Extraction of chitosan from crustacean waste using seaweed and bivalve associated bacteria and its application

A Dissertation for
Mid: dissertation
Number of Credits: 08

Submitted in partial fulfilment of
Master of Science in Microbiology
By

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21P042016

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Extraction of chitosan from crustacean waste using seaweed and bivalve associated bacteria and its application**" is based on the results of investigations carried out by me in the Department of Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Lata Gawade and the same has not been submitted elsewhere for the award of a 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 CERTIFICATE

This is to certify that the dissertation "**Extraction of chitosan from crustacean waste using seaweed and bivalve associated bacteria and its application**" is a bonafide work carried out by **Ms. Kunjlata Suresh Naik** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Microbiology at the School of Biological Sciences and Biotechnology, Goa University.

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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 Sciences and Biotechnology for extending necessary facilities.

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

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

My sincere thanks to Prachi, Esther, Sherley, Sharwani, Rhutuja, Simran, Nikita 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 lord almighty who kept blessings on me and took me to the path success.

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AIM AND OBJECTIVES

AIM:

To extract chitosan from crustacean waste using sea weed and bivalve associated bacteria and its application in dye adsorption and antibacterial activity.

OBJECTIVES:

1. To collect Seaweed sample and juvenile mussel sample from the intertidal region of Goa Coast
2. Isolation of seaweed and bivalve associated bacteria
3. To check the ability of seaweed and mussel associated bacteria for chitin extraction
4. Extraction of chitin using bacteria isolated from seaweed and mussel and deacetylation of Chitin to chitosan by using chemical method.
5. Applications of extracted chitosan:
    a) To adsorb dye from textile industry;
    b) To determine antibacterial activity of extracted chitosan

INTRODUCTION

1.1. CRUSTACEAN WASTE

All the coastal countries are involved in fishing and fish processing. During processing, particularly of crustaceans and shellfish, only the meat part is utilised as food and shell and head portion are discarded as the waste. Therefore fish processing industries generates approx. 6-8 million tonnes of waste annually (Marina et al., 2021;(Tarafdar & Biswas, 2013). Sea food processing industries face a major problem to dispose this waste. The waste is usually landfilled, dumped into sea or left as it is, hence it causes adverse effects on the environment as well as on the humans and other animals (Lanka & Pradesh, 2021). The seafood waste is rich in many valuable compounds which after proper processing can add to overall benefits, but the high perishability of the shrimp biomaterial can cause a major problem. Decay of the waste leads to the production of biogenic amines which are responsible for the very offensive smell leading to the real waste of the material (Kandra et al., 2012).

Figure 1: Crustacean waste a) Crab waste b) Shrimp waste

Shells of crustaceans like crabs and shrimps are the main sources of chitin, containing 20-40% protein, 20-50% calcium carbonate and 15-40% pigments and lipids (Suryawanshi et al., 2019). These wastes can be utilised to derive the industrially important compounds thus

reducing the waste in the environment. Chitin can be obtained from this waste by using deproteinisation and demineralisation processes. By further deacetylation, chitosan, a substance with wide range of applications can be produced from the chitin (Tarafdar & Biswas, 2013).

1.2 CHITIN

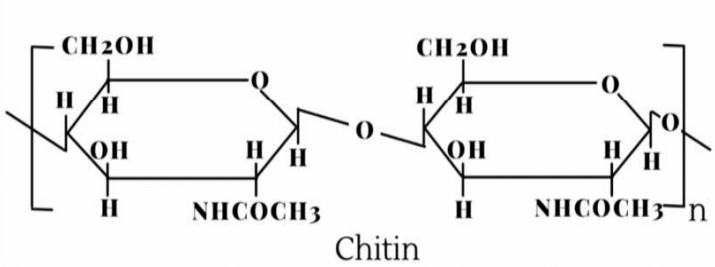


Figure 2: Structure of Chitin (Dave et al., 2021)

Chitin is a long-chain polymer of N-acetyl D-glucosamine linked by β-1-4 glycosidic bond. In abundance chitin is next to cellulose, a natural biopolymer (Pal et al., 2014). It gives strength to the exoskeleton of the insects, algal and fungal cell wall and the shells of crustaceans (Dave et al., 2021). Chitin is found in three different crystalline forms α, β and γ (Yadav et al., 2019). α chitin contains antiparallel arrangement of the polysaccharide chains allowing maximum bonds hence it is most stable form containing 80% crystallinity index. Crab and lobster tendons, shrimp shells, yeast and fungal cell wall and insect cuticles are made up of α chitin. Polymer chains of β chitin are found in parallel arrangement having crystallinity index of 70%. This form of chitin is more reactive and soluble in the solvents due to the presence of more distance between adjacent polymer chains (Tan et al., 2020). This form is generally found in squid pens (Filipe et al., 2015). γ Chitin is the combination of both α- and β type arrangement. It contains two parallel polymer chains arranged alternatively with one antiparallel (Yadav et al., 2019); Filipe et al., 2015).

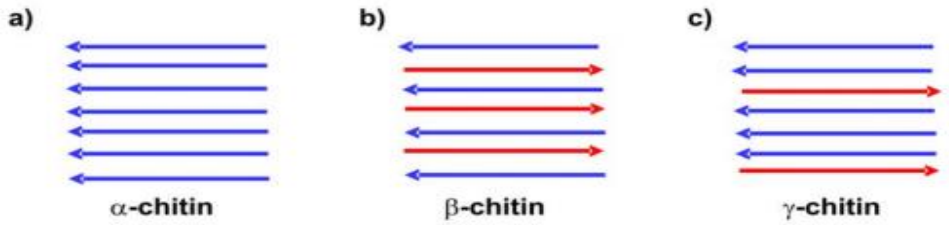


Figure 3: Three polymorphic forms of Chitin (Kumirska et al., 2011)

1.3 CHITOSAN

Chitosan is a linear polysaccharide containing D-glucosamine and N-acetyl-D-glucosamine linked by β-(1–4) glycosidic bond (Fatullayeva et al., 2022). It is produced by deacetylation of chitin by alkali treatment or by enzyme hydrolysis. The degree of deacetylation in chitosan is based on the ratio of glucosamine and N-acetyl glucosamine. Chitosan contains higher glucosamine percentage than N-acetyl glucosamine (Yadav et al., 2019).

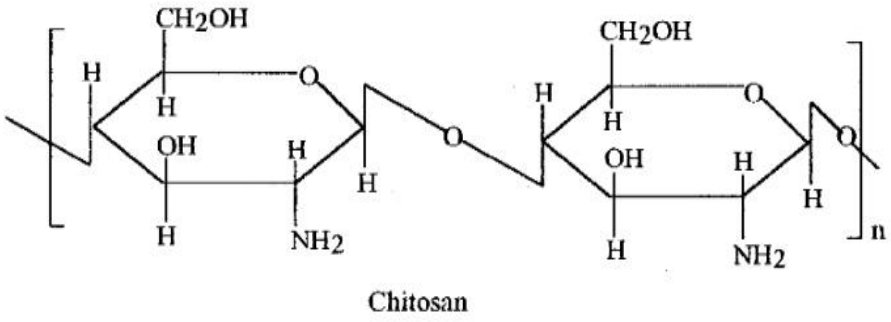


Figure 4: Structure of Chitosan (Ibram et al., 2019)

1.4 PROPERTIES OF CHITIN AND CHITOSAN

Chitin and Chitosan are the basic polysaccharides (Pal et al., 2014). Chitin contains 5-8% Nitrogen depending on the extent of deacetylation. Due to presence of amino group at C-2 position, chitosan is more versatile than chitin (Dutta et al., 2004). Due to strong intermolecular hydrogen bonds, chitin is hydrophobic and insoluble form. The molecular

weight and degree of deacetylation defines the physical and chemical properties of chitosan. With increase in $–NH_2$ group solubility of chitosan increases. Chitosan is biodegradable, safe and non-toxic and soluble in dilute acids. Due to presence of excess –OH and –NH2 groups it is an excellent sorbent for trace metals and contains highly flexible structure (Whitacre, 2015).

2. CHITIN EXTRACTION

Chitin extraction can be done by either using chemical or biological method. Biological extraction includes fermentation and enzyme based extraction. The commercial process of extraction of chitin from crustacean shell waste includes demineralisation by acid and deproteinisation by treatment of alkali. As an alternative to alkali treatment several enzymatic deproteinisation processes have been introduced. Extraction of chitin using purified enzymes, proteolytic microorganisms and fungi is an eco- friendly, low cost technology (Pal et al., 2014). The shells of shrimps are thinner than the shells of crabs and lobster hence the extraction efficiency is more using shrimp waste than the other crustacean waste (Percot et al., 2003;Tan et al., 2020).

2.1.1 CHEMICAL METHOD

The shell waste is treated with 4% sodium hydroxide in deproteinisation step at high temperature 70-120^0 C (Rao et al., 2000; Yang et al., 2000). The protein is removed from the solid component of the shells. By filtration the protein hydrolysate is easily removed and can be used as protein supplement in feed after drying. After deproteinisation the shells contains chitin, calcium carbonate and pigments (Kandra et al., 2012).

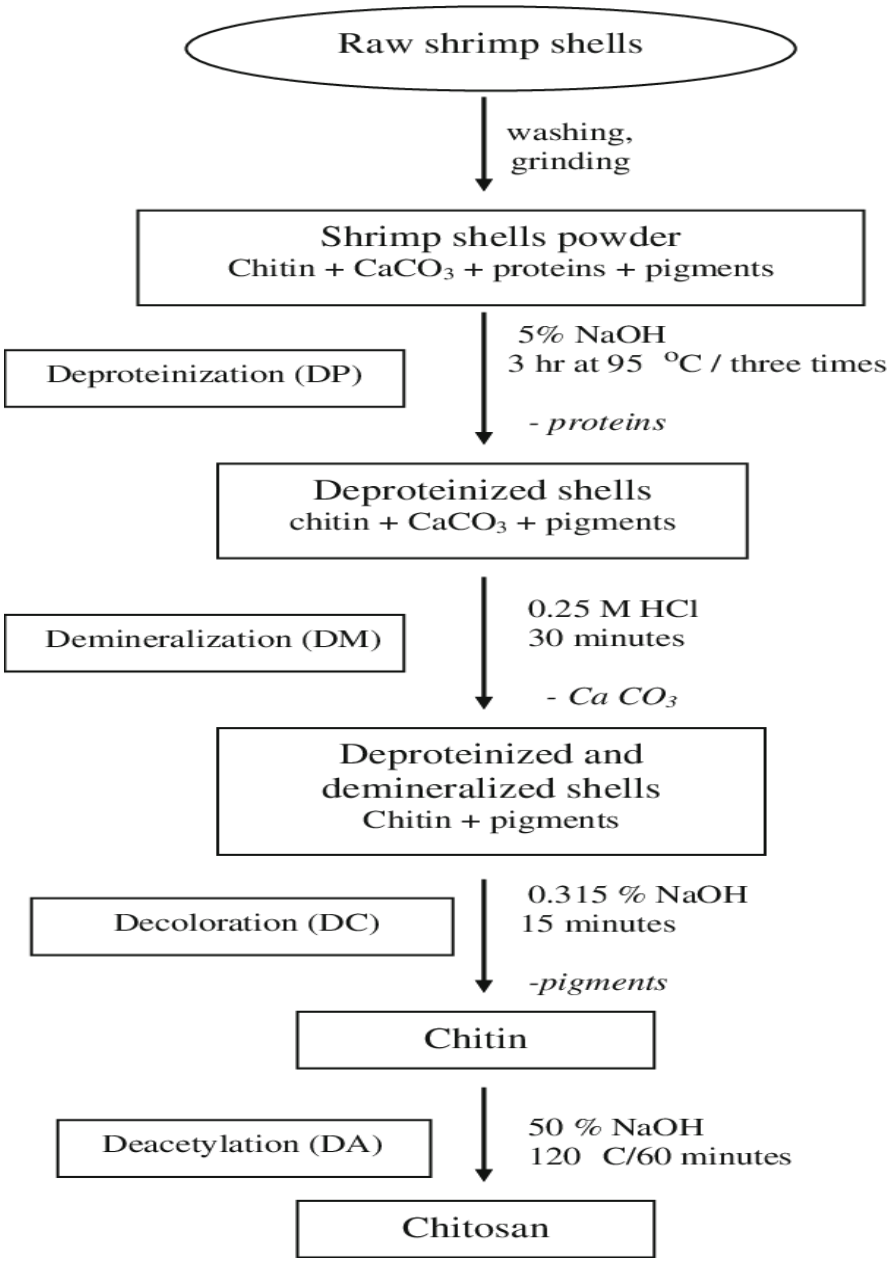


Figure 5: Extraction process of Chitin and Chitosan

The solid fraction is treated with 4% hydrochloric acid in the demineralisation step which converts insoluble calcium carbonate into soluble calcium chloride. It can be further removed by washing (Kandra et al., 2012). Chitin is insoluble in acids, alkali and organic acids hence it is deacetylated to chitosan. It is deacetylated using 50% NaOH at high temperature of 70-90^0 C. A good chitosan can dissolve in 1-2% acetic acid and forms highly viscous solution.

2.1.2 DISADVANTAGES OF CHEMICAL METHOD

In chemical method there are chances of denaturation of chitin. Chitin extracted using organic solvents is not suitable for medicinal uses due to toxicity, mutagenicity and corrosiveness of organic solvents and shell waste processing cost also increases (Pal et al., 2014). Use of sodium hydroxide for chemical deproteinization resulted in partial deacetylation, lower molecular weight and hydrolysis of biopolymer. Hydrochloric acid used for chemical demineralisation resulted in the production of chitin with higher residual ash content and degradation of polymer (Tan et al., 2020). It is expensive and not an environmental friendly process (Kandra et al., 2012).

2.2.1 BIOLOGICAL EXTRACTION METHOD

Green extraction process using microorganisms and enzymes for extraction of chitin is gaining more attention. Enzymatic extraction and fermentation using microorganisms are the biological methods which are commonly used (Yadav et al., 2019). Enzymatic method leads to the extraction of chitin with high molecular weight. Cost of extraction can be minimized in biological method by reducing the cost of carbon sources (Pal et al., 2014). Chitin structure is preserved with high molecular weight and high degree of deacetylation in biological extraction method with total removal of proteins and minerals. The solvent input is less which results in simpler manipulation, less energy consumption and higher reproducibility rate in short time. Hence chemical discharge is less (Tan et al., 2020).

2.2.2 ENZYMATIC DEPROTEINISATION

For the removal of proteins during chitin extraction the enzymes such as proteases are required. Proteolytic enzymes are produced by microorganisms, plants and animals. For protein removal, proteases like trypsin, pepsin and papain are used. Crude enzymes as well as

purified enzymes can be used for deproteinisation but due to higher cost of purified enzymes, crude enzymes obtained from bacteria and fish viscera are commonly used. The efficiency of enzymatic method is lower as compared to chemical method. Even after extraction 5%- 10% protein still remained in association with chitin (Synowiecki & Al-Khateeb, 2000). For increasing purity, additional NaOH treatment can be used. In chemical process order of deproteinization and demineralization does not influence the quantity and yield of the product (Kaur & Dhillon, 2015).

2.2.3 FERMENTATION

The price of using enzyme can be reduced by using microbial strains for deproteinisation in fermentation process. Two classes of fermentation include lactic acid fermentation and non-lactic acid fermentation (Yadav et al., 2019). *Lactobacillus sp.* that produces lactic acid and proteases are used for fermentation of crustacean shells. Lactic acid is obtained when pH of the silage decreases due to alteration of glucose. The inoculum composition, pH, carbon source and temperature affect the productivity of lactic acid fermentation. Non-lactic acid fermentation of crustacean shells can be done using both fungi and bacteria for example *Aspergillus* sp., *Bacillus* sp. and *Pseudomonas* sp. (Yadav et al., 2019).

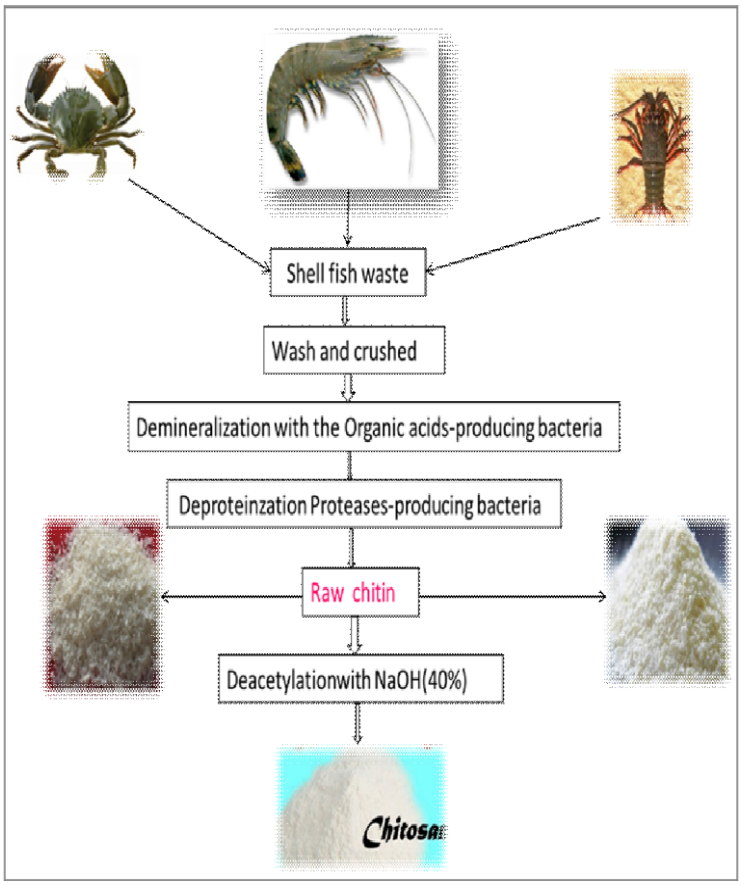


Figure 6: Biological process of Chitin extraction

3. DEACETYLATION OF CHITIN

Chitosan can be prepared from chitin by using either chemical or biochemical (deacetylation using enzymes) method (Younes & Rinaudo, 2015). Deacetylation of chitin can also be done using thermo-chemical process. It is further classified into homogenous and heterogeneous method. The chitin was reacted with concentrated sodium hydroxide at 100^0 C in heterogeneous method resulting in degree of deacetylation between 85-99%. The chitin was allowed to react with concentrated sodium hydroxide at 20^0 C and then dissolved it in grated ice at 0^0 C in homogenous method. Water soluble chitosan was produced with degree of deacetylation between 48% and 55% (Younes and Rinaudo 2015). N-acetylation of chitosan using acetic anhydride was done to prepare water- soluble chitosan (Qin et al., 2006).

Chitosan can also be prepared by enzymatic method. Chitin deacetylase enzyme specifically cleaves the acetyl group which is attached to the second carbon atom of chitosan (Suryawanshi et al., 2019).

4. APPLICATIONS OF CHITIN AND CHITOSAN

To produce derivatives of chitin such as chitosan, glucosamine hydrochloride and oligosaccharide, the chitin is used as the raw material. Chitosan has application in water purification and wine clarification. It is also used in paper industry as the ideal material. Chitosan also has applications in agricultural field (Pal et al., 2014).

Chitin provides substrate for matrix material in bone regeneration to promote osteogenesis in mesenchymal stem cells. It has rapid wound healing properties. As the repeating units N-acetylglucosamine is the fundamental unit of hyaluronate and keratin sulphate, it acts as a biomaterial to help in cartilage regeneration. Chitin and chitosan scaffolds are used in tissue repair that involves the degradation of products in vivo because they are associated with important biological activity (Sethulekshmi, 2014).

Chitosan is used as flocculating agent in processing of food waste during recycling. It is also used in textile industries for removal of dye from effluents. In medical related textile sutures, fibres and threads chitin and chitosan both has remarkable contribution. It is used as non-absorbable carrier of concentrated food ingredients (Dutta et al., 2004).

In agriculture field the accelerated growth is shown by the seeds treated with chitin. Addition of chitin in the soil resulted in the fungal pathogen suppression and reduction in root knot worm infestation. Chitosan has applications in colour photography as a fixing agent for the acid dye in gelatin. It also helps to improve diffusion while developing photographs. In chromatographic separation chitin and chitosan has wide variety of applications. For the

separation of nucleic acids by thin layer chromatography use of chitosan is reported (Dutta et al., 2004). Chitosan exhibits antibacterial and antifungal activity. It also helps in increasing the shelf life of the fruits and vegetables (Tarafdar & Biswas, 2013).

5. SEAWEED ASSOCIATED EPIPHYTIC BACTERIA

Bacterial communities associated with seaweed vary in different species as well as based on different temporal variations. Bacteria utilise seaweed surface as a substratum and utilise their organic substances for its growth and multiplication (Singh & Reddy, 2014). Using those organic compounds they synthesize various enzymes such as amylases, phosphatases, agarase, protease (Gonzalez NB et al., 2016) This enzymes can be used for various industrial applications, degradation of shell fish waste and also for extraction of chitin from crustacean shells.

6. BIVALVE ASSOCIATED BACTERIA

Being filter feeders, bivalve harbors Bacteria from the water column within themselves. They support healthy functioning of ecosystem, improving quality of water and provide habitat for other microscopic organisms. The composition of surrounding water determine the microbiome composition of bivalves (Howells & Brosnahan, 2022)

MATERIALS AND METHODS

1. SAMPLE COLLECTION:

Prawns and crabs were purchased from local fish market. Immediately the meat was removed and shells were separated. Shells were thoroughly washed to remove remaining dust and meat and dried in sun for 4 hours.

2. SAMPLE COLLECTION FOR BACTERIAL ISOLATES

Sea weed sample (Sargasssum) was collected from the intertidal regions of Anjuna beach, Mapusa Goa and Sea weed sample *Ulva intestinalis* and bivalve sample (Juvenile Mussel) was collected from Bambolim beach, Goa (Fig:7). The samples were collected in zip lock bags and were taken to laboratory for further analysis.

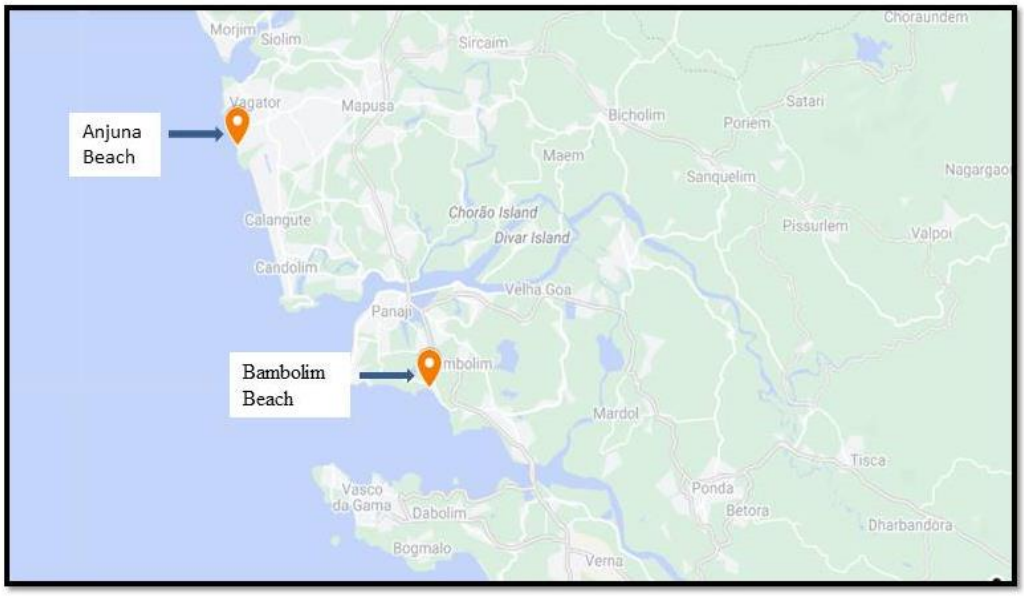


Figure 7: Map showing sample collection sites

a b

Figure 8: Sampling site a) Anjuna rocky intertidal region b) Bambolim rocky intertidal region

3. ISOLATION OF BACTERIA

3.1. Isolation of epiphytic bacteria from Sea weeds

Sea weeds were washed with sterile seawater and placed into test tube containing 10ml sterile sea water. Tube was vortexed for 5 minutes. Serial dilutions were prepared and dilutions till 10^{-5} were spread plated on Zobell Marine Agar (ZMA) (Appendix I-1) and incubated at room temperature for 48 hours. Morphologically distinct colonies were selected and purified (Kumar et al., 2011).

3.2. Isolation of bivalve associated bacteria

Bivalve samples (Juvenile Mussel) attached to intertidal rocks were collected in ziplock bags and taken to the laboratory for further analysis. Sample washed using sterile seawater. Inner tissues were removed from the shells using forceps. Tissue was homogenised using mortar and pestle. Tissue homogenate was added in the tube containing 9ml of autoclaved seawater. Vortexed for 2-3 minutes and serial dilutions were prepared. 0.1ml of the sample was spread

plated on the ZMA plates and incubated at room temperature for 48 hours. Morphologically different colonies were purified by streaking on the ZMA plates.

4. SCREENING OF ENZYMES

4.1. Protease activity

Seaweed and bivalve associated bacteria was spot inoculated on the plates containing sea water agar with 2% skim milk (Appendix I-3). Plates were incubated at room temperature for 48-72 hours. Cultures showing protease activity were identified by the formation of zone of clearance around the colony.

4.2. Calcium Carbonate Solubilisation

Seaweed and bivalve associated bacterial cultures were ~~as~~ spot inoculated on the plates containing sea water-based media with 1% calcium carbonate (Appendix I-4). Plates were incubated at room temperature for 48- 72 hours. Calcium Carbonate solubilisation activity was indicated by the clear zone around the colonies.

4.3. Chitinase activity

Chitinase activity of the seaweed and bivalve associated bacterial cultures was checked by spot inoculating bacterial colonies on the plates containing sea water based media with 1% colloidal chitin (Appendix I-5). Plates were incubated at room temperature for 48-72 hours. Plates were flooded with 1% Congo red (Appendix II-1) for 15 minutes followed by decolourisation using 1M NaCl (Appendix II-2). Excess NaCl was poured out (Samant et al., 2019). Zone of clearance was shown by the chitinase producing bacteria.

BIOCHEMICAL TESTS

A. Gram staining

On clean grease free slide, bacterial smear of 18 hour old culture was made. Slide was air dried and heat fixed. It was flooded with 1% Grams Crystal Violet for 1 minute followed by draining and flooding with Grams Iodine for 1 minute. Slide was drained and decolourisation was done using 95% ethanol for 30 seconds. Slide was rinsed with water and counter stained with Saffranine for 30 seconds. Slide was washed with water, air dried and observed under oil immersion lens of microscope.

B. Motility Test

Nutrient agar butt containing 0.4% agar was prepared (Appendix I-12). 18 hour old culture was stabbed and tube was incubated for 24 hours at room temperature (28^0C±2). Growth throughout the agar butt indicates positive results.

C. Indole Test

Tryptone broth (Appendix I-11) was prepared and was inoculated with loopful of culture and incubated for 24 hours at room temperature (28^0C±2). Few drops of Kovac's reagent was added. Red ring formation indicated the positive result.

D. Methyl Red (MR) and Voges- Proskaeur (VP) Test

Glucose phosphate broth (Appendix I-10) was prepared and a loop full of culture was inoculated and incubated for 24 hours at room temperature (28^0C±2). After incubation the broth was divided into two test tubes. One test tube was used for MR test and other for VP test. 2-3 drops of methyl red reagent (Appendix II-10) was added for MR Test. Positive result is indicated by the red colouration of the broth. For VP Test, 2 drops of Omeara's reagent was added, mixed and allowed to stand for 30 minutes. Positive test is indicated by the pink colouration.

E. Catalase Test

Suspension of 18 hour old culture was prepared on glass slide using saline (Appendix II-8). A drop of hydrogen peroxide was added to it. Production of effervescence indicates the positive result.

F. Hugh leifson Test

Hugh leifson medium (Appendix I-13) was prepared and added 5ml each in two tubes. The 18 hour old culture was stab inoculated. One tube was covered with the layer of paraffin oil. Both the tubes were incubated for 24 hours at room temperature (28^0C±2). If both tubes show growth then the culture is facultative anaerobe. Isolate is aerobe if the growth is only present in tube without liquid paraffin. Isolate is anaerobe if growth is there in only liquid paraffin containing tube.

G. Sugar fermentation

Isolates of 18 hour old were inoculated in peptone water (Appendix I-14) based medium containing phenol red as an indicator, Inverted Durham's tube and 0.5% of sugars (Glucose, Sucrose, Maltose and Lactose) and incubated at room temperature (28^0C±2) for 24 hours. Sugar fermentation is indicated by colour change of Phenol red from red to yellow and presence of gas bubble in Durham's tube.

Based on the results of Gram staining and biochemical tests, colonies were partially confirmed at genus level.

5. BIOLOGICAL METHOD OF CHITIN EXTRACTION

5.1. PREPROCESSING OF SHELL FISH WASTE

Prawn and crab shell waste was collected from the local fish market. Shells were thoroughly washed with distilled water and dried in hot air oven at 60^0 C. shells were grinded into powder using mortar and pestle. This powder was used for extraction of Chitin.

5.2. EXTRACTION PROCESS

The cultures showing good proteolytic activity were selected and inoculated in the flasks containing 50ml of sea water based broth composed of 1% glucose and 1% prawn/crab shell waste. The flasks were incubated on rotary shaker at 100 rpm at 30^0 C for 48 hours.

For chitin extraction, sea water based broth was prepared containing 10% (w/v) glucose and 5% (w/v) sterilised prawn and crab waste (Appendix I-7). 10% (v/v) of the previously grown culture in broth was inoculated in the flasks and incubated on rotary shaker at 100 rpm at 30^0C for 6 days. Control was maintained containing sea water based broth with 5% prawn/ crab waste and 10% glucose. After 6 days of incubation the solid phase was separated from the broth by filtration using Whatman No. 1 filter paper (Suresh et al., 2023). The extracted polymer was washed with distilled water followed by washing with 70% ethanol. It was then dried in hot air oven at 60^0 C for 24 hours.

6. PREPARATION OF CHITOSAN FROM CHITIN

40% NaOH solution (Appendix II-3) was added in the extracted chitin and kept in water bath at 90^0C with constant stirring for 6 hours. The solution was filtered using Whatman filter

paper and washed continuously till the pH was neutralised. It was then dried in the hot air oven for 24 hours.

7. SOLUBILITY TEST FOR EXTRACTED CHITOSAN

Extracted chitosan was added in 1% acetic acid (Appendix II-4) and stirred on the magnetic stirrer for 2-3 minutes. Chitosan has the ability to dissolve in dilute acid (Tarafdar & Biswas, 2013; Varun et al., 2017).

8. ESTIMATION OF CHITOSAN

8.1. COLORIMETRIC METHOD

The reaction mixture containing chitosan solution prepared using 1% acetic acid and 0.1ml $NaNO_2$ (0.5M) (Appendix II-6) in a test tubes were heated in water bath at 80^0 C for 45 minutes for complete depolymerisation- deamination reaction and cooled under tap water. pH was adjusted to 4 by adding 1N HCl (Appendix II-5) followed by addition of 1ml DNSA (Appendix II-7). The tubes were kept in water bath for 15 minutes at 75^0 C. The tubes were cooled and 5ml of distilled water was added. Absorbance was measured at 540nm (Mojumdar et al., 2019).

9. APPLICATIONS OF EXTRACTED CHITOSAN

9.1. Dye adsorption

Two different concentrations of methylene blue dye stock solutions 5mg/500ml and 0.5mg/500ml were prepared. 0.2g of different chitosan samples extracted from the prawn and crab shells were taken in the beakers and 20 ml of dye solution was added in each beaker. The mixture was stirred till the chitosan was settled. Control was kept without adding chitosan sample. The beakers were kept at room temperature (28^0 C±2) for 24 hours. After 24 hours all the dye solutions containing chitosan were centrifuged for 10 minutes at 8000 rpm and OD of supernatant was taken at 664nm (Arunachalam, 2021).

The adsorption capacity of Chitosan was calculated using following formula

$$\text{Adsorption capacity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

9.2. ANTIBACTERIAL ACTIVITY OF EXTRACTED CHITOSAN

Escherichia coli and *Staphylococcus aureus* cultures were inoculated in the nutrient broth (Appendix I-8) and incubated at 37^0 C.

9.2.1. DISC DIFFUSION METHOD:

Chitosan solution of 10mg/ml, 5mg/ml, 2mg/ml and 1mg/ml were prepared in phosphate buffered saline. *Escherichia coli* and *Staphylococcus aureus* cultures of 18 hour old duration were spread plated on the Mueller- Hinton agar (Appendix I-9) plates. The discs were impregnated in the different concentrations of chitosan and placed on the surface of the Spread plated MHA plates. The disc impregnated with phosphate buffered saline (Appendix

II-9) was used as the control. The plates were incubated at 37^0 C for 24 hours. The plates were checked for the inhibition zone around the disc.

9.2.2. WELL DIFFUSION METHOD

Chitosan solution of 10mg/ml, 5mg/ml, 2mg/ml and 1mg/ml were prepared in phosphate buffered saline. 18hr old *Escherichia coli* and *Staphylococcus aureus* cultures were spread plated on the Mueller- Hinton agar plates. The wells were formed using cork borer. The different concentrations were added in the wells. Sterile phosphate buffered saline is used as the control. The plates were incubated at 37^0 C for 24 hours. The plates were checked to see if there is zone of inhibition around the wells. The test was performed in duplicates (Piegat et al., 2021).

RESULTS AND DISCUSSION

1. SAMPLE COLLECTION FOR BACTERIAL ISOLATES

Seaweed sample one and bivalve sample was collected from Bambolim beach, Goa and other seaweed sample was collected from Anjuna beach, Mapusa Goa.

a. *Ulva intestinalis* b. Sargassum c. Juvenile Mussels

Figure 9: Seaweed and Juvenile mussel sample

2. ISOLATION OF BACTERIA

After Pre-processing of sample, Serial dilutions were prepared and spread plated on ZMA plates. Different types of colonies were sub-cultured on ZMA and purified (Fig: 9). Six different types of bacterial colonies were isolated from *Ulva intestinalis*, six colonies from Sargassum and 11 bacterial colonies from Bivalve tissue sample. The colony characteristics of isolates were noted.

The colonies isolated from *Ulva intestinalis* were named as S1C1 to S1C8. The colonies isolated from Sargassum were named as S2C1 to S2C6 and from Bivalves as B1 to B11.

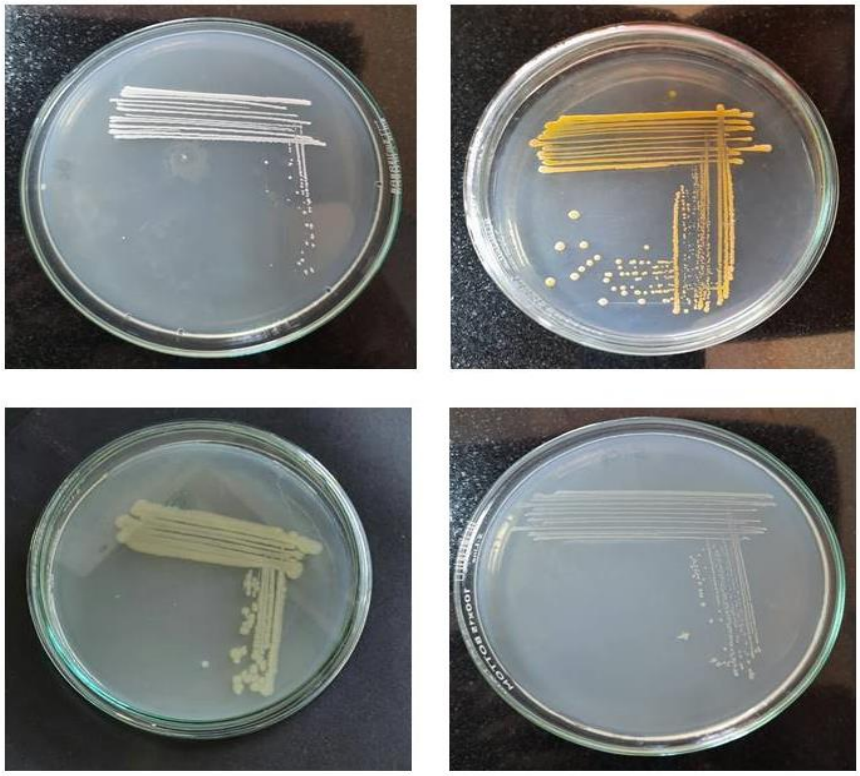


Figure 10: Bacterial isolates sub cultured on ZMA

3. SCREENING OF ENZYMES

3.1. Protease production by bacteria

Skim milk agar plates allow qualitative determination of protease activity. The degradation of substrate casein by the bacteria shows the zone of hydrolysis around the colony. All the colonies were spot inoculated on the sea water based media containing 2% Skim milk. Out of 23 isolates, 7 isolates showed zone of clearance. They are S1C6, S1C8, S2C3, B2, B5, B6, and B7. Isolate B7 showed highest zone of clearance (Fig: 10). Isolates B7, B6, S1C6 and S1C8 were further used for the extraction of chitin from prawn and crab shells.

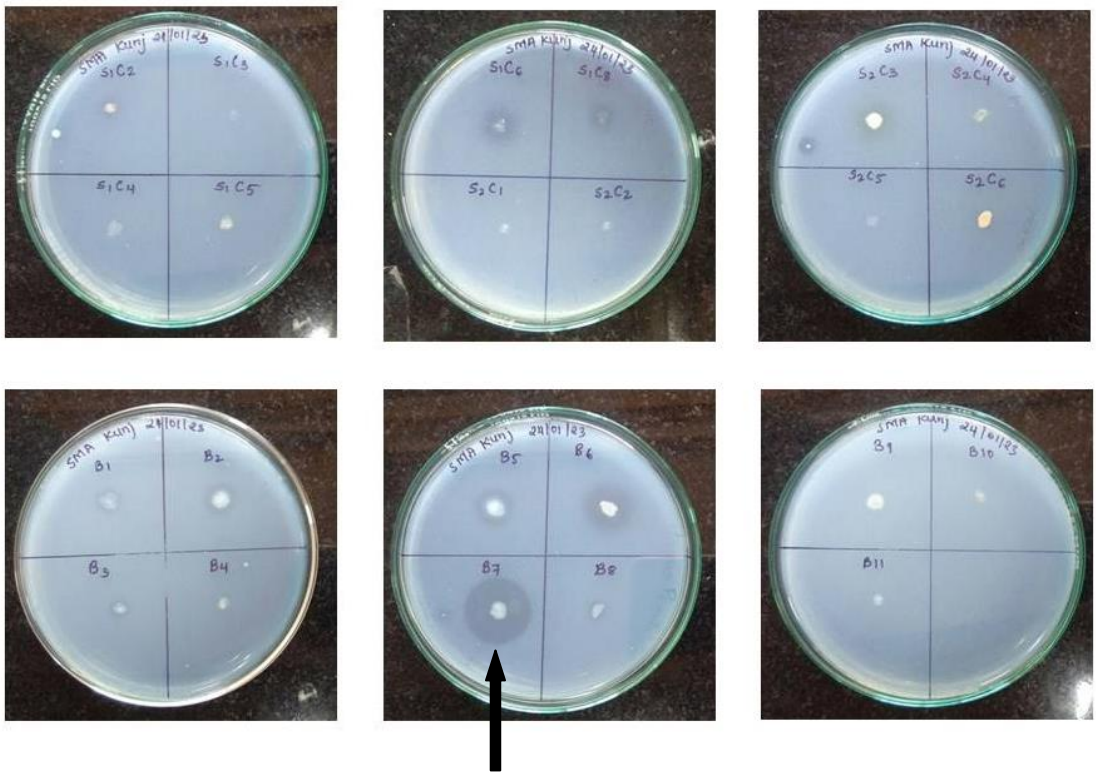


Figure 11: Proteolytic activity by B7, B6, B5 and B2 isolated from Bivalves; S1C6, S1C8 isolated from *Ulva intestinalis* and S2C3 isolated from Sargassum

3.2. Calcium Carbonate Solubilisation

The shells of prawn and crab waste contain 20-50% $CaCO_3$. Demineralisation is one of the steps during chitin extraction. The bacterial isolates capable of Calcium carbonate solubilisation can be used during chitin extraction process. On solubilisation of calcium carbonate bacterial colony forms zone of clearance around the colony. All the isolates were spot inoculated on the plates containing sea water based media with 1% $CaCO_3$. Only 3 bacterial isolates, B2, B5 and B9 showed slight zone of clearance (Fig: 11).

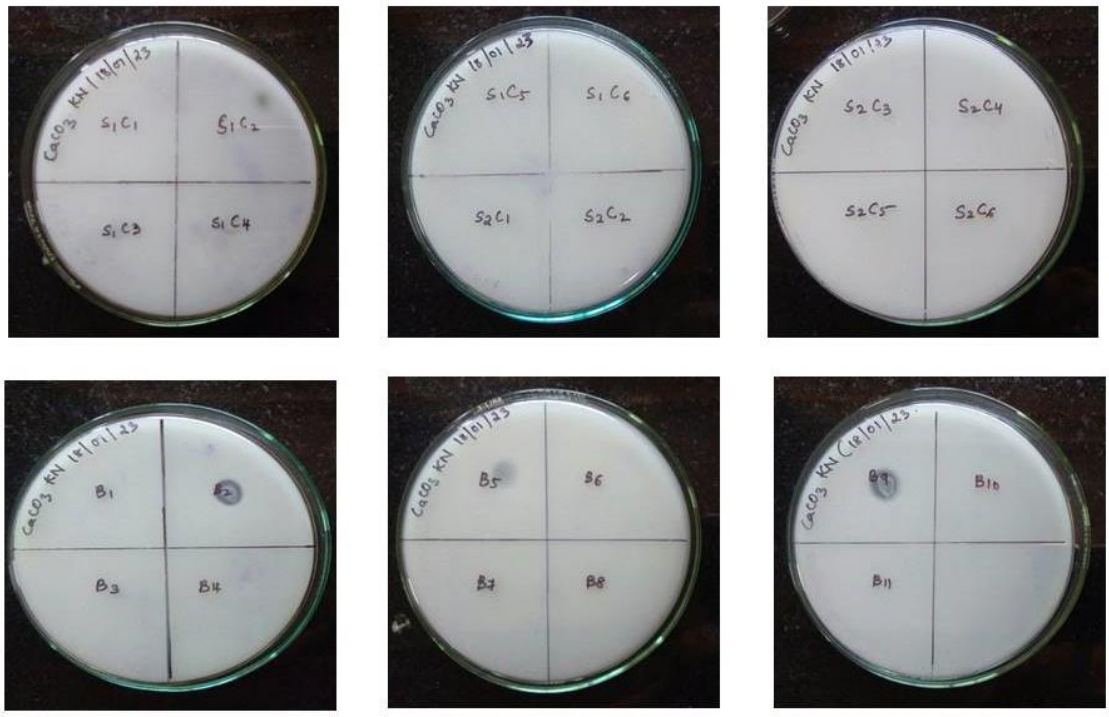


Figure 12: Calcium Carbonate solubilisation by B2, B5, B9 isolated from Bivalve sample

3.3. Chitinase Production

Chitinase activity is indicated by the formation of clear zone surrounding colony on breakdown of chitin compound in the medium. Bacterial isolates showing chitinase activity can be used for degradation of chitin. Those isolates can be further used for deacetylation of chitin into chitosan by first screening for Chitin deacetylase enzyme. All the isolates were spot inoculated on the plates containing sea water based agar with 1% Colloidal chitin. Only one colony, B7 showed zone of clearance around the colony on addition of 0.1% Congo red and rest of the plate was stained red (Fig: 12).

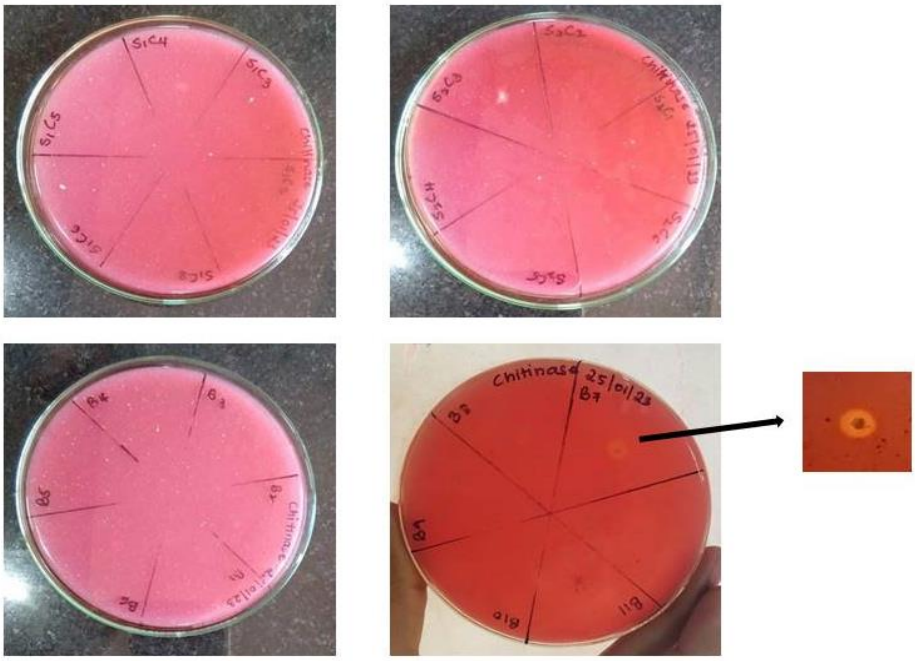


Figure 13: Chitinase activity by B7 isolated from Bivalve

4. COLONY CHARACTERISTICS

Table 1: Colony characteristics of isolates showing good protease activity

	B6	B7	S1C6	S1C8
SHAPE	Circular	Irregular	Circular	Irregular
SIZE	1mm	2mm	2mm	5mm
COLOUR	White	Off white with opaque centre	Off white	Yellow
OPACITY	Opaque	Opaque	Opaque	Opaque
ELEVATION	Convex	Convex	Flat	Convex
CONSISTANCY	Dry	Dry	Butyrous	Butyrous
MARGIN	Entire	Irregular	Entire	Entire

B6, B7 – Isolated from Bivalve (Juvenile mussel) sample

S1C6, S1C8 – Isolated from seaweed (*Ulva intestinalis)*

4.1 BIOCHEMICAL TESTS

Table 2: Biochemical tests

BIOCHEMICAL TEST	B7	B6	S1C6	S1C8
GRAM CHARACTER	Gram Positive, Cocci	Gram Positive, Cocci	Gram Positive, rods	Gram Positive, rods
MOTILITY	+	-	+	-
INDOLE	-	-	-	-
METHYL RED	-	+	-	-
VOGES PROSKAUER	-	-	-	-
CATALASE	+	+	+	+
HUGH-LEIFSON	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic
GLUCOSE UTILISATION	+	+	+	+
LACTOSE UTILISATION	-	-	-	-
SUCROSE UTILISATION	-	-	-	-
MALTOSE UTILISATION	+	-	+	+

For sugar fermentation tests:

Negative test : -

Production of acid: +

Production of acid and gas: + +

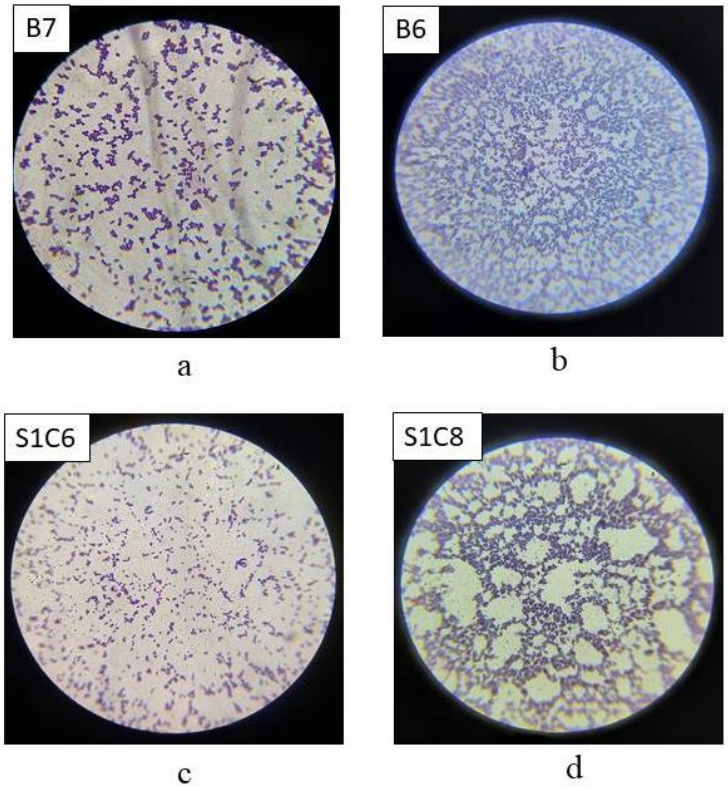


Figure 14: Gram staining a) Gram positive rods b) Gram positive cocci c) Gram positive rods d) Gram positive rods

Based on the results of Gram staining and biochemical tests, colony B6 was partially identified as *Staphylococcus.* Data of biochemical tests was insufficient for the identification of other three bacterial isolates.

5. BIOLOGICAL METHOD OF CHITIN EXTRATION

5.1. PREPROCESSING OF SHELL FISH WASTE

Prawn and crab shells were washed with water, dried at 60^0 C overnight and grinded into fine powder.

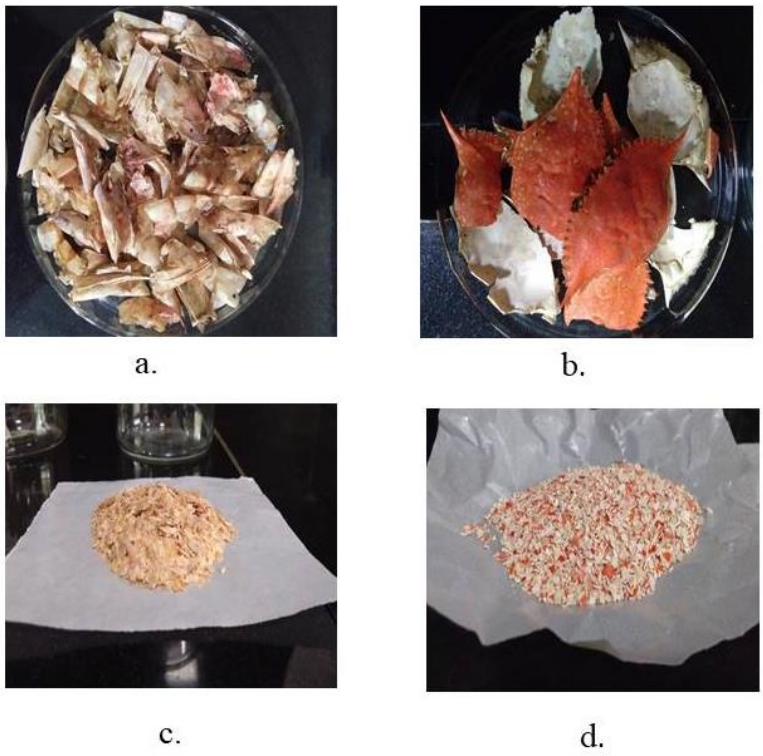


Figure 15: a. Prawn shells b. Crab shells c. powdered prawn shells, d. powdered crab shells

5.2. EXTRACTION PROCESS

Isolates B7, B6, S1C6 and S1C8 showing good proteolytic activity were used for chitin extraction from Prawn and crab shells. Chitin from 5 grams each of prawn waste and Crab waste was extracted by one- step fermentation process using four different isolates separately. Growth of protease producing bacteria indicates that it can hydrolyse protein present in prawn shell and crab shell waste for its nitrogen and carbon requirements. During the process, the

presence of high sugar percentage in media results in the formation of lactic acid leading to the conversion of calcium carbonate to calcium lactate along with decrease in pH. pH changed from near neutral on day 1of inoculation to pH 5±0.5 on sixth day of incubation.

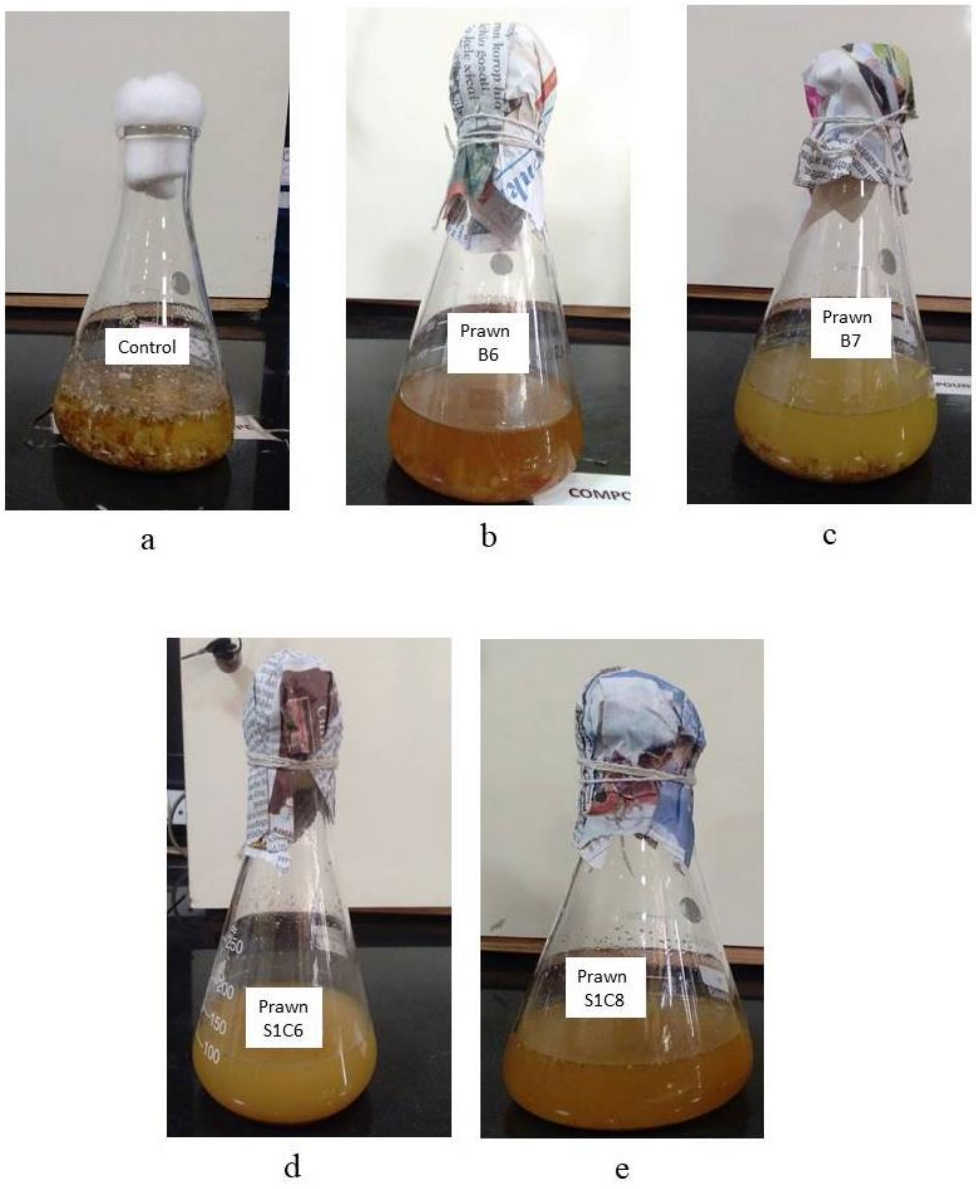


Figure 16: a) Control; Chitin extraction from prawn shells using b) B6, c) B7, d) S1C6, e) S1C8

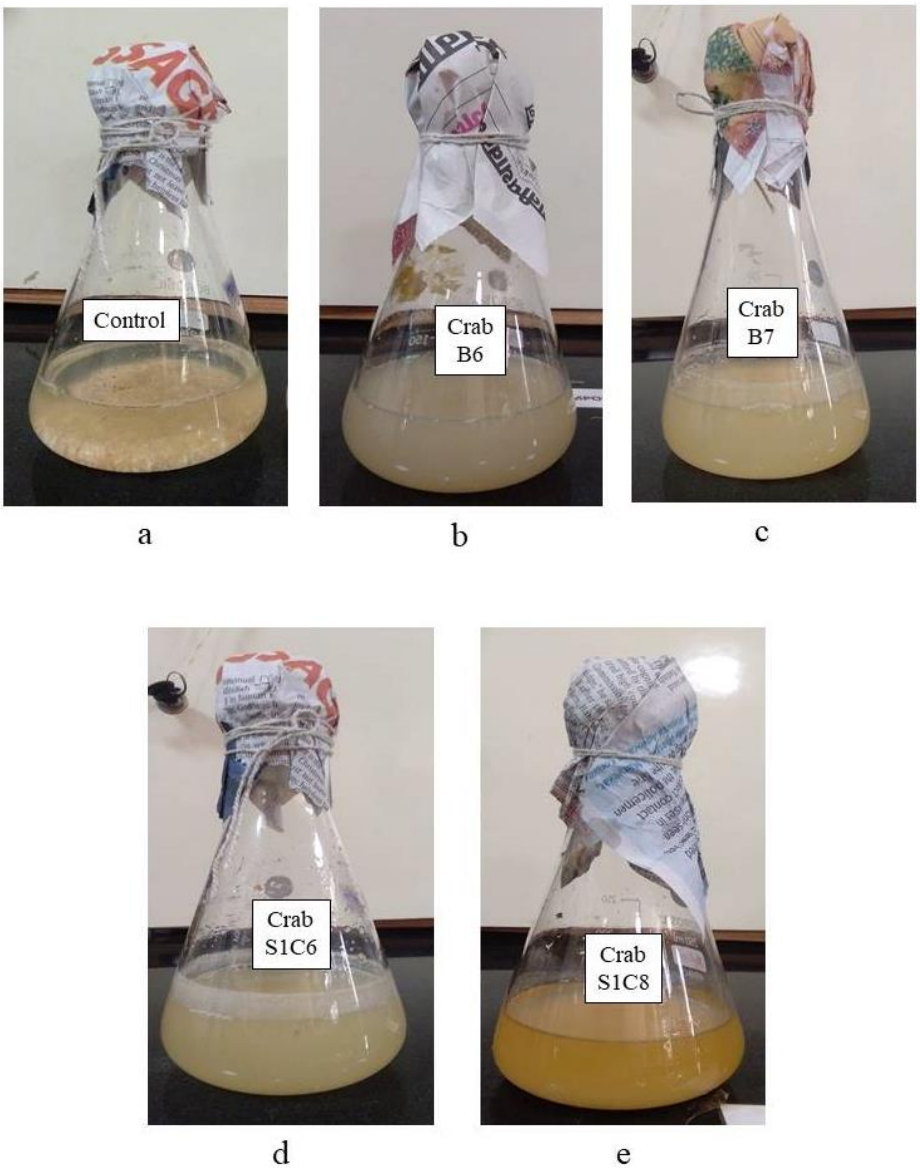


Figure 17: a) Control; Chitin extraction from crab shells using b) B6, c) B7, d) S1C6 and e) S1C8

Table 3: Weight of extracted chitin

SAMPLE USED FOR EXTRACTION	ISOLATE USED	WEIGHT OF EXTRACTED CHITIN (g)
PRAWN SHELLS	B6	3.5
	B7	2.9
	S1C6	2.05
	S1C8	2.08
CRAB SHELLS	B6	3.36
	B7	2.3
	S1C6	3.27
	S1C8	3.10

6. PREPARATION OF CHITOSAN FROM CHITIN

Chitin is insoluble hence has very less applications as compared to its derivative chitosan. Chitosan is soluble in 1% acetic acid and due to its solubility it can be widely used for different industrial as well as environmental and medical applications. Chitin extracted using all four isolates from prawn and crab shell waste was treated with 40% NaOH at 90^0 C for 6 hours to get chitosan and pH was adjusted to neutral by constant washing with distilled water.

Extracted chitosan samples were named as follows: Chitosan extracted from prawn shell chitin using B6, B7, S1C6 and S1C8 were named as CH1, CH2, CH3 and CH4 respectively. Chitosan extracted from crab shell chitin using B6, B7, S1C6 and S1C8 were named as CH5, CH6, CH7 and CH8 respectively.

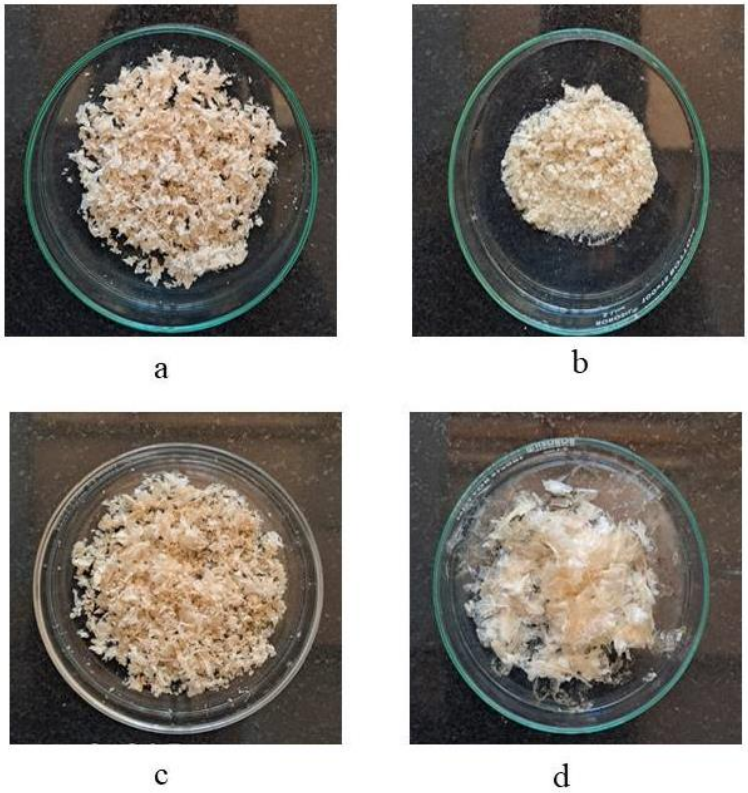


Figure 18: Chitosan prepared from Prawn shells chitin a) CH1 b) CH2 c) CH3 d) CH4

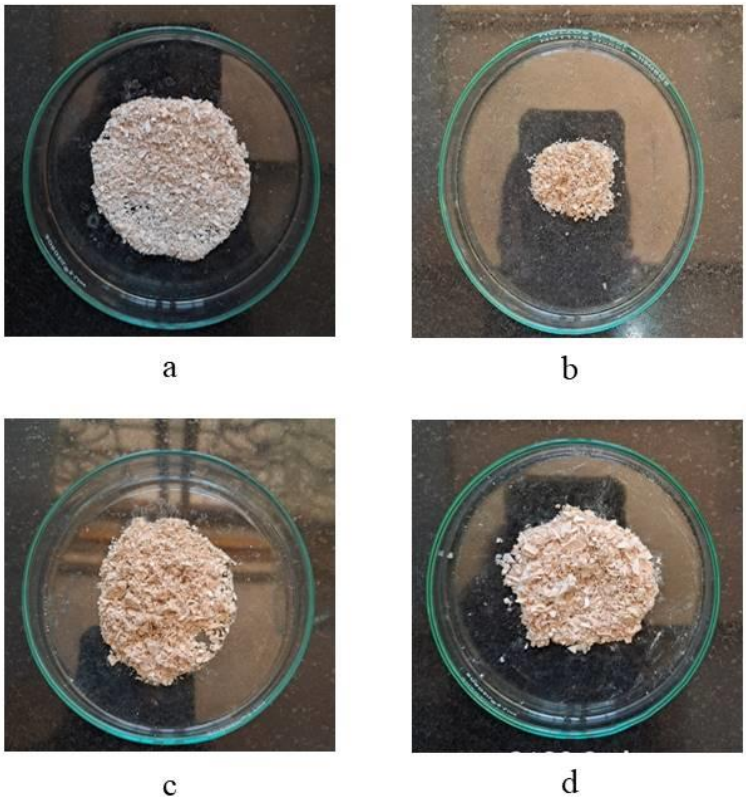


Figure 19: Chitosan prepared from crab shells chitin a) CH5 b) CH6 c) CH7 d) CH8

7. SOLUBILITY TEST FOR EXTRACTED CHITOSAN

Chitosan dissolves in acetic acid because it is a strong base having primary amino groups. It becomes polyelectrolyte when those amino groups get protonated. Solubility of chitosan defines its quality.

Chitosan CH1, CH2, CH3, CH4, CH6 were fully soluble in 1 % acetic acid solution and chitosan CH5, CH7 and CH8 were partially soluble in 1% acetic acid.

8. ESTIMATION OF CHITOSAN

8.1. COLORIMETRIC METHOD

Chitosan is converted into 2, 5-anhydro-D-mannose by depolymerisation and deamination using Sodium Nitrite. 2, 5-Anhydro-D-mannose is the sum of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc). The deamination of (1 → 4)-linked 2-acetamido-2-deoxyβ-D-glucopyranose unit takes place on depolymerisation and forms 2, 5-anhydro-D-mannose as the reducing end. 2, 5-Anhydromannose molecules is the end product which is determined colorimetrically using DNSA. The standard curve was plotted using standard chitosan purchased from HIMEDIA having Degree of Deacetylation: >=75.00% and using that curve concentration of unknown chitosan sample was determined.

Stock chitosan solution – 5mg/ ml in 1% acetic acid

Diluent – Distilled Water

Table 4: Standard for Chitosan

Conc. of Chitosan (mg/ml)	Volume of stock (ml)	Volume of Diluent (ml)	Total Volume	0.5M $NaNO_2$		DNSA		OD at 540 nm
1	0.2	0.8	1ml	0.1ml	Keep in water bath at 80^0C for 45 minutes and cool under tap water. Adjust pH to 4 using 1N HCl	1ml	Keep in water bath at 75^0C for 15 minutes. Cool under tap water + Add 5ml of distilled water	0.235
2	0.4	0.6						0.620
3	0.6	0.4						0.930
4	0.8	0.2						1.172
5	1.0	0						1.370
Blank	0	1						0

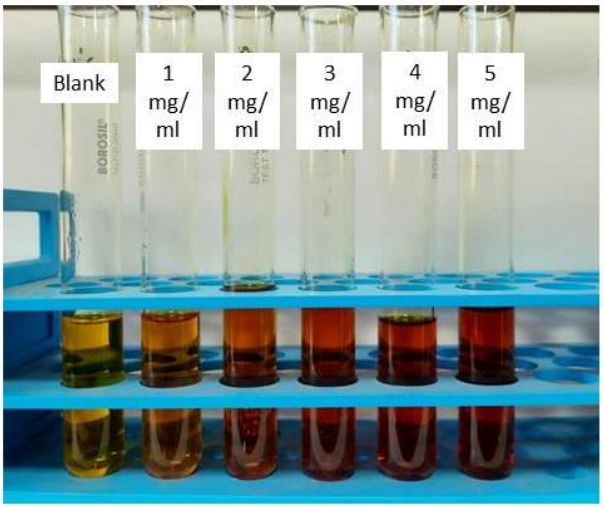


Figure 20: Assay using standard chitosan

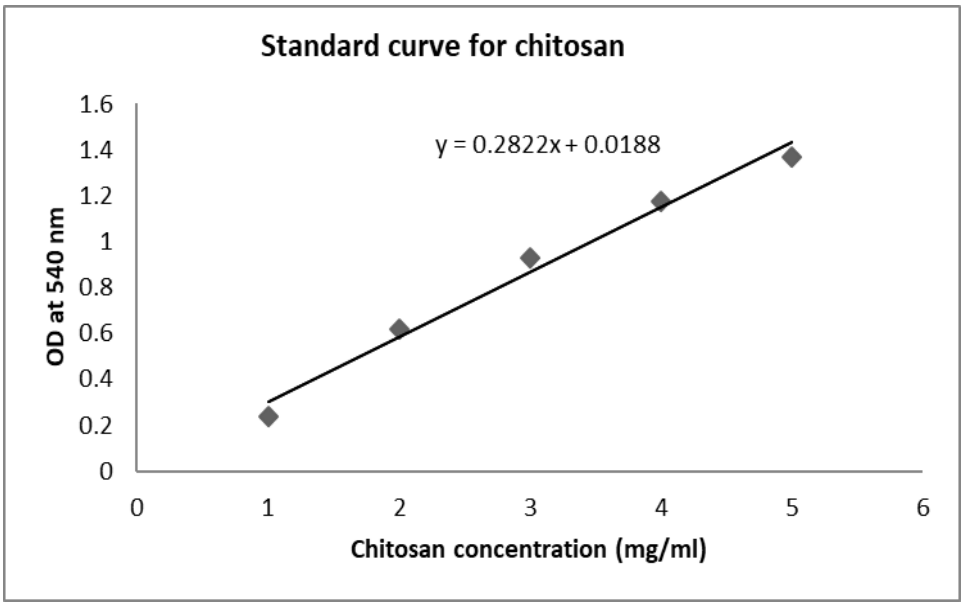
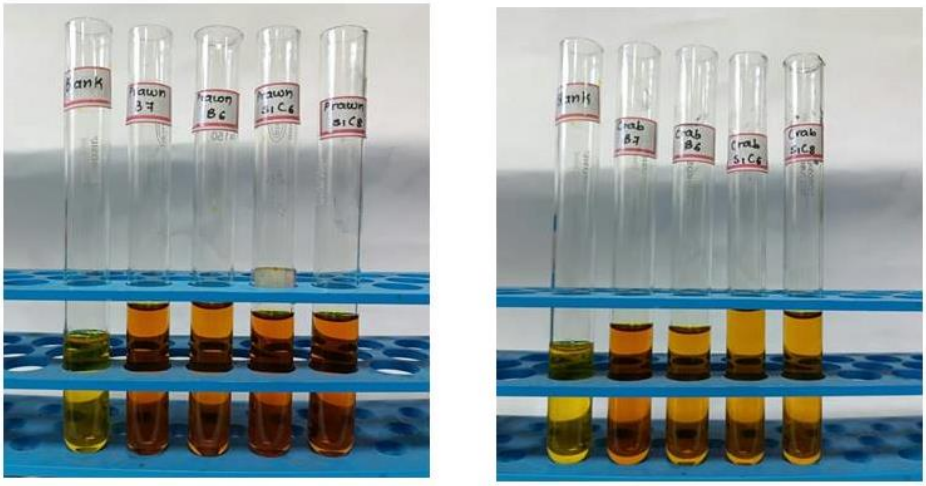


Figure 21: Standard curve of Chitosan

Table 5: Assay table for extracted Chitosan

Concentration of Chitosan (mg/ml)	Volume of stock (ml)	Volume of Diluent (ml)	Total Volume	0.5M $NaNO_2$		DNSA		OD at 540 nm
CH1	1	-	1ml	0.1ml	Keep in water bath at 80^0C for 45 minutes and cool under tap water. Adjust pH to 4 using 1N HCl	1ml	Keep in water bath at 75^0C for 15 minutes. Cool under tap water +Add 5ml of distilled water	0.338
CH2	1	-						0.422
CH3	1	-						0.482
CH4	1	-						0.475
CH5	1	-						0.184
CH6	1	-						0.356
CH7	1	-						0.185
CH8	1	-						0.163



a b

Figure 22: Assay for extracted a) Prawn Chitosan b) Crab Chitosan

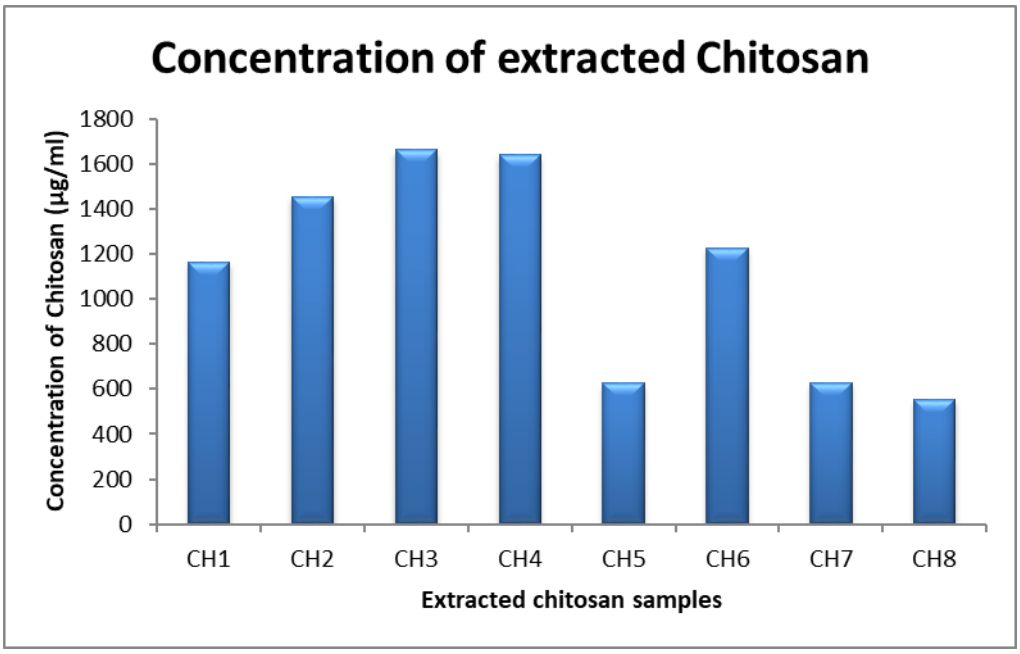


Figure 23: Concentration of extracted chitosan

In the above bar graph, the concentration of each extracted chitosan sample was determined using DNSA assay. X- axis contains different extracted chitosan samples and Y- axis has the concentration of chitosan. The concentration of Chitosan CH3 and CH4 was highest and lowest concentration is shown by CH5, CH7 and CH8. This implies that the concentration of chitosan extracted from prawn shells using S1C6 and S1C8 was highest but its efficiency of extraction from crab shells is low. Only B7 isolate showed good extraction efficiency from both prawn and crab shells. All chitosan samples extracted from prawn shells has the higher concentration than chitosan extracted from crab shells.

9. APPLICATIONS OF EXTRACTED CHITOSAN

9.1. Dye adsorption

Methylene blue is most commonly used cationic dye in the textile, ink and paper industries. Discharge of effluent from this industries leads to the pollution of the natural water bodies. For prevention of pollution adsorption is one of the efficient methods. Adsorption is referred as surface phenomenon because the dye gets attached to the adsorbent's surface. The dye adsorption capacity of chitosan is induced by its $–NH_2$ and –OH functional groups. Adsorption capacity of different extracted chitosan samples was determined by checking OD of dye solutions after 24 hours of chitosan addition.

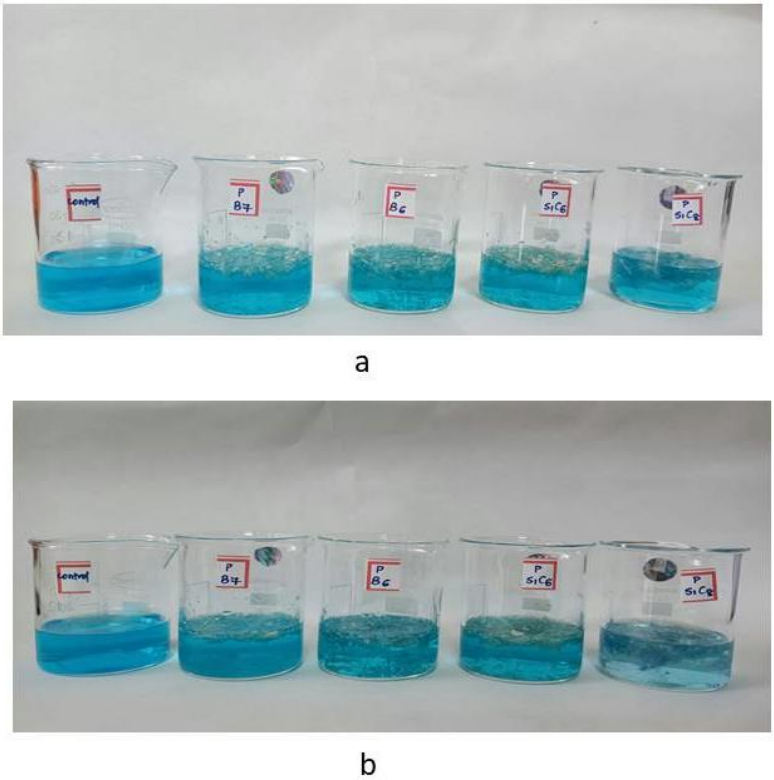
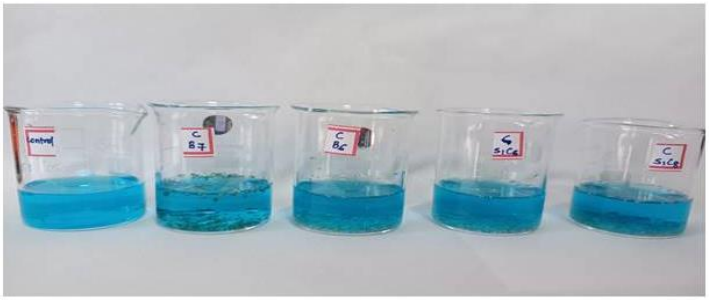


Figure 24: Dye adsorption using extracted prawn Chitosan a) Day of Chitosan inoculation b) After 24 hours of inoculation



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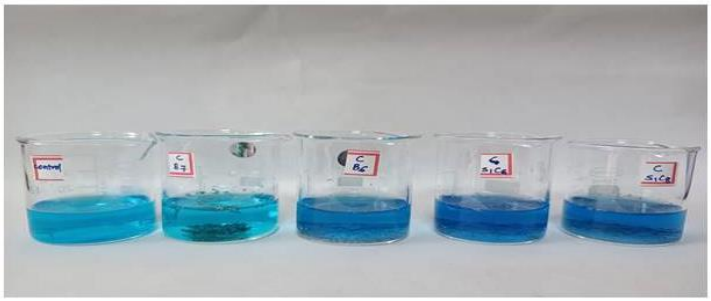


b

Figure 25: Dye adsorption using extracted crab Chitosan a) Day of Chitosan inoculation b) After 24 hours of inoculation

Table 5: Adsorption capacity of extracted chitosan for 1mg/100ml dye solution

CHITOSAN SAMPLE	ADSORPTION CAPACITY (%)
CH1	48.29
CH2	21.42
CH3	41.6
CH4	82.65
CH5	72.33
CH6	53.4
CH7	86.73
CH8	88.32

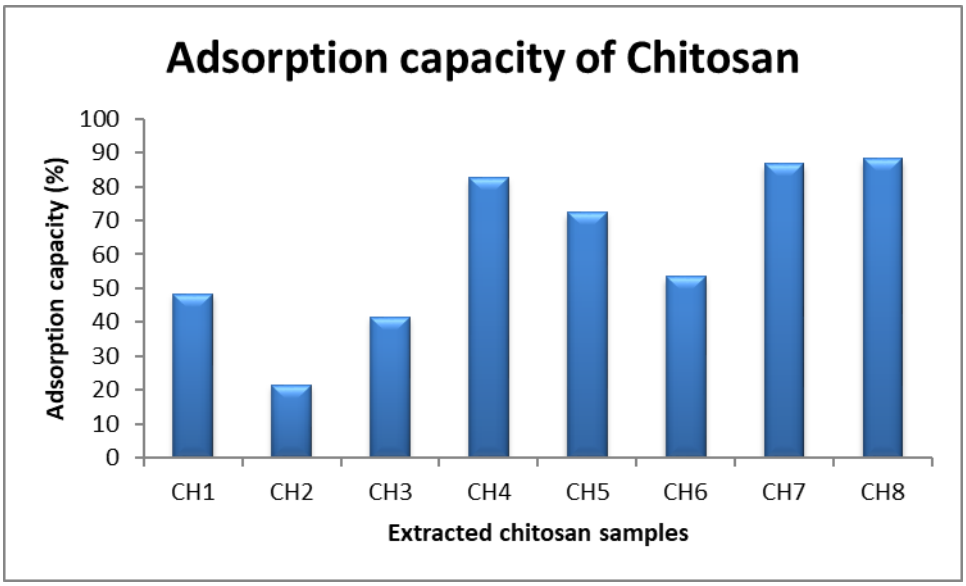
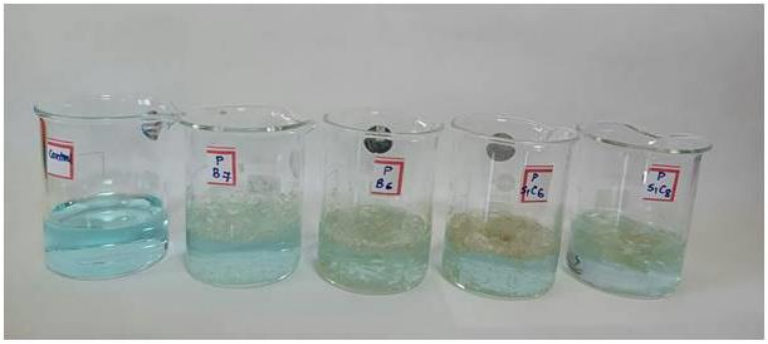


Figure 26: Dye adsorption capacity of extracted chitosan for dye concentration 1mg/100ml

The above graph shows the adsorption capacity of different extracted chitosan samples for the dye concentration of 1mg/100ml. CH7, CH8, CH4 and CH5 showed good adsorption capacity of 86.73%, 88.32%, 82.65% and 72.33%. CH2 showed the lowest dye adsorption capacity of 21.42%. This implies that chitosan extracted from prawn as well as crab sample using S1C8 showed good adsorption capacity. As a whole crab chitosan showed more adsorption capacity as compared to prawn chitosan.



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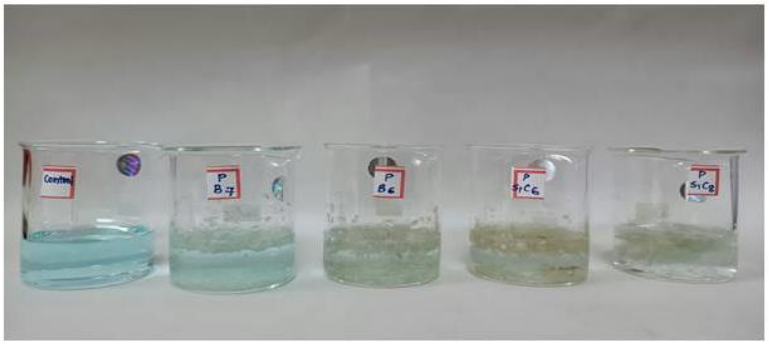
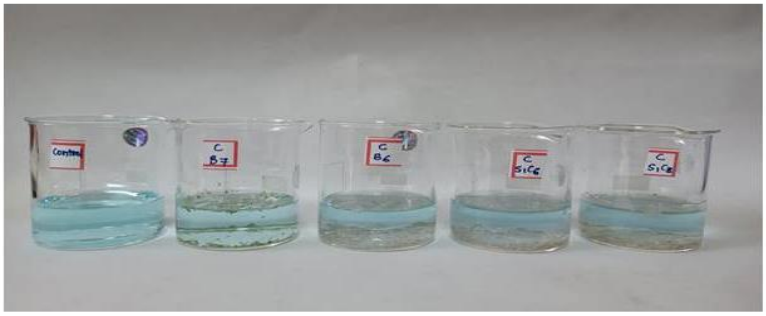


b

Figure 27: Dye adsorption using extracted prawn Chitosan a) Day of Chitosan inoculation b) After 24 hours of inoculation

a



b

Figure 28: Dye adsorption using extracted crab Chitosan a) Day of Chitosan inoculation b) After 24 hours of inoculation

Table 6: Adsorption capacity of extracted chitosan for 0.1mg/100ml dye solution

CHITOSAN SAMPLE	ADSORPTION CAPACITY (%)
CH1	79.56
CH2	43.01
CH3	74.19
CH4	96.77
CH5	74.19
CH6	27.95
CH7	90.32
CH8	92.47

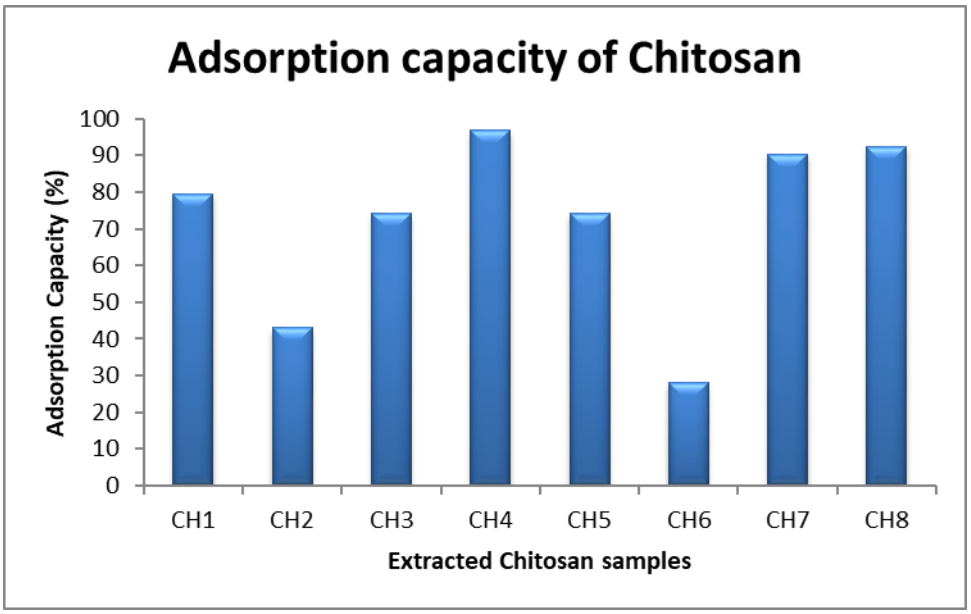


Figure 29: Adsorption capacity of chitosan for dye concentration of 0.1mg/100ml

The above graph shows the adsorption capacity of different extracted chitosan samples for the dye concentration of 0.1 mg/100ml. CH4, CH7 and CH8 showed good adsorption capacity of 96.77%, 90.32% and 92.47% respectively. CH6 showed the lowest dye adsorption capacity of 27.95%. The chitosan extracted from both prawn and crab shell waste using isolates B6, S1C6 and S1C8 showed good dye adsorption capacity for lower dye concentration. CH4 resulted in complete decolourisation of the dye solution.

9.2. ANTIBACTERIAL ACTIVITY OF CHITOSAN

Due to antibacterial activity of the chitosan, it can be used as the antimicrobial coating for fruits, vegetables, grains etc. which protects those items from infestation from the microorganisms.

Antibacterial activity of chitosan depends on the type of bacteria that is Gram negative and Gram positive. The molecular weight and the degree of deacetylation also affect the antibacterial activity. Chitosan with higher molecular weight creates an impermeable coating

with the outer cell wall of bacteria thus preventing the exchange of metabolic by products and nutrients causing cell death.

The different concentrations of extracted chitosan tested for its antibacterial activity against *Escherichia coli* and *Staphylococcus aureus.* None of the Chitosan concentrations showed the inhibition zones by Well diffusion and Disc diffusion method. According to (Piegat et al., 2021) Chitosan acts only when it is in direct contact with microorganisms. This may be the possible reason for not showing antibacterial activity against used pathogens.

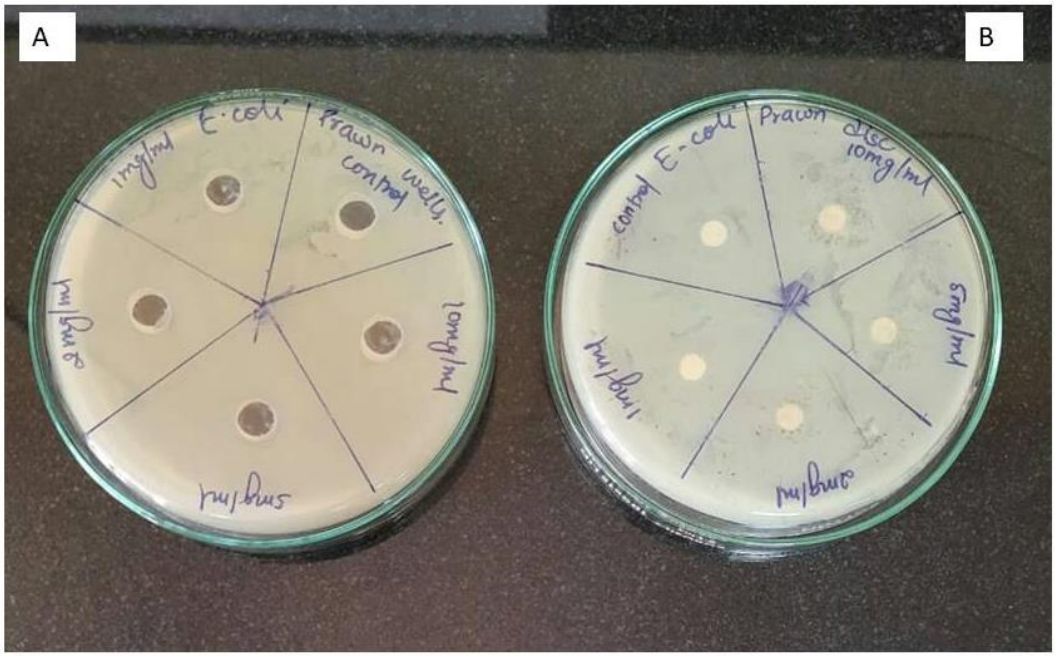


Figure 30: Antibacterial activity of chitosan extracted from Prawn shells against *E. coli* using A) Well diffusion method B) Disc diffusion method

Figure 31: Antibacterial activity of chitosan extracted from Prawn shells against *S. aureus* using A) Well diffusion method B) Disc diffusion method

SUMMARY

Shells of crabs and shrimps are the main sources of chitin, containing 20-40% protein, 20-50% calcium carbonate and 15-40% pigments and lipids. These wastes can be utilised to derive the industrially important compounds such as Chitin and its derivatives thus reducing the waste in the environment which otherwise is responsible for pollution. Chitin and chitosan has wide number of applications in medical fields, Waste treatment, food processing, industrial products and also in agriculture. The Non- toxicity, biodegradability, biocompatibility and adsorption properties make it more useful to the society. Biological extraction using bacteria makes it cheaper and less time consuming process as well as eco-friendly process. In this study, seaweeds, *Ulva intestinalis*, Sargassum and Juvenile Mussels were used for the isolation of bacteria. Six bacterial cultures were isolated from *Ulva intestinalis,* six from Sargassum and 11 isolates from the juvenile mussel sample. Among all the isolates, four isolates showed good proteolytic activity. Hence these four isolates were used for extraction of chitin from prawn and crab shell waste.

Extracted chitin was deacetylated at high temperature using 40% NaOH to get Chitosan which has wide industrial applications. Extracted Chitosan samples were checked for its solubility in 1% acetic acid solution. Chitosan has ability to dissolve in dilute acids. Standard curve for Chitosan was prepared using DNSA Assay and concentration of unknown Chitosan was determined. Based on standard curve, Chitosan extracted from prawn shells using isolates S1C6 and S1C8 has the highest concentration that is 1664µg/ml and 1640µg/ml. Dye adsorption capacity was checked of all the extracted Chitosan samples for two different concentrations of dye solutions. Chitosan samples extracted using S1C6 and S1C8 showed good dye adsorption capacity of 86.73% and 88.32% for dye concentration of 1mg/100ml and 90.32 and 92.47 for dye concentration of 0.1mg/100ml. The different concentrations of Chitosan were also used to check for antibacterial activity against *E. coli* and *S. aureus* and

none of the concentrations showed antibacterial activity against tested pathogens.

Based on the results of Gram staining and biochemical tests, colony B6 was partially identified as *Staphylococcus*. Data acquired from the biochemical tests was insufficient for the identification of other three bacterial isolates.

CONCLUSION

Chitin extraction using biological method is eco-friendly, cost effective and not a time consuming method. Four bacterial colonies isolated out of 23 from Seaweed and juvenile mussel samples were showing good proteolytic activity and were efficient in extraction of chitin. Due to more applications and solubility of chitosan in dilute acids, chitin was deacetylated to chitosan using 40% NaOH at high temperature. Extracted chitosan was soluble in 1% acetic acid. The concentration of chitosan was checked using colorimetric method. Chitosan extracted from prawn shells showed higher concentration of chitosan. Extracted chitosan samples showed good dye adsorption capacity. Chitosan extracted using isolates S1C6 and S1C8 from *Ulva intestinalis* showed best results for dye adsorption.

Present work has good potential to reduce the fish industry waste in to the environment thus making safe environment for the life as well as it is a sustainable eco-friendly process. Derived chitosan has wide application in various sectors.

FUTURE PROSPECTS

1. Comparison of the efficiency of Chemical and biological method of Chitin and Chitosan extraction.
2. To check for the more industrially important applications of chitin and chitosan
3. Degradation of other crustacean and fish scale waste.

APPENDIX

APPENDIX-I

(MEDIA)

1. Zobell Marine Broth

Composition	Grams/Litre
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	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 (at 25°C)	7.6±0.2

40.25 g is suspended in 1000 ml distilled water. Dissolve the media by heating. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes. Mix well and dispense as desired.

2. Zobell Marine Agar

Composition	Grams/Litre
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	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
Agar	15.000
pH (at 25°C)	7.6±0.2

55.25 g is suspended in 1000 ml distilled water. Dissolve the media completely by heating. Sterilize by Autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes. Mix well and pour in sterile Petri plates.

3. Sea water based agar containing 2% Skim milk

Composition	Grams/L
Sodium chloride	28.13
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	40ml
Agar	20
Final pH (at 25^0 C)	7.5±0.5

Sterilised 40 ml Skim Milk separately and added to 960ml of the sterile media before pouring plates.

4. Sea water based agar containing 1% Calcium Carbonate

Composition	Grams/L
Sodium chloride	28.13
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Agar	20
Calcium Carbonate	10

5. Sea water based media containing 1% Colloidal chitin

Composition	Grams/L
Sodium chloride	28.13
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Agar	20
Colloidal Chitin	10

All the contents dissolve in volumetric flask and make up the volume to 1000ml and sterilise by autoclaving at 15lbs pressure at 121^0 C.

6. Sea water based broth containing 1% Glucose and 1% Prawn/ Crab shells

Composition	Grams/L
Sodium chloride	28.13
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

Sterilise 10g Prawn / Crab shells separately and then add to the sterilised media

7. Sea water based broth containing 5% prawn/ Crab shells, 10% Glucose

Composition	Grams/L
Sodium chloride	28.13
Potassium chloride	0.77
Calcium chloride 2H2O	1.60
Magnesium chloride 6H2O	4.80
Sodium bicarbonate	0.11
Magnesium sulphate 7H2O	3.50
Glucose	100

Sterilise 50g prawn/ crab shells separately and add to 1000ml sterilised media

8. Nutrient broth

Composition	Grams/litre
Peptone	10
Beef extract	10
Sodium Chloride	5
Final pH	7.3±0.1

Suspend 25 grams in 1000ml distilled water. Sterilise by autoclaving at 15lbs pressure (121^0C) for 15 minutes.

9. Mueller Hinton Agar

Composition	Grams/litre
Beef extract	300
Casein Acid hydrolysate	17.5
Starch	1.5
Agar	17
Final pH (25^0 C)	7.3±0.1

Suspend 38 grams in 1000ml distilled water. Boil to dissolve the medium completely. Sterilise by autoclaving at 15lbs pressure (121^0C) for 15 minutes.

MEDIA FOR BIOCHEMICAL TESTS

10. Glucose Phosphate broth (MR-VP Medium)

Composition	Grams
Peptone	7
Dipotassium phosphate	5
Dextrose	5
Distilled Water	100ml
pH	7.4±0.2

11. Tryptone broth (Indole Test)

Composition	Grams
Tryptone	1
NaCl	0.5
Distilled water	100ml
pH	7.2±0.2

12. Nutrient Agar (Motility)

Composition	Grams
Nutrient Broth	2.5
Distilled water	100ml
Agar	0.4
pH	7.4

13. Hugh leifson medium

Composition	Grams/litre
Peptone	2
Sodium Chloride	5
Dipotassium phosphate	0.3
Glucose	10
Bromothymol blue	0.03
Agar	20
pH	7.1±0.2

Suspend 20.33 grams in 1000ml distilled water. Boil to dissolve the medium completely. Dispense into test tubes in duplicate for aerobic and anaerobic fermentation.

14. Peptone Water (Sugar fermentation)

Composition	Grams/litre
Peptone	10
Sodium chloride	5
Phenol red	10
Final pH	7.2±0.2

APPENDIX 2

(REAGENTS)

1. 1% Congo red solution

Composition	Grams
Congo Red	1
Distilled Water	100ml

2. 1M NaCl solution

Composition	Grams
NaCl	5.844
Distilled water	100ml

3. 40% NaOH solution

Composition	Grams
NaOH pallets	40
Distilled water	100ml

4. 1% Acetic acid

Composition	Quantity
Acetic acid	1ml
Distilled water	100ml

5. 1N HCl

Composition	Quantity
Concentrated HCl	8.33ml
Distilled water	91.67ml

6. 0.5M $NaNO_2$

Composition	Grams
$NaNO_2$	3.45
Distilled water	100ml

7. 3,5 Dinitrosalicylic acid (DNSA) reagent

Composition	Grams
NaOH	2
Crystalline Phenol	0.2
DNSA	0.2
Distilled Water	100ml

Dissolve NaOH in 50ml of distilled water and add Phenol, DNSA and stir on magnetic stirrer to completely dissolve DNSA powder.

8. Saline (0.85%)

Composition	Grams
NaCl	0.85g
Distilled water	100ml

9. Phosphate buffered saline

Composition	Quantity
NaCl	137mM
KCl	2.7mM
Na_2HPO_4	10mM
KH_2PO_4	1.8mM

10. Methyl Red reagent (for MR test)

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

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