Biological extraction of chitin and chitosan using zooplankton associated bacteria

A Dissertation for

Course code and course title: MIC-651 Discipline specific dissertation

Credits:16

Submitted in partial fulfilment of

Master of Science in Microbiology

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DECLARATION BY STUDENT

I hereby declare that the data presented in this dissertation report entitled, "Biological extraction of chitin and chitosan using zooplankton associated bacteria" is based on the result of investigations carried out by me in the Department of Microbiology at the School of Biological Science and Biotechnology, Goa university under the supervision of Dr. Lata Gawade and the same has not been submitted elsewhere for the award of degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CETIFICATE

This is to certify that the dissertation **"Biological extraction of chitin and chitosan using zooplankton associated bacteria"** is a bonafide work carried out by **Ms. Manaswi Yashwant Band** under my supervision/ mentorship in partial fulfillment of the requirements for the award of the degree of **Master of Science** in the Discipline Microbiology at the School of Biological Science and Biotechnology, Goa University.

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PREFACE

This report has been prepared as part of my dissertation, as part of my Master's degree. The report is prepared with the view to include all the details regarding the project work that I carried out. Biological extraction of chitin and chitosan using zooplankton-associated bacteria offers several advantages. It is a rapidly evolving field with significant potential for innovation and contribution to sustainable biotechnological processes. It's a more environmentally friendly method compared to chemical extraction processes, as it reduces the need for harsh chemicals. It can potentially yield higher-quality chitin and chitosan due to the specificity of bacterial enzymes involved in the process. After the biological degradation step, the chitin is typically purified through processes such as filtration, centrifugation, and chemical treatments to remove any remaining impurities and obtain a high-purity chitin product.

The initial portion is the description, methodology result, and discussion after that scanning electron microscopy (SEM) analysis. In this project, I took various types of crustacean sources and isolated zooplankton-associated bacteria for further extraction of chitin and chitosan. In this research, you can see the amount of chitin & chitosan extracted from crustacean sources & its associated bacteria. Finally, it provides a platform for addressing real-world challenges related to waste management, resource utilization, and the development of novel biomaterials with diverse applications in industries such as healthcare, agriculture, and food packaging.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my guide Dr. Lata Gawade, for her guidance throughout my project.

I am grateful to Dr. Lakshangy Charya, Programme Director, Microbiology, School of Biological Science and Biotechnology for extending necessary facilities.

I would like to extend gratitude to all my teachers, Dr. Trupti Asolkar, Dr. Sandeep Garg, Dr Milind Naik, Dr. Judith Noronha, and Dr. Bhakti Salgaonkar.

Special thanks to the non-teaching staff Mr. Surendra Velip, Mrs. Robertina Fernandes, Mr Lakshman sir, Mr. Bhagwant Karpe, Ms Domingos Dins for their constant help while carrying out this work.

My sincere thanks to Nikita, Sarvasvi, Rachel, Bhavana, Kareena, Apoorva, Marlon, and all my friends for their constant support and help.

I would like to thank my parents. No words would be adequate to express my gratitude for their immeasurable understanding and encouragement. Lastly, I would like to thank the almighty who kept blessing on me and took me to the path of success.

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ABBREVIATION

Entity	Abbreviations
CDA	Chitin degradative activity
POM	Particulate organic matter
DES	Deep Eutectic Solvents
DSC	Differential Scanning Calorimetry
FT-IR	Fourier Transform Infrared Spectroscopy
XRD	X-ray Diffraction
TGA	Thermogravimetric Analysis
NMR	Nuclear Magnetic Resonance Spectroscopy
BSA	Bovine Serum Albumin
ZMA	Zobell Marine Agar
MR-VP	Methyl red and Voges–Proskauer test
SEM	Scanning electron microscopy

`ABSTRACT

This study aims to explore a sustainable approach for the extraction of chitin and chitosan from crustacean shells and eggshell waste through a biological method utilizing zooplankton-associated bacteria. Zooplankton, ubiquitous in marine environments, possess chitinous structures and harbour a diverse array of associated bacteria. It includes the collection of zooplankton samples, isolation of zooplankton-associated bacteria, assessment of their enzymatic activity for chitin extraction, biological extraction of chitin, and subsequent deacetylation to chitosan.

Although lactic acid bacteria are mainly applied, other microbial species including proteolytic bacteria have also been successfully implemented, as well as mixed cultures involving lactic acid-producing bacteria and proteolytic microorganisms. Since lactic acid combines with calcium carbonate, the primary mineral component, to make calcium lactate, the generated lactic acid permits shell demineralization. This study offers a streamlined, one-step method for biologically extracting chitin from fish scales via sequential fermentation. The fermentative approach for chitin extraction from fish scales using zooplankton-associated bacteria. The enzyme chitin deacetylase hydrolyses the acetamido groups of N-acetylglucosamine in chitin, resulting in the deacetylated product known as chitosan. When a regulated and well-defined process is needed, an alternate or complementary method that takes use of chitin's enzymatic deacetylation may be used. The extracted polymer was characterized using scanning electron microscopy (SEM). This current research states that a sustainable and eco-friendly approach gives value to waste materials, potentially offering a renewable source of chitin and chitosan for various industrial applications.

Chapter 1.1

INTRODUCTION

INTRODUCTION

1.1.1. FISH WASTE THAT GENERATED INTO THE ENVIRONMENT:

Fish wastes may be used to make value-added goods since they are abundant in the environment. Every year, 18–30 million tons of fish waste are disposed of worldwide. Because of their high levels of fat, oil, and grease, total suspended solids, chemical oxy gen demand, pathogens, organic materials, additional nutrients, and other contaminants, fishery wastes are extremely dangerous (Sapkota et al., 2008). Currently, the primary source of industrial chitin is the waste from the manufacturing of marine food, mostly the shells of crustaceans, such as krill, shrimp, and crab (Mojarrad., et al 2007, Xu 2008 Xuemei 2002). 40 to 50 percent of the entire mass is wasted while preparing shrimp for human consumption (Gimeno et al., 2007). One of the biggest pollutants in coastal regions is the garbage that is discharged into the sea, with a tiny portion being dried and utilized as chicken feed (Wang et al., 2010). The disposal of shellfish wastes has been suggested as an alternative to conventional waste treatment methods, in addition to being used to address environmental issues (Crini et al., 2009). The primary components of crustacean shell waste are 20–30% chitin, 30–50% calcium carbonate, and 30–40% protein. Eggshells are also a major source of chitin with as high as 74% in chicken eggs. Likewise, Battampara et al. (2020) reported (2020) that the dormant eggs of *Ceriodaphnia quadrangular* could yield 16–17% chitin and 11% chitosan.

Figure 1.1: crustacean waste green mussels shell waste and fish scales waste

1.1.2. CHITIN:

First discovered in 1884, chitin, also known as poly (b-(1-4)-N-acetyl-D-glucosamine), is a significant natural polysaccharide (Rinaudo, 2006). The main component of squid cartilage, shrimp and crab shells, and insect exterior coverings is chitin. In general, it has been reported that 30–40% protein, 30–50% calcium carbonate and calcium phosphate, and 20–30% chitin make up a crustacean's shell (Fernandez-Kim, 2004). Chitin has three allomorph which are α, β & γ- chitin. α-chitin has antiparallel chains, while β-chitin has parallel chains for, and γ-chitin is a combination of α- and β-chitin (Jampafuang et al., 2019). Water and most other organic solvents do not dissolve chitin. The most often acquired kind of chitosan is α–chitosan derived from crustaceans, namely chitin from leftover shrimp and crab shells. Approximately 70% of the chemical components found in these shells are made up of chitin. Additional chitin sources, such as β-chitin from squid pens, have also been proposed as potentially useful for chitosan synthesis (Martău et al., 2019).

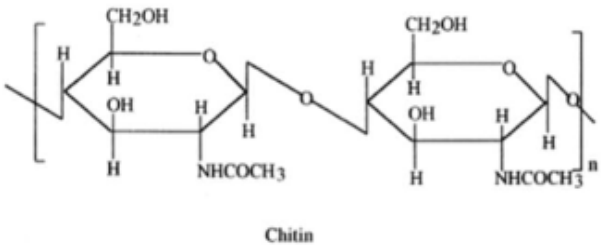


Figure 1.2: Structure of Chitin (Dave et al., 2021)

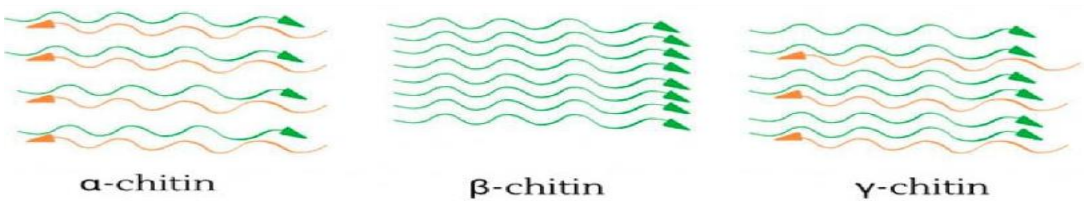


Figure 1.3: illustration of the parallel and antiparallel chain piles seen in β- and α-chitin and the parallel one found in β-chitin (Dave et al., 2021).

1.1.3. CHITOSAN:

Chitosan is a naturally linear polysaccharide containing D -glucosamine and N-acetyl-D-glucosamine linked by β-1-4 glycosidic bond that is produced by partially deacetylating chitin. Chitosan is often insoluble in aqueous solutions above pH7 when it is in its crystalline state. Chitosan is insoluble in phosphoric and sulfuric acids but soluble in 1% hydrochloric acid. Starch and cellulose powders have a smooth texture and are white in color, however chitosan powder is flabby and ranges in color from pale yellow to white. High molecular weight biopolymers include chitosan. The sources of raw materials and production techniques have an impact on the molecular weight of chitosan, much like on its content. Chitin is the source of chitosan, a material that resembles fiber. Chitosan is a high molecular weight, non-toxic, biodegradable polymer that resembles plant fiber cellulose. The enzymatic or alkaline technique of deacetylating chitin can yield chitosan. During this process, some of the polymer's N-acetyl linkages are broken, resulting in a generation of D-glucosamine units, which have a free amine group and increase the polymer's solubility in aqueous solutions. (Kalut, 2008).

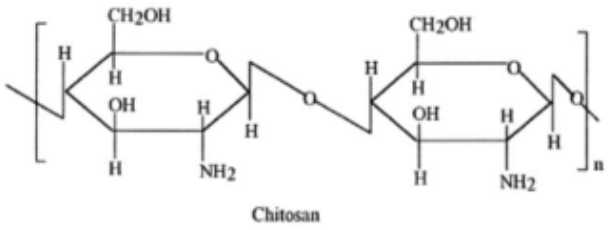


Figure 1.4: Structure of Chitosan (Dave et al., 2021)

1.1.4. PROPERTIES OF CHITIN & CHITOSAN:

Chitin possesses biological qualities such as biocompatibility, promoting the growth of osteoblasts, which create bones, and acting as a haemostatic, fungistatic, spermicidal, anticancer, and anticholestatic agent. Biodegradability and biocompatibility: Because of its

excellent biocompatibility and low toxicity, chitosan has the potential to be a valuable medicinal ingredient when used as a fat binder in formulations for cholesterol reduction and weight loss. Lysozyme and other human enzymes break down chitosan, which is regarded as biodegradable. In addition, it has antibacterial, anticholesterolemic, and permeation-enhancing properties. Absorption of Chitosan: Chitosan has garnered a lot of interest lately as a possible mucosal epithelia absorption enhancer, particularly for peptide medications. chitosan offers a few benefits Because of its mucoadhesive qualities, it can stay concentrated in the drug's absorption zone. pH sensations: Because it contains a lot of amino groups, chitosan behaves as a weak base and is Ph-sensitive.

1.1.5. CHITIN EXTRACTION:

The primary sources of chitin are the shells of shrimp and crabs. The principal constituents of the shell consist of chitin, proteins, lipids, pigments, and trace elements. About 90% of the dry shell weight is made up of proteins, chitin, and calcium carbonate (Ferrer et al., 1996). Chitin needs to be separated from proteins, minerals, and other substances to become pure chitin. Three steps are primarily used to accomplish this separation: Three steps are involved: 1) Demineralization to remove calcium carbonate 2) Deproteinization and 3) Lipid and color removal. There are two basic ways to accomplish the chitin extraction steps: chemically and biologically. Fermentation and enzyme-assisted extraction are the two known techniques for biological extraction.

1.1.6. CHEMICAL METHOD:

The shell is demineralized by inorganic acid (HCl) treatment and deproteinized by alkali treatment with NaOH in the chemical technique of chitin extraction. Lipids and colour are removed with an organic solvent treatment. From the perspective of biological activity, this

approach primarily forms monomers to trimers which are preferable (Shahidi et al., (1999). The potential denaturation of the chitin presents another issue.

1.1.7. DISADVANTAGES OF CHEMICAL METHOD:

The intrinsic properties of purified chitin are negatively impacted by extraction by chemical treatments, which has several negative effects. These include: (i) a decrease in MW and Da (molecular weight and Daltan), that compromises the physico-chemical properties of chitin; (ii) a negative impact on wastewater effluent that contains certain chemicals; and (iii) an increase in the cost of chitin purification procedures. The biological approach—which makes use of microorganisms preserves the structure of chitin, it is superior than the chemical approach.

Large amounts of dangerous chemicals (NaOH and HCl) are needed for the traditional extraction of chitin, which is done using an antiquated chemical process and discharged into the environment (Younes et al., 2014). According to (Mao et al. (2017), chemical extraction has several detrimental effects on the environment and does not yield a greater recovery of the protein components. Green extraction methods have been used to pre-treat chitinous biomass in order to get around these problems.

1.1.7. BIOLOGICAL EXTRACTION OF CHITIN:

Biological techniques provide an alternate approach to difficulties involving chemical extraction. Alkali treatment of crustacean shells might be avoided by using proteases to deproteinize them (Jung et al., 2007). Proteolytic bacteria were employed in addition to exoenzymes to deproteinize demineralized shells. Deproteinization procedures utilizing mechanical, enzymatic, and microbiological techniques have been documented for the manufacture of chitin mostly from shrimp waste. It has been reported that the production of chitin from crustacean's shell occurs through biological demineralisation (Synowiecki, 2000)

1.1.8. ENZYMATIC DEPROTEINIZATION:

Enzymes like proteases are needed for the extraction of chitin in order to remove the proteins. Proteases such as trypsin, pepsin, and papain are used to remove proteins.. When compared to the chemical process, the enzymatic method's efficiency is lower. 5%–10% of the protein was retained associated with chitin even after extraction (Synowiecki & Al-Khateeb, 2000). Additional NaOH treatment can be used to increase purity. The amount and yield of the product are unaffected by the sequence in which demineralization and deproteinization occur in a chemical process (Kaur & Dhillon, 2015). Younes et al. enhanced enzymatic deproteinization prior to demineralization in a different investigation. The minerals in the cuticles, however, may reduce the accessibility of the proteases and impact the deproteinization effectiveness of shrimp shells if enzymatic deproteinization is used. Therefore, the initial step should be demineralization.

1.1.9. ENZYMATIC FERMENTAION:

According to Yadav et al. (2019), there are two types of fermentation: lactic acid fermentation and non-lactic acid fermentation. Crustacean shell fermentation uses *Lactobacillus sp*., a producer of lactic acid and proteases. When glucose is altered in silage, the pH drops and lactic acid is produced. The pH, temperature, carbon supply, and makeup of the inoculum all influence how productive lactic acid fermentation is. Both fungi and bacteria, such as *Aspergillus sp., Bacillus sp., and Pseudomonas sp.,* can be used to ferment crustacean shells without the need of lactic acid (Yadav et al., 2019).

1.1.10. CHITIN DEACETYLASE (CDA):

Chitin deacetylase (CDA) is the enzyme that catalyses the deacetylation of N-acetyl-D-glucosamine residues in chitin to convert it to chitosan. The enzyme is a ~75 kDa acidic

glycoprotein that contains 30% (w/w) carbohydrates. It has an impressive heat stability at 50 °C and a broad pH optimal range (Kafetzopoulos c 1993). A characteristic with potential biotechnological applications is their resistance to inhibition by acetate, which is a byproduct of the deacetylation process. Acetyl groups are taken out of the chitin molecule by the deacetylation process, resulting in the product chitosan, which has a highly reactive amino group (-NH2). Because of this, the level of deacetylation is a crucial component in the physicochemical characteristics of chitosan synthesis, which influences its suitability for various uses (Rout, 2001). According to (Tolaimate et al. 2000), deacetylation also influences immunological activity and biodegradability. The degree of deacetylation of chitosan ranges from 56% to 99% with an average of 80%, depending on the crustacean species and the preparation methods (No and Meyers, 1995).

1.1.11. APPLICATION OF CHITIN AND CHITOSAN:

Chitin is the primary ingredient utilized in the production of chitin-derived products, including glucosamine hydrochloride, chitosan, and oligosaccharides. Chitosan has several significant uses in the food industry, such as cleaning wine, regaining protein from fish wash water and meat processing facilities, adding it to animal feed, and purifying drinking water. In thin-layer chromatography, chitosan is employed to separate nucleic acids Takeda, (1961). Chitosan satisfies every need to be the perfect substance used in the paper industry Muzzarelli, (1977). Additionally, it was noted that chitosan-coated duplex board cartons worked well for packing frozen fish instead of wax-coated ones. Research on animal nutrition has demonstrated that a modest quantity of chitin added to food improves the animal's consumption of whey by altering its gut flora (Spreen et al.,1980). Derivatives of chitosan are used in dental pastes, nail polishes, emollients, moisturizers, and antistatic agents Radhakrishnan et al., (1991).

1.1.12. ZOOPLANKTON ASSOCIATED BACTERIA:

In aquatic systems, zooplankton serve as dynamic microhabitats for bacteria. They frequently sustain bacterial concentrations of 107 to 1011 cells ml^{-1} body volume, which is comparable to or higher than those in the surrounding water (Tang et al. 2010). The bacterial population associated with zooplankton can colonized both internal and external surfaces of zooplankton. Additionally, the zooplankton can release and exude complex organic compounds like taurine and chitin, which the bacterial community can digest. Particulate organic matter (POM), which comes from phytoplankton, heterotrophic microzooplankton, and detritus, is released in large quantities into the surrounding water by crustacean zooplankton (Heinle et al., 1977; Calbet, 2001). By colonizing zooplankton's exoskeleton and gut, microorganisms can make use of it as a microhabitat that is high in nutrients and carbon (Carman and Dobbs, 1997; Tang et al. 2010). Aside from the nutrient-rich conditions, the zooplankton's stomach provides a hypoxic environment that may mimic important anaerobic processes including denitrification, dissimilatory nitrate or nitrite reduction, and methanogenesis in oxygenated open seas. (Tang et al., 2011). Bacteria associated with zooplankton can have tens of order of magnitude higher life than free-living (Tang et al.,2006; Tang et al., 2010; Tang et al., 2011; Schmidt et al., 2016).

Chapter 1.2

1.2 Hypothesis

Zooplankton are drifting plankton/marine planktons which have chitinous structure & have lots of associated bacteria, so these chitinaceous zooplankton-associated bacteria will/may have efficiency for chitin-chitosan extraction & good enzymatic activity.

Chapter 1.3

AIM

AND

OBJECTIVES

1.3 AIM:

To extract chitin and chitosan by biological method from different crustacean shells & eggshell waste using zooplankton-associated bacteria.

OBJECTIVES:

1. To collect zooplankton sample and isolate zooplankton-associated bacteria.
2. To check the enzymatic activity of zooplankton-associated bacteria for chitin extraction.
3. Biological extraction of chitin using zooplankton-associated bacteria.
4. Biological deacetylation of chitin to chitosan by zooplankton-associated bacteria.

Chapter 2

LITERATURE

REVIEW

Literature review

- Chitin, a widely abundant polymer in nature, boasts diverse applications and favourable material properties, including biocompatibility. A biopolymer, chitin plays a significant role in the supporting tissues of many different types of creatures, including fungi, insects, and crustaceans. The natural carbohydrate polymer is called chitin. It is a glucose derivative and a long-chain polymer of N-acetyl D-glucosamine. Whereas chitosan is poly-β-(1-4)-D-glucosamine, chitin is poly-β-(1-4)-N-acetyl-D-glucosamine.
- Currently, the primary source of industrial chitin is the waste from the manufacturing of marine food, mostly the shells of crustaceans, such as krill, shrimp, and crab (Mojarrad., et al 2007, Xu 2008, Xuemei 2002). In general, it has been reported that 30–40% protein, 30–50% calcium carbonate and calcium phosphate, and 20–30% chitin make up a crustacean's shell (Fernandez-Kim, 2004).
- (Khanafari et al.'s 2008) comparison of chemical and biological approaches for chitin extraction from shrimp shells was conducted. According to (Mao et al. (2017), chemical extraction has several detrimental effects on the environment and does not yield a greater recovery of the protein components.
- Additional chitin sources, such as β-chitin from squid pens, have also been proposed as potentially useful for chitosan synthesis (Martău et al., 2019). This study introduces a novel method for extracting β-chitin from fishery by-products using alkaline Deep Eutectic Solvents (DES), specifically potassium carbonate: glycerol (KGLY). The resulting β-chitin showed high purity, with an acetylation degree between 77 and 88%, high crystallinity, and a maximum degradation temperature of around 350 °C, while the DES system demonstrated recyclability over multiple cycles, making it a promising approach for the production of chitosan and biomaterials (McReynolds et al., 2022).

- The biological method it has been reported that, of chitin extraction from crustacean shells, using lactic acid bacteria, is eco-friendly and cost-effective, producing high-quality chitin and a protein-rich liquid fraction suitable for feed. However, its application is limited to laboratory settings due to challenges in achieving desired demineralization and deproteinization yields when compared to chemical methods (Pal et al., 2014).
- Traditional chemical extraction methods, relying on high concentrations of alkali, are environmentally unfriendly and economically impractical. Consequently, there is a growing need for eco-friendly extraction techniques, particularly from cost-effective sources. This study introduces a simplified, one-step biological extraction method from fish scales` using successive fermentation with *Stenotrophomonas koreensis* enzymes, showcasing a novel approach not documented elsewhere. With a chitin yield of 28% (w/w), the extracted polymer underwent thorough characterization through DSC, FTIR, XRD, and TGA analysis, highlighting a significant advancement in green chitin extraction methods (Suresh et al., 2023).
- Chitin and chitosan were successfully extracted from the resting eggs of *Ceriodaphnia quadrangula*, with chitin comprising 16-17% and chitosan 11% of the dry weight. The extracted chitosan exhibited a degree of deacetylation of 80%, also concluded that is alpha chitin suggesting that these materials can serve as an alternative source of chitin and chitosan (Kaya et al., 2014).
- The purpose of this work is to extract chitin from *Doryteuthis sibogae*, which is found along the Indian coast. The squid pens were treated according to the standard protocol, which included demineralization and deproteinization, but because the squid pens had a low amount of inorganic compounds, a two-step alkaline treatment was sufficient to

produce ß-chitin. Acid and alkaline treatments were used to manufacture squid chitin; the yield of chitin was 33.02% of the total weight of the dried pens (Barwin et al., 2011).

- Chitosan, a valuable natural polymer with applications in biotechnology and various industries, is traditionally produced from chitin via a harsh thermochemical process, resulting in environmental concerns and heterogeneous product outcomes. However, this study highlights the potential for an alternative enzymatic deacetylation approach, leveraging specific bacterial strains with chitin deacetylase activity, offering a controlled and well-defined process for chitosan production. Applying chitin as the only carbon source resulted in yields of 0.1 and 0.16 g/L of chitosan, respectively. (Kaur et al., 2012).
- During the deacetylation process, some of the polymer's N-acetyl linkages are broken, resulting in a generation of D-glucosamine units, which have a free amine group and increase the polymer's solubility in aqueous solutions. (Kalut, 2008).
- The degree of deacetylation in chitin can vary from 30% to 95% (Martino et al., 2005), depending on the source and the processing method used to generate it (Khora and Limb, 2003).
- The study determined the amounts of inorganic salt, protein, fat, and chitin in the exoskeleton *of Parapenaeus longirostris* from Moroccan sources. Chitin was extracted from the shells using treatments with HCl and NaOH, and then converted into chitosan. Various spectroscopic and microscopy techniques were used to characterize the chemical structure and physico-chemical properties of chitin and chitosan, including FT-IR, NMR, XRD, and SEM. Additionally, the molecular weight of chitosan was determined by viscometric methods, and the degree of acetylation was assessed using 1H NMR, marking the first report on the extraction and characterization of chitin and chitosan from *Parapenaeus longirostris* (Dahmane et al., 2014).

- Chitin and chitosan were both detected in Scanning Electron Microscopy (SEM) to have relatively uniform surfaces with lamellar organization and dense structures, while the surface of chitosan seems less crystalline (Dahmane et al., 2014).
- The spectra of chitin and chitosan showed various distinctive peaks in Fourier Transform-Infrared (FT-IR) spectroscopy. The peak at 3400 cm^{-1} is ascribed to the stretching vibration of the -NH2 and -OH groups as well as intermolecular hydrogen bonding. The amide I stretch is represented by the two bands in chitin spectra, 1659 and 1624 cm^{-1}. The stretching of the C-O group hydrogen bonded to the neighbouring intra-sheet chain is assigned to the band at 1659 cm^{-1}, and the 1624 cm^{-1} band may represent a particular hydrogen bond of C-O with the hydroxyl-methyl group of the subsequent chitin residue of the same chain. Amide II (N-H bending) and Amide III (C-N stretching) are represented by the absorption bands at 1556 and 1315 cm-1, respectively. The peak at 1556 cm-1 vanished when chitin was deacetylated, and a new, strong peak at 1595 cm-1 (NH2 bending) formed (Dahmane et al., 2014).

Chapter 3

METHODOLOGY

1.2. SAMPLE COLLECTION:-

Debris/waste of crab, prawns, fish scales, squids, and green mussel shells that was gathered from the fish market before the meat was cut off and the shells were separated. Then shells were carefully cleaned to get rid of any meat or dust particles. Cuttlefish bone was discovered on the shore and egg shells were discovered among the trash/garbage. Everything was then dried in an oven for overnight.

3.2.1 COLLECTION OF ZOOPLANKTON SAMPLE: -

Zooplankton-associated bacteria were isolated following Wang et al. (2021) with some modifications. 20 liters of seawater samples were filtered through the 200 µm mesh to collect the zooplankton samples.

3.2.2 ISOLATION OF ZOOPLANKTON ASSOCIATED BACTERIA: -

Zooplankton retained on the mesh were cleaned with the filter-autoclaved seawater, collected with a clean spatula, and dispensed into the tube containing filter-autoclaved seawater. The same sample was sonicated and dilutions up to 10-5 were prepared and plated on ZMA agar. Plates were incubated at 37°C for 48 hours. Growth was counted on all the plates.

3.3 SCREENING OF ENZYMES: -

3.3.1 PROTEASE ACTIVITY: -

protease activity of zooplankton-associated bacteria was performed by Spot inoculation on seawater agar plates with 2% skim milk. For 6 days, plates were incubated at room temperature. Protease-active cultures were distinguished from others by the development of a clearing surrounding the colony (Samant et al.,2019).

3.3.2 CALCIUM CARBONATE SOLUBILIZATION: -

Plates containing seawater-based media with 1% calcium carbonate were spot-inoculated with bacterial cultures associated with zooplankton, and the plates were incubated at room temperature for 10 days. The colonies' clear zone around them indicated the presence of calcium carbonate solubilization activity.

3.3.3 CHITIN ACTIVITY: -

Spot-inoculating bacterial colonies on plates with seawater-based medium with 1% colloidal chitin allowed researchers to assess the chitinase activity of the bacterial cultures associated with zooplankton. For 10 days, plates were incubated at room temperature. After 15 minutes of flooding with 1% Congo red, plates were decoloured with IM NaCl. Excess NaCl was poured out. The bacteria that produced chitinase had a zone of clearance (Samant et al,.2019).

3.4. VOGE-PROSKAUER (MR) & METHYL RED (VP):

A loop full of culture was infected with glucose phosphate broth (Appendix 1-10) and incubated for 24 hours at room temperature (28°C+2). The soup was split into two separate tubes after incubation. For the Mit test, one test tube was utilized, and for the VP test, another. For the MR test, add two to three drops of the methyl red reagent. The broth's crimson coloring indicates a successful outcome. for the VP Exam. After adding two drops of Omeara's reagent, stirring, and letting it stand for thirty minutes. The pink color indicates a positive test.

3.5. BIOLOGICAL EXTRACTION OF CHITIN

3.5.1 BIOLOGICAL EXTRACTION OF CHITIN FROM CRUSTACEANS:

Collection of Fishery waste

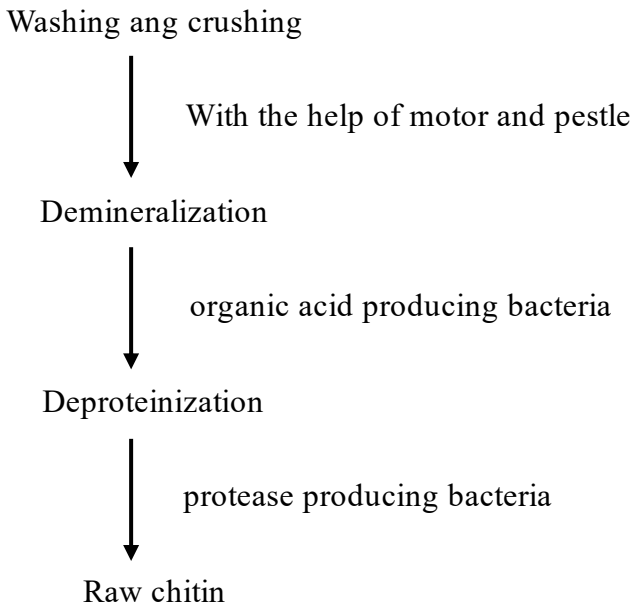


Figure3.5: Flow chart for biological extraction of chitin

Preparing inoculum by incubating the lactobacillus cell in 5 ml MRS broth at 30°C for 24 hours. Then, take 2 mL of the starter culture. Transfer to 100 ml of sterile MRS broth and continue incubating at 30°C for another 24 hours. The inoculums are ready to fermentation. Fermentation involves properly grinding shellfish waste. Then, combine 10% carbon sources with 10% culture inoculums. After 180 hours of incubation, sift and dry the solid cake in a hot air oven. then, Proteases are released into the fermentation medium to aid in protein separation. Exo-proteases and proteolytic bacteria may both deproteinize cells. Calcium carbonate separation has done by Lactic acid-producing bacteria utilize an additional carbon source (Pal et al., 2014).

3.5.2 BIOLOGICAL EXTRACTION OF CHITIN FROM FISH SCALES:

Fish scales were washed to remove sticky flesh and unwanted impurities several times by using distilled water and then dried at 60^0 C in a hot air oven, overnight. The media that was used to extract fish scale biologically was made up of 5% (w/v) fish scale and 10% (w/v) glucose. The media was autoclaved / sterilized at a temperature of 121^0 for 30 mins, subsequently addition

of inoculum 10% (v/v) and incubated at 37^0 C. In the shaking incubator (150 rpm) for 6 days. The solid phase was then separated by using Whatman filter paper No. 1 and then washed conscientiously by using distilled water. The extracted polymer then dried in a hot air oven at 60^0 C for 24 hrs (Suresh et al., 2023).

3.5.3 PROTEIN ESTIMATION TO CHECK DEPROTEINIZATION:

According to the working range given in table, 1ml different conc. of protein sample (BSA)was prepared and distilled water was kept as blank. Then 5ml of reagent A was added in each tube. Then test tubes were kept in the dark for 10 min. 0.5 of 1:1 folin-ciocalteau reagent was added to each tube. The tube was again incubated in the dark for 30 mins. The absorbance was recorded at 660nm. Then graph was plotted using conc. of protein mg/ml on X axis against absorbance at 660nm on Y axis. (Arunima, 2022).

3.6. BIOLOGICAL DEACETYLATION OF CHITIN TO CHITOSAN:

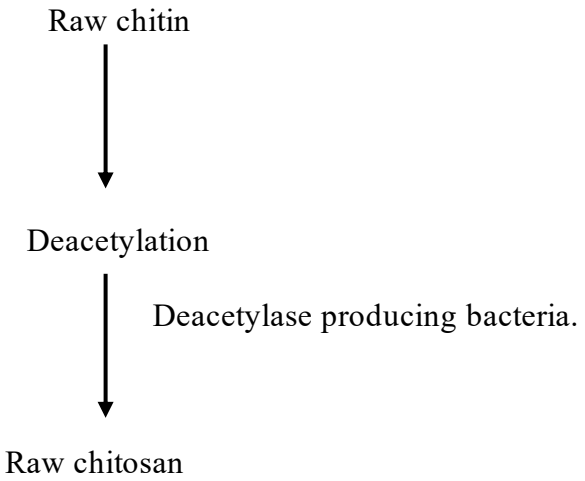


Figure 3.6: Flow chart for biological extraction of chitosan

3.6.1 All cultures are screened for CDA (chitin degradative activity) by preparing a solution. 5 g of p-nitroacetanilide was dissolved in 100 mL of ethanol to create a solution. Whatman #1 filter paper was cut into 5cm× 1cm to make small strips. Then these strips were dipped into the

p-nitroacetanalide. And removed and air-dried. This was repeated twice & thrice for sufficient to make concentrated. Then these stripes were used further for the test tube containing the media which is composed of 0.15g of potassium dihydrogen phosphate, 1 g of yeast extract, and 0.4g of ammonium sulphate (pH 8.0) then inoculated that individual isolated colony in a separate test tube and one kept as a control then incubated at 25 ^{0}C for 2 days. After the incubation period, 2mL aliquots were further transferred to another set of sterile test tubes which has diagnostic strips. These tubes were then cultured for ten days at 25 ^{0}C. After incubation yellow colour has observed that indicates that respective bacterial isolates have deacetylase activity (Srinivasan, 1998).

3.6.2. Transformation for chitin to chitosan.

The production media for CDA containing 0.15g of potassium dihydrogen phosphate, 1g of yeast extract, and 0.4g of ammonium sulphate (pH 8.0) containing 50mg of chitin which is used fermentation media. 50 ml of fermentation media were taken into 250ml capacity of flask. which were inoculated with 0.1 O. D $_{600}$ suspension of positive isolates & one flask kept as control then incubated on rotary shaker at 25 ^{0}C for 10 days. (Kaur et al., 2012).

3.6.3. Recovery of chitosan

Each fermentation broth was centrifuged at 12000 rpm for 15 mins. The pellet contains chitin chitosan & bacteria and supernatant is discarded. Add 10ml of 0.1N NaOH to each pellet mix thoroughly and taken into the clean test tube the it is autoclaved for 15min then allowed to come to room temperature. During alkaline treatment, most cell was solubilized. Then again centrifuged at 12000rpm for 15 min. Solubilize chitosan pellet contain chitin, chitosan, small amount of cell debris mixed with 10ml 2% acetic acid then kept on shaker overnight for RT. Then centrifuged at 12000rpm for 15 min. Then 10ml of supernatant were collected white

precipitate upon neutralization with 1N NaOH indicates presence of chitosan (Srinivasan, 1998).

3.6.4. Chitosan Qualitative Estimation.

After recovery, the white precipitate was centrifuged for 15 minutes at 5000 rpm. It was twice cleaned in pH 7 distilled water. A new suspension of the precipitate was then made in 0.5 milliliters of pH 7 distilled water and placed in a watch glass. It was left to dry for two to four hours at 55°C. The confirmatory test was conducted using the dehydrated precipitate. Two to three drops of an iodine/potassium iodide solution were added to the dried precipitate, stirred, and then acidified with two to three drops of 1% H_2SO_4. The precipitate turns dark brown and the solution turns colorless upon the addition of iodine/potassium iodide solution. When sulfuric acid is added, the dark brown hue changes to dark purple. This suggests the presence of chitosan (Kaur et al., 2012).

3.6.5. Quantitative estimation of chitosan:

The precipitate was washed twice by distilled water and resuspended in 1 ml of distilled water. Then weight has been taken of empty Petriplates. Then that 1ml suspension has taken in empty Petriplates and kept for drying for 55^0C for 2-3 hrs, plates were weighted after drying. (Kaur et al., 2012).

3.7. QUANTITATIVE SPECTROPHOTOMETRIC ESTIMATION BY DNSA METHOD:

A portion of the polymer's N-acetyl linkages are broken during deacetylation, resulting in the synthesis of D-glucosamine units, which have a free amine group and increase the polymer's solubility in water. 1 g of DNSA, 200 mg of crystalline phenol, and 50 mg of sodium sulphite were dissolved in 100 mL of 1% NaOH to create the Dinitrosalicylic Acid Reagent (DNSA

Reagent), which was then kept at 4°C. Because sodium sulphite causes the reagent to degrade, it is added at the time of use to allow for longer storage. 40% Rochelle salt solution (potassium sodium tartarate) is then added. In distilled water, stock solutions (mg/mL) of the fermentation medium were made. After adding 0.5 mL of DNSA reagent, the water bath was heated to 80° to 85°C for 15 minutes. when the tubes' contents were still warm. After cooling the reaction mixture, a spectrophotometer was used to detect the colored complex's absorbance at 540 nm. A standard graph was created by plotting the absorbance on the Y-axis and the monosaccharide concentration (microgram) on the X-axis (Rajbhar et al., 2015).

Chapter 4

RESULT

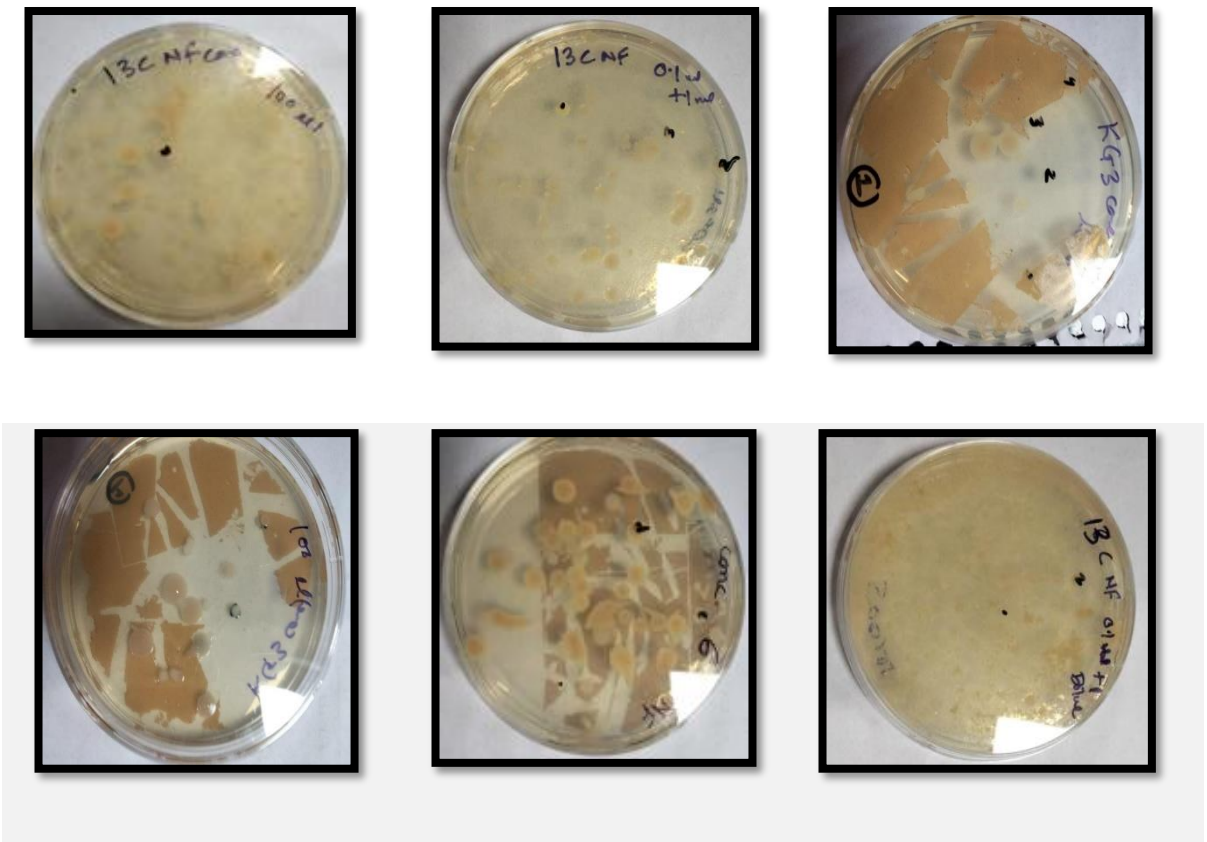
AND

DISCUSSION

RESULT & DISSCUSSION

COLLECTION OF ZOOPLANKTON SAMPLE & ISOLATION OF ZOOPLANKTON ASSOCIATED BACTERIA:

Zooplankton associated bacteria were isolated following Wang et al. (2021) with some modifications. 20 litres of seawater sample were filtered through the 200 µm mesh to collect the zooplankton samples. Zooplanktons retained on the mesh were cleaned with the filter autoclaved seawater, collected with clean spatula and dispensed in to the tube containing filter autoclaved seawater. Same sample was sonicated and dilutions up to 10^{-5} were prepared and plated on ZMA agar. Plates were incubated at 37°C for 48 hours. Growth was counted on all the plates.



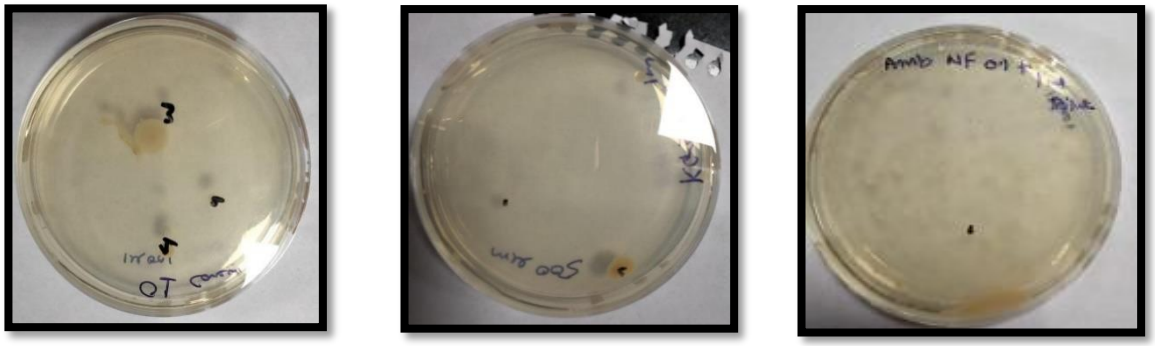
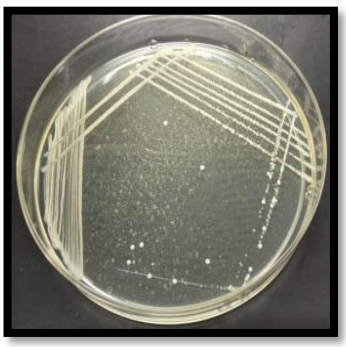
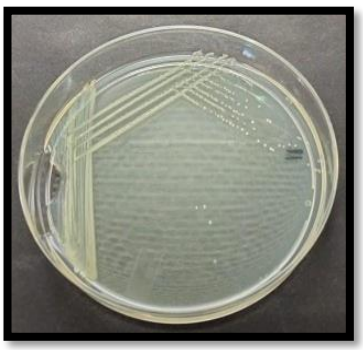


Figure 4.7: Isolation of Zooplankton associated bacteria on sea water based ZMA plates

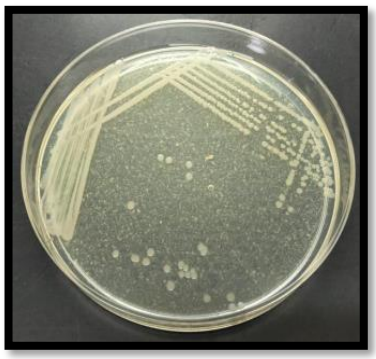
KG3-NF-13T-8Z



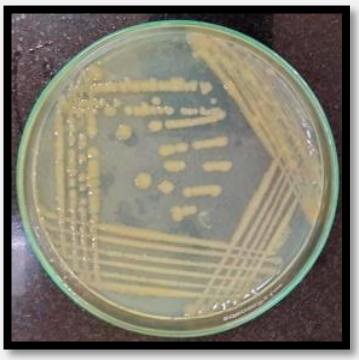
KG3-NF-6T-11Z



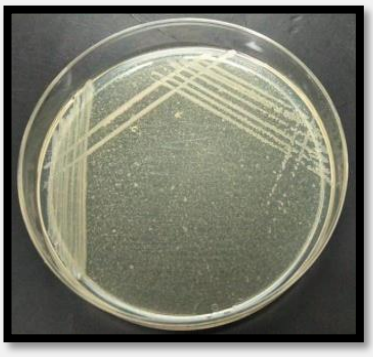
KG3-NF-6T-4Z



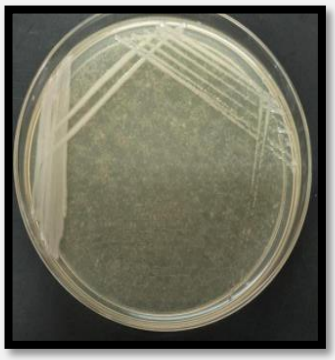
KG3-NF-13T-26Z



KG3-NF-13T-16Z



KG3-NF-13T-8Z



KG3-NF-6T-15Z

Figure 4.8: Zooplankton associated bacterial culture, sub-cultured on sea water based ZMA plates.

1. SCREENING OF ENZYMES

1.1. PROTEASE PRODUCTION:

Protease activity may be qualitatively determined using skim milk agar plates. The zone of hydrolysis around the colony is demonstrated by the bacteria's breakdown of the substrate casein. Spot inoculations were performed on all colonies using a seawater-based medium containing 2% skim milk. Nine isolates out of fifty exhibited a zone of clearing. Those isolates are KG3-NF-6T-1Z, KG3-NF-6T-4Z, KG3-NF-13T-6Z, KG3-NF-13T-8Z, KG3-NF-6T-11Z, KG3-NF-RT-14Z, KG3-NF-6T-15Z, KG3-NF-RT-21Z, KG3-NF-13T-26Z. Isolate KG3-NF-6T-1Z, KG3-NF-6T-4Z, KG3-NF-6T-11Z, and KG3-NF-13T-26Z had the maximum zone of clearing, therefore prawns, crabs, fish shells, green mussel shell, eggshells, squids, and cuttlefish bones were among the materials from which chitin was extracted.

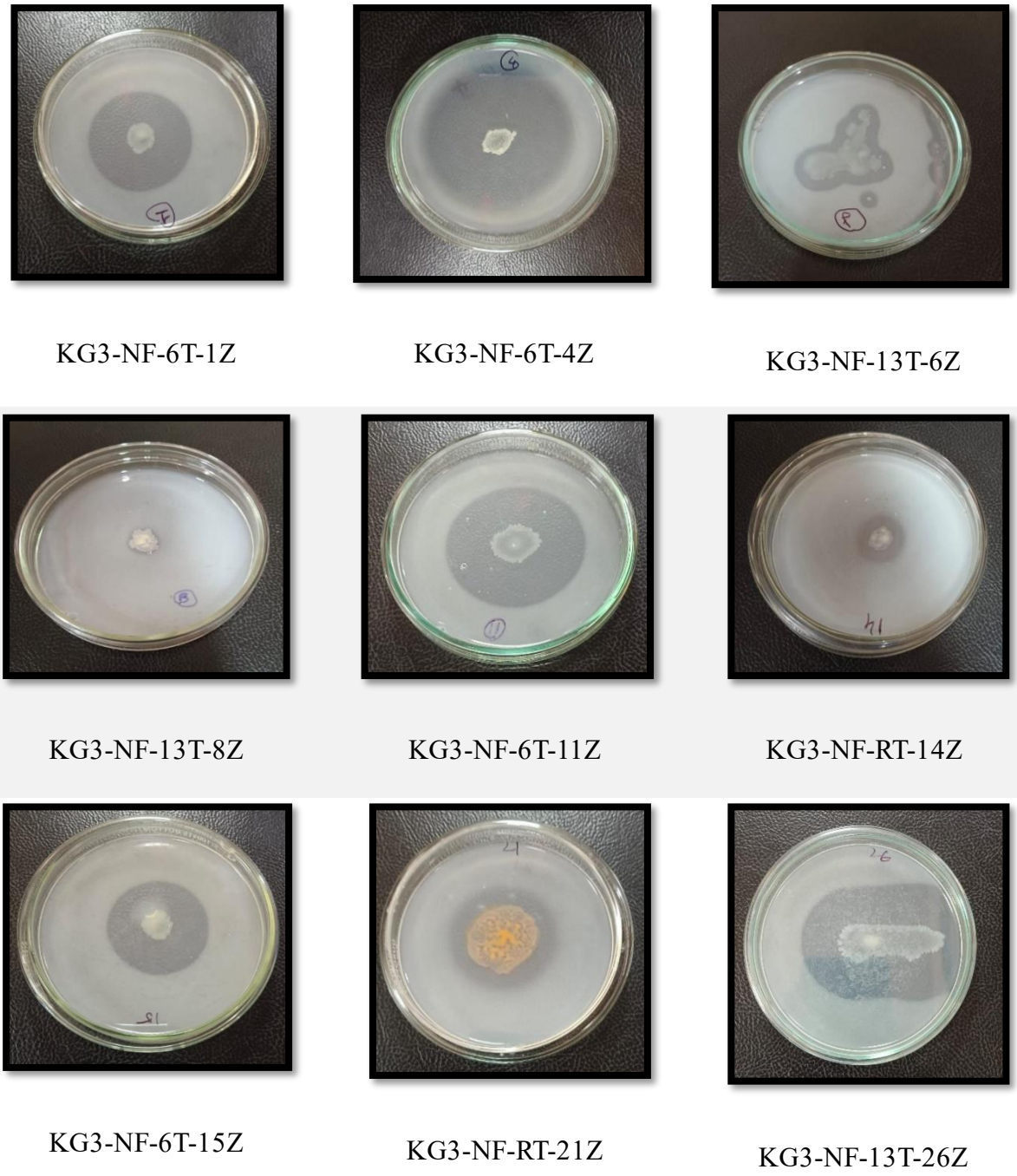


Figure 4.9: Proteolytic activity by KG3-NF-6T-1Z, KG3-NF-6T-4Z, KG3-NF-13T-6Z, KG3-NF-13T-8Z, KG3-NF-6T-11Z, KG3-NF-RT-14Z, KG3-NF-6T-15Z, KG3-NF-RT-21Z, KG3-NF-13T-26Z isolates from zooplankton associated bacteria.

1.2. CALCIUM CARBONATE SOLUBILIZATION:

Demineralization is one of the processes involved in the extraction of chitin, and the shells of leftover prawn and crab contain 20–50% CaCO. The chitin extraction procedure can make use

of bacterial isolates that are able to solubilize calcium carbonate. A zone of clearing surrounds the bacterial colony when calcium carbonate is dissolved. On plates with seawater-based medium containing 1% CaCO, each isolate was spot-injected. Just one bacterial isolate — isolate no 16 exhibited a small clearing zone.

KG3-NF-13T-16Z

Figure 4.10: Calcium carbonate solubilization activity by KG3-NF-13T-16Z isolates from zooplankton associated bacteria.

1.3. CHITINASE PRODUCTION:

The creation of a clear zone around the colony upon the breakdown of the chitin complex in the medium is a sign of chitinase activity. Chitin may be broken down by using bacterial isolates that exhibit chitinase activity. By initially screening for the enzyme chitin deacetylase, such isolates may be utilized to deacetylate chitin into chitosan. On plates with agar based on seawater and 1% colloidal chitin, each isolate was spot-injected. When 0.1% Congo red was added, only two isolates KG3-NF-RT-13Z, KG3-NF-6T-15Z showed a zone of clearing surrounding the colony; the remainder of the plate was stained red.

KG3-NF-RT-13Z, KG3-NF-RT-14Z,

KG3-NF-6T-15Z, KG3-NF-13T-16Z

Figure 4.11: chitinase activity by KG3-NF-RT-13Z, KG3-NF-6T-15Z, isolates from zooplankton associated bacteria

BIOCHEMICAL TEST ONLY MR-VP TEST:

Table 4.1: MRVP test for all 50 isolates

Isolate no.	KG3-NF-6T-1Z	KG3-NF-6T-2Z	KG3-NF-6T-3Z	KG3-NF-6T-4Z	KG3-NF-13T-5Z	KG3-NF-13T-6Z	KG3-NF-13T-7Z	KG3-NF-13T-8Z	KG3-NF-RT-9Z	KG3-NF-RT-10Z
MRVP +/-	-	-	-	-	-	-	-	+	-	-
Isolate no.	KG3-NF-6T-11Z	KG3-NF-6T-12Z	KG3-NF-RT-13Z	KG3-NF-RT-14Z	KG3-NF-6T-15Z	KG3-NF-13T-16Z	KG3-NF-6T-17Z	KG3-NF-13T-18Z	KG3-NF-6T-19Z	KG3-NF-6T-20Z
MRVP +/-	-	-	-	-	-	-	-	-	-	-
Isolate no.	KG3-NF-RT-21Z	KG3-NF-RT-22Z	KG3-NF-RT-23Z	KG3-NF-RT-24Z	KG3-NF-6T-25Z	KG3-NF-13T-26Z	KG3-NF-6T-27Z	KG3-NF-6T-28Z	KG3-NF-6T-29Z	KG3-NF-6T-30Z
MRVP +/-	-	-	-	-	-	-	-	-	-	-
Isolate no.	KG3-NF-13T-31Z	KG3-NF-13T-33Z	KG3-NF-13T-33Z	KG3-NF-13T-34Z	KG3-NF-13T-35Z	KG3-NF-13T-36Z	KG3-NF-RT-37Z	KG3-NF-6T-38Z	KG3-NF-13T-39Z	KG3-NF-6T-40Z
MRVP +/-	-	-	-	-	-	-	-	-	-	-

Isolate no.	KG3-NF-6T-41Z	KG3-NF-13T-42Z	KG3-NF-6T-43Z	KG3-NF-6T-44Z	KG3-NF-6T-45Z	KG3-NF-RT-46Z	KG3-NF-RT-47Z	KG3-NF-RT-48Z	KG3-NF-6T-49Z	KG3-NF-RT-50Z
MRVP +/-	-	-	-	-	-	-	-	-	-	-

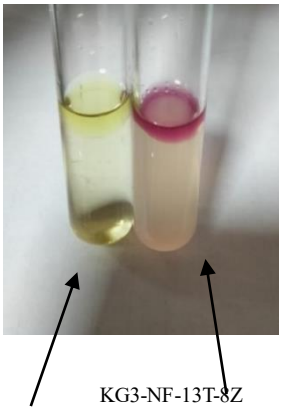
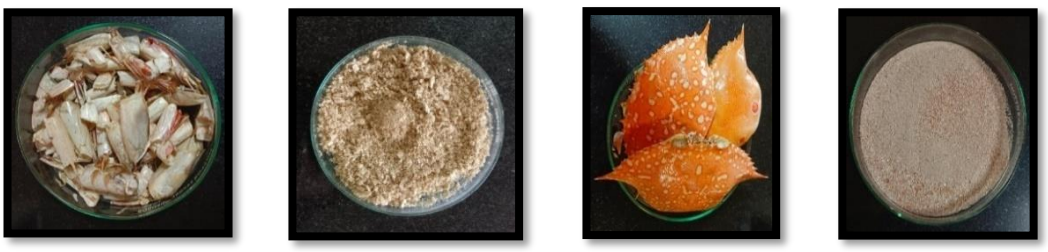


figure 4.12: MRVP by KG3-NF-13T-8Z isolated from zooplankton associated bacteria.

2. BIOLOGICAL EXTRACTION OF CHITIN:

2.1. PREPARATION OF FISH WASTE:

Prawns, crab, fish scales, green mussel shell, egg shell & squids and cuttle fish bone has washed properly and dried at 60^0C for overnight and make a powder by using motor and pestle



Prawns shell Prawns shell powder Crab shell Crab shell powder

Fish scales

Fish scales powder

Squids bone

Squids bone powder

Green mussel shell

Green mussel shell powder

Cuttle fish bone

Cuttle fish bone powder

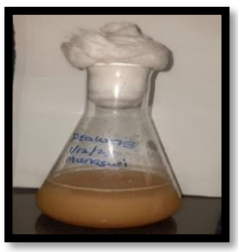
Egg shell

Egg shell powder

Figure 4.13: crustacean shell waste & egg shell waste and its respective powder

2.2. EXTRACTION PROCESS:

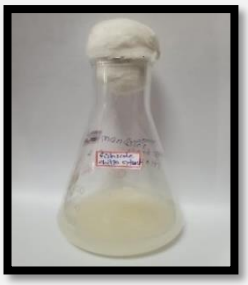
Control for Prawns shell



Prawns shell after inoculation

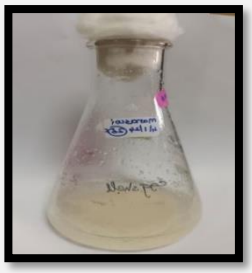
Control for Crab shell

Crab shell after inoculation



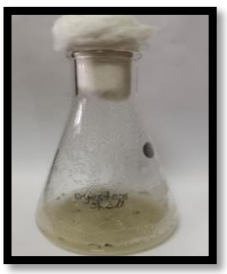
Control for Fish scales

Fish scales after inoculation



Control for Eggshell

Eggshell after inoculation



Control for Green mussel shell

Green mussel shell after inoculation

Control for Cuttle fish bone

Cuttle fish bone after inoculation

Control for Squid shells

Squid shells after inoculation

Figure 4.14: Control for each crustacean shell & eggshell waste and its respective after inoculation for chitin extraction

PROTEIN ESTIMATION USING FOLIN CIOCALTEU METHOD:

STANDARD CURVE FOR PROTEIN ESTIMATION:

Table 4.2: standard for Protein estimation by Folin Ciocalteu method

Working range	Volume stock	Volume diluent	Reagent A ml	Reagent B ml	OD at 660nm
50	0.17	0.83	5	0.5	0.127
100	0.33	0.67	5	0.5	0.210
150	0.5	0.5	5	0.5	0.291
200	0.67	0.33	5	0.5	0.380
250	0.87	0.17	5	0.5	0.461
300	1	0	5	0.5	0.573

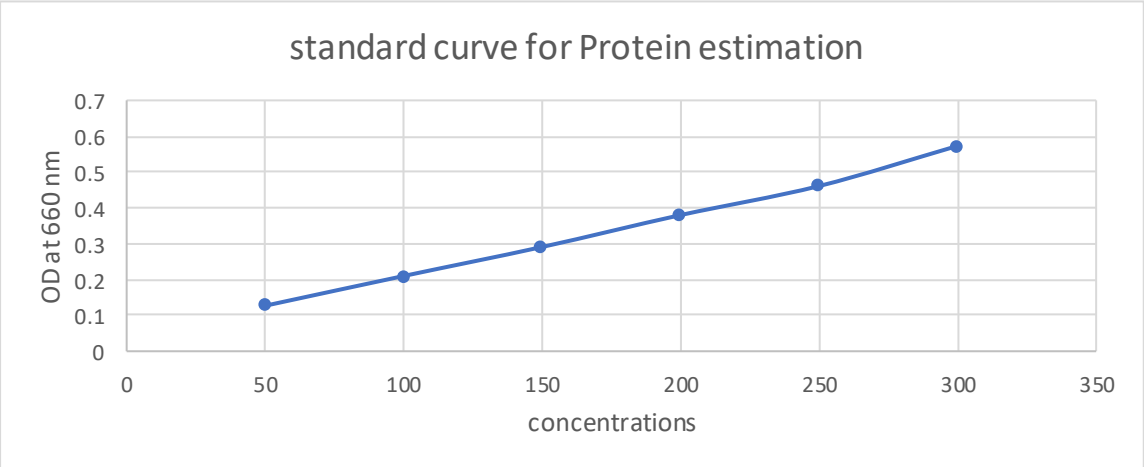


Figure 4.15: Standard curve for protein estimation

PROTEIN ESTIMATION OF FERMENTATION MEDIA:

Table 4.3: Protein estimation by Folin Ciocalteu method for fermentation media to check deproteinization

Source/days	OD at 660 nm							
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 9
Crab	3	2.899	2.664	2.345	2.169	1.921	1.672	1.172
Fish	3	2.925	2.789	2.458	2.154	1.998	1.763	1.468
Prawns	3	2.731	2.449	2.389	2.226	1.879	1.623	1.123
Squids	3	2.908	2.789	2.389	2.145	2.021	1.863	1.363
Green mussels	3	2.916	2.893	2.649	2.415	2.149	1.961	1.587
Eggshell	3	2.892	2.786	2.575	2.351	2.208	1.899	1.379
Cuttle fish bone	3	2.85	2.765	2.611	2.389	2.111	1.738	1.271

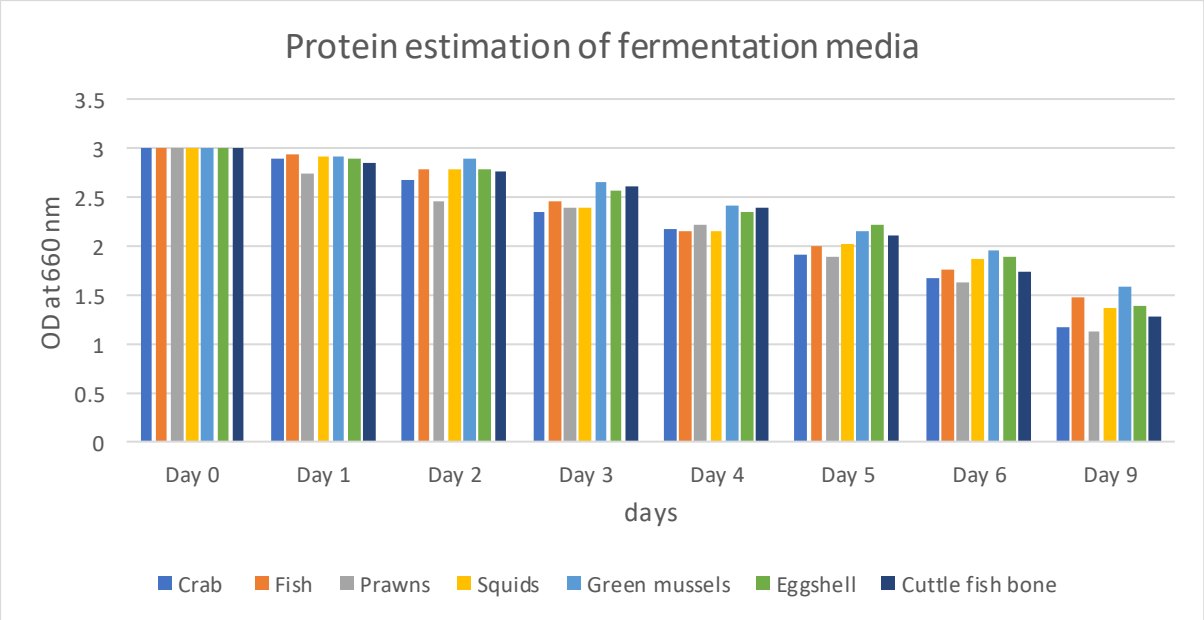


Figure 4.16: Graph for protein estimation of fermentation media to check deproteinization

After deproteinization, the absorbance at 650 nm should decrease. This is because deproteinization removes proteins from the sample, which can contribute to absorbance at that wavelength.

a. EXTRACTED CHITIN:

Prawns – KG3-NF-6T-4Z

Prawns – KG3-NF-6T-11Z

Prawns –KG3-NF-13T-26Z

Crab – KG3-NF-6T-4Z

Crab – KG3-NF-6T-11Z

Crab – KG3-NF-13T-26Z

Fish scale – KG3-NF-6T-4Z

Fish scale – KG3-NF-6T-11Z

Fish scale – KG3-NF-13T-26Z

Green mussels–KG3-NF-6T-4Z

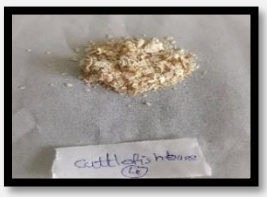
Green mussels–KG3-NF-6T-11Z

Green mussels – KG3-NF 13T-26Z

Egg shell – KG3-NF-6T-4Z

Egg shell – KG3-NF-6T-11Z

Egg shell – KG3-NF-13T-26Z



Cuttle fish bone – KG3-NF-13T-26Z

Squids – KG3-NF-6T-4Z

Squids – KG3-NF-6T-11Z

Figure 4.17: extracted chitin from prawns, crab, green mussels' shells, fish scale, squids & cuttle fish bone waste etc.

b. TABLE OF EXTRACTED CHITIN:

Table 4.4: Weight of extracted chitin from prawns, crab, green mussel shells, fish scale, squids & cuttle fish bone waste etc.

Sample used for extraction	Which isolate has used	Weight of extracted chitin	%
Prawns shell	KG3-NF-6T-4Z KG3-NF-6T-11Z KG3-NF-13T-26Z	1.9049 1.6491 1.63	19% 16.4% 16.3%
Crab shell	KG3-NF-6T-4Z KG3-NF-6T-11Z KG3-NF-13T-26Z	1.8617 1.6265 1.753	18.6% 16.2% 17.5%
Fish scales	KG3-NF-6T-4Z KG3-NF-6T-11Z KG3-NF-13T-26Z	3.401 3.879 3.365	34% 38.7% 33.6%
Green mussel shells	KG3-NF-6T-4Z KG3-NF-6T-11Z	2.2305 1.6708	22.3% 16.7%

	KG3-NF-13T-26Z	1.6870	16.8%
Egg shells	KG3-NF-6T-4Z KG3-NF-6T-11Z KG3-NF-13T-26Z	2.4453 2.172 1.831	24.4% 21.7% 18.3%
Squids bone	KG3-NF-6T-4Z KG3-NF-6T-11Z	3.0012 3.1301	30% 31.3%
Cuttle fish bone	KG3-NF-6T-4Z	1.5580	15.5%

3. EXTRACTION OF CHITOSAN FROM EXTRACTED CHITIN:

1. SCREENING FOR CHITIN DEGREDATIVE ACTIVITY:

According to prior studies by Alexander in 1985, it was assumed that since the above 2 isolates are strong chitin degraders, they would also generate the enzyme chitin deacetylase to liberate chitosan. As a result, the diagnostic strip test was used to screen these isolates for chitin deacetylase activity to determine if the enzyme, which is thought to be unique in and of itself, could convert p- nitroacetanilide by enzyme.

Figure 4.18: Deacetylase activity for all 50 isolates, KG3-NF-RT-13Z & KG3-NF-6T-15Z which is isolated from zooplankton associated bacteria

2. TRANSFORMATION OF CHITIN TO CHITOSAN BY ISOLATES:

Chitosan production: the findings of this investigation, which involved cultivating the two bacterial isolates, KG3-NF-RT-13Z & KG3-NF-6T-15Z, on a chitin-containing production medium, are reported.

DNSA: The percentage of glucosamine monomer residues in chitin is known as the deacetylation degree. The most common technique for converting chitin into chitosan is alkali deacetylation, which may also be done enzymatically. The creation of D-glucosamine units, which have a free amine group and break part of the polymer's N-acetyl linkages during deacetylation, increases the polymer's solubility in aqueous solutions (Chen & Tsaih, 1998).

Table 4.5: DNSA for all chitosan extracted media for each crustacean and eggshell when it converting extracted chitin to chitosan

Conc. Of Glucose (mg/ml)	Vol. of stock (ml)	Vol of diluent (ml)	Total volume (ml)	DNSA		Absorbance at 540 nm
0.2	0.2	0.8	1	1		0.1192
0.4	0.4	0.6	1	1		0.2148
0.6	0.6	0.4	1	1		0.3785
0.8	0.8	0.2	1	1		0.4341
1	1	0	1	1		0.5913
Prawns- KG3-NF-13T-26Z - KG3-NF-RT-13Z	1	0	1	1		2.736

Prawns- KG3-NF-13T-26Z - KG3-NF-6T-15Z	1	0	1	1		0.567
Crab- KG3-NF-13T-26Z - KG3-NF-RT-13Z	1	0	1	1	keep in boiling water bath for 5-8 min	2.591
Crab- KG3-NF-13T-26Z - KG3-NF-6T-15Z	1	0	1	1		2.435
Fish- KG3-NF-13T-26Z - KG3-NF-RT-13Z	1	0	1	1		2.769
Fish- KG3-NF-13T-26Z - KG3-NF-6T-15Z	1	0	1	1		2.673
Oyster- KG3-NF-6T-11Z - KG3-NF-6T-15Z	1	0	1	1		2.553
Egg shell- KG3-NF-13T-26Z - KG3-NF-RT-13Z	1	0	1	1		2.643
Squid bones- KG3-NF-6T-4Z - KG3-NF-RT-13Z	1	0	1	1		2.642
Cuttle fish- KG3-NF-6T-4Z - KG3-NF-RT-13Z	1	0	1	1		2.516

Without inoculation of culture (Control):

Prawns shell:1.040 Crabs hells: 1.085 Fish scale: 1.008 Oyster shells:1.123

Egg shells:1.257 Squids bone:1.201 Cuttle fish bone:0.978

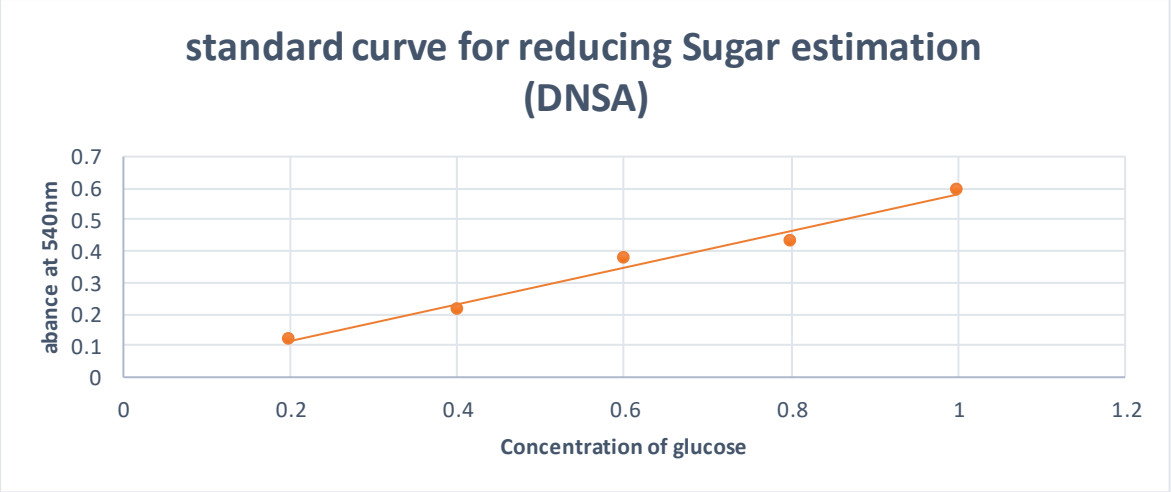


Figure 4.19: standard curve for reducing sugar estimation (DNSA)

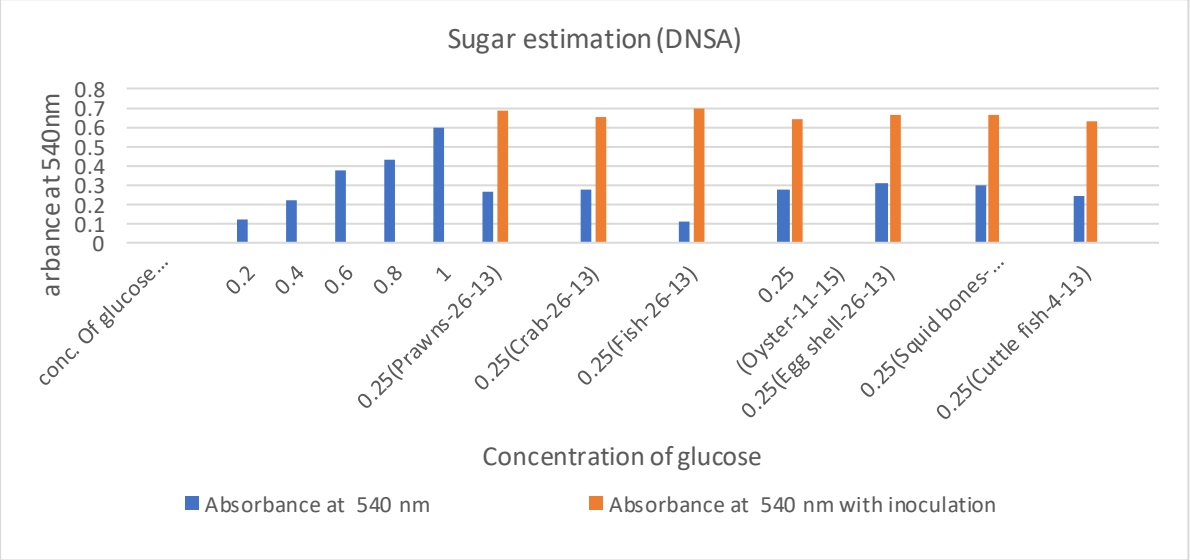


Figure 4.20: Graph for reducing sugar estimation in fermentation media (DNSA)

Then DNSA were performed to check while chitin is converting into chitosan or not, by calorimetric test. When chitin is converting into chitosan it releases glucose into the fermentation media. On that basis of increase in absorbance at 660nm, we can conclude that chitin is converting into the chitosan.

3. RECOVERY OF CHITOSAN:

When 1 N NaOH was added to it, white precipitate formed, indicating the presence of chitosan in the mixture.

Figure 4.21: Recovery step of extracted chitosan from prawns, crab, green mussel shells, fish scale, squids & cuttle fish bone etc

4. QUANTITATIVE ESTIMATION OF ISOLATES:

After the designated incubation period, the fermented broth was analyzed for the presence of chitosan. The findings show that isolates 13 and 15 release chitosan from raw chitin. The reaction that resulted in a dark purple tint indicated that the precipitate was, in fact, chitosan.

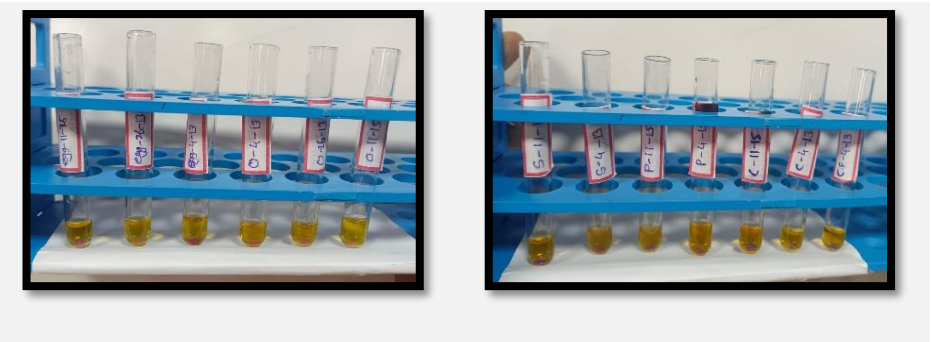


Figure 4.22: Qualitative estimation of extracted chitosan from prawns, crab, green mussel shells, fish scale, squids & cuttle fish bone etc.

Eventually all samples which are extracted have some quality of chitosan. The reaction that resulted in a dark purple tint indicated that the precipitate was, in fact, chitosan so, both KG3-NF-RT-13Z & KG3-NF-6T-15Z have efficiency of chitosan extraction. But, Prawns shell – KG3-NF-13T-26Z – KG3-NF-RT-13Z, Prawns shell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Crab shell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Crab shell – KG3-NF-6T-11Z – KG3-NF-6T-15Z, Crab shell – KG3-NF-13T-26Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-6T-11Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-13T-26Z – KG3-NF-6T-15Z, Eggshell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Eggshell – KG3-NF-13T-26Z – KG3-NF-RT-13Z,Green Mussel shell – KG3-NF-13T-26Z – KG3-NF-RT-13, Squids – KG3-NF-6T-11Z – KG3-NF-6T-15Z, Cuttle fish – KG3-NF-6T-4Z – KG3-NF-RT-13Z shows good quality of chitosan that means KG3-NF-RT-13Z bacteria isolate have good efficiency of chitosan extraction than KG3-NF-6T-15Z.

5. QUANTITATVIE ESTIMATION OF CHITOSAN:

Determining the yield became crucial once it was established that the isolates release chitosan. For this, a gravimetric approach was used.

P – KG3-NF-6T-4Z – KG3-NF-RT-13Z

P – KG3-NF-6T-11Z – KG3-NF-6T-15Z

P – KG3-NF-13T-26Z – KG3-NF-RT-13Z

P – KG3-NF-13T-26Z – KG3-NF-6T-15Z

C – KG3-NF-6T-4Z – KG3-NF-RT-13Z

C – KG3-NF-6T-11Z – KG3-NF-6T-15Z

C – KG3-NF-13T-26Z – KG3-NF-RT-13Z

C – KG3-NF-13T-26Z – KG3-NF-6T-15Z

F – KG3-NF-6T-4Z – KG3-NF-RT-13Z

F – KG3-NF-13T-26Z – KG3-NF-6T-15Z

F – KG3-NF-6T-4Z – KG3-NF-6T-15Z

F – KG3-NF-6T-11Z – KG3-NF-6T-15Z

GM – KG3-NF-6T-4Z – KG3-NF-RT-13Z

GM– KG3-NF-6T-11Z – KG3-NF-6T-15Z

GM – KG3-NF-13T-26Z – KG3-NF-RT-13Z

E – KG3-NF-6T-4Z – KG3-NF-RT-13Z

E – KG3-NF-6T-11Z – KG3-NF-6T-15Z

E – KG3-NF-13T-26Z – KG3-NF-RT-13Z

Squids – KG3-NF-6T-4Z – KG3-NF-RT-13Z

Squids – KG3-NF-6T-11Z – KG3-NF-6T-15Z

Cuttle fish – KG3-NF-6T-4Z – KG3-NF-RT-13Z

Figure 4.23: extracted chitosan from prawns, crab, green mussels' shells, fish scale, squids & cuttle fish bone waste etc.

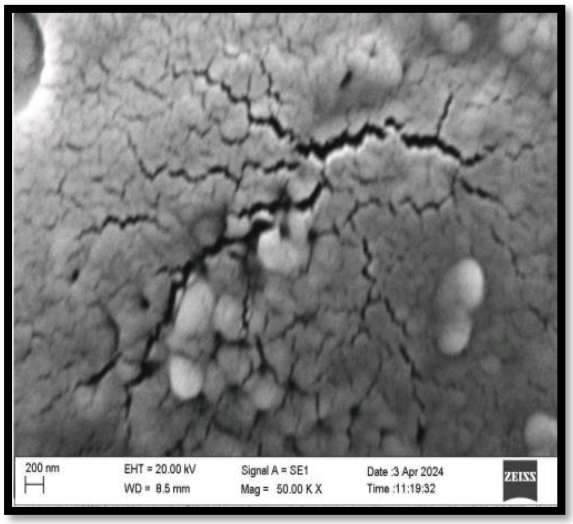
6. TABLE FOR EXTRACTED CHITOSAN

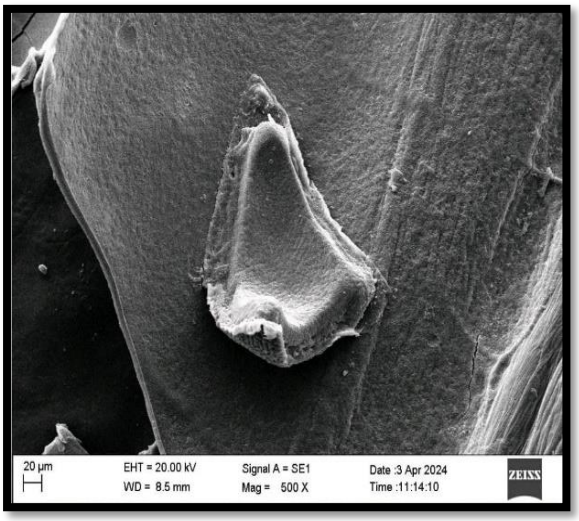
Table 4.6: Amount of chitosan produced & its percentage of extracted chitosan from prawns(P), crab(C), green mussels' shells (GM), fish scale(F), squids(S) & cuttle fish bone (CF) waste etc.

Isolates no.	Amount of chitosan produced in g/L	%
P – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.082	8%
P – KG3-NF-6T-11Z – KG3-NF-6T-15Z	0.0858	8.58%
P – KG3-NF-13T-26Z – KG3-NF-RT-13Z	0.0848	8.48%
P – KG3-NF-13T-26Z – KG3-NF-6T-15Z	0.1023	10.23%
C – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.1164	11.64%
C – KG3-NF-6T-11Z – KG3-NF-6T-15Z	0.0944	9.44%
C – KG3-NF-13T-26Z – KG3-NF-RT-13Z	0.0324	3.24%
C – KG3-NF-13T-26Z – KG3-NF-6T-15Z	0.032	3.2%
F – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.0846	8.46%
F – KG3-NF-6T-4Z – KG3-NF-6T-15Z	0.0899	8.99%
F – KG3-NF-6T-11Z – KG3-NF-RT-13Z	0.0501	5.01%
F – KG3-NF-6T-11Z – KG3-NF-6T-15Z	0.1257	12.57%
F – KG3-NF-13T-26Z – KG3-NF-RT-13Z	0.1233	12.33%
F – KG3-NF-13T-26Z – KG3-NF-6T-15Z	0.1633	16.33%

G M– KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.0999	9.99%
G M– KG3-NF-6T-11Z – KG3-NF-6T-15Z	0.1003	10.03%
G M– KG3-NF-13T-26Z – KG3-NF-RT-13Z	0.1049	10.49%
E – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.0585	5.85%
E – KG3-NF-6T-11Z – KG3-NF-6T-15Z	0.0652	6.52%
E – KG3-NF-13T-26Z – KG3-NF-RT-13Z	0.0832	8.32%
S – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.0735	7.35%
S – KG3-NF-6T-11Z - KG3-NF-6T-15Z	0.0536	5.36%
C.F – KG3-NF-6T-4Z – KG3-NF-RT-13Z	0.0935	9.35%

Scanning Electron Microscopy (SEM) analysis:





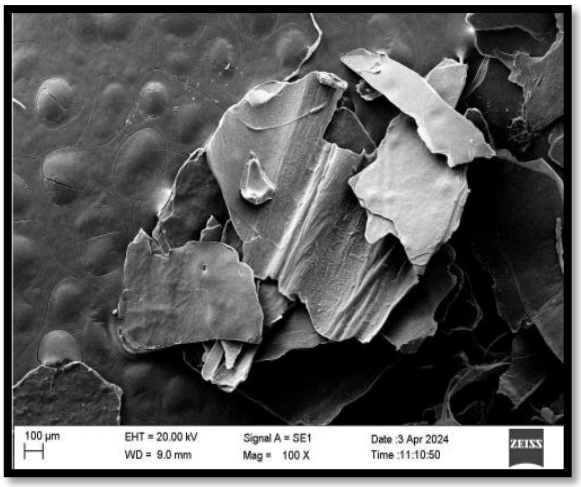


Figure 4.24: scanning electron microscopy of prawn's chitin

Result and observation: Alpha chitin often appears smoother and more homogeneous, Alpha chitin typically has a more ordered and compact crystal structure, Alpha chitin Fiber may be more tightly packed and aligned.

S2 fish chitin:

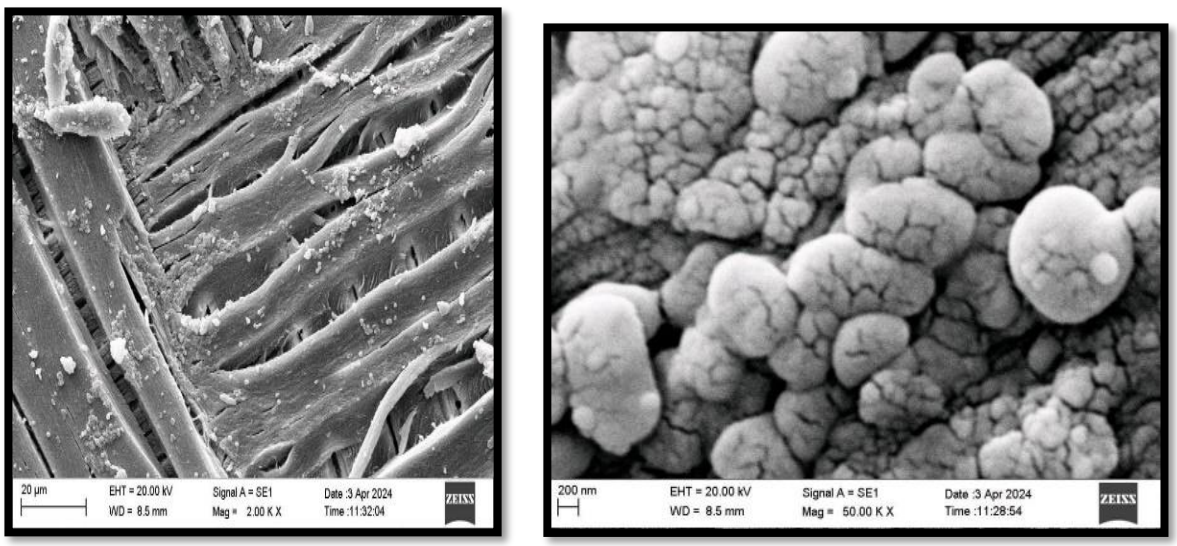


Figure 4.25: scanning electron microscopy of fish scale chitin

Results: The morphology of the chitin sample was studied using a scanning electron microscope. SEM images with different magnifications and different area of the chitin sample are present. It was observed that the biopolymer has porous and fibril structure.

S3 squid's chitin

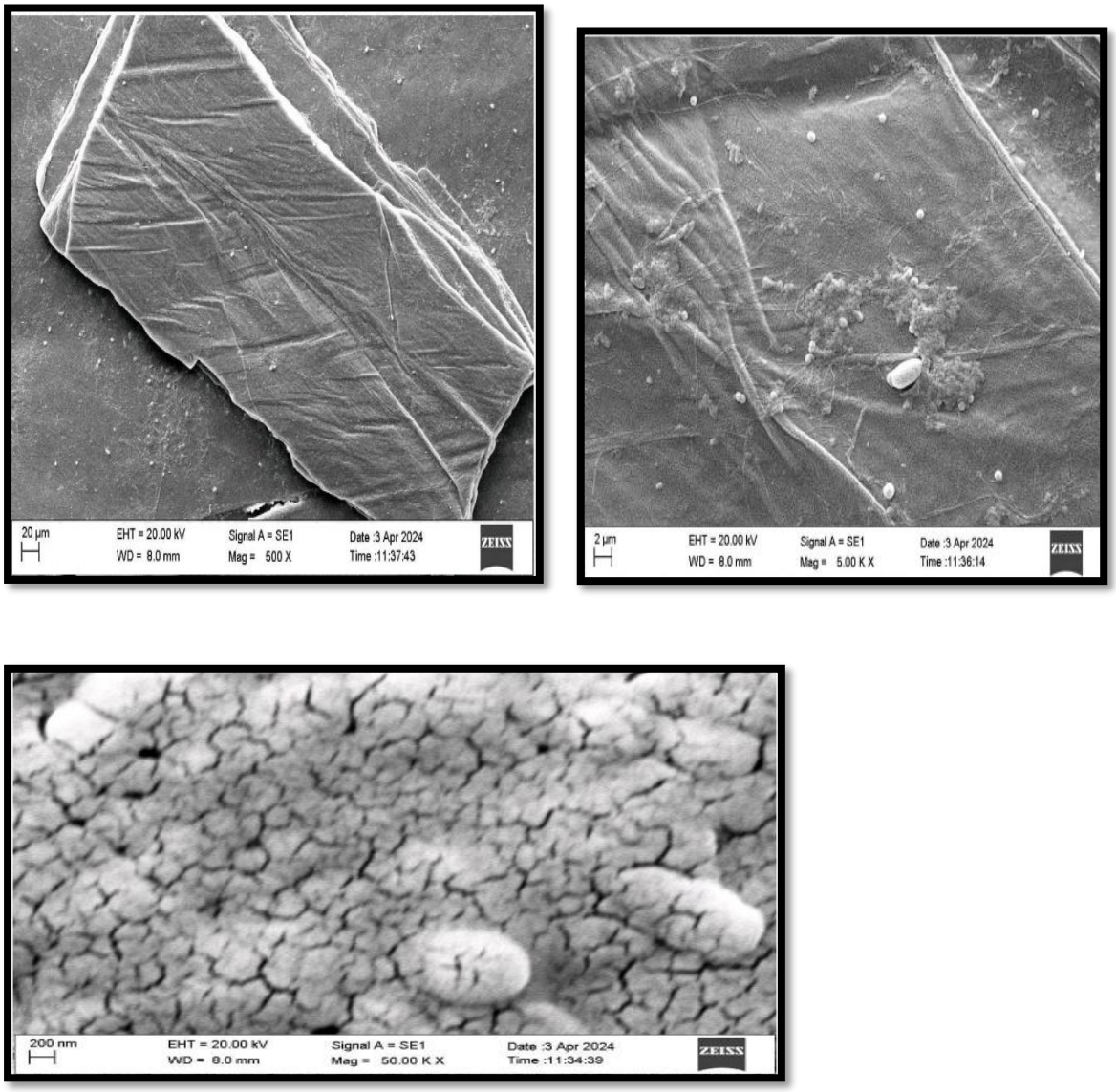


Figure 4.26: scanning electron microscopy of squid's chitin

Result and observation: Beta chitin may display a rougher or more irregular surface texture, which may appear less organized, fibres may exhibit a more random or disorganized

arrangement, may have slightly different microfibril diameters, which can be observed under high-resolution SEM imaging than Alpha chitin.

S4 prawn chitosan

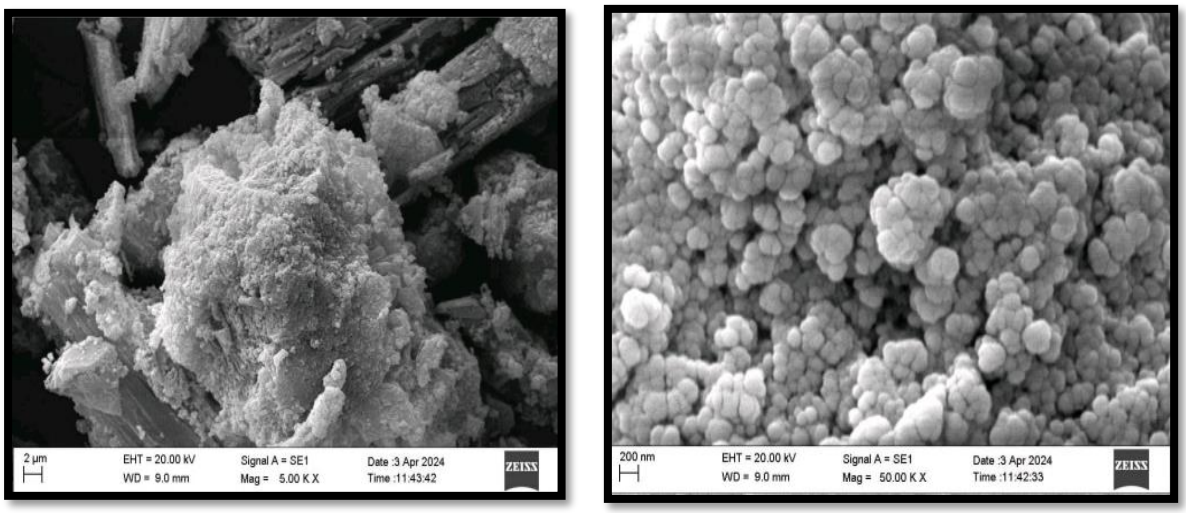


Figure 4.27: Scanning Electron Microscopy (SEM) analysis of prawn's chitosan typically reveals its fibrous structure, characterized by interconnected networks of fibers with varying diameters and surface roughness. Chitosan appears as a porous material under SEM.

S5 Fish Chitosan

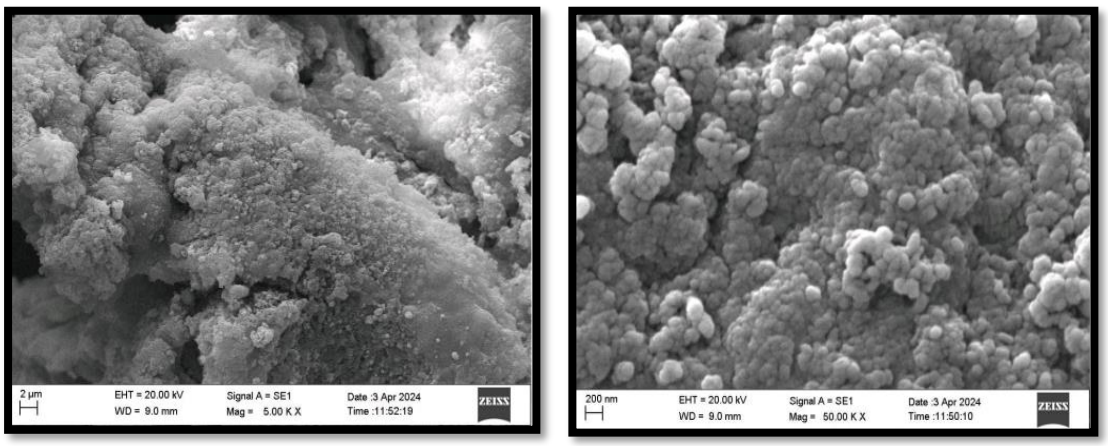


Figure 4.28: scanning electron microscopy of fish scale chitosan

Scanning Electron Microscopy (SEM) analysis of chitosan typically reveals its fibrous structure, characterized by interconnected networks of fibers with varying diameters and surface roughness. Chitosan appears as a porous material under SEM, with the morphology influenced by factors such as molecular weight, degree of deacetylation, and processing conditions.

S6 Squids Chitosan

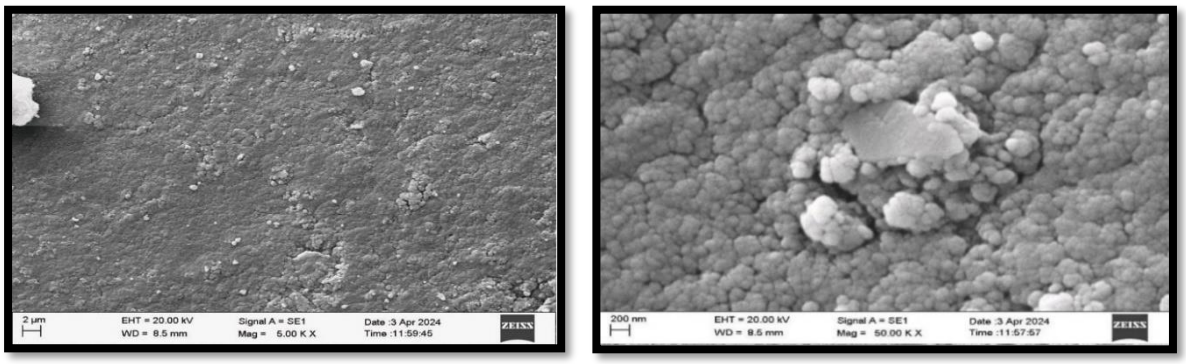


Figure 4.29: scanning electron microscopy of squid's chitosan.

Scanning Electron Microscopy (SEM) analysis of chitosan typically reveals its fibrous structure, characterized by interconnected networks of fibers with varying diameters and surface roughness. Chitosan appears as a porous material under SEM, with the morphology influenced by factors such as molecular weight, degree of deacetylation, and processing conditions

COMPARISION BETWEEN VARIOUS STUDIES AND RESULTS

Table 4.7: comparison between study of result and conclusion which is done before (literature review) & present result and conclusion (my present conclusion)

Sr. No	Chitin extracted %	Chitosan extracted %	source	Method	Organism used / source	Reference
1	5%	1.5%	Shrimp	Biological method	*Bacillus spp.* / seaweed associated bacteria.	Samant et al 2011.
2	11.73%.	77.78%	blue crab (Callinectes sapidus)	Chemical method	Acid alkaline treatment	Bölgen et al., 2016
3	33.02%	-	squid pens *Doryteuthis sibogae*,	Chemical method	two-step alkaline treatment	(Barwin et al., 2011).
4	28%	-	fish scales	Biological method	*Stenotrophomonas koreensis* enzymes,	(Suresh et al., 2023)
5	16% to 17%	11%	resting eggs	Biological method	*Ceriodaphnia quadrangula*,	(Kaya et al., 2014)
6	- -	(11.60%) (6.51%)	Green mussel's crab	Chemical method	high concentration of an alkaline solution	Pratama et al., 2023
7	19%, 16.4%, 16.3%	8% to 10.23%	Prawns shell	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024

8	18.6% 16.2% 17.5%	3.2 To 11.64%	Crab shell	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024
9	22.3% 16.7% 16.8%	5.01 To 16.3%	Fish scales	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024
10	22.3% 16.7% 16.8%	9.99% To 10.49%	Green mussel's	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024
11	24.4% 21.7% 18.3%	6.52% To 8.32%	Egg shells	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024
12	15.5%	5.36% To 7.35%	Cuttle fish bone	Biological method	Protease producing, Chitinase producing, And glucose fermentative	Present study 2024

					zooplankton associated bacteria	
13	30% 31.3%	9.35%	Squids bone	Biological method	Protease producing, Chitinase producing, And glucose fermentative zooplankton associated bacteria	Present study 2024

Chapter 5

SUMMARY

SUMMARY

In this study, zooplankton samples were used for the isolation of zooplankton associated bacteria. Fifty bacterial cultures were isolated from zooplankton sample. Among all the isolates eight isolates showed good proteolytic activity hence three isolates were used for extraction of chitin from prawns, crab, green mussel shell, eggshells, squids, cuttle fish bone & fish scale waste, and protein estimation has done to check deproteinization by folin-ciocalteau method i.e. decrease in absorbance. Chitin was extracted from prawns, crab, fish scale, eggshell, green mussel shell, squid's pen, fish bone was extracted using KG3-NF-6T-4Z, KG3-NF-6T-11Z & KG3-NF-13T-26Z bacterial isolate. Whare as chitin was extracted from 7 different sources- prawn shells, crab shells, fish scales, eggshells, green mussel shells, squids, cuttle fish bone. And for chitosan extraction all fifty cultures were used for chitin degradative activity in which only two bacterial isolates KG3-NF-RT-13Z & KG3-NF-6T-15Z showing presence of deacetylase in respective bacterial isolate. Before the recovery of chitosan DNSA assay has done to check whether the D-glucosamine units were released into the media . After extraction of chitosan precipitation will indicates the presence of chitosan. Then quantitative estimations have done by taking weight of chitosan. After that qualitative estimation of has done by checking colour change of precipitate white to purple after adding 2-4 drops of iodine solution & 2-4 drops of 1% H_2SO_4.

In SEM analysis: Alpha chitin often appears smoother and more homogeneous, has a more ordered and compact crystal structure, more tightly packed and aligned, while beta chitin may display a rougher or more irregular surface texture, which may appear less organized, fibers may exhibit a more random or disorganized arrangement slightly different microfibril diameters, which can be observed under high-resolution SEM image And for chitosan typically fibrous structure, characterized by interconnected networks of fibers with varying diameters and surface roughness. Chitosan appears as a porous material under SEM

Chapter 6

CONCLUSION

CONCLUSION

Biological extraction method for chitin and chitosan is environment friendly, mild processing condition, high purity, resource efficiency, biocompatibility, versatility, cost effective and not time-consuming method. Chitin was extracted from 7 different sources, i.e.-prawn shells, crab shells, fish scales, eggshells, green mussel shells, squids, cuttle fish bone etc to compare which source will give you the more production of chitosan. Eight zooplankton associated bacterial isolates KG3-NF-6T-1Z, KG3-NF-6T-4Z, KG3-NF-13T-6Z, KG3-NF-13T-8Z, KG3-NF-6T-11Z, KG3-NF-RT-14Z, KG3-NF-6T-15Z, KG3-NF-RT-21Z, KG3-NF-13T-26Z, out of 50 showed good proteolytic activity for effective extraction of chitin. We have used KG3-NF-6T-4Z, KG3-NF-6T-11Z, KG3-NF-13T-26Z for extraction process, among these KG3-NF-6T-4Z, KG3-NF-6T-11Z, gives good production rate than KG3-NF-13T-26Z. The same can be confirm using Folin-Ciocalteu method to check deproteinization by taking absorbance at 540nm, on that basis a decrease in absorbance was seen, through which we can conclude that deproteinization is taking place.

Due to many applications & solubilities of chitosan in dilute acid, Chitin was deacetylated to chitosan by using deacetylase producing bacteria/ microorganisms having chitin degradative activity. Two bacterial isolates KG3-NF-RT-13Z & KG3-NF-6T-15Z have shown deacetylase producing / chitinase activity. Eventually all chitosan samples which are extracted have some quality of chitosan. The reaction that resulted in a dark purple precipitate indicated that precipitate was, in fact, chitosan, so both KG3-NF-RT-13Z & KG3-NF-6T-15Z have efficiency of chitosan extraction.

Then DNSA was performed to check whether chitin is converting into chitosan or not, using calorimetric test. When chitin is converting into chitosan it releases glucose into the fermentation media. On that basis of increase in absorbance at 660nm, we can conclude that

chitin is converting into chitosan. In Recovery of chitosan step, we get white precipitate, on that basis we can conclude that chitosan is recovered. In qualitative estimation we get colour changes of precipitate to purple so we can conclude that chitosan is of good quality. But, Prawns shell – KG3-NF-13T-26Z – KG3-NF-RT-13Z, Prawns shell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Crab shell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Crab shell – KG3-NF-6T-11Z – KG3-NF-6T-15Z, Crab shell – KG3-NF-13T-26Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-6T-11Z – KG3-NF-RT-13Z, Fish scales – KG3-NF-13T-26Z – KG3-NF-6T-15Z, Eggshell – KG3-NF-6T-4Z – KG3-NF-RT-13Z, Eggshell – KG3-NF-13T-26Z – KG3-NF-RT-13Z,Green Mussel shell – KG3-NF-13T-26Z – KG3-NF-RT-13, Squids – KG3-NF-6T-11Z – KG3-NF-6T-15Z, Cuttle fish – KG3-NF-6T-4Z – KG3-NF-RT-13Z shows good quality of chitosan that means KG3-NF-RT-13Z bacteria isolate have good efficiency of chitosan extraction than KG3-NF-6T-15Z. And on basis of quantitative estimation of chitosan KG3-NF-6T-15Z have shown good efficiency of extraction comparatively more amount of chitosan than KG3-NF-RT-13Z.

So, these isolated zooplankton associated bacteria have good efficiency for production of protease, chitinase enzyme, and calcium carbonate solubilization. Hence are efficient for biological extraction of chitin and chitosan. Bacteria associated with zooplankton help degrade this chitin, breaking it down into simpler compounds. This process is crucial for nutrient recycling and maintaining ecosystem health. Through enzymatic processes, these bacteria break down chitin into chitosan, a valuable biopolymer with various industrial applications. Zooplankton-associated bacteria aid in the decomposition of organic matter, releasing nutrients such as carbon, nitrogen, and phosphorus back into water. This recycling of nutrients is essential for the growth of other marine-organisms in the ecosystem. Additionally, these bacteria contribute to nutrient recycling in aquatic ecosystems by decomposing organic matter, thus playing a vital role in maintaining ecosystem balance and health.

The current work has a fair chance of reducing the trash from the fish companies entering the environment, creating a safe habitat for life and being an environmentally friendly, sustainable process. Chitosan derived from fish waste is widely used in many industries.

FUTURE PROSPECT

1. To look for the more significant industrial use of chitin and chitosan like nano particles, biodegradability, antimicrobial activity, solvent in chemical reactions, antibiotics, antioxidant, that has potential use in a wide range of industries, including agriculture, food and textile manufacturing, medicine, and cosmetics.

Chapter 8

Bibliography

Bibliography

1. Arunima, S., & Verulkar, S. (2022). Comparative analysis of different protein estimation methods. *The Pharma Innovation Journal*, *11*(4), 2091-2095.
2. Battampara, P., Sathish, T. N., Reddy, R., Guna, V., Nagananda, G. S., Reddy, N., ... & Radhakrishna, P. G. (2020). Properties of chitin and chitosan extracted from silkworm pupae and egg shells. *International Journal of Biological Macromolecules*, *161*, 1296-1304.
3. Bölgen, N., Demir, D., Öfkeli, F., & Ceylan, S. (2016). Extraction and characterization of chitin and chitosan from blue crab and synthesis of chitosan cryogel scaffolds. *Journal of the Turkish Chemical Society Section A: Chemistry*, *3*(3), 131-144.
4. Crini, G., Guibal, E., Morcellet, M., Torri, G., & Badot, P. (2009). Chitin and Chitosan. Preparation properties and main applications Chitin and Chitosan In the biopolymer application. Besançon.
5. De Corte, D., Srivastava, A., Koski, M., Garcia, J. A. L., Takaki, Y., Yokokawa, T., ... & Herndl, G. J. (2018). Metagenomic insights into zooplankton-associated bacterial communities. *Environmental microbiology*, *20*(2), 492-505.
6. Dave, U., Somanader, E., Baharlouei, P., Pham, L., & Rahman, M. A. (2021). Applications of chitin in medical, environmental, and agricultural industries. Journal of Marine Science and Engineering, 9(11), 1173.
7. Dahmane, E. M., Taourirte, M., Eladlani, N., & Rhazi, M. (2014). Extraction and characterization of chitin and chitosan from Parapenaeus longirostris from Moroccan local sources. *International Journal of Polymer Analysis and Characterization*, *19*(4), 342-351.

8. Ferrer, J., Paez, G., Marmol, Z., Ramones, E., Garcia, H., & Forster, C. F. (1996). Acid hydrolysis of shrimp-shell wastes and the production of single cell protein from the hydrolysate. *Bioresource Technology*, *57*(1), 55-60.
9. Fernandez-Kim, S.O. (2004) 'Physicochemical and Functional Properties of Crawfish Chitosan as Affected by Different Processing Protocols'.
10. G. Y. Zhou, Y. H. He, and H. Y. Zhang, "Screening and 16S rRNA analysis of the bacteria of producing chitin deacetylase," in Proceedings of the 4th International Conference on Bioinformatics and Biomedical Engineering (iCBBE '10), June 2010.
11. Gimeno, M., Ramírez-Hernández, J. Y., Mártinez-Ibarra, C., Pacheco, N., García-Arrazola, R., Bárzana, E., & Shirai, K. (2007). One-solvent extraction of astaxanthin from lactic acid fermented shrimp wastes. *Journal of agricultural and food chemistry*, *55*(25), 10345-10350.
12. Jampafuang, Y., Tongta, A., & Waiprib, Y. (2019). Impact of crystalline structural differences between α-and β-chitosan on their nanoparticle formation via ionic gelation and superoxide radical scavenging activities. *Polymers*, *11*(12), 2010.
13. Jung, W. J., Jo, G. H., Kuk, J. H., Kim, Y. J., Oh, K. T., & Park, R. D. (2007). Production of chitin from red crab shell waste by successive fermentation with Lactobacillus paracasei KCTC-3074 and Serratia marcescens FS-3. *Carbohydrate polymers*, *68*(4), 746-750.
14. Kalut, S. A. (2008). *Enhancement of degree of deacetylation of chitin in chitosan production* (Doctoral dissertation, UMP).
15. Kaur, K., Dattajirao, V., Shrivastava, V., & Bhardwaj, U. (2012). Isolation and characterization of chitosan-producing bacteria from beaches of Chennai, India. *Enzyme research*, *2012*. doi:10.1155/2012/421683
16. Kaya, M., Baran, T., Saman, I., Asan Ozusaglam, M., Cakmak, Y. S., & Menteş, A. (2014). Physicochemical characterization of chitin and chitosan obtained from resting eggs of

Ceriodaphnia quadrangula (Branchiopoda: Cladocera: Daphniidae). *Journal of Crustacean Biology*, *34*(2), 283-288.

17. Khanafari, A., Marandi, R. E. Z. A., & Sanati, S. H. (2008). Recovery of chitin and chitosan from shrimp waste by chemical and microbial methods.
18. Kafetzopoulos, D., Thireos, G., Vournakis, J. N., & Bouriotis, V. (1993). The primary structure of a fungal chitin deacetylase reveals the function for two bacterial gene products. *Proceedings of the National Academy of Sciences*, *90*(17), 8005-8008.
19. Linden, J. C., Stoner, R. J., Knutson, K. W., & Gardner-Hughes, C. A. (2000). Organic disease control elicitors. *Agro food industry hi-tech*, *11*(5), 32-34.
20. Mao, X., Guo, N., Sun, J., & Xue, C. (2017). Comprehensive utilization of shrimp waste based on biotechnological methods: A review. *Journal of Cleaner Production*, *143*, 814-823.
21. Mojarrad, J. S., Nemati, M., Valizadeh, H., Ansarin, M., & Bourbour, S. (2007). Preparation of glucosamine from exoskeleton of shrimp and predicting production yield by response surface methodology. *Journal of agricultural and food chemistry*, *55*(6), 2246-2250.
22. Muzzarelli RAA. Enzymatic synthesis of chitin and chitsosan, Pergamon Press, Oxford New York, 1977; 5-17.
23. Miller, C. B., Johnson, J. K., & Heinle, D. R. (1977). Growth rules in the marine copepod genus Acartia 1, 2. *Limnology and Oceanography*, *22*(2), 326-335.
24. Mohammed Kuddus, M. K., & Saima, S. (2016). Microbial chitinase: production and potential applications. In *The handbook of microbial bioresources* (pp. 331-360). Wallingford UK: CABI.
25. Martău, G. A., Mihai, M., & Vodnar, D. C. (2019). The use of chitosan, alginate, and pectin in the biomedical and food sector—biocompatibility, bioadhesiveness, and biodegradability. *Polymers*, *11*(11), 1837.

26. McReynolds, C., Adrien, A., de Fraissinette, N. B., Olza, S., & Fernandes, S. C. (2022). Deep eutectic solvents for the extraction of β-chitin from Loligo vulgaris squid pens: a sustainable way to valorize fishery by-products. *Biomass Conversion and Biorefinery*, 1-13
27. No, H. K., & Meyers, S. P. (1995). Preparation and characterization of chitin and chitosan—a review. *Journal of aquatic food product technology*, *4*(2), 27-52.
28. Naik-Samant, S., Naik, M. M., Vaingankar, D., Mujawar, S. Y., Parab, P., & Meena, S. N. (2019). Biodegradation of seafood waste by seaweed-associated bacteria and application of seafood waste for ethanol production (Chapter 10). Elsevier.
29. Pal, J., Verma, H. O., Munka, V. K., Maurya, S. K., Roy, D., & Kumar, J. (2014). Biological method of chitin extraction from shrimp waste an eco-friendly low cost technology and its advanced application. *International Journal of Fisheries and Aquatic Studies*, *1*(6), 104-107.
30. Pratama, G., Munandar, A., Surilayani, D., Rizky, J. A., Hasanah, A. N., Haryati, S., ... & Aditia, R. P. (2023). Characteristics of crab shells and green mussel shells as potential chitosan material from Karangantu, Banten, Indonesia. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1137, No. 1, p. 012033). IOP Publishing.
31. Rinaudo, M. (2006) 'Chitin and chitosan: Properties and applications', Progress in Polymer Science, pp. 603-632.
32. Radhakrishanan VV, Vijayan MS, Sambasivan M, Jallaldhin RBS. Haemostatic potential of chitosan, Biomedicine 1991, 23-26.
33. R. S. Vadake, Biotransformation of Chitin to Chitosan, United States Patent 5739015, 1998.
34. Rajbhar, K., Dawda, H., & Mukundan, U. (2015). Quantitative spectrophotometric estimation of specific monosaccharides by DNSA method. *Journal of Biological Science (IJRDO)*, *2*(1), 112-126.
35. Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., & Lawrence, R. (2008). Aquaculture practices and potential human health risks: current knowledge and future priorities. *Environment international*, *34*(8), 1215-1226.

36. Synowiecki, J., & Al-Khatech, N. A. A. Q. (2000). The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp Crangon crangen processing discards. Food Chemistry, 68(2), 147-152. Mtps://doi.org/10.1016/50308-8146(99)00165-X

37. Shahidi, F., & Abuzaytoun, R. (2005). Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. *Advances in food and nutrition research*, *49*(4526), 49003-8.

38. Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosans. *Trends in food science & technology*, *10*(2), 37-51.

39. Srinivasan, V. R. (1998). *U.S. Patent No. 5,739,015*. Washington, DC: U.S. Patent and Trademark Office.

40. Suresh, S., Umesh, M., & Santosh, A. S. (2023). Biological extraction of chitin from fish scale waste using proteolytic bacteria Stenotrophomonas koreensis and its possible application as an active packaging material. *Biomass Conversion and Biorefinery*, 1-11.

41. Sedaghat F, Yousefzadi M, Toiserkani H, Najafpour S (2016) Chitin from Penaeus merguiensis via microbial fermentation processing and antioxidant activity. Int J Biol Macromol 82:279–283.

42. Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer*, *41*(7), 2463-2469.

43. Takeda M. In porc. First Int. Conf. chitin/chitosan. MIT. Cambridge, 355 Townshey, P.M. 1961, Nature, 191, 626.

44. Tang KW, Freund CS, Schweitzer CL (2006a) Occurrence of copepod carcasses in the lower Chesapeake Bay and their decomposition by ambient microbes. Estuar Coast Shelf Sci 68:499–508

45. Tang KW, Hutalle KML, Grossart HP (2006b) Microbial abundance, composition and enzymatic activity during decomposition of copepod carcasses. Aquat Microb Ecol 45: 219–227

46. Wang, S. L., Chang, T. J., & Liang, T. W. (2010). Conversion and degradation of shellfish wastes by Serratia sp. TKU016 fermentation for the production of enzymes and bioactive materials. *Biodegradation*, *21*, 321-333.

47. W. F. Fang, L. G. Quiang, Z. Yan, H. Y. Hao, and Z. G. Ying, "Screening and identification of A4 starin for producing chitin deacetylase," Journal of Central South University of Forestry & Technology, no. 9, 2010

48. Xu, Y., Gallert, C., & Winter, J. (2008). Chitin purification from shrimp wastes by microbial deproteination and decalcification. *Applied Microbiology and Biotechnology*, *79*, 687-697.

49. Xuemei, Z., & Hawkins, S. J. (2002). Interactions of aquaculture and waste disposal in the coastal zone. *Journal of Ocean University of Qingdao*, *1*, 8-12.

50. Younes, I., Ghorbel-Bellaaj, O., Nasri, R., Chaabouni, M., Rinaudo, M., & Nasri, M. (2012). Chitin and chitosan preparation from shrimp shells using optimized enzymatic deproteinization. *Process Biochemistry*, *47*(12), 2032-2039.

51. Yadav, M. Goswami, P. Paritcod, K., Kumur, M. Pareek, N., & Vivekanand, V. (2019) Seabed waste a source for prepanation of courierially eniployable chitin chaosan zusturiali. Brewurces and Booprocessing (1) htqsdoi.org/10.118640643-019- 0543-y

Chapter 9

APPENDIX

APPENDIX -I

(MEDIA)

1. Zobell Merine Broth:

Composition	Grams/ L
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium sulphate	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
pH(25^0C)	7.6=/-0.2

40.25 g suspended in 1000ml filter sterilized sea water. Then autoclave the media at 15 lbs pressure for 121^{0}C for 15 mins.

2. Zobell Merine Broth:

Composition	Grams/ L
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium sulphate	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
pH(25^{0}C)	7.6=/-0.2

55.25 g suspended in 1000ml filter sterilized sea water. Then autoclave the media at 15 lbs pressure for 121^{0}C for 15 mins. Well mixed and pour in sterile petri plate.

3. Sea water-based agar containing 2% skimmed milk:

Composition	Grams/ L
Sodium chloride	28.0
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Skim milk	20ml
Agar	20
Final pH (at 25^0C)	7.5=/-0.5

20 ml of skim milk sterilized separately and add 960ml of sterile media before pouring the plates.

4. Sea water-based agar containing 1% Calcium Carbonate:

Composition	Grams/ L
Sodium chloride	28.0
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Calcium carbonate	10
Agar	20

All the contain dissolve in flask & make the volume to 1000ml and sterilize by autoclaving at 15 lbs pressure for 121^{0}C for 15 mins.

5. Sea water-based agar containing 1% colloidal chitin:

Composition	Grams/ L
Sodium chloride	28.0
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Skim milk	10
Agar	20

All the contain dissolve in flask & make the volume to 1000ml and sterilize by autoclaving at 15 lbs pressure for 121^{0}C for 15 mins.

6. Sea water-based broth containing 1% glucose & 1% Prawns, fish scale, crab, oyster shell, egg shell, egg shell & squids and cuttle fish bone

Composition	Grams/ L
Sodium chloride	28.0
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50

Glucose	10
Agar	20

Then the media supplemented with 1% respective shell fish powder then all the contain mixed in conical flask and sterilize by autoclaving at 15 lbs pressure for 121^{0}C for 15 mins.

7. Sea water-based media for chitin degradative activity:

Composition	**Grams/ L**
Yeast extract	1
Ammonium sulphate	0.4
Potassium dihydrogen phosphate	0.15
pH	8.0

8. Production media for chitosan:

Composition	**Grams/ L**
Yeast extract	10
Ammonium sulphate	4
Potassium dihydrogen phosphate	1.5
Extracted Chitin	0.05
pH	8.0

Then the media supplemented with 50mg respective extracted chitin then all the contain mixed in conical flask and sterilize by autoclaving at 15 lbs pressure for 121^{0}C for 15 mins.

9. Glucose phosphate broth (MR-VP medium):

Composition	**Grams/ L**
Peptone	7

Dipotassium phosphate	5
Dextrose	5
Distilled water	100ml
pH	7.4+/-0.2

APPENDIX -II

(REAGENTS)

1. 1% Congo red solution:

Composition	**Grams**
Congo red	1
Distilled water	100

2. 1M NaCl solution:

Composition	**Grams**
NaCl	5.844
Distilled water	100

3. Reagent A for Folin-ciocalteau:

50 ml of solution-1 + 1 ml of solution-2 = 51ml

3.1. Solution 1 (protein estimation)

Composition	**Grams**
NaOH	0.1
Na_2CO_3	2

Distilled water	100

3.2. Solution 2 (protein estimation)

Composition	**Grams**
NaK	1
$CuSO_4$	0.5
Distilled water	100

Weight 1g NaK of dissolve in 100 ml of distilled water then add 0.5 g of $CuSO_4$.

4. Reagent B for Folin-ciocalteau:

Composition	**Grams**
Folin Ciocaltau reagent	5 ml
Distilled water	5 ml

5ml of chemical Folin Ciocaltau reagent+ 5 ml of distilled water =1:1 ratio

5. 2N NaOH

Composition	**Grams**
NaOH	8
Distilled water	100ml

6. 3,5 Dinitrosalicylic acid (DNSA) reagent:

Composition	**Grams**
NaOH	2
Crystalline Phenol	0.2
DNSA	0.2
Distilled water	100ml

7. Methyl Red reagent (for MR-VP test):

Composition	Grams
Methyl red	0.2
Ethanol	600ml
Distilled water	400ml

8. 0.1N NaOH:

Composition	Grams
NaOH	1
Distilled water	250

9. 2%Acetic acid:

Composition	Grams
Acetic acid	2
Distilled water	198

10.1N NaOH:

Composition	Grams
NaOH	40
Distilled water	1000

11.1% H_2SO_4:

Composition	Grams
H_2SO_4	1
Distilled water	99ml