AMYLASE PRODUCTION AND ACTIVITY IN HALOTOLERANT BACTERIA 0F RIBANDAR SALT PANS

Dissertation

Submitted To

GOA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MARINE MICROBIOLOGY BY

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May 2023

DECLARATION

I hereby declare that the work incorporated in this dissertation, titled as "Amylase production and activity of halotolerant bacteria from Ribandar salt pans" which is in partial fulfillment 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

Candidates Signature

Place: Goa University,

Taleigao, Goa

Sumeedha Harmalkar

COMPLETION CERTIFICATE

This is to certify that the dissertation entitled as "Amylase production and activity of halo tolerant bacteria from Ribandar Salt pans" is a bonafide and an authentic record of the research carried out by Ms. Sumeedha Harmalkar, student of M.Sc. Marine Microbiology under the supervision and guidance of Dr. Nikita 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.

Dr. Nikita Lotlikar

Marine Microbiology

Date: 02/05/2023

Chandrashekher U. Rivonkar

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Date:

Sumeedha harmalkar

ACKNOWLEDGEMENT

I am overjoyed as I express my gratitude towards all the people who contributed towards the completion of my project. Firstly, I would like to thank my guide Dr. Nikita Lotlikar for her encouragement, guidance and for having the time and patience to solve my queries throughout the duration of the project.

I would also like to thank all the staff (teaching and non-teaching) of school of Earth Ocean and Atmospheric Sciences, Goa university for helping me with everything that was required whenever approached. I extend my science thanks to our Lab Assistant- Ms Vaishali and Ms Sitam for constantly helping me throughout my dissertation.

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1. INTRODUCTION

Marine environment covers more than 70% of the earth's surface and includes a vast array of diverse habitats ranging from tropical, shallow water coral reefs to sub zero deep ocean trenches (Jensen & Fenical 1996). One of the widely distributed aquatic ecosystems is the saline environments (Al-Rubaye et al 2017). Hypersaline environments are widely distributed world wide where they exist as natural water bodies such as saline lakes, saltpans, salt marshes,or as artificial solar salterns (Burhan et al 2003). Despite the high concentration of salt, a great diversity of life forms is observed in this environment and they are found in all the three domains of life, Bacteria, Archaea, and Eukarya (Al-Rubaye et al 2017). The domain bacteria typically contain many types of halophilic and halotolerant microorganisms that spread over a large number of phylogenetic subgroups. Most of them are moderate rather than extreme halophiles (Purohit et al 2014). Microorganisms that thrive in these environments have been broadly classified into halophilic microorganisms that require salt for their viability and halotolerant microorganisms which are able to grow in the absence as well as in the presence of NaCl (Purohit et al 2014).

Halophiles can be classified into three groups on the basis of their response to NaCl.

- Slight halophiles which grow optimally at 2-5% NaCl (0.2-0.85 M).
- The moderate halophiles show rapid growth at 5-20% NaCl (0.85-3.4 M).
- The extreme halophiles which optimally grow at 20-30% NaCl (3.4-5.1 M).

The non-halophiles grow optimally at less than 2%NaCl (0.2M). Halophiles and halotolerant microorganisms can grow over a wide range of salt requirements or salt tolerance at times depending on environmental and nutritional factors (Oren, A. 2020). Halophiles also include prokaryotic and eukaryotic salt-loving microorganisms of saline environments which are able to

balance the high osmotic pressure. To adapt to high level saline, they have developed a number of biochemical strategies to maintain cell structure and function. Halophiles play an important role in the carbon and phosphorus cycles in saline environments (Al-Rubaye et al 2017).

1.1 Salt pans of goa

Traditional salt farming in Goa, India has been practiced for the past 1,500 years by a few communities (Mani et al 2012). Goa's riverine estuaries, easy access to sea water and favorable climatic conditions makes salt production attractive during summer. The salt pans in Goa experience three phases; namely the monsoon ceased phase, the salt pan preparatory phase and the salt harvesting phase. Sea water from the salterns is drained using motor pumps. Once the water has been completely drained, the preparation of salt pan beds begins. All the pans are interconnected through an opening at the corners. The reservoir pan is connected with the creek. It supplies seawater during tidal influxes, through a sluice gate.

Solar salterns are extreme environments that provide a suitable environment for organisms which thrive over a range of extreme salinities, temperatures, pH, nutrient concentrations, oxygen availability, water activity and solar radiation (Mani et al 2012). Microorganisms which survive in such high salinities are known as halophilic extremophiles, which include bacteria, archaea and fungi. Solar salterns are man-made ecosystems showing diversity of microbial populations during different stages of salt crystallization. Microorganisms play a vital role in the recycling of materials such as nutrients and other substances in the saltern ecosystem and are important members of biogeochemical cycles (Mani et al 2012).

1.2 Adaptation

To survive in high salt concentrations, halophilic and halotolerant microorganisms must maintain a cytoplasm that is osmotically isotonic with the outside medium (Oren, A. 2020).

Osmoregulation is the active regulation of the osmotic pressure of an organism's fluids to maintain the homeostasis of the organism's water content which helps to keep the organism's fluids from becoming too diluted or too concentrated. The concentrations of these osmotic solutes are regulated according to the salt concentration in which the cells are found and can be rapidly adjusted as required when the outside salinity is changed. The amount of compatible solutes synthesized by accumulation from the medium keeps steadily growing. In either case intracellular sodium concentrations are kept as low as possible and outward-directed sodium pumps in the cytoplasmic membranes are of most importance, both in maintaining the proper intracellular ionic environment, and in pH regulation. Adaptation and adaptability of halophilic bacteria depend on the regulation and synthesis of such organic osmolytes such as glycine, betaine, ectoine and glucosylglycerol (Oren, A. 2020). Halophiles have developed two different adaptive strategies to cope with the osmotic pressure induced by the high NaCl concentration of the normal environments they inhabit. The halobacteria and some extremely halophilic bacteria accumulate inorganic ions in the cytoplasm (K+, Na+) to balance the osmotic pressure of the medium. They have developed specific proteins that are stable and active in the presence of salts(Mani et al 2012). Moderate halophiles accumulate high amounts of specific organic osmolytes, which function as osmoprotectants, providing osmotic balance without interfering with the normal metabolism of the cell (Mani et al 2012).

1.3 Microbial diversity

- Diversity studies on bacteria have shown the presence of members belonging to genera *Aeromonas, Pseudomonas, Vibrio, Desulfobacter , Desulfovibrio , Desulfococcus* and *Chromohalobacter.* These bacteria play an important role in the cycling of substances within the saltern ecosystem.

- Haloarchaeal diversity studies in the salterns of Goa showed that *Halococcus sp.* are the dominant haloarchaeal members during less saline conditions.
- During the salt harvesting phase, members belonging to the genera *Halococcus, Halorubrum, Haloarcula* and *Haloferax* are identified.
- Archaeal members belonging to phylum *Crenarchaeota* and *Euryarchaeota* are found in various salterns of Goa.
- Fungal communities in salt pans of Goa were dominated by members belonging to the genera *Aspergillus* and *Penicillium* such as *Aspergillus versicolor, A. wentii, A. candidus, A. penicilloides A. flavus, A. sydowii, Penicillium chrysogenum, P. corylophilum, P. griseofulvum, Eurotium amstelodami* and *Horta Werneckii* (Mani et al 2012).

1.4 Applications

Marine bacteria have successfully drawn the attention of the scientific community for potential applications of their bioactive molecules to be used in medicine, agriculture, bioenergy and other industries (Dutta et al 2022).

In recent years, halophilic microorganisms have been explored for their biotechnological potential in different fields. The applications range from the use of different products, such as the compatible solutes, biopolymers or carotenoids in a variety of industries or the use of these microorganisms in environmental bioremediation processes Besides being essentially stable and active at high salt concentrations. Halophilic enzymes offer opportunities in biotechnological applications, such as food processing, environmental bioremediation and biosynthetic processes (de Lourdes Moreno et al 2013). Carotenoid rich strains of halophilic bacteria are used for the commercial production of beta carotene for use as health food and food additive. The extraction of ectoine from moderately halophilic bacteria, to be used as enzyme protectant and as a moisturizer in the cosmetic industry. Halophilic bio-molecules are also used for specialized applications, e.g. bacteriorhodopsin for

biocomputing, gas vesicles for bioengineering floating particles, pigments for food coloring and compatible solutes as stress protectants (Kerkar, S 2004).

1.5 Enzymes

Halophilic and halotolerant bacteria secrete a wide range of hydrolytic enzymes into their surrounding environment. Several of these enzymes include amylases, proteases, xylanases and cellulases display polyextremophilic properties. These are generally haloalkaliphilic and thermotolerant and play an important role in industrial processes (Jensen & Fenical 1996).

Amylases

Amylases are amongst the most studied enzymes. These enzymes are amylolytic enzymes that catalyze the hydrolysis of internal alpha 1-4 glycosidic bonds in polysaccharides. They are found in all forms of living things, plants and microbial amylases have been used in the brewing industry. Fungal amylases are widely used in the preparation of oriental cuisine. Amylases from bacteria and fungi are increasingly in demand for industrial application.These enzymes are amylolytic enzymes that catalyze the hydrolysis of internal alpha 1-4 glycosidic bonds in polysaccharides. They are found in all forms of living things, plants and microbial amylases have been used in the brewing industry. Fungal amylases are widely used in the preparation of oriental cuisine. Amylases from bacteria and fungi are increasingly in demand for industrial application.(Saini et al 2017).

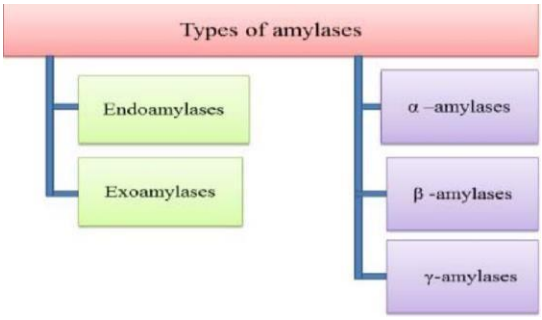


Fig.1 Different types of amylases

Different types of amylases

Endo Amylase cleaves the α-1, 4-bonds in starch in the starch molecule's inner regions by passing the amylopectin α-1, 6-branching points. The most widely recognized endoamylase is the α-amylase. This allows the starch solution to rapidly lose viscosity. These enzymes are classified into two groups according to the degree of substrate hydrolysis: liquifying (30-40%) and saccharifying (50-60%) (Kumari et al 2019).

Exo Amylases also cleave the α-1, 4-bonds, e.g. β- amylase but some of them are able to attack the α-1, 6-bonds, e.g.glucoamylase.These enzymes act externally on substrate bonds from the nonreducing end of starch and hence produce only low molecular weight products from starch, e.g. maltose and glucose, respectively (Kumari et al 2019).

α-amylase (alpha-1,4-glucan glucanohydrolase) is an extracellular enzyme. This enzyme breaks α-1,4glycosidic bonds in starch to produce oligosaccharides. β-Amylase (1,4-α-D-glucan maltohydrolase; glycogenase; saccharifying enzyme amylase) is another form of amylase. They are synthesized by bacteria, fungi and plants. β-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, simultaneously cleaving two glucose units (maltose) (Saini et al 2017).

γ-amylase (Glucan 1, 4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomes α-glucose). γ-amylase cleaves glycosidic α(1-6) linkages. γ-Amylase is most efficient in an acidic environment, with an optimum pH of 3 (Saini et al 2017) .

1.6 Application of amylase

Amylase from plants and amylase from microbial sources has been used in the brewing and fermentation industries for the conversion of starch to fermentable sugars.Fungal amylase is widely used in the preparation of oriental cuisine. The textile industry for designing textiles, the laundry industry for mixtures of proteases and lipases for washing clothes, the paper industry for sizing, the food industry for the production of sweet syrups, increasing the diastase content of flour, baby food. For processing

and removing starch Amylases of bacteria, fungi are in increasing demand in industrial Application (Saini et al 2017).

The first industrially produced enzyme was amylase from a fungal source in 1894 and was used as a pharmaceutical adjuvant to treat indigestion (Mostafa et al 2021).

Amylase can be used in several medical conditions: acute inflammation of the pancreas, perforated gastric ulcer, strangulated ileus, torsion of ovarian cyst, macroamylasemia, and mumps. The level of amylase activity in human body fluids is of clinical importance in the study of diabetes, pancreatitis and cancer. Amylases are obtained from a variety of bacteria, yeasts, fungi and actinomycetes. Enzymes from fungi and bacteria are gaining importance in industry. Industrial amylase applications rely on unique properties such as: B. Their effect pattern, substrate specificity, optimum temperature, incubation time, and optimum pH (Gupta et al 2017).

In (Mishra et al. 2008) investigation a pure strain of *Bacillus* was isolated from soil samples collected from different sites receiving kitchen waste. Plates with bacterial colonies were flooded with Gram's iodine reagent.selection was done as per colonies with and without clear and transparent zone as amylase producing and amylase non producing strains respectively. (Padma and Pallavi 2016) Soil sample was obtained from Roorkee, Haridwar district, Uttarakhand state, India. All the isolated colonies were screened for active zone technique with iodine solution. Out of 10 bacterial isolates only 5 showed positive results for amylase production. Out of these five strains b3 strain showed the widest diameter and was further chosen for amylase potential screening. (Khunt et al 2011) 24 isolates were obtained from excreta sample of wild ass after enrichment. Amylase producers were screened on medium containing starch as an inducer. 9 isolates were secreting amylase on the bases of zone clearance on solid media. *Bacillus macquariensis* was found to be the potent amylase producer. (Aygan et al 2008) study deals with the isolation of alkaline and highly thermostable alpha amylase bacillus sp. from Van soda lake and characterization of alpha amylase. A total of 226 bacterial isolates were screened for amylase

production on starch agar plates containing minimal medium, from which 88 amylase-positive strains were obtained by flooding the agar plates with an iodine solution for maximum activity. 5 amylase positive isolates were selected and stored for enzyme production.

To select the optimal starch concentration, isolates were exposed to five different concentrations of starch media without agar (0.15, 0.5, 1, 1.5, 2%). Maximum enzymatic activity was observed at 2% starch concentration (Mishra et al. 2008). (Khunt et al 2011) examined the effect of substrate on amylase at different starch concentrations (0.3, 0.6, 0.9, and 1.2%) and showed optimal enzyme production at 1.2% starch.

In (Mishra et al. 2008) selected the optimal temperature range by culturing the inoculum at different temperatures In (Mishra et al. 2008) selected the optimal temperature range by culturing the inoculum at different temperatures (RT 37, 40, 45 and 50 °C). The optimum temperature for the Bacillus strain was 37°C. (Padma and Pallavi 2016) Study b3 stems exhibited the largest diameter and were further selected to determine the optimum temperature. The amylase activity at different temperatures from 35 to 45°C decreased gradually from 0.992 to 0.545 U/ml. (Khunt et al. 2011), the maximum effect of temperature (20, 30, 40, 50 and 60 °C) on amylase production was observed at 30 °C. In (Aygan et al. 2008) The enzyme was active over a wide temperature range from 20-90 °C and optimal activity at 50 °C.

In (Padma and Pallavi 2016) investigation b3 strain showed the widest diameter and was further chosen for determination of optimum incubation time. Amylase activity decreased from 0.981 to 0.215U/mL as the incubation time increased from 24 to 72 hours. In (Khunt et al 2011) *Bacillus macquariensis* produces maximum amylase after 72 hours of incubation.

SDS-PAGE 10% was performed to determine molecular weight homogeneity. After electrophoresis the gel was destained to detect protein bands. SDS-PAGE showed a single band of 66 kDa. Enzyme activity was inhibited by 38-34% by SDS or EDTA(Aygan et al 2008).. Analysis of the enzyme by SDS-PAGE revealed a single band indicative of amylolytic activity.

Starchdegrading activity was detected on a starch gel and the molecular weight of the partially purified enzyme was estimated to be 94500 Da. Thus, this study showed that the enzyme is alkaline, thermostable, thermopile- and chelator-resistant, and useful for detergents, textiles, and other industrial applications(Burhan et al(2003).

2. AIM AND OBJECTIVES

AIM:

The present study was conducted to screen halotolerant bacterial strains from Ribandar salt pans and to check enzyme activity, specific activity of the amylase positive cultures.

OBJECTIVES:

- To screen halotolerant bacterial strains for amylase enzyme.
- To check specific activity and enzyme activity of the positive amylase cultures.

3. MATERIALS AND METHODS

3.1 Sample collection

Sample was collected from the salt pan site: Ribandar (15° 30.166N and 73° 51.245 E). Water sample was serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and water sample was serially diluted to 10^{-2},10^{-3} and 10^{-4}. 100µl of the dilution were spread plated on three different media i.e., Nutrient agar, Malt extract and Yeast extract agar. They were prepared using 15% filtered crude salt solution and 2% agar agar. Isolation of the culture was done by the previous batch student with a count of 196. The cultures were spot inoculated on nutrient agar plates with 2% Nacl in a grid format with each plate having 18 isolates. Plates were incubated at room temperature and growth was observed after 24 - 48 hours.

3.2 Screening of amylase enzyme

All the sub-cultured cultures were screened on starch (2%) nutrient agar plates. Bacterial cultures streaked onto agar plates were incubated at room temperature for 48 hours and the positive one will show an opaque zone around the colony when flooded with iodine solution, and observed for clearance zone indicating hydrolysis of starch(Raiz et al 2021).

3.3 Isolation of amylase from bacterial cultures

Amylase positive bacterial isolates were aseptically inoculated into 250 mL nutrient broth containing soluble starch and were incubated at RT at 100 RPM for 48 hours. After incubation, the culture was centrifuged at 8000 rpm for 10 minutes, the supernatant was transferred in a clean 50 ml centrifuge tube and used for protein precipitation(Kanimozhi et al 2014)

3.4 Protein precipitation using ammonium sulfate

This method is used to precipitate proteins 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 10 min. Supernatant was discarded and precipitate was resuspended with 4 ml of phosphate buffer and further purification of proteins was done using dialysis method.

3.5 Desalting of protein by dialysis

Treatment of dialysis tubing was done by placing the dialysis tubing (catalogue number: LA39510MT, HIMEDIA) in running tap water for 3- 4 hours. The tubing was rinsed with distilled water at 60°C 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 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. Dialysate was removed from the tubing and was used for enzyme assay and protein estimation.

3.6 Estimation of protein concentration By Folin Lowry's method

Series of BSA conc in six tubes were prepared (50, 100, 150, 200, 250, and 300mg/ml) using BSA working std of 100µg/ml. To that 4.5ml 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.

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

Glucose standards (100-700 µg) were prepared from glucose stock solution. To each tube's substrate, buffer and enzymes were added according to the appropriate amount. Tubes were incubated at 37°C for 15 min and 1 mL of DNSA reagent was added and the tubes were kept in boiling water for 5 min. Ten mL of distilled water was added to each tube and absorbance was measured at 540 nm (Mishra et al. 2008).

3.8 Characterization of enzyme

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

3.9 Determination of protein using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Assemble of glass plates was done in a gel casting apparatus and the components of resolving gel 10% were mixed and added into the gel plates to a level of 2 cm. The gel was allowed to stand for 30 min at RT. Stacking gel 4% was then poured into the gel plates and comb was inserted on the top of the spacers, gel was allowed to solidify overnight and next day the gel was run at 100V for 3 hrs. Staining (Coomassie brilliant blue) and destaining of the gel was carried out.

3.10 Gram staining

On a clean glass slide a thin smear was prepared and was heat fixed. Immediately the slide was flooded with Gram's iodine for 1 min. The stain was drained and it was then flooded with Gram's iodine for 1 min. Alcohol was used as a decolourizer. The slide was then washed under tap water and was counter stained with Safranin for 1 min, the slide was washed and air dried to examine

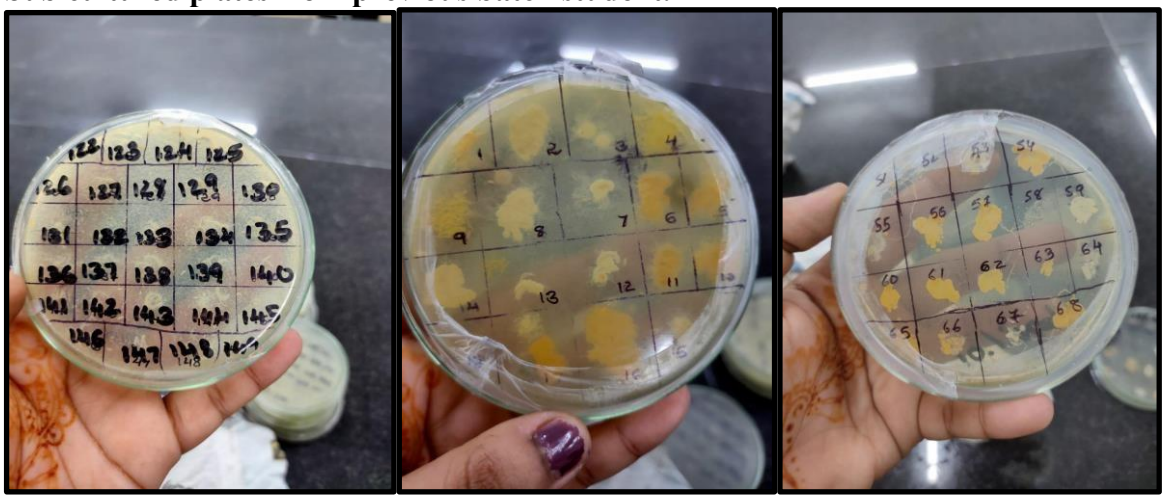
under oil immersion objective. Gram characters of only 5 cultures which were showing positive results for starch hydrolysis were studied.

3.11 Biochemical tests

Biochemical tests are used to identify bacterial species by differentiating them on the basis of biochemical activities. Three tests were conducted due to the less availability of chemicals. Indole test : 24 hours culture was inoculated into a sterilized test tube containing 5ml of tryptophan broth. Tubes were incubated at RT for 24 hours. Next day 0.5ml of Kovac's reagent was added to the broth culture and observed for the presence of the ring. Catalase test : Small amount of bacterial cultures were taken on the slide and a drop of 3% hydrogen peroxide solution was added to the bacterial cultures and was checked for evolution of oxygen bubbles. Motility test : 24 hours old culture was stabbed using a needle loop once to a depth ½ of inch in the middle of the tube containing agar medium. Tubes were incubated at RT for 24 hours. After incubation, the tubes were observed for a diffuse zone of growth flaring out from the line of inoculation.

4. RESULTS AND DISCUSSION

4.1 Sub cultured plates from previous batch student.



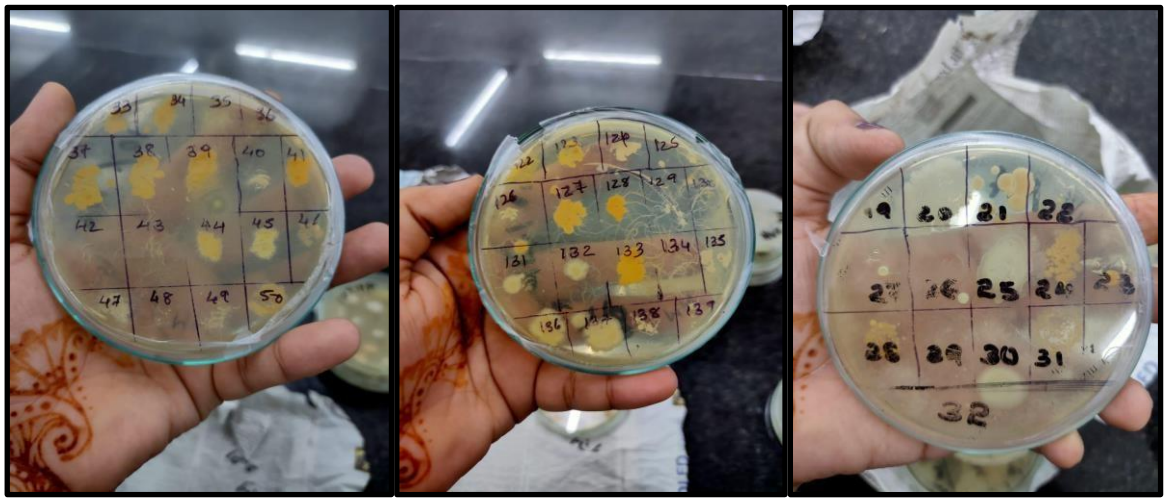
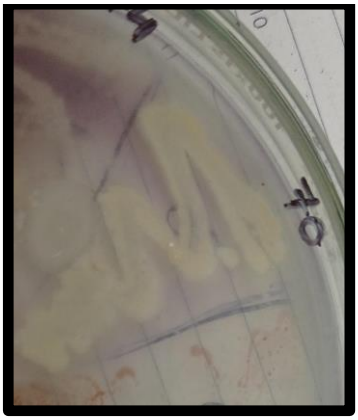


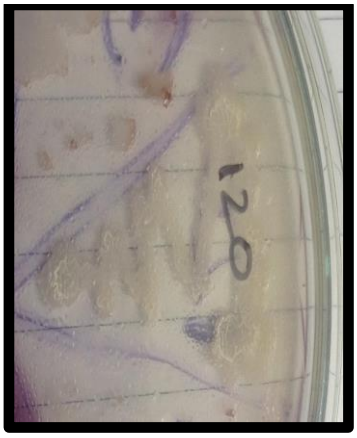
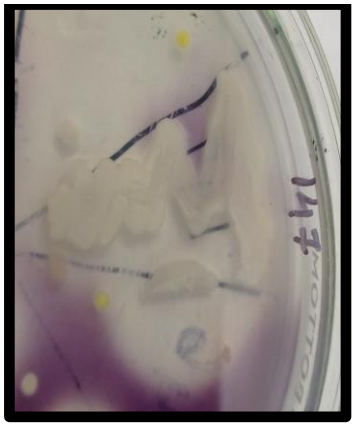
Fig 2: Plates revived on nutrient agar + 2% NaCl

➢ From 196 cultures 94 were revived on the plates containing nutrient agar with 2% NaCl and were used for the screening of amylase enzyme.

4.2 Screening of amylase producing bacteria

Table 1 : Bacterial isolates showing hydrolysis of starch

Bacterial isolates No.	Growth after 48 hours	Zone of hydrolysis
70		

62		
120		
147		

106	Positive culture	No picture hence the bacterial isolate was showing zone of clearance

- Among the 94 bacterial cultures 5 were showing positive results for amylase enzymes after 48 hours. These cultures showed a clear zone around the colony when flooded with iodine solution indicating hydrolysis of starch. All these 5 cultures (70, 62, 120, 147, 106) were showing positive results for amylase enzymes. Showing zone of clearance, indicating hydrolysis of starch.
- (Padma and Pallavi 2016) isolated 10 soil sample. 5 showed positive results for amylase production. Out of this five B3 was chosen for amylase potential screening. (Khunt et al 2011) 24 isolates were obtained from an excreta sample of wild ass from which 9 isolates were secreting amylase on the bases of clearance. *Bacillus macquariensis* was found to be a potent amylase producer. (Aygan et al 2008) isolated 226 alkaline and highly thermostable alpha amylase *Bacillus* spp. Out of which 88 amylolytic isolates were selected and the best five were selected among them.

4.3 Isolation of amylase from bacterial cultures.

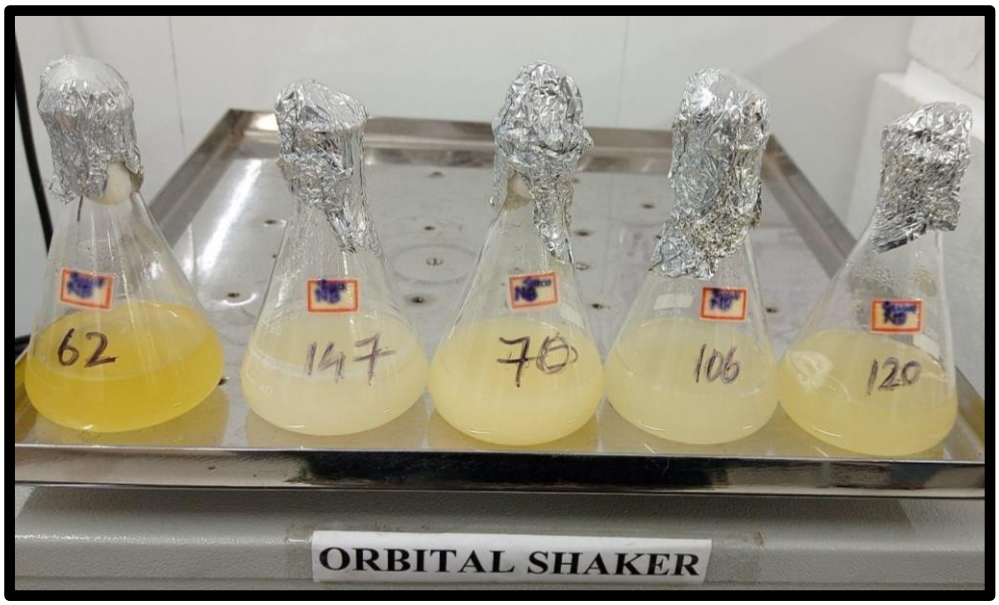


Fig 3: Bacterial cultures showing growth after 48 hours on orbital shaker.

- All the 5 cultures were grown in nutrient broth with 2% starch and growth was observed after 48 hours.
- This culture broth was added to 50ml centrifuge tubes and centrifuged at 8000 RPM for 10 min. Supernatant was separated and stored at 4°C.

4.4 Precipitation of protein using ammonium sulfate

Fig 4: Tubes after adding ammonium sulfate

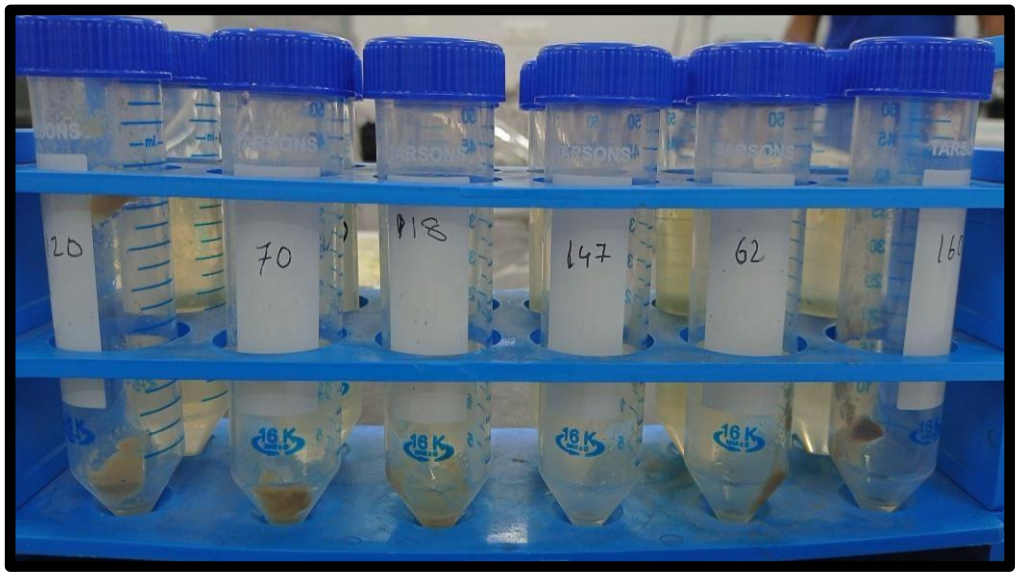


Fig 5: Precipitate after centrifugation at 8000 RPM

➢ Precipitation was obtained in addition to Crystalline ammonium sulfate to achieve 70% saturation.

4.5 Desalting of protein by dialysis

Fig 6 : Dialysis tubing placed in phosphate buffered saline

➢ Purification of protein was done using the dialysis method.

4.6 Estimation of protein concentration By Folin Lowery's method

Table 2: Protein estimation of unknown samples

Cultures	Protein concentration (µg/ml)
70	410.5
62	255.2
106	195
147	77.5
120	37.5

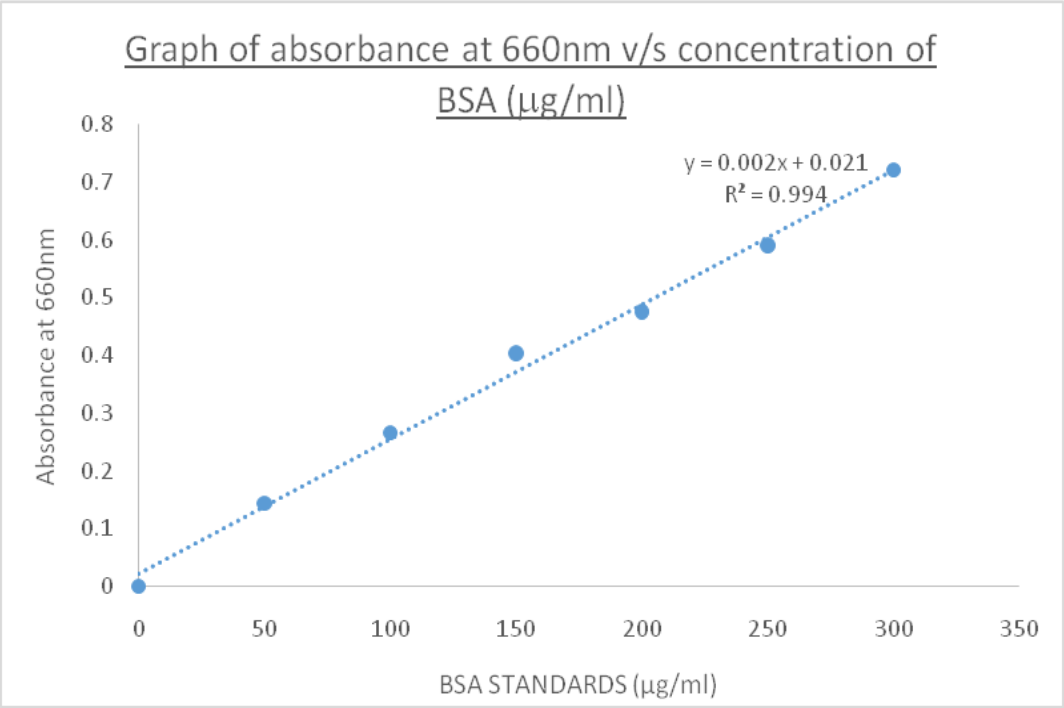


Fig 7 : Estimation of protein concentration By Folin Lowery's method at 660 nm

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

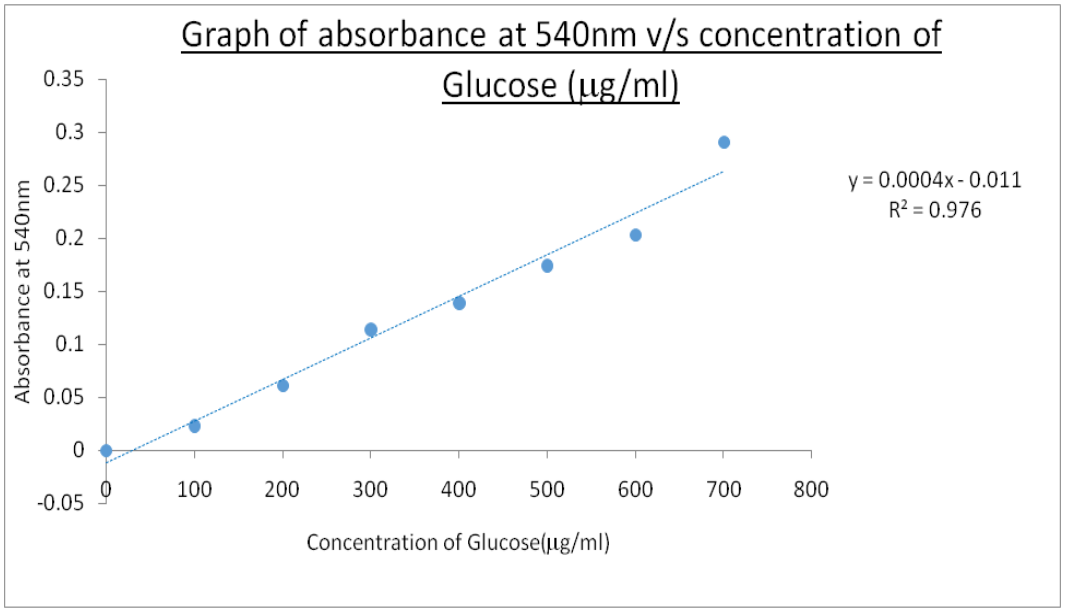


Fig 8 : Estimation of sugar by 3-5 dinitrosalicylic acid (DNSA) method at 540 nm.

Calculations :

$$\text{Enzyme activity} = \frac{\mu g \text{ of product released} \times 1000}{\text{Molecular weight of product} \times \text{incubation time}}$$

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Product concentration}}$$

$$\text{Enzyme unit} = \frac{\text{Product concentration}}{\text{Incubation time}}$$

Table 3 : Enzyme unit, enzyme activity and specific activity of bacterial isolates

Isolate no.	Enzyme unit (µg/min)	Enzyme activity (µg/ml/min)	Specific activity
70	22	31.45	0.535
62	27.1	37.69	0.57
106	26.3	36.54	0.56
147	23.3	32.37	0.541
120	37	52.03	0.642
EC	10	13.87	0.349
SC	8.8	12.25	0.322

➢ As the enzyme activity increases there is a decrease in specific activity and enzyme unit.

4.8 Characterisation of amylase activity

Table 4, 5, 6 : Results of enzyme activity

CULTURES	INCUBATION TIME IN (Min)							
	5	15	30	45	60	120	150	180
120	8.09	31.22	25.67	28.21	28.67	18.96	3.00	4.85
106	8.32	36.54	19.19	21.27	24.74	16.88	5.31	5.08
62	15.72	27.29	44.63	**46.02**	30.29	18.73	7.63	4.62
147	9.01	34.69	21.74	21.97	25.47	19.42	3.70	3.46
70	10.17	32.61	23.59	25.20	25.90	17.57	3.70	6.93
SC	5.78	29.37	15.26	9.25	6.24	5.08	5.31	4.62
EC	9.25	192.88	13.87	59.90	3.46	4.62	5.08	3.00

- Among all the cultures, isolate no 62 was showing high enzyme activity for 45 min. As the time increases the enzyme activity decreases.
- In (Padma and Pallavi 2016) investigation the maximum incubation time for enzyme activity was determined after 24 hours. In (Khunt et al 2011) *Bacillus macquariensis* produce amylase after 72 hours. In order to compare all the results my data showed the best enzyme activity (42.06) at 45 min for culture 62.

Table : 5

CULTURES	'EMPERATURES				
	4°C.	28°C.	37°C.	55°C.	70°C.
120	6.47	5.088	36.5	**40.01**	9.01
106	7.57	6.01	35.15	**41.63**	9.48
62	8.32	7.63	30.06	30.06	7.40
147	10.87	8.09	61.51	33.30	8.55
70	6.93	10.63	32.61	**34.69**	6.47
SC	5.08	9.25	29.60	32.37	6.94
EC	41.16	46.25	37.69	116.56	21.74

- The best enzyme activity was seen at 55°C for 120, 106 and 70 respectively. As the temperature increases enzyme activity decreases.
- In (Mishra et al 2008) the enzyme activity was seen at 37°C. Whereas (Padma and Pallavi 2016) investigation 35°C showed the maximum enzyme activity. (Aygan et al 2008) enzyme activity was optimum at 50°C. In comparison, my study showed better enzyme activity at 55°C than the other three.

Table : 6

CULTURES	SUBSTRATE CONCENTRATION IN %					
	0.5%	1%	2%	3%	4%	5%
120	28.447	59.43	132.06	211.39	214.45	283.78
106	24.747	64.75	102.68	231.04	268.05	295.57
62	34.692	38.85	97.60	175.77	268.517	250.24
147	25.209	57.58	109.62	233.64	284.47	**342.29**
70	27.985	54.35	108.70	209.30	239.60	293.03
SC	6.244	62.67	103.61	230.35	185.94	237.98
EC	3.469	21.27	16.88	16.88	64.06	45.09

- The best enzyme activity was seen at 5% substrate concentration for all the cultures. Also from the above observation, culture 147 was giving the maximum product (342.29) at 5% . As the substrate concentration is increased more the product is formed.
- (Mishra et al 2008) maximum enzyme activity was observed at 2% starch concentration whereas (Khunt et al 2011) studies showed that maximum enzyme production was at 1.2% starch. In comparison, these two studies show much better results than my observations.
- As the substrate concentration is increased more the product is formed.

4.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

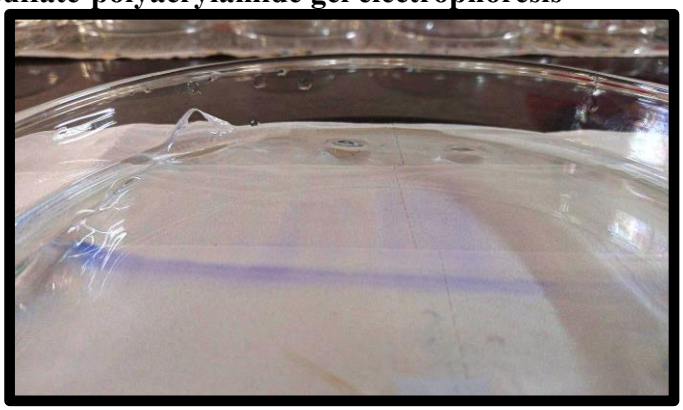
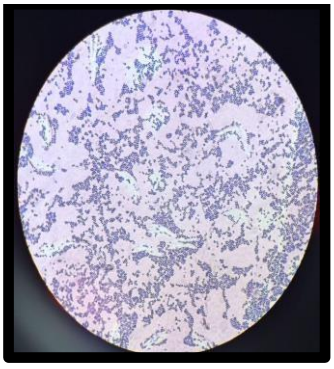


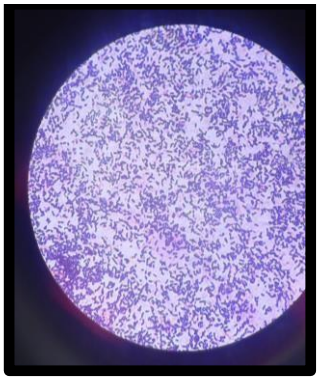
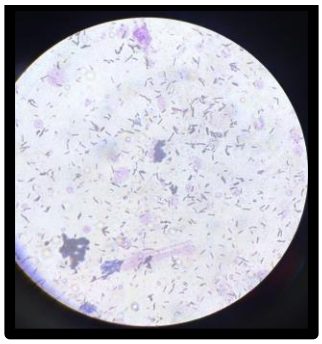
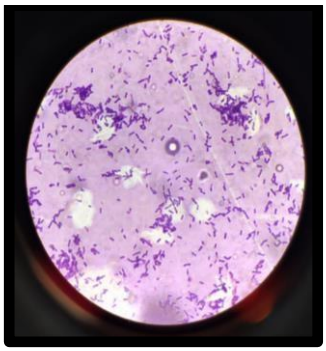
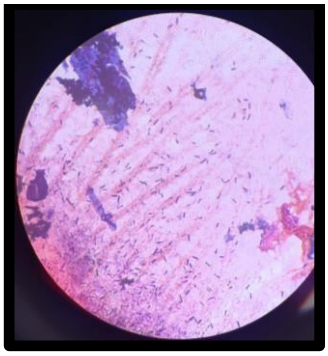
Fig 9 : Gel showing bands

- SDS-PAGE is used to separate proteins in the mass range 1–100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. The concentrations of acrylamide used in the gels are lower than in other electrophoretic systems (Schägger, H. 2006).
- SDS-PAGE was not successful as the bands were not clearly visible on the loaded sample, also the standard protein marker showed only one band.(experiment was repeated 2 times)

4.10 Gram's staining

Table 7 :Identification of bacterial cultures

ISOLATE NO.	GRAM CHARACTER	IMAGE
70	Gram positive cocci in clusters and chains	

62	Gram positive rods	
106	Gram positive rods	
147	Gram positive rods	
120	Gram positive rods	

➢ Gram staining is the differential staining used in microbiology. It is an important criteria for identifying all types of bacterial isolates. Gram character of each isolates was studied.

4.11 Biochemical test

Table 8 : Biochemical detection for catalase,indole and motility.

Isolates No.	Test		
	CATALASE	**INDOLE**	**MOTILITY**
70	Negative	Negative	Negative
106	Positive	Negative	Negative
147	Negative	Negative	Negative
120	Positive	Positive	Negative
62	Negative	Negative	Negative

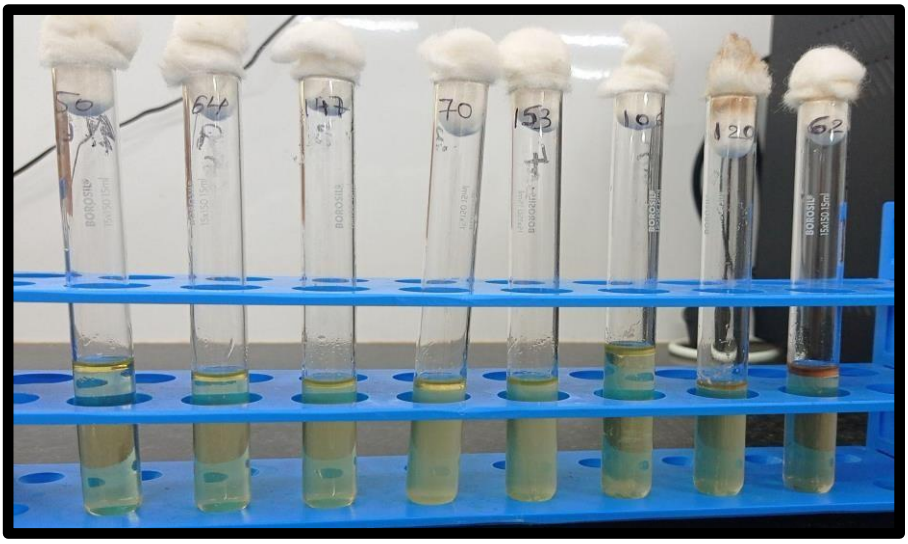


Fig 10 : Culture 62 showing red colouration of oily layer indicating indole positive test

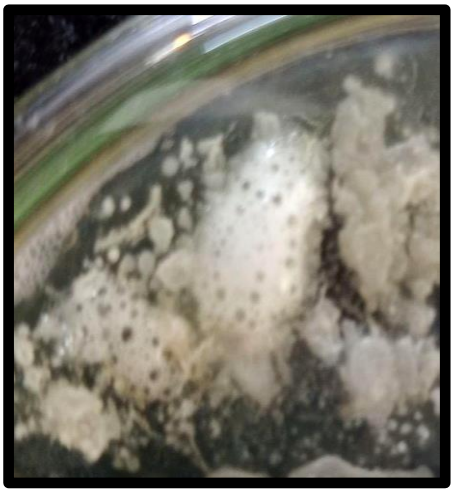


Fig 11 : Culture 106 showing active bubbling indicating positive test for catalase.

Fig 12 : Culture 120 showing active bubbling indicating positive test for catalase.

- Overall the bacterial isolates 120 and 106 were showing active bubbling indicating positive test for catalase.
- Culture 62 showed red coloration of oily layer indicating indole positive test.
- Motility test was negative for all the five cultures
  (only 3 test were performed due to less availability of chemicals)

5. CONCLUSION

Extremely halophilic bacterial strains were pre-isolated from the salt pans of Ribandar (15° 30.166N and 73° 51.245 E) , Goa and were screened for amylase enzyme which gave 5 positive cultures showing an opaque zone around the colonies. Protein estimation by Folin Lowry method and sugar estimation by DNSA method were carried out to determine the enzyme activity, specific activity and enzyme unit of the amylase enzyme.

Characterization of enzyme was carried out by subjecting the pure enzyme to different parameters such as incubation time, temperature and substrate concentration. The enzyme activity was maximum at incubation time of 45min showing 46.02µg/ml/min for culture 62. The enzyme activity for temperature was maximum at 55°C having 40.01, 41.63 and 34.69µg/ml/min for 120, 106 and 70 cultures respectively. The best enzyme activity was seen at 5% substrate concentration i.e. 342.29 for culture 147. Characterization of different parameters is necessary to ensure purity, identity and activity of enzyme product.

SDS-PAGE was not successful as the bands were not clearly visible, because the concentration of protein in the sample was below the detection level or the proteins must have degraded. Standard protein marker showed only one band.

6. SUMMARY

Extremely halophilic bacterial strains were isolated from the salt pans of goa by the previous batch student having 194 isolates out of which 94 were revived on nutrient agar plates with 2% Nacl. These cultures were screened for amylase enzyme which gave 5 positive cultures showing an

opaque zone around the colonies and each of these were isolated. Protein precipitation was done for each of these 5 cultures to get 70% saturation using ammonium sulfate. Dialysis was performed for desalting the proteins. Protein concentration was estimated for BSA standards and samples by Folin Lowry's method. Sugar concentration was determined by the DNSA method followed by enzyme activity and specific activity. Characterization of each of the isolates producing amylase activity was carried out at different parameters such as time, temperature and substrate concentration. SDS-PAGE was performed to determine protein conc. In each of the samples. Gram character and biochemical test was determined for each of the isolates.

7. ANNEXURE

SDS-PAGE solutions

40% Acrylamide	
Acrylamide	116.8g
N,N-Methylene bisacrylamide	3.2g
DDI water	To 300ml
Filter and store in a dark bottle at 4°C	

RG buffer- 1.5M trisCl, pH 8.8	
DDI water	300ml
Tris-free base	90.75g
Conc. HCl	800ml
Adjust to pH 8.8 with conc. HCl, and bring the final volume to 500ml with DDI water.	

4x SDS-PAGE sample buffer	
125mM Tris HCl, pH 6.8 1M	5ml
20% glycerol	8ml
4% SDS	8ml
10% beta-mercaptoethanol	4ml
0.5mg/ml bromophenol blue	20mg
DDI water	15ml
total	40ml

Destain solution	
Ethanol	1200ml
Glacial acetic acid	400ml
DDI water	2.4l

Coomassie stain solution	
Ethanol	150ml
Glacial acetic acid	50ml
DDI water	300ml
Coomassie brilliant blue	1g

30% Ammonium Persulfate

Ammonium Persulfate	1.5g
DDI water	5ml
Store at 4°C	

SG Buffer-10 MM TrisCl, pH6.8	
DDI Water	300ml
Tris free base	60.54g
Conc.HCl	36ml
Adjust to pH 6.8 with conc.HCl and bring final volume to 500ml with DDI water	

10x SDS-PAGE running buffer	
30.3g	Tris base
144.0g	Glycine
10.0g	SDS
Dissolve and bring the total volume to 1000 ml with DDI water. Do not adjust pH with acid or base	

Media for amylase screening (Nutrient agar + starch)

Peptone	5g
Sodium chloride	5g
HM peptone B	1.5g
Yeast extract	1.5g
Agar	15g
pH	7.0
Starch	2%

Media for enzyme extraction (Nutrient broth + starch)

Beef extract	5g
Peptic digest of animal tissue	5g

Sodium chloride	1.5g
Yeast extract	1.5g
Distilled water	1000ml
Starch	2%

Phosphate buffered saline

Disodium hydrogen phosphate	20.214g
Sodium dihydrogen phosphate	3.394g
Distilled water	1000ml
Sodium chloride	8.5g
pH	7.4

Phosphate buffer

Disodium hydrogen phosphate	20.214g
Sodium dihydrogen phosphate	3.394g
Distilled water	1000ml
pH	7

Folin lowery ‘s reagent

Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide.

Solution B: 0.5% copper sulphate (CuSO4.5H2O)

Solution C: 1% potassium sodium tartrate in water.

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

Reagent 2: 1 part of Folin Ciocalteu reagent + 1 part of distilled water

BSA standard solution : 1 mg/ml

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