficient protection from the environment and shall be of sufficient size for the work to be carried out under hygienic conditions.
- ✚ The design and layout shall be such as to preclude contamination and protect against accumulation of dirt, contact with toxic materials, shedding of particles into fish products and formation of condensation or undesirable mould on surfaces.
- ✚ Layout of different sections shall be such as to facilitate smooth and orderly flow of work and to prevent possible cross contamination of finished product from raw materials.
- ✚ The layout shall also ensure sufficient space in different sections for machinery, equipment, personnel etc. without congestion. Also, flow of drain should be opposite to the flow of the production.
- ✚ Non-operative areas (residential areas, machine room, toxic chemical storage areas, dry storage of food grade chemicals and packaging materials, offal and waste storage areas) inside the establishment shall be properly separated from operative areas (fish products handling areas) to avoid possible cross-contamination.
- ✚ The processing areas should have separation between clean and dirty sections and should be organised.
- ✚ Adequate working space for satisfactory performance of all operations
- ✚ There shall be a raised platform for receiving the material and the sides and roof of the platform shall be sufficiently protected from extraneous contamination.
- ✚ Separate and adequate facilities should be provided to prevent contamination: Hazardous substances; Cleaning chemicals Dry storage of materials, packaging etc.

EQUIPMENT AND CONTAINERS

Equipment, Utensils and Machinery that come in direct contact with fish and fish products shall be hygienically designed, constructed, located and, if necessary, installed to ensure that they can be adequately cleaned, sanitized and maintained to avoid contamination.

Equipment's and containers in contact with exposed fish and fish products should:

- ✚ have smooth impervious surface,
- ✚ be resistant to corrosion.

- ✦ made of material which is non-toxic,
- ✦ does not transmit odour or taste,
- ✦ is free from pits and crevices
- ✦ be non-absorbent,
- ✦ be of Food grade material
- ✦ capable of withstanding repeated exposure to normal cleaning and disinfection, be easily cleaned, disinfected and maintained.
- ✦ Permanently mounted equipment shall either be installed sufficiently away from wall and above the floor.
- ✦ Adequate supply and / or water storage facilities
- ✦ Adequate supply of potable water with appropriate facilities for its storage and distribution should be available wherever necessary to ensure the safety and suitability of food.
- ✦ Own supply of fresh water or other water sources and use chlorine, the residual content of chlorine should not exceed that of potable water.
- ✦ Surface of Water tanks shall be easily cleanable & Sanitizable
- ✦ Storage tanks and water pipes should be protected against contamination. They shall be adequately designed, made of material that is on toxic, corrosion resistant material, and periodically cleaned and maintained.
- ✦ The records of the same shall be maintained. The tanks shall be covered to prevent access by pests and other extraneous matter.
- ✦ Where water filters are used, they shall be regularly monitored or effectively maintained.
- ✦ Water including ice or steam (including culinary steam) used as a product ingredient or in contact with fish and fish products or used for equipment and plant cleaning shall be potable and should not constitute a threat to the safety or suitability of the product.
- ✦ Provide sufficient supply of hot water wherever necessary in the establishment.
- ✦ Non-potable water can be used for cleaning of those equipment which does not come in contact with food and food contact surfaces. It can also be used for firefighting, refrigeration equipment, lavatory etc.
- ✦ Non-potable water pipes shall be clearly distinguished from those in use for potable

water and shall not connect with, or reflux into, potable water systems.

- ✦ Colour coding is recommended for different type of water lines.

4.1.2 SANITATION IN PROCESSING PLANTS PERSONNEL HYGIENE FACILITIES

Fish and Fish Product handlers shall maintain a high degree of personal cleanliness with adequate, suitable and clean:

- Protective clothing,
- head cover,
- face mask
- gloves (if worn) and
- gumboots

• Workers shall be provided with neat, clean and hygienic uniform/protective gears. The facility for cleaning and sanitation can be in house or outsourced.

• The personal protective clothing shall be worn in such an order to avoid any cross-contamination of dust/dirt, etc.; i.e. starting from head to foot. Head caps/headgears to be worn first and foot wears to be worn at the last.

• The facility for cleaning and sanitation can be inhouse or outsourced.

Hand washing facilities

• Hand washing facility should be provided with potable water at adequate temperature, fitted with dispensers for liquid soap and other hand cleansing agents (sanitizer) and suitable hygienic means for drying hands.

• of Taps non-hand



operable type are preferable like foot/elbow/sensor/knee /Automatic type etc.

Hand washing and drying facility

- Non- Perfumed liquid soap should be used.
- Where hand driers were installed should be in working condition at all time during working hours.



- Where paper towels are used, a sufficient number of dispensers and receptacles should be provided near to each washing facility.
- Dustbins to throw used paper towels should be foot-operated.
- Posters directing personnel how to wash their hands effectively near hand wash stations.
- Hand sanitizer should be provided and should be used after drying of hands. This is the next step of disinfecting hands after cleaning.

Provision of toilets:

- Sufficient number of latrines, urinals, for each gender shall be provided. Generally, 1:25 is followed for facility: employee ratio
- Toilets should be well lit and ventilated and should not open directly on to food handling areas.
- Adequate supply of water should be provided in toilets and urinals.
- Potable water should be used at the toilet wash basin stations

Changing rooms/lockers:

•



Suitable and sufficient facilities (for e.g. lockers, gum boots stand, apron stand etc.) for persons working in the fish processing units should be provided for changing their clothes, keeping their personal belongings and Street footwear.

- Adequate facility for lockers shall be provided. Separate lockers should be provided for home personal clothes and

company



uniforms. Lockers shall be made of such material that can be cleaned and sanitized effectively.

- Foot dip to be present at the entry level of the processing unit (Recommended Chlorine concentration – 100-200 ppm depending on the hygiene requirement of the establishment).

Rest and refreshment room

- Rest & Refreshment Rooms shall be separate from other areas.
- These areas shall not open directly to fish handling areas,
- Only personal belongings to be kept in lockers,

Note: A display board mentioning 'Dos' and 'Don'ts' for workers should be posted in a prominent place inside the premises, in English or local language, for all to understand

4.2 Quality Control of fish and fishery products – pre-processing control, control during processing and control after processing.

4.2.1 PRE-PROCESSING CONTROL

Pre - processing control is important in the following sections or areas of the processing units they are

1. Receiving of Incoming Material
2. Storage of Raw Material & Packaging Materials

Receiving of Incoming Material

- There should be written specifications/quality standards for all incoming materials –ingredients and packaging materials.

- Suppliers of raw materials and ingredients should be evaluated regularly and must be FSSAI registered/ Licensed.
- Materials on receipt shall be inspected and those complying with the specifications should be accepted into the processing facility and non-conforming materials should be rejected and proper records should be maintained thereof.
- Immediately on receipt, Fish/Shell Fish shall be washed or cleaned to remove soil or other contaminants using potable water.
- Recommended that temperature of fish at the time of delivery should be in the range of 0°C to +4.0°C in case of chilled and -18°C or below in case of frozen.
- All packaged raw materials shall be checked for 'expiry date'/'best before'/'use by date'.
- The incoming vehicles that bring the raw material should be checked for cleanliness and hygiene i.e. the trucks are clean, with no pests or dirt, with no strong odour and does not contain materials other than the raw material.
- To prevent cross contamination, the raw material receiving station shall be separate with adequate space to receive and store raw material in chilled conditions.
- Aqua Inputs used in aquaculture farms should be on CAA approved list and Aqua Feed from BIS.
- For e.g. In case of Shrimps: to be procured from farmers registered under:
- Marine Products Export Development Authority (MPEDA),
- Coastal Aquaculture Authority (CAA) or
- State Fisheries Department.



Receiving of Incoming material

Storage of Raw Material & Packaging Materials

- Fish/ Shellfish should be stored such that damage from over stacking or overfilling of boxes will be prevented and should be kept in shallow layers surrounded by sufficient finely divided ice or with a mixture of ice and water before processing.
- The temperature of the fish should be maintained between 0°C and +4°C in case of chilled and -18°C or below in case of frozen.
- Ingredients and packaging materials should also be stored appropriately in terms of temperature and humidity and protected and segregated to prevent cross contamination.
- A fish processing establishment shall store raw material and packaging materials in appropriate dry and ventilated areas for effective protection from dust, condensation, drains, waste and other sources of contamination during storage.
- Packaging material storage room should be closed from all sides to restrict entry of flies, rodents, birds, insects/pests etc.
- Storage of raw material/ ingredient, /packaging material shall be done as per FIFO (First in First Out) / FEFO (First Expire First Out) stock rotation system, as applicable.
- The food materials/ ingredient/ packaging material shall be stored on racks/ pallets such that they are stored off the floor on pallets and off the walls to ensure easy and adequate cleaning and prevent harbouring of any insects, pests or rodents.
- The storage of raw, processed, semi processed, rejected, recalled or returned materials or products, shall be made separately and properly segregated. These areas shall be marked for identification and shall be secured.
- All raw materials/food additives and ingredients shall be stored separately from



Fish Storage



Packing Material Storage

printed packaging materials, sanitary, hardware and cleaning materials/chemicals.

4.2.2. FISH AND FISHERY PRODUCTS QUALITY CONTROL DURING PROCESSING

All operation in connection with the preparation or packing of fish and fish products shall be carried out under hygienic conditions.

- ✚ Particular attention needs to be given to temperature control.
- ✚ The temperature of the processing and packaging rooms shall be controlled so as to maintain the temperature of the product in chilled or frozen condition as desired.
- ✚ Special care of food safety control measures related to cooking, handling and packing shall be taken for cooked fish and fish products.
- ✚ Cooked fish and fish products are those products that are subjected to heat treatment, wherein minimum thermal core temperature of 75°C is achieved.
- ✚ The cooking should be adequate to eliminate and reduce hazards to an acceptable level which might have introduced at raw food level.
- ✚ Products which are heated below 75°C but above 60°C there may be a microbiological safety risk, such type of products shall be treated as semi- cooked products. These products shall be re-heated above 75°C before consumption. Special instruction shall also be given on the product label stating re-heating of the product above 75°C before consumption.
- ✚ Products which are exposed to heating but below 60°C shall be treated as raw processed products. Such type of products shall be cooked above 75°C before consumption. Special instruction shall also be given on the product label stating cooking of the product above 75 degrees Celsius before consumption.

ALLERGEN CONTROL PROGRAM (ACP)

Employee awareness through product and utensil identification

- ✚ Hand washing in between non-allergic and allergic materials
- ✚ Clothing- change of clothes wore while handling allergen materials.
- ✚ Rework control- Utmost care to be taken to handle allergen materials to avoid any accidental cross-contamination.
- ✚ Waste control- Allergen material wastes should not be allowed to pile up or spill which can result in environment cross contamination

CLEANING:

- ✚ Effective cleaning to avoid risk of cross contamination
- ✚ Cleaning of equipment shall be done before using same equipment's for allergen and non-allergen material.
- ✚ Cleaning schedule to be developed keeping in mind all the chances of cross contamination
- ✚ Regular cleaning of spillages of allergen materials throughout processing

PRODUCTION:

- ✚ Minimize movement of materials
- ✚ Scheduling of production runs with appropriate cleaning between the runs
- ✚ Physical barriers between allergen and non-allergen materials
- ✚ Schedule allergen containing product last in production plan or necessary cleaning shall be done during shifting of production from non-allergen containing products from allergen containing products.
- ✚ Control and trace reworked products

4.2.3 FISH AND FISHERY PRODUCTS QUALITY CONTROL AFTER PROCESSING

After processing of fishery products consists of two important stages i.e., packing and storage. Quality control parameters for those two stages include the following:

- ✚ Packing material should confirm FSSAI regulation i.e. Food Safety and Standards (Packaging and Labelling) Regulations, 2011 and regulation made there under.
- ✚ Packing should be covered and secured to prevent spoilage and contamination during transit and storage.
- ✚ Packaging section to be considered high care zone & access restricted & controlled via changing facility
- ✚ Clean protective clothing & footwear to be worn before entry
- ✚ Daily internal Calibration & recording of packaging equipment like weighing scales.
- ✚ Packaging Process steps to be performed without any delay to avoid contamination
- ✚ Non-toxic PM or gases to be used to not pose threat to the safety and suitability of processed product

LABELLING: Product labelling must confirm the requirement laid down by Food Safety and Standards (Packaging and Labelling)

Regulations, 2011 and regulation made there under.

- Product Label minimum should contain below information as required by FSSAI.



1. The Name of Food
2. List of Ingredients
3. Food Category number & Name
4. Nutritional information
5. Declaration regarding Veg or Non veg
6. Declaration regarding Food Additives
7. Name and complete address of the manufacturer with FSSAI Numbers
8. Net quantity in terms of Weight, Number or litres
9. Lot/Code/Batch identification
10. Date of manufacture or packing
11. Best Before and Use by Date
12. Country of origin for imported food
13. Instructions for use etc.

Fish and Fish Products Storage including Warehousing

- It is recommended to follow best practices for warehousing.
- All packed goods should be stored 18" away from walls, preferably stocks to be kept on pallets.
- The warehouses should be kept clean, ventilated, lighted and under hygienic condition to avoid pest infestation, dirt, dust, smell.
- Where specified for a particular kind(s), temperature and humidity control systems should be introduced and must be carried out with calibrated recording equipment with appropriate maintenance of records. For e.g. for chilled condition, it is 0-4°C and for frozen it is -18°C or below
- All end products should be stored at right temperature in a clean, sound and hygienic environment.
- Severe fluctuations in storage temperature (more than 3 °C) should be avoided.

- Too long storage time (depending on fat content of species used and type of coating) should be avoided.
- Products should be properly protected from dehydration, dirt and other forms of contamination.
- In chilled storages, direct blowing of cold air to iced fish in crates should be avoided to prevent dehydration of fish. Extra ice may be placed on the top crate along with proper covering.
- In the case of frozen storage there shall be suitable defrosting system and suitable arrangements to remove melt water from the frost.
- It shall be ensured that during defrosting, variation in temperature to be minimized so as to ensure that product temperature may not rise above -18°C.

UNIT V: Quality Assurance, Management and Certification

5.1. Seafood Quality Assurance and Systems: Good Manufacturing Practices (GMPs); Good Laboratory Practices (GLPs); Standard Operating Procedures (SOPs); Concept of Hazard Analysis and Critical Control Points (HACCP) in seafood safety.

Fish and fishery products are invaluable food commodity for human being. A high-quality protein and vital nutrients available resulting increment in consumption every year.

There are several quality assurance programmes evolved to keep fish and fishery products safe. Various kinds of quality standards like Codex standards, USFDA (United States Food and Drug Administration) standards, BSI (British Standards Institution) standards, FSSAI (Food Safety and Standards Authority of India) etc., HACCP (Hazard Analysis Critical Control Points) system of USA, European Commission Norms aimed to ensure safety and quality of fish and fishery products.

Quality control began in India as a pre-shipment inspection before the products were exported to the other country. In the initial stages of export of fishery products from India, heavy losses resulted since entire consignments were rejected by some countries due to bacterial contamination and spoilage. These prompted the Government of India to set up a fully equipped fish processing

technology laboratory at Cochin (Kerala) in 1957 to initiate research on quality control and thus provide the much-needed support for the seafood processing industry.

5.1.1 Seafood Quality Assurance Good Manufacturing Practices (GMPs)

Good Manufacturing Practice (GMP)/Good Hygienic Practices (GHP) GMP/GHP is defined as all practices regarding conditions and measures necessary to ensure safety and suitability (quality) of food at all stages of the food chain. The important elements of Good Manufacturing Practice are listed below:

Plant design, construction and layout:

- ✚ The plant design shall be such that the movement of edible materials from raw materials stage to the finished products stage is unidirectional and opposite to the movement of waste materials like solid wastes and liquid effluents.
- ✚ All materials used shall be water resistant, washable and with a smooth surface.
- ✚ Construction shall be such that there is no sharp corners, and all wall to wall, wall to floor and wall to roof joints are round and smoothened.
- ✚ The design shall take care to provide fly proofing of all external openings like doors, windows, ventilators, chute doors and drain outlets.
- ✚ Safety at drain outlets shall be such that there is no chance for any solid particles to go out as well as no fly can enter into the food handling areas.
- ✚ The plant will also need several electrical and mechanical fittings. All such items shall be washable and laid out in such a way that there is no scope for pest/microbial harbourage.

Machinery design, construction and layout

- ✚ Machinery shall be designed, constructed and installed to facilitate unidirectional movement of food materials and that the machinery is water resistant, washable and subitizable.
- ✚ All the machinery shall also be in a position to achieve criteria for good manufacturing practice.
- ✚ Equipment's for cooking shall be able to attain the validated cooking temperature and time without causing under or over cooking.

- ✚ Selection and installation of processing machinery in this way will exclude all possible health risks from machinery.

Provision for pest control

- ✚ Pest can be the cause for both dirt and contamination with microbes of public health significance.
- ✚ Exclusion of pests is best done by providing fly proof netting for all windows and ventilators as well as providing automatic air curtain and self-closing shutters for all doors and chutes directly opening to outside.
- ✚ There shall also be fly proof netting for drain outlets.
- ✚ To take care of any pest by-passing these facilities, there can be electrical fly catchers and rodent traps at strategic locations.
- ✚ For pest control, there shall not be any chemical-based pest control procedures.
- ✚ In case, there is any unusual fly population, fumigation with formaldehyde followed by defumigation with ammonia can be followed.

Personnel Involved in Production

- ✚ Workers or plant personnel are the most dynamic source of various type of microbial contamination in any food-processing establishment.
- ✚ In case of food materials from land and inland water bodies, there is every chance of occurrence of organisms of public health significance.
- ✚ But in case of seafood, the occurrence of Public Health Indicator organisms is a sure indication of poor hygiene and sanitation.
- ✚ To exclude such contamination from workers, all personnel in the production unit shall follow good hygiene practice.

Medical Fitness of workers-

- ✚ Medical examination to certify the workers is an exercise to be done without failure once in a year. Workers must be examined by a qualified medical practitioner to rule out that the worker is not suffering from any disease.
- ✚ Use of clean uniform including gum boots, head cover, face mask and gloves
- ✚ Removal of ornaments and other beauty aids
- ✚ Scrubbing of hands with soap and clean water
- ✚ Sanitized footwear using a foot dip containing 100 ppm
- ✚ Hand sanitizing by dipping the full palm of both hands in 20 ppm of chlorine water

Cleanliness of Direct/Indirect Food Contact Surfaces

The cleanliness of direct and indirect food contact surfaces is responsible for contamination of food.

There shall be identification and listing of all food contact and non-contact surfaces followed by a cleaning procedure and cleaning schedule.

All these operations are popularly known as SOPs—. The following are the main elements of SOP:

- Safety of the water that comes into contact with food or food contact surfaces, or is used in the manufacture of ice
- Condition and cleanliness of food contact surfaces, including utensils, gloves and outer garments
- Prevention of cross-contamination from insanitary objects to food; food packaging material; and other food contact surfaces including utensils, gloves and outer garments; and from raw product to cooked product
- Maintenance of hand washing, hand sanitizing and toilet facilities
- Protection of food, food packaging material and food contact surfaces from adulteration with lubricants, fuel, pesticides, cleaning compounds, sanitizing agents, condensate and other chemical, physical and biological contaminants
- Proper labelling, storage and use of toxic compounds
- Control of employee health conditions that could result in the microbiological contamination of food, food packaging materials and food contact surfaces
- Exclusion of pests from the food plant

Risk/Hazard Monitoring Facilities

The success of all the above processes and procedures in a food plant will depend on the facilities of the laboratory.

In fact, the laboratory shall have all test methods and testing equipment in par with the national and international requirements.

Traceability and recall procedures

A system for tracing all raw materials and finished products is a necessary component in a prerequisite programme.

No process is fail-safe and traceability that includes lot identification is essential to an effective recall procedure.

A crisis response plan should be in place to handle any incidents. Appropriate records of processing, production and

distribution should be kept and retained for a period that exceeds the shelf life of the product. Where there is a health hazard, products produced under similar conditions may be withdrawn.

The need for public warning should be considered. Once retrieved, products must be held under supervision until the manner of product disposition e.g. rework or destruction has been determined.

Training

All employees should receive documented training on personal hygiene, GHP, cleaning and disinfection procedures, product handling and protection, the HACCP-system and process control. Periodic refresher training should be part of the overall training programme.

Training in basic food hygiene is fundamentally important. All personnel should be aware of their roles and responsibilities in protecting fish and the fish products from contamination and deterioration.

5.1.2 Seafood Quality Assurance Good Laboratory Practices (GLPs);

Good Laboratory Practices (GLPs) Good Laboratory Practices (GLPs) define the rules and criteria for a quality system concerned with the organizational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded and reported. General Principles of the (good laboratory practices)

Management 's Responsibilities Most of the responsibilities of test facility management are of a general nature, such as the requirements that test facility management has to ensure the availability of qualified personnel and of appropriate facilities and equipment for the timely and proper conduct of the study.

Furthermore, it has to ensure that health and safety precautions are applied according to national and/or international regulations; appropriate Standard Operating Procedures are established and followed, etc.

b) Laboratory Head 's Responsibilities

The study director continues to be the single point of study control and has the responsibility for the overall conduct and reporting of the study. He/she should agree to the study plan and ensure that the procedures specified in the study plan are followed.

c) Personnel Responsibilities

Personnel should exercise safe working practice and health precautions; the chemicals should be handled with suitable caution until their hazard(s) has been established.

Personnel known to have a health or medicinal condition that is likely to have an adverse effect on the study should be excluded from operations that may affect the study.

d) Facilities

The GLP Principles mandate in general that test facilities should be of suitable size, construction and location to meet the requirements of the studies performed therein, and an adequate degree of separation should be provided between the different activities to ensure the proper conduct of each study.

e) Apparatus, material, and reagents

Apparatus should be suitably located, be of appropriate design and adequate capacity, and should be periodically inspected, cleaned, maintained and calibrated according to SOPs.

Apparatus and materials should not interfere with the test systems, and reagents should be properly labelled.

F) Test Systems

All the methods used by the lab shall be approved methods by national or international agencies, like BIS standards (Bureau of Indian Standards), EU (European Union) Norms, US FDA (United States Food and Drug Administration) Guides and Codex.

Under no circumstances, unapproved procedures shall be used for monitoring any process/quality parameter.

In case of physical/chemical test systems, the used apparatus should be properly located and have appropriate design and capacity.

Reference substances should be used to ensure the integrity of the test systems and considering biological test systems the housing, handling and care of animals, plants, microbial as well as other cellular and sub-cellular systems should be carried out under proper conditions to ensure the quality of the data.

Test and Reference Substances All the records referring to test and reference substances should be maintained; handling, sampling, and storage procedures as well as the test and reference substances should be identified.

The stability of test and reference substances under storage and test condition should be known.

5.1.3 Seafood Quality Assurance Standard Operating Procedures (SOPs);

SOPs contains details of in-plant procedures to be followed by a food processing firm to control, monitor and correct the key conditions and practices.

The objectives of SOP are to:

- ✚ Describe the procedures to be used in a food processing facility.
- ✚ Provide the schedule for the different procedures.
- ✚ Provide a training tool for employees.
- ✚ Identify trends and prevent re-occurring problems.
- ✚ Ensure understanding of acceptable operating practices by everyone involved from management to production workers.
- ✚ Provide a foundation to support a routine monitoring program.
- ✚ Encourage prior planning to implement corrections when necessary.
- ✚ Demonstrate commitment to buyers and inspectors.
- ✚ Establish improved operating practices and conditions in the plant.

Implementation of SOP

The processing plants should implement SOP to ensure quality of food being processed. The SOP developed should be specific to each processing unit and describe procedures associated with proper handling of food, cleanliness of the plant environment, and activities conducted to meet them.

Types of SOP

The SOP developed by the food processing facility can be of two types,

- **Informal SSOP**
- **Formal SSOP.**

Informal SOP outlines the frequency and procedures to be followed to control, monitor and correct deficiencies for a specific task/of plant operation.

Formal SOP is a written document in a standard format containing standard information for implementation of SOP at each stage.

Requirements of SOP

Irrespective of the type of SOP developed by a processing unit, it should meet two important requirements.

1. It should provide enough detail for someone to carry out the task in question.
2. The procedures used should accurately reflect the activities being conducted.

Standard Operating Procedures (SOPs) should be elaborated for test facilities, and there should be immediately available SOPs for each separate laboratory unit.

- Performance of the Study Prior to initiation of a study, a study plan should exist.

It should be retained as raw data, and the study should be conducted according to it.

The proper form and content of a study plan is specified in the Principles of GLP.



SOP in a shrimp processing plant

5.1.4 Seafood Quality Assurance -Concept of Hazard Analysis and Critical Control Points (HACCP)

Application of good manufacturing practices often fails to ensure zero tolerance required for many pathogenic microorganisms. The classical approach to microbiological quality control has relied heavily on determination of microbiological parameter of both raw material and product.

This requires too long time for getting results, offers no control over the process and often foods fail to meet quality standards. Thus, there is a need for fool proof approaches to ensure production of safe products.

The newer approach for ensuring product quality is through the adoption of **HACCP** concept as a method of choice for ensuring the safety of foods from farm to table.

HACCP: HACCP is a statement of prevention system of controls based on hazard analysis and critical control point.

HACCP involves application of more systematic rule-based approach for ensuring production of microbiologically safe food.

Hazard analysis: Hazard analysis involves identification of ingredients and products which might have pronounced effect on food safety.

HACCP was originally developed by **Pillsbury Company** to **produce zero defect food** for astronauts of NASA in USA.

Since 1973, it has been adopted in food industry to produce quality food by identifying stages of hazards and applying control points to reduce /eliminate hazards.

HACCP has been recommended by Codex Alimentarius and has become international reference system for food safety assurance.

Critical control point (CCP)

Any point or procedure in a food processing system where control can be exercised and hazard can be minimized or prevented. Therefore, loss of control over processing parameters would result in unacceptable risk to consumers.

Principles of HACCP

HACCP as a natural and systematic approach to food safety is based on seven basic principles.

These include,

1. Conducting hazard analysis.
2. Determining critical control points.
3. Establishing critical limit.

4. Establishing monitoring procedures.
5. Establishing corrective actions.
6. Establishing verification procedures.
7. Establishing record keeping and documentation procedures.

Conducting hazard analysis

This involves assessing hazards and risks associated with growing, harvesting, raw material, ingredients, processing, distribution, marketing, preparation and consumption of food. It helps to determine hazard which could pose threat to the safety of those consuming the product. Hence, hazards must be controlled by production process.

Determining critical control points

CCPs are identified after hazards are analysed. This involves identifying potential hazards and their occurrence in process line (where they occur), and measures to control them.

CCP is defined as a location, step or procedure at which some degree of control can be exercised over a microbial hazard thereby the hazard can be prevented, eliminated or reduced to acceptable levels

Establishing critical limits

A critical limit is one or more prescribed tolerances that must be met to ensure that a CCP effectively controls a microbiological hazard. The critical limits (with tolerance limits where appropriate) established should include physical parameters, chemical parameters, level of preservatives etc.

Establishing monitoring procedures

Monitoring of CCPs involve the scheduled testing or observation of a CCP and its limits. This helps to confirm and record that the control is maintained on CCPs. This will help in process verification, analyse trends which could lead to loss of process control and to take easy remedial measures.

Establishing corrective actions

This involves establishing corrective actions whenever deviations occur in CCP monitoring. Actions taken must eliminate hazard created by deviation in plan/process. The actions taken varies widely depending on the product being processed, and action taken must bring the CCPs under control.

Establishing procedures for verification

This involves establishing procedures for verification that the HACCP system is working correctly. Verification consists of methods, procedures and tests used to

determine that the system is in compliance with the plan.

Establishing effective record keeping system

Establishing effective record keeping system is necessary to document the HACCP plan. The HACCP plan must be on file at the food establishment and made available to official inspectors.

5.2 National and International standards – ISO 9000: 2000 Series of Quality assurance System, Codex Alimentarius

5.2.1 ISO 9000: 2000 SERIES OF QUALITY ASSURANCE SYSTEM

The International Standards Organization (ISO) is located in Geneva, Switzerland, and is a federation of national standard bodies representing almost 100 countries. Today, more than 50 countries have adopted the ISO 9000 series which is equivalent to the BS 5750 standards as just mentioned. In the United States, the standards are published as the ANSI/ASQC Q 90 series and in the European Community they are published as the European Norm (EN) 29000 Series.

ISO 9001, 9002 and 9003 are three specific standards describing the elements and requirements of a quality system to be implemented in a company in connection with a contractual situation, i.e. the supplier - customer relation.

The various elements of the ISO 9000 standards are shown in Table and shall be commented briefly in the following.

Management responsibility is the first and overall, most important system requirement mentioned. Responsibility and authority of all personnel who manage, perform and verify work affecting quality, shall be defined by the management, and adequate resources should be provided.

Quality System, refers to the documented system ensuring that products comply with the specified requirements.

Contract review it is laid down that the producer shall review and evaluate all contracts to ensure that he can supply a product which meets the customer's specified requirements and expectations.

Product development the supplier shall establish and maintain procedures which control and verify all phases of product development to ensure that the specified requirements are met.

Documentation is a vital part of the system, and so is Document Control. This control shall ensure that all necessary documents (procedures, instructions, forms, etc.) are available where needed, and obsolete documents promptly removed from all

Procedures for product identification and traceability during all stages shall be established, maintained and recorded as stated in. If required, each batch, package, etc. shall be provided with a unique identification which shall be recorded.

Process Control shall ensure that all processes influencing the quality of the final product shall be specified and documented to ensure and verify that they are carried out under controlled conditions.

A schedule for **testing and inspection** of raw materials, intermediate and final products shall be established. For the HACCP system, the schedule must be based on CCPs as identified in the hazard analysis. Test methods must be defined.

The **test equipment** used shall be selected to demonstrate acceptable compliance with the defined specifications for the products and shall be calibrated at regular intervals against nationally recognized standard references (Requirement No. 10).

Proper identification of the inspection and test status shall be ensured with untested, tested, approved or rejected products being clearly marked (Requirement No. 11).

Training is a vital part of the ISO 9000 standards.

Table: Following are the quality requirements for ISO 9000-2000 series.

Quality System Requirements		Contents
1	Management responsibility	Define and document commitment, policy and objectives, responsibility and authority, verification resources and personnel. Appoint a management representative and conduct regular reviews of the system
2	Quality system	Establish and maintain a documented quality system ensuring that products conform to specified requirements

3	Contract Review	Ensure that customer's contractual requirements are evaluated and met
4	Product development	Plan, control and verify product development to ensure that specified requirements are met
5	Document control	System for control and identification of all documents regarding quality, e.g. procedures, instructions, and specifications
6	Purchasing	Ensure that purchased products conform to specified requirements
7	Product identification and traceability	System to identify and control traceability of product at all stages from raw materials through production to the final product as delivered to the customer
8	Process control	Ensure and plan the control of production which directly effects quality by documented work instructions, monitoring and control of processes
9	Inspection and testing	Inspect and test incoming products, intermediate and final product; establish product conformance to specified requirements and identify non-conforming products; maintain inspection and test records
10	Inspection, measuring and test equipment	Selection and control of equipment to ensure reliability and accuracy in measuring data
11	Inspection and test status	For the whole process the products shall be identified and clearly marked concerning test status, including indication of conformance or non-conformance
12	Control of non-conforming products	Identification, documentation, evaluation, isolation (if possible) and disposition

		of non-conforming products
13	Corrective actions	Prevention of reoccurrence of failures (non-conformance)
14	Handling, storage packaging and delivery	Protection of the quality of the product during handling, storage, packaging and delivery
15	Quality records	Records, including those which demonstrate that the specified requirements have been met, shall be controlled and maintained
16	Internal Quality Audits	Regular, planned internal audits shall be carried out, documented and recorded to verify the effectiveness of the quality system
17	Training	Training requirements at all levels shall be identified and the training planned, conducted and recorded
18	Cleaning and Disinfection	Although not required by the ISO 9000 standards, these two points should be given special attention in all food companies
19	Personal hygiene	ISO- 9000 SOP for the personal hygiene is to be followed

5.2.2 Codex Alimentarius

International food trade has existed for thousands of years but until not too long-ago food was mainly produced, sold and consumed locally. Over the last century the amount of food traded internationally has grown exponentially, and a quantity and variety of food never before possible travels the globe today

The CODEX ALIMENTARIUS

International food standards, guidelines and codes of practice contribute to the safety, quality and fairness of this international food trade. Consumers can trust the safety and quality of the food products they buy and importers can trust that the food they ordered will be in accordance with their specifications.

Purpose of the Codex Alimentarius

The Codex Alimentarius is a collection of internationally adopted food standards and related texts presented in a uniform manner.

The publication of the Codex Alimentarius is intended to guide and promote the elaboration and establishment of definitions and requirements for foods to assist in their harmonization and in doing so to facilitate international trade.

Scope of the Codex Alimentarius

The Codex Alimentarius includes standards for all the principal foods, whether processed, semi-processed or raw, for distribution to the consumer.

Nature of Codex Standards

Codex standards and related texts are not a substitute for, or alternative to national legislation. Every country's law and administrative procedures contain provisions with which it is essential to comply.

Revision of Codex Standards

The Codex Alimentarius Commission and its subsidiary bodies are committed to revision as necessary of Codex standards and related texts to ensure that they are consistent with and reflect current scientific knowledge and other relevant information.

The Codex Alimentarius covers various aspects of food production, safety, and trade.

Codex Alimentarius process:

Initiation: The process begins with the identification of a need for a new standard or the revision of an existing one.

Assignment to Committee: The proposal is assigned to the relevant Codex Committee based on the subject matter.

Working Groups: Committees may establish working groups composed of experts from member countries to develop draft standards. These working groups meet to discuss and draft the proposed standards.

Consensus Building: The draft standards undergo a process of review and consensus building. Member countries provide input, and discussions aim to achieve broad agreement on the content of the standards.

Adoption: Once consensus is reached, the draft standards are presented to the full committee for adoption. Adoption requires a consensus among member countries, and the standards are then forwarded to the Codex Alimentarius Commission for final approval.

Approval by the Commission: The Codex Alimentarius Commission, which is the highest decision-making body, reviews and approves the standards. The Commission is

composed of representatives from member countries and meets annually.

Publication: Approved standards are published in the Codex Alimentarius, which is a collection of food standards and related texts. The publication is regularly updated to reflect new standards and revisions.

Implementation: Member countries are encouraged to incorporate Codex standards into their national regulations to ensure uniformity in food safety measures and facilitate international trade.

The Codex Alimentarius plays a crucial role in promoting food safety and fair practices in international trade by providing a basis for harmonizing standards and reducing barriers to trade.

Step 1

CAC approves suggested Project Document as **New Work** considering critical review from CCEXEC

Step 2

Proposed Draft Standard is prepared

Drafting & Elaboration

Step 3

Comments of circulated proposed draft standard are received from member countries

Step 4

Comments are reflected & **Committee** present the document to **CAC** for adoption at Step 5



Concluding & Finalization

Step 5

CAC adopts presented document at **Step 5** as **Draft Text**

Step 6

Comments of circulated Draft Text are received from member countries

Step 7

Comments are reflected & **Committee** present the document to **CAC** for final adoption at Step 8

Step 8

CCEXEC make **critical reviews** and **CAC** adopts presented document at **Step 8** as **Codex Standard**



Welcome: Dr. N. SREENIVAS (Self Published Author)

Book Details

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SREENIVAS- CHAKRAPANI- ANILKUMAR

POSTHARVEST TECHNOLOGY OF FISH & FISHERIES

PAPER VII A-ZOOLOGY



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II SEMESTER



BIOLOGY OF FIN FISH & SHELL FISH

(COURSE-IV)

LABORATORY MANUAL



Dr. SREENIVAS- Dr. CHAKARPANI-Dr. ANIL KUMAR

1. Length-weight relationship of fishes
2. Gut content analysis in fishes and shrimp
3. Mouth parts and appendages of cultivable prawns, shrimps and other crustaceans
4. Study of eggs of fishes, shrimps, prawns and other crustaceans
5. Study of oyster eggs
6. Embryonic and larval development of fish
7. Study of gonadal maturity and fecundity in fishes and shellfish
8. Observation of crustacean larvae
9. Study of nest building and brooding of fishes

Assessing fish length and weight is a crucial aspect of fisheries management, ecological studies, and aquaculture practices.

Protocol outlining steps for accurately assessing fish length and weight:

Equipment Preparation:

- Obtain a measuring board or tape measure calibrated in centimeters or inches.
- Have a scale capable of measuring the weight of the fish accurately. Make sure the scale is properly calibrated.
- Prepare a container or bag for temporarily holding the fish during measurement.
- Use gloves to handle the fish to minimize stress and potential harm to the fish.

Preparation of the Fish:

- Ensure the fish is fresh and handled carefully to avoid any damage.
- Rinse the fish gently with water if necessary, but avoid excessive handling to prevent removal of slime coat or scales.

Measuring Length:

- Place the fish on the measuring board or flat surface with its nose against the zero end of the measuring scale.
- Straighten the fish's body along the measuring surface, ensuring the tail is fully extended.
- Measure the total length of the fish from the tip of its snout to the tip of its tail fin.
- Record the length to the nearest millimetre or centimeter, depending on the precision required.

Weighing the Fish:

- Place the fish in the container or bag suitable for weighing.
- Calibrate the scale to zero with the container on it.
- Carefully place the fish in the container without causing harm or stress.
- Record the weight displayed on the scale to the nearest gram or ounce, depending on the scale's precision.

Data Recording:

- Document the species of fish, date, and location of the measurement.
- Record both the length and weight measurements accurately.
- Store the data in a secure and organized manner for further analysis or reporting.

Data Analysis:

- Analyze the collected data to identify patterns in fish size and weight distribution.
- Use statistical methods to infer population parameters and trends if conducting ecological studies.
- Compare the measurements with known standards for the species to evaluate growth rates and health.

Reporting:

- Prepare reports summarizing the findings from the length and weight assessments.
- Include details of the methodology used, sample sizes, and any limitations or uncertainties.
- Present the data in a clear and understandable format, using tables, graphs, or charts as appropriate.

Quality Control:

- Regularly calibrate measuring instruments and scales to ensure accuracy.
- Implement quality control measures to minimize errors in measurement and recording.
- Consider inter-observer reliability tests if multiple individuals are involved in data collection.

Ethical Considerations:

- Handle fish with care and minimize stress during measurements to ensure their welfare.
- Follow ethical guidelines and regulations regarding the treatment of animals in research and fisheries management.
- By following these steps, you can establish a robust protocol for accurately assessing fish length and weight, essential for various scientific, conservation, and management purposes.

Mathematical Modelling:

- Fish length-weight equations are mathematical models used to estimate the weight of a fish based on its length. These equations are species-specific and are often developed through empirical studies where lengths and weights of numerous individuals of the same species are measured and recorded. The relationship between length and weight is typically non-linear, and thus, length-weight equations are often expressed in a power law form:

$$W=a \times L^b$$

Where:

W is the weight of the fish (in grams or kilograms).

L is the length of the fish (in centimetres or meters).

a and b are constants specific to the species and the population being studied.

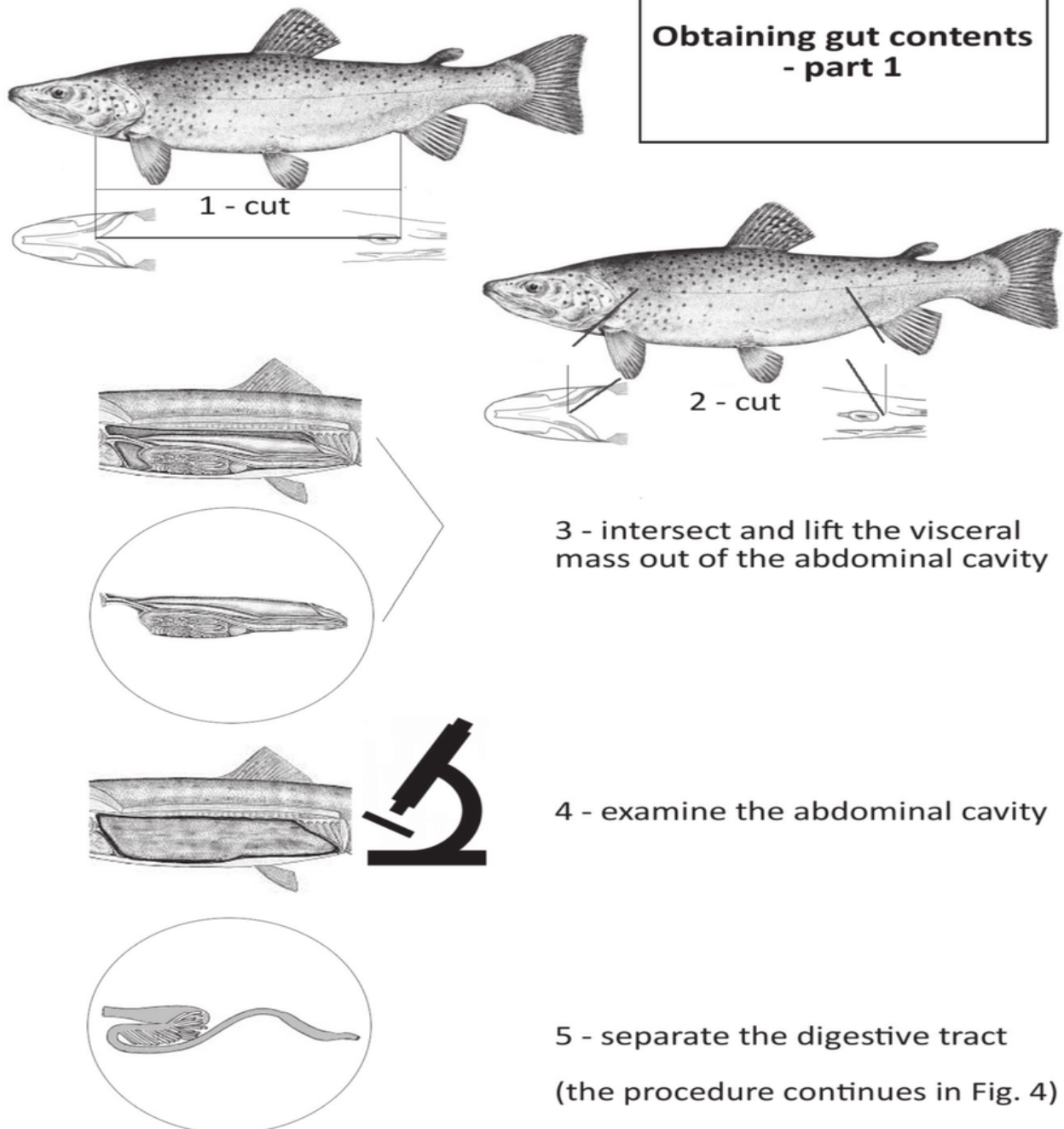
Interpretation:

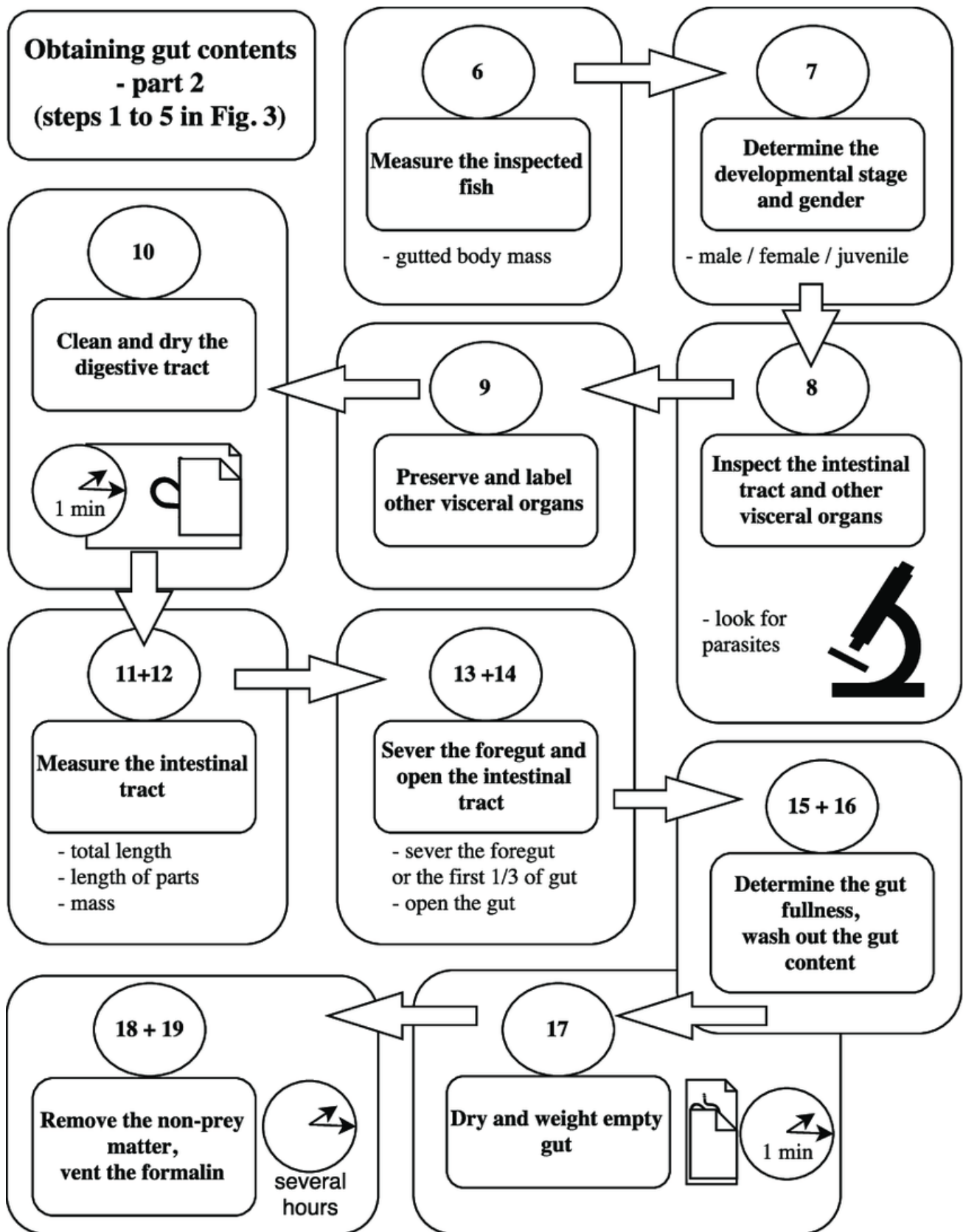
- Interpret the equation in the context of the species being studied.
- Pay attention to the value of b, which represents the allometric growth coefficient.
- A b value close to 3 indicates isometric growth (where weight increases with the cube of length), while values significantly different from 3 suggest either negative or positive allometry.

Reporting: Report the developed length-weight equation along with the estimated parameters and any relevant statistics (e.g., R-squared value, standard error of the estimate) in scientific publications or management reports.

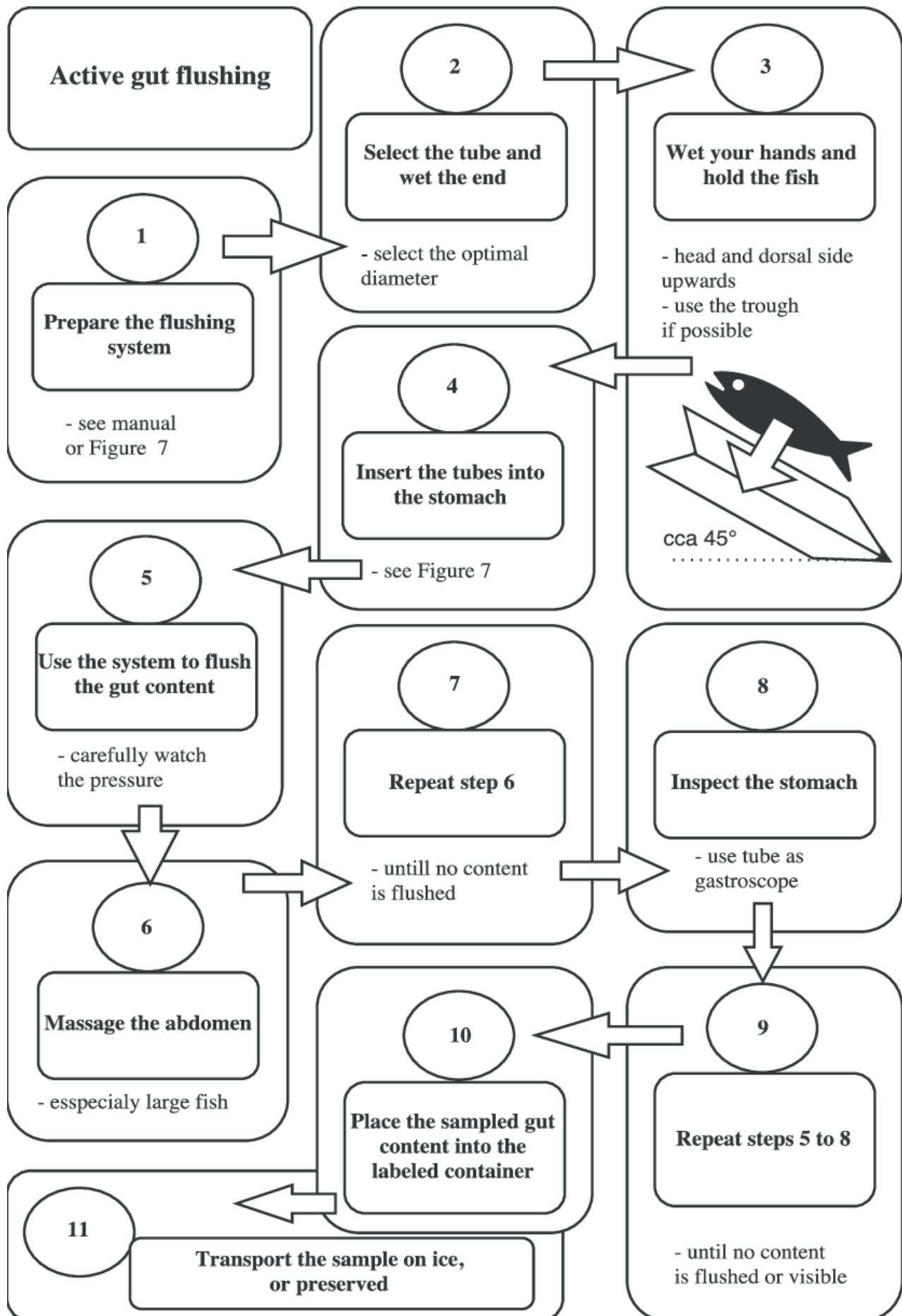
It's important to note that length-weight relationships can vary among populations and may change over time due to factors such as environmental conditions, habitat quality, and fishing pressure. Therefore, periodic updates or validation of length-weight equations may be necessary to ensure their accuracy and reliability for fisheries management and research purposes.

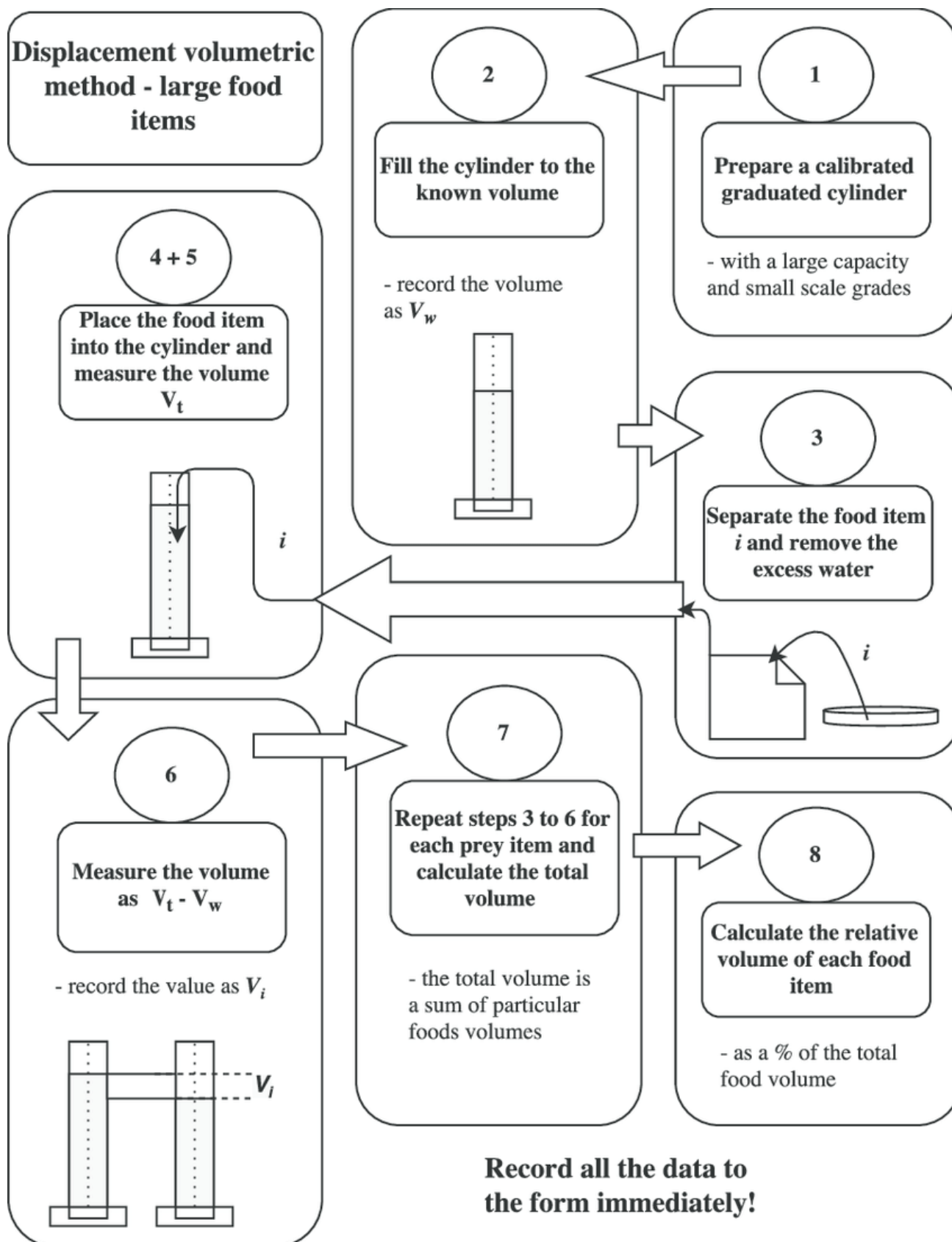
2. Gut Content Analysis of Fish:

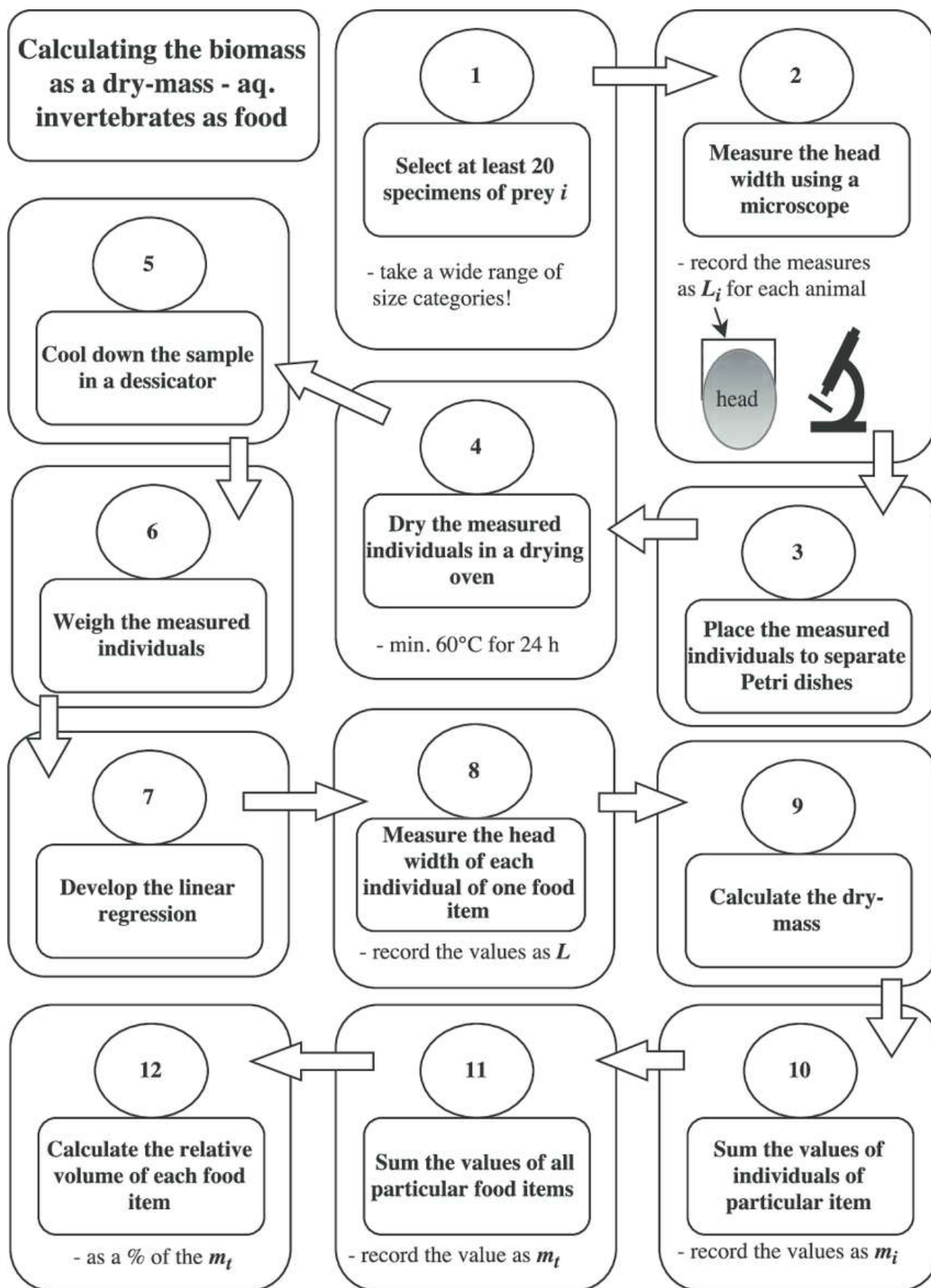


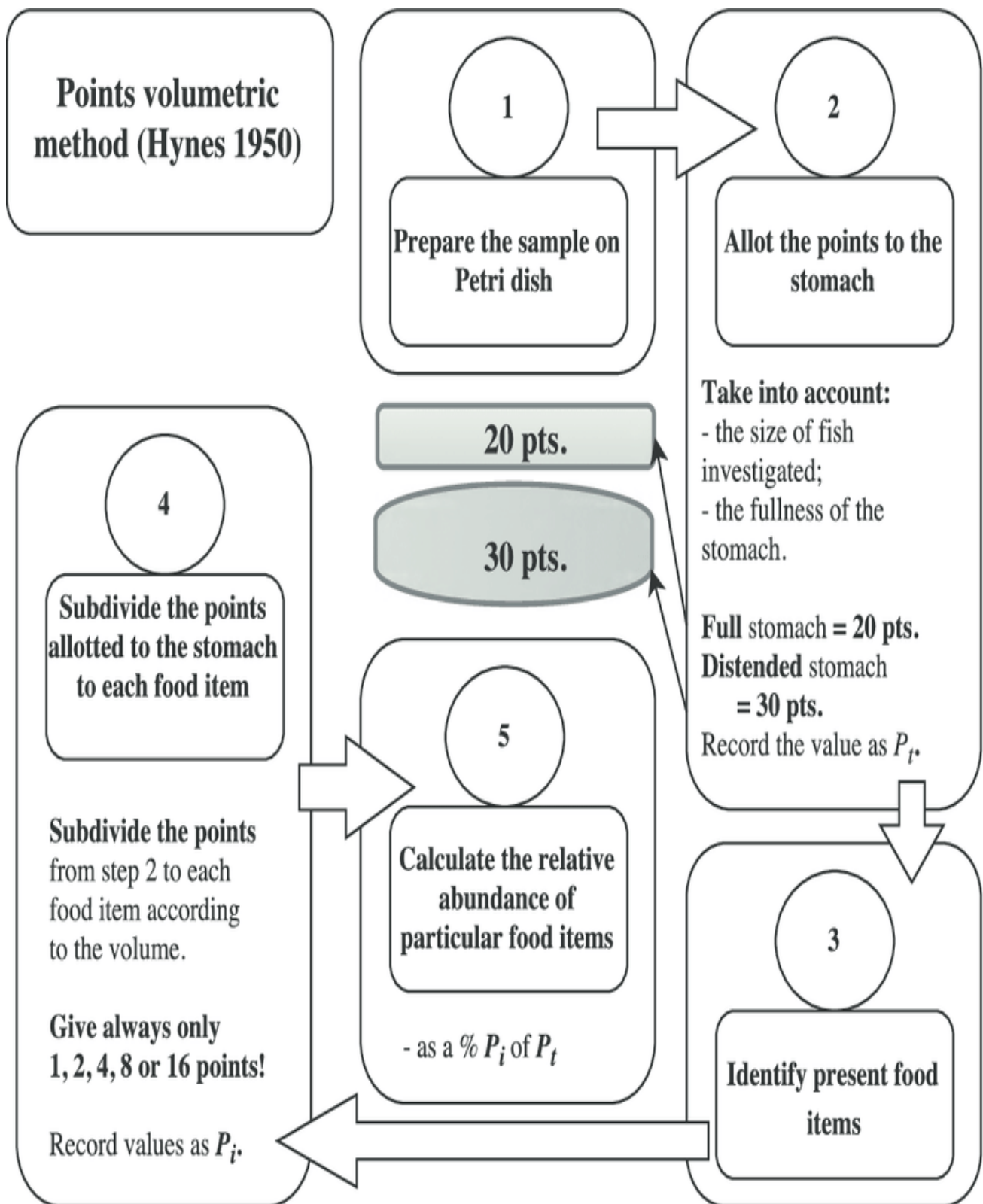


Record all the data in the form immediately!









2 B. Gut content analysis of shrimp:

"Gut content analysis of shrimp" typically refers to a scientific method used to study the diet of shrimp species by examining the contents of their digestive tracts. This analysis provides valuable insights into their feeding habits, ecological roles, and interactions within their ecosystems. Here's a basic protocol for conducting gut content analysis of shrimp:

Sample Collection:

Collect shrimp specimens from their natural habitat using appropriate sampling methods such as nets or traps. Ensure that specimens are handled carefully to minimize stress and damage to their digestive tracts.

Specimen Preservation:

Preserve collected specimens in a suitable preservative solution (e.g., ethanol) to prevent decomposition and maintain the integrity of gut contents.

Dissection:

In a laboratory setting, carefully dissect each shrimp specimen under a dissecting microscope or magnifying glass. Use fine dissecting tools to open the shrimp's abdomen and access its digestive tract.

Gut Extraction:

Extract the entire digestive tract from the shrimp's body cavity using forceps or tweezers. Handle the gut carefully to avoid rupturing or damaging it.

Content Removal:

Gently flush the digestive tract with a suitable flushing solution (e.g., saline solution or distilled water) to remove any loosely attached or semi-digested food particles.

Content Examination:

Place the extracted gut contents on a petri dish or microscope slide for examination. Use a stereomicroscope or compound microscope to observe and identify the ingested food items.

Food Item Identification:

Identify and record the types of food items present in the gut contents. This may involve visual inspection, morphological analysis, and reference to existing literature or taxonomic keys.

Quantification:

Estimate the quantity or abundance of each food item by counting the number of occurrences or measuring their dimensions (e.g., length or area) using image analysis software.

Data Analysis:

Analyze the collected data to determine the dietary preferences, feeding behaviors, and trophic interactions of the studied shrimp species. Statistical methods such as frequency of occurrence, percent composition, and diversity indices may be employed for data interpretation.

Reporting:

Compile the results of the gut content analysis into a scientific report or publication, including detailed descriptions of the methods, findings, and implications for understanding the ecology of shrimp populations and their ecosystems.

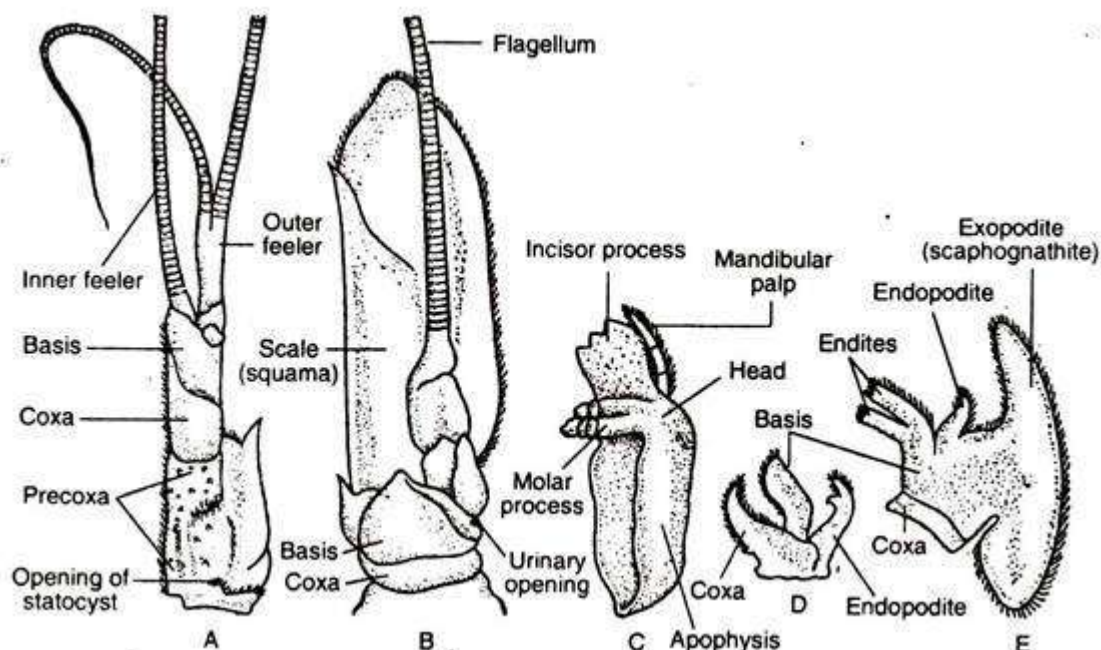
Precautions:

It's important to note that the specific details of the protocol may vary depending on the research objectives, target species, and available resources. Additionally, researchers

should adhere to ethical guidelines for animal experimentation and obtain necessary permits or approvals for collecting and handling shrimp specimens.

3. Mouth parts of (Crustacean) Prawn:

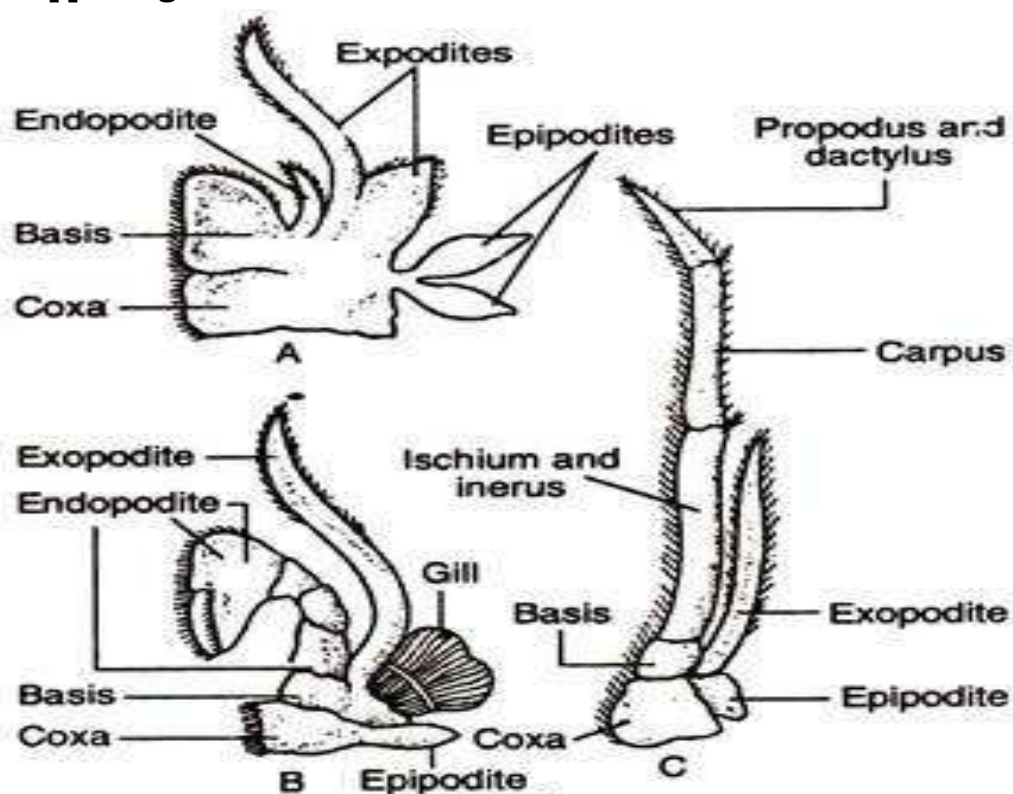
3.1 CEPHALIC APPENDAGES OF PRAWN



A. ANTENNULE B. SECOND ANTENNA C. MANDIBLE. D. FIRST MAXILLA E. SECOND MAXILLA

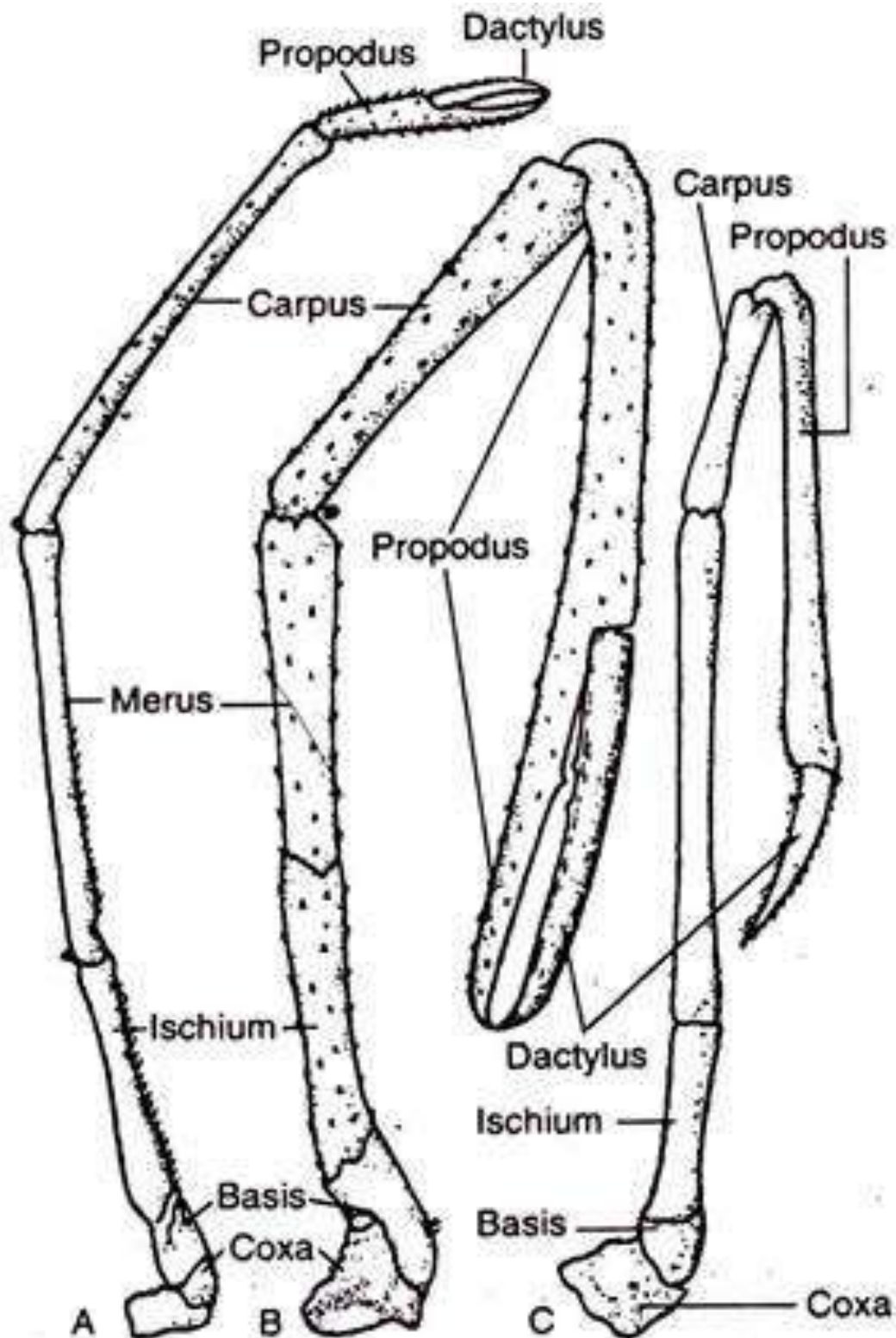
3.2 Thoracic appendages

FIRST



A. MAXILLIPED B. SECOND MAXILLIPED C. THIRD MAXILLIPED

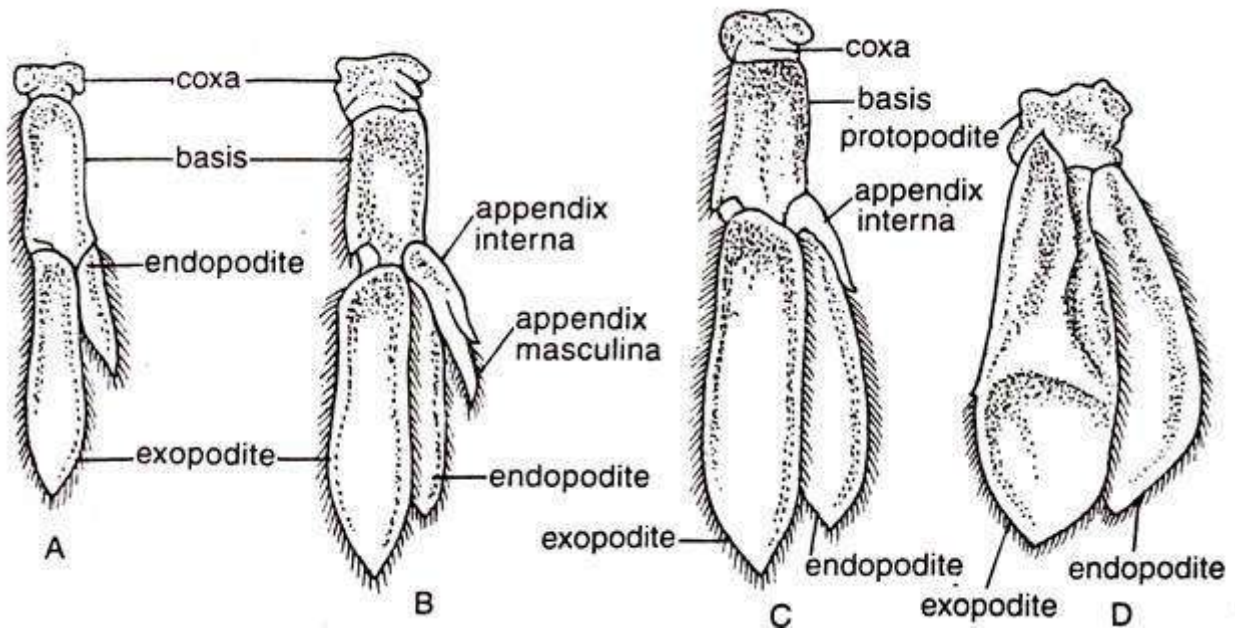
Thoracic appendages WALKING LEGS



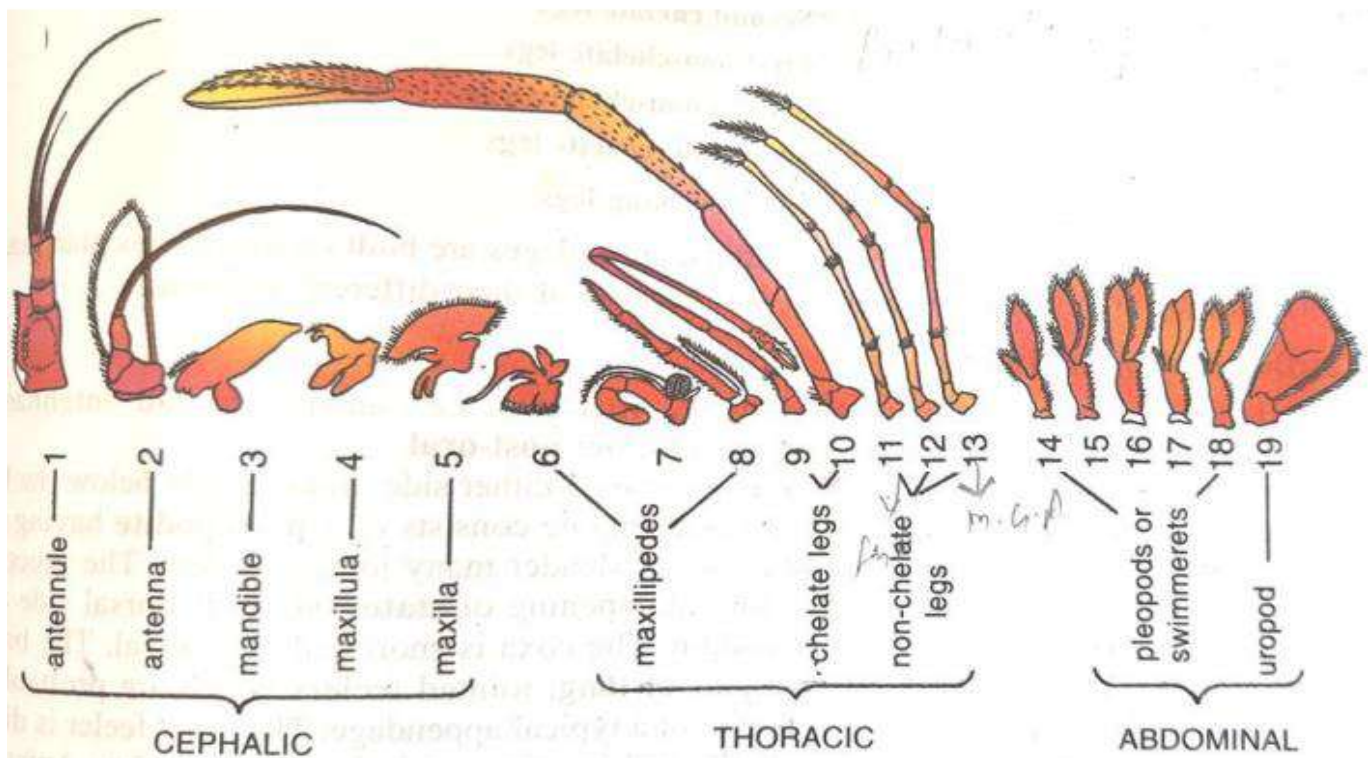
FIRST WALKING LEG B. SECOND WALKING LEG C. THIRD WALKING LEG

Abdominal Appendages:

First Swimmeret B. Second swimmeret C. Third Swimmeret D. Uropod



Appendages (19 Pairs)



4. 1. Study of Fish Eggs:

Stage I:

The primitive egg cells (ovogonium or arch ovogonium) are very small, their size being hardly bigger than that of other cells (8–12 microns). They multiply by normal mitosis.

Stage II:

The egg cells grow to a size of 12–20 microns, and a follicle begins forming around each egg cell. The follicle, whose function is to nurture and protect the developing egg, eventually becomes a double layer of cells.

Stage III:

During this stage, the egg cell grows significantly larger to attain a size of 40–200 microns and becomes enclosed by the follicle.

These first three stages mark the period prior to the accumulation of nutrients in the developing eggs.

Stage IV:

During this stage the production and accumulation of the yolk begins; this is a process known as vitellogenesis. The egg continues to grow to a size of 200–350 microns with the accumulation of drops of lipid materials in its cytoplasm.

Stage V:

This marks the second phase of vitellogenesis. The cytoplasm is now full of lipid drops and yolk production begins. The egg size reaches 350–500 microns.

Stage VI:

This is the third phase of vitellogenesis, during which the yolk plates push the lipid drops toward the edge of the cell where two rings begin forming. The nucleoli, which take part in protein synthesis and the accumulation of nutrients are seen adhering to the membrane of the nucleus. The size of the egg is now 600–900 microns.

Stage VII:

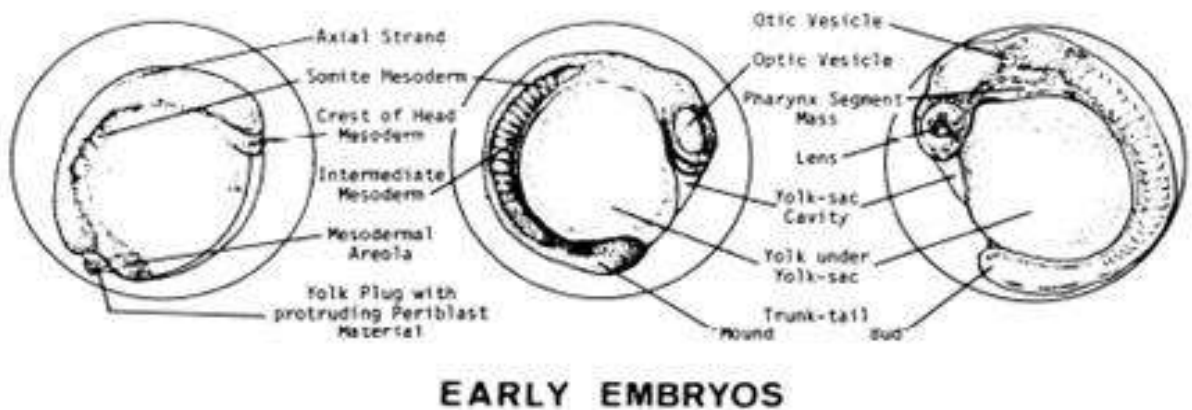
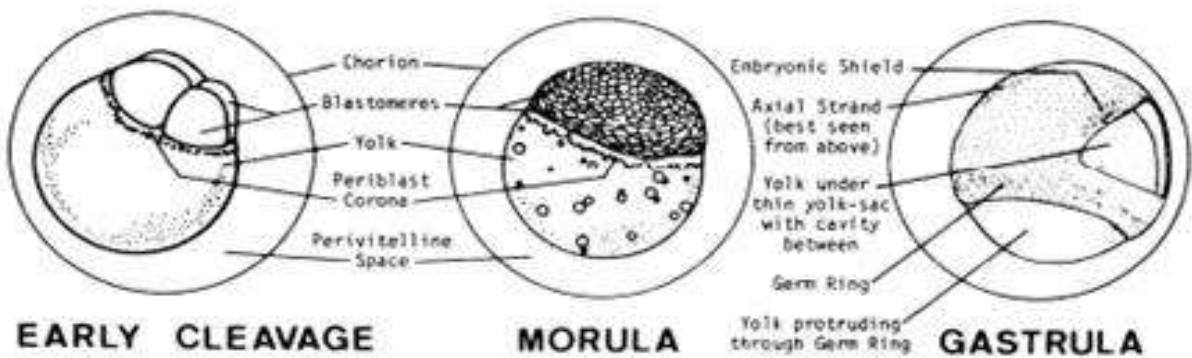
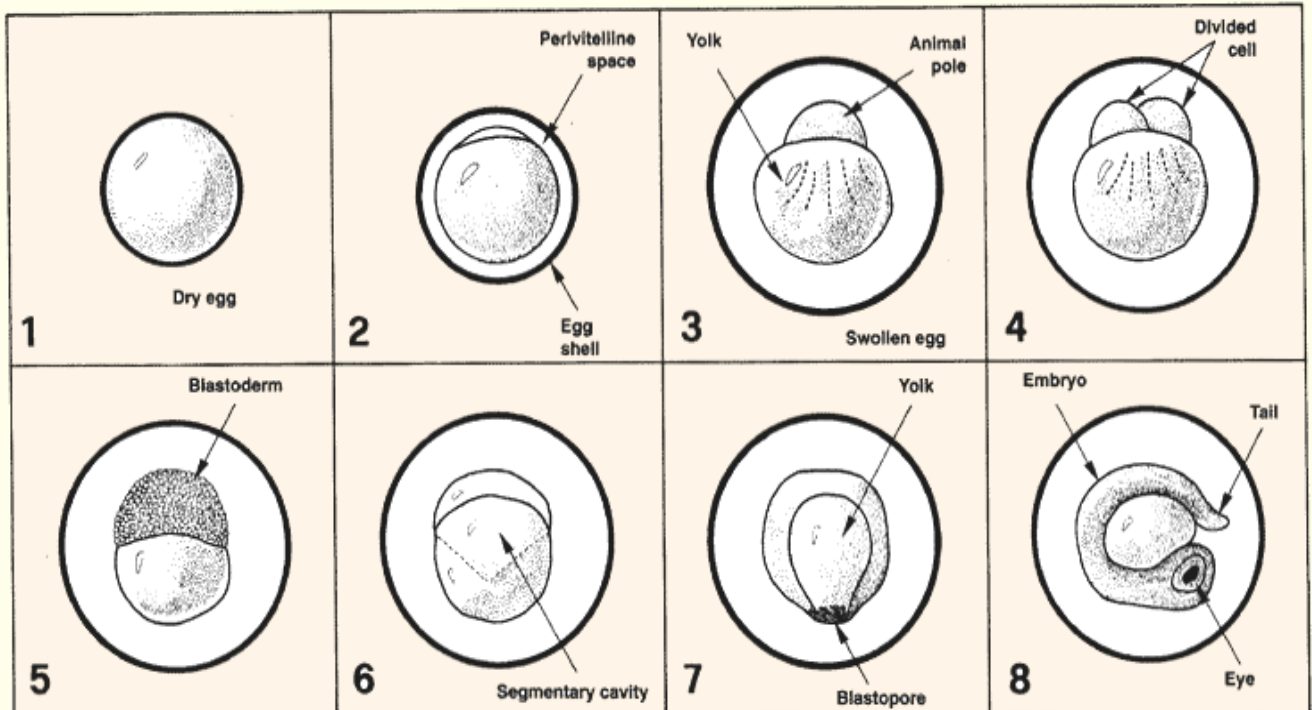
The process of vitellogenesis is completed during this stage and the egg attains a size of 900–1 000 microns. When the yolk accumulation ends, the nucleoli withdraw into the centre of the nucleus. The micropyle (a small opening on the egg shell) develops during this stage.

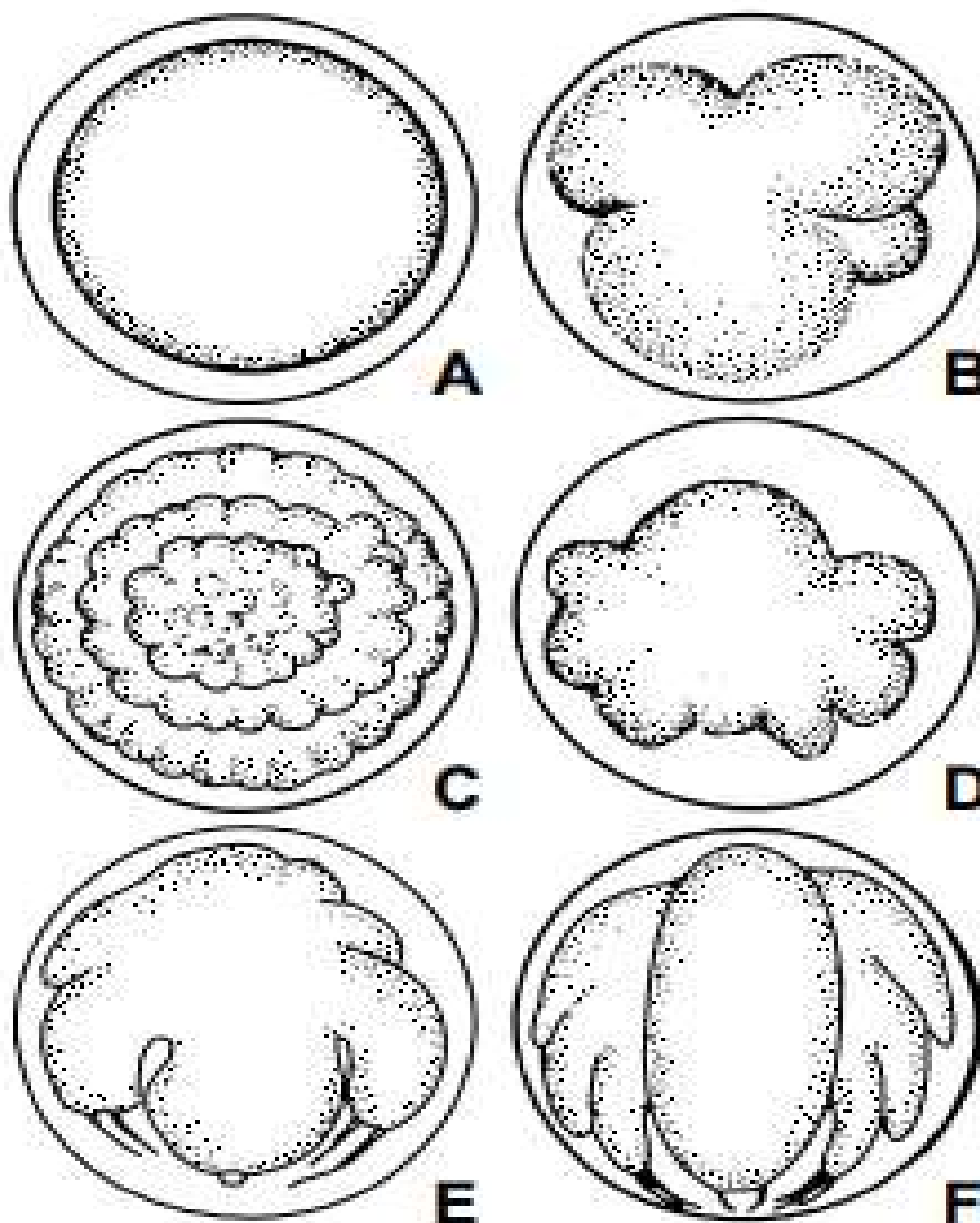
Stages IV, V, VI, and VII are the stages of vitellogenesis, when yolk is synthesized and accumulated in the egg cell. The egg is now materially ready. To reach this stage of development, the female fish needs a lot of protein in its food and a favourable temperature range.

On the completion of stage VII, the egg may remain as such for several months without any change, and this forms the “dormant” or “resting” phase.

Some phases of
fish egg development

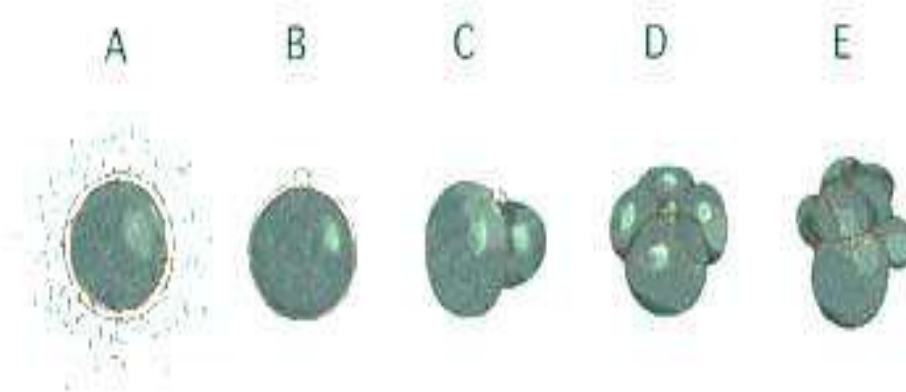
0 1 2 3 4 5 mm



4 b SHRIMP EGGS:

Eggs of *Penaeus monodon* at various embryonic developmental stages. A: newly spawned egg; B: 4-cell stage (about 1 hr after spawning); C: morula stage (about 1.8 hr after spawning); D: early embryonic nauplius; E: late embryonic nauplius; F: embryonic nauplius immediately before hatching.

5. OYSTER EGGS



Stages in the early development of eggs;

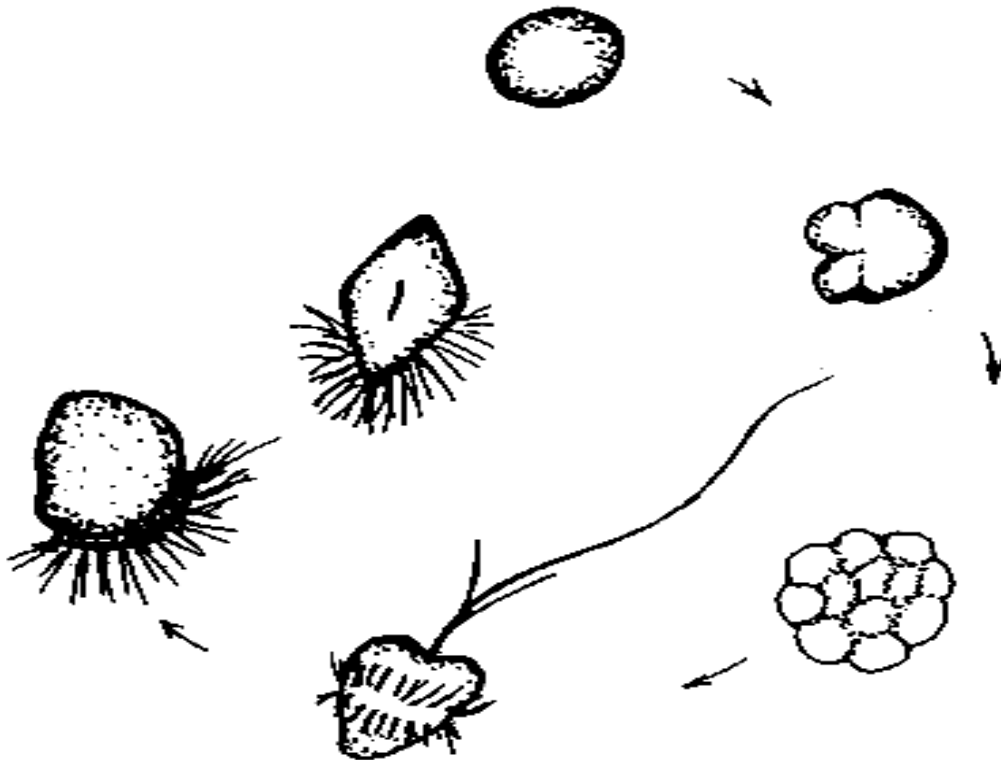
A - sperm swarming around a rounded-off egg;

B - extrusion of the first polar body following fertilization;

C - two-cell stage also showing the second polar body;

D - four-cell stage;

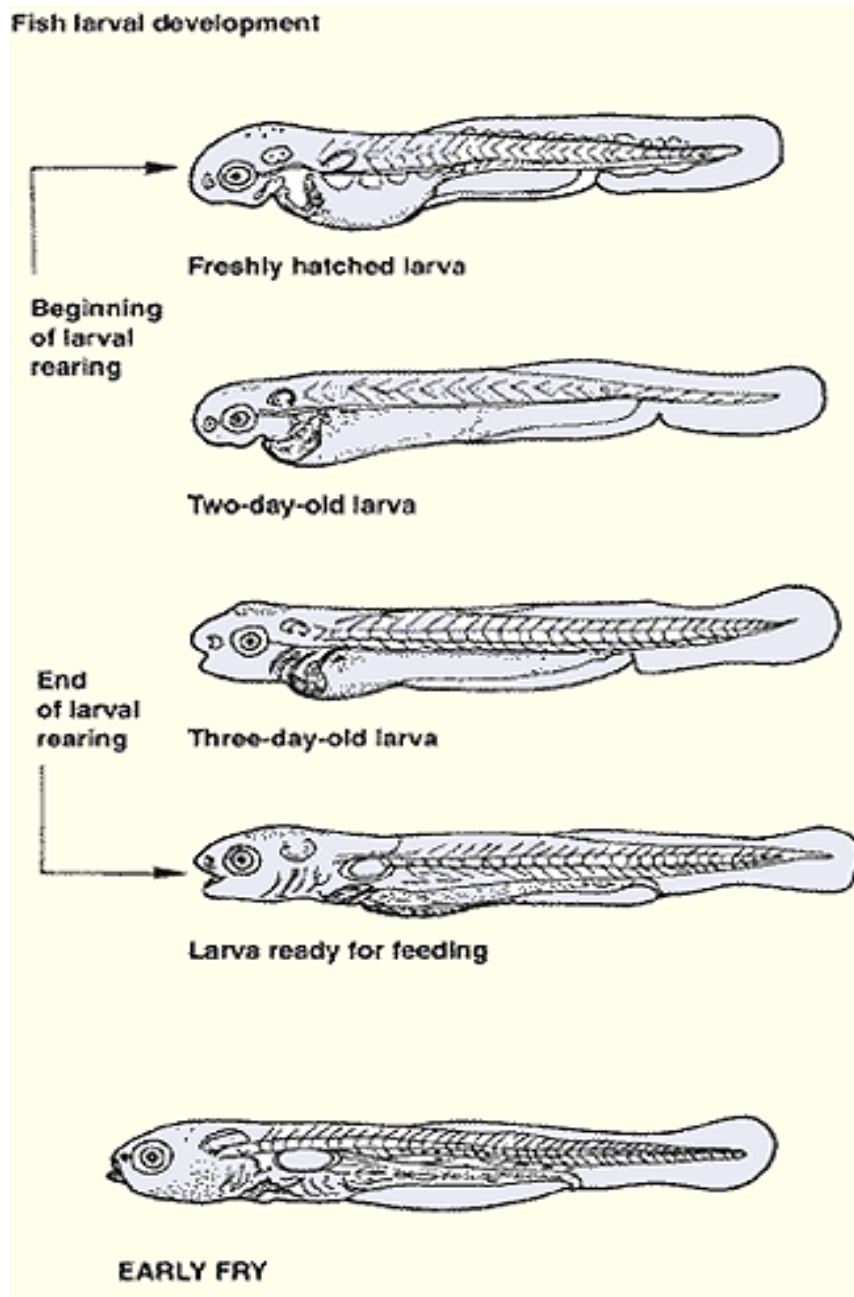
E - eight-cell stage. The eggs of most oviparous bivalves' range in size from about 60 to 80 μm , depending on species. The time from fertilization to the various developmental stages is species and temperature dependent.

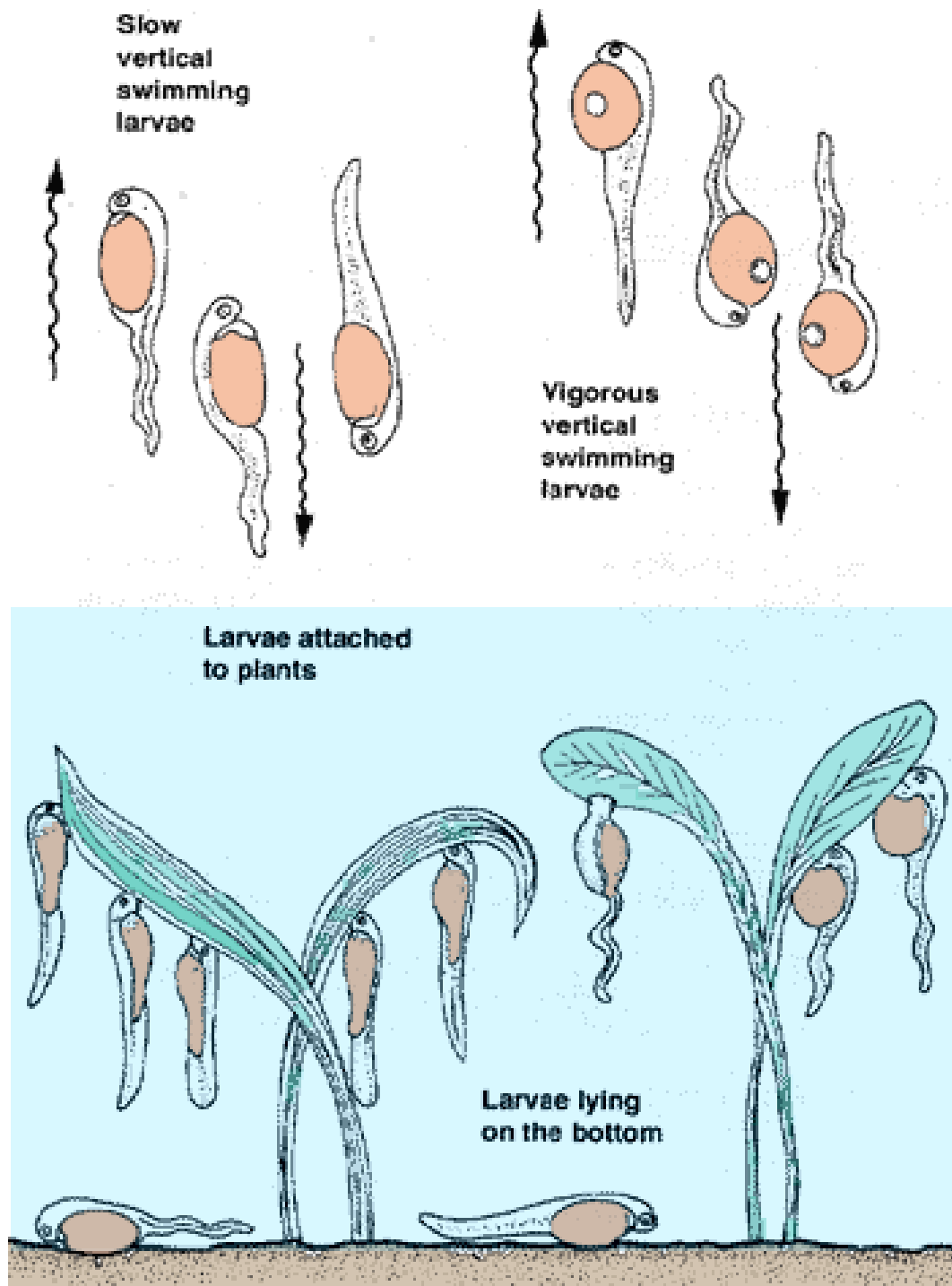


Developmental stages of an oyster larva: (1) fertilized egg, (2) early cleavage, 4-cell stage, (3) Morula stage, (4) ciliate trochophore, (5) Veliger and (6) Straight-hinge larva.

6. LARVAL DEVELOPMENT OF FISH:

Fish larvae mostly develop their **alimentary and respiratory organs**. **The yolk sac** provides the material for growth and development. Its size slowly decreases until complete reabsorption, soon after the larva has started to ingest external food. The length of this period therefore greatly depends on **the initial size of the yolk sac**, which varies from species to species, and on **the rate of larval development**, which mainly varies with **water temperature**. For each species, there is an **optimal thermal range**, similar to the one defined for egg incubation (The length of the larval rearing period is similarly defined in **degree-days (dd)**, as is the incubation period).





7.A. Gonadal maturity and fecundity of Fish

Stage of maturity	Male	Female	
	Nature and extent of testis in body cavity	Nature and extent of ovary in body cavity	Appearance of ova under microscope
I Immature	Small, transparent, pale, occupying a very small portion up to 1/3 of body cavity	Small, transparent, pale, occupying a very small portion up to 1/3 of body cavity, ova not visible to naked eye	Irregular, small, yolkless/ yolk deposit just started, transparent with partially visible/clearly visible nucleus
II Maturing 1	Whitish, translucent, occupying about 1/2 of body cavity	Pale yellow, granular ova visible to naked eye, occupying about 1/2 of body cavity	Medium-sized, assuming a round shape, opaque, with fair amount of yolk
III Maturing 2	Creamy white, occupying about 3/4 of body cavity	Pale yellowish, blood vessels visible on dorsal side, ova clearly visible, occupying about 3/4 of body cavity	Medium-sized, opaque, fully yolked
IV Mature	Creamy white, soft, occupying full length of body cavity	Pinkish-yellow, blood vessels prominent, large ova prominently visible, occupying full length of body cavity	Large, mature, transparent at periphery
V Ripe/Running	Not encountered	Not encountered	-
VI Spent	Flabby, reddish, occupying about 1/2 of body cavity.	Flaccid, reddish, occupying about 1/2 of body cavity	Medium-sized ova present with disintegrating ripe ova

Stage	Female gonads	Male gonads
I	 1cm	 1cm
II	 1cm	 1cm
III	 1cm	 1cm
IV	 1cm	 1cm
V	 1cm	 1cm
VI	 1cm	 1cm

7.B. Gonadal maturity and fecundity of Prawn

Male genital organ.

The internal reproductive organ of the male consists of paired testes, vasa deferentia, and terminal ampoules located in the cardiac region dorsal to the hepatopancreas.

The testis is translucent and composed of six lobes, each connected in the inner margins leading to the vas deferens.

The vas deferens consists of four portions, namely: the short narrow proximal vas deferens, a thickened larger median portion or the medial vas deferens, the relatively long narrow tube as the distal vas deferens, and the muscular portion referred to as terminal ampoule.

The terminal ampoule contains the spermatophore and opens at the base of the coxopod of the fifth pereopods.

The spermatozoa of *P. monodon* are minute globular bodies composed of the head of about 3 microns in diameter and a short spike.

The petasma is a pair of endopods of the first pleopods formed by the interlocking hook-like structures. The appendix masculina is oval and is located on the endopod of the second pleopod.

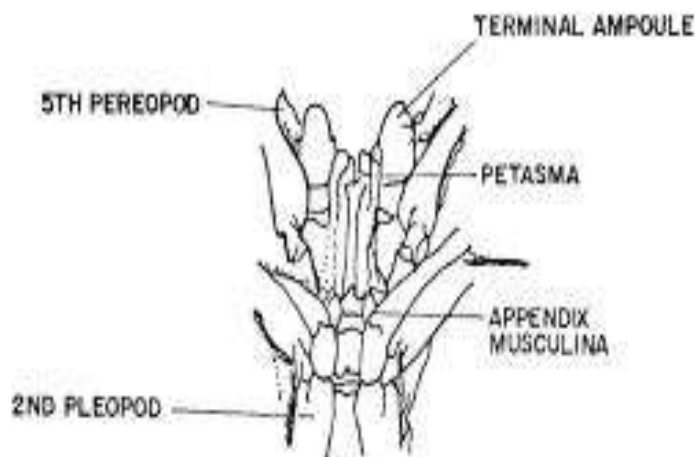
Female genital organ.

The internal reproductive organ of the female consists of paired ovaries and oviducts.

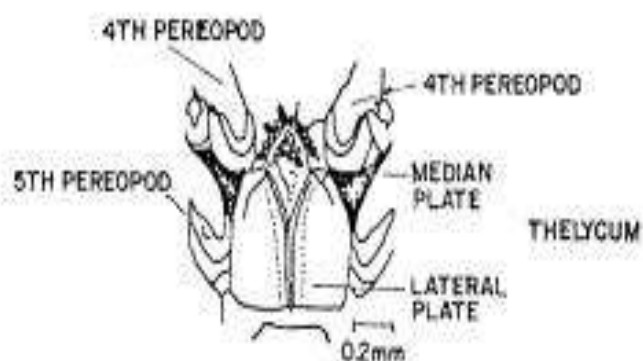
The ovaries are bilaterally symmetrical, partly fused, and extend almost the entire length of the mature female. It is composed of the anterior lobe located close to the esophagus and the cardiac region of the stomach; the lateral lobes located dorsal to the hepatopancreas; and the abdominal lobe which lies dorso-lateral to the intestine and ventro-lateral to the dorsal abdominal artery.

The oviducts originate at the tips of the sixth lateral lobe and lead to the external genital opening at the coxopods of the third pair of pereopods. The thelycum, located between the fifth pair of pleopods, consists of an anterior and a pair of lateral plates. It receives the spermatophores during mating. In penaeids, the thelycum may be classified as closed or open type, and *P. monodon* belongs to the closed type.

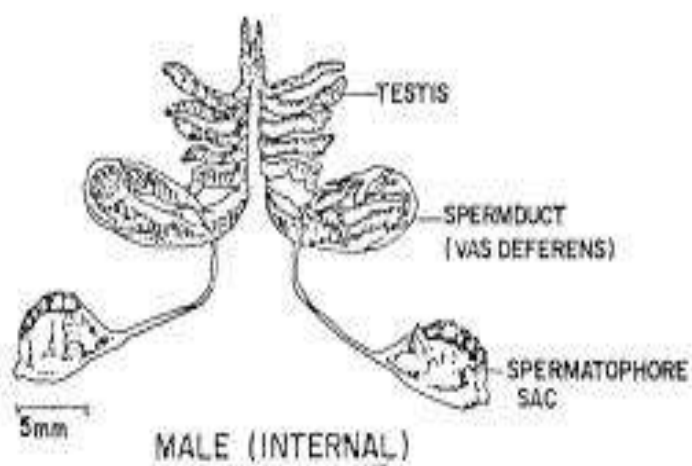
P. monodon males possess spermatozoa at 37 mm carapace length (CL) (about 35 g body weight or BW) and females at 47 mm CL (about 67.7 mm BW) although pond-reared prawns were mature only at 31 mm CL (about 20 g BW) and 39 mm CL (about 41.3 g BW), respectively.



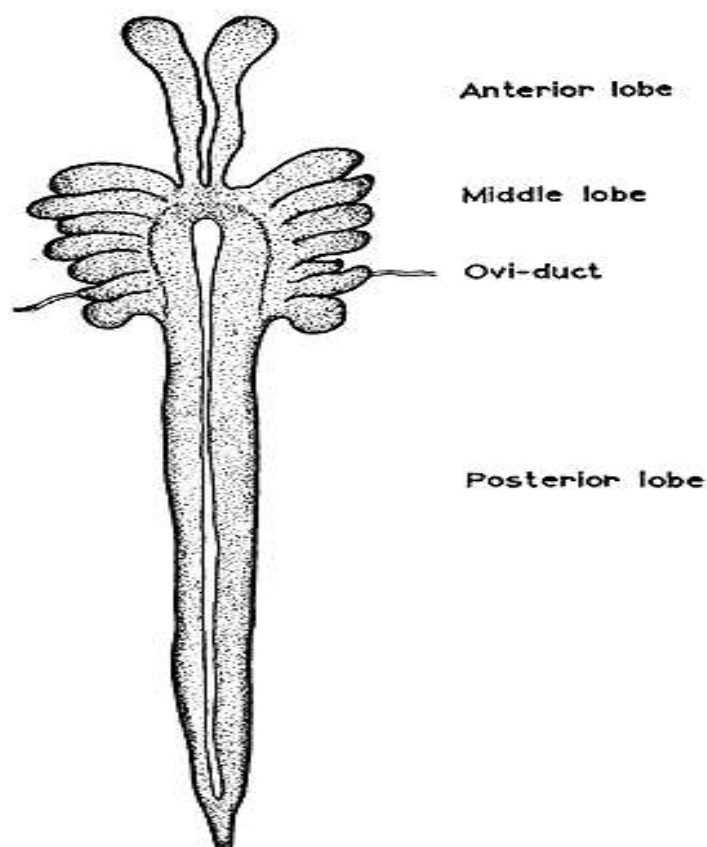
EXTERNAL GENITALIA OF MALE



EXTERNAL GENITALIA OF FEMALE



MALE (INTERNAL)



Five maturation stages are recognized for the four species of Penaeid and they may be distinguished on the following points:

Immature stage: The ovaries of immature prawns are thin, translucent, unpigmented and confined to the abdomen. They contain oocytes and small spherical ova with clear cytoplasm and conspicuous nuclei.

Early maturing stage: The ovary is increasing in size and the anterior and middle lobes are developing. The dorsal surface is light yellow to yellowish green. Opaque yolk granules are formed in the cytoplasm and partly obscure the nuclei. The developing ova are clearly larger than the immature stock.

Late maturing stage: The ovary is light green and is visible through exoskeleton. The anterior and middle lobes are fully developed. The maturing ova are opaque, due to the accumulation of yolk.

Mature stage: The ovary is dark green and clearly visible through the exoskeleton. The ova are larger than in the preceding stage and the peripheral region becomes transparent. This stage is believed to be the last stage of maturity before actual spawning as the largest ova are encountered only in this stage.

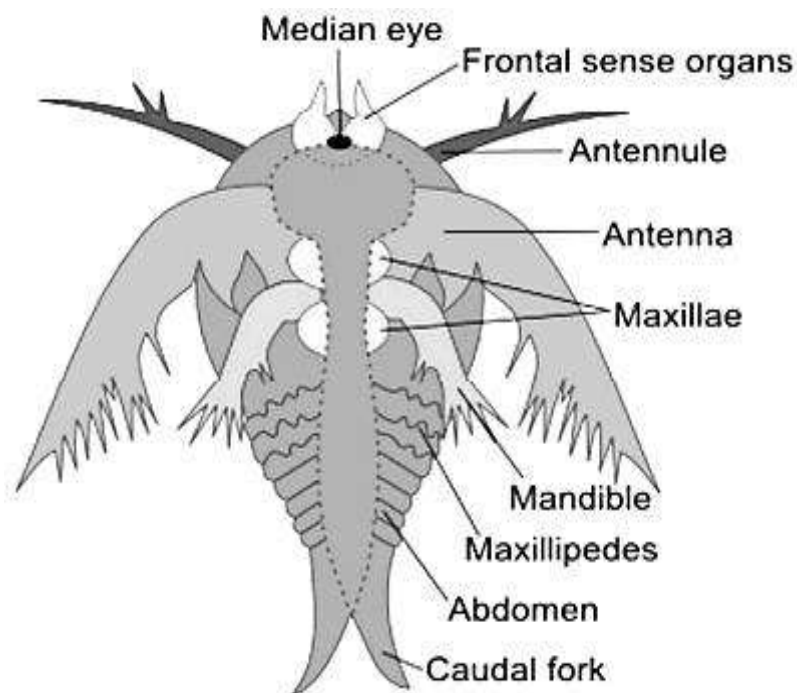
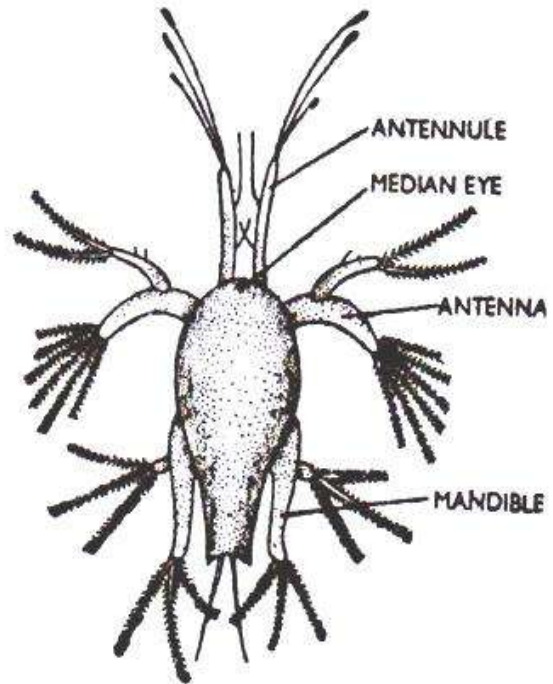
Spent-recovering: It is probable that after the extrusion of eggs, the gonad reverts almost immediately to the immature condition. The present stage is therefore distinguishable from that found in immature virgin females only on the size of the prawn.

The number of eggs produced by mature females of each species was determined and the relationship between the length of the prawn and fecundity was estimated. It is found that the number of eggs produced varies with the size of the prawn in a logarithmic manner.

8. CRUSTACEAN LARVAE

1. NAUPLIUS

- The simplest, commonest and earliest larval form in crustacea.
- It is a microscopic, oval or pear shaped with an unsegmented body having a broad anterior head region, an intermediate trunk and a posterior bilobed anal region.
- It has 3 pairs of unjointed appendages bearing seae for swimming.
- The first pair is uniramous and becomes the antennules of the adult.
- Second is antennary and third is mandibular to become the respective parts of the adult.
- The head region bears a noticeable sessile median eye.
- The mouths open anteriorly between the bases of antennary and mandibular feet.
- The anus lies at the extremity of the caudal region.
- The alimentary canal is straight and made of foregut, midgut and hindgut.
- However, mouth and alimentary canal are lacking in nauplius of Cirripedia.
- The larva is without a heart and has a segmented ventral nerve cord.



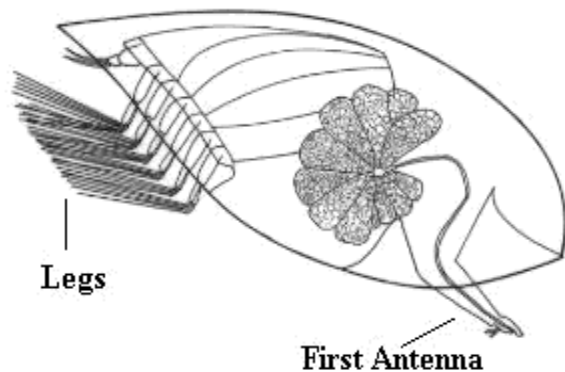
METANAUPLEUS LARVA OF APUS

2. METANAUPLIUS

- It is indistinctly defined stage following the nauplius.
- It consists of an oval cephalothorax, an elongated trunk-region and an abdomen terminating in a caudal fork provided with setae.
- Dorsal shield of the head grows back to form the carapace.
- In addition to the basic appendages of nauplius, it also develops the rudiments of four pairs of appendages, which become the maxillulae the maxillae and first two pairs of maxillipedes of the adult.
- Branchiopoda, Cephalocardia and perhaps some Stomatopoda hatch at this stage.

3. CYPRIS

Cypris Larva

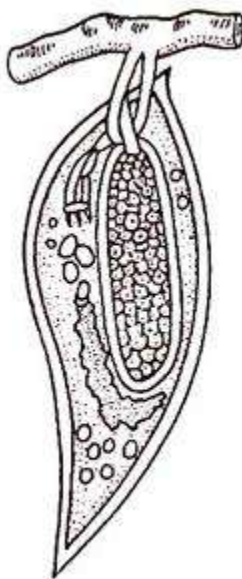


cypris form.

In some Cirripedia like Lepas, the nauplius passes into the cypris, meaning mussel stage, in which the body and the appendages are enclosed within bivalved carapace with an adductor muscle to close it.

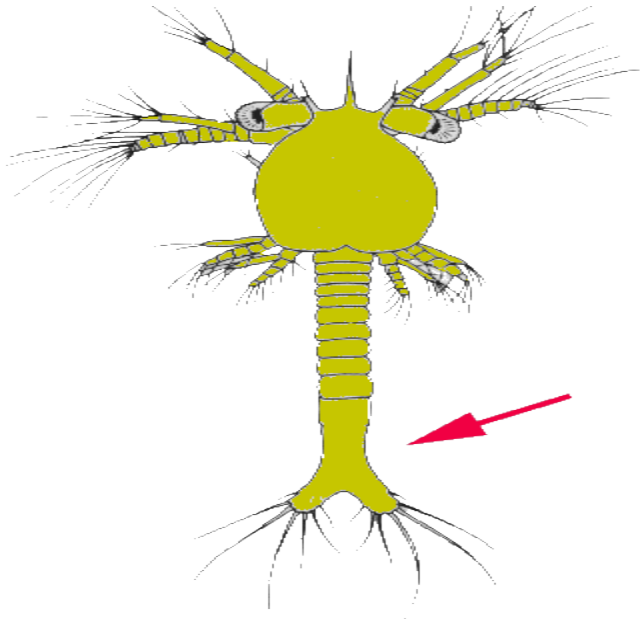
- Its modified antennules have **cement glands** situated at their bases.
- It undergoes a remarkable series of metamorphoses to become the sessile adult form.
- **Ostracods** eggs typically hatch in the

4. KENTROGEN LARVAE



- It is the larva of sacculina an exclusive parasite of crab.
- It is attached to the body of the host crab.
- It looks like a sac covered with a cuticle.
- The bag encloses a mass of germ cells.
- The larva has a chitinous tube called dart.
- At a later stage the dart pierces through the body of the crab and the germ cells of the larva pass into the body of the host.
- Inside the host crab the kentrogen develops into the next larva
- sacculina interna.
- It ultimately develops rhizoids and matures to adult parasitic sacculina.

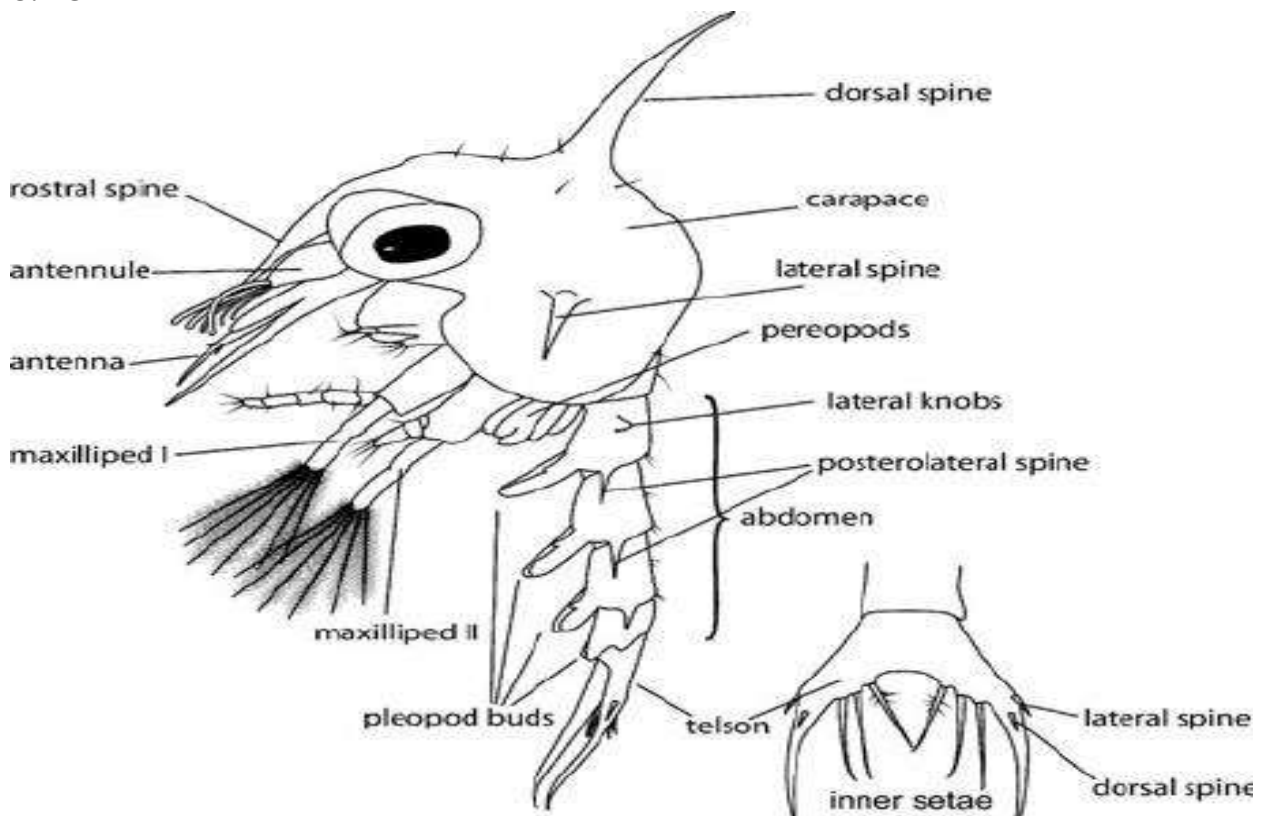
5. PROTOZOEAE



- The metanauplius larva is succeeded by the protozoaea stage with seven pairs of appendages and the beginning of segmentation.
- The carapace become enlarged and covers the dorsal surface anteriorly.
- The seven pairs of appendages present in the metanauplius, up to 2nd maxillipede, become well-developed and capable of movements.
- The rudiments of paired lateral eye begin to appear near the median eye.
- The rudiments of the remaining posterior six thoracic segments are also marked off, but the abdomen still remains unsegmented and limbless.
- The protozoaea swims by antennae.

- **Penaeus** hatch in the **protozoaea** stage.

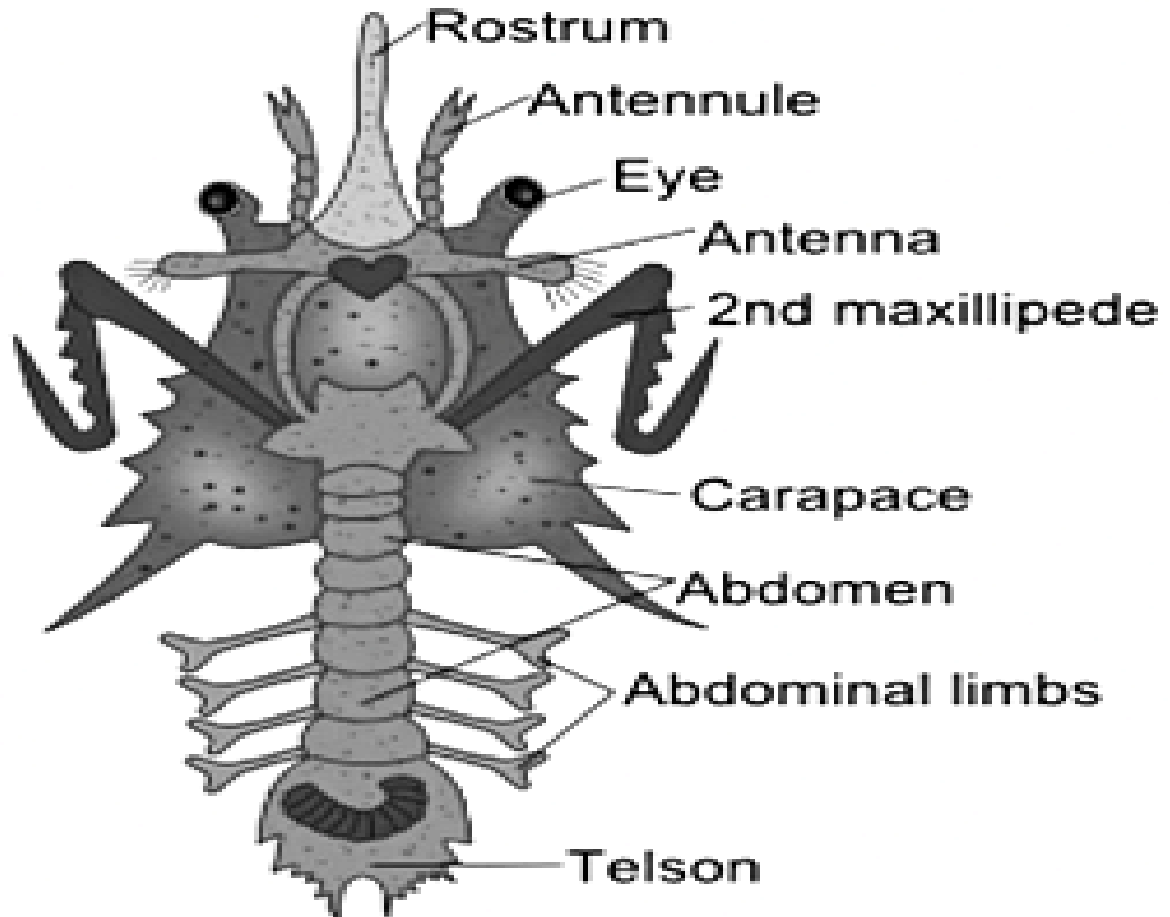
6. ZOEAE



- Zoea is the second important larvae of the Crustacea, the first being the nauplius. 2. Protozoaea stage is succeeded by the zoea stage characterized with a distinct cephalothorax and abdomen, eight pair of appendages and buds of six more, it looks like adult Cyclops.
- The cephalothorax is highly developed and covered by a helmet-like carapace, which is produced into two long spines, an anterior median rostral and a posterior median dorsal. Sometimes two lateral spines are also present.

- The paired lateral and stalked compound eyes become well formed.
- The long abdomen is clearly made of six segments, and terminates in a caudal fork.
- Abdomen still lacks appendages and hence larvae swim by means of thoracic limbs.

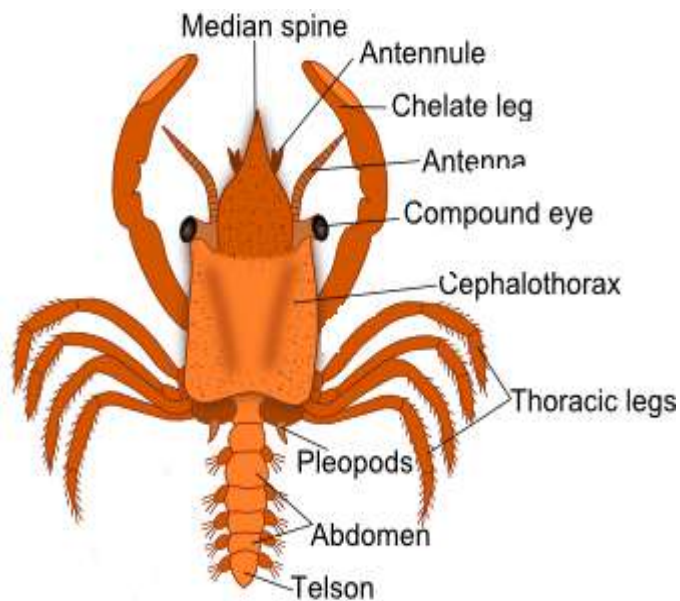
10.ALIMA



ALIMA LARVA OF SQUILLA

- The so-called alima larva of Squilla which hatch out from the egg directly, is a modified zoea.
- Being pelagic and having a glass-like transparency it occurs in large numbers in the plankton.
- It has a slender form, and a sort and broad carapace.
- All the head appendages are present.
- Abdomen is six-segmented, having 4 or 5 pairs of pleopods i.e. swimming legs.
- The alima larva differs from the zoea larva in the armature of the telson and a very large raptorial second maxillipede.

11.MEGALOPA

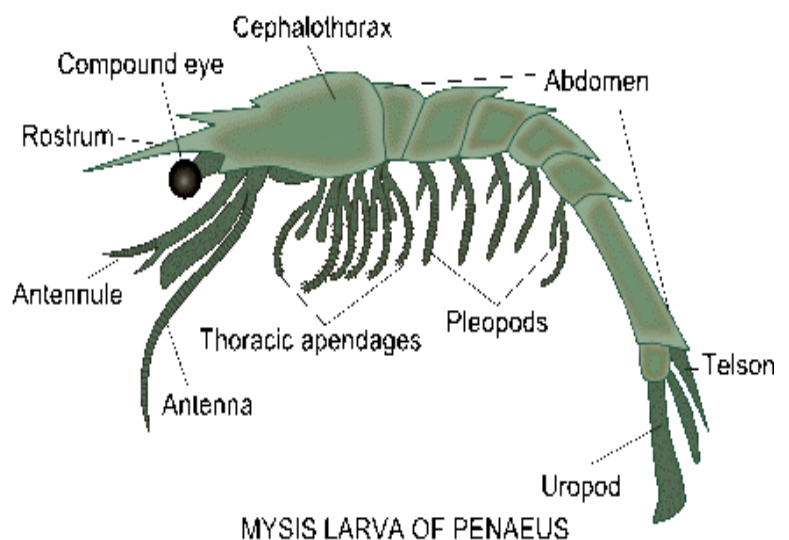


MEGALOPA LARVA OF CRAB

- All the true crabs with the zoea larva or metazoea passing through successive moults develop into the post larval megalopa stage.
- It has a broad and a crab like unsegmented cephalothorax.
- The carapace is produced anteriorly into a median spine.
- The eyes are large, stalked and compound.
- All the thoracic appendages are well formed of which the last five pairs uniramous.
- The abdomen is also well formed, straight and bears biramous pleopods.

13. MYISIS

- In *Penaeus*, the zoea, instead of converting into the megalopa stage, moults into the post larval mysis larva within thirteen pairs of appendages.
- All thoracic appendages are biramous.
- Even the five pairs of posterior thoracic legs are biramous with flagellar exopodites which take up the locomotory function (Which is chiefly by the antennae in other larvae.)
- The abdomen develops similar to that of the adult form, with five pairs of biramous pleopods and a pair of uropods and telson.
- The mysis larva metamorphoses into the adult prawn, by losing the exopodites of the thoracic legs.

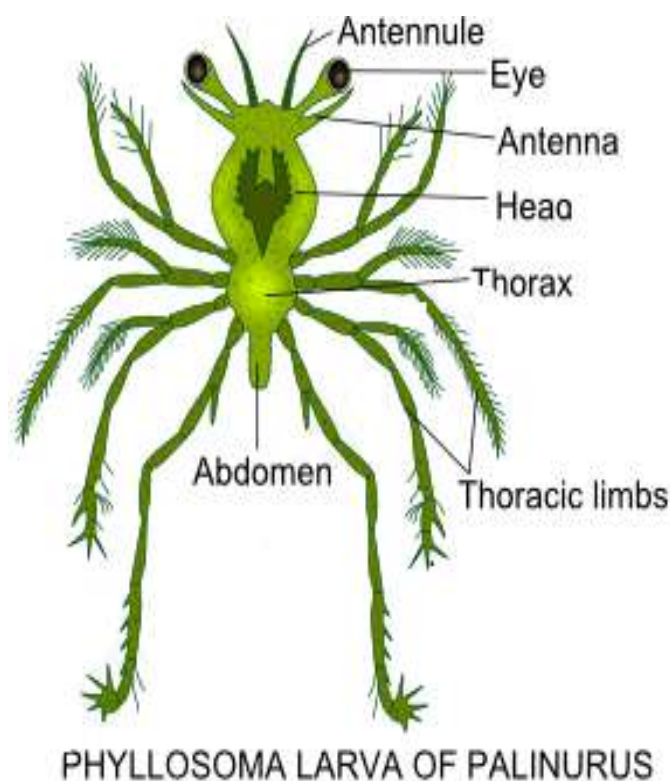


MYISIS LARVA OF PENAUEUS

4. PHYLLOSOMA

- It is the newly hatched larva of the rock- lobster, *Palinurus*, it is also called glass-crab and is a greatly modified mysis stage.
- It is remarkable for its large size, extremely flattened and a leaf-like delicate form and glassy transparency.
- A narrow constriction demarcates the head from the thorax.

- A large oval carapace covers the head and the first two thoracic segments.
- The eyes are largely stalked and compound.
- Only anterior six pairs of thoracic appendages are present in the newly hatched larva.
- The first thoracic appendages or maxillipedes are rudimentary in case of *Palinurus* or absent in case of *Scyllarus*.
- The second are uniramous and succeeded by four pairs of very long biramous legs with notary exopodites.
- Last two pairs of thoracic appendages are usually absent.
- Abdomen, though indistinctly segmented is very small and limbless.
- Phyllosoma undergoes several moults before reaching the adult form.



9. Nest builders & Brooding in Fish

The common nest builders are Gourami, Siamese fighter and Angelfish. They are bubble nest builders and incubate their eggs in floating nests, specially made by the male fish.

Gourami (*Osphronemus spp.*): Among the nest builders gouramis are the most popular. For breeding purposes males and females are kept separately in different tanks for a week and fed with live food. When the abdomen of female becomes grossly distended with eggs it is transferred to a smaller breeding tank with water level of 5-6" at 28o-30o C. The tank should contain plenty of fine-leaf plants such as *Cabomba* and some floating plants. The water hardness of 100-200 ppm and pH of 7.0-7.5 are ideal.

After 1-2 days, mature male is introduced in the breeding tank. A transparent perforated plastic sheet or a glass is covered over the tank to keep the humidity and temperature at high level, which help to maintain the bubble nest in good condition. The male soon begins to build the bubble nest. This is possible by engulfing a large gulp of air at the water surface and converting it into many smaller bubbles that are passed into gill chamber and coated with an anti-burst agent before release.

After making the nest, the female deposits a large number of eggs in the nest. After breeding, female is removed, while males guard the nest. Hatching takes place within 24-36 hr and the moment fry swim freely from the nest, males are removed from the tank. The young ones are given infusorians at this stage and after a week newly hatched artemia and small cladocerans are provided. As they grow, they accept all kinds of prepared feeds.

Siamese fighter (*Betta splendens*): Adult fish attain sexual dimorphism at a length of 6 cm. It is best to attempt breeding with fishes that are about 9-12 months old. Allow one male to every 2 or 3 females. Females should be at least the same size as the male.

Males are kept in small aquaria of 2-5 litre capacity, while females are kept in tanks containing 25-50 litres of water. Another breeding tank containing 50 litres water (depth 15 cm) and having leaf plants like *Myriophyllum* and *Cabomba*, is required. The tank has to be partitioned into two using fine mesh net. In one half females and other half male fish is placed. Water temperature is maintained around 27°C.

The male starts building a bubble nest quite quickly and once this is underway, the partition net is removed. At this crucial stage male should accept the female, otherwise male starts vigorous display of chasing which ultimately leads to fin tearing of female. Fighters often spawn in early morning and within a few hours 200-300 eggs are laid. As the eggs are shed and fertilized, they sink to the bottom.

Males then collect them in his mouth and spit them into the bubble nest. At the end of spawning females are removed and male is left to guard the nest for 3 days after which it is removed. The eggs hatch after 36-48 hr. The smaller fry become free swimming after 5 or 6 days during when they can be fed with infusorium and egg-yolk. After 3 or 4 days, fries generally accept fine dry foods. The temperature of the water should be warm at around 27° C.

Bubblenest of siamese fighter fish



Angelfish (*Pterophyllum* spp.): The mature angelfish having straight top and bottom fins without any bowing or bend is selected. They should be healthy, strong, robust and active. Unfortunately, it is very difficult to differentiate between a male and female angelfish externally. In the beginning, 6-8 potential breeders are selected which can be set in a 100-litre tank and they are fed well with live food. The fish soon make pairs and start displaying breeding and courtship behavior. They spawn on broad-leaved Amazon sword plants in the aquarium. Angelfish prefer water with a 6.0–8.0 pH, with 6.5-7.4 being ideal, a water hardness of 50–130 ppm, and a temperature range of 24–30° C.

The female will deposit a line of eggs on the spawning substrate, followed by the male who will fertilize the eggs. This process will repeat itself until there are a total of 100-600 eggs. The pair will take turns maintaining a high rate of water circulation around the eggs by swimming very close to the eggs and fanning the eggs with their lateral fins. In a few days, the eggs will hatch and the fry will remain attached to the spawning substrate. During this period, the fry will not eat and will survive by consuming the remains of their yolk sacs. At one week, the fry will detach and become free-swimming. Fry can now feed on brine shrimp and after 2 weeks feed on powdered artificial feed.



Welcome: **Dr. N. SREENIVAS** (Self Published Author)

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[Back](#)

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BIOLOGY OF FIN FISH & SHELL FISH



LABORATORY MANUAL
(AQUACULTURE MAJOR)



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ENVIRONMENTAL STUDIES



e-CONTENT



SREENIVAS-SANJEEVAKUMAR
CHENNARAO-CHAKRAPANI

INDEX

Unit 1: Environment and Natural Resources

Pages: 1-16

1. Multidisciplinary nature of environmental education; scope and importance.
2. Man as an integral product and part of the Nature.
3. A brief account of land, forest and water resources in India and their importance.
4. Biodiversity: Definition; importance of Biodiversity - ecological, consumptive, productive, social, ethical and moral, aesthetic and option value.
5. Levels of Biodiversity: genetic, species and ecosystem diversity.

Unit-2: Environmental degradation and impacts

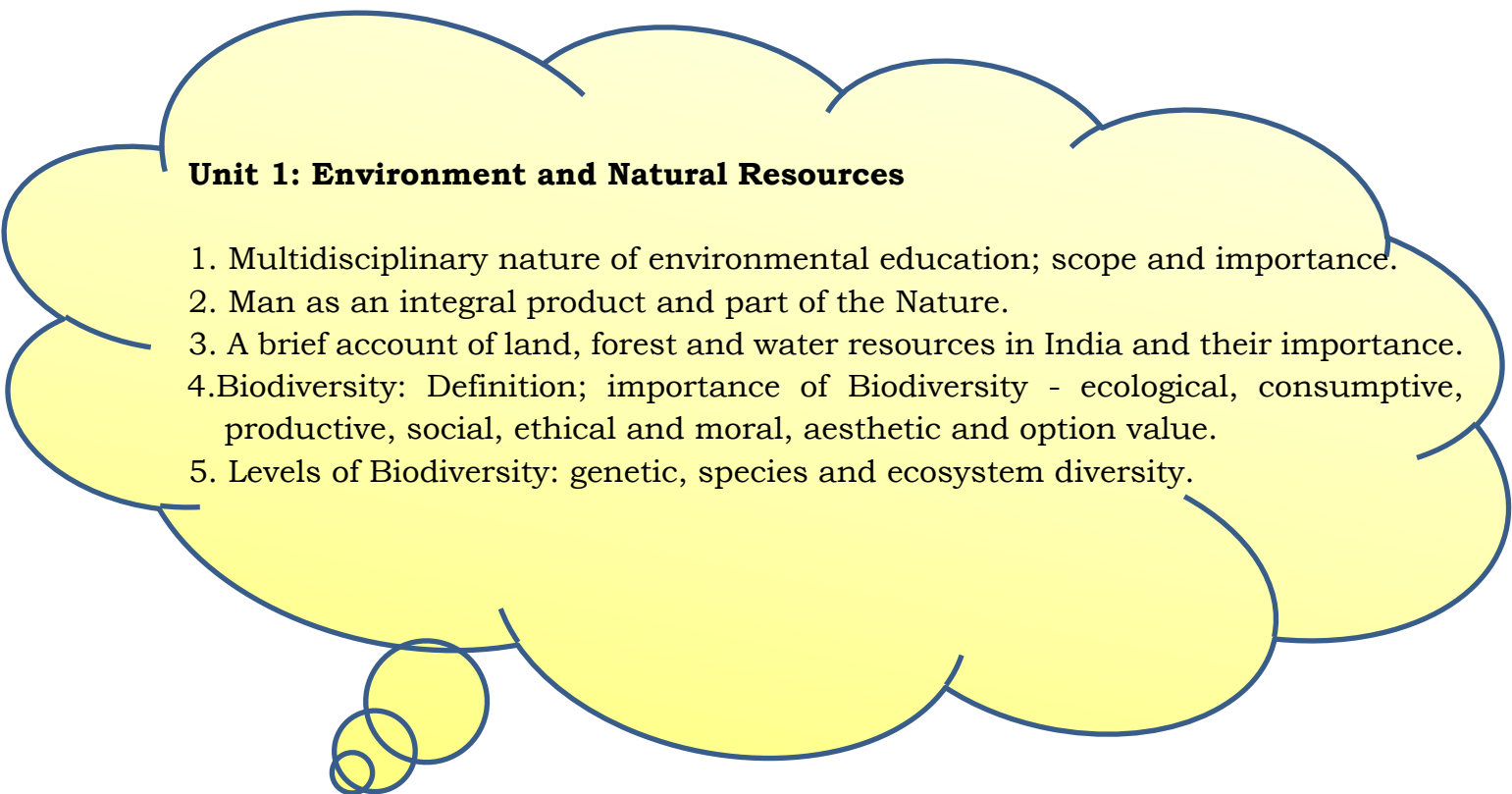
Pages: 17-29

1. Human population growth and its impacts on environment; land use change, land degradation, soil erosion and desertification.
2. Use and over-exploitation of surface and ground water, construction of dams, floods, conflicts over water (within India).
3. Deforestation: Causes and effects due to expansion of agriculture, firewood, mining, forest fires and building of new habitats.
4. Non-renewable energy resources, their utilization and influences.
5. A brief account of air, water, soil and noise pollutions; Biological, industrial and solid wastes in urban areas. Human health and economic risks.
6. Greenhouse effect - global warming; ocean acidification, ozone layer depletion, acid rains and impacts on human communities and agriculture.
7. Threats to biodiversity: Natural calamities, habitat destruction and fragmentation, over exploitation, hunting and poaching, introduction of exotic species, pollution, predator and pest control.

Unit 3: Conservation of Environment

Pages: 30-38

1. Concept of sustainability and sustainable development with judicious use of land, water and forest resources; afforestation.
2. Control measures for various types of pollution; use of renewable and alternate sources of energy.
3. Solid waste management: Control measures of urban and industrial waste.
4. Conservation of biodiversity: In-situ and ex-situ conservation of biodiversity.
5. Environment Laws: Environment Protection Act; Act; Wildlife Protection Act; Forest Conservation Act.
6. International agreements: Montreal and Kyoto protocols; Environmental movements: Bishnoi of Rajasthan, Chipko, Silent valley.



Unit 1: Environment and Natural Resources

1. Multidisciplinary nature of environmental education; scope and importance.
2. Man as an integral product and part of the Nature.
3. A brief account of land, forest and water resources in India and their importance.
4. Biodiversity: Definition; importance of Biodiversity - ecological, consumptive, productive, social, ethical and moral, aesthetic and option value.
5. Levels of Biodiversity: genetic, species and ecosystem diversity.

Module 1.1 Multidisciplinary nature of environmental studies

'There is a sufficiency in the world for man's need but not for man's greed'
~Mohandas Gandhi

Environment

The word environment is derived from the French word 'environner', which means surrounding. Thus, Environment can be defined as the combined interaction of physical, chemical and biological conditions affecting an individual or community in the complex of social and cultural conditions.

Environment consists of surroundings which includes abiotic and biotic environment. Environment refers to sum of all i.e., water, air and land, along with their inter-relationships among themselves and also other living organisms. Environment can be studied by interpreting the knowledge from all the disciplines.

Global environment constitutes important segments i.e. atmosphere, hydrosphere and lithosphere. Nitrogen, oxygen and argon are major gases which account for 99% of air. Hydrosphere consists of sea, ocean, rivers, glaciers, lakes, reservoirs, polar ice caps, and shallow water ground bodies and about 70% of earth's surface covered with water. In the modern era, human beings have exploited natural resources making the use of latest techniques in fields of information technology, genetic engineering, and others technologies for enhancing productivity and income which may lead to brighten our future. Over exploitation of natural resources may lead to starvation for resources and can create an environment which is not suitable for living organisms. So, there is a need to keep the balance to sustain

production level and maintain environment. Polluted air, water, and soil have negative impact on health and economic opportunities for the humans. Polluted environment gives birth to harmful biological and chemical agents which have direct or indirect threats to human health.

To face these threats, there is a growing realization at national and international level that proper utilization of environmental endowments of life support systems such as air, water and soil is a must for sustainable development. So, there is need to identify environment problems and to find solution. Individuals should be involved in the process of improving the environment.

Academic disciplines are created to help us understand the universe better. While nature can be understood using the disciplines, but not be 'divided' into disciplines. For instance, a certain phenomenon may be referred to as a chemical change while another as a physical one. But these categories are only perceptions. 'Environmental studies' is study about the environment. Environment cannot be studied from the point of any one particular discipline, how one action is connected to another, how the same thing can be understood from different perspectives, perspectives of ten rooted in different disciplines.

There is need to create a consensus at national level to give emphasis to environmental science involving the application of scientific and technical information to understand, manage and conserve the environment and its resources.

Environmental Science is interconnected to many branches of sciences. Some of these are:

Environmental science and biology

Biology mainly deals with life and environmental factors have effect on living organisms. Interaction between living things and different components of environment that affect the efficiency and combined effect can be known as environmental biochemistry. Biological processes in nature profoundly influenced by chemical species existing and these processes will determine the nature of species, their degradation, and synthesis, both in the aquatic and soil environments. Study of such phenomena are the basis of biochemistry of environment

Environmental science and chemistry

Chemistry deals with chemical processes occurring in nature. When these processes are studied in the shape of reaction which affects the existence of different species, with special reference to air, soil and water environment, this relationship is termed as environmental chemistry. One of environmental chemistry's major challenges is the determination of the nature and quantity of specific pollutants in the environments

Environment and economics

Economic environment refers to all factors or forces, which contribute to economic impact on the man, his activities and his region.

Resources such as agriculture, dairying, fisheries poultry, horticulture, floriculture and machinery help to improve economic condition. Economic conditions, internal and external factors such as export and import balances create favorable economic environment for fast development of the country.

Society and environment

Social environment of the society indicates the mental makeup of man's activities and helps the individual to decide his occupation and use of resources for his development. Infrastructure such as roads, buildings, settlements, communication setup plantation of horticulture and cropped fields are major component which helps to create social environment.

Using the combination of tools of modern technology indigenous technical knowledge (ITK) can help to fasten the change of physical environment into cultural environment.

1.2 Man as an integral product and part of the Nature.

The history of mankind has revealed that the present configuration of human life is determined and affected by social as well as natural environment. International Encyclopedia of the Social and Behavioural Science (2001) defines environment as the total external conditions and influences affecting the life and fortune of organisms, effectively the 'parameters of life'. 'According to the biophilia hypothesis, the human species evolved in the company of other life forms, and we continue to rely — physically, emotionally, intellectually - on the quality and richness of our affiliations with natural diversity' (Saunders 2003).

Healthy and diverse natural environment is necessary pre-condition for human lives of satisfaction and fulfillment. So, it is the technology of man which has drastically changed the man-environment relationship from prehistoric period to the present most advanced industrial period.

In fact, 'the industrial and scientific revolutions' have led to rapid changes in our environment, but all technology, from the most primitive to the most advanced, causes some changes in the environment. Underlying cause of the depletion of natural resources in a large-scale social dilemma is the unrestricted access to natural resources - either renewable or nonrenewable - that people have, such as electricity, water, oil, clean air, etc. Though there is a traditional debate between conservationist and non-conservationist, still people in general are highly concerned with the quality of the environment. In spite of this general concern, people greatly differ in the level of their environmental involvement and in the amount of time and energy they are willing to invest in behaviours aimed at conserving or improving the quality of the environment. A comparatively recent study on motivation towards the environment suggests that these behaviours are not necessarily equal in terms of their perceived level of difficulty. Many environmental problems have the structure of a social dilemma, namely a conflict between private interests and the interests of the collective at large; how individual affinity to consumerism contradicts with concept of social solidarity of global citizenship. 'The effects and side-effects of human activity become less and less predictable', and 'any judgement about environmental damage is based on value assumptions. Understanding the nature of environment problems and how they might be solved requires much more than a scientific appreciation of environmental processes. It demands an understanding of how societies work and how collective action within those

societies is both organized and constrained. Uninformed environmental decisions and behaviours could be very costly at the ecological, economic, and social levels. The human systems are divided into socio-political activities (decision making, institutional organization, culture and values); and to physical activities (demography, consumption, production). This model provides at least a very general framework for cross-disciplinary discussions. Three basic dimensions of the role of humans can be differentiated

- The interactions between human and environmental systems (the sources of global changes, the consequences of these changes).
- The choices that individuals, governments and organizations make in efforts to manage interaction (public perceptions, options, values).
- The underlying elements of social structure or culture that shape these interactions and choices. According to the report prepared by IPCC (2007), the human influences on the issue of climate change may have-
 - very likely contributed to sea level rise during the latter half of the 20th century
 - likely contributed to changes in wind patterns, affecting extra-tropical storm tracks and temperature patterns
 - likely increased temperatures of extreme hot nights, cold nights and cold days more likely than not increased risk of heat waves, area affected by drought since the 1970s and frequency of heavy precipitation events. IPCC-2007 At this juncture where in one hand, we are accelerating the exploitation of nature indiscriminately, on the other, people have felt the need of eco-friendly

development which is further manifested through different sphere of life.

The environment might be managed, identified three main positions:

- Dry green, reflecting the prevalent scientific prediction within an 'enlightened' market economy;
- Shallow green, reflecting 'eco-auditing' in which production, marketing and consumption would be adjusted to a more 'ethically acceptable' environmental philosophy; and
- Deep green, reflecting a more sustainable approach to the planet as an entity, through emphases such as more 'self-reliant communities' (what might be called communalism) and a greater emphasis on 'global co-existence' and 'green rights' (what might be called Gaianism).

Only an environmentally literate society will be able to adequately and constructively participate in the on-going discussions and reflection. The present generation of students, and likely several successive generations, will inherit the Earth's environmental problems and be faced with addressing them. Therefore, humanity needs a new relationship with nature, a healthier bond between the 'Self and the 'Surroundings', a new set of cultural values and a 'paradigmatic shift' in the global vision of earth-man relationship. Development of healthy personal and social attitudes in learners will go a long way towards environmental sustenance, building a vigilant society, and promoting sustainable development, as well as maintaining a standard of health, hygiene and sanitation. One indicator of environmentalism is environmental citizenship, which refers to support and acceptance of public policies that may require material

sacrifice in order to reach environmental goals.

A strong sense of environmental citizenship may help people to overcome potential barriers to environmentally benign behaviour. However, when a specific behaviour is received as easy to perform there is less need for a strong environmental citizenship to motivate a behavioural change. Thus if we look at the historical progression of man-environment relationships it become clear that purely natural relationship between 'physical primitive man' and natural environment during prehistoric period has changed to hostile relationship between 'technological man' and the environment at present This substantial change and shift in the nature and magnitude of man's interactions with the natural environment has given birth to numerous environmental problems of serious consequences because the changes effected by man in the environment have become un adjustable by the in-built self-regulatory mechanism of the natural environmental system / ecosystem.

"O earth! Pleasant be thy hills and snow-clad mountains and thy woodlands on the earth-brown, black, ruddy and of all colours – the firm earth, the earth protected by the deity (Indra), upon this earth I – unconquered, un slain, unwounded, have set my foot."

1.3 A brief account of land, forest and water resources in India and their importance

1.3.1 Land resources in India and their importance

Land resources form the most important natural wealth of the country and their proper utilization is a matter of

utmost concern to its people. The utilization of the land according to its use capability ensures that this resource is utilized to the best advantage. Its improper use leads to wastage and can lead to progressive deterioration and loss of productivity of this vital resource. It is the moral obligation of the present generation to pass this valuable resource on to future generations as nearly unimpaired and over-exploited as possible, calligraphically.

One of the most significant features of land use in India is the large proportion of area suitable for agriculture that already has been brought under cultivation. About 11 per cent of the surface area is made up of mountain land over 7,000 ft. in elevation almost all of which is too steep or too cold or agriculture.

Another 18 per cent of the surface is hill land between 1,000 and 7,000 ft. above sea level, three quarters of which is too steep to farm. Plateaus of relatively low relief, between 1,000 and 3,000 ft. form 28 per cent of all land in India, all but a quarter of which is topographically usable. In the low lands, less than 1,000 ft. in elevation, which make up 43 per cent of the total area in India, only one acre in 20 is unusable because of roughness of topography.

Classification of Land:

Classification of land is “a process which assigns each body or tract of land in an area to its proper class in a system of classes. The classes in the systems are defined in terms of the qualities or characteristics with which the classification is concerned.” In India, the classification of land has had its roots in agricultural statistics.

Till 1950, the land in India was broadly classified into five categories:

- (i) Area under forests,
- (ii) Area not available for cultivation;
- (iii) Uncultivated lands including current fallows;
- (iv) Area under current fallows; and
- (v) Net area sown.

But then it was realized that such a classification did not give a clear picture of the actual area under different categories of land use required for agricultural planning. Hence, a reclassification was adopted from March 1950.

Under it, land in India is now classified under nine different categories, viz.:

- (i) Forests,
- (ii) Barren and uncultivable lands,
- (iii) Land put to non-agricultural uses,
- (iv) Culturable wastes,
- (v) Permanent pastures and other grazing lands;
- (vi) Miscellaneous tree crops and groves not included in the net area sown;
- (vii) Current fallows;
- (viii) Other fallows; and
- (ix) Net area sown.

The total geographical area of India is 328.88 million hectares, but land use statistics are available for 304.1 m. hectares or 93.6 per cent of the total geographical area. The net area sown is 138.3 m. hectares (or 44.7 per cent) of which only 25.5 m. hectares or about 8.1 per cent bear crop more than once. 52.8 per cent of the land area is under crops and fallow. It means that among large nations of the Orient, India is one of the best endowed with agricultural land.

Wasteland, land under urban or village settlement, and land put to other non-agricultural uses comprise 14.3 per cent of the total area; forest land makes up

another 22.0 per cent. The remainder 10.9 per cent is made up of village pastures and grazing land, and firewood groves, jungle scrub and bamboo, private rests and culturable waste.

Within the total geographical area, it is the arable land area which is of particular importance in an agricultural country like India. For all the countries of the world 32 per cent of the total geographical area falls under this category. The corresponding figure of India is larger at 46.3%. In USSR, it is 27.4%; in USA 40.0%; in Canada 6.4% cent and in Brazil 16.1% respectively.

Classification of land use

Item	(Million Hectares)	
	1950-51	1998-99
1. Area under forests	40.5	69.0
2. Area not available for cultivation	47.6	42.4
(i) Area under non-agricultural uses	9.4	22.8
(ii) Barren and uncultivable land	38.2	19.6
3. Other uncultivated land	49.4	28.7
(excluding fallow land)		
(i) Permanent pastures and other grazing land	6.7	11.1
(ii) Land under misc. Free crops and groves not including in net area sown	19.8	3.6
(iii) Culturable waste land	22.9	14.0
4. Fallow Land	28.1	24.4
(i) Fallow land other than current fallows	17.4	9.9
(ii) Current fallow	10.7	13.5
5. Net area sown	118.7	142.6
6. Total cropped area	131.9	192.6
7. Area sown more than once	13.1	50.0
8. Cropping intensity*	111.1	135.1
9. Total reporting area	284.3	306.0
10. Area for which no return exists	44.4	22.8
11. Total geographical area	328.8	328.8

* Cropping intensity = (Total Cropped Area/net sown area) × 100

Source: https://www.geographynotes.com/wp-content/uploads/2018/04/clip_image004-12.jpg

Further, on every hectare of arable land, India has to support 3 persons. The corresponding figure for the U. K. and West Germany is 7; for Belgium it is 11, for the Netherlands it is 14 and for Japan the figure is 17; i.e., about six times as large as that of India. Thus, while India is highly populated, the arable land available in relation to the population is much better than in countries like U. K., West Germany and Japan. However, the per capita cultivable land available in India is only 0.27 hectares as against 0.90 hectares in U. S. A., 0.92 hectares in USSR, 1.01 hectares in Argentina, 1.95 hectares in Canada and 3.40 hectares in Australia.

There has been a tremendous increase in population with the result that not only the total land per capita but also the proportion of cultivated land per capita has been steadily shrinking in size. The per capita cultivated land went down from 1.09 acres (0.44 hectares) in 1921, to 1.04 acres (0.42 hectares) in 1931, to 0.94 acres (0.34 hectares) in 1941, to 0.84 acres (0.34 hectares) in 1951, to 0.74 acres (0.30 hectares) in 1961; and to 0.27 hectares in 1971, with this decline in the cultivated land per capita, the share of food and other produce of cultivation available to each individual is declining. The population density per hectare of gross area sown varies considerably from 7.7 in Kerala, 5.7 in West Bengal, 5 in Tamil Nadu to 1.8 in M. P., 2.2 in Maharashtra, 2 in Punjab, 4.2 in Bihar, 3.5 in U. P. and 1.5 in Rajasthan, all India average being 3. The population density per hectare of gross area sown is higher in about half the states than All-India basis in Gujarat, M. P., Maharashtra, Karnataka, Punjab and Rajasthan, the population density is lower than All-India

average. Andhra Pradesh and Orissa have the same density as All-India.

If the cultivated area per capita is computed the situation remains similar. The cultivated area per capita varies from 0.14 hectares in West Bengal to 0.11 hectares in Kerala, 0.17 hectares in Assam, 0.21 hectares in U. P., 0.31 hectares in Orissa, 0.3 hectares in Punjab, 0.68 hectares in Rajasthan, 0.41 hectares each in Maharashtra and Karnataka, 0.19 hectares in Tamil Nadu and 0.48 hectares in M. P.

With the increase in population the net area shown has increased during the year 1950-51 to 1982-83 by about 23.1 million hectares, i.e., the net cultivated area increased from 118.7 million hectares to 141.8 million hectares. This increase has been due to increased facilities of irrigation, reduction in fallow lands, and improved methods of cultivation, besides expansion in multiple cropping, use of better strains of seed and the reclamation of land for agricultural purposes.

1.3.2 Types of Forest Resources in India:

Because of unique geographical locations and climatic diversities, the forest types of India are highly variable. A total of fourteen major classes of forest are found in diverse region of the Indian territories.

There are as follows:

1. Tropical dry deciduous forest
2. Tropical moist deciduous forest
3. Tropical evergreen forest
4. Tropical semi evergreen forest
5. Tropical rainforest
6. Subtropical Forest
7. Temperate broad-leaved forest
8. Temperate conifer forest
9. Subalpine Forest
10. Alpine Forest

11. Desert thorny forest
12. Coastal sand dune forest
13. Estuarine evergreen forest
14. Grasslands

All these forest types are further subdivided to subclass/association for further characterisation. At present, a little over 21 per cent of India's land area is classified as forest. India has about 64 million hectare of forest cover (of which 59% forest is dense, 40% is open and 1% is coastal mangrove) [FAO, 2005].

It is further repeated that 65 per cent of the India's forest wealth is administered solely by the government; another 27 per cent reserved for community and indigenous groups, (through joint forest management) and remaining 8 per cent of forest land is managed by private individuals on farms. The average stocking level of forest in India is 74 cubic meter per hectare much lower than 113 cubic meter per hectare in other developing countries.

The pressure on India's forest come from a variety of sources, including—the increase in population, from 390 million in 1950 to 1 billion in 2001, the loss of the 4.5 million hectares forest area since 1950 through agricultural conversion and other uses, the high percentage (78%) of forest subject to heavy grazing, exposure of half of all forests to risk from fires, shifting cultivation affect over 10 million hectares and encroaching of forest area by environmental victims/refuges. The demand of timber and fuel-wood has increased tremendously over past couple of decades and thus pressure of wealth cover is extremely high.

In spite of such constrains, during 1990-2000, India is the only country in south Asia with a positive increase in forest cover of 38,000 hectares.

Deforestation is global concern, over 60% of the original natural forest has been cleared and the remaining forest is threatened by logging, mining and other large scale development projects. The rate of deforestation in tropics is extremely high. Being a tropical country with high population growth, India showed massive deforestation.

During past 50 years more than 40% forest cover has been lost during this period. This kind of deforestation is connected with severe loss of biodiversity. It is estimated that the active ingredients for 25% of the world's prescriptive drugs are substances derived from plants, most of which grow in tropical forests and the estimated value of such commercial drugs is around \$100 billion per year.

A large population of tribals all over the world are forest dependent. In many countries they are deprived of forest wealth due to eviction from the native areas as a consequence of various developmental projects viz., multipurpose dam, industrial development, urbanization and so on.

In India, Narmada dam project alone caused the displacement of over 86,000 people. Mining in forest cover hill tracts also causes the displacement of fairly a good numbers of village settlement in different states of India.

The devastating effects of deforestation in India leads to soil loss, water deficit, forest loss, biodiversity loss and associated local climate changes. These events lead to immense expenditure for management. The degradation cost of India is estimated to be around 16,400 crores every year.

Forest fire in India is around 3 million hectare per year with a loss of forest cover valuing around Rs 440 crores.

On the whole, in India, forests contribute 1.7 per cent of GDP of the country excluding the fuel-woods and other NTFP material values.

Importance of Forest Resources:

In general forests have three important functions viz., protective function, productive function and accessory function. The productive function includes the fact that forests are the sources of various materials of human livelihood support system.

The protective functions include the fact that forests are performing a number ecological support system viz., climate control, soil conservation, drought and cyclone mitigation. In addition, there are other functions too, viz., the significance of forest in recreation, aesthetics, wildlife conservation.

It is an estimate that during the lifetime of a typical grown tree, 200,000 US\$ worth of ecological benefits can be available annually. The benefits include oxygen production, CO₂ sink, air purification, soil fertility enhancement, soil erosion control, water recycling, humidity control, and wild-life habitat management. But the benefit of same tree as timber is worth about some US\$ only. Only 10,000 years ago, agriculture began at the expenses of deforestation, and consequently global forest cover reduced to only 25 to 35% on an average. In many areas of tropics forests are disappearing and fragmented.

Forests as Carbon Sinks

The vegetation and soils of the world's forests contain a vast quantity of carbon—more than one-and-a-quarter times the amount stored in the atmosphere. CO_2 is released when forests are cleared or degraded, and vegetation is burned or decays. Burning forest vegetation also releases other greenhouse gases, including methane (CH_4), nitrous oxide (N_2O), carbon monoxide (CO), and oxides of nitrogen (NO_x).

When forests grow, CO_2 is withdrawn from the atmosphere through photosynthesis and stored as carbon in the vegetation. Levels of carbon in the soil may be increased by reforestation and other forest management practices. Currently, the world's forests are estimated to be net sources of CO_2 , primarily due to deforestation and forest degradation in the tropics. Temperate and boreal forests, which overall are slightly increasing in area and in some places in biomass per unit area, are net carbon sinks. The following three forest-related strategies have been proposed as means to influence CO_2 emissions:

1. maintaining existing carbon stocks through forest management and conservation;
2. increasing storage of carbon in forests (by increasing forest area or biomass per unit area) and in forest products; and
3. substituting fossil fuels with fuelwood from sustainably managed forests, and substituting energy-expensive products (for example, steel, aluminium, concrete) with industrial wood products.

Credits: https://www.biologydiscussion.com/wp-content/uploads/2016/12/clip_image0024.jpg

1.3.3 Water resources in India and their importance

Availability and Potential of Water Resources in India

India covers around 2.45% of the world's surface area and has 4% of the world's water resources. In India, rainfall is the primary source of freshwater. For a country of its size, India receives the second most amount of rainfall.

India receives an average of 1,170 millimeters (46 in) of rain per year, which equates to around 4,000 cubic kilometers (960 cu miles) of rain per year, or about 1,720 cubic meters (61,000 cu feet) of freshwater per person.

Water Resources of India can be classified into:

- Surface water Resources
- Groundwater Resources

Surface Water Resources

In India, there are four significant surface water resources. They are rivers, lakes, ponds, and tanks.

In India, there are around 10,360 rivers and their tributaries with a length of more than 1.6 kilometers.

The total yearly flow in India's river basins is estimated to be 1,869 cubic kilometers. However, only roughly 690 cubic (37%) kilometers of accessible surface water can be used.

This is because:

- A four-month period accounts for roughly 90 percent of the annual flow of Himalayan rivers.
- Capturing such resources is difficult and constrained because of the unavailability of adequate storage reservoir sites.
- The amount of water that flows through rivers is determined by the catchment basin, the size of the river basin, and the amount of rainfall that falls within the catchment basin.

Within the catchment areas of the Ganga, Brahmaputra, and hence the Barak rivers, precipitation is abundant. These three rivers cover less than a third of India's total land area. However, it controls 60% of India's surface water resources.

The southern rivers, on the other hand, have significant flow variability throughout the year.

Groundwater Resources

The country's total replenishable groundwater resources are around 432 cubic kilometers.

About 46% of the total replenishable groundwater resources are found in the **Ganga and Brahmaputra basins**. In the river basins of the northwestern area and sections of south India, groundwater utilization is relatively high. India also relies heavily on groundwater resources, which account for more than half of all irrigated land and serve 20 million tube wells.

To conserve river waters and improve groundwater recharging, India has built about 5,000 major or medium dams, barrages, and other structures.

Lagoons and Lakes

India contains a wide coastline and because of this, a diversity of lagoons and lakes have developed. States like Kerala, Odisha, and West Bengal have huge surface water resources in these lagoons and lakes. The water in these locations is habitually brackish. This brackish water is utilized to irrigate specific types of paddy, coconuts, and other crops, the water bodies are also used for fishing.

Dams And Rivers

To store river waters and improve ground water recharging, India has built nearly 5,000 major or medium dams, barrages, and other structures. The major dams (59 in total) have a total storage capacity of 170 billion cubic meters.

About 15% of India's food is produced with groundwater resources that are rapidly depleting or being mined.

The end of an era of massive groundwater use expansion will necessitate a greater reliance on surface water supply systems.

Usage of Water

Irrigation is by far the biggest consumer of India's water reserves, accounting for 78 percent of total water reserves, followed by the household sector (6%), and the industrial sector (5%). In both urban and rural India, groundwater is a key supply of drinking water. Groundwater reserves provide 45 percent of total irrigation and 80 percent of household water. In some states, overuse of groundwater has led to a major scarcity of water.

Availability of Water

Per Capita availability of Water in India

Area of the country as % of World Area	2.4%
Population as % of World Population	17.1%
Water as % of World Water	4%
Rank in per capita availability	132
Rank in water quality	122
Average annual rainfall	1160 mm (world average 1110 mm)
Range of distribution	150-11690 mm
Range Rainy days	5-150 days, Mostly during 15 days in 100 hrs
Range PET	1500-3500 mm
Per capita water availability (2010)	1588 m ³

Water Availability facts in India

Water availability of fewer than 1700 m³ per capita per year is defined as 'water strained,' while less than 1000 m³ per capita per year is classified as 'water scarcity,' according to international standards.

In India, surface water availability was 2309 m³ in 1991 and 1902 m³ in 2001, respectively.

However, by the years 2025 and 2050, per capita surface water availability is expected to be reduced to 1401 m³ and 1191 m³, respectively.

In 2010, per capita, water availability in the country was 1588 m³, compared to 5200 m³ in 1951.

Water Pollution and Scarcity

India's surface water resources are polluted to the tune of 70%. Wastewater from various sources, intensive agriculture, industrial output, infrastructural development, and untreated urban runoff are all major contributors to water contamination.

According to the WHO, water is responsible for half of India's sickness. In India, 54 percent of rural women had to walk between 200 meters and 5 kilometers every day to get drinking water in 2012.

Overharvesting of groundwater resources is causing a water crisis in villages. Groundwater supplies over 80% of the country's drinking water.

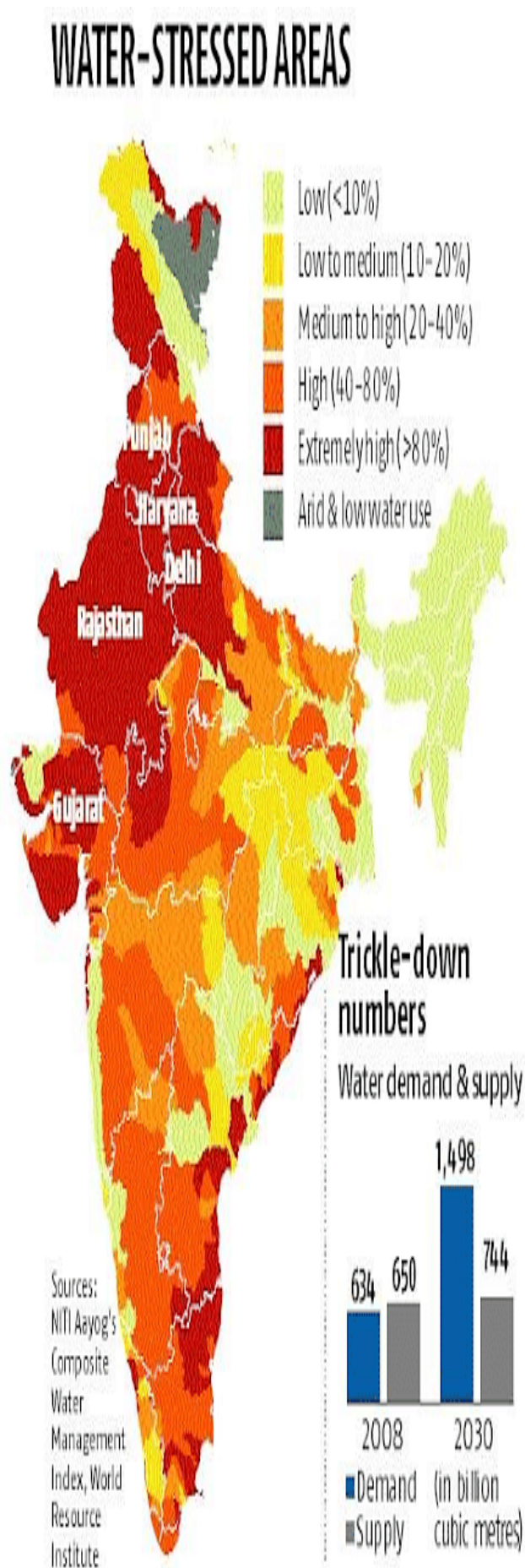
Water Stress in India: What Causes It?

Households and agriculture both require a lot of water as the population grows.

India's large agricultural sector consumes the majority of the country's water, leaving less for industry and homes.

Rapid urbanization: The high-water demand of India's densely populated cities is putting a strain on groundwater and surface water resources.

Climate change: will have a huge impact on Himalayan water resources as well as monsoonal rainfall.



Rising temperatures: will boost evaporation and precipitation, while rainfall will vary more dramatically across the country.

Droughts and floods: may become more common in different places at different periods, and mountainous areas can expect major variations in snowfall and snowmelt.

Aquifer depletion: As the world's population grows, many of the world's major aquifers are becoming exhausted. owing to direct human consumption as well as groundwater irrigation for agriculture.

Pollution and water protection: Many pollutants endanger water supplies, but the discharge of raw urban sewage, untreated industrial waste, and

Government Measures

Government Measures to alleviate the stress of the water crisis Include the initiation of the 'Jal Shakti Abhiyan,' a campaign for water conservation and security, by the Ministry of Jal Shakti. Citizens are encouraged to participate in the program, which focuses on water-stressed districts and blocks around the country.

'Har khet ko pani' and 'More Crop per Drop' are two slogans for the Pradhan Mantri Krishi Sinchay Yojana (PMKSY), which focuses on increasing water efficiency.

Other initiatives include the National Water Mission, National Ganga Mission, Dam Improvement and Rehabilitation Programme, Groundwater Management, Flood Control and Forecast, Biodiversity Conservation, Wetland

Conservation, Green India Mission, CAMPA, and others.

Jal Kranti Abhiyan: Through block-level water conservation programs, the government is actively working to modernize communities and towns. Its goal is to turn one water-scarce village in each district of the country into a water-surplus village by using a holistic and integrated approach that includes conservation and management approaches.

The National Water Mission was started by the Indian government with the goal of conserving water, reducing waste, and ensuring more equitable distribution across and within States through integrated water resource development and management.

One of the Mission's goals is to double the efficiency of water use by 20 percent. The new 'Jal Shakti Ministry is the result of the merger of two previous ministries: the Ministry of Water Resources, River Development, and Ganga Rejuvenation and the Ministry of Water Resources, River Development, and Ganga Rejuvenation.

Drinking-Water and Sanitation Ministry In certain over-exploited and water-stressed areas, the Atal Bhujal Yojana aim to promote sustainable groundwater management with community engagement.

National Rural Drinking Water Program Restructured — Expanding access to piped drinking water in rural areas. Increase the quality of service provided. The focus on water quality coverage has an impact on people's lives. **Jal Jeevan Mission:** The continuing National Rural Drinking Water Programme (NRDWP) has been reformed and integrated into the Jal Jeevan

Mission (JJM) to supply Functional Family Tap Connection (FHTC) to every rural household, i.e. Har Ghar Nal Se Jal (HGNSJ) by 2024.

Conclusion

Water is one of the most basic, widely used, and exploited natural resources, and it is perhaps on par with the other essential natural resources for life's survival. Although India has abundant water resources, most of it is getting wasted due to mismanagement, pollution, lack of wastewater management, and other such reasons. Water, being the most important resource for survival, should be conserved. The Government as well as the common masses has to work hand in hand in order to conserve water.

1.4 Biodiversity: Definition; importance of Biodiversity - ecological, consumptive, productive, social, ethical and moral, aesthetic, and option value

1.4.1 The Concept of Biodiversity

The term biodiversity is a relatively new term. It is a contraction of 'biological diversity'. Simply stated, biodiversity is the variety of life on earth and its myriad of processes. It includes all life forms – from the unicellular fungi, protozoa and bacteria to complex multicellular organisms such as plants, birds, fishes and mammals. According to the World Resources Institute – "Biodiversity is the variety of the world's organisms, including their genetic diversity and the assemblage they form. It is the blanket term for natural biological wealth that undergirds human life and well-being.

The breadth of the concept reflects the inter-relatedness of genes, species

and ecosystems. Because genes are the components of species, and species are the components of ecosystems. Therefore, altering the make-up of any level of this hierarchy can change the others – species are central to the concept of biodiversity'.

Since biodiversity covers a wide range of concepts and can be examined at different levels; therefore, it has now become customary to study the concept of biodiversity at three hierarchical levels.

Genetic diversity

Within any given species, there can be several varieties, strains or races which slightly differ from each other in one or more characteristics such as size, shape, resistance against diseases, pests, insects, etc., and resilience to survive under adverse environmental conditions. Such diversity in the genetic make-up of a species is termed as the 'genetic diversity'. In other words, 'genetic diversity', is the variety of building blocks found within individuals of a species. The species having large number of varieties, strains or races are considered as rich and more diverse in its genetic organization. The differences between individual organisms arise from variation in the genetic material possessed by all organisms and passed on to successive generations (heritable variation), and from environmental influence on the growth and development of each individual organism. Heritable variation serves as the raw material for both, evolution by natural selection and by artificial selection, and is ultimately the basis for all biodiversity.

Opportunities for evolutionary change, the survival of species and the formation of new species are in part a function of the amount of genetic

diversity in populations. The various applications of biotechnology, such as crop or animal breed improvement, depend on the identification of genetic material that give rise to desirable traits and the incorporation of this genetic material in appropriate organisms.

Species diversity

In practice, most attention is generally given to 'species diversity'. It refers to the number of different kinds of organisms found at a particular place, and how it varies from place to place and even seasonally at the same place. In terms of species diversity, it must be noted that merely counting the number of species is not enough to describe biological diversity. Diversity has to do with the relative chance of seeing species as much as it has to do with the actual number present. A community in which each species has the same number of individuals as all others would be the most diverse; whereas, a community with one species making up most of the individuals would be least diverse. Further, species that are very different from each other contribute more to overall diversity than species which are similar to each other.

Ecosystems diversity

Ecosystem diversity is generally assessed in terms of the global or continental distribution of broadly-defined ecosystem types, or in terms of the species diversity within ecosystems. It is the distinctive assemblage of species that live together in the same area and interact with their physical environment in unique ways. A system having the component species present in nearly equal abundance is considered as more

diverse than one having extremes of high and low abundance.

1.4.2 Values of biodiversity

Environmental economics (or ecological economics) provides methods of assigning economic values to species, communities and ecosystem. These values include the harvest (or market place) value of resources, the value provided by un-harvested resources in their natural habitat, and the future value of resources. For example, the Asian wild guar could be valued for the meat could be harvested from its current populations, its value for eco-tourism, or its future potential in cattle breeding.

The values can be divided as:

Direct values

Direct values, also known as use values and commodity values, are assigned to the products harvested by people. Direct values can be readily estimated by observing the activities of representative groups of people, by monitoring collection points for normal products and by examining the export/import statistics. These values can be further sub-divided as:

a) Consumptive use value

It can be assigned to goods such as fuel wood and goods that are consumed locally and do not figure in national and international market

b) Productive use value

It is assigned to products that are derived from the wild and sold in commercial markets, both national as well as international markets.

Indirect values

Indirect values are assigned to benefits provided by biodiversity that do not involve harvesting or destroying the natural resource. Such benefits include

ecological benefits such as soil formation, nutrient cycling, waste disposal, air and water purification, education, recreation, future options for human beings, etc. Indirect value can be further sub-divided as:

a) Non-consumptive use value

It is assigned to benefits such as soil formation/ protection, climate regulation, waste disposal, water and air purification, eco-tourism, medical research, etc.

b) Aesthetic, social and cultural value

The diversity of life on Earth brings us many aesthetic and cultural benefits. It adds to the quality of life, providing some of the most beautiful and appealing aspects of our existence.

Biodiversity is an important quality of landscape beauty. Many species of birds, large land mammals, sea animals and flowering plants are appreciated for their beauty. Millions of people enjoy hiking, camping, picnics, fishing, wildlife watching, and other recreational activities based on nature. These activities provide invigorating physical exercise and allow us to practice pioneer living skills. Contact with nature can also be psychologically and emotionally restorative. In many cultures, nature carries spiritual connotations, and a particular plant or animal species or landscape may be inextricably linked to a sense of identity and meaning.

Today we continue to imbue certain animals and plants with cultural significance; for instance, in India tiger and peacock, which are endangered, are especially valued because they have been adopted as national animal and bird respectively.

c) Option value

The option value of a species is its potential to provide our economic benefit to human society in the near future. For instance, there are several plant species which are edible and superior than those which are currently in use; e.g. Katemfe, a plant found in W. Africa, produces proteins that are 1,600 times sweeter than sucrose.

d) Existence value

It is assigned to protect wildlife. Since, for many people, the value of wildlife goes beyond the opportunity to photograph or even see a particular species. They argue that 'existence value', based on simply knowing that a species exist, is a sufficient reason to protect and preserve it. This right to exists was also stated in the U.N. General Assembly World Charter for Nature, 1982.

e) Ethical value

Moral justification for conservation of biodiversity is based on the belief that species have a moral right to exist, independent of our need for them. Consequently, the argument follows that in our role as the most intelligent species on Earth we have a responsibility to try as much as possible for the continuance of all forms of life.

Ethical values are deep rooted within human culture, a religion and society, but those who look on cost benefit analysis, they overlook these ethical values. International boycotts of furs, teak and ivory are the good examples of moral justification.

Unit-2: Environmental degradation and impacts

1. Human population growth and its impacts on environment; land use change, land degradation, soil erosion and desertification.
2. Use and over-exploitation of surface and ground water, construction of dams, floods, conflicts over water (within India).
3. Deforestation: Causes and effects due to expansion of agriculture, firewood, mining, forest fires and building of new habitats.
4. Non-renewable energy resources, their utilization and influences.
5. A brief account of air, water, soil and noise pollutions; Biological, industrial and solid wastes in urban areas. Human health and economic risks.
6. Greenhouse effect - global warming; ocean acidification, ozone layer depletion, acid rains and impacts on human communities and agriculture.
7. Threats to biodiversity: Natural calamities, habitat destruction and fragmentation, over exploitation, hunting and poaching, introduction of exotic species, pollution, predator and pest control.

2.1 Human population growth and its impacts on environment; land use change, land degradation, soil erosion and desertification.

Human population growth is the increase in the number of people living on Earth over time. It has been one of the most significant drivers of environmental change in recent history, with a range of impacts on the natural world.

One of the major impacts of human population growth has been the expansion of human settlements and infrastructure, leading to land use change. As populations grow, people need more space to live and work, which often involves clearing forests, grasslands, and other natural habitats to make way for buildings, roads, and agricultural fields. This process of land use change can have negative impacts on wildlife, ecosystems, and the climate.

Another consequence of land use change is land degradation. As natural habitats are destroyed or altered, the soil can become less fertile and less able to support plant growth. This can lead to reduced crop yields, food insecurity, and even famine in some areas.

Soil erosion is also a major impact of human population growth. When forests and other vegetation are removed from an area, the soil becomes more susceptible to erosion by wind and water. This can lead to the loss of topsoil and nutrients, making it difficult for plants to grow and reducing the soil's ability to support agriculture.

Desertification is another consequence of human population growth, particularly in arid and semi-arid regions. As populations grow, people often need more land for agriculture, grazing, and other uses. This can lead to overuse of the land, which can cause the soil to become dry and infertile. As a result, the land may become decertified, meaning that it is no longer able to support plant life and may become a barren wasteland.

Overall, human population growth has had a significant impact on the environment,

particularly through land use change, land degradation, soil erosion, and desertification. These impacts are likely to continue as the human population continues to grow, highlighting the importance of sustainable development and responsible land use practices.

2.2 Use and over-exploitation of surface and ground water, construction of dams, floods, conflicts over water (within India).

Water is one of the most important natural resources on Earth, and its use and management have significant impacts on both human and environmental systems. In India, water is a crucial resource that is used for a wide range of purposes, including agriculture, industry, and domestic use. However, the country faces a range of challenges related to the use and management of water, including over-exploitation of surface and ground water, construction of dams, floods, and conflicts over water.

One of the most significant challenges facing India is the over-exploitation of both surface and ground water resources. As the population grows, there is increasing demand for water for domestic, agricultural, and industrial purposes. This has led to the overuse of groundwater resources in many parts of the country, which has resulted in a decline in groundwater levels, reduced water availability, and increased salinity in some areas.

Another challenge is the construction of dams, which can have significant impacts on the natural environment and local communities. Dams are often built to store water for irrigation, hydropower, and other purposes. While dams can provide important benefits, such as flood control and electricity generation, they can also have negative impacts on the environment and local communities. For example, the construction of dams can alter the natural flow of rivers, disrupt ecosystems, and displace people from their homes and communities.

Floods are also a major challenge in India, particularly during the monsoon

season. While floods are a natural part of the hydrological cycle, they can be devastating when they occur in populated areas. Flooding can lead to loss of life, damage to infrastructure and homes, and disruption to agricultural activities.

Conflicts over water are another significant challenge facing India. With increasing demand for water resources, there are often disputes between different groups over access to water. These conflicts can occur between different regions, between rural and urban areas, and between different user groups, such as farmers and industry. Water disputes can be complex and difficult to resolve, and can lead to tension and even violence.

To address these challenges, India has implemented a range of policies and initiatives aimed at improving water management and addressing water-related issues. These include measures to promote sustainable use of water resources, such as rainwater harvesting and groundwater recharge, as well as initiatives to improve water governance and resolve conflicts over water resources.

In conclusion, the use and management of water resources in India is a complex and multifaceted issue that requires careful attention and management. The challenges of over-exploitation of surface and ground water, construction of dams, floods, and conflicts over water are significant, but with effective policies and management practices, it is possible to ensure the sustainable use of water resources for the benefit of both human and environmental systems.

2.3 Deforestation: Causes and effects due to expansion of agriculture, firewood, mining, forest fires and building of new habitats.

Deforestation refers to the permanent removal of trees from forests, leading to significant environmental impacts. It is a complex issue, driven by a range of factors, including expansion of agriculture, demand for firewood, mining, forest fires, and building

of new habitats. In this essay, we will explore the causes and effects of deforestation in more detail.

One of the main drivers of deforestation is the expansion of agriculture. As populations grow and demand for food increases, more land is needed for farming, leading to the clearance of forests for agricultural land. This process is particularly prevalent in developing countries, where subsistence farming is a common practice. However, the growth of large-scale commercial agriculture is also a significant factor in deforestation.

Demand for firewood is another significant driver of deforestation. In many parts of the world, particularly in developing countries, firewood is a primary source of energy for cooking and heating. As demand for firewood increases, forests are cleared to meet this demand, leading to deforestation. Mining is also a significant factor in deforestation. Mining operations often require large areas of land, and forests may be cleared to provide access to mineral deposits. This can have significant impacts on local ecosystems and communities.

Forest fires, both natural and human-caused, are also a significant driver of deforestation. Forest fires can be caused by lightning strikes, but they are also often started deliberately to clear land for agriculture or to remove vegetation that is considered a fire hazard. Uncontrolled forest fires can quickly spread and cause extensive damage to forests, leading to deforestation. Finally, the building of new habitats, including roads, urban areas, and infrastructure, is another significant factor in deforestation. As populations grow and demand for housing and infrastructure increases, more land is needed for construction. This can lead to the clearance of forests, leading to deforestation.

The effects of deforestation are wide-ranging and significant. Deforestation can lead to a loss of biodiversity, as many plant and animal species are dependent on forests for their survival. Deforestation also

contributes to climate change, as trees absorb carbon dioxide from the atmosphere and release oxygen. When trees are cut down, this carbon dioxide is released back into the atmosphere, contributing to the greenhouse effect and climate change.

Deforestation can also lead to soil erosion, as the removal of trees can lead to increased soil runoff and reduced soil quality. This can make it more difficult for crops to grow, leading to food insecurity and famine in some areas.

Deforestation can also have significant social impacts. Forests are often home to indigenous communities, and the clearance of forests can displace these communities, leading to loss of land, resources, and cultural heritage.

In conclusion, deforestation is a complex issue driven by a range of factors, including the expansion of agriculture, demand for firewood, mining, forest fires, and building of new habitats. The effects of deforestation are significant, including loss of biodiversity, climate change, soil erosion, and social impacts. Addressing deforestation requires a multi-faceted approach, including efforts to promote sustainable land use practices, protect forests, and promote alternative energy sources.

2.4 Non-renewable energy resources, their utilization and influences.

Non-renewable energy resources refer to energy sources that are finite and will eventually be depleted. These resources include fossil fuels such as coal, oil, and natural gas. These energy sources have been the backbone of industrial development and economic growth over the past century, but their utilization has come with significant environmental, economic, and social influences.

The primary use of non-renewable energy resources is to generate electricity, power transportation systems, and provide heat for industrial processes and buildings. Fossil fuels are burned in power plants to generate electricity, and refined to produce gasoline and diesel fuel for transportation.

The use of non-renewable energy resources has allowed for the rapid expansion of industry and economic growth, but it has also had significant negative impacts.

One of the most significant environmental impacts of non-renewable energy resources is air pollution. Burning fossil fuels releases a range of pollutants into the air, including carbon dioxide, sulphur dioxide, and nitrogen oxides. These pollutants contribute to smog, acid rain, and climate change. They can also have significant health impacts, particularly for those living near fossil fuel power plants or transportation systems.

Another significant impact of non-renewable energy resources is the destruction of habitats and ecosystems. Mining for coal and oil can lead to the removal of forests and other ecosystems, leading to loss of biodiversity and habitat destruction for wildlife. Additionally, the extraction of these resources can lead to water pollution, land degradation, and soil erosion.

The use of non-renewable energy resources also has significant economic impacts. The availability of these resources has driven economic growth and development, but it has also led to dependence on foreign sources of energy, creating geopolitical tensions and economic instability.

Additionally, the reliance on non-renewable energy resources can create price volatility, as fluctuations in supply and demand can lead to price spikes and economic shocks.

Finally, the use of non-renewable energy resources can have significant social impacts. The extraction of these resources can lead to displacement of communities and disruption of traditional ways of life. Additionally, the pollution and health impacts associated with the use of these resources can have significant impacts on public health and quality of life.

In conclusion, non-renewable energy resources have been critical to economic growth and development, but their utilization

has come with significant environmental, economic, and social impacts. Addressing these impacts requires a shift towards renewable energy sources and increased energy efficiency, along with policies to promote sustainable land use and protect ecosystems. Additionally, addressing the social impacts of non-renewable energy resources requires policies to support affected communities and promote social justice.

2.5 A brief account of air, water, soil and noise pollutions; Biological, industrial and solid wastes in urban areas. Human health and economic risks.

2.5.1 Air Pollution

Air pollution is the presence of harmful substances in the air that we breathe. These substances can be gases, particulate matter, or biological molecules, and they can be natural or human-made. Air pollution has a range of negative impacts on human health, the environment, and the economy.

There are a number of causes of air pollution, both natural and human-made. Natural sources of air pollution include wildfires, dust storms, and volcanic eruptions. However, the majority of air pollution is caused by human activities, such as transportation, industrial processes, and energy production. Fossil fuel combustion in cars, trucks, and power plants is a major contributor to air pollution, releasing pollutants such as carbon monoxide, nitrogen oxides, and particulate matter into the air. Other human-made sources of air pollution include construction and demolition activities, agriculture, and waste disposal.

The negative impacts of air pollution on human health are well-documented. Exposure to air pollution can cause respiratory diseases, heart disease, stroke, and lung cancer, and it can exacerbate existing conditions such as asthma. Air pollution also has negative impacts on the environment, contributing to climate change, acid rain, and damage to crops and forests. Additionally, air pollution has economic

impacts, reducing worker productivity and increasing healthcare costs.

Preventing air pollution requires a range of measures, both at the individual and societal level. At the individual level, there are a number of actions that individuals can take to reduce their contribution to air pollution. These include using public transportation or carpooling, reducing energy use at home, and properly disposing of waste. Additionally, individuals can advocate for policies that promote clean air, such as regulations on industrial emissions and incentives for renewable energy.

At the societal level, there are a number of policies that can be implemented to reduce air pollution. These include setting emissions standards for vehicles and industrial processes, promoting energy efficiency and the use of renewable energy, and investing in public transportation infrastructure. Additionally, policies that promote sustainable land use and reduce waste can also contribute to reducing air pollution.

Major primary pollutants produced by human activity

i. Sulphur Oxides (SO_x): SO₂ is produced by volcanoes and in various industrial processes. Since coal and petroleum often contain sulphur compounds, their combustion generates sulphur dioxide. Further oxidation of SO₂, usually in the presence of a catalyst such as NO₂, forms H₂SO₄, and thus acid rain. This is one of the causes for concern over the environmental impact of the use of these fuels as power sources.

ii. Nitrogen Oxides (NO_x): Especially nitrogen dioxide is emitted from high temperature combustion. Nitrogen dioxide is the chemical compound with the formula NO₂. It is responsible for photochemical smog, acid rain etc.

iii. Carbon monoxide (CO): It is a colourless, odourless, non-irritating but very poisonous gas. It is a product by incomplete combustion of fuel such as natural gas, coal or wood.

Vehicular exhaust is a major source of carbon monoxide.

iv. Carbon dioxide (CO₂): A greenhouse gas emitted from combustion but is also a gas vital to living organisms. It is a natural gas in the atmosphere.

In conclusion, air pollution is a significant public health, environmental, and economic challenge that requires action at both the individual and societal level. Preventing air pollution requires a range of measures, including promoting sustainable transportation, reducing energy use, and implementing policies to reduce emissions from industrial processes and energy production. By taking action to reduce air pollution, we can protect public health, preserve the environment, and support economic growth and development.

2.5.2 Water Pollution

Water pollution is the contamination of water bodies such as lakes, rivers, oceans, and groundwater by harmful substances. These substances can include chemicals, pathogens, and other pollutants, and can have negative impacts on human health, aquatic life, and the environment.

There are many different causes of water pollution, both natural and human-made. Natural causes of water pollution include soil erosion, sedimentation, and the runoff of natural materials such as animal waste and decaying vegetation. However, the majority of water pollution is caused by human activities, including industrial processes, agricultural activities, and the discharge of untreated sewage and wastewater.

Industrial activities can be a significant source of water pollution, releasing pollutants such as heavy metals, solvents, and other chemicals into water bodies. Agricultural activities, such as the use of fertilizers and pesticides, can also contribute to water pollution, as these substances can run off into nearby water bodies. Additionally, the discharge of untreated sewage and wastewater can introduce pathogens and

other contaminants into water bodies, posing a risk to human health and aquatic life.

Preventing water pollution requires a range of measures, both at the individual and societal level. At the individual level, there are a number of actions that individuals can take to reduce their contribution to water pollution. These include properly disposing of hazardous materials, reducing water use, and avoiding the use of harsh chemicals that can contaminate water bodies. Additionally, individuals can advocate for policies that promote clean water, such as regulations on industrial pollution and the protection of water sources.

At the societal level, there are a number of policies that can be implemented to reduce water pollution. These include setting standards for industrial discharge and agricultural activities, investing in water treatment infrastructure, and promoting sustainable land use practices. Additionally, policies that promote the conservation and protection of water sources can also contribute to reducing water pollution.

In conclusion, water pollution is a significant environmental and public health challenge that requires action at both the individual and societal level. Preventing water pollution requires a range of measures, including reducing the discharge of pollutants into water bodies, promoting sustainable land use practices, and investing in water treatment infrastructure. By taking action to reduce water pollution, we can protect public health, preserve aquatic ecosystems, and support sustainable economic development.

2.5.3 Soil Pollution

Soil pollution refers to the presence of harmful substances in soil that can have negative impacts on the environment and human health. These substances can include heavy metals, pesticides, and other chemicals, and can enter the soil through a range of human activities.

There are many causes of soil pollution, including industrial activities, agricultural practices, and waste disposal.

Industrial activities can be a significant source of soil pollution, releasing chemicals and other contaminants into the soil. Similarly, agricultural practices such as the use of pesticides and fertilizers can contribute to soil pollution. Additionally, the improper disposal of waste materials such as chemicals, batteries, and electronics can also contaminate soil.

Soil pollution can have a range of negative impacts on the environment and human health. Contaminated soil can harm plant and animal life, and can also lead to the loss of soil fertility and reduced agricultural productivity.

Additionally, exposure to contaminated soil can have negative impacts on human health, such as respiratory problems, skin irritation, and increased risk of cancer.

Preventing soil pollution requires a range of measures, both at the individual and societal level. At the individual level, there are a number of actions that individuals can take to reduce their contribution to soil pollution. These include properly disposing of hazardous materials, reducing water use, and avoiding the use of harsh chemicals that can contaminate soil. Additionally, individuals can advocate for policies that promote clean soil, such as regulations on industrial pollution and the protection of soil quality.

At the societal level, there are a number of policies that can be implemented to reduce soil pollution. These include setting standards for industrial discharge and agricultural practices, investing in waste disposal infrastructure, and promoting sustainable land use practices. Additionally, policies that promote the conservation and protection of soil quality can also contribute to reducing soil pollution.

In conclusion, soil pollution is a significant environmental and public health challenge that requires action at both the individual and societal level. Preventing soil pollution requires a range of measures, including reducing the discharge of pollutants into soil, promoting sustainable

land use practices, and investing in waste disposal infrastructure. By taking action to reduce soil pollution, we can protect public health, preserve the environment, and support sustainable economic development.

2.5.4 Noise Pollution

Noise pollution is a form of environmental pollution caused by the excessive presence of unwanted and disruptive sound. It is a growing concern in urban areas and can have negative impacts on physical and mental health, communication, and quality of life.

The sources of noise pollution are varied, including transportation, construction, industrial activities, and outdoor recreational activities. Transportation noise pollution, for example, is caused by the sound of cars, trucks, buses, and other vehicles, and can be particularly pronounced in areas with heavy traffic. Similarly, construction and industrial activities can generate high levels of noise that can be disruptive to nearby residents.

Noise pollution can have a range of negative impacts on physical and mental health. Exposure to high levels of noise can lead to hearing loss, tinnitus, and other auditory problems. It can also contribute to cardiovascular disease, stress, and sleep disturbances. Additionally, noise pollution can have negative impacts on communication and quality of life, making it difficult to hear and communicate with others and interfering with leisure activities such as reading and relaxation.

Preventing noise pollution requires a range of measures, both at the individual and societal level. At the individual level, there are a number of actions that individuals can take to reduce their contribution to noise pollution. These include reducing the use of noisy equipment and vehicles, using noise-cancelling headphones or earplugs, and advocating for policies that promote quieter transportation and industrial practices.

At the societal level, there are a number of policies that can be implemented to reduce noise pollution. These include

setting standards for noise levels in transportation and industrial activities, investing in noise-reducing infrastructure such as sound barriers, and promoting land use policies that separate residential areas from noisy industrial activities.

In conclusion, noise pollution is a significant environmental and public health challenge that requires action at both the individual and societal level. Preventing noise pollution requires a range of measures, including reducing the use of noisy equipment and vehicles, setting standards for noise levels, investing in noise-reducing infrastructure, and promoting land use policies that separate residential areas from noisy industrial activities. By taking action to reduce noise pollution, we can protect public health, improve quality of life, and support sustainable urban development.

2.5.5 Biological, industrial and solid wastes in urban areas

As the world's population continues to grow, urban areas are faced with the challenge of managing an increasing amount of waste. Urban waste can be broadly categorized into three types: biological waste, industrial waste, and solid waste.

Biological waste is generated from human and animal waste products, as well as food waste. This type of waste is often organic in nature and can decompose rapidly, leading to the production of greenhouse gases such as methane. In urban areas, biological waste is typically managed through a combination of wastewater treatment, composting, and landfilling. Proper management of biological waste is important for public health, as it can contain harmful pathogens and pollutants.

Industrial waste is generated from a variety of industrial activities, including manufacturing, construction, and mining. This type of waste can include hazardous materials such as chemicals, heavy metals, and radioactive waste. Proper management of industrial waste is important for protecting the environment and public health, as exposure to hazardous materials can cause a range of negative health effects.

Solid waste is generated from a variety of sources, including households, businesses, and public spaces. This type of waste can include a wide range of materials, such as plastics, paper, glass, and metals. In urban areas, solid waste is typically managed through a combination of recycling, composting, and landfilling. Proper management of solid waste is important for reducing the environmental impact of urban areas, as well as promoting resource conservation and reducing the amount of waste that ends up in landfills.

Managing urban waste requires a range of strategies and technologies. Recycling and composting programs can help to reduce the amount of waste that ends up in landfills, while wastewater treatment facilities can help to manage biological waste. Landfilling and incineration are also common methods for managing urban waste, although these methods can have negative environmental impacts if not properly managed.

In conclusion, urban waste is a significant environmental and public health challenge that requires effective management strategies. Biological waste, industrial waste, and solid waste each present unique challenges, and managing these waste streams requires a range of strategies and technologies. By implementing effective waste management strategies, urban areas can reduce their environmental impact, promote resource conservation, and protect public health.

2.5.6 Human health and economic risks.

Human health and economic risks are closely linked, as environmental hazards can have negative impacts on both individual and societal well-being. Some of the key risks associated with environmental hazards are:

Exposure to air pollution: Air pollution is a significant risk factor for a range of health problems, including respiratory diseases, cardiovascular disease, and cancer. In addition to its negative impacts on human health, air pollution can also have economic

impacts, such as increased healthcare costs and lost productivity due to illness.

Contaminated water: Exposure to contaminated water can lead to a range of health problems, including gastrointestinal illness, neurological damage, and cancer. Contaminated water can also have economic impacts, such as the cost of treating illnesses caused by waterborne pathogens.

Exposure to toxic chemicals: Exposure to toxic chemicals, such as pesticides and industrial chemicals, can have negative impacts on human health, including neurological damage, cancer, and reproductive problems. Toxic chemical exposure can also have economic impacts, such as lost productivity due to illness and the cost of treating illnesses caused by toxic exposures.

Climate change: Climate change can have significant impacts on human health, including increased incidence of heat-related illnesses, respiratory problems due to poor air quality, and the spread of vector-borne diseases. Climate change can also have economic impacts, such as the cost of treating illnesses and the cost of damage caused by extreme weather events.

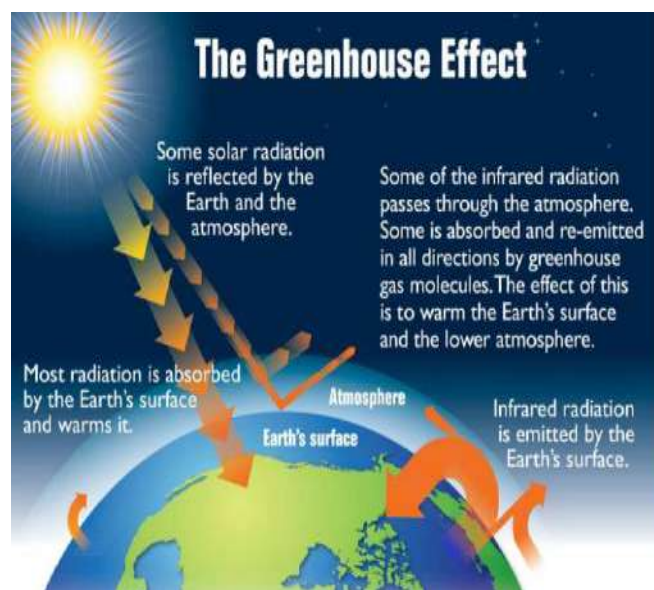
Food safety: Food safety hazards, such as foodborne pathogens and chemical contaminants, can have negative impacts on human health, including gastrointestinal illness, neurological damage, and cancer. Food safety hazards can also have economic impacts, such as the cost of treating illnesses caused by contaminated food and the cost of food recalls.

Natural disasters: Natural disasters, such as floods, hurricanes, and earthquakes, can have significant impacts on both human health and the economy. These events can cause physical injuries, displacement, and psychological trauma, as well as damage to infrastructure and economic activity.

In conclusion, human health and economic risks are closely linked, as environmental hazards can have negative impacts on both individual and societal well-being. Addressing environmental risks

requires a range of strategies, including improving air and water quality, reducing exposure to toxic chemicals, addressing climate change, improving food safety, and improving disaster preparedness and response. By addressing environmental risks, we can protect human health, promote economic prosperity, and support sustainable development.

2.6 Greenhouse effect - global warming; ocean acidification



The greenhouse effect is a natural phenomenon that occurs when certain gases in the Earth's atmosphere, such as carbon dioxide, methane, and water vapor, trap heat from the sun and prevent it from escaping into space. This process helps to maintain a stable temperature on Earth that is suitable for life. However, human activities, such as burning fossil fuels, deforestation, and agriculture, have significantly increased the concentration of greenhouse gases in the atmosphere, leading to a phenomenon known as global warming.

Global warming refers to the long-term increase in the Earth's average surface temperature due to the increase in greenhouse gases. This increase in temperature has a range of negative impacts, including more frequent and severe heatwaves, droughts, storms, and wildfires. It also contributes to the melting of glaciers and

ice caps, leading to rising sea levels that threaten coastal communities.

In addition to global warming, the increase in greenhouse gases also leads to ocean acidification. As the concentration of carbon dioxide in the atmosphere increases, a portion of it is absorbed by the ocean, leading to an increase in the acidity of the water. This has negative impacts on marine life, particularly organisms that rely on calcium carbonate to build shells and skeletons, such as coral reefs and molluscs.

Acidification also disrupts the food chain, as it affects the ability of zooplankton and other small organisms to survive, which impacts larger predators.

To address the impacts of global warming and ocean acidification, it is necessary to reduce greenhouse gas emissions. This can be done through a range of strategies, including improving energy efficiency, promoting renewable energy sources, and shifting to low-carbon transportation. It also involves reducing deforestation and adopting sustainable agriculture practices. In addition, it is essential to support research and development of technologies that can help to mitigate the impacts of global warming and ocean acidification, such as carbon capture and storage and ocean alkalinity enhancement.

In conclusion, the greenhouse effect and global warming have significant impacts on the environment, including ocean acidification. It is crucial to address these issues through a range of strategies to reduce greenhouse gas emissions and promote sustainable development. By taking action to address global warming and ocean acidification, we can help to protect the planet and ensure a sustainable future for all.

Global Warming and Greenhouse Effect The history of the greenhouse effect and global warming. First of all, predicted by Svante Arrhenius was a Swedish scientist that was the first to claim in 1896 that fossil fuel combustion may eventually result in enhanced global warming. He proposed a

relation between atmospheric carbon dioxide concentrations and temperature. When we burn organic materials (i.e. carbon-containing) fuels, or organic matter decomposes, carbon dioxide is released into the air. It is transparent to incoming solar radiation, but opaque to some wavelengths of heat radiated from the warmed surface of the Earth, and so traps heat, leading eventually to a warming of the lower atmosphere. This is known as the greenhouse effect, as in principle, the atmosphere behaves in a similar manner to a garden greenhouse, it allows sunlight to penetrate, but heat is trapped within the atmosphere in the same way that it is trapped within the glass walls of a greenhouse. This trapped hot air is causing the earth to heat up, resulting in global warming, and ultimately climate change.

Greenhouse gases include naturally occurring gases, such as carbon dioxide, methane, and even water vapor. In fact, water vapor is the most abundant greenhouse gas. However, human activities, such as burning of coal, exhaust fumes from vehicles, and burning of trees during deforestation activities, are contributing huge amounts of additional greenhouse gases into the atmosphere, where they enhance the greenhouse effect further and contribute to global warming.

The Albedo Effect Snow-capped mountains and ice sheets reflect radiation away from the Earth which is a phenomenon that is known as the albedo effect. This helps reduce the amount of heat absorbed by the Earth, and therefore plays a vital role in keeping the Earth cool. When ice sheets melt, it exposes dark rock or vegetation that doesn't have the same reflective properties as ice, and thus tends to absorb rather than reflect heat. Life sustains on Earth by depending on the energy coming from the sun. About 60 percent of the energy and light reaching the surface of the Earth passes through the air and clouds where the harmful gases get segregated and absorbed. These gases are radiated upwards in the form of infrared heat. About 89 percent of this heat is then taken by

the greenhouse gases and radiated back to the surface.

2.6.2 Ozone layer depletion

The ozone layer is a layer of gas in the Earth's atmosphere that contains high concentrations of ozone molecules, which absorb harmful ultraviolet (UV) radiation from the sun. However, the ozone layer is being depleted at an alarming rate, primarily due to human activities. Ozone depletion is a serious environmental issue that has far-reaching impacts on human health and the environment.

The primary cause of ozone layer depletion is the release of man-made chemicals, such as chlorofluorocarbons (CFCs), hydrochlorofluorocarbons (HCFCs), and halons, into the atmosphere. These chemicals are widely used in refrigeration, air conditioning, foam insulation, and fire extinguishers. Once these chemicals are released into the atmosphere, they can take several years to reach the ozone layer. Once they reach the ozone layer, they react with ozone molecules and cause them to break down, resulting in a depletion of the ozone layer.

The depletion of the ozone layer has several negative impacts, including an increase in skin cancer, cataracts, and other health problems. Exposure to UV radiation can also weaken the immune system, making individuals more susceptible to infections and diseases. In addition, the depletion of the ozone layer can have negative impacts on the environment, including changes in the distribution and behaviour of plants and animals, and changes in the climate.

To address the issue of ozone layer depletion, the international community has taken several steps, including the signing of the Montreal Protocol in 1987. This international agreement aims to phase out the production and consumption of ozone-depleting substances. As a result of this agreement, the production of CFCs and other ozone-depleting chemicals has decreased significantly, and the ozone layer is expected to recover by the middle of this century.

In conclusion, the depletion of the ozone layer is a serious environmental issue that has significant impacts on human health and the environment. It is essential to continue to take action to reduce the production and consumption of ozone-depleting substances to ensure the protection of the ozone layer and the health of individuals and the environment. By taking action today, we can ensure a sustainable future for generations to come.

2.6.3 Acid rains and impacts on human communities and agriculture

Acid Rain Acid rain refers to a mixture of deposited material, both wet and dry, coming from the atmosphere containing more than normal amounts of nitric and sulphuric acids. Simply put, it means rain that is acidic in nature due to the presence of certain pollutants in the air due to cars and industrial processes.

It is easily defined as rain, fog, sleet or snow that has been made acidic by pollutants in the air as a result of fossil fuel and industrial combustions that mostly emits Nitrogen Oxides (NO_x) and Sulphur Dioxide (SO₂). Acidity is determined on the basis of the pH level of the water droplets. Normal rain water is slightly acidic with a pH range of 5.3-6.0, because carbon dioxide and water present in the air react together to form carbonic acid, which is a weak acid. When the pH level of rain water falls below this range, it becomes acid rain.

When these gases react with water molecules and oxygen among other chemicals found in the atmosphere, mild acidic chemical compounds such as sulphuric and nitric acid are formed resulting to acid rain. Acid rain generally leads to weathering of buildings, corrosion of metals, and peeling of paints on surfaces.

Erupting volcanoes contains some

Causes of Acid Rain

Both natural and man-made sources are known to play a role in the formation of acid rain. But it is mainly caused by combustion of fossil fuels which results in

emissions of sulphur dioxide (SO₂) and nitrogen oxides (NO_x).

Natural Sources

The major natural causal agent for acid rain is volcanic emissions. Volcanoes emit acid producing gases to create higher than normal amounts of acid rain or any other form of precipitation such as fog and snow to an extent of affecting vegetation cover and health of residents within the surrounding. Decaying vegetation, wildfires and biological processes within the environment also generate the acid rain forming gases. Dimethyl sulphide is a typical example of a major biological contributor to sulphur containing elements into the atmosphere. Lightning strikes also naturally produces nitric oxides that react with water molecules via electrical activity to produce nitric acid, thereby forming acid rain.

Man-made sources

Human activities leading to chemical gas emissions such as sulphur and nitrogen are the primary contributors to acid rain. The activities include air pollution sources emitting sulphur and nitrogen gases like factories, power generations facilities, and automobiles. In particular, use of coal for electrical power generation is the biggest contributor to gaseous emissions leading to acid rain. Automobiles and factories also release high scores of gaseous emissions on daily basis into the air, especially in highly industrialized areas and urban regions with large numbers of car traffic. These gases react in the atmosphere with water, oxygen, and other chemicals to form various acidic compounds such as sulphuric acid, ammonium nitrate, and nitric acid. As a result, these areas experience exceedingly high amounts of acid rain.

- **Effect on Aquatic Environment:** Acid rain either falls directly on aquatic bodies or gets run off the forests, roads and fields to flow into streams, rivers and lakes. Over a period of time, acids get accumulated in the water and lower the overall pH of the water body. The aquatic plants and animals need a particular pH level of about 4.8 to survive. If

the pH level falls below that the conditions become hostile for the survival of aquatic life. Acid rain tendency of altering pH and aluminum concentrations greatly affects pH concentration levels in surface water, thereby affecting fish as well as other aquatic life-forms. At pH levels below 5, most fish eggs cannot hatch. Lower pH can also kill adult fishes.

- **Effect on Forests:** It makes trees vulnerable to disease, extreme weather, and insects by destroying their leaves, damaging the bark and arresting their growth. Forest damage due to acid rain is most evident in Eastern Europe – especially Germany, Poland and Switzerland.

- **Effect on Soil:** Acid rain highly impacts on soil chemistry and biology. It means, soil microbes and biological activity as well as soil chemical compositions such as soil pH are damaged or reversed due to the effects of acid rain. The soil needs to maintain an optimum pH level for the continuity of biological activity. When acid rains seep into the soil, it means higher soil pH, which damages or reverses soil biological and chemical activities.

- **Vegetation Cover and Plantations:** The damaging effects of acid rain on soil and high levels of dry depositions have endlessly damaged high-altitude forests and vegetation cover since they are mostly encircled by acidic fogs and clouds. Besides, the widespread effects of acid rain on ecological harmony have led to stunted growth and even death of some forests and vegetation cover.

- **Effect on Architecture and Buildings:** Acid rain on buildings, especially those constructed with limestone, react with the minerals and corrode them away. This leaves the building weak and susceptible to decay. Modern buildings, cars, airplanes, steel bridges and pipes are all affected by acid rain. Irreplaceable damage can be caused to the old heritage buildings.

- **Effect on Public Health:** When in atmosphere, sulphur dioxide and nitrogen oxide gases and their particulate matter derivatives like sulphates and nitrates,

degrades visibility and can cause accidents, leading to injuries and deaths. Human health is not directly affected by acid rain because acid rain water is too dilute to cause serious health problems. However, the dry depositions also known as gaseous particulates in the air which in this case are nitrogen oxides and sulphur dioxide can cause serious health problems when inhaled. Intensified levels of acid depositions in dry form in the air can cause lung and heart problems such as bronchitis and asthma.

- **Other Effects:** Acid rain leads to weathering of buildings, corrosion of metals, and peeling of paints on surfaces. Buildings and structures made of marble and limestone are the ones especially damaged by acid rain due to the reactivity of the acids in the rain and the calcium compounds in the structures. The effects are commonly seen on statues, old grave stones, historic monuments, and damaged buildings. Acid rain also corrodes metals like steel, bronze, copper, and iron

In conclusion, acid rain is a significant environmental issue that has significant impacts on human communities and agriculture. It is essential to take action to reduce the emissions of sulphur dioxide and nitrogen oxides to ensure the protection of human health and the environment. By taking action today, we can ensure a sustainable future for generations to come.

2.7 Threats to biodiversity: Natural calamities, habitat destruction and fragmentation, over exploitation, hunting and poaching, introduction of exotic species, pollution, predator and pest control.

The following are the threats to the biodiversity are

Natural Calamities:

Events like earthquakes, tsunamis, volcanic eruptions, floods, droughts, and wildfires can cause significant damage to ecosystems and habitats, leading to the loss of species.

Habitat Destruction and Fragmentation:

Human activities like deforestation, urbanization, agricultural expansion, and mining can lead to the destruction, degradation, and fragmentation of natural habitats, making it difficult for species to survive and migrate.

Over-exploitation:

Unsustainable exploitation of natural resources, such as overfishing, overhunting, and excessive logging, can deplete populations of species and disrupt ecosystems.

Hunting and Poaching:

Illegal hunting and poaching of animals for their body parts, trophies, or the wildlife trade can lead to the extinction of many species, especially those that are already endangered.

Introduction of Exotic Species:

The introduction of non-native species, either intentionally or accidentally, can disrupt ecosystems and outcompete or prey on native species, leading to their decline or extinction.

Pollution:

Various forms of pollution, such as air pollution, water pollution, soil contamination, and plastic waste, can have detrimental effects on ecosystems and the health of many species.

Predator and Pest Control:

Indiscriminate use of pesticides, herbicides, and other chemicals to control predators and pests can have unintended consequences on non-target species and disrupt food chains.

Addressing these threats through conservation efforts, sustainable practices, and policy interventions is crucial for protecting biodiversity and ensuring the well-being of ecosystems and the species that inhabit them.

Unit 3: Conservation of Environment

1. Concept of sustainability and sustainable development with judicious use of land, water and forest resources; afforestation.
2. Control measures for various types of pollution; use of renewable and alternate sources of energy.
3. Solid waste management: Control measures of urban and industrial waste.
4. Conservation of biodiversity: In-situ and ex-situ conservation of biodiversity.
5. Environment Laws: Environment Protection Act; Act; Wildlife Protection Act; Forest Conservation Act.
6. International agreements: Montreal and Kyoto protocols; Environmental movements: Bishnoi of Rajasthan, Chipko, Silent valley.

3.1 Concept of sustainability and sustainable development with judicious use of land, water and forest resources; afforestation

Sustainability refers to the ability to meet the needs of the present without compromising the ability of future generations to meet their own needs. It involves the careful and responsible use of natural resources, including land, water, and forests, to ensure that they are not depleted or damaged beyond repair.

Sustainable development is a concept that seeks to balance economic development with environmental protection and social responsibility. It involves meeting the needs of the present generation while ensuring that the natural resources are available for future generations to use.

One important aspect of sustainable development is the judicious use of land, water, and forest resources. This involves using these resources in a way that ensures their long-term availability and productivity. For example, agricultural land can be managed in a way that preserves soil fertility and prevents erosion, while water resources can be managed to ensure that they are not depleted or polluted.

Another important aspect of sustainable development is afforestation. This involves planting trees in areas where they have been cut down or where there is no forest cover. Afforestation can help to combat climate change by absorbing carbon dioxide from the atmosphere and providing habitats for wildlife.

Overall, the concept of sustainability and sustainable development involves a long-term approach to the use of natural resources. It requires careful planning, responsible management, and a commitment to protecting the environment for future generations.

Sustainability and sustainable development with regard to water and forest resources are critical for ensuring the long-term availability and productivity of these resources.

Water is a finite resource, and it is essential for human life, agriculture, industry, and ecosystems. Sustainable water management involves balancing the needs of humans and the environment while ensuring that water resources are used efficiently and not overexploited.

Some ways to promote sustainable water management include improving water-use efficiency, reducing water pollution, promoting water conservation, and managing water resources in an integrated and equitable manner.

Forests are also a vital resource that provide a range of ecosystem services, such as carbon storage, biodiversity, and watershed protection. Sustainable forest management involves ensuring that forests are used in a way that maintains their productivity, biodiversity, and ecological functions.

Some ways to promote sustainable forest management include protecting forests from deforestation, promoting reforestation and afforestation, managing forests for multiple benefits, and promoting sustainable forest-based industries.

Overall, sustainability and sustainable development with regard to water and forest resources involve a long-term approach to managing these resources in a way that ensures their continued availability and productivity for future generations. It requires a commitment to protecting the environment and balancing the needs of humans and the natural world.

Afforestation is the process of establishing a forest, or stand of trees, in an area where there was no forest cover previously. The concept of afforestation is an important aspect of sustainability because it helps to mitigate the negative impacts of deforestation, which can lead to soil erosion, loss of biodiversity, and other environmental problems.

Afforestation has several benefits, including providing habitat for wildlife, improving soil health and water quality, sequestering carbon from the atmosphere, and mitigating the effects of climate change.

Afforestation can also provide economic benefits, such as timber and non-timber forest products, as well as recreational and cultural benefits.

Sustainable afforestation involves careful planning and management to ensure that the forest is established and maintained in a way that balances ecological, social, and economic considerations. This may involve selecting appropriate tree species for the local environment, controlling invasive species, protecting biodiversity, and involving local communities in the management of the forest.

Overall, the concept of sustainability afforestation involves a long-term approach to establishing and managing forests in a way that benefits both humans and the environment. It requires a commitment to protecting the natural world, promoting biodiversity, and mitigating the negative impacts of deforestation.

3.2 Control measures for various types of pollution; use of renewable and alternate sources of energy

3.2.1 Control measures for various types of pollution

Pollution is the introduction of harmful substances into the environment, which can have negative impacts on human health, wildlife, and the natural world. There are several types of pollution, including air pollution, water pollution, and soil pollution. Following are control measures for each type of pollution:

Air Pollution:

- Reduce emissions from industries and vehicles through the use of cleaner fuels and technologies
- Implement regulations on the use of pollutants such as nitrogen oxide, sulphur dioxide, and particulate matter
- Encourage the use of public transport, cycling, or walking instead of private vehicles

- Promote the use of energy-efficient appliances and devices
- Plant trees and vegetation to absorb pollutants and improve air quality

Water Pollution:

- Implement regulations on the discharge of pollutants into water bodies
- Promote the use of wastewater treatment plants to treat sewage and industrial effluent
- Encourage the use of organic farming practices to reduce agricultural runoff
- Promote the use of biodegradable and eco-friendly cleaning products
- Monitor and control industrial waste disposal practices
- Educate the public about the proper disposal of hazardous waste, chemicals, and pharmaceuticals

Soil Pollution:

- Implement regulations on the disposal of hazardous waste, including contaminated soil
- Promote the use of organic farming practices and the use of natural fertilizers and pesticides
- Monitor and control industrial waste disposal practices
- Promote the use of sustainable land use practices, such as crop rotation and intercropping
- Encourage the remediation of contaminated sites through phytoremediation, microbial remediation, and other techniques

Controlling pollution requires a combination of regulatory measures, technological innovations, and public awareness and education. By implementing these measures, we can help to protect human health and the environment from the negative impacts of pollution.

3.2.2 Use of renewable and alternate sources of energy

Renewable and alternate sources of energy are essential for achieving sustainability and reducing our dependence on fossil fuels, which are finite resources and contribute to climate change. The following are some examples of renewable and alternate sources of energy:

Solar Energy: Solar energy involves capturing the energy from the sun and converting it into electricity using photovoltaic cells (PV) or solar thermal systems. Solar energy is a clean and renewable source of energy that can be used to power homes, businesses, and communities.

Wind Energy: Wind energy involves capturing the energy from the wind and converting it into electricity using wind turbines. Wind energy is a clean and renewable source of energy that can be used to power homes, businesses, and communities.

Hydro Energy: Hydro energy involves capturing the energy from flowing water and converting it into electricity using hydroelectric power plants. Hydro energy is a clean and renewable source of energy that can be used to power homes, businesses, and communities.

Geothermal Energy: Geothermal energy involves tapping into the heat energy stored beneath the earth's surface and converting it into electricity using geothermal power plants. Geothermal energy is a clean and renewable source of energy that can be used to power homes, businesses, and communities.

Biomass Energy: Biomass energy involves converting organic matter, such as wood, crops, and agricultural waste, into biofuels or electricity. Biomass energy is a renewable source of energy that can be used to power homes, businesses, and communities.

Use of renewable and alternate sources of energy is essential for achieving sustainability and reducing our dependence

on fossil fuels. By promoting the use of these sources of energy, we can help to protect the environment, reduce greenhouse gas emissions, and promote economic development.

3.3 Solid waste management: Control measures of urban and industrial waste.

Solid waste management involves the collection, transportation, treatment, and disposal of solid waste, which includes both urban and industrial waste. Some control measures for urban and industrial waste:

Urban Waste Management:

- Implement a waste hierarchy, which prioritizes waste reduction, reuse, and recycling before disposal
- Promote the use of composting and other organic waste treatment methods to reduce the amount of waste sent to landfills
- Establish effective collection and transportation systems for solid waste, including separate collection of recyclable materials
- Implement regulations and incentives to encourage waste reduction and proper disposal practices
- Educate the public about waste reduction and proper disposal practices
- Industrial Waste Management:
- Implement pollution prevention measures to reduce the amount of waste generated in the first place
- Establish effective collection and transportation systems for industrial waste, including hazardous waste
- Promote the use of waste-to-energy technologies to recover energy from waste
- Implement regulations and incentives to encourage waste reduction and proper disposal practices
- Monitor and control industrial waste disposal practices to prevent pollution and protect human health and the environment

Effective solid waste management requires a combination of regulatory measures, technological innovations, and public

awareness and education. By implementing these measures, we can help to reduce the amount of waste generated, recover resources, and protect human health and the environment from the negative impacts of solid waste.

3.4 Conservation of biodiversity: In-situ and ex-situ conservation of biodiversity

Conservation of biodiversity is crucial for maintaining the balance of the ecosystem and sustaining life on Earth. It involves the protection, preservation, and management of biological diversity, including species, ecosystems, and genetic diversity. The following are two approaches to conservation of biodiversity:

In-situ conservation: In-situ conservation involves the protection and management of biodiversity in its natural habitat. This can be done through the establishment of protected areas, such as national parks, wildlife reserves, and sanctuaries, where biodiversity is conserved in its natural state. In-situ conservation also involves the restoration and management of degraded habitats to restore biodiversity and ecosystem functions. This approach is crucial for preserving the genetic diversity of species, maintaining natural ecological processes, and ensuring the long-term survival of species.

Ex-situ conservation: Ex-situ conservation involves the conservation of biodiversity outside of its natural habitat. This can be done through the establishment of gene banks, seed banks, zoos, and botanical gardens, where biodiversity is conserved in captivity. Ex-situ conservation can also involve the reintroduction of endangered or extinct species back into their natural habitat. This approach is crucial for protecting species that are at risk of extinction, preserving genetic diversity, and providing a backup for in-situ conservation efforts.

Both in-situ and ex-situ conservation approaches are essential for the conservation of biodiversity. By protecting biodiversity, we can maintain the natural balance of ecosystems, promote sustainable

development, and ensure the survival of species for future generations.

3.5 Environment Laws: Environment Protection Act; Wildlife Protection Act; Forest Conservation Act.

3.5.1 Environment Protection Act:

The Environment Protection Act (EPA) is a legislation enacted by the Government of India in 1986 to protect and improve the quality of the environment and prevent and control environmental pollution. The EPA is a comprehensive legislation that provides for the establishment of various institutions, such as the Central Pollution Control Board (CPCB) and State Pollution Control Boards (SPCBs), to oversee and regulate environmental protection measures.

Some key provisions of the Environment Protection Act:

Regulation of Pollution: The EPA provides for the regulation of all sources of environmental pollution, including air pollution, water pollution, and noise pollution. It empowers the CPCB and SPCBs to set standards for emissions and effluent discharge, and to enforce these standards through monitoring and inspection.

Environmental Impact Assessment: The EPA mandates the preparation of environmental impact assessments (EIAs) for all development projects that are likely to have significant impacts on the environment. The EIAs are used to identify potential environmental impacts and to recommend measures to mitigate these impacts.

Hazardous Substances: The EPA provides for the regulation of hazardous substances, including their manufacture, storage, transportation, and disposal. It empowers the CPCB and SPCBs to enforce safety standards for hazardous substances and to require the use of appropriate protective measures.

Penalties: The EPA provides for penalties for violations of its provisions, including fines and imprisonment. It also allows for the closure of polluting industries and the confiscation of equipment and materials used for environmental pollution.

Environment Protection Act is a crucial piece of legislation that provides for the regulation and protection of the environment in India. It has been instrumental in promoting sustainable development and ensuring the conservation of natural resources for future generations.

3.5.2 Wildlife Protection Act;

The Wildlife Protection Act (WPA) is a legislation enacted by the Government of India in 1972 to protect wildlife in India and regulate hunting and poaching activities. The WPA is a comprehensive legislation that provides for the protection of wild animals, birds, and plants, and establishes penalties for offenses related to wildlife.

Key provisions of the Wildlife Protection Act:

Protected Species: The WPA provides for the protection of endangered species of wild animals, birds, and plants, and regulates their hunting, trapping, and trade. The Act provides for the establishment of a network of protected areas, including national parks, wildlife sanctuaries, and conservation reserves, for the conservation of wildlife and their habitats.

Prohibitions and Penalties: The WPA prohibits the hunting, trapping, poaching, or trade of protected species without a permit, and provides for severe penalties for offenses. The Act also provides for the forfeiture of any property used in the commission of an offense, including vehicles, weapons, and other equipment.

Wildlife Crime Control Bureau: The WPA established the Wildlife Crime Control Bureau (WCCB) to combat organized wildlife crime and enforce the provisions of the Act. The WCCB is responsible for investigating wildlife crimes, coordinating with law enforcement agencies, and promoting awareness about wildlife conservation.

Amendments: The WPA has been amended several times to strengthen its provisions and address emerging issues related to wildlife conservation. The most recent amendment in 2006 introduced stricter penalties for wildlife

offenses and provided for the establishment of a National Tiger Conservation Authority.

Wildlife Protection Act is a crucial piece of legislation that has played a significant role in the conservation of wildlife in India. It has helped to protect endangered species, regulate hunting and poaching activities, and promote sustainable development.

3.5.3. Forest Conservation Act

The Forest Conservation Act (FCA) is a legislation enacted by the Government of India in 1980 to regulate the diversion of forest lands for non-forestry purposes. The FCA is a crucial piece of legislation that provides for the conservation of forests and the sustainable use of forest resources.

Provisions of the Forest Conservation Act:

Prior Approval: The FCA requires prior approval from the central government for the diversion of forest lands for non-forestry purposes, such as mining, industrial development, and infrastructure projects. The approval process involves a detailed examination of the impact of the proposed diversion on the environment and the local community, and requires the submission of a compensatory afforestation plan.

Compensatory Afforestation: The FCA mandates the implementation of a compensatory afforestation plan for every hectare of forest land diverted for non-forestry purposes. The plan should ensure the replacement of the lost forest cover with an equivalent area of afforested land, and should be implemented within a specified time frame.

Conservation of Forests: The FCA emphasizes the conservation of forests and the sustainable use of forest resources. It provides for the regulation of forest management practices, including logging and grazing, and encourages the participation of local communities in forest conservation and management.

Penalties: The FCA provides for penalties for violations of its provisions, including fines and imprisonment. It also allows for the cancellation of approvals for forest land diversion and the forfeiture of any benefits derived from such diversion.

Forest Conservation Act is a crucial legislation that has played a significant role in the conservation of forests in India. It has helped to regulate the diversion of forest lands for non-forestry purposes, promote compensatory afforestation, and encourage sustainable forest management practices. The Act has been instrumental in promoting sustainable development and ensuring the conservation of natural resources for future generations.

3.6 International agreements: Montreal and Kyoto protocols; Environmental movements: Bishnoi's of Rajasthan, Chipko, Silent valley.

3.6.1 Montreal and Kyoto protocols

The Montreal Protocol and the Kyoto Protocol are two important international agreements aimed at addressing global environmental issues.

The Montreal Protocol is an international treaty signed in 1987 to protect the Earth's ozone layer. The ozone layer is a thin layer of gas in the Earth's atmosphere that absorbs most of the sun's harmful ultraviolet (UV) radiation. The Montreal Protocol aims to phase out the production and consumption of substances that deplete the ozone layer, such as chlorofluorocarbons (CFCs) and hydrochlorofluorocarbons (HCFCs). The treaty has been successful in reducing the production and consumption of these substances and has helped to prevent further damage to the ozone layer.

The Kyoto Protocol is an international agreement signed in 1997 to address climate change. The Protocol aims to reduce greenhouse gas emissions, such as carbon dioxide (CO₂), from industrialized countries. The treaty established legally binding targets for reducing emissions and created a mechanism for trading emissions credits between countries. The Kyoto Protocol has been an important step in global efforts to address climate change, although its effectiveness has been limited by the lack of

participation by some countries, such as the United States.

Montreal Protocol and the Kyoto Protocol are important international agreements aimed at addressing global environmental issues. The Montreal Protocol has been successful in protecting the Earth's ozone layer, while the Kyoto Protocol has been an important step in global efforts to address climate change. However, further international cooperation and action are needed to address these and other environmental challenges facing the planet.

3.6.2 Environmental movements: Bishnoi's of Rajasthan

The Bishnoi community of Rajasthan is one of the oldest environmental movements in India. The Bishnoi's are a community of nature lovers who are deeply committed to the protection of wildlife and the preservation of the environment. Their founder, Guru Jambheshwar, established 29 principles or 'bis' that provide guidelines for living in harmony with nature.

The Bishnoi's are known for their strong belief in the sanctity of plant and animal life. They have a tradition of hugging trees to protect them from being cut down, and they also protect wildlife from harm. The community has a long history of conservation efforts, dating back to the 18th century when they fought against the Maharaja of Jodhpur's decision to cut down trees for the construction of a new palace. The Bishnoi's refused to allow the trees to be cut down and many of them lost their lives protecting the trees.

Today, the Bishnoi community continues to play an important role in the conservation of wildlife and the environment in Rajasthan. They have set up community

conservation areas where wildlife is protected, and they have also established a network of sacred groves where trees are protected. The Bishnoi's have also been instrumental in the reintroduction of several endangered species in Rajasthan, including the blackbuck antelope.

The Bishnoi's environmental movement is an inspiring example of the power of community action in the conservation of nature. Their efforts have helped to protect wildlife and preserve the environment for future generations, and their principles of living in harmony with nature continue to inspire environmentalists around the world.

3.6.3 Environmental movements: Chipko

The Chipko movement, also known as the Chipko Andolan, was a forest conservation movement that originated in the Indian state of Uttarakhand in the 1970s. The movement was led by a group of women who hugged trees to prevent them from being cut down by loggers. The Chipko movement was one of the earliest environmental movements in India and played a significant role in raising awareness about the importance of forests and the need for their conservation. The Chipko movement was triggered by the felling of trees in the Alaknanda valley in the Himalayas. The local villagers were dependent on the forests for their livelihoods, and the indiscriminate cutting of trees was leading to soil erosion, landslides, and floods. In 1973, a group of women led by Gaura Devi protested against the cutting of trees in the Chamoli district. They formed a human chain around

the trees and prevented the loggers from felling them. The women continued their protest for several days until the government agreed to halt the logging operations in the region.

The success of the Chipko movement inspired similar movements in other parts of the country. The movement also led to the formulation of policies and laws for the conservation of forests in India. The Forest Conservation Act, which was passed in 1980, was a direct result of the Chipko movement. The Chipko movement also highlighted the important role that women play in conservation efforts. Women in the local communities were often the primary caregivers of the forests and had a deep understanding of the ecological systems in the region. The movement gave women a platform to voice their concerns and play an active role in forest conservation.

The Chipko movement was not without its challenges. The loggers and the government initially dismissed the protests as the work of ignorant villagers. The protesters faced violence and intimidation from the loggers and the police. However, the women persisted, and their efforts paid off in the end. In conclusion, the Chipko movement was a landmark event in the history of environmental movements in India. The movement brought attention to the need for forest conservation and highlighted the important role that women play in conservation efforts. The movement also paved the way for the formulation of policies and laws for the conservation of forests in India. The Chipko movement serves as an

inspiration for future generations to continue the fight for the protection of the environment.

3.6.4 Environmental movements: Silent valley.

The Silent Valley movement was a protest against the proposed construction of a hydroelectric dam in the Silent Valley, a pristine forest area located in the Western Ghats of Kerala, India. The movement was led by environmental activists and local tribal communities who were concerned about the impact of the dam on the fragile ecosystem of the valley.

The Silent Valley is home to several endangered species of plants and animals, including the lion-tailed macaque, a critically endangered primate found only in the Western Ghats. The proposed dam would have flooded a large part of the valley, displacing thousands of tribal people and destroying the unique biodiversity of the region.

The Silent Valley movement gained momentum in the late 1970s when the government announced plans to build the dam. Environmental activists and local tribal communities joined forces to oppose the project. The movement received widespread support from scientists, conservationists, and other concerned citizens.

The protesters argued that the construction of the dam would destroy the fragile ecosystem of the Silent Valley and displace thousands of tribal people who depend on the forest for their livelihoods. They also pointed out that the dam was unnecessary, as there were several other

sources of power available that would not have such a devastating impact on the environment.

The Silent Valley movement gained international attention and support, and in 1985, the Indian government announced that it would abandon the project. The decision was a significant victory for the environmental movement in India and highlighted the importance of public participation in decision-making processes.

The Silent Valley movement had several positive outcomes. It raised public awareness about the importance of biodiversity conservation and the need to balance economic development with environmental protection. The movement also highlighted the importance of local communities in environmental decision-making and paved the way for the recognition of tribal rights in India.

Silent Valley movement was a landmark event in the history of environmental movements in India. The movement succeeded in preventing the construction of a hydroelectric dam in a fragile ecosystem and raised awareness about the importance of biodiversity conservation. The movement also demonstrated the importance of public participation and the role of local communities in environmental decision-making.



Welcome: Dr. N. SREENIVAS (Self Published Author)

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POSTHARVEST TECHNOLOGY OF FISH AND FISHERIES LAB PROTOCOLS

SREENIVAS-CHAKRAPANI-ANILKUMAR

POSTHARVEST TECHNOLOGY OF FISH AND FISHERIES LAB

Practical (Laboratory) Syllabus:

- 1. Evaluation of fish/ fishery products for organoleptic, chemical and microbial quality.**
- 2. Preparation of dried, cured and fermented fish products**
- 3. Examination of salt, protein, moisture in dried / cured products**
- 4. Examination of spoilage of dried / cured fish products, marinades, pickles, sauce.**
- 5. Preparation of isinglass, collagen and chitosan from shrimp and crab shell.**
- 6. Developing flow charts and exercises in identification of hazards – preparation of hazard analysis worksheet**
- 7. Corrective action procedures in processing of fish- flow chart- work sheet preparation**

1. Evaluation of fish & fishery products for organoleptic, chemical and microbial quality

Aim: To evaluate the fish and fishery products for organoleptic, chemical and microbial quality.

Introduction:

Fish is low in fat but high-quality protein filled with omega-3 fatty acids and vitamins such as D and B diet.

Fish is an important source of minerals such as calcium and phosphorus, iron, zinc, iodine, magnesium, and potassium.

Demand for fish and its products are consumed in large numbers worldwide. As many countries, do not have the conditions to cultivate the desired fishes so are imported from coastal countries.

Fish being a highly perishable product, the quality of the fish starts to deteriorate after the fish is brought to the landing sites. The deterioration of the fish occurs due to:

- (1) Bacterial invasion and putrefication,
- (2) enzymatic autolysis,
- (3) chemical oxidation and
- (4) Mechanical damage and environmental optimization aggregating growth of microbes.

EXISTING TECHNIQUES FOR QUALITY CONTROL

ORGANOLEPTIC METHODS

- Sensory methods are used to assess the degree of freshness based on organoleptic characteristics such as general consistency of flesh, odour, color also eye and gill condition etc.
- These characteristics are judged by panel members. i.e. subjective judgment is made by individuals.
- Various numerical scoring or ranking systems have been developed to evaluate the judgments or results.
- Sensory methods have advantages that it can be adapted by the human being easily and the quality can be assessed by Oduor or visual inspection for quality defects. Human senses indeed are more efficient in some complex tasks than the instruments.
- There is, however, no single satisfactory test developed for the quality assessment of fish. A combination of chemical, biological and organoleptic tests would be the best procedure to assess such freshness.

MICROBIAL ASSESSMENT/BIOLOGICAL EVALUATION:

Fish decomposition starts after catch starts and changes occur in pH, atmosphere, nutrient composition have effects on micro flora.

Raw fish consists of its own unique flora, determined by the microbial content of the surrounded water, which still remain despite food processing and subsequent cooling.

Categorization of fish with similar microflora is considered when assuring the quality of Fish and its products. Microbes play a vital role in the shelf life of fish as gram-negative, fermentative bacteria (such as *Vibrionaceae*) spoil unpreserved fish, whereas psychrotolerant gram negative bacteria (*Pseudomonas* spp. and *Shewanella* spp) can continue growth in chillers. Several standards are utilized to judge its freshness and adequacy.

Total viable counts or TVC

Total viable counts or TVC s are utilized widely as a part of microbiological allowing one to identify the active number of growing/dividing microbes.

TVC ranges from 10^2 – 10^6 cfu/g in case of fish and the range determines its acceptability.

TVC limit varies greatly upon its conditions (temp, atmosphere, pH etc).

The maximum microbiological limit for the TVAC is 5×10^5 cfu/g and TVAC mostly ranged from 2.8×10^5 to 4.9×10^5 cfu/g (Colony forming units/g) for exported frozen fish which was below the maximum acceptable.

II. MICROBIAL EVALUATIONS:

Total plate count (TPC): Total number of microbial flora is changed with the time in fish or fish products.

The numbers per gram of fish or fish products or per square centimeter of the surface area indicate the quality of fish from the microbiological view point.

Total plate count or viable bacterial count is determined by the culture of bacteria present in fish sample using a suitable bacteriological media that could recover maximum number of bacteria in fish tissue.

A known weight of fish sample is minced aseptically and serial decimal dilutions are pour-plated with the media.

For marine fish agar agar and for processed fish products tryptone glucose beef extract agar media are commonly used.

Inoculated plates are incubated at 37^0 C for 24 hours and the bacterial colonies are counted.

From the colony counts, TPC is calculated by multiplying with appropriate dilution factor.

TPC does not strictly indicate the edibility of the fish.

Fish with low TPC may bear pathogen that would have more dangerous if consumed. Therefore, qualitative analysis is done to determine the presence of any pathogenic or health hazard bacteria.

Different types of selective or ordinary media are used for the identification of pathogenic organisms in fish.

For example, SS-agar or XLD agar medium are used to detect coliform bacteria like *Salmonella*, *Shigella*, *E. Coli*.

III. NON-SENSORY METHOD

Since sensory assessment may require the use of a taste panel, non-sensory methods can be cheaper and often quicker.

Non- sensory assessments should give the same result no matter where they are carried out, whereas sensory evaluations may depend on subjective responses of the panellists to the fish being examined.

Non-sensory methods can appear more objective and reliable than sensory methods, although this need not be the case. When specifications are being prepared it is easier to insert numerical limits based on non-sensory tests than on sensory tests, especially in international trade, and this is widely done.

There are different kinds of non-sensory or instrumental method, they are described below:

1. Biochemical methods

A variety of chemical compounds or groups of compounds accumulate post-mortem fish muscles. These chemicals are either intermediate or end products of biochemical changes occurring in the muscles of fish after death.

1. Proximate composition: Because of influence of chemical composition on keeping quality, proximate chemical composition like moisture, lipid, protein and ash contents of fish samples from the time or day of harvest to different storage periods or conditions are often investigated. Proximate composition may vary with species, sex, season, place of harvest, feeding condition, etc. So, conclusive results are very difficult to obtain.

2. Hypoxanthin value: As a consequence of post-mortem changes, breakdown of ATP to ADP, AMP, IMP and finally to hypoxanthine takes place. Hypoxanthin content of muscle increases on storage of fish. Estimation of hypoxanthin is an objective test of freshness of fish. However, the estimation of hypoxanthin is too cumbersome and it is seldom employed in practice. Fish with a hypoxanthin value of 7-8 micro moles/g is considered spoiled.

3. Histamine content: Histamine develops in freshly caught fish after 40-50 hours of death, if the fish is not properly iced. To avoid histamine formation in tuna, skipjack and mackerel, care is taken to ice or freeze fish as quickly as possible. Histamine is a major problem in warm water pelagic species that causes a form of food poisoning known as scombroid poisoning, as the name derived from the family name of tuna and mackerel, scombroidae.

The symptoms of scombroid poisoning are facial flushing, rashes, headache and gastro-intestinal disorders. Histamine is produced from histidine which is one of the constituents of muscle protein of all fishes. Generally, pelagic fishes contain sizable amount of histidine in free state as well as bound state within protein. Upon death, histidine in fish muscle is converted to histamine by bacterial enzymes. Histamine content over 20mg/100g in canned fish is prohibited by the USFDA standard.

4. pH: Change in pH of the fish muscle is a usual good index for freshness assessment.

5. Trimethylamine: Marine fish contains sizable amount of trimethylamine oxide (TMAO) which is reduced to trimethylamine (TMA) during the spoilage of fish. TMA determination is a useful index to measure the quality of stored fish. Fish with a level of 1.5 mg TMA nitrogen/100 g fish is considered acceptable, while 10-15 mg/100 g limit is set for moderately spoilt fish and beyond this range is set for highly spoilt fish.

6. Ammonia: Bacteria can generate small amounts of ammonia in spoiling fish, mainly from free amino acids; the amount of ammonia can give an indication, though not a particularly accurate one, of the extent of spoilage. Much larger amounts of ammonia are produced during spoilage of the elasmobranch fishes, skate and dogfish for example, because they have large amounts of urea in their flesh. Shellfish, also, may develop more ammonia than most marine fish and at an

earlier stage. There are several chemical and enzymic methods for measuring ammonia.

7. Peroxide value: Oxidative rancidity developed in fish tissue is determined by the estimation of peroxide value. Good quality fish should have a peroxide value quite less than 10. Peroxide value above 20 for any fish is considered rancid.

8.Thiobarbituric acid value (TBA): It is also determined as an index of oxidative rancidity in fish. For a good quality moderate lipid fish, TBA value of less than 2 is usually accepted.

9.K value: High level of adenosine related compounds or inosine mono phosphate in fish muscle imparts sweet, meaty flavour and is regarded as a reliable index of freshness. Postmortem accumulation of inosine or hypoxanthin generally reflects poor quality. The conversion of ATP to IMP is very fast and is usually complete within a day.

Subsequent accumulation of inosine or hypoxanthin is related to both autolytic and microbial action. Based on hypoxanthin values and their correlation with freshness of fish, Saito *et al* first proposed the K value as an index of the freshness of fish.

In freshly caught fish K value would be as low as zero, in moderate quality fish the value could be 10 to 20 but in spoilt fish it can go up to 90. The K values are found to have excellent agreement with sensory data of fish. K values also rise with the ice storage period of fish.

EXPERIMENT 2. PREPARATION OF DRIED, CURED AND FERMENTED FISH PRODUCTS

2A: PREPARATION OF DRIED FISH PRODUCTS

AIM: To understand the process of dried fish products preparation

INTRODUCTION: Drying is the removal of water from fish, and like salting, is a very common method of preserving fish, particularly in tropical countries. When sufficient water has been removed fish will be preserved because water is essential for bacteria and enzymes to survive and work to spoil fish. Drying is often used in combination with salting and or smoking for additional preservation.

Drying of fish takes place in two phases:

1. Initially, water on or near the surface of the fish evaporates. The rate of drying depends on:
 - i) surface area of the fish (size)
 - ii) speed of air movement over the fish,
 - iii) relative humidity of the air.
2. The second phase occurs when the surface of the fish has evaporated. The drying rate in this phase depends on:
 - i) the nature of the fish. Fat in fish flesh retards water movement
 - ii) fish shape. The thicker the fish, the longer the time of drying
 - iii) temperature. Drying will proceed more rapidly at higher temperatures
 - iv) water content.

How should fish be prepared for drying?

As with the preparation of fish for salting, fish selected for drying must be as fresh as possible. If ice is available, the freshly-caught fish should be placed on ice until it is required for processing. If no ice is available, clean the fish with fresh or sea water, place it in a clean box and cover it with a wet cloth or mat to protect it from sunlight, dust and flies.

However, without ice, the fish will need to be processed as soon as possible. The fish most suitable for drying are lean fish and fish with a low-fat content, such as tilapia, shark and most white-fleshed fish.

Fish must always be gutted before drying. Depending on the size of the fish it can be prepared in several ways:

- Large fish, like shark, should be cut into strips or small rectangles to ensure adequate drying
- Small fish need only be gilled and gutted before drying
- Small fish and fish which have been cut into strips can be dried, hanging from a rod
- Racks (flat wire-mesh racks and vertical hanging racks) can be used to dry fish which have been split down the back bone. Such racks ensure that the fish is evenly dried all over.

Methods of drying

Drying of fish is most often done using sun drying or mechanical dryers. Very small, thin fish can be dried straight away in the sun if they are brought in early enough in the morning, and the sun is shining. If these conditions are not fulfilled, the fish must be put for one night in brine, or dry salted.

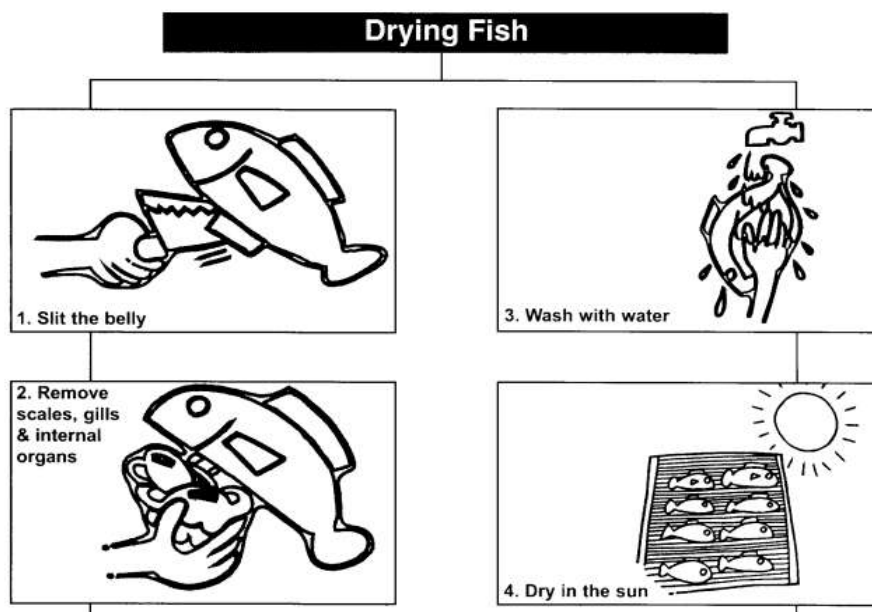
They can then be dried the next morning. If it happens to be raining the next day, it is necessary to wait until the weather has cleared up. In this latter case it will be necessary to wash the salt away from the fish by soaking it in fresh or salt water for a couple of hours before drying; this depends again on the tastes of the consumers and on the purpose for which the fish is cured

Measuring the drying rate of fish

When drying unsalted fish, an easy way to check water loss is to weigh the fish before and after drying:

- lean fish, such as shark and grouper, should lose 75 - 76% of their original weight
- medium, fatty fish, such as tilapia should lose 72 - 73% of their original weight
- fatty fish, such as sardine and mackerel, should lose 67 - 68% of their original weight.

If the weight reduction takes longer than 2 - 3 days, under good drying conditions, the fish is too thick. It must be filleted or cut into strips before drying. If weight reduction is so slow that the fish spoil, the fish will have to be discarded.



2B: PREPARATION OF CURED FISH PRODUCTS

AIM: To Know various fish curing methods for preservation of fish

Introduction: Curing is a process by which the fish is preserved by sun drying, salting, smoking, artificial drying etc. This can be done either by any single method or a combination of these methods. Simple sun drying was the widely practiced traditional method of fish preservation. By this, preservation was achieved by removing the water in the fish, thereby retarding the activity of bacteria and fungi. The heat was able to destroy the bacteria to a certain extent. Later on, a combination of salting and drying or salting, smoking and then drying were developed.

Indian Standard Specification for Common Salt for Fish Curing

Physical properties – The material shall be crystalline, white, pale pink or light grey in colour, free from visible contamination with clay, grit and other extraneous adulterants and impurities.

Microbial quality: Salt shall be free from halophilic microorganisms, the most common of them being the red halophilic bacteria.

Particle Size: The material shall be between 2.36 mm and 5.00 mm in size.

Moisture Content: The moisture content of the material shall be not more than 6.0 percent by mass

Dry salting or Kench curing

In this method the fish is salted, but the juices, and brine (pickle) are allowed to drain away. For 2 parts of fish, 1 part of salt is needed. Layers of fish are separated by layers of salt and placed into a wooden box that has slats cut out of the sides, enabling the draining of juice. It is important to layer the fish with the first layer being flesh side upwards, and the next layer of fish being flesh-side down. The final layer should be salt. The box has a lid placed on top of the stack and weighted to press the fish down. This encourages faster salt penetration and water removal. Salting time varies from three days to a week, depending on the type and size of fish

Pickle salting:

Pickle curing is a type of wet salting where the fish is layered by granular salt (fish-to-salt ratio of 1-part fish to 0.3 or 0.4 parts salt by weight) which, dissolves in the surface moisture of the fish forming solution which penetrates into the fish removing moisture from the fish. The fish is allowed to remain in this self-brine. If the self-brine is not sufficient, saturated brine is added to immerse the fish.

Mona curing:

Mona curing is mainly adopted for medium to small size fishes. Before salting, the intestine and entrails are removed by pulling out through the gill region without split opening the fish. The flesh is not exposed during salting thereby causing less contamination and the product has a shelf stability of about two months. The yield obtained by this method is about 70%

Pit curing:

In this method, fish is mixed with salt (4:1) and placed in pits dug on beaches. The pits may be lined with palmyra / coconut leaves. After 2-3 days of maturation, the fish is taken out for marketing in wet condition and packed in bamboo baskets and transported to markets without drying. The quality of fish cured by this technique is poor with a shelf stability of up to three weeks only.

Prevention of spoilage in cured fish products Raw material

Fish must be as fresh as possible. Fatty fish is best wet salted, while lean fish is best dry salted. Take care not to damage fish during handling. Salt must be clean and dry. Note: mix of 1/3 small crystals:2/3 large crystals for dry salting. Fine crystals are usually better for wet salting

- Use clean water
- Processing methods
- Ensure that hands, clothing, cooking utensils and work surfaces are perfectly clean.
- Take note of the time required for each step of the salting process. ✓ Pay attention to the amount of salt or brine/fish weight ratio.
- Containers must be clean, and possess a secure lid.
- Handling of finished products
- Dry salted fish can be enclosed in clean/dry plastic bags or wrapped and secured inside dry banana leaves.
- The salted fish must be stored in a clean, and if possible, cool place. Keep it away from dust, insects, rodents and direct sunshine. Not only is it important to obtain a good product, but it is also important to keep it in good condition

2C: PREPARATION OF FERMENTED FISH PRODUCTS

AIM: To Acquire the practical knowledge on the fermented fish products

Introduction: Fermentation of fish is practiced in different parts of the world and is most popular in Southeast Asian countries, including India. Fish and fishery products have been associated with the socio-economic life of the people since many years. Fermentation is one of the ancient and most economical curing methods adopted for preserving fish.

Why to ferment fish?

Fish should be consumed in fresh condition, but fish due to its rich nutrients composition deteriorates very quickly. Therefore, there is a need to delay their degradation by application of some preservation technique. Fermentation is one such technique which is applied for the following reasons.

1. Preservation of fish/ to handle surplus catch/ prevent spoilage
2. To overcome fishing off-season
3. Flavour development
4. Nutrient enhancement
5. Value addition
6. To develop product variety
7. To develop unique taste (savory/umami)
8. Fish fermentation is still existing because the consumers enjoy the taste



Fermentation is an ideal example of hurdle technology.

It works as preservative technique by lowering the pH, redox potential (Eh) and water activity (aw) of the substrate. In modern technique, fermentation is sometime referred to as bio-preservation by addition of lactic acid bacteria (LAB) to the fish to be fermented. LAB produces antimicrobials such as lactic, acetic acid, antimicrobial nisin, hydrogen peroxide and peptide bacteriocins. These active substances prevent pathogenic and spoilage bacteria to proliferate and thus helps to preserve the fish.

TRADITIONAL FERMENTED FISH PRODUCTS OF INDIA:

- Shidal is commonly consumed in all Northeastern states of India.
- Also known as seppa, hidal & shidal in Assam, Tripura & Mizoram.
- Principle involved is moistened fish are packed into an oil processed pot & sealed air tight.
- Fish allowed to get fermented anaerobically by some resident bacteria for about 6 months,
- Fish protein & lipid are broken down to peptides, amino acids, fatty acid, indole, sketole etc.
- Produce a strong characteristic odour

Raw materials:

- (i) Fish: Dry *Puntius sp* is used. • Fish should be bigger & uniform in size, no insect infestation.
- (ii) Mutka (vat): Round bottom & narrow necked earthen vats, Capacity 8 to 40 kg.,
- (iii) Fish oil/veg oil: Fish oil extracted from entrails of fish or other edible oil used for mutka processing.
- (iv) Cover paste: A soft dough prepared from semi-dried or fully dried fish (broken or smaller *Puntius sp.* & waste of other dry fish), After grinding them into powder & by adding water
- (v) Cover-leaf: Broad plant leaf e.g. Catesu or banana leaves or creepers like bottle-guard leaves commonly used. Used to cover the cover paste temporarily to restrict fly infestation. A piece of newspaper may also serve the purpose if leaves are not available.
- (vi) Mud/Clay-seal: Humas rich & fine coarse soil is ground into powder & thick mud is prepared from it. Cover leaf removed from mouth & a layer of mud is applied on surface.

This acts as permanent seal to ensure anaerobic condition inside pot & also to prevent insect infestation.

PROCEDURE (shidal from raw fish):

- Gutting, descaling & washing of raw fish (Puntius sp.)
- Drying under sun (to reduce moisture content by half)
- Repeated oil smearing & drying of mutkas under sun.
- Packing of fish in mutkas layer by layer
- Tight packing so that no air gap in between layers
- Filling up to neck of mutka
- Rest part is filled with cover paste (from neck to rim of mutka)
- Paste covered with cover leaf
- Mutkas kept in sheltered place undisturbed for 3-4 days.
- Cover leaf removed & clay-seal is put in 2-3 layers keeping no air gap
- Kept under dry & sheltered place
- If crack appears on mud seal, put another layer of mud till a crack less seal obtained.
- Mutkas are kept for fermentation underground for 5-6 months
- After required period mutkas taken out from ground.
- Mouth not opened. While retailing mouth is opened by removing clay- seal & putrefied paste.
- Ready shidal is taken out.

3. EXAMINATION OF SALT, PROTEIN, MOISTURE IN DRIED / CURED PRODUCTS

3A. EXAMINATION OF SALT IN DRIED / CURED PRODUCTS

Principle:

This method determines the chloride ion concentration of a solution by titration with silver nitrate. As the silver nitrate solution is slowly added, a precipitate of silver chloride forms. The end point of the titration occurs when all the chloride ions are precipitated

Apparatus/ Instruments

- 1) Weighing balance
- 2) Pipettes
- 3) Burettes
- 4) Erlenmeyer flasks
- 5) Standard flasks
- 6) Beaker
- 7) Hotplate / Sand bath

Materials and Reagents

- 1) Silver nitrate standard solution (0.1M)
- 2) Ammonium thiocyanate standard solution (0.1M)
- 3) Ferric Indicator

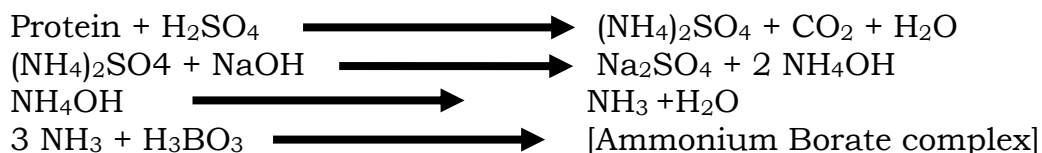
Method of analysis

- 1) Add known volume 0.1M AgNO₃ solution, more than enough to precipitate all Cl as AgCl & then add 20ml of HNO₃, boil gently on hot plate or sand bath until all solids except AgCl dissolve (usually 15 min).
- 2) Cool, add 50ml water & 5ml indicator & titrate with 0.1M NH₄SCN solution until becomes permanent light brown.
- 3) Subtract mL 0.1M NH₄SCN used from the volume of 0.1M AgNO₃ added and calculate difference as NaCl.

Calculation with units of expression with 10g test portion each mL 0.1N AgNO₃= 0.0058% NaCl

3B. EXAMINATION OF PROTEIN IN DRIED / CURED PRODUCTS

Principle: The nitrogenous compound in the sample are converted in to ammonium sulfate following digestion with concentrated sulfuric acid. The ammonia from the formed ammonium sulfate is liberated upon distillation with excess alkali. The liberated ammonia is absorbed in boric acid solution and titrated with a standardized acid for determination of nitrogen content. The nitrogen content is multiplied by a sample-specific protein factor (6.25 for fish products) to obtain the protein content.



Materials and Reagents

- 1) Kjeldahl catalyst 2) Concentrated Sulphuric acid (Nitrogen free, AR grade)
- 3) NaOH Solution (40%) 4) Standard 0.1 N sodium carbonate solution
- 5) Standard acid solution (HCl 0.1N or H₂SO₄) 6) Boric acid solution (4%)
- 7) Tashiro's indicator 8) Methyl orange indicator
- 9) Phenolphthalein indicator 10) Pumice stones

Preparation of Reagents

- 1) Kjeldahl catalyst- Mix 8 part of K₂SO₄ with 1-part of CuSO₄
- 2) NaOH Solution (40%)-Dissolve 40 g NaOH pellets in 100 ml distilled water and allow to cool
- 3) 0.1 N Na₂CO₃ Solution-Dissolve 530 mg of Na₂CO₃ in 100 ml of distilled water
- 4) Standard acid solution (HCl 0.1N or H₂SO₄)- if HCl, 0.1N (3.646g/L) or H₂SO₄, 0.05M or 0.1N (4.9 g/L). Actual strength of the acid was determined by titrating against the 0.1 N Na₂CO₃ primary standard solution, with methyl orange as indicator. The end point is indicated as red.
- 5) Boric acid solution-Dissolve 40 g boric acid in 500 ml hot distilled water, cool and make up to 1 L.
- 6) Tashiro's Indicator-Stock solution
A: 0.2% ethanolic methyl red and Stock solution
B: 0.2% ethanolic methylene blue.
Mix 100 ml "A" with 50 ml "B". Mix 1 part of the mixture with 1 part of ethanol and 2 part of water and use as working solution.

Method of analysis

- 1) Transfer with pipette a known volume (V₂) of the diluted digested solution in to the reaction chamber of the micro-Kjeldahl distillation apparatus.
- 2) Rinse down with distilled water, add two drops of phenolphthalein indicator and 40% NaOH till the indicator turns pink.
- 3) The receiver end of the distillation unit should be dipped into 10 ml boric acid solution (4%) containing a drop of Tashiro's indicator.
- 4) Perform distillation for 4 min and absorb the liberated ammonia in the boric acid solution. Lower the flask, taking care that the receiver tip is not touching the solution, continue heating for another 1 min, wash the tip with distilled water.
- 5) Determine the amount of ammonia absorbed by titrating with standardized acid solution till the green colour of the solution turns light pink

Calculation with units of expression

$$\text{Nitrogen} \left(\text{mg} \frac{N}{100g} \right) = 14 \times (b - a) \times N \times V_1 \times \frac{100}{V_2 \times W} = "X"$$

Where:

b= volume (ml) of standard acid used in sample titration

A= volume (ml) of standard acid used in reagent blank titration

N= corrected normality of the standardized acid for titration

V1= made up volume (ml) of the digested solution

V2= volume of diluted digested solution taken for distillation

W= sample weight

14 is the atomic weight of Nitrogen

Protein content (%) = $X \times 6.25 / 1000$

Where 6.25 is the nitrogen to protein conversion factor for fish and fish products;

1000 is the factor to covert mg N to g

3C. EXAMINATION OF MOISTURE IN DRIED / CURED PRODUCTS

Principle

The principle of the thermogravimetric method of moisture content determination is defined as the weight loss of mass that occurs as the material is heated. The sample weight is taken prior to heating and again after reaching a steady-state mass subsequent to drying.

Apparatus/ Instruments

- 1) Weighing balance
- 2) Hot air oven
- 3) Silica crucible

Sample Preparation

Cut the large pieces into small sizes and mix. Grind the pieces as finely as possible preferably using an electric grinder so that a homogenous sample is obtained. Keep the material in an airtight container in order to prevent the loss of moisture during subsequent handling. Use this material for testing.

Method of analysis

Weigh about 5g of the prepared sample into tared silica crucible or dish. Dry the dish in an air oven $100 \pm 1^\circ\text{C}$ for 6h. Cool in a desiccator and weigh.

Calculation with units of expression

$$\text{Moisture}(w/w\%) = \frac{M1 \times 100}{M2}$$

Where,

M1= loss of mass in g in sample

M2= mass in g of sample taken for test

EXPERIMENT NO. 4. EXAMINATION OF SPOILAGE OF DRIED / CURED FISH PRODUCTS, MARINADES, PICKLES, SAUCE

4A: EXAMINATION OF SPOILAGE OF DRIED / CURED FISH PRODUCT- MARINADES

Introduction:

Fish marinade: Salted or brine cured fish is called marinated fish.

Purpose: Extended shelf life & flavor.

Ingredients:

Three basic ingredients used to marinate fish are : a combination of acids, oils and herbs & spices.

The acids can be lemon, vinegar, fruit juices, wine, cider, milk or Yogurt, Orange, pineapple etc. White wines are good -- they won't discolor the fish like red wines.

The oil or fat -- olive oil, vegetable oil or peanut oil.

seasonings obtained from herbs & spices make up the flavors Salt and pepper are the commo

Others include garlic, basil, thyme or bay leaves.

Species used for marinades: Pelagic fatty fishes e.g. Sardine, herring, pony fish, Indian mackerel, lizard fish, Croakers, shellfishes etc. are used.

SPOILAGE IN MARINATED FISH:

(i) Physical spoilage:

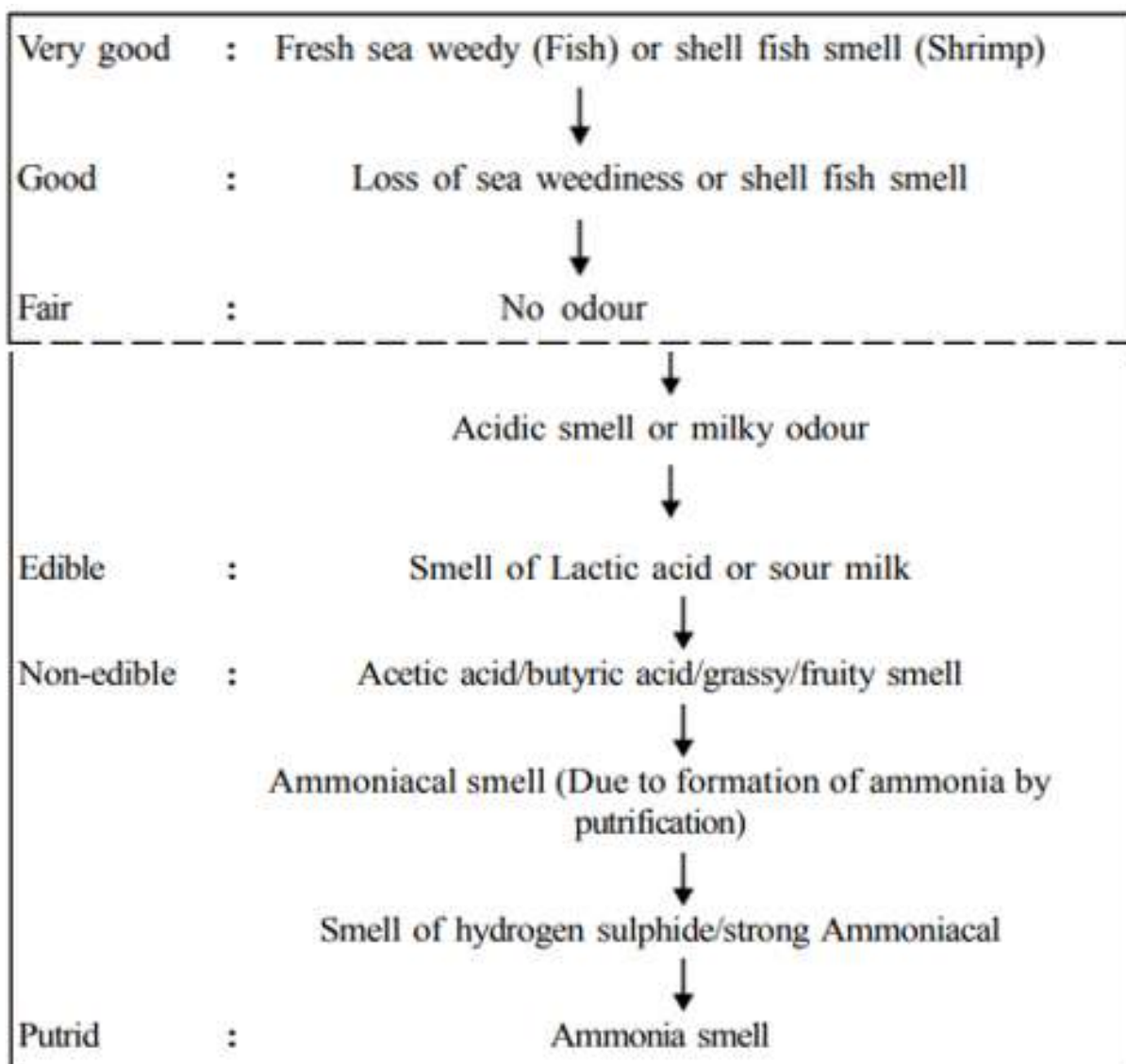
- Pack of marinades if stored below 0° C, contents will freeze. Water expands on freezing, so may damage glass jar or tin can. Cooked marinades – str. of jelly will be damaged by ice crystals. Heat damage occurs if cans are filled when too cold & product stored at warm temp. Swelling of cans if cooked marinades are sealed in cans before swelling of can is completed.

(ii) Chemical spoilage:

- Caused by action of acetic acid on metal cans. If cans are badly lacquered, due to corrosive action of acid on metal H₂ gas liberated, cause cans to swell. Imparts metallic taste to product– unacceptable.

• (iii) Biological spoilage:

Simple flipping to hard swell & even bursting of cans Microbial degradation of proteins in fish. Fermentation of added sugar or sugar containing additives. Results in production of CO₂ gas & degradation of product quality.



Sl. No.	Parameters	Spoilage Rate	
		Slow	Fast
1.	Size of the fish	Big fish	Small fish
2.	pH (acidity of muscle)	Low pH (6-7)	High pH (alkaline range, above 7)
3.	Lipid content of muscle	Lean fish	Fatty fish
4.	Thickness of the skin	Thick skinned fish	Thin skinned fish
5.	Muscle glycogen content	Large	Small

4B: EXAMINATION OF SPOILAGE OF DRIED / CURED FISH PRODUCT- FISH PICKLE

There are two basic methods of pickling of fish. During pickling of fish, only high- grade distilled vinegar, course pickling salt, fresh spices, and earthen crock or glass jars should be used as metal containers may cause discoloration of “tinny” taste. Fish pickle when carefully prepared under most hygienic conditions with the addition of the required quantity of salt, preservatives and spices will have generally an average shelf life of one year.

Standard recipe for fish pickle preparation

Ingredient name	Amount	Ingredient name	Amount
Fish muscle	500g	Vinegar	50ml
Chili powder	20g	Black pepper	2g
Turmeric powder	2g	Panchphoron (mixture of five spices such as Cumin, Brown Mustard, Fenugreek, Nigella and Fennel)	5g
Cumin	10g	Sugar	50g
Onion	20g	Salt	30g
Garlic	80g	Tomato sauce	30g
Ginger	10g	Tamarind	20g
Cloves	2g	Sodium benzoate	1g
Mustard	150ml		

Quality Analysis: The quality parameters of fish pickle samples stored at room temperature were analyzed at every 3 days interval. On the other hand, the quality parameters of the pickle samples stored at refrigeration temperature were analyzed initially at 7 days interval and then analyzed around 15 days interval whereas the analysis of quality parameters of the pickle samples stored at frozen temperature was done initially at 15 days interval and then at 30 days interval. Triplicate samples were taken to carry out the experiment.

4C: EXAMINATION OF SPOILAGE OF DRIED / CURED FISH PRODUCT- FISH SAUCE

Aim: Evaluating the spoilage of fish sauce involves assessing its sensory characteristics, chemical composition, and microbiological quality.

Methods to evaluate spoilage in fish sauce:

Sensory Evaluation:

Color: Check for any unusual changes in color. Fresh fish sauce is usually clear to light amber. If it becomes dark or cloudy, it may indicate spoilage.

Odor: Fresh fish sauce has a characteristic savory and umami odor. Spoiled fish sauce may have off-putting, foul, or putrid smells.

Taste: The taste of fish sauce should be salty and umami. Any bitter or rancid taste could indicate spoilage.

Chemical Analysis:

pH Level: Fresh fish sauce typically has a pH level ranging from 4.0 to 5.0. An increase in pH may suggest the growth of spoilage microorganisms.

Total Nitrogen Content: Spoilage can lead to changes in nitrogen content. A significant increase in total nitrogen may indicate degradation.

Salt Content: The salt concentration in fish sauce acts as a preservative. A decrease in salt content might indicate spoilage.

Microbiological Analysis:

Total Viable Count (TVC): Measure the total number of viable microorganisms. A high TVC indicates microbial contamination and potential spoilage.

Test for the presence of harmful bacteria such as Salmonella, Escherichia coli (E. coli), and other pathogens.

Packaging Integrity:

Check for Leaks or Swelling: Ensure that the packaging is intact and there are no leaks. Swelling may indicate gas production by spoilage microorganisms.

Storage Conditions:

Temperature Control: Fish sauce should be stored in cool and dark conditions. Exposure to high temperatures or sunlight can accelerate spoilage.

Expiration Date: Always check the expiration date on the packaging. Using fish sauce beyond its expiration date can pose risks.

Visual Inspection:

Presence of Sediment or Particles: Examine the fish sauce for any unusual sediment or particles. Sediment may indicate microbial growth or breakdown of components.

Organoleptic Evaluation:

Appearance, Smell, and Taste: Regularly perform organoleptic evaluations by trained sensory panels to detect any deviations from the normal characteristics of fresh fish sauce.

Regular monitoring using a combination of these methods will help ensure the quality and safety of fish sauce and identify any signs of spoilage. It's essential to follow good manufacturing practices and storage recommendations to prevent spoilage in the first place.

EXPERIMENT NO.5 PREPARATION OF ISINGLASS, COLLAGEN AND CHITOSAN FROM SHRIMP AND CRAB SHELL

5A. PREPARATION OF ISINGLASS

Aim: To study preparation by-product from fish called isinglass. This is obtained from an organ of the fish called air bladder.

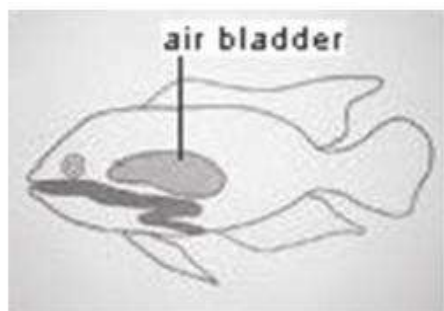
Introduction:

Fig. 3.8: Generalized location of air bladder in fish



Fig. 3.9: Air bladder

If you cut open a fish, above the intestine and digestive organs, you will notice a whitish sac filled with air. This is the air bladder; also called swim bladder, sound, fish maws, etc.

Preparation of Isinglass Procedure:**Collection and preservation of bladder**

Slit open the belly wall of the fish using a knife. You can easily locate the bladder. Now, cut off the membranes that attach it to the belly wall. Peel off the membrane covering the bladder. In case you see blood stain, mucus, etc., you must wash them off with mild scrubbing.

We can prepare isinglass straight from the wet bladder. But, quite often, there can be some delay in processing of the bladder. In such cases, you can preserve the fresh bladders for a few days by salting. For this, apply powder salt at the rate of one kg for 3 to 4 kg bladder. Then, pack them tightly in bags or boxes.

Drying

This is the effective method of preserving air bladders for a long time. Slit open the bladder lengthwise. The material is then unfolded. Sometimes, drying may be done without slitting open. If you adopt sun drying, the material must be kept in shade. Mechanical drier is convenient for drying at temperatures not exceeding 45°C. But, take care to prevent overheating as it may affect the protein, colour, etc. The moisture content of bladder must be reduced from about 80% to approximately 8%. You'll notice that the moist, soft and flabby material becoming stiff, hard and slightly yellowish in colour. You can pack dried fish maws in suitable containers

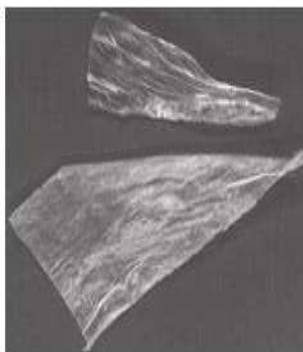
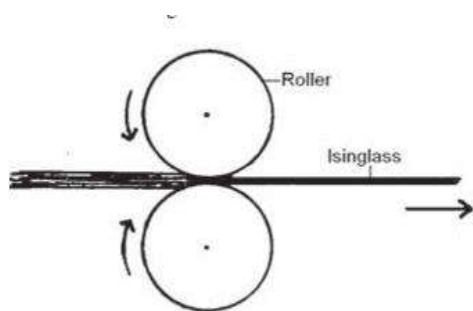


such as plastic bags and plastic lined sacks and store them for several months.

Processing fish maws to isinglass

The dried material must be made soft again by soaking the fish maws in water. Next, cut them into small pieces, mix and compress between rotating metal rollers. The material gets pressed to a sheet. The pressing action is repeated until the required thickness is obtained. This can be anywhere between

inch. In order to prevent heating of the material during pressing, the rollers are made hollow and cold water is circulated through them. Now, dry the thin material in a drier or by sun drying once again to a moisture content below 8%. You get the isinglass as thin transparent films. You can cut them in the form of strips called 'leaves' or can be pulverized to powder called 'finings'. The product must be suitably packed.



5.B: PREPARATION OF CHITOSAN

Aim: To know the process of preparation of chitosan.

Introduction: Chitosan is a natural polymer derived from chitin, which is found in the exoskeletons of crustaceans like shrimp and crabs. The preparation of chitosan involves several steps, primarily deacetylation of chitin.

Materials and Equipment:

Chitin Source: Shrimp shells, crab shells, or other crustacean exoskeletons.

Chemicals:

- Sodium hydroxide (NaOH)
- Acetic acid
- Hydrochloric acid (HCl)
- Equipment:
- Blender or grinder
- Reaction vessel
- Stirring equipment
- Filtration setup
- Distillation apparatus
- pH meter

Procedure:

1. Collection and Cleaning of Shells:

Collect and thoroughly clean crustacean shells to remove any impurities or residues.

2. Demineralization:

Grind or crush the cleaned shells into small pieces.

Treat the crushed shells with a dilute acid (e.g., HCl) to remove minerals. This step is known as demineralization.

Wash the demineralized shells thoroughly with water.

3. Deproteinization:

Treat the demineralized shells with a dilute solution of sodium hydroxide (NaOH) to remove proteins. This step is called deproteinization.

Stir the mixture for a specific duration.

Wash the deproteinized shells with water.

4. Deacetylation:

Dissolve the deproteinized chitin in a concentrated NaOH solution. This process, called deacetylation, converts chitin into chitosan.

Maintain the reaction at an elevated temperature for a specific period.

Stir the mixture to ensure uniform deacetylation.

Adjust the pH of the reaction mixture using acetic acid.

Continue stirring until chitosan precipitates.

5. Filtration and Washing:

Filter the chitosan precipitate to separate it from the liquid phase.

Wash the chitosan with water to remove any remaining impurities.

6. Drying:

Dry the chitosan thoroughly. This can be done by air-drying or using an oven at a low temperature.

7. Milling or Grinding:

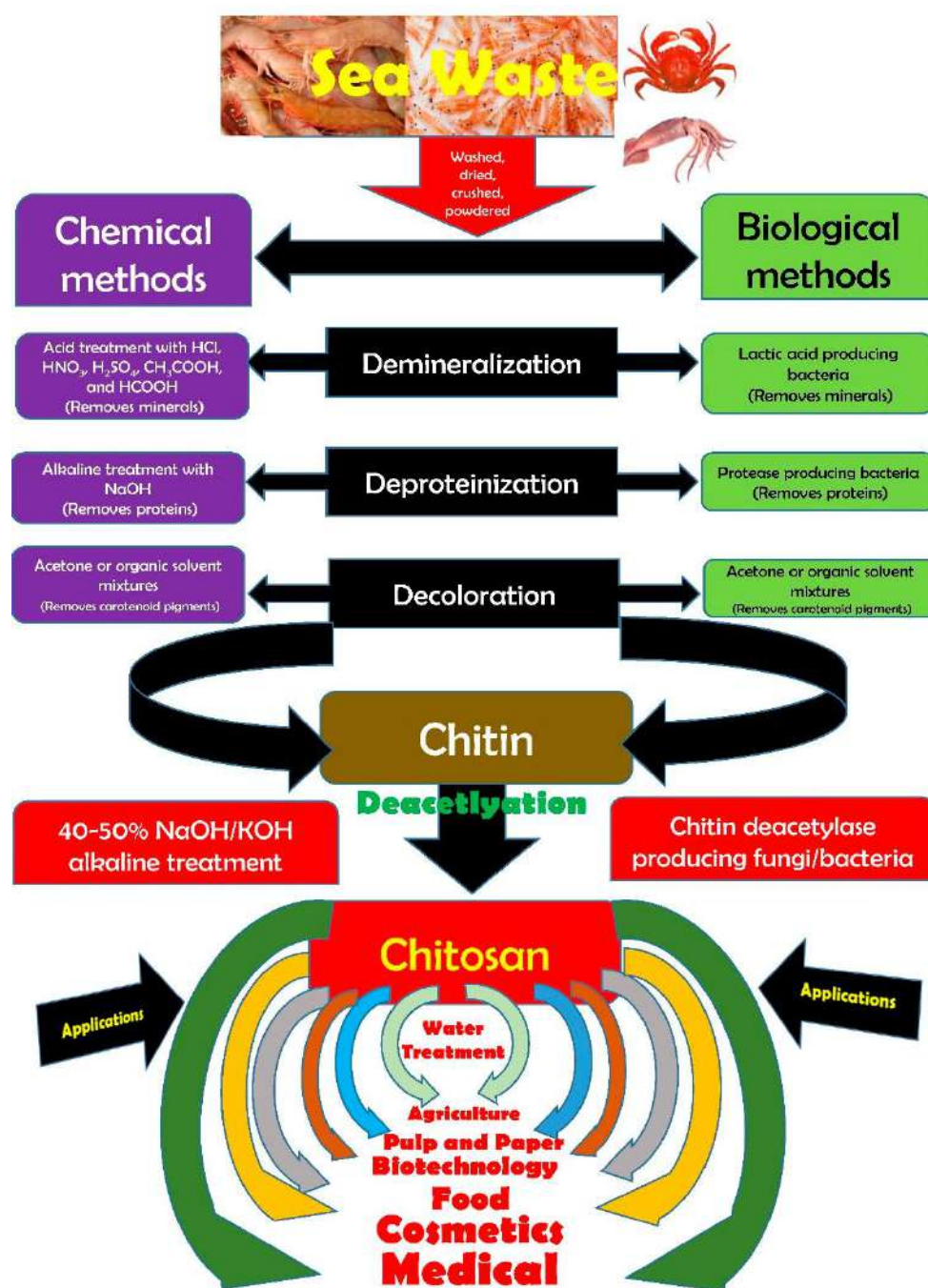
Optionally, mill or grind the dried chitosan into a fine powder for better handling and increased surface area.

8. Characterization:

Optionally, characterize the prepared chitosan using techniques such as Fourier-transform infrared spectroscopy (FTIR) or nuclear magnetic resonance (NMR) to confirm its structure.

Precautions:

- The specific concentrations, temperatures, and durations for each step may vary depending on the source material and desired properties of the chitosan.
- Safety precautions should be followed when handling chemicals, especially NaOH and HCl.
- The pH of the reaction mixture is crucial during deacetylation, and it is usually monitored using a pH meter.



5C PREPARATION OF COLLAGEN

Aim: To know the process of preparation of Collagen

Introduction: The preparation of collagen from crustacean shells involves several steps, including demineralization and deproteinization similar to the process for chitosan.:

Materials and Equipment:

- Crustacean Shells: Shrimp, crab, or other crustacean exoskeletons.
- Chemicals:
 - Hydrochloric acid (HCl)
 - Sodium hydroxide (NaOH)
- Ethanol
- Equipment:
 - Blender or grinder
 - Reaction vessel
 - Stirring equipment
 - Filtration setup
 - Dialysis tubing
 - Centrifuge
 - Distillation apparatus
 - pH meter

Procedure:

1. Collection and Cleaning of Shells:
Collect and thoroughly clean crustacean shells to remove impurities.
2. Demineralization:
Grind or crush the cleaned shells into small pieces.
Treat the crushed shells with a dilute acid (e.g., HCl) to remove minerals. This step is known as demineralization.
Wash the demineralized shells thoroughly with water.
3. Deproteinization:
Treat the demineralized shells with a dilute solution of sodium hydroxide (NaOH) to remove proteins. This step is called deproteinization.
Stir the mixture for a specific duration.
Wash the deproteinized shells with water.
4. Extraction of Collagen:
Dissolve the deproteinized material in a dilute acid (e.g., acetic acid or citric acid) to extract collagen.
Stir the mixture at a controlled temperature for a specific duration.
Adjust the pH of the solution to facilitate collagen solubility.
5. Centrifugation:
Centrifuge the collagen solution to separate insoluble components.
Collect the supernatant, which contains soluble collagen.
6. Dialysis:
Dialyze the collagen solution against distilled water to remove residual acids and salts.
Use dialysis tubing with an appropriate molecular weight cutoff.
7. Concentration:

Concentrate the dialyzed collagen solution using methods such as ultrafiltration or centrifugation.

8. Sterilization:

Sterilize the collagen solution by filtration through a sterile filter or other appropriate methods.

9. Characterization:

Optionally, characterize the prepared collagen using techniques such as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to confirm its purity and molecular weight.

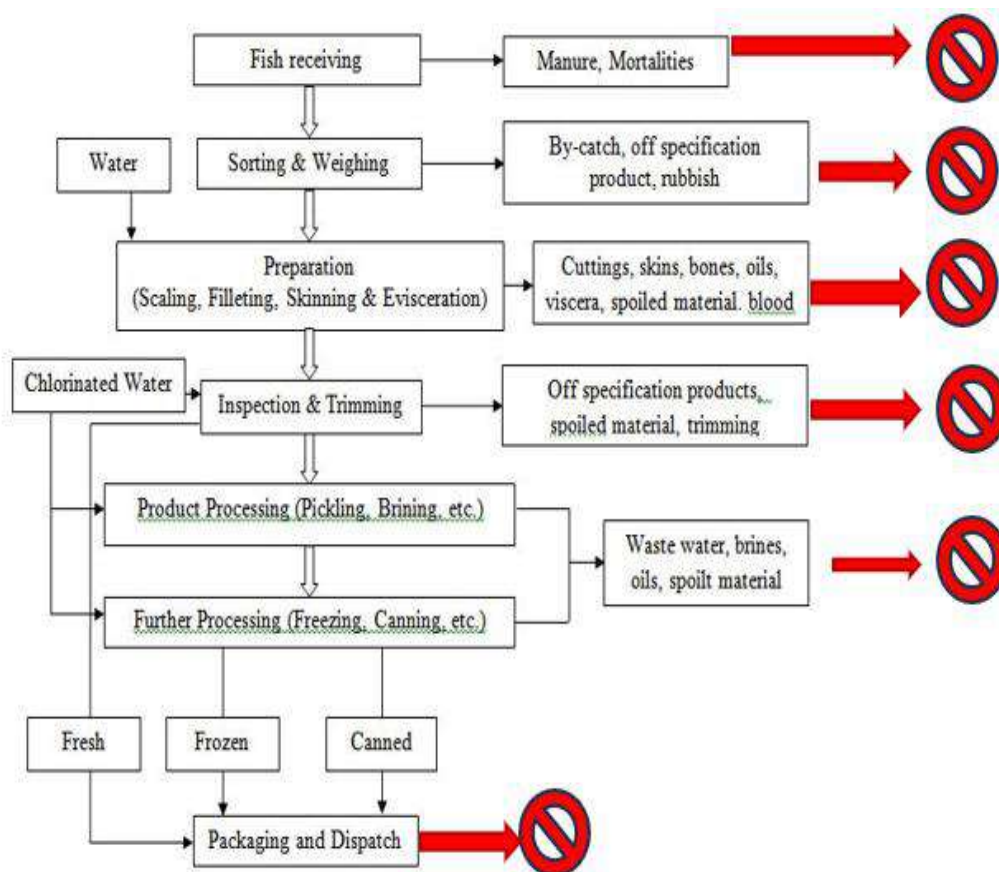
Precautions:

The specific concentrations, temperatures, and durations for each step may vary depending on the source material and desired properties of the collagen. Safety precautions should be followed when handling chemicals, especially HCl and NaOH.

The pH of the reaction mixture is crucial during deproteinization and collagen extraction.

This procedure provides a general overview of collagen preparation from crustacean shells, but specific details may vary based on the desired characteristics of the collagen and the source material used. Always refer to literature or specific protocols for more precise instructions.

EXPERIMENT NO.6 DEVELOPING FLOW CHARTS AND EXERCISES IN IDENTIFICATION OF HAZARDS – PREPARATION OF HAZARD ANALYSIS WORKSHEET

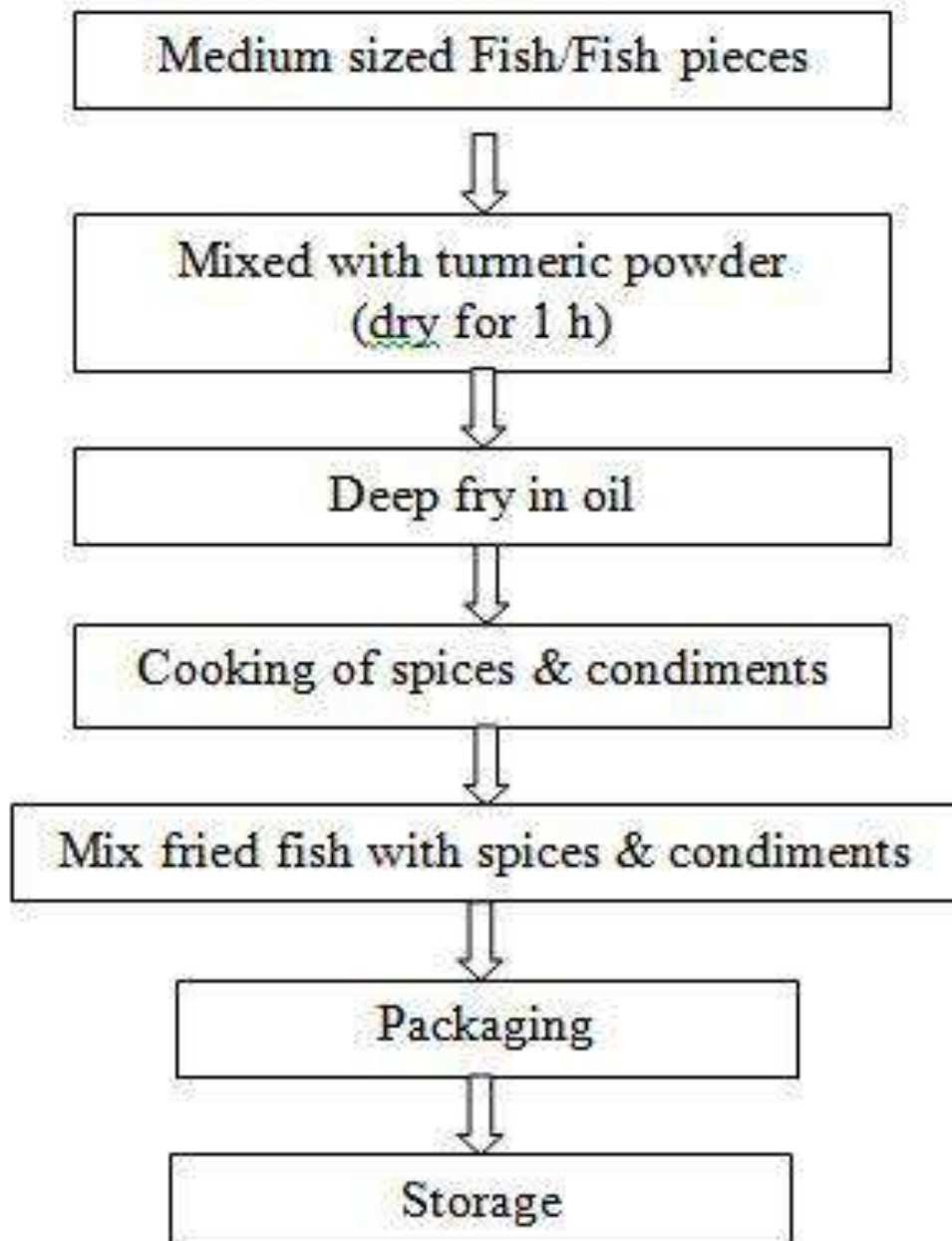


Flow chart with the areas under hazard in a fish processing industry

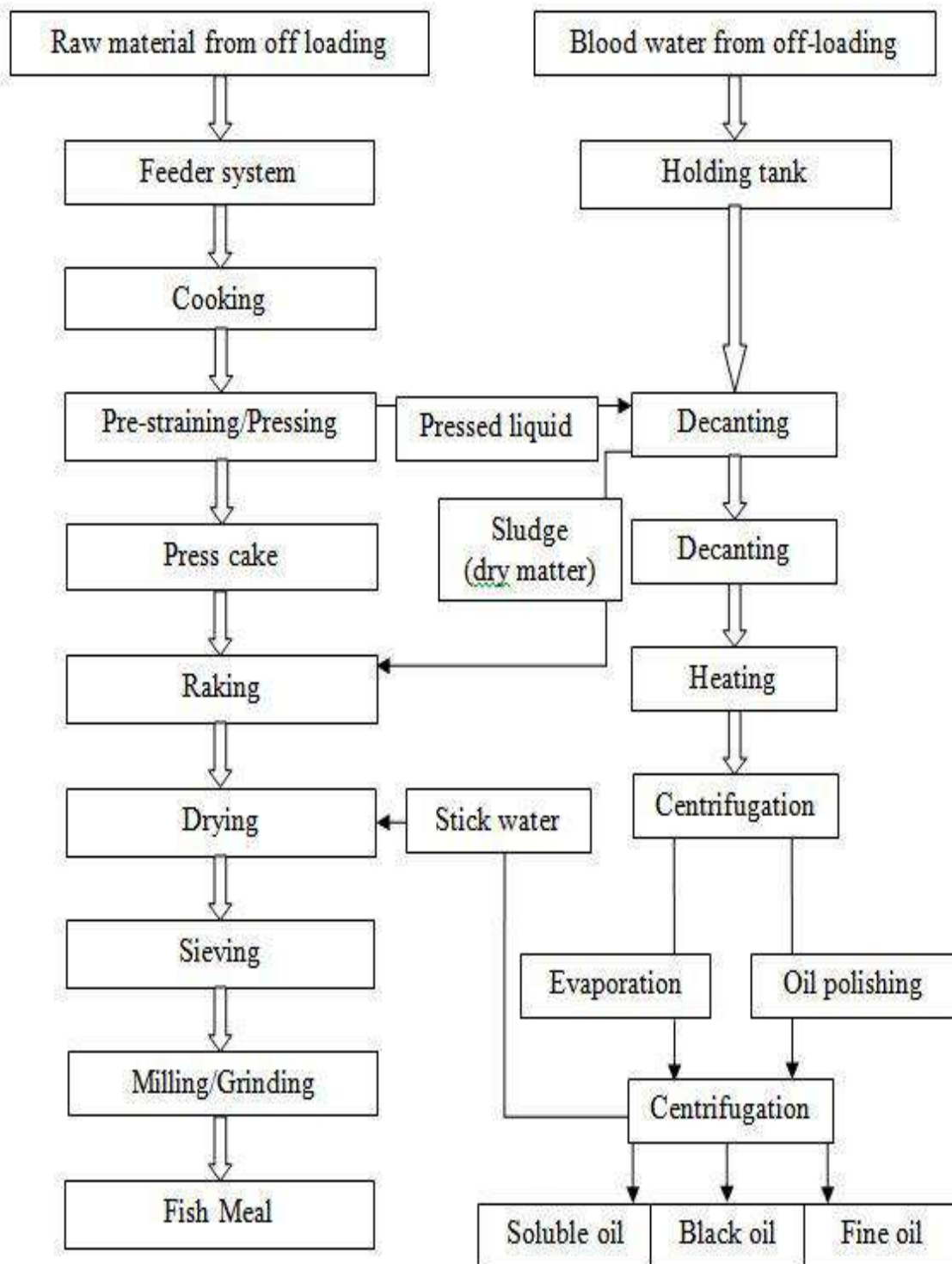
EXPERIMENT NO.7: CORRECTIVE ACTION PROCEDURES IN PROCESSING OF FISH- FLOW CHART- WORK SHEET PREPARATION

7A: FISH PICKLING FLOW CHART

Flow Sheet of Fish Pickling

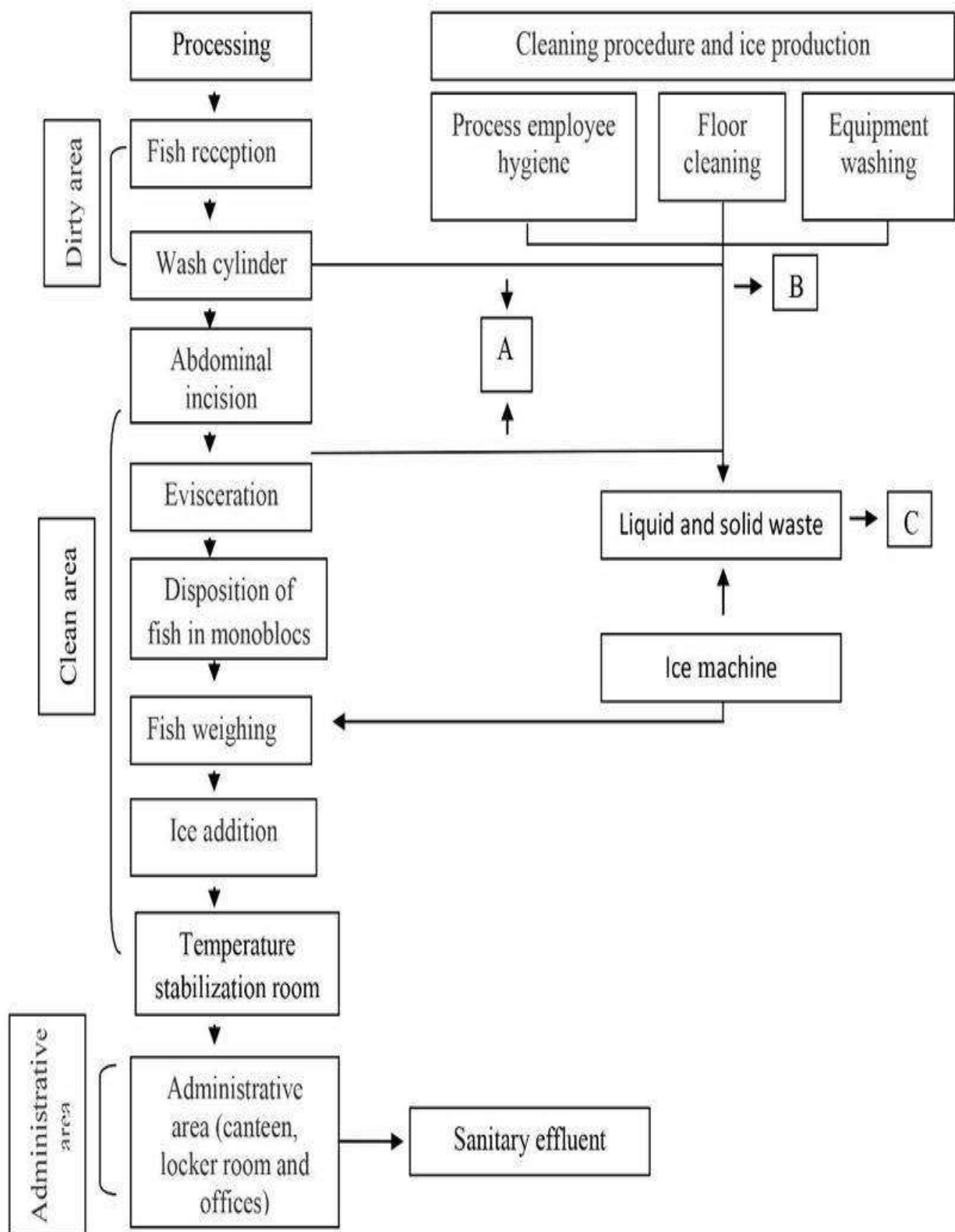


7B FISH MEAL, FISH OIL FLOW CHART

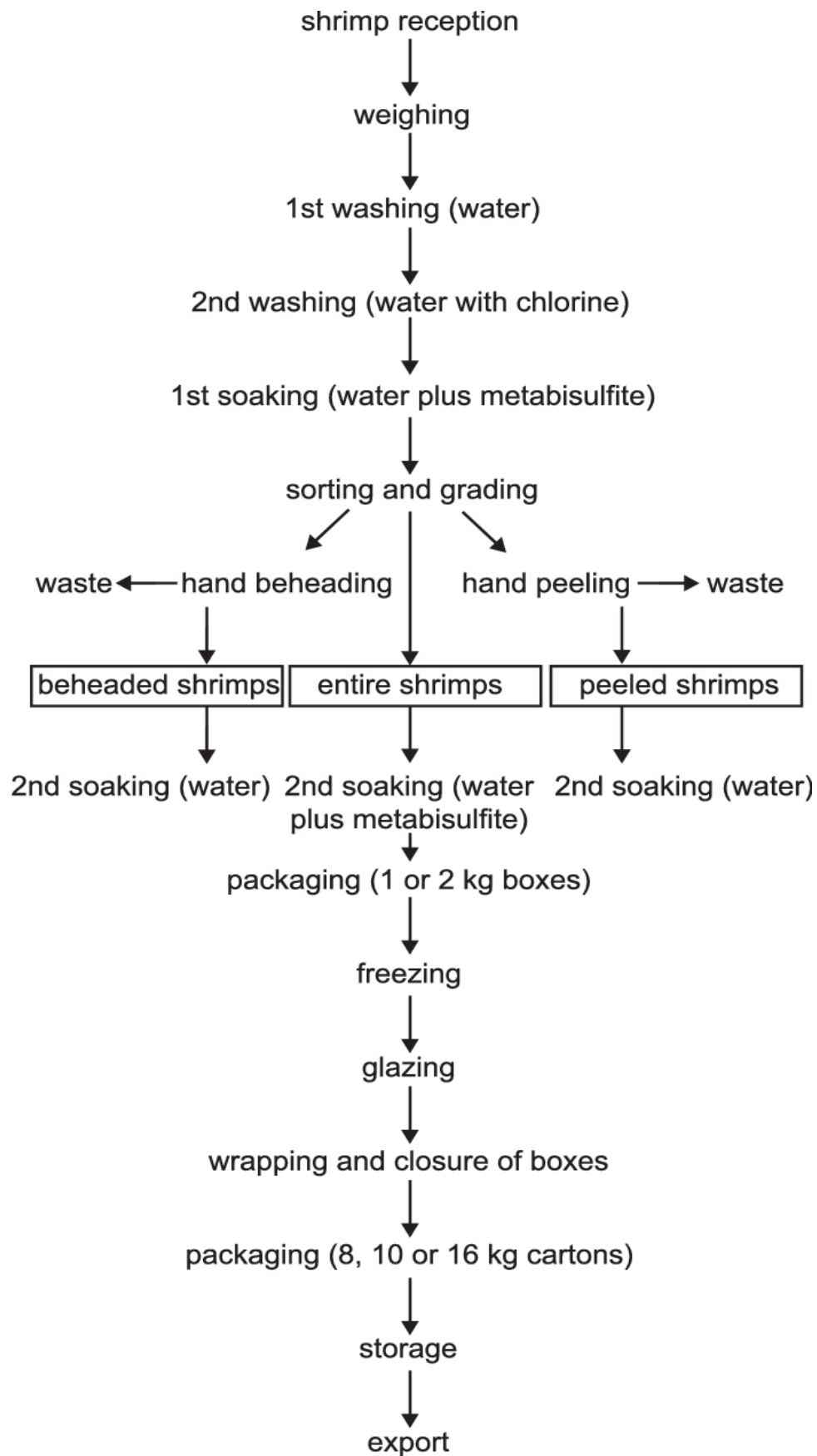


Flow Sheet of Processing of Fish Meal and Oil

7.C FLOW CHART COMPLETE FISH PROCESSING



7D. SHRIMP PROCESSING FLOW CHART





Welcome: Dr. N. SREENIVAS (Self Published Author)

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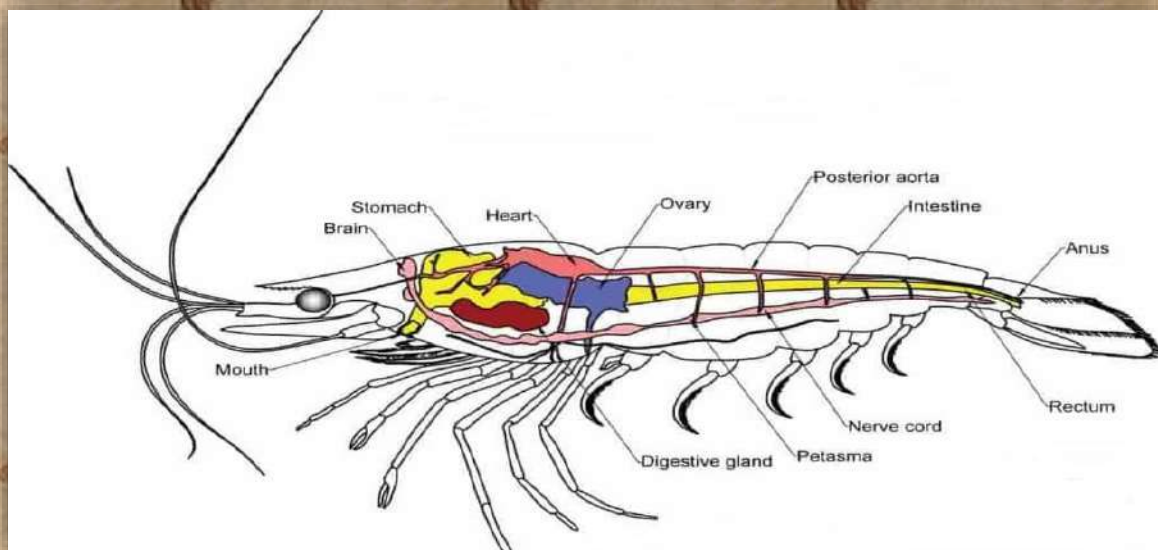


TAXONOMY AND FUNCTIONAL ANATOMY OF FIN FISH AND SHELL FISH

(AQUACULTURE MAJOR -COURSE -3 LAB MANUAL)



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Dr. SREENIVAS- Dr. CHAKARPANI-Dr. ANIL KUMAR

COURSE III- TAXONOMY AND FUNCTIONAL ANATOMY OF FIN FISH AND SHELL FISH

SYLLABUS

1. Study of mouth parts in herbivorous and carnivorous fishes
2. Comparative study of digestive system of herbivorous and carnivorous fishes
3. Demonstration of brain of fish
4. Demonstration of cranial nerves of fish
5. Demonstration of Nervous system of prawn
6. Exposure of gills of prawn
7. Exposure of gills of fish

1. STUDY OF MOUTH PARTS IN HERBIVOROUS AND CARNIVOROUS FISHES

TERMINAL MOUTH The mouth is located in the front of the head of the fish pointing forward where the lower jaw and upper jaw are the same length.

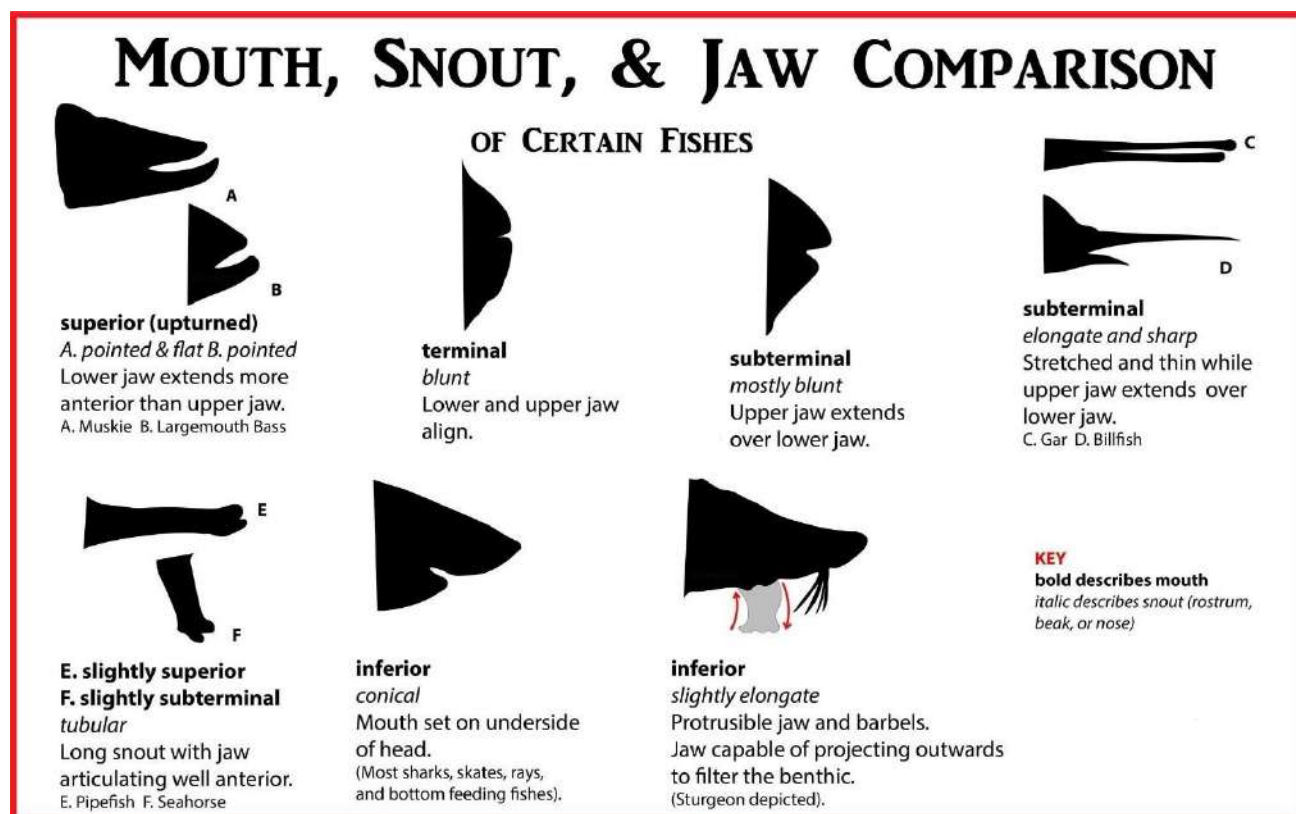
SUPERIOR MOUTH The mouth is located on top of forward tip of the fish with the upper jaw being shorter than the lower jaw.

INFERIOR SUB-TERMINAL MOUTH The mouth is located below the forward tip of the fish with lower jaw being shorter than the upper jaw.

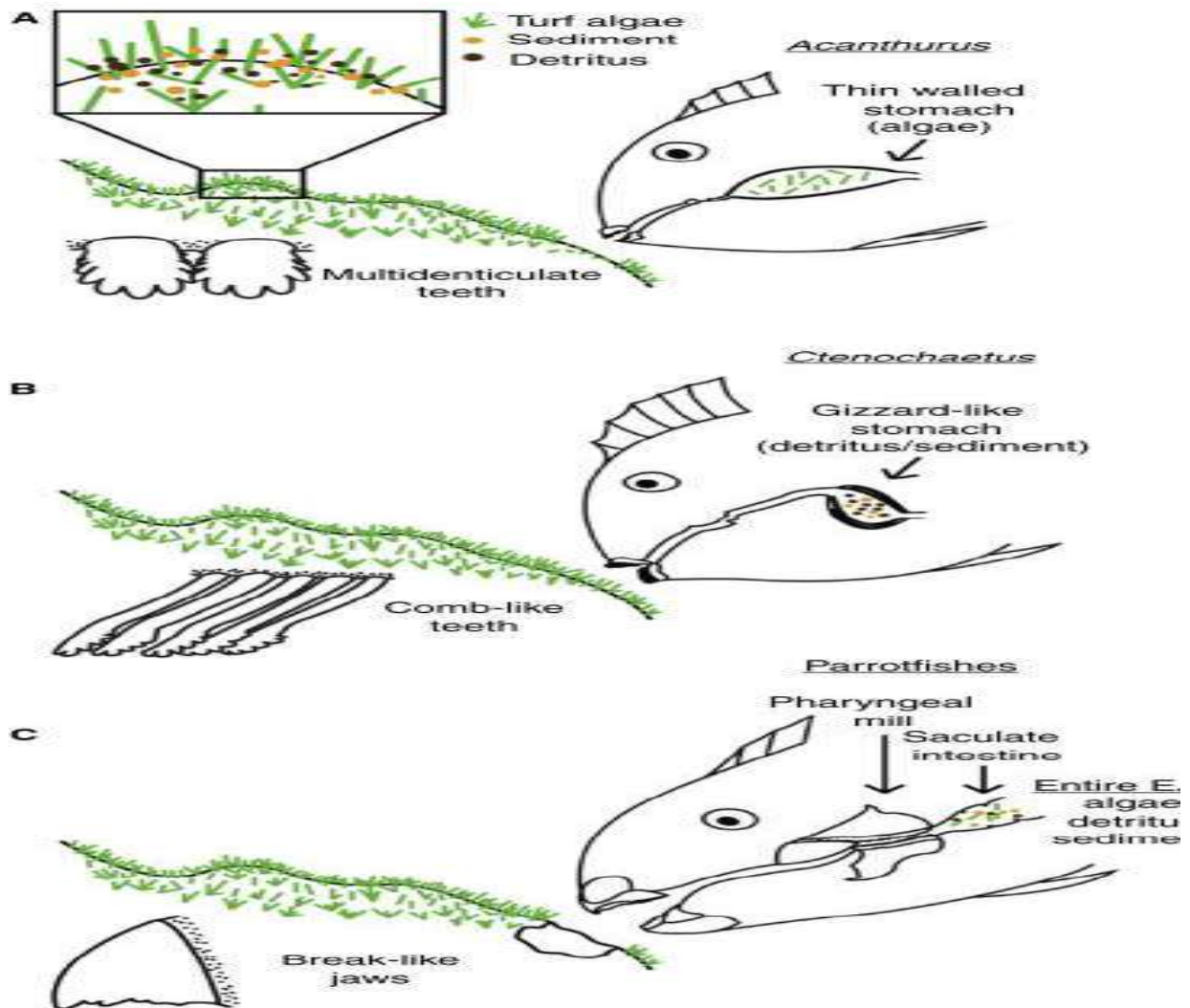
BARBELS Sensory organs with a whisker-like in appearance. Typically found on the sides or surrounding the mouth which may be used to locate their food source.

Allows fish to extend their reach by creating a vacuum or suction to bring in their **SUCTION MOUTH** prey or food. Most commonly associated with fish that have an Inferior Sub-Terminal mouth.

PROTRUSIBLE MOUTH Also called a suction mouth.



1. HERBIVOROUS MARINE FISH MOUTH

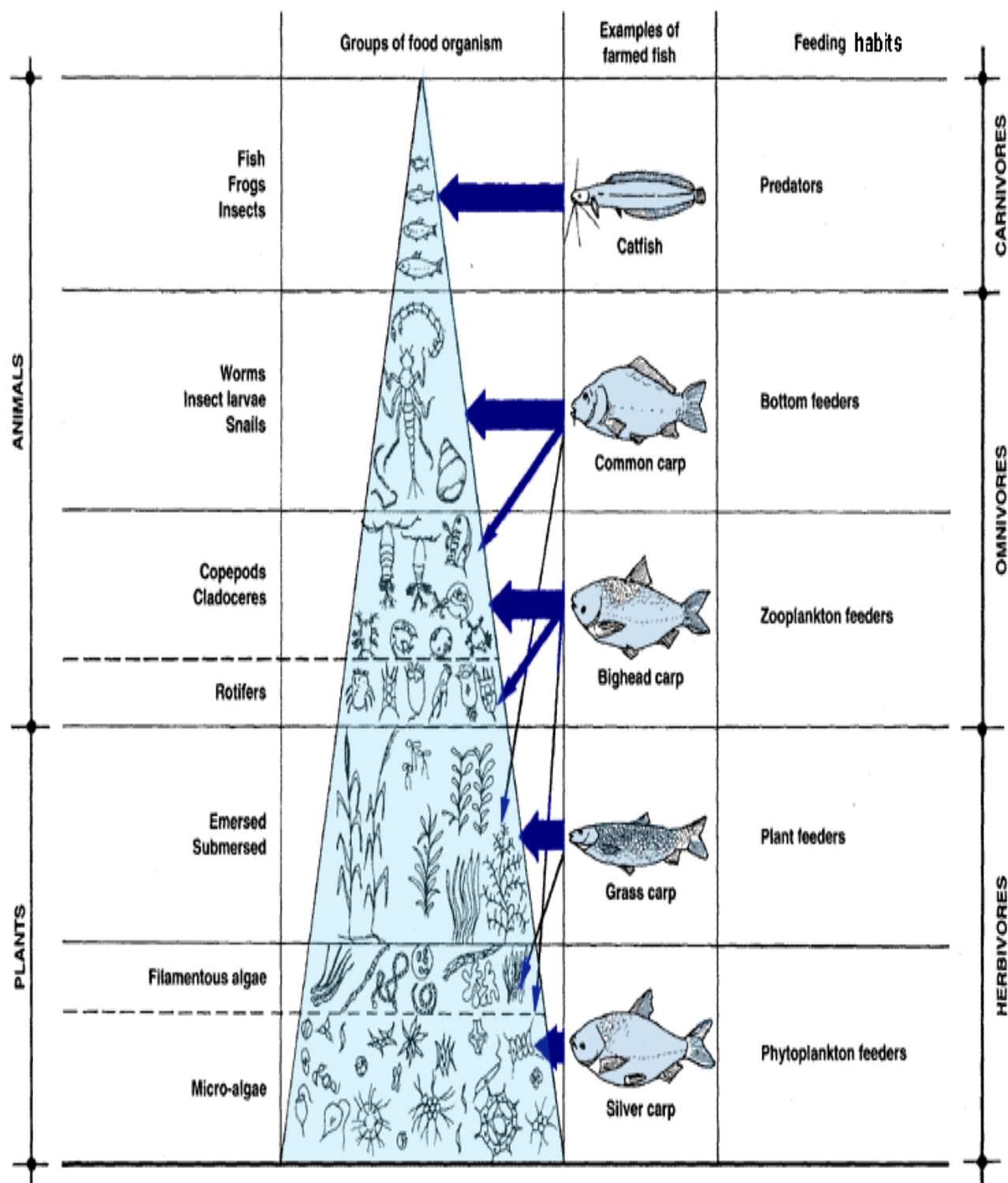


2. Carnivorous fish Mouth

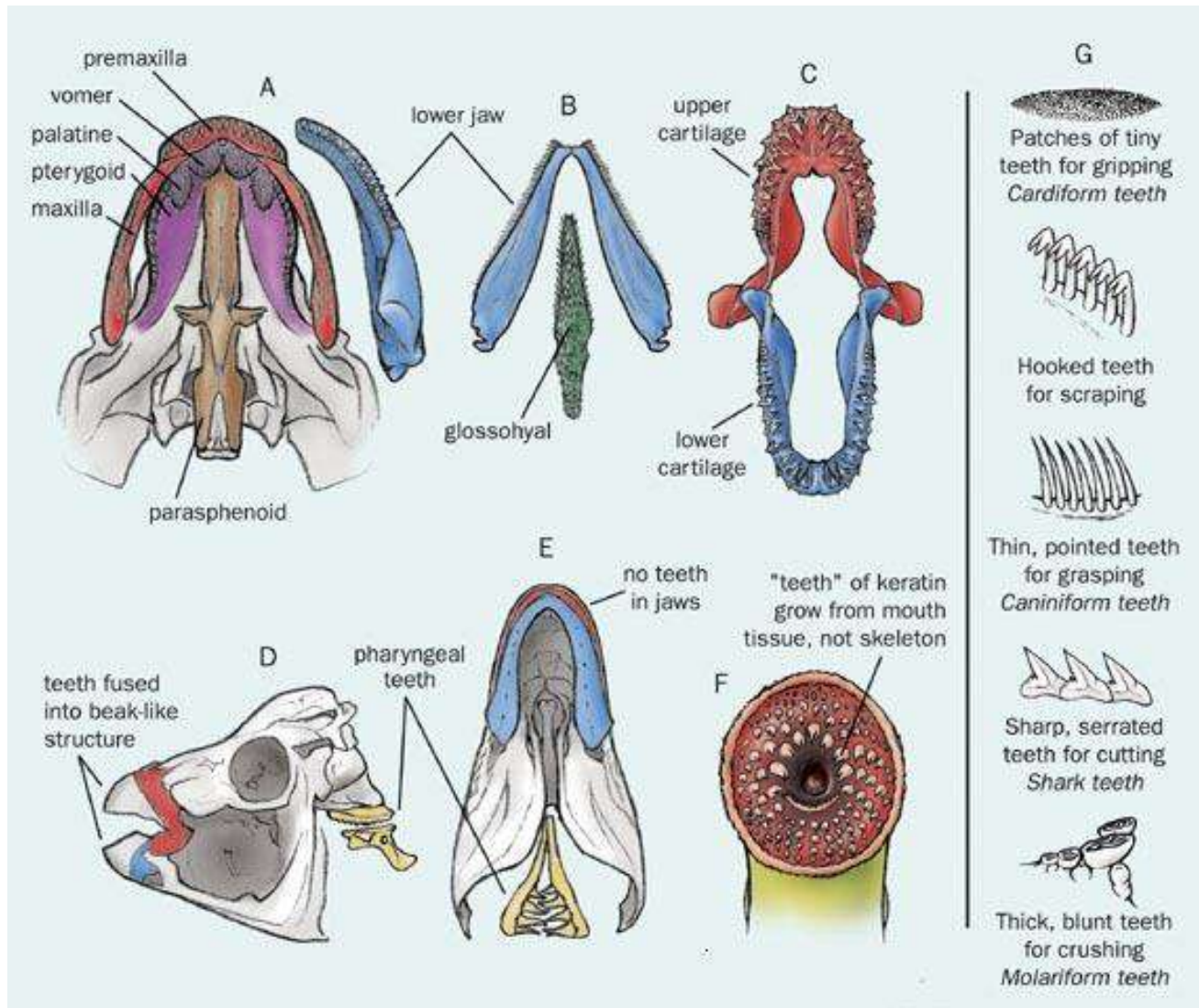
- Carnivores mouth
 - Have many teeth that point inwards.
 - Not used for chewing.
 - Used to capture and hold prey.
- Other fish
 - Few or no teeth.



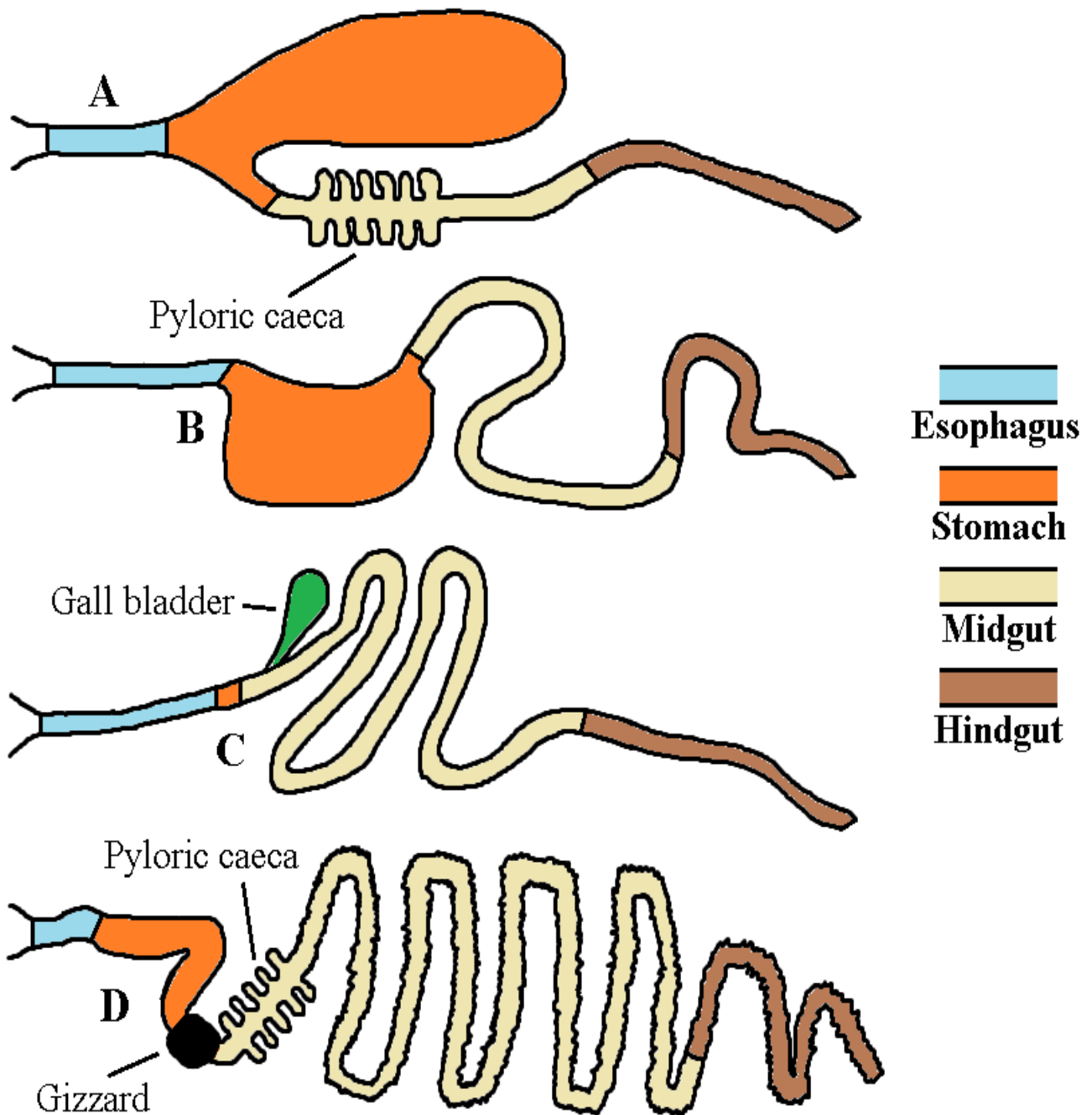
3. Feeding Habits of Fishes



4. Mouth Parts of Fish



5. COMPARATIVE STUDY OF DIGESTIVE SYSTEM OF HERBIVOROUS AND CARNIVOROUS FISHES

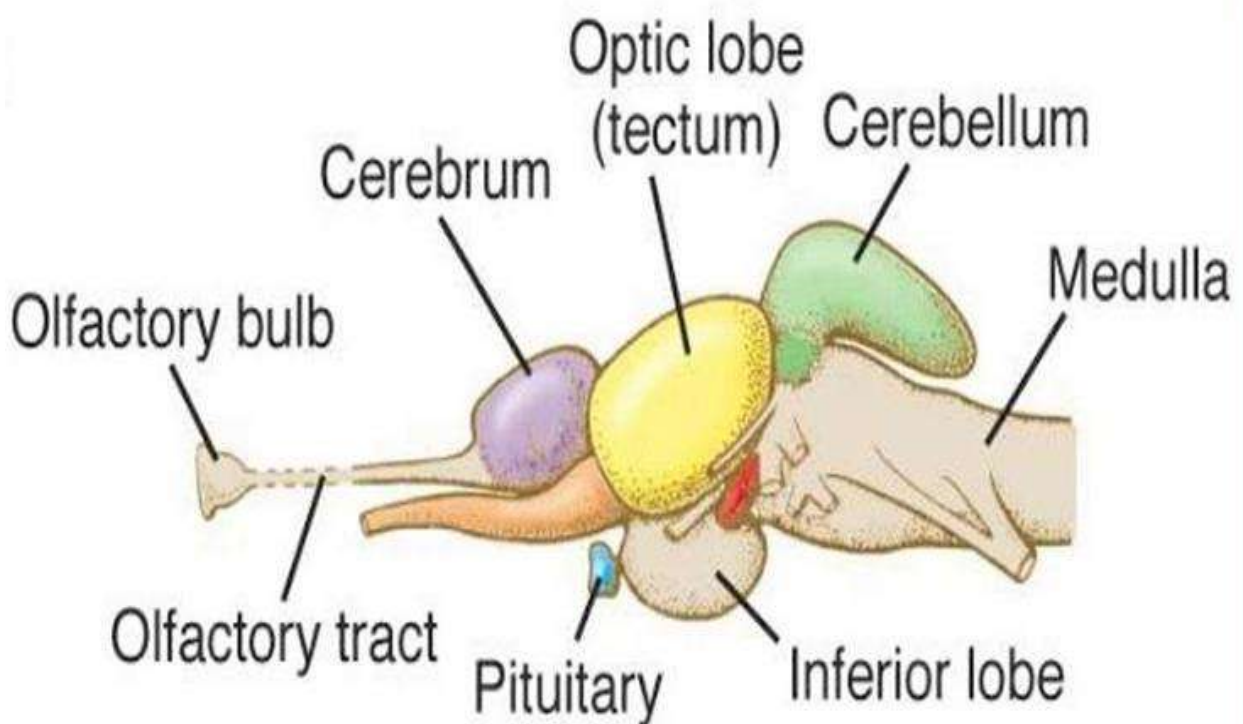
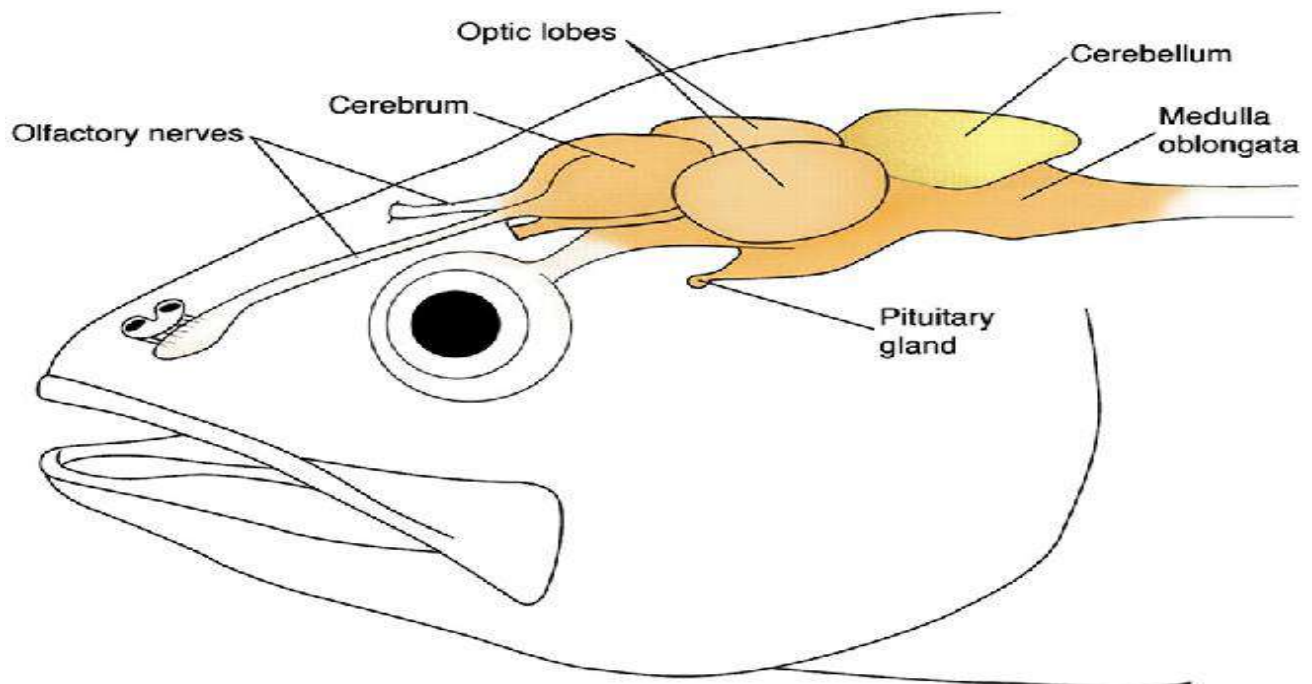


A: Carnivore.

B & C: Omnivores with different strategies.

D: Herbivore.

6. DEMONSTRATION OF BRAIN OF FISH



Fish Brain

7. CRANIAL NERVES OF FISH

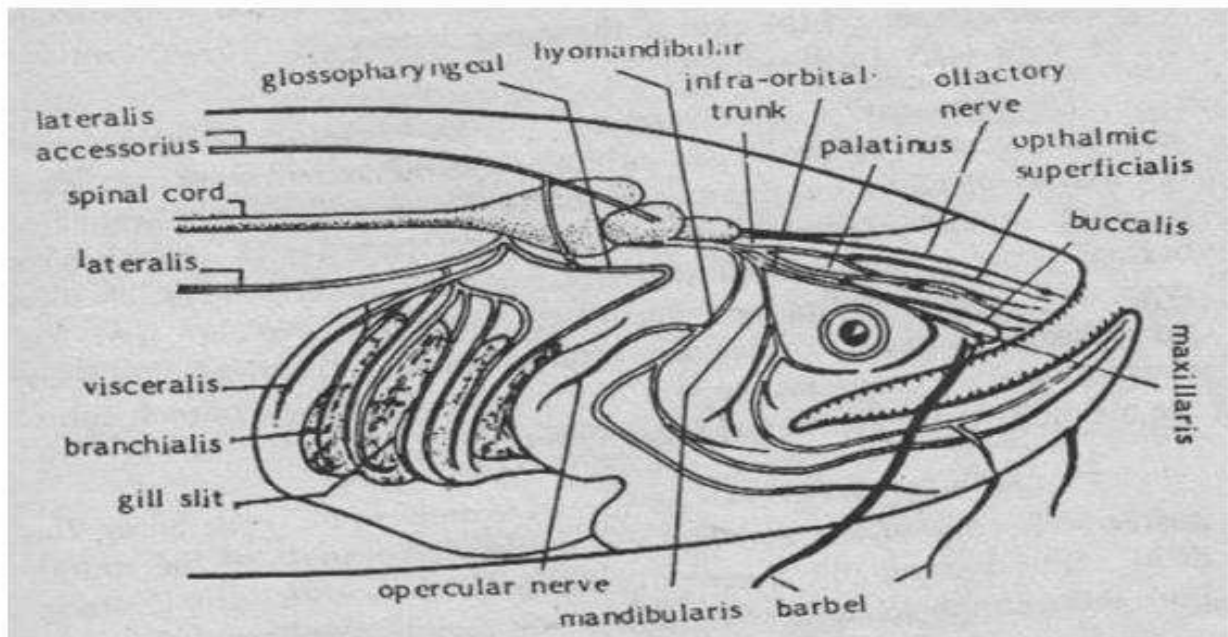


Fig: Cranial nerves of *Wallago attu*.

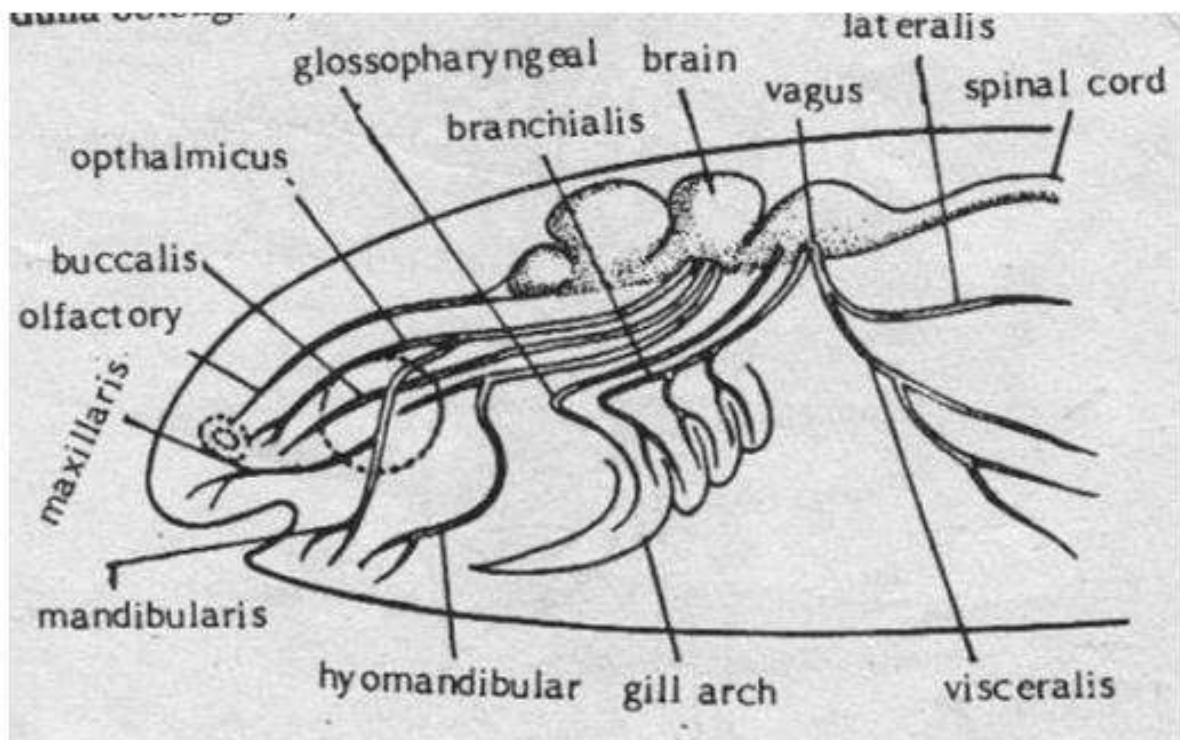
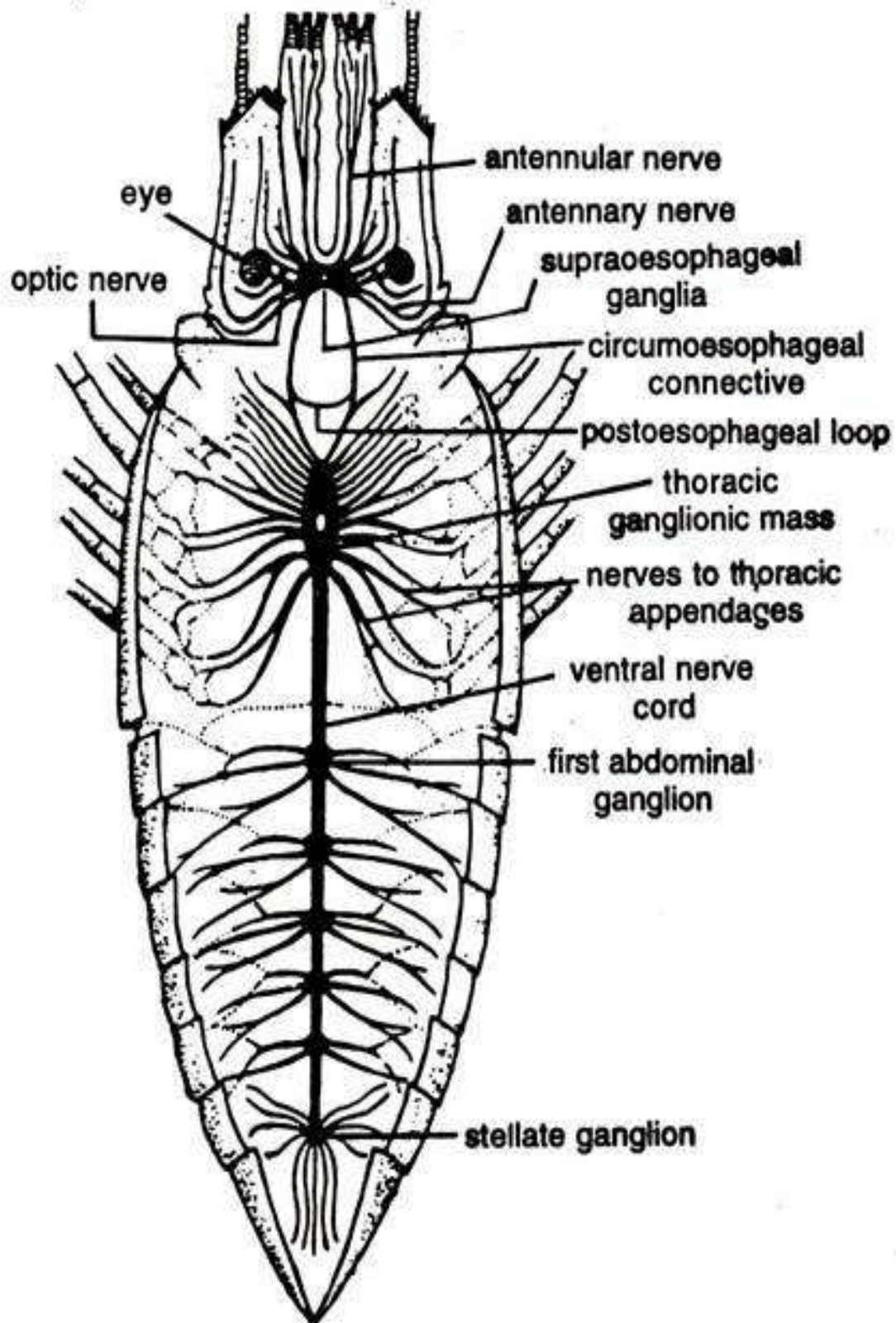
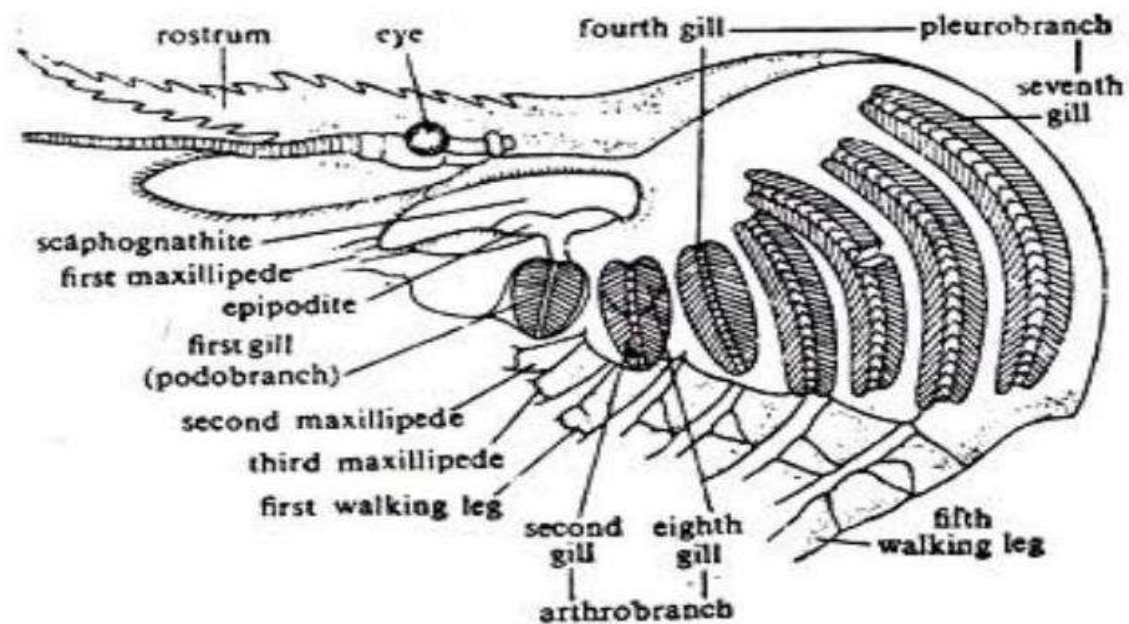


Fig: Cranial nerves of *Labeo*.

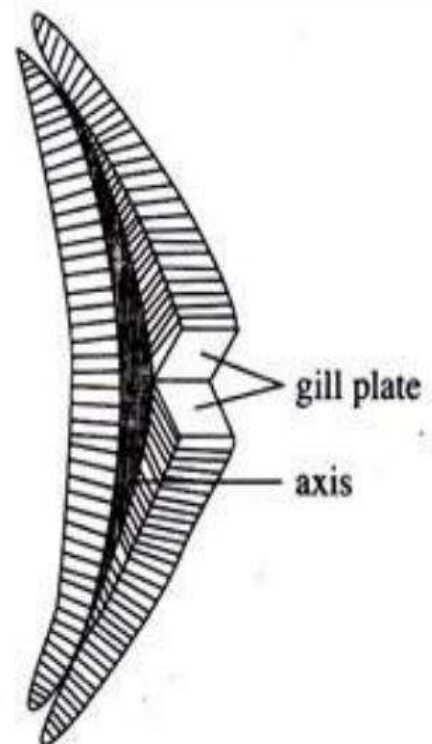
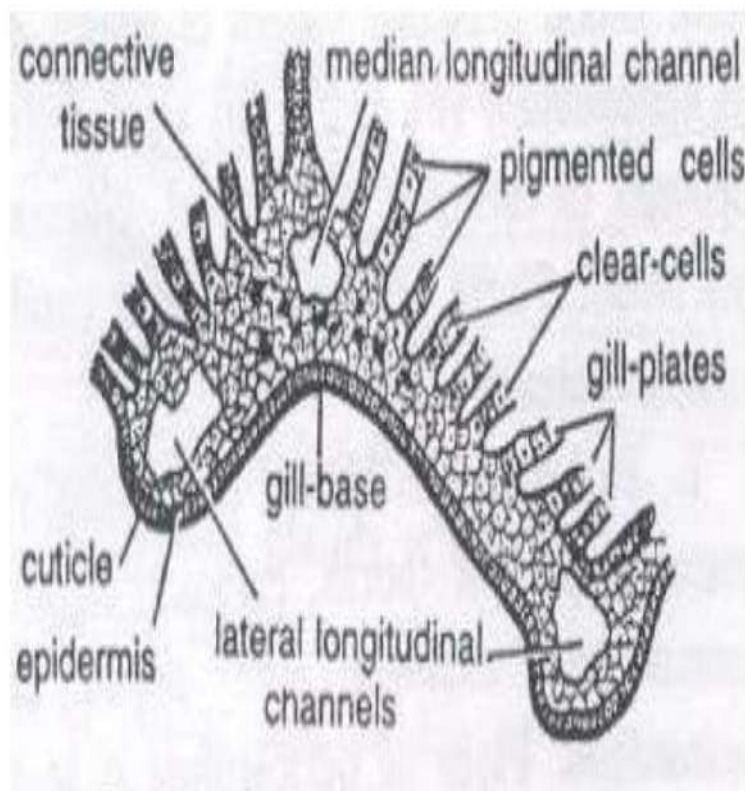
8. NERVOUS SYSTEM OF PRAWN



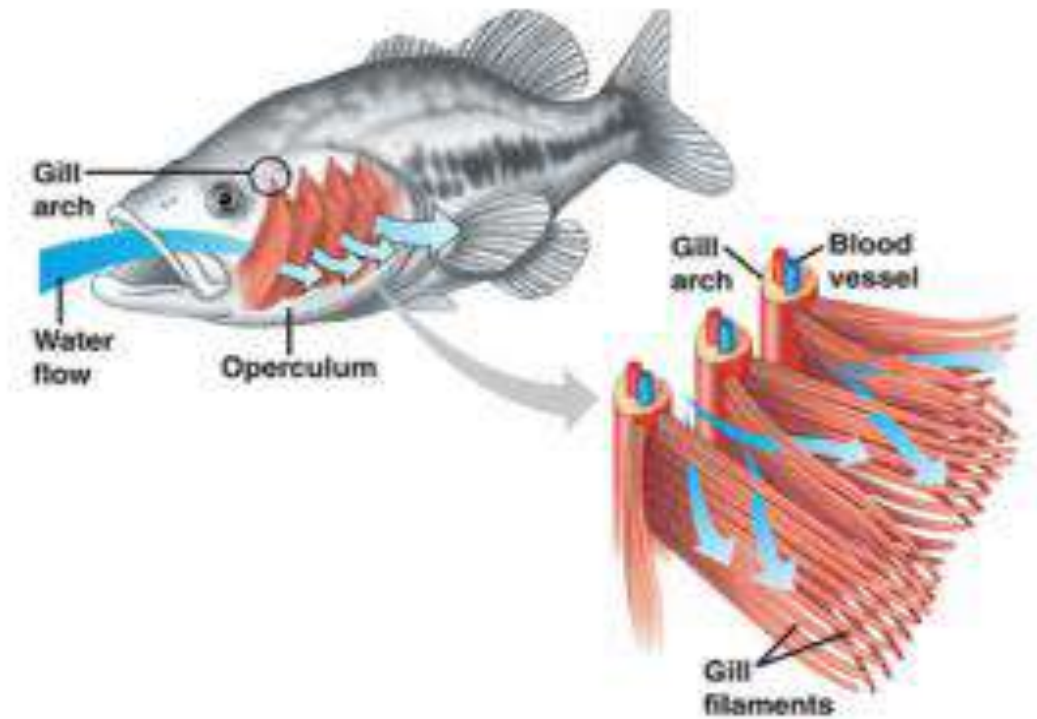
9. GILLS OF PRAWN



9a Oblique Section of prawn gill

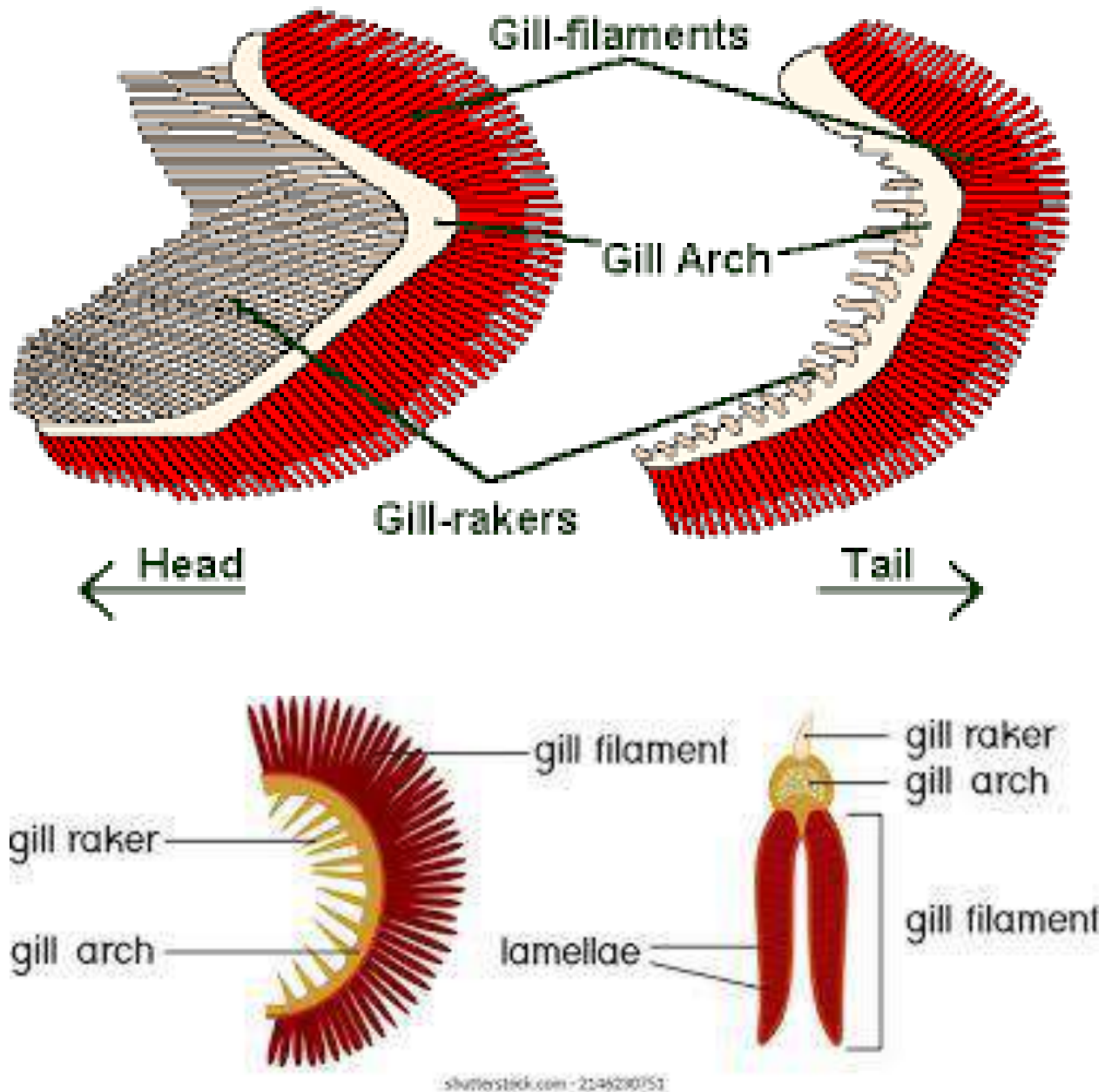


10. GILLS OF FISH



10. A. GILLS OF FISH

Diagrammatic Representation of Two Forms of Fish Gills



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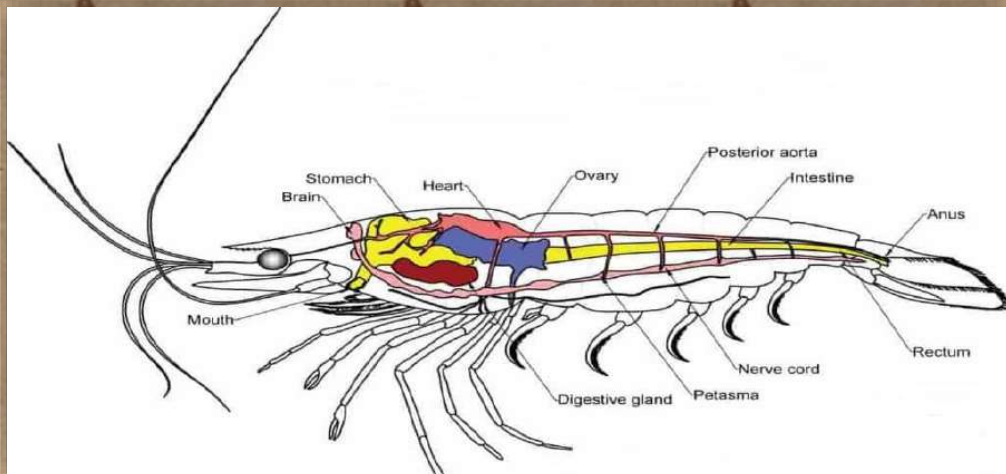
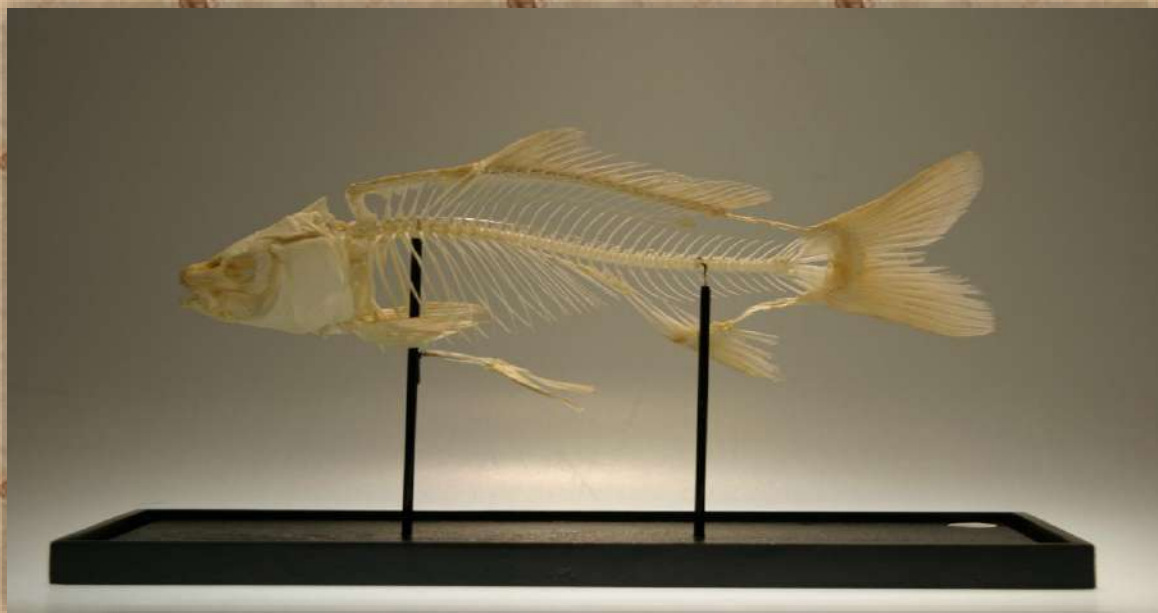
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