PRODUCTION OF CELLULOLYTIC ENZYMES

FROM MARINE BACTERIA

Dissertation

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IN

MARINE MICROBIOLOGY

BY

PAMILA FERNANDES

(21P039005)

School of Earth, Ocean and Atmospheric Sciences

Goa University, Taleigao Plateau

Goa, India

APRIL, 2023

DECLARATION

I hereby declare that the work incorporated in this dissertation, titled as “Production of Cellulolytic Enzymes From Marine Bacteria” which is in partial fulfilment of the M.Sc., degree course, is original and carried out in the School of Earth, Ocean and Atmospheric Sciences, Goa University, Goa, and it has not been submitted in any part or in full for any other degree or diploma of any other university.

Date: Candidate’s Signature

Place: Goa University, Pamila Fernandes

Taleigao, Goa

COMPLETION CERTIFICATE

This is to certify that the dissertation report "Production of Cellulolytic Enzymes from Marine Bacteria" is a bonafide work carried out by Ms Pamila Fernandes 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.

Dr. Nikita P. Lotlikar
Marine Microbiology

Date: 02/05/2023

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School Stamp

Dr. Chandrashekher U. Rivonker
Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences
Date:
Place: Goa University

Dr. C. U. Rivonker
Senior Professor and Dean,
School of Earth, Ocean
& Atmospheric Sciences,
Goa University,
Goa - 403 206.

CERTIFICATE

This is to certify that the dissertation entitled as "Production of Cellulolytic Enzymes From Marine Bacteria" is a bonafide and an authentic record of the research carried out by Ms. Pamila Fernandes, student of M.Sc. Marine Microbiology under the supervision and guidance of Dr. Nikita P. Lotlikar, Assistant Professor, at the School of Earth, Ocean and Atmospheric Sciences, Goa University, Goa, in partial fulfilment of the requirement of M.Sc. Marine Microbiology Degree of the University and that no part has been submitted before for any other degree or diploma in any other university.

Date:

Dr. Nikita P. Lotlikar

Assistant Professor,

Marine Microbiology

School of Earth, Ocean and Atmospheric Sciences,

Goa University

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TABLE OF CONTENTS

CHAPTER 1

1. INTRODUCTION

Microorganisms that exist/live in salt water or oceans are called marine microorganisms. They are ubiquitously found in lakes and coastal areas worldwide. Microbes account for more than 90% of the ocean biomass (Smithsonian 2019; Hall, 2019) and out of which only 1% can be grown in laboratory and most of them are non-culturable. The habitats are extremely diverse, which include open waters, estuaries, and hydrothermal vents (Hunter et al. 2005).

Microbes are engines of ecosystems without which there wouldn't be access to food and nutrients. They play a role in the biogeochemical cycling of different elements in the ocean (York, 2018). Microbes can be bacteria, archaea, fungi and even viruses. Bacteria are most abundant and are unicellular prokaryotes, shaped as spheres, rods, or spirals. They are very small, with cell diameters in microns (Smithsonian, 2019; Hall, 2019). Marine bacteria are predominantly Gram negative and motile and occur in low concentrations in water and higher in sediments (Johnson et al. 1968).

Prokaryotes can survive a broad range of ecological stresses, like extremes of temperatures, pressure, salinity, pH, radiation, etc. 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. Halophilic bacteria are the most commonly isolated and studied organisms. Halophiles are microorganisms that require salt (NaCl) for growth, and they can be found in

hypersaline soils, springs, salt lakes, salt pans, and other naturally-occurring coastal saline habitats, marshes, marine abyssal sediments to endophytes (Corral et al. 2019).

- Halophilic organisms: are organisms that live in highly saline environment, and require salinity to survive. Salt concentration: Slight halohiles prefer 0.2-0.5% M NaCl, Moderate halophiles prefer 0.5-2.5 M NaCl, and Exreme halophiles prefer 2.5-5.2 M NaCl (Didari et al. 2020).
- Halotolerant organisms: are organisms that grow in saline conditions, but do not require elevated concentrations of salt for growth (Didari et al. 2020)

Saline Soils: A number of bacterial species dominant, found in such habitats belong to the *Bacillus*, *Pseudomonas*, *Micrococcus* and *Alcaligenes* genera. Saline water: Water with salinity of 3% or above is said to be saline water. Brackish water, sea and oceanic water and water from salt lakes and salterns are all considered as saline water (Ali et al. 2016). These bacteria need low nutritional requirements and resistance to high concentrations of salt with the capacity to balance the osmotic pressure of the environment (Corral et al. 2019). Halophilic bacteria exist in various forms of colonies, ranging from pigmented to non-pigmented, according to the salt concentration in the media. They are slow growing compared to non-halophile or normal bacteria.

1.1. Applications of Halophiles

Halotolerant and Halophilic bacteria are essential for salty foods and have many commercial applications, such as in fermented food products, cosmetics, preservatives, manufacturing of bioplastics, photoelectric devices, biosensors, etc. (Corral et al. 2019). Because of their adaptation to extreme conditions these halophilic/halotolerant bacteria are useful in fields such as agriculture, medicine and biotechnology. These bacteria also produce enzymes like lipases, hydrolases,

nucleases, which have applications in pharmaceutical and food industry (Barzkar & Sohail, 2020).

1.2.History

The first known account of halophilic microorganisms dates back to 2700 BC (Bass-Becking 1931) and reported the isolation of red brine microbes that were found in hypersaline environments. Later in 1936, Benjamin Elazari isolated the extreme halophiles, *Halobacterium trapanicum* and *Micrococcus morrhuae* and the moderate halophiles *Chromohalobacterium marismortui*, *Pseudomonas halestrogus,* and *Flavobacterium halmephium* from waters and shorelines of the Dead Sea (Gunjal & Bandodekar, 2021)

1.3. Adaptive Strategies in extreme marine habitats

Halophiles have developed different adaptive strategies to support the osmotic pressure induced by the high NaCl concentrations in the environments. Halophilic bacteria accumulate inorganic ions (K^+, Na^+, Cl^-) in the cytoplasm, which is a type of "salt-in" strategy to balance the osmotic pressure of the environment, and they have also developed specific proteins that are stable and active in the presence of salts (Poli et al. 2017)

Halophilic proteins bind significant amounts of salt and water. This characteristic is dependent on the number of acidic amino acids on the surface of the protein (Poli et al. 2017).

Another haloadaptations based on biosynthesis and/or accumulation of high amounts of specific organic osmolytes in the cytoplasm, which function as osmoprotectant providing osmotic balance and maintaining low intracellular salt concentrations without interfering in the normal metabolism of the cell (Poli et al. 2017).

1.4. Cell wall degrading hydrolases from marine extremophiles

1.4.1. Cellulase

Hydrolases are enzymes that catalyze reactions with the substrate through the hydrolysis of chemical bonds. And 65% of hydrolases enzymes are used in industrial processes in detergent, textile, pulp, paper and starch industries and 25% are used for food processing.

Cellulose ($C_6H_{10}O_5$) is an organic compound, a polysaccharide containing a linear chain of hundreds to ten thousand β (1→4)-linked D-glucose units. Cellulose is the most available saccharide in nature and is about 50% of all plant matter and hemicellulose is ~20-30% while the remainder is mainly lignin. Bacteria including *Vibrio, Clostridium, Cellulomonas, Streptomyces, cytophaga* can produce cellulase and fungal genera include *Aspergillus, Trichoderma*, *Chrysosporium*, *Penicillium* can also produce cellulase (Dalmaso & Ferreira, 2015). Due to its compactness and crystalline nature of cellulase, it is very resistant to hydrolysis and degradation. Cellulases are group of enzymes that are capable of degrading insoluble cellulose polymers, present in plants, bacteria and fungi, having molecular weight of 20-80 kDa with hydrophilic properties. They hydrolyze cellulose to cellobiose and glucose molecules. Cellulase enzymes are mostly produced for industrial purposes. There are three types of cellulases named according to the position of the glycosidic linkage they hydrolyzeand they are; Endoglucanase, Exoglucanase and Beta Glucosidases (Dalmaso & Ferreira, 2015).

Enzyme activity

Enzyme activity refers to catalytic properties of an enzyme, and enzyme assays are standardized procedures for measuring the amounts of specific enzymes in a sample. The objective of measuring enzyme activity is normally to determine the amount of enzyme present under defined conditions.

The factors that affect the activity of an enzyme are pH, substrate concentrations(s), ionic strength, temperature and nature of salts present (Scopes, 2002).

1.5. Applications of cellulase enzyme

Cellulolytic- biotechnological potential

Cellulase can be used for bio-textile auxiliaries, cotton and linen products processes and biofertilizer processing. Cellulases degrade seaweed processing waste to low molecular fragments which can be easily absorbed by plants as bio-fertilizer (Zhang & Kim, 2010). Cellulases from marine sources exhibit high specific activity, thermostability, high salt tolerance, cold adaptivity and other important biochemical properties (Barzakar & Sohail, 2020).

CHAPTER 2

2. LITERATURE REVIEW

Several characteristics are found in marine bacteria which are rare among non-marine bacteria. The majority of the heterotrophic motile bacteria of seawater were non-pigmented, straight or slightly curved rods. And most were psychrophilic and halophilic. Many of these organisms grow very poorly on the usual media (Leifson, 1963).

A haloarchaeon, *Haloferax sulfurifontis* GUMFAZ2 producing cellulase was isolated from marine *Haliclona* sp., a sponge inhabiting the rocky intertidal region of Anjuna, Goa. This enzyme from haloarchaea have great potential for biotechnological application because of their stability at high salinity (upto 5M) and is therefore worth pursuing (Malik & Furtado, 2019). They also isolated another bacteria in Goa from sediments of Dead Sea-Israel, named *Halomicroarcula pellucida* GUMF5 (Accession number: MH244431), globally, is the only bacteria isolated from the sediments of Dead Sea producing haloextremozyme cellulase, and hence is an important biotechnological resource (Malik & Furtado, 2021). Asha and Sakthivel isolated *Bacillus subtilis* that exhibit halophilic, alkalophilic and solvent-tolerant properties in it (Asha & Sakthivel, 2014). Sulyman et al. isolated cellulase producing fungi that were cultured on *Arachis hypogaea* shells by fermentation using *hypogaea* shells as carbon source. The cellulase was purified by ammonium precipitation, dialysis and gel filtration chromatography. Munoz et al. selected 9 strains having endoglucanase activity and they were characterized on how well they can degrade the cell walls of microalgae by the production of cellulase, when whole-cell organisms were used in experiment and this resulted in increased biogas production. Munoz et al. used cellulolytic bacteria for the

pretreatment of microalgae that degrades the microalgae. And these bacteria were isolated from mollusk species, in a medium containing 1% Carboxymethyl cellulose agar (Munoz et al. 2014).

Nursyirwani et al. 2020, demonstrated that cellulolytic bacteria have ability to degrade cellulose and have potency to inhibit growth of pathogenic bacteria. They isolated bacteria from soil and mangrove ecosystems in Dumai Marine station, Indonesia and examined antagonism against pathogenic bacteria (*Escherichia coli, Pesudomonas aeruginosa,* and *Vibrio alginolyticus*. Nursyirwani et al. used Zobell marine agar with CMC and checked for hydrolysis. Disc diffusion agar method was used to examine antagonism of selected isolates against pathogens. Elsababty et al. 2022, isolated cellulase producing bacteria, *Bacillus licheniformis* strain Z9 from soil in Egypt.

Malik & Furtado, 2019, the culture was grown in CMC-Na for production of xylanase-free cellulase. Malik & Furtado took 0.1 g of sediment sample from Dead Sea and grew that strain on mineral salts medium with 20% NaCl and 0.5% CMC-Na and incubated for 1 week. Malik & Furtado 2021. Asha and Sakthivel, took the soil suspension and spread plated onto carboxymethyl cellulose-Congo red agar medium and incubated at 30ºC for 72 hours. Colonies were examined for clearing zones (Asha & Sakthivel, 2014). The method used for production and extraction of cellulase was done in flasks containing 100 ml medium and this medium composed of *A. hypogaea* shells as carbon source. Flasks shaken on orbital shaker for 7 days at 30ºC (Sulyman et al. 2019). Elsababty et al. 2022, performed DNSA for sugar estimation and carried out optimization, characterization tests in order to know the enzyme activity.

In Elsababty et al. 2022, the reaction mixture while characterization of enzyme, the incubation time was 30 min, at 50ºC, using phosphate buffer. In Sulyman et al. 2019, the reaction mixture containing enzyme and substrate, were incubated for 30 min at 50ºC, along with 0.5 M citrate buffer (pH 4.0).

In Malik & Furtado, 2019 the maximum activity was reported using CMC-Na in 3.5 M NaCl containing medium at pH 7and at 40ºC. The Culture *Haloferax sulfurifontis* GUMFAZ2 from Malik & Furtado, 2019, produced maximum activity of 11.7 U/ml. In the reports of Malik and Furtado 2021, the purified cellulase had the optimum activity at 20% NaCl, at 40ºC, 0.5% CMCNa, and at pH 7. Malik and Furtado 2021.The yield of cellulase was 78.53% with enzyme activity of 131.13 U/mg (Malik & Furtado, 2021). The enzyme activity by *Bacillus subtilis* was determined using 2% CMC, and was active over a broad range of pH and Temperatures. The optimum pH was 8 having enzyme activity 500 U/ml and the optimum temperature was 45ºC yielded 633.3 U/ml. The substrate specificity of enzyme was determined in a reaction mixture of 1% CMC gave activity 675 U/ml (Asha & Sakthivel, 2014). The endonuclease activity of purified cellulase by *A. niger* at optimal pH was 4 having activity of 48.78 u/ml whereas the optimal temperature was 40ºC having 85 U/ml activity and substrate concentration that obeyed Michaelis-Menten type having activity 9.26 U/ml, Sulyman et al. 2019. The optimum temperature of enzyme was 30ºC having enzyme activity of around 100 U/ml. and optimum pH was 7.4 having activity of around 110 u/ml Elsababty et al. 2019.

In Malik & Furtado, 2019, the SDS-PAGE results gave molecular weight of cellulase as 19.6kDa.When the SDS-PAGE was carried out, the molecular weight of the cellulase was 240 kDa, 40 kDa and 17.4 kDa.The molecular weight of the cellulase determined by SDS-PAGE, was 51.6 kDa (Asha & Sakthivel, 2014). The purified enzyme from *A. niger* had molecular weight of 13.5 kDa (Sulyman et al. 2019). The SDS results gave molecular weight as 54.4 kDa in Elsababty et al. 2022.

Since the best enzyme activity of cellulase produced by marine microbes is not best known or still not studied much, the following experiments were carried out to check for the cellulolytic production and its enzyme activity.

3. AIMS AND OBJECTIVES

Aim: The project was conducted to check the Cellulase production and its activity from pre-isolated marine bacteria.

Objectives:

- **To screen pre-isolated marine bacteria for cellulolytic production**
- **To check the enzyme activity of marine derived cellulase under various physiological parameters**

CHAPTER 3

4. METHODOLOGY

4.1. Materials: Bacteria already isolated from salt pans of Goa, i.e., from Ribandar (15º 30.166 N and 73º 51.245) and from Curca (15º 27'27.8" N and 73º52'58.3" E), by growing them on the nutrient agar media and malt and yeast extract agar containing 10% salt concentration, were used for screening of the cellulase production.

4.2. Isolation and Screening of Cellulase producing Bacteria

The pre-isolated bacteria were streaked on plates containing CMCA (Carboxymethyl cellulose agar) media (Sethi et al. 2013), having CMC as a carbon source. The plates incubated at room temperature for 24-48 hours. The plates having bacterial growth were stained with 1% Congo red solution for 15 minutes at room temperature and washed with 1 M NaCl for de-staining (Potprommanee at al. 2017). Clear zones indicated the cellulose hydrolysis.

Note: CMC (Carboxymethyl cellulose) is not easily dissolved and its preparation is greatly affected by factors including temperature and rotational speed. CMC is water absorbent and has very high water retention and therefore clumps easily.

4.3. Identification of cellulolytic bacteria by morphological and biochemical Characterization

4.3.1. Morphological Characterization

Gram staining method (Islam & Roy, 2018) was performed on bacterial isolates that were CMCase positive, to differentiate between gram positive and Gram negative bacteria. And then observed under compound microscope for morphological identification.

4.3.2. Biochemical Characterization

Biochemical tests (Islam & Roy, 2018) performed on the CMCase positive cultures were Motility test, Catalase test and indole test.

- Motility Test: soft agar of 0.4 % was prepared in test tube and the culture was stabbed in the center of the medium, incubated and observed for the growth.
- Catalase test: On a clean glass slide, loop full of culture was placed and a drop of 3% H_2O_2 was added and observed for effervescence.
- Indole Test: culture was inoculated in Tryptophan broth and incubated for 24 hours. Few drops of Kovac's reagent were added and observed for pink ring.

4.4. Preparation of crude enzyme (Cellulase)

The isolates that were found to be CMCase positive were cultured in Luria-Bertani broth medium containing 0.25 % CMC and incubated at 37ºC for 48 hours on orbital shaker (Islam and Roy, 2018).

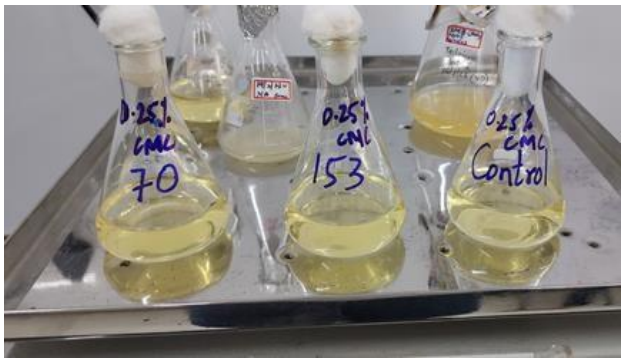


Fig 1: Cultures grown in LB broth containing 0.25% CMC

These cultures transferred to 50 mL centrifuged tubes and centrifuged at 10,000 rpm for 10 min at 4ºC to remove the bacterial cells. The supernatant was used as the crude enzyme and pellet was discarded containing bacterial cells.

4.5. Purification of crude Cellulase

- **Ammonium Sulphate Precipitation**

The crude enzyme (Cellulase) in centrifuge tube, that is the supernatant, was brought to 80% saturation with crystalline ammonium sulphate. Gently mixed and kept overnight at 4ºC. The mixture centrifuged at 10,000 rpm for 20 min and the pellet was dissolved in Phosphate buffer (pH 7.0) and dialyzed overnight in phosphate Buffer (Potprommanee et al. 2017; Islam and Roy, 2018). This is partially purified enzyme.

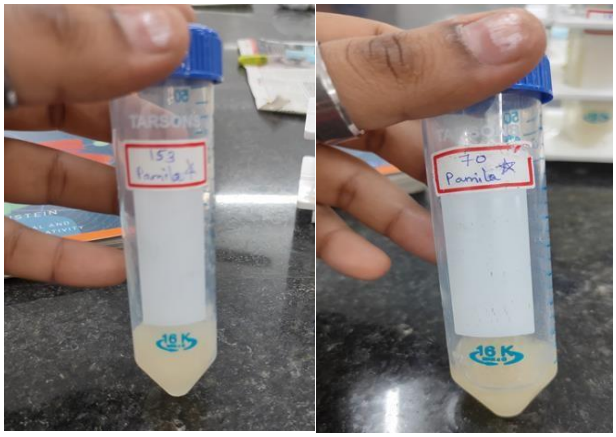


Fig 2: Protein precipitation using crystalline Ammonium Sulphate

- **Protein purification Using dialysis Method**

Requirements: dialysis tubing, 1 L beaker, phosphate buffered saline, phosphate buffer, glass rod, cold room.

Treatment of dialysis tubings is done prior to the purification step, wherein the tubings were cut of desired length and kept under running water for 2-3 hours, and then rinsed in distilled water at 60ºC for 2 min and then place the tubings in 0.2% H_2SO_4 for 2 min to acidify, followed by rinsing with hot water.

For the Dialysis Method: The tubings were then filled with partially purified enzyme i.e precipitate dissolved in phosphate buffer, and kept in phosphate buffered saline (pH 7.4) at 4ºC. This buffer needs to be changed in intervals. Next day transfer the dialysate (purified enzyme) into fresh new tubes. This dialysate was used for further estimation of protein and sugar present.

Fig 3: Dialysis done using phosphate buffer

4.6. Protein Estimation by Folin Lowry Method

Folin Lowry method was performed to determine the protein concentration in the samples (culture 153 and culture 70). The protein concentration lies in the reactivity of the peptide nitrogen with the copper (II) ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteau phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. And the color absorbance was measured at 660 nm.

BSA (Bovine Serum Albumin) standards were prepared along with a blank and the unknown protein concentration of samples were measured.

To the test tubes containing BSA standards that were prepared by using stock of 1000 µg/ml, 4.5 ml of reagent I was added and incubated for 10 min in dark and 0.5 ml of Reagent II was added and incubated for 30 min. Absorbance measured at 660 nm.

4.7. Enzyme Assay and Sugar Estimation by DNS (3, 5-dinitrosalicylic Acid) method

Enzyme assay involves measurement of enzymatic activity using specific substrate and is carried out by DNSA method to estimate reducing sugars released by CMC. DNSA is yellow in color and when boiled with reducing sugars produce an orange red color, of which absorbance is measured at 540 nm. Cellulase production was estimated by glucose calibration curve by using Glucose standards having 1000 µg/ml concentration of stock solution.

Determination of cellulase activity of samples in test tubes: 0.5 ml of purified enzyme is added to 0.5 ml of 1% CMC solution in phosphate and incubated at 45ºC for 30 min. The reaction was stopped using 3 ml of DNS reagent and boiled for 10 min. Add 5 ml of distilled water. The enzyme activity was expressed as the amount of reducing sugar released/ml of the sample/unit time (Sulyman et al. 2020).

NOTE: Since the purified cellulase enzyme using dialysis method did not give proper results during the DNSA reaction, so further estimation was carried out using directly the precipitate formed by ammonium precipitation (partially purified cellulase enzyme), and dissolved in little amount of phosphate buffer and the characterization of cellulase was done.

Calculation:

Enzyme Unit: Product Concentration/Incubation Time

CMCase Activity (U/ml): µg of product released * 1000/Molecular weight of the product *

Incubation Time

Specific Activity: Enzyme Activity/Protein Concentration

4.8. Characterization of Cellulase enzyme

4.8.1. Effect of incubation time on the enzyme catalyzed reaction by partially purified cellulase

The activity of partially purified cellulase was measured by varying the incubation time between 5-150 min, after which cellulase activity was determined. Where in the substrate concentration and temperature were kept unchanged.

0.5 ml of partially purified enzyme was added to 0.5 ml of 1% CMC prepared in phosphate buffer and incubated at 5, 15, 30, 60, 120 & 150 min. Add 3 ml of DNS reagent and place in boiling water for 10 min. Add 5 ml of distilled water and absorbance at 540 nm measured.

4.8.2. Effect of Temperature on the enzyme catalyzed reaction by partially purified cellulase

The partially purified cellulase was incubated with substrate at different temperatures of 4ºC, 28ºC, 37ºC, 45ºC & 60ºC. Where in Incubation time and Substrate concentration were kept unchanged.

0.5 ml of partially purified enzyme was added to 0.5 ml of 1% CMC prepared in phosphate buffer and incubated at selected temperature for 1 hour. Add 3 ml of DNS reagent and place in boiling water for 10 min. Add 5 ml of distilled water and absorbance at 540 nm measured.

4.8.3. Effect of Substrate concentration on the enzyme Catalyzed reaction by partially purified cellulase

The Michaelis-Menten kinetic constants, Km and Vmax for purified cellulase was determined by varying concentration of Carboxymethyl cellulose ranging from (0.5%, 1%, 1.5%, 2%, 2.5%). 0.5 ml of varying concentration of CMC added to 0.5 ml of partially purified enzyme. The reaction mixture incubated for 1 hour and at 60ºC. Here the temperature and incubation time was kept constant and substrate concentration was changed.

4.9. Molecular weight Determination by SDS-PAGE

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on 10% resolving gel and 4% stacking gel to determine the molecular weight of the partially purified cellulase. This technique is rapid and sensitive and thus only small amounts of protein is needed to carry out this procedure. This technique is based on the separation of proteins based on their size and thus is used to determine the molecular mass of the protein. The protocol follows below:

- The glass plates were assembled and spacers were placed between the glass plates and fixed.
- The borders of plates were sealed using 1.2% agar.
- The components of 10% resolving gel were mixed and poured into plates and allowed it to set at room temp.
- The components of 4% stacking gel were mixed and poured on top of resolving gel and comb was placed and allowed to set.
- 1X sample buffer was prepared and mixed with the protein sample.
- The assembly was then placed into the electrophoresis chamber and 1X running buffer was poured into the chamber.
- The comb was removed and the wells were loaded with the samples containing sample buffer (1X)
- And the gel was ran at 100 V until the dye front migrated into the running gel.

- After running the gel against 1 X Assay Buffer, the gel was removed carefully and stained with Coomassie stain so that the bands were visible and then again destained using destaining solution to remove the excess stain.

CHAPTER 4

5. RESULTS AND DISCUSSION

5.1. Isolation of Cellulase producing bacteria by pre-isolated bacteria from salt pans

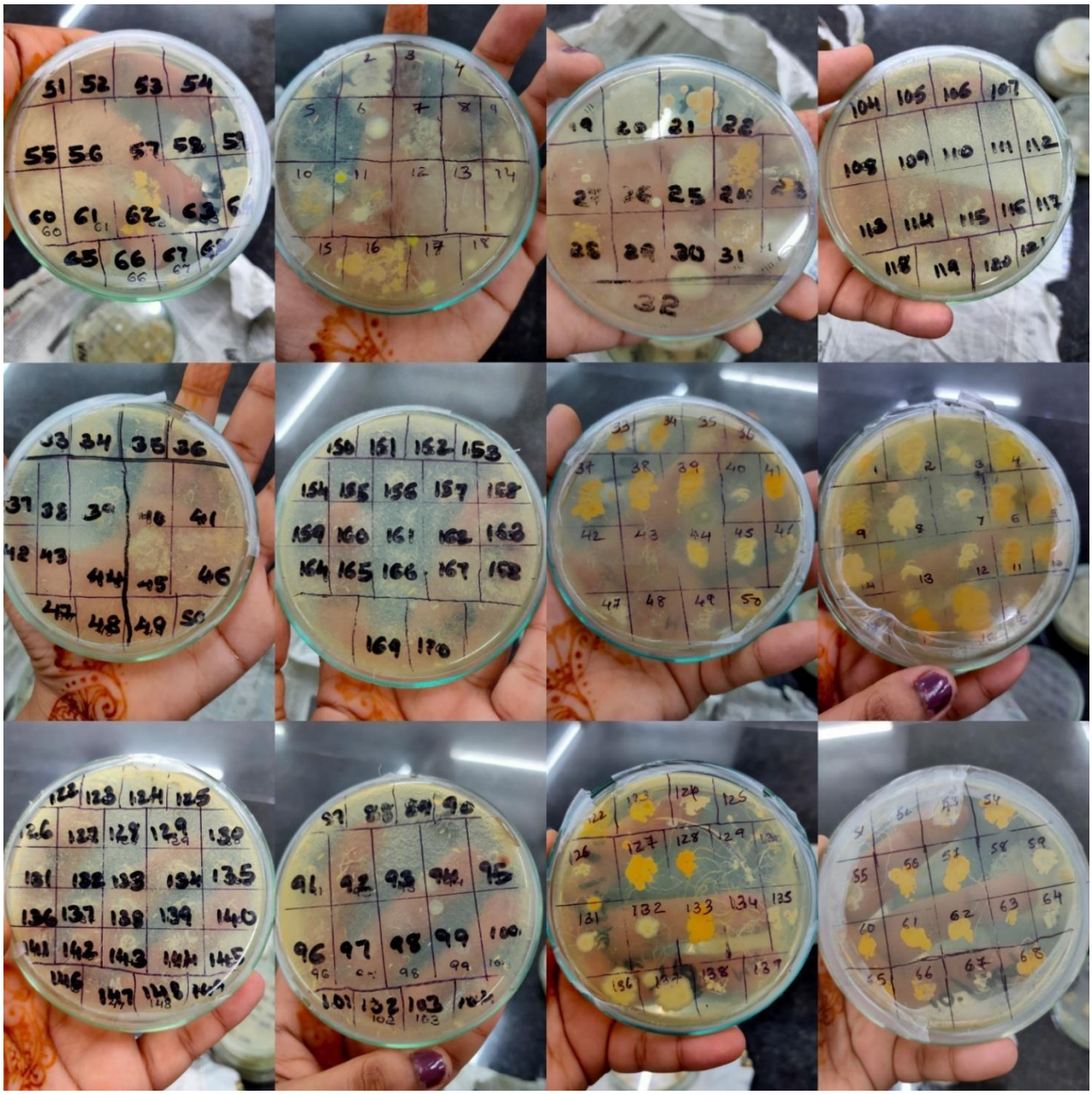
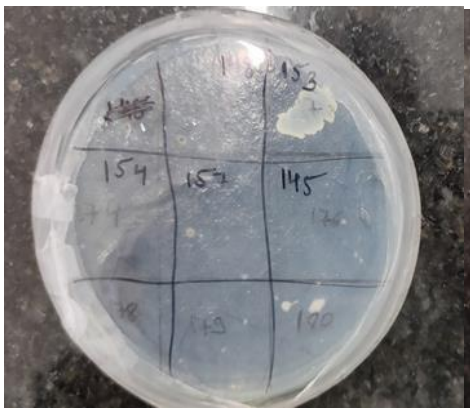


Fig 4: More than 150 cultures which were pre-isolated from salt pans, were revived on media containing 10% NaCl

Fig 5: Screening of cellulolytic bacteria from pre-isolated marine bacteria.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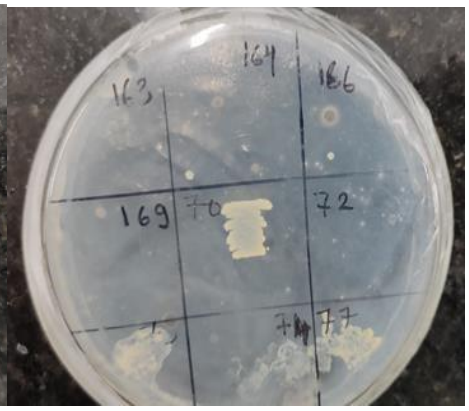
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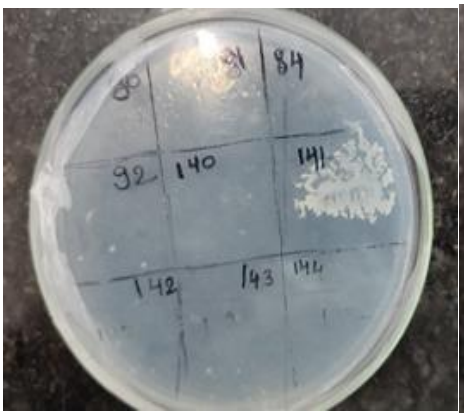
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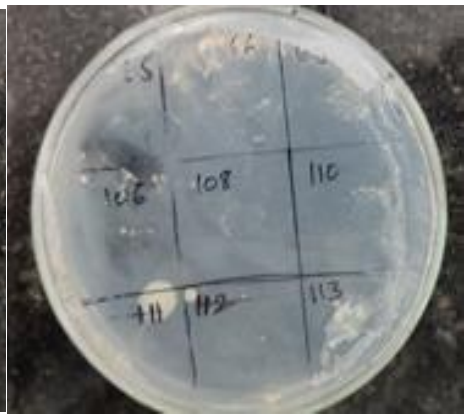
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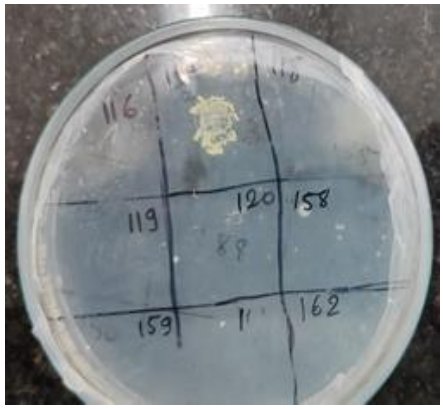
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5.2. Screening of CMCase Positive

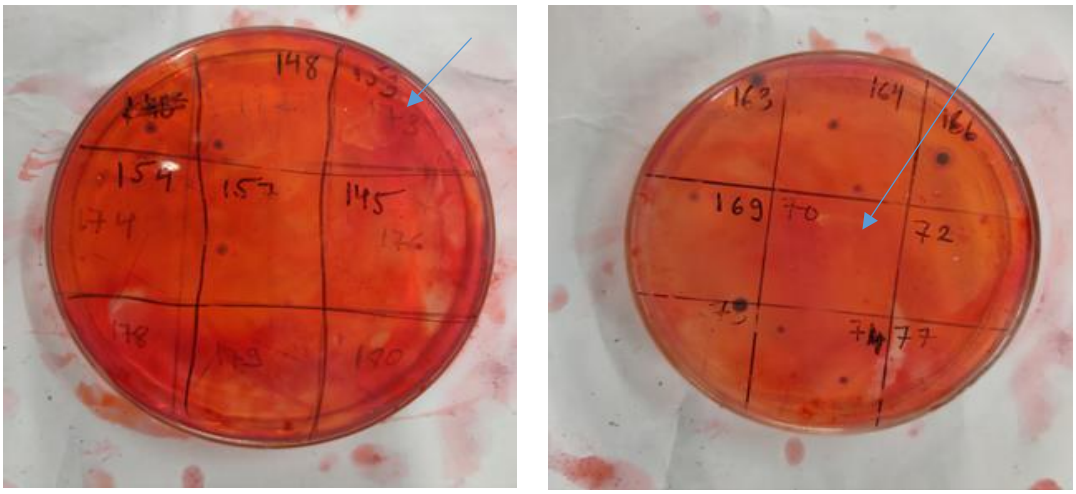

Fig 6: Only 2 isolates were found to be positive, i.e. Culture 153 and Culture 70 showing clearance.

5.3. Morphological and Biochemical identification

5.3.1. Morphological identification by gram staining method Fig

7 (a and b): Identification of CMC positive isolates

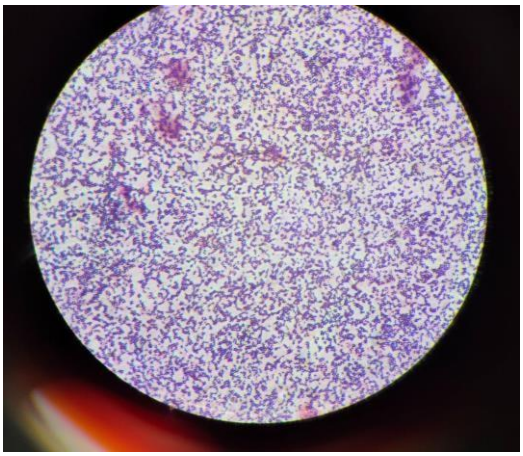

Figure 7 (a): Isolate 153 showing gram positive cocci morphology

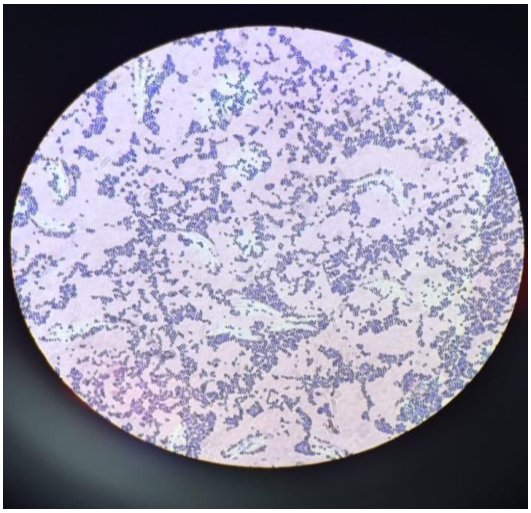

Figure 7 (b): Isolate 70 showing gram positive cocci morphology

5.3.2. Biochemical Tests

- Biochemical tests done for the detection of Motility, Catalase and Indole test Table 1: Biochemical test results

Culture	Motility	Catalase	Indole
153	Non-Motile	positive	negative
70	Non-Motile	positive	negative

5.4.

Protein Estimation by Folin Lowry Method

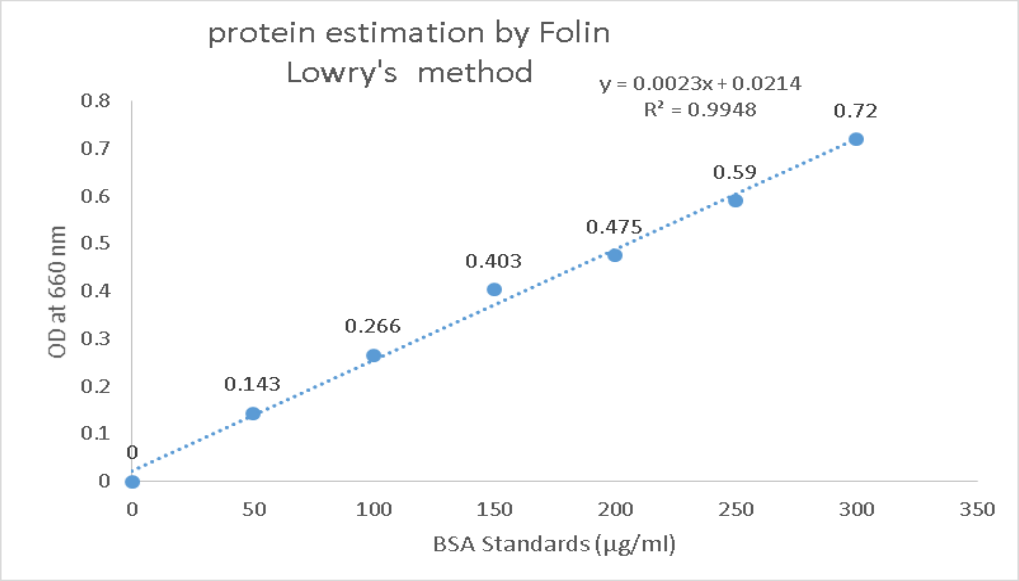

Figure 8: Standard graph of Protein estimation by Folin Lowry Method

Cultures	Protein concentration (µg/ml)
70	125.47
153	132.43

Table 2: of Protein estimation of the unknown samples

5.5.

Enzyme Assay and Sugar Estimation by DNSA method

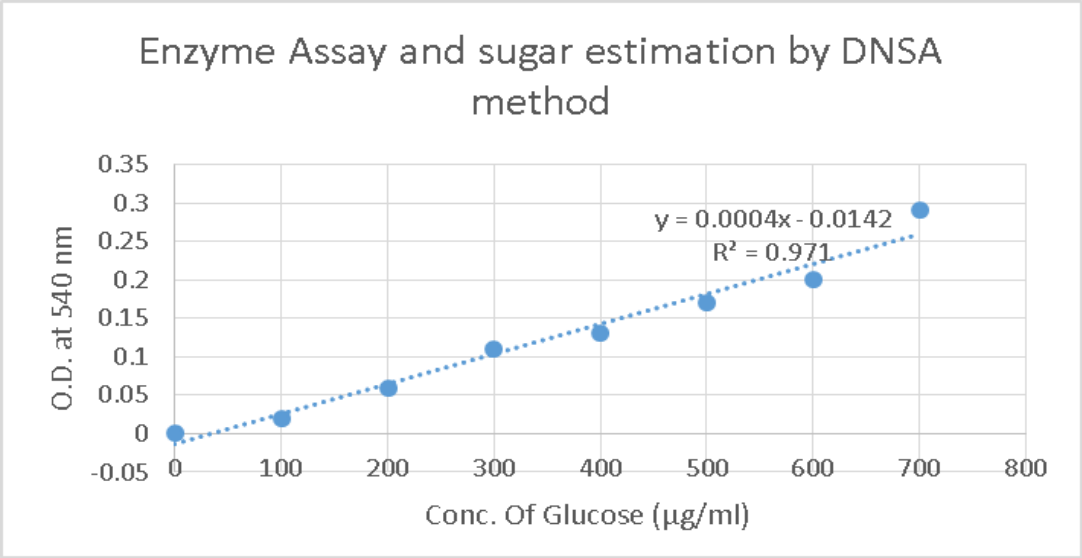

Figure9: Standard graph of Sugar estimation by DNSA method

Sample No.	Enzyme unit (µg/min)	Enzyme activity	Specific activity (units/mg)
Sample no. 153	4.88	54.12	0.433
Sample no. 70	5.13	56.98	0.43

Table 3: Table of Sugar estimation by DNSA method of the samples

5.6.

Characterization of Cellulase enzyme

The Reaction mixture contained:

Sign (+) = Added

Sign (-) = Not added

Test sample	Enzyme	Substrate	DNS
153	+	+	+
70	+	+	+
153 Enzyme Control	+	-	+
70 Enzyme control	+	-	+

5.7.

Substrate control	-	+	+

Table 4: Reaction mixture taken in test tubes

5.6.1. Effect of incubation time on enzyme catalyzed reactions

Enzyme activity (U/ml) at various Incubation Time (min)						
Culture	5 min	15 min	30 min	60 min	120 min	150 min
153	158.75	49.15	35.89	57.45	13.43	86.22
70	164.3	38.52	39.59	63.46	12.41	59.39
153 EC	189.28	33.12	34.27	53.28	13.8	23.31
70 EC	205.93	44.62	44.22	65.31	13.15	60.31
SC	180.95	54.33	25.71	58.37	11.76	30.71

Table 5: Effect of varying incubation period on the enzyme-catalyzed reactions when the substrate concentration and temperature were kept unchanged

The incubation time showed better results when the reaction mixture was incubated for 5 min for both the isolates i.e 70 and 153, and hence better enzyme activity was reported. This was an unexpected result and can be by any error. Whereas the least enzyme activity was shown when the reaction mixture was incubated for 120 min. The Enzyme controls of both the isolates also showed maximum activity at 5 min incubation time. Again there was an increase in the enzyme activity at 150 min incubation time, which can be an error. The enzyme activity of culture 70 was better than that of culture 153.

The longer the incubation time, the more the product will be formed. However at a certain point all proteins suffer denaturation and hence loss of catalytic activity with time.

In Elsababty et al. 2022 and in Sulyman et al. 2019, the most commonly used incubation time was 30 min. If I compare it with my results then, the result shows best activity at 60 min followed by 15 min incubation time.

5.6.2. Effect of temperature on Enzyme catalyzed reactions

Enzyme activity (U/ml) at various Temperatures (ºC)					
Cultures	4ºC	28ºC	37ºC	45ºC	60ºC
153	24.79	22.01	27.1	17.16	31.26
70	44.4	33.11	24.05	31.26	43.75
153 EC	25.2	18.31	28.72	17.39	36.43
70 EC	39.3	35.89	35.89	51.62	49.17
SC	14.15	12.02	19.7	12.3	26.64

Table 6: Effect of varying Temperature on the enzyme catalyzed reaction when the optimum Incubation period (60 min) and substrate concentration were kept the same.

- The best temperature which gave the best result was 60ºC, when incubated for 60 min, for both the isolates i.e 70 and 153, and hence better enzyme activity. Whereas the reaction showed moderate results when incubated at 4ºC which was unexpected. The least activity was observed at 28ºC. The Enzyme Controls of both isolates showed maximum activity as compared to normal enzyme and substrate reaction. The enzyme activity of culture 70 was more than that of culture 153 when incubated at 60ºC for 60 min.

The rate of an enzyme catalyzed reactions increases as the temperature increases but even at high temperatures the rate decreases again as the enzyme becomes denatured.

In paper Asha and Sakthivel, 2014, the enzyme from *Bacillus subtilis*, was active over broad range of temperatures and the optimal temperature was 45ºC, giving enzyme activity of 633.3 U/ml. Where as in Sulyman et al. 2019, the optimum temperature was 40ºC, having activity of 85 U/ml. When the results compared with this study, the best results obtained are at 60ºC, having activity between 20-40 U/ml. Hence it can be concluded that their enzyme yielded more than this present enzyme and hence their enzyme more powerful than this study.

5.6.3 Effect of substrate concentration on Enzyme catalyzed reactions

Enzyme activity (U/ml) at various Substrate Concentration (%)					
Cultures	0.5%	1%	1.5%	2%	2.5%
153	31.03	31.5	34.04	33.12	43.29
70	37.97	43.75	40.28	44.45	55.78
153 EC	30.57	39.13	34.28	49.08	49.3
70 EC	37.98	34.5	37.05	28.5	55.09
SC	30.11	28.72	29.41	35.2	40.98

Table 7: Effect of Substrate concentration on the Enzyme reaction mixture, when the optimum incubation time (1 hour) and optimum temperature (60ºC) were kept constant.

- The best results of enzyme activity obtained were at the substrate concentration 2.5%, in both the isolates i.e. 70 and 153. The Enzyme controls of both isolates also showed the

maximum activity at 2.5% as well. The least was observed at the substrate concentration 0.5%. Again the enzyme activity of culture 70 was higher than that of culture 153.

The enzyme activity increases with increase in substrate concentration but at a certain point the enzyme will become saturated and no more substrate can react even if plenty of substrate is available.

In Asha & Sakthivel, 2014, the substrate specificity of enzyme by *Bacillus subtilis*, gave activity of 675 U/ml when 1% of CMC was used. Where as in Sulyman et al. 2019, at concentration 5%, the activity reported was 9.26 U/ml. When compared with this study, the enzyme activity was highest at 2.5% substrate concentration giving activity between 40-60 U/ml. Hence this enzyme can be better than Sulyman et al. but not more than Asha & Sakthivel enzyme.

5.7. SDS-PAGE

No Bands were visible on the Polyacrylamide Gel even after staining with Coomasie. The standard protein markers were also not visible. Hence the molecular weight couldn't be determined.

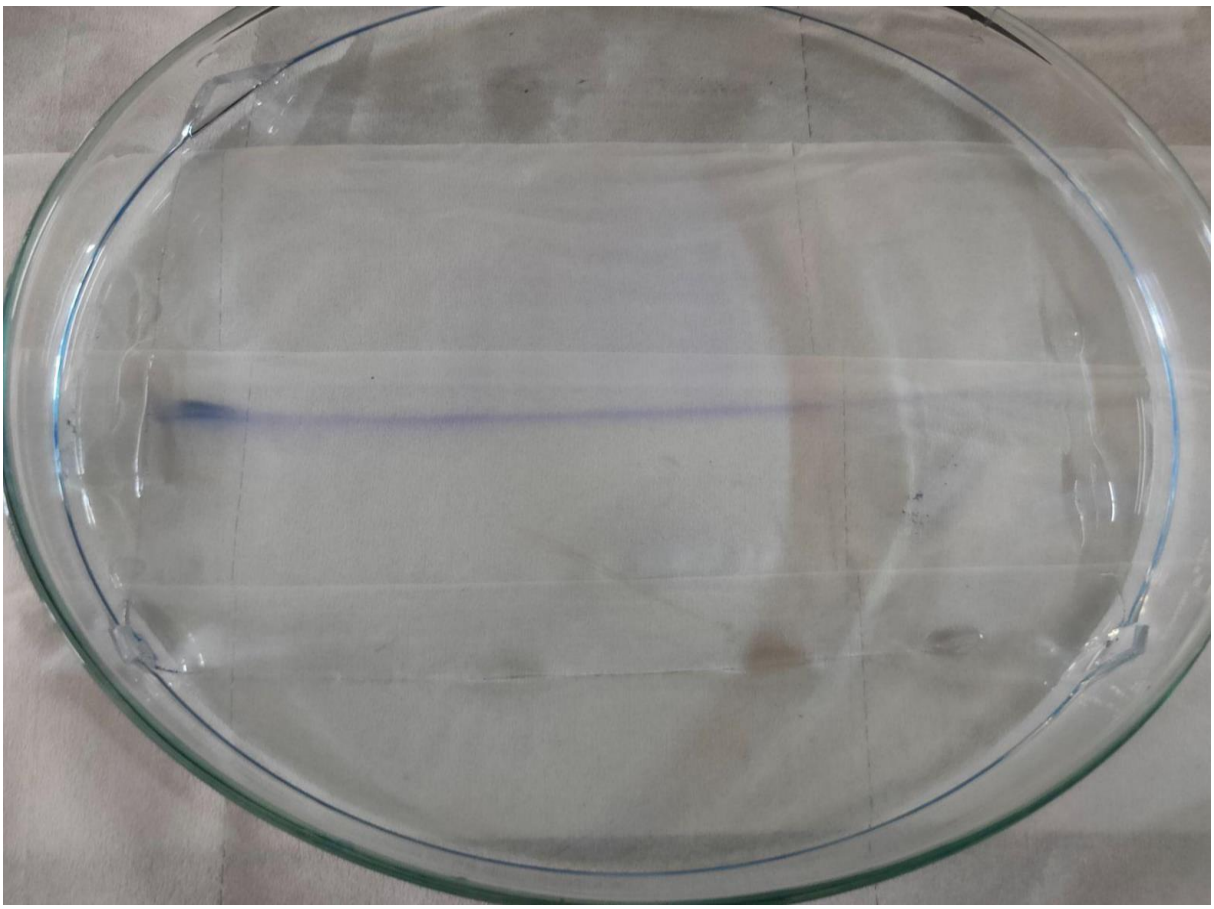

Fig 10: The SDS gel containing the sample buffer and enzyme.

The reason the bands not visible in the Gel must be because the concentration of the protein in the sample was too less or below detection level or may be the proteins might have degraded. Also there was no DDI water available at that time so only distilled water was used for the preparation of reagents.

Since the molecular weight of the cellulase in this study was not determined, but the studies reported in Malik and Furtado, 2019, the cellulase enzyme had 19.6 kDa molecular weight. Where as in Asha & Sakthivel, 2014, the molecular weight of cellulase was 51.6 kDa and by Elsababty reports the cellulase had 54.4 kDa molecular weight.

6. CONCLUSION

Cellulose is the most abundant polysaccharide present on earth. Marine bacteria were tested for their ability to produce cellulase when grown on solid media containing CMC. The protein estimation by Folin Lowry method and sugar estimation by DNSA method were carried out to determine the enzyme activity and specific activity of the cellulase enzyme. Characterization of the enzyme to ensure the identity, purity, structural and activity of an enzyme product. The longer an enzyme is incubated with the substrate, the more the product will be formed. Also that proteins suffer denaturation, hence loss of catalytic activity with time is also reported and also, maintaining a constant temperature is necessary as high temperatures will denature the protein. The best enzyme activity reported is at 60 min incubation time at 60ºC and having 2.5% substrate concentration, and that culture 70 had higher enzyme activity than that of culture 153 in overall.

7.

SUMMARY

More than 150 bacteria isolated from salt pans, were screened for the production of Cellulase enzyme. Out of which only 2 isolates (culture 70 and culture 153) were found to be positive showing hydrolysis on agar plates containing CMC (Carboxymethyl cellulose) when stained with Congo red. Morphological and Biochemical Characterization tests were performed on isolates. Crude enzyme extraction procedures were carried out in order to estimate protein concentration and enzyme activity of the cellulase produced by the isolates. Characterization of the enzymes with varying Incubation Time, Temperatures and Substrate concentration was done. The isolate no. 70 showed better results than isolate no.153 and hence showed better CMCase activity. The best enzyme catalyzed reactions showed at 60 minutes incubation, at 60ºC, and at 2.5% substrate concentration and more enzyme activity was seen by the culture 70 than culture 153.

8.

ANNEXURE

1. CMCA (Carboxymethyl cellulose agar) Composition

Part I	
Distilled water	500 ml
$NH_4H_2PO_4$	0.5 g
KCl	0.1 g
$MgSO_4.7H_2O$	0.5 g
Yeast extract	0.5 g
Agar	7.5 g
Part II	
CMC	13 g
Nutrient agar	1.5 g

Mix Ingredients from part I and add to part II.

2. Phosphate Buffer

Disodium Hydrogen Phosphate	20.214 g
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.

3. Folin Reagents

Solution A: 2% Na_2CO_3 in 0.1 N NaOH (0.4 g in 100 ml D/W)

Solution B: 1% Sodium Potassium Tartrate in D/W

Solution C: 0.5% $CuSO_4.5H_2O$ in H_2O

9.

Reagent I: 48 ml of A + 1 ml of B + 1 ml of C

Reagent II: 1 part of Folin Ciocalteau reagent + 1 part of D/W

4. DNSA Reagent

Solution 1: 20 ml of 2 N NaOH (1.6 g in 20 ml D/W) + 1 g of DNS powder

Solution 2: Dissolve 30 g of sodium tartrate in 50 ml of D/W

Slowly pour solution2 in solution 1 and make up the volume to 100 ml with D/W.

5. SDS-PAGE Solutions

40% Acrylamide (37.5.1)

Acrylamide	116.8 g
N, N'-Methylene bisacrylamide	3.2 g
DDI	300 ml Filter

and store in dark bottle at 4ºC.

30% Ammonium Persulphate

Ammonium persulphate	1.5 g
DDI H_2O	5 ml

Store at 4ºC. Use freshly prepared.

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

DDI H_2O	300 ml
Tris-free base	90.75 g
Conc. HCl	8 ml

Adjust pH to 8.8 with conc. HCl and bring final volume to 500 ml with DDI H_2O.

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

DDI H_2O	300 ml
Tris-free base	60.54 ml
Conc. HCl	36 ml

Adjust pH to 6.8 with conc. HCl and bring volume to 500 ml with DDI H_2O.

4 X SDS-PAGE Sample Buffer

125 mM Tris HCl, pH 6.8	5 ml
20% glycerol	8 ml
10 % Beta-Mercaptoethanol	4 ml
4% SDS	8 ml
0.5 mg/ml Bromophenol blue	20 mg

DDI H_2O	15 ml
Total	40 ml

10X SDS-PAGE Running Buffer

Tris base	30.3 g
Glycine	144 g
SDS	10 g

Dissolve and bring volume to 1000 ml with deionized water.

10% Resolving Gel

DDI H_2O	1.6 ml
1.5 M Tris HCl, pH 8.8 (R.G. Buffer)	1.3 ml 40%
Acrylamide stock	1 ml
20% SDS	100 µl
30% Ammonium persulphate	10 µl
TEMED	4 µl

4% Stacking Gel

DDI H_2O	3.9 ml
1.0 M Tris HCl, pH 6.8 (S.G. Buffer)	500 µl
40% Acrylamide stock	500 µl
20% SDS	100 µl
30% Ammonium persulphate	16 µl
TEMED	8 µl

Coomasie Stain Solution

Ethanol	150 ml
Glacial acetic acid	50 ml
DDI H_2O	300 ml
Coomasie Brilliant Blue-R 250	1 g

Destain solution

Ethanol	1200 ml
Glacial acetic acid	400 ml
DDI H_2O	2.41

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