Chitinase Production And Activity Of Halotolerant Bacteria Isolated From Ribandar Salt Pans

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MARINE MICROBIOLOGY

BY

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DECLARATION

I hereby declare that this work titled “Chitinase Production And Activity Of Halotolerant Bacteria isolated from Ribandar salt pans” incorporated in this dissertation, which is in partial fulfillment of the requirement of the M.Sc. Marine Microbiology Degree, is a record of the original work done by me under the guidance of Dr. Nikita Lotlikar, Assistant Professor, Marine Microbiology, School of Earth, Ocean and Atmospheric Sciences, Goa university, Taleigao, Goa and has not formed the basis for the award of any Degree/ Diploma/ Associateship/ Fellowship or any similar title to any candidate of any university.

Date:

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Chitinase Production and Activity of Halotolerant Bacteria Isolated from Ribandar Salt Pans" is a bonafide work carried out by Ms Samixa Jana Sawant under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

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CERTIFICATE

This is to certify that the project titled "Chitinase Production And Activity Of Halotolerant Bacteria isolated from Ribandar salt pans" is a bonafide and authentic record of the research carried out by Samixa J. Sawant, student of M.Sc. Marine Microbiology , during the period of January 2023- May 2023, under my supervision and guidance at the School of Earth, Ocean and Atmospheric Sciences, Goa university, Taleigao, Goa, in partial fulfilment for the award of the degree M.Sc. Marine Microbiology of the university has not been submitted before any other degree or diploma in any other university.

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CHAPTER 1

1. INTRODUCTION

The term "marine microbe" covers various microorganisms which includes microalgae, bacteria and archaea, protozoa fungi, viruses and also can be prokaryotes and eukaryotes. These organisms are exceedingly small, only 1/8000th the volume of a human cell and spanning about 1/100th the diameter of human hair (https://ocean.si.edu/ocean-life/microbes/marinemicrobes). Marine bacteria are single celled organisms having sphere or rod shape or (less commonly)

spirals. All sorts of chemical processes are performed by them in the open ocean, including most of the steps in the nitrogen cycle. Marine bacteria are predominantly gram negative and motile. They occur at low concentrations in water and higher ones are in the bottom sediments. Based on the optimal salt concentration in order to live, microorganisms are classified as halotolerant and halophilic bacteria and they inhabit broad ranges of salty habitats. Halotolerance is a term that refers to the ability to survive or thrive at salt concentrations higher than those necessary for growth. A microorganism is called extremely halotolerant when the growth range is above 2.5 M salt (Antón, 2011). In nature, halotolerant organisms can be found in saline waters and soils that are inhabited by autochthonous halophilic microbiota or even in association with animals (Josefa, 2011). Halotolerant species tend to live in areas such as hypersaline lakes, coastal dunes, saline deserts, salt marshes and inland salt seas (Josefa, 2011). Halophiles are organisms that live in highly saline environments and require salinity to survive ("Halophiles and Their Biomolecules: Recent Advances and Future Applications in Biomedicine - PMC"). Halotolerant microorganisms are considered to be of biotechnological interest. Halophiles are organisms represented by archaea, bacteria and eukarya for which the main characteristic is the salinity environment "salt loving"(Ma et al., 2010). They constitute the natural microbial communities of hypersaline ecosystems and are widely distributed around the world, they also require sodium ions for their growth and metabolism (Corral et al., 2019). Thus, based on the NaCl optimal requirement for the growth the halophiles are classified into three categories: slight, moderate and extreme (1-3%, 3-15% and 15-30% respectively) (Corral et al., 2019). The tolerance and salt requirements are based on certain parameters such as temperature, pH, and growth medium and in such a way halophiles are adapted and limited by specific environmental factors. A halophilic microorganism can also be alkaliphile, known as haloalkaliphilic, growing optimally or very well at pH values above 9.0, but cannot grow at the neutral pH value of 6.5 (Corral et al.2019).

Some common features of halophilic microorganisms are 1) the low nutritional requirements, 2) resistance to high concentrations of salt with the capacity to balance the osmotic pressure of

the environment. They are physiologically diverse, aerobic as well as anaerobic, heterotrophic, phototrophic and chemoautotrophic (Corral et al.2019). In the ecosystem the halophilic microorganisms inhabit salinity higher than seawater i.e. 3.5% NaCl , these niches go from hypersaline soils, springs , salt lakes and other naturally occurring coastal saline habitats, marshes, marine abyssal sediments to endophytes (Corral et al.2019).

The marine microorganisms produce many and various types of enzyme systems to adapt to the complicated marine environment. Marine microorganisms attract more attention as a resource for new enzyme because the microbial enzyme are more stable and active than the enzymes derived from plants or animals (Zhang and Kim,2010). In recent years, researchers have isolated many enzymes with special activities from microorganisms such as marine bacteria, actinomycetes, fungi and more (Zhang and Kim, 2010). Enzymes such as protease, lipase, esterases, nucleases, hydrolases, chitinases, xylanases have application in food and pharmaceutical industries, biofuel production and bioremediation of waste water.

Chitin, which is found in the cell walls of some fungi and the shells of crustaceans, is the second most prevalent polysaccharide in nature after cellulose. Because chitinolytic enzymes called "Chitinases" are present, chitin does not accumulate in the environment (Mesbah, 2022). Four different forms of chitinases are known to be produced by a wide variety of bacteria, including Serratia and Bacillus (Rathore and Gupta,2015). It is known that the majority of filamentous fungi manufacture 20 distinct chitinases. N-acetylglucosamine is a polymer that is joined by 1,4-glycosidic linkages to form chitin. There are three different types of this crystalline polysaccharide that are found in nature: : α-chitin, β-chitin, and γ-chitin.. Because the chitin chains are arranged in an antiparallel manner, which encourages strong hydrogen bonding αchitin is the most prevalent, isomorphic, and more compact version of the protein. β-chitin that is loosely packed, and that is placed in parallel with weaker intermolecular interactions results in a less stable structure. The third polymorphic form, γ-chitin, is a combination of α- and βchitins (Rathore and Gupta, 2015). Since chitin is closely linked to lipid pigments, proteins, and minerals like calcium carbonate, demineralization and deproteinization of

chitinous waste by a strong acid or base are required steps in the production of chitin. "Endo-chitinase" and "exo-chitinases" are the two primary classifications of chitinolytic enzymes. Endo Chitinases are a group of chitinases that randomly cleave internal chitin sites to produce low molecular mass multimers of glucosamine residues such chitotriose, chitobiose, and diacetylchitobiose. Chitotriosidase, which catalyses the gradual release of diacetylchitobiose from the terminal non-reducing end, and N-acetylglucosaminidase, which breaks down the oligomeric products produced by endo chitinases into monomers of N-acetyl glucosamine, are the two groups into which exo chitinases have been divided (Mohan et al. 2020).

1.1 Role of Chitinases in Various Organisms

These chitinolytic enzymes are necessary to organisms for three distinct processes. (A) Organisms have a thick covering of chitin, and during developmental stages, chitinases are expressed to aid in modifying the structure of their exoskeleton in order to support and maintain body size and shape. (b) The insoluble chitin polymer is broken down into absorbable metabolites, which provide energy, by creatures that ingest other chitin-containing organisms as a source of nutrition. (c) Organisms that are prone to chitin-coated microorganism infection express chitinases to break down the invading pathogens' protective covering, resulting in immunity (Rathore and Gupta, 2015).

1.2 Applications of Chitinases

There are several practical uses for chitinases. In the field of biotechnology used in waste management, agricultural pest control, and human health care, chitinases are becoming more significant.

- Waste management: Chitinous biomass, or the chitinous waste of marine life, can be processed by recombinant chitinases into simpler, more profitable depolymerized components, thereby reducing water pollution. Additionally, chitinase can be utilised to turn chitinous waste into biofertilizers. (Rathore and Gupta ,2015).

- Biocontrol agents: Chitinase is a direct biopesticide that can be used against a variety of fungus and insects in place of chemical pesticides. Chitin plays a significant part in insect metamorphosis as well as in the gut of insects, therefore chitinase can be employed as a target for biopesticide in addition to being a biocontrol agent (Rathore and Gupta, 2015).
- Medical application: In the treatment of various fungal infections, chitinase is utilised as an antifungal agent in conjunction with antifungal medications. It has also been proposed that chitinase be utilised to identify invasive human fungal infections.

Because of their anticancer, wound-healing, and antihypertensive properties, chitooligosaccharides also offer a significant amount of pharmacological potential for application in human therapeutics (Rathore and Gupta 2015).

1.3 Literature Review

Two chitinases from marine bacterium *Paenibacillus barengoltzii* have been reported in (Fu et al. 2016) studies. *Paenicibacillus barengoltzii* CAU904 was maintained on colloidal chitincontaining agar plate at 4◦C and transferred every 4–5 weeks. Chitinase was produced on incubation for 3 days, the culture broth was centrifuged at 10000 rpm and the supernant was collected and used as crude enzyme for further analysis. In (Saima et al. 2013) screening was performed with bacterial isolates on the colloidal chitin agar medium incubated at 37◦C . Bacterial isolates were selected on the basis of a larger hydrolysis zone after 96 h of incubation. The cultures were centrifuged at 10000 rpm for 15 min at 4◦C and the crude was used for further analysis. Chitinase production was (Annamalai et al. 2010) studied by culturing the bacteria into the production medium which contains 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH2PO4 and 0.01% MgSO4.7H2O. The culture conditions were as follows: temperature 37°C, and 1.0% chitin and 150 rpm. After 72 hours, the shaking flask's culture was harvested, and the cells were separated by centrifuging it at 6000 RPM for 30 minutes at 4°C. In (Saima et al. 2013) cultures were inoculated at 37 ◦C and harvested after 24

hours, centrifuged at 10,000 rpm for 15 min and the supernatant were used. The crude chitinase was precipitated with ammonium sulphate at 60% saturation. 80% saturation ammonium sulphate was used to precipitate the enzyme, which was then dissolved in 50 mM Tris±HCl and dialysed (S.N. Zhou et al. 1999). In the SDS page analysis Protein bands were visualized after staining with Coomassie Brilliant Blue. The molecular mass of the chitinase was approximately 67.0 kDa on SDS-PAGE (Fu et al. 2016). The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 24 kDa. The pattern of enzyme production by each of the four strains inoculated in minimal chitin media at different hours of incubation till 120 hrs (V. Anuradha et al, 2013).

ISaima (et al. 2013) reported the effects of incubation time on chitinase production of two isolates; *A. hydrophila* HS4 produced highest chitinase after 24 h and remains constant up to 48 h, while *A. punctata* HS6 produced maximum chitinase after 48 h of incubation.

Nawani (et al. 2002) reported that *B. cereus*, *B. alvei* and *B. sphaericus* produced highest chitinase after 48 h of incubation. Faramarzi (et al.2009) reported maximum chitinase production at 36 h of incubation by *M. timonae*. The optimal temperature of PbChi67 was determined by its activity at different temperatures (40–80◦C) in 50 mM phosphate-citrate buffer (pH 3.5). Purified chitinase was pre-incubated at different temperatures (40–80◦C)in 50 mM phosphatecitrate buffer (pH 3.5) for 30 min. The optimal temperature of PbChi67 was 60◦C and it was stable up to 55◦C with a thermal denaturing half-life of 43 min at 65◦C(Fu et al. 2016). Optimum temperature for enzyme activity was determined by incubating the reaction mixture at different temperature levels, was incubated at 35, 40, 50, 60, 70, 75, 80, 85, 90 and 95°C for 1 h. The enzyme was active at temperatures between 30 to 50°C and optimum being at 40°C. The enzyme was 100% stable even up to 60°C.and also it exhibited 83% activity at 70°C. At 90°C and above, it completely lost its activity (Annamalai et al. 2010). The enzyme showed optimum temperature at 50 °C. It was stable in the temperature below 40 °C, during 60 and 120 min . On storing at 4 °C, the enzyme was stable for at least 15 days. (S.N.

Zhou et al. 1999). The optimum temperature of enzyme was around 35°C and chitinase was stable at 40°C (Lee et al. 2000). The effect of temperature on activity of the enzyme was determined by

subjecting them to various temperatures ranging from 10°C to 50°C. the temperature optimum for chitinase enzyme is 40°C for the strains *V.aestuarianus* and *S. putrefaciens*. These isolates showed a similar temperature optimum to those of other marine bacteria. the chitinase from *F.odoratus* and *Exiguobacterium* has the temperature optima at 30°C and 10°C (V. Anuradha et al, 2013). In (Saima et al. 2013) different concentrations of substrates (0.1–1.2%) were applied in optimized media and condition to determine the best substrate concentration. In (Souza et al.,2005) the chitinase production was carried out using various substrates concentrations (0.1-1%) in order to determine the best substrate for enzyme production. The colony of the strain was smooth, circular and dirty white. The strain was Gram positive Cocci and it grows between the temperature range of 25 to 45°C. The strain gram positive cocci was negative for indole, methyl red, Vogus Proskuer, catalase test and it is positive for oxidase and nitrate reduction test (Annamalai et al. 2010).

CHAPTER 2

2. Aim And Objectives

2.1. Aim

The present study was conducted to check the chitinase production and activity of halotolerant bacteria.

2.2. Objectives

The experiment has been divided into two objectives:

- To screen pre isolated marine bacteria for chitinase production.
- To check the enzyme activity of chitinolytic bacteria under various physiological parameters.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Sample Collection

Samples were collected from two salt pan sites: Ribandar (15° 30.166 N and 73° 51.245) and Curca (15° 27’27.8” N 73°52’58.3” E), Goa, India situated in Tiswadi taluka and the isolates were inoculated on nutrient agar plates with 2% NaCl. Plates were incubated at room temperature and growth was observed after 24-48 hours.

3.2 Preparation of Colloidal Chitin

Colloidal chitin was prepared from the chitin powder (GRM1356-500G, HIMEDIA). Chitin powder was slowly added with concentrated HCl and kept for overnight at room temperature. Next day chitin was precipitated as colloidal suspension by adding chilled water and kept overnight at 4°C. Then suspension was filtered by using filter paper and making the pH 7. After pH became 7 the sample was freeze dried, (Lab conco 2.5 L freeze dryer, Benchtop). After the above treatment, the loose colloidal chitin was used as a substrate.

3.3 Screening of pre-isolated marine bacteria for chitinolytic activity.

All the sub-cultured cultures were screened on colloidal chitin agar medium incubated at 37°C. Bacterial cultures streaked onto agar plates were incubated at room temperature for 6-7 days and observed for the hydrolysis zone around the colony.

3.4 Isolation of Chitinase from bacterial cultures

Chitinase positive bacterial isolates retrieved after screening were inoculated into 250ml minimal media containing colloidal chitin and were incubated at room temperature at 120 RPM for 24 hours. Culture broths were centrifuged at 6000 RPM for 20 mins and supernatant was separated in a clean 50 ml centrifuge tube and was used for protein precipitation.

3.5 Protein precipitation using ammonium sulfate

In this method proteins are precipitated based on their solubility in varying salt concentration . Crystalline ammonium sulfate was added to the collected supernatant to achieve 70% saturation and was mixed thoroughly and was placed at 4°C overnight. Next day the supernatant was centrifuged at 8000 RPM for 20 mins. Supernatant was

discarded and precipitate was resuspended with 4 ml of phosphate buffer pH 7.0 and further purification of proteins was done using dialysis method.

3.6 Desalting of protein by dialysis

Treatment of dialysis tubing was done by placing the dialysis tubing in running tap water for 2-4 hours. The tubing was rinsed with distilled water at 60℃ for 2 min and was directly placed into 0.2% sulphuric acid for 2 min to acidify followed by rinsing with hot water. Further the tubing was placed into fresh distilled water for further use. One end of the tubing was tied with thread and the supernatant was added. Other end of the tubing was tied to a glass rod and the glass rod was placed into a 1 liter beaker containing phosphate buffer saline. Buffer was changed after 3 hours and the entire experiment was conducted in cool condition. Dialysis was removed from the tubing and was used for enzyme assay and protein estimation.

Fig 3.6.1: Dialysis done using phosphate buffer

3.7 Estimation of protein concentration by Folin Lowery's method

Series of Bovine Serum Albumin (BSA) concentrations in six tubes were prepared (50, 100, 150,200, 250, and 300 mg/ml) using the BSA working standard so that 4.5 ml of reagent 1 was added and was incubated for 10 min. After incubation 0.5ml of reagent 2 was added and incubated for 30 min. Absorbance was measured at 660nm.

3.8 Estimation of sugar by 3-5 dinitrosalicylic acid (DNSA) method

Glucose standards (100-700µg) were prepared from glucose stock (1000μg/ml) solution to each tube's substrate, buffer and enzymes were added according to the appropriate amount. Tubes were incubated at 37℃ for 15 min and 1 ml of DNSA reagent was added and the tubes were kept in boiling water for 5 min. 10 ml of distilled water was added to each tube and absorbance was measured at 540 nm.

➔ **Calculations:**

Chitinase Activity(U/ml)= μg of product released ✕1000 ➗ Molucular weight of the product ✕ Incubation Time

Specific Activity= Enzyme Activity ➗ Protein concentration

3.9 Characterisation of Chitinase enzyme

Characterisation of enzymes was carried out with different parameters such as temperature, incubation time and substrate concentrations. Followed by DNSA protocol and absorbance was measured at 540 nm.

3.10 Determination of protein molecular weight using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Thoroughly clean and dry glass plate of the electrophoresis unit and wipe it clearly with alcohol. Three spacers were taken from the kit and inserted between the plates and then clamp the plates with clips. Put the tape on the glass plate to seal the sides of the plate. Take 1.2% 50 ml agar agar and pour into the plate, then add resolving gel and wait for 30 min, then add 1 ml butanol in order to get fine layers. Once the gel solidify remove butanol and add a little amount of distilled water to clean it properly and remaining will clean using blotting paper. Then pour stacking gel and immediately insert comb and allow to set for 30-40 minutes. After 40 minutes assemble the plates on the vertical gel

electrophoretic unit, and running buffer and remove the comb carefully. load the sample into wells and in one well add a standard marker. Then connect to the powerpack and switch on the powerpack, keeping voltage at 80-100 V. When the blue line of the sample reaches the end of the plate, stop the powerpack and remove the plates carefully. Remove the gel carefully in a separate plate containing a staining solution(coomassie brilliant blue) and keep it on an orbital shaker overnight. Next day remove the staining solution carefully and add destaining solution and keep it on the shaker till the bands can be seen. At last wash it with distilled water and observe the bands.

3.11 Gram Staining

Loopful of suspension was taken on a clean grease slide. Suspension then air dried and heat fixed. A primary stain (crystal Violet) added for 1 min. Water wash is given to remove unbound stains. Gram's iodine is added and kept for 1 min then decolorized for 30 sec. with alcohol to remove the crystal violet stain. Later the secondary stain (safranin) was added for 1 min. and water wash was given. Air dry the slide, Take a drop of oil on the slide and observe the slide under oil immersion lens.

3.12 Biochemical Tests

Biochemical tests were performed as described in Bergey's Manual of Determinative Bacteriological.

- **Catalase test**

  In a clean test tube pour 1-2 ml of hydrogen peroxide solution. Then using a nichrome loop, take several colonies of 18-24 hours test organism and immerse in the hydrogen peroxide solution. observe for immediate bubbling.

- **Indole test**

  Take sterile test tubes containing 4 ml of tryptophan broth, inoculate the tube aseptically by taking the growth from 18-24 hours cultures. Then incubate the

tubes at 37℃ for 24 hours. Then add 0.5 ml of kovac's reagent to the broth culture. Observe the presence and absence of rings.

- **Motility test**

  Touch a straight needle to a colony of 18-24 hours culture growing on agar medium, stab once to a depth of half inch in the middle of the tube. Be sure to keep the needle in the same line it entered as it is removed from the medium.

  Incubate at 35℃- 37℃ and examine. Observe a diffuse zone of growth flaring out from the line of inoculation.

CHAPTER 4

4.RESULTS AND DISCUSSIONS

4.1. Subcultures plates from previous batch student

➔ More than 150 cultures were pre-isolated from salt pans.

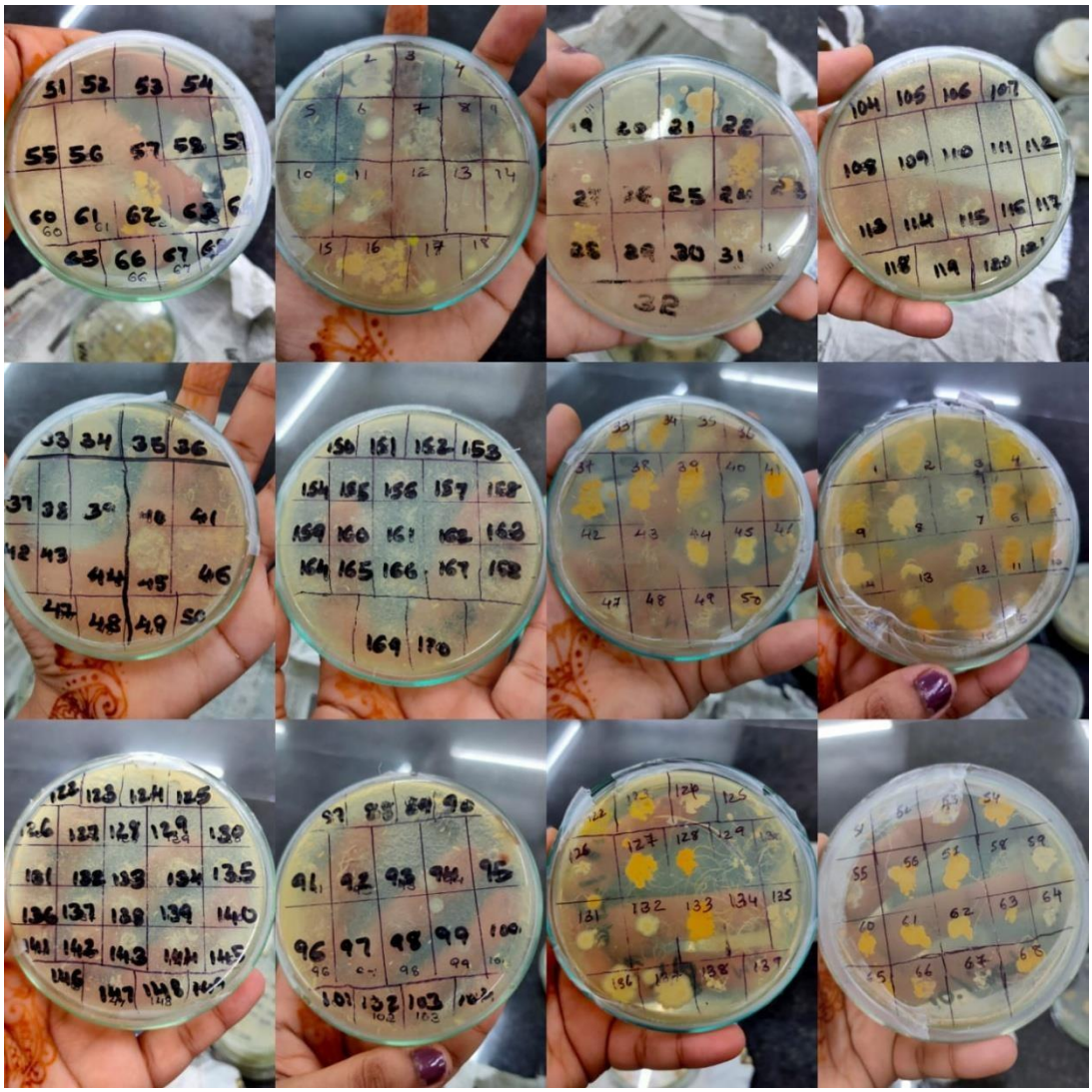
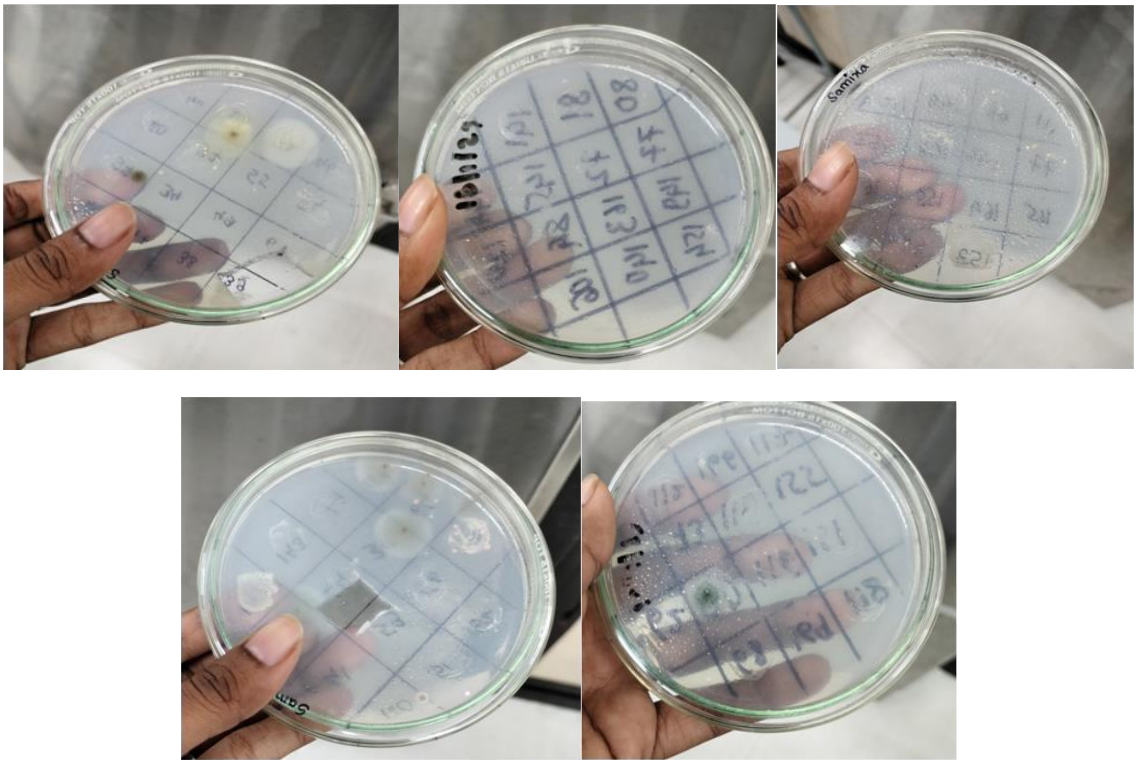


Fig 4.1.1: Subcultures plates.

4.2Preparation of colloidal chitin

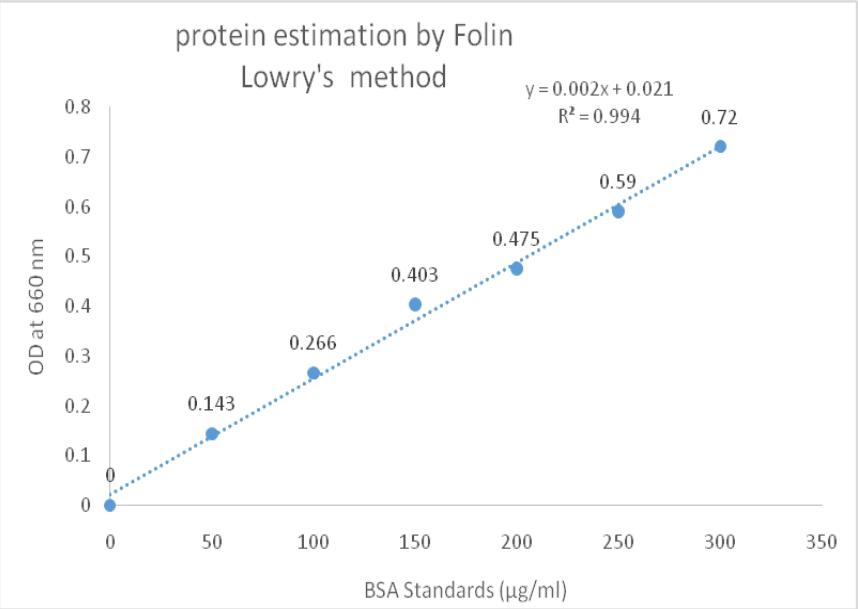
Fig 4.2.1: Colloidal chitin was obtained by freeze drying

4.3.Screening for chitinolytic production from pre- isolated marine bacteria



→ So among 150 bacterial cultures 5 were showing growth positive for chitinase enzymes after 6-7 days i.e. 153, 147, 120, 64, 50. In (Fu et al. 2016) the cultures showing positive for chitinase enzyme after 3-4 days. In (Saima et al, 2013.) two bacterial isolates were showing hydrolysis zone after 4 days of incubation.

4.6 Protein concentration by Folin Lowry's method

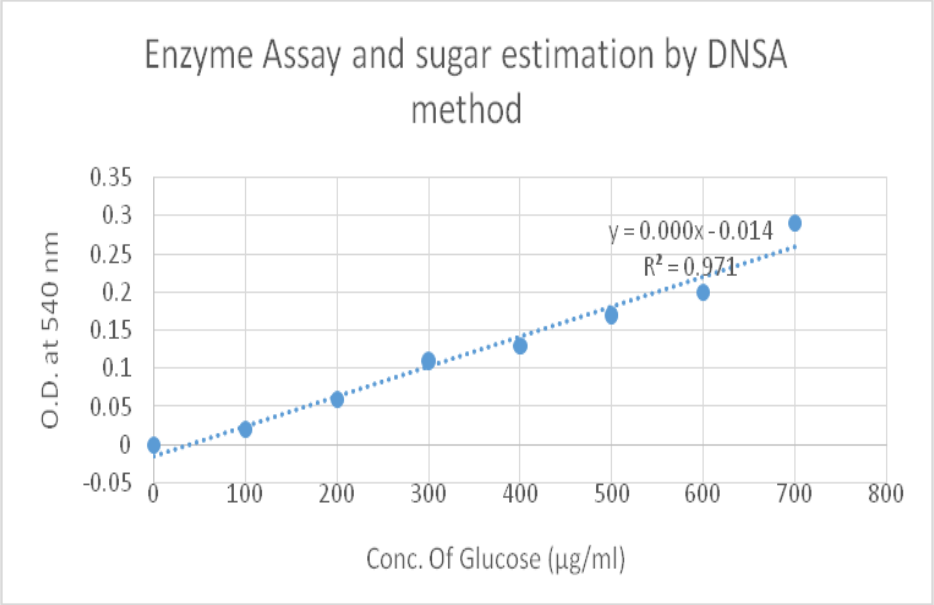


Graph 4.6.1: Graph of Protein estimation by Folin Lowry method.

Cultures	Protein Concentration (µg/ml)
153	6.347
147	29.826
120	20.260
64	18.956
50	10.260

Table 4.6.1: Protein estimation of the samples.

4.7 Estimation of sugar by 3-5 dinitrosalicylic acid (DNSA) method



Graph 4.7.1: Graph of standard v/s absorbance at 540 nm

Cultures	Enzyme unit	Enzyme activity	Specific activity
153	0.3394	0.4261	0.1386
147	0.3398	0.4452	0.1388
120	0.3396	0.4452	0.1384
64	0.3398	0.4452	0.1381
50	0.3406	0.4322	0.1388

Table 4.7.1: Enzyme unit, enzyme activity and specific activity of the cultures.

4.8 Characterisation of enzyme

● **Incubation time in minutes**

Enzyme activity of the cultures over incubation time

Cultures	**5**	**15**	**30**	**60**	**120**	**180**
153	32.27	60.24	98.68	21.55	13.66	8.64
147	69.93	23.31	112.40	16.23	7.77	10.34
120	44.96	102.87	81.50	56.43	18.52	13.81
64	61.61	90.84	110.64	34.04	18.87	14.58
50	69.93	102.87	56.52	18.78	12.85	9.80
EC	158.75	79.74	54.67	21.09	13.66	7.57
SC	167.07	76.97	30.62	20.39	8.81	8.49

Table 4.8.1: Enzyme activity of cultures and its incubation time.

➔ The incubation time showed better results when the reaction mixture was incubated for 30 min or 15 min for isolates and hence better enzyme activity was reported. Where as least enzyme activity was shown when the reaction mixture was incubated for 60 min, 120 min, and 180 min. In (Saima etal.2013) the incubation time on chitinase production of two isolates is highest after 24 h- 48 h. In (Nawani et al. 2002) maximum chitinase production at 36 h of incubation. In order to compare all the results my data showed the best enzyme activity at 30 mins.

• **Temperature ˚C**

Enzyme activity of the cultures over temperatures

Cultures	**18**	**22**	**37**	**40**	**50**	**55**

153	0.0429	0.0435	0.0551	0.0475	0.0542	0.0485
147	0.0441	0.0445	0.0511	0.0479	0.0538	0.0625
120	0.0452	0.0454	0.0549	0.0468	0.0530	0.0498
64	0.0460	0.0462	0.0612	0.0473	0.0525	0.542
50	0.0469	0.0469	0.0599	0.0479	0.0521	0.486
EC	0.0477	0.0450	0.0407	0.0437	0.0536	0.0633
SC	0.0452	0.0431	0.0609	0.0500	0.0506	0.0479

Table 4.8.2: Enzyme activity of the cultures and its temperature.

➔ The best temperature which gave the best result was 37℃ for isolates and hence shows better enzyme activity. The least activity was observed at 18℃ and 50℃. In (Fu et al. 2016) the optimal temperature of PbChi67 was 60◦C and it was stable up to 55◦C. In (Annamalai et al. 2010) the enzyme was active at temperatures between 30 to 50°C and optimum being at 40°C. The enzyme was 100% stable even up to 60°C.

- **Substrate Concentration(%)Enzyme activity of the cultures over a substrate concentration (%)**

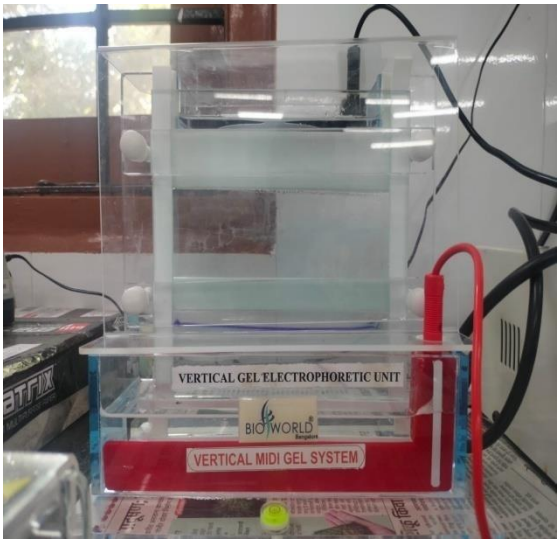
Cultures	**0.5**	**1**	**2**	**3**	**4**	**5**

153	0.0513	0.0492	0.0664	0.0283	0.0342	0.612
147	0.0528	0.0538	0.0587	0.0559	0.0532	0.0549
120	0.0576	0.0492	0.0574	0.0342	0.0456	0.0449
64	0.0563	0.0568	0.0603	0.0371	0.0403	0.0587
50	0.0494	0.0570	0.0532	0.0291	0.0361	0.0285
EC	0.0534	0.0513	0.0662	0.0291	0.0361	0.0285
SC	0.0540	0.0502	0.456	0.0304	0.0567	0.0407

Table 4.8.6: Enzyme activity of cultures and its substrate concentration (%)

The best results obtained were at the substrate concentration 2%, in isolates. The least was observed at the substrate concentration 3%. Enzyme activity increase with increase in substrate concentration. In (saima et al.2013) showed *A. hydrophila* HS4 and *A. punctata* HS6 strains produced enzyme maximally at 0.3% of colloidal chitin. In (Souza et al.,2005) maximum chitinase production at 0.3% colloidal chitin.

4.9.Determination of protein by SDS-PAGE



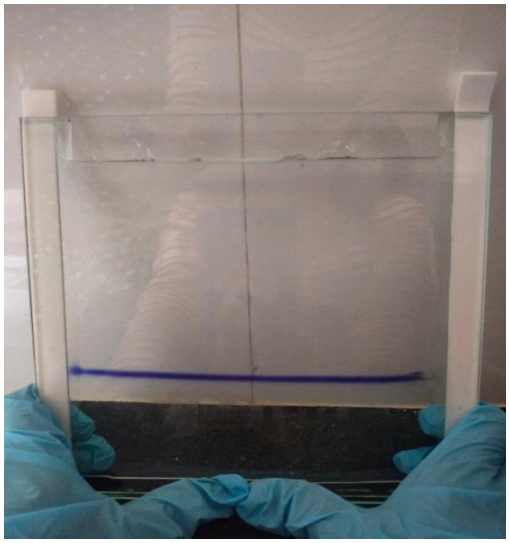
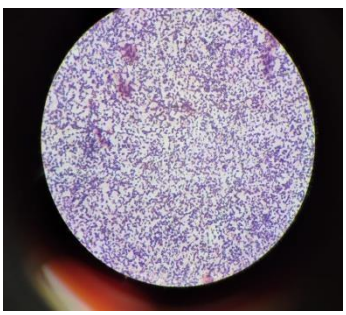
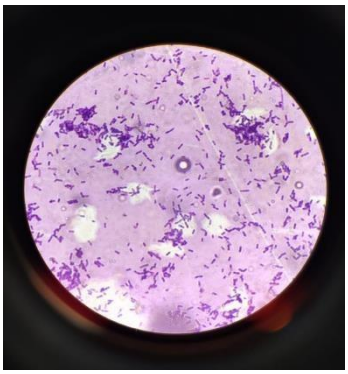
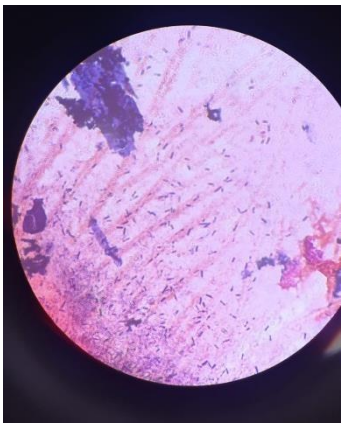
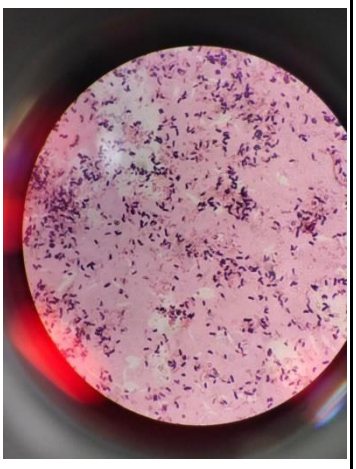


Fig 4.9.1: Experimental setup of SDS-PAGE

➔ Bands were not visible on the gel and only one band of the standard protein marker was visible because protein concentration is too less. In (V. Anuradha et al, 2013) purified enzyme revealed a single band on SDS-PAGE gel.

4.10 Gram staining

Cultures	**Microscopic images**	**Gram characters**
153		Gram positive cocci in clusters

147		Gram Positive short rods.
120		Gram Positive short rods
64		Gram positive short rods

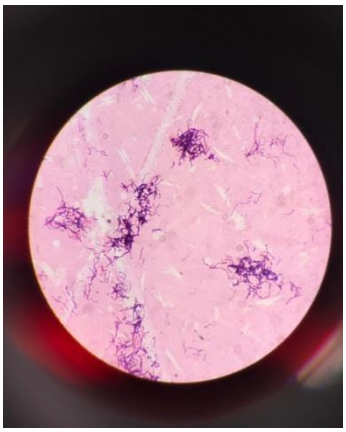
50		Gram Positive bacilli

Table 4. 10.1: Gram characters of the bacterial cultures observed under microscope.

➔ Among 5 cultures 3 were gram positive rods (147,120,64), one is gram positive cocci (153) with clusters and one is gram positive bacilli(50).

4.11 Biochemical test

Cultures	**Catalase test**	**Indole test**	**Motility test**
153	Positive	Negative	Non -motile
147	Negative	Negative	Non -motile
120	Negative	Negative	Non –motile
64	Negative	Negative	Non –motile
50	Positive	Negative	Motile

Table 4.11.1: Biochemical tests (Catalase, Indole, Motility) were performed and the results observed.

In catalase test culture 153 and 50 is positive whereas 147,120 and 64 is negative. In the indole test all cultures were negative and in the motility test only culture 50 is motile and others are non motile. In (Annamalai et al. 2010) the strain gram positive cocci was negative for indole, methyl red, Vogus Proskuer, catalase test and it is positive for oxidase and nitrate reduction test. In order to my catalase test for gram positive cocci with cluster showing positive.

CHAPTER 5

SUMMARY

The objective of the project was to isolate chitinolytic bacteria from marine acquired bacteria. Protein estimation and sugar estimation methods were carried out by Folin Lowry and DNSA respectively to determine the enzyme activity and specific activity of the chitinase enzyme. The longer the time for an enzyme incubation with the substrate the more the product formed. Protein suffers from denaturation so there is loss of catalytic activity observed, also that the maintenance of the stable temperature is obligatory if not than protein will be denatured with higher temperatures. In SDS-PAGE , the reason for the invisibility of the bands could be due to the low concentration of the protein in the sample. It must be too less or maybe degradation of the protein might have occurred.

CONCLUSION

Chitinase plays an important role in the degradation of chi-tin and potentially in the utilization of chitin as a renewable resource. Halophilic bacteria which contain chitinase enzymes are able to grow in the minimal media containing colloidal chitin as they break down the chitin and utilise it for its growth and development. From the results obtained, we can conclude that it takes around 6-7 days for the bacterial culture having chitinase enzyme to utilize chitin. Among all the 5 cultures, culture 147,120,and 50 showed the highest enzyme activity which implies

that it contains higher concentration of chitinase enzymes which break down the chitin (substrate) present. Halophilic bacteria containing chitinase enzyme show highest enzyme activity at incubation time of 30 minutes, temperature 55℃ and having substrate concentration of 2%. Chitin-containing halophilic bacteria can be utilised to depolymerize and simplify chitinase waste from marine creatures, hence minimising water pollution.

CHAPTER 6

6. Annexure

- **Minimal media composition**

Composition	**Concentration(g/l)**
Potassium Nitrate	4.002
Monopotassium Phosphate	4.083
Disodium Phosphate	7.119
Magnesium Sulfate	0.197
Calcium Chloride	0.00077
Ferrous Sulfate	0.0011
Manganeses Sulfate	0.00067
Na-EDTA	0.00148

Table 6.1: Composition of minimal media and its concentrations

- **Folin Lowery's method reagents**

  A. 2% Na_2CO_3 in 0.1 N NaOH

  B. 1% NaK Tartrate in H_2O

  C. 0.5% $CuSO_4.5\ H_2O$ in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water ● **Phosphate Buffer**

Disodium Hydrogen Phosphate 20.214g

Sodium dihydrogen phosphate 3.394 g

mix the above ingredients in a minimum amount of distilled water, adjust the pH by using HCl and NaOH and bring the volume to 1 L.

● **DNSA Reagent**

Solution 1: 20 ml of 2N NaOH (1.6g in 20 ml distilled water) + 1g of DNS powder.

Solution 2: Dissolve 30 g of sodium tartrate in 50 ml of distilled water.

slowly pour solution 2 in solution 1 and make up the volume to 100 ml of distilled water.

● **SDS- PAGE Reagent**

40% Acrylamide (37.5.1)

Acrylamide 116.8g

N,N- Methylene bis acrylamide 3.2g

DDI water 300ml

Filter and store in dark bottle at 4°C

30% Ammonium Persulphate

Ammonium Persulphate 1.5g DDI

water 5 ml

store at 4°C. use freshly prepared

RG (Running Gel) Buffer 1.5M Tris HCl, pH 8.8

DDI Water 300ml

Tris- free base 90.75g

Conc. HCl 8 ml

Adjust pH to 8.8 with conc. HCl and bring final volume to 500ml with DDI water

SG (Stacking gel) 1.0M Tris HCl, pH 6.8

DDI Water 300 ml

Tris free base 60.54ml

Conc. HCl 36ml

Adjust pH to 6.8 with conc. HCl and bring volume to 500ml with DDI water.

4X SDS-PAGE Sample Buffer

125mM Tris HCl, pH 6.8 5ml

20%Glycerol 8ml

10% Beta Mercaptoethanol 4ml

4% SDS 8ml

0.5mg/ml Bromophenol blue 20mg

DDI Water 15ml total

40 ml

10X SDS-PAGE Running Buffer

Tris base 30.3g

Glycine 144g

SDS 10g

Dissolve and bring volume to 1000 ml with deionised water

10% Resolving Gel

DDI WATER 1.6ml 11.5M

Tris HCl, pH 8.8 (RG Buffer) 1.3ml

40% Acrylamide stock 1ml

20% SDS 100**µ**l

30% Ammonium persulphate 16**µ**l

TEMED 8**µ**l

4% Stacking Gel

DDI water 3.9ml

1.0M Tris HCl, pH 6.8 (SG Buffer) 500**µ**l

40% Acrylamide stock 500**µ**l

20% SDS	100µl
30% Ammonium persulphate	16µl
TEMED	8µl

Coomassie Stain solution

Ethanol	150ml
Glacial acetic acid	50ml
DDI water	300ml
Coomassie Brillient Blue	1g

Destain Solution

Ethanol	1200ml
Glacial acetic acid	400ml
DDI water	2.4 L

CHAPTER 7

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