PRODUCTION OF PULLULANASE FROM HALOPHILIC CULTURES USING SEAWEED AS A SUBSTRATE

A Dissertation for

MBPD 501 & Dissertation, Credits: 16

Submitted in partial fulfilment of Master's Degree

MSc. In Marine Biotechnology

By

SHWETAKSHI MISHRA

22P0500015

Under the Supervision of

DR. MEGHANATH PRABHU

School of Biological Sciences and Biotechnology

GOA UNIVERSITY

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Examined by: Seal of the School

08/04/2024

8/4/24

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Production of Pullulanase from Halophilic Cultures using Seaweed as a Substrate"** is based on the results of investigations carried out by me in the discipline of **Marine Biotechnology** at the **School of Biological Sciences and Biotechnology**, Goa University under the Supervision of **Dr. Meghanath Prabhu** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Production of Pullulanase from Halophilic Cultures Using Seaweed as a Substrate"** is a bonafide work carried out by **Ms. Shwetakshi Mishra** under my supervision in partial fulfilment of the requirements for the award of the degree of **MSc. Marine Biotechnology** in the Discipline of **Marine Biotechnology** at the **School of Biological Sciences and Biotechnology, Goa University**.

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PREFACE

Marine biotechnology is a fascinating field that offers tremendous potential for commercial ventures across multiple industries, including pharmaceuticals, biomedicine, cosmetics, nutraceuticals, food, feed, agriculture, and related fields. Further, this marine environment is home to diverse life forms including seaweed, planktons, fishes, crustaceans and other creatures, all having unique adaptations to sustain in the marine ecosystem.

Seaweed are fascinating biomass loaded with many unique sugars having unexplored potential. Many pieces of research have recently gained attention on utilizing these seaweed sugars for fermentation to produce enzymes of commercial importance. Following with the trend, this work is also aimed at using seaweed hydrolysates for production of pullulanase enzymes. Pullulanase is an extracellular, de-branching enzyme that has high demand in industry, especially food and beverage sector. The enzyme however has lower yield when produced using plant based sources. So, the present work is an attempt to use various halophilic cultures that can grow in seaweed hydrolysate and then use them to produce pullulanase by utilizing the seaweed sugars.

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TABLES

FIGURE CAPTIONS

ABBREVIATION USED

No.	Abbreviation	Full form
1.	°C	Degree Celsius
2.	µ mol	Micro moles
3.	µL	Microliter
4.	BSA	Bovine Serum Albumin
5.	Cfu	Colony forming units
6.	CHNS	Carbon, Hydrogen, Nitrogen, Sulphur
7.	Cm	Centimeter
8.	CMC	Carboxy Methyl Cellulose
9.	CO_2	Carbon Dioxide
10.	DNSA	3,5-dinitrosalicylic acid
11.	DW	Distilled water
12.	EC	Electrical Conductivity
13.	EDTA	Ethylenediaminetetraacetic acid
14.	Fig.	Figure
15.	g	Grams
16.	H_2O_2	Hydrogen Peroxide
17.	HCl	Hydrochloric acid
18.	K_2HPO_4	Di-Potassium hydrogen phosphate
19.	kDa	Kilo Daltons
20.	KH_2PO_4	Potassium di-hydrogen phosphate
21.	Km	Kilometer

22.	M	Molar
23.	Mbar	Mega Bars
24.	Mg	Milligram
25.	Min	Minutes
26.	Ml	Milliliter
27.	mm	Millimeter
28.	N	Normal
29.	NaCl	Sodium Chloride
30.	NaOH	Sodium Hydroxide
31.	NIO	National Institute of Oceanography
32.	Nm	Nanometer
33.	PBS	Phosphate Buffer Saline
34.	Ppm	Parts per million
35.	Ppt	Parts per thousand
36.	Psi	Pound-force per square inch
37.	Rpm	Revolutions per minute
38.	SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
39.	SSF	Solid State Fermentation
40.	TDS	Total Dissolved Solids
41.	TS	Total Solids
42.	TVS	Total Volatile Solids
43.	U	International Units

ABSTRACT

The pullulanase production is a global need as it has been widely used food and beverage sectors to break glycosidic linkages and make simpler sugars for various applications. However, lower yield and relatively lesser stability of currently produced pullulanase, brings up opportunity to explore for other substrates and cultures that can increase yield of the enzyme. Production of pullulanase was carried out using seaweed hydrolysates as a substrate in this study. The work involved characterization of six different seaweed found in Goa viz. *Fucus, Sargassum, Gracillaria, Padina, Ulva* and *Solieria* and preparing their acid and alkaline hydrolysate. Further, halophilic cultures were isolated from Curca Saltpan, Goa and screened for pullulanase activity. The selected cultures were inoculated in seaweed hydrolysate and optimized for pH and temperature to get better pullulanase activity. The produced pullulanase was further processed using ammonium salt precipitation (single fraction 80%) followed by dialysis. The results revealed that the culture GUPM2 gave best pullulanase activity of 5.584 U when inoculated in acid hydrolysate of *Sargassum* at pH 7.5 and temperature of 30°C. Likewise, the cultures GUPM5 and GUPM22 gave best pullulanase activity of 5.359 U and 7.440 U, when inoculated in base hydrolysate of focus at pH 7 and pH 7.5 and temperature of 37°C respectively. Further, SDS-PAGE analysis tentatively suggests that molecular mass of produced pullulanase is approximate 59.40 kDa (for GUPM2), 75.75 kDa (GUPM5) and 103.14 kDa (for GUPM22). Likewise, thermal stability analysis showed that enzyme activity was stable up to 50°C for all the pullulanase. Thus, the study concludes the possibility of using seaweed as a source of carbon for production of pullulanase using microorganisms.

Keywords: Seaweed Hydrolysate, Pullulanase, Pullulan, Saltpan, Halophiles

INTRODUCTION

"The key to growth is the introduction of higher dimensions of consciousness into our awareness."

- Lao Tzu

Chapter 1

INTRODUCTION

1.1 Background

Marine macro-algae, commonly called seaweeds, refer to a group of weeding plants of the sea. These are forms of macro-algae that thrive in intertidal marine waters (*Maryam Kokabi et al. 2016)* and are marked by structures with holdfasts, stipes and blades resembling the plant's root, stem and leaves, respectively (*Samuel Starko, et al. 2016*). The seaweeds are grouped into three main types based on their pigmentation: *Chlorophyceae*, *Rhodophyceae*, and *Phaeophyceae*, which depict green, red and brown colouration to seaweeds (*Hossam S. El-Beltagi et al. 2022).* All groups of seaweed are photosynthetic and thus contribute significantly to the ocean's primary productivity, generating 50% of the world's total oxygen production. It is suggested that seaweed has a role in the global coastal carbon cycle (*Albert Pessarrodona, et al. 2022).*

Seaweeds have been used as food since ancient times around the world, especially in Asian countries (*Rocío Peñalver et al. 2020).* They are fascinating biomass packed with many essential minerals, vitamins, proteins, lipids, and unique polysaccharides (mannitol, laminarin, alginate, fucoidan, ulvan, carrageenan, starch, cellulose), sugars, and metabolites (pigments, terpenoids and phenolic compounds), which are highly exploited as a means for medicines, biofuels, cosmetics, bioplastics, bioremediation, etc. (*Bhat et al. 2021; Cikoš et al. 2022; Satyabrata Dash Sharma, et al. 2016).* For instance, *Agardhiella subulata* and *Ulva lactuca* seaweed possess antiviral properties showing effects against human viruses like Human Immunodeficiency Virus and Herpes Simplex Virus. Also, *lectin griffithsin* and the phycocolloid carrageenan from red seaweed, ulvan from green and fucoidan from brown seaweed can possess antiviral activity for COVID-19 (*Leonel Pereira and Alan T Critchley 2020)*.

Likewise, *Ecklonia cava*, a brown seaweed, produces phlorotannins to protect its cells from radiation-induced injury and is thus exploited to form solar-protectant formulations *(Rocio Penalver et al. 2020).* Many Asian countries are fond of food delicacies of well-known red *algae Palmariapalmata, Porphyratenera* and *Eisenia bicyclis* seaweeds (*Stephen Bleakley and Maria Hayes 2017)*. Seaweeds have recently been used as an additive in making fabric construction material and for methane reduction.

Seaweed biomass is also a significant part of blue carbon ecosystems as it can rapidly sequester CO_2 due to its rapid growth rate and is important for biodiversity and habitat monitoring (*World Bank Report, 2023).* The global seaweed industry's full potential is still untapped, yet its market value is projected to grow to $11.8 billion by 2030 (*World Bank Report 2023).* In general, there are two major research group distributions related to seaweeds; the former involves the direct use of seaweed properties for many pharmaceutical and industrial uses. These may include the extraction of agar, alginate, carrageenan, fucoidan, β-carotenoid, etc., as direct commercial products in the market (*N. Kaliaperumal, 2003).*

However, with increasing global human demands, seaweeds have become a trending research candidate as biomass to produce value-added products. Furthermore, studies conducted to find such commercial and industrial potential are not restricted to just seaweed as a component; instead, a lot more research is emerging around finding seaweed and associated microbial forms to provide an even more valuable and wider range of valuable products that are not found in seaweeds *(Andrea Gomez Zavaglia et al. 2017).* The seaweed-associated bacteria, for instance, help induce growth-promoting substances that help in the overall growth and development of seaweed in its habitat (*Ravindra Pal Singh and CRK Reddy, 2014)*.

Not only its natural microflora but artificially inducing certain organisms to carry forward seaweed fermentation is also a strategy to prepare prebiotics and other value-added products (*Gabriele Maiorano et al. 2022).*

1.2 Aim and Objectives

The present work is aimed to touch upon one of the aspects of enzyme production for industrial application using seaweed as substrate.

AIM: To produce Pullulanase from Halophlic Cultures Using Seaweed as a Substrate

OBJECTIVES:

- Isolation of Halophilic cultures from saltpan
- Screening of cultures for pullulanase activity
- Seaweed collection and biomass characterization
- Seaweed hydrolysate preparation and its characterization
- Pullulanase production using seaweed hydrolysate.
- Partial characterization of produced pullulanase enzyme

1.3 Hypothesis

Pullulanase is mainly produced using bacterial cultures belonging to the genus of *Klebsiella (Takashi Kuroiwa et al.2005) Bacillus, Streptomyce*s, etc. It is also been produced using fungi like *Aspergillus flavus* and archaea. Pullulanase is usually stable up to 45ºC, but the use of extremophilic microbes, including *Pryococcus, Halobacterium, Thermococcus*, etc., increases its stability up to 110ºC *(Bindu Naik et al. 2023).* However, pullulanase has a lower yield when produced via these cultures. Goa being a western coastal state of the Indian Peninsula, it is a hub for many salt pans that may have many cultures that can potentially produce pullulanase. Cultures, including halophiles, have been tested previously to produce pullulanase using various artificial sources like starch and cellulose, as well as natural sources like agro-waste and food waste (*Vijay Kumar et al. 2022).*

However, to the best of our knowledge, seaweed as a substrate for producing pullulanase has not been reported. Thus, we propose to carry out the dissertation to produce pullulanase using seaweed as a substrate.

1.4 Scope

Bioactive compounds' commercial production has received wider attention with increasing interest in biomolecules, genetics and molecular biology analysis. Also, since bioactive compounds are directly related to biological life forms, they are under constant exploitation for therapeutic and pharmaceutical applications (*Anderson Junger Teodoro, 2019).* Bioactive compounds are mostly metabolites secreted in small quantities, and they have properties like antioxidants, inflammation, immune modulation, etc. They may include enzymes, organic acids, essential oils, flavonoids, carotenoids, co-enzymes, etc. (*Aytul Hamzalioglu et al. 2016).*

Since the late nineteenth century, rapid advances have been made in the extraction, characterization, and commercial exploitation of enzymes in diverse industries (*Peter K. Robinson 2015).* Enzymes such as hydrolases, especially glycoside hydrolases, are reported a lot for industrial applications. They are widely utilized in food and fermentation industries to break down complex sugars into simpler forms for easy utilization *(Smriti Shrivastava, 2020).* Pullulanase is one such hydrolysing enzyme that functions up by breaking open α-1, 6 glucosidic linkages present in starch, amylopectin, pullulan, and related oligosaccharides to form simpler sugars like maltotriose. This de-branching enzyme has an international market in the food and fermentation industries during the process of saccharification *(Bindu Naik et al. 2023).*

LITERATURE REVIEW

"Literature provides us with experiences it would not be otherwise possible to introduce into our own world and thus enlarges our understanding of the world."

- Louise Rosenblatt

Chapter 2

LITERATURE REVIEW

The core basis for this dissertation revolves around three key constituents viz. seaweed, bioactive compound and source culture for production. Seaweed have become increasingly become popular since last few decades as a research candidate and for commercial exploitation. Seaweeds being abundant, and loaded with many unique compounds are useful in wide range of application including pharmaceutical, cosmetics, bio plastics, biofuels, bioremediation etc. *(Silvia Lomartire1 and Ana M. M. Gonçalves, 2022)*.

Md. Shirajul Islam Sarkar et al. (2017) have carried out many experiments of preparing commercial application-based food products using seaweed including soups, jellies, ice-cream, curd, samosa etc. These products can pose a healthier snack and also promote better eating habits. Not only direct products, but seaweed are into exploitation for bioactive compounds. *Bahare Salehi, et al. (2019)*, has aimed to provide chemical constituents of different types of seaweed and correlate with its industrial uses. *Hossam S El-Beltagi, et al. (2022)* have categorized seaweed bioactive compounds into eight main groups. Previously a lot many pieces of researches are oriented to provide optimized conditions for culturing, production and extraction of constituents from seaweed.

For instance, *Dennis J. McHugh, (2003)* in his a book entitled 'A guide to seaweed industry' provides an overview to seaweed, its morphology, types, scope, culturing and products. Likewise, *Sangil Kim et al. (2022)* details about sampling method for seaweed and what parameters to check while handing seaweed. Recently, *Yogesh Kumar et al. (2021)* describes many vitamins, minerals, and bioactive metabolites in *Sargassum* and *Ulva*. Similarly, *Silvia Lomartire1 and Ana M. M. Gonçalves (2022)*, provided an overview to various bioactive compounds in *Phaeophyceae, Rhodophyta* and *Chlorophyta* as valuable source for the food, cosmetic, pharmaceutical and nutraceutical industries.

Extraction of such various molecules from seaweeds demands a suitable technology. However, the recent developed technologies for extracting molecules from terrestrial biomass are not suitable for macroalgae biomass. For instance, the seaweed contains many unique carbohydrates compared with terrestrial plants, so the existing methods to extract sugars from plants cannot be used to saccharify these seaweed sugars (*Monteiro P et al. 2021).*

However, to meet global needs from seaweed molecules, the extraction processes should be time saving, non-destructive, quantitative and cost effective. Also, the extraction efficiency must be high. Several factors such as pH, solvent, temperature, particle size, extraction time are responsible for extraction efficiency. *Andrew J. Lorbeer et al. (2017)*, have also worked on acid treatment to efficiently extract fucoidans and alginates from various brown seaweed like *Ecklonia radiata, Macrocystis pyrifera, Durvillaea potatorum* etc.

Joseph S. Pechsiri et al. (2016), emphasized the need to optimize biorefinery methods by showcasing an example of Kelp seaweed to make biogas and fertilizer. It suggests that optimizing in anaerobic digestion process would lead to a large reduction in energy use and emissions. For green seaweed too, biorefinery contributes to maximum extraction of products. *Glasson et al. (2017),* demonstrated that *Ulvan* (a sulphated polysaccharide) can be extracted with higher yields from *Ulva ohnoi* seaweed by following a sequential method of aqueous pre-treatment and Hydrochloric acid extraction.

However, apart from the harsh method of biorefinery, green methods for the extraction of these products are emerging and becoming more popular. *Meiron Zollmann et al. (2019),* provide a comprehensive overview of different green methods for the biorefinery of green seaweeds. Some of them would include ultrasound, pulsed electric field, use of green solvents, anaerobic digestion, etc.

Similarly, *José Alberto Herrera Barragán et al. (2022),* describe enzyme-assisted extraction as an efficient green method for biorefinery of seaweeds. But, despite extensive work in this area, the utilization of products directly obtained from seaweed is limited.

Increasing global human population is leading to production of resources in more efficient and effective ways with use of efficient biomass *(World Bank Report, 2023).* Seaweeds offer several advantages over terrestrial biomass. Seaweeds are easy to grow without fertilizers and freshwater, have high growth rate, low impact on the environment, and are hardly affected by disease which makes them a better substitute as a biomass for producing high value products. This opens an opportunity to formulate novel products that can provide new insights for research in seaweeds (*Maiorano G, Ramires FA, et al. 2022).*

Not only bioactive metabolites from seaweed are into application, rather many other sources even before use of seaweeds are utilized for extracting different metabolites. *Mrigya Bansal et al. (2023)*, describes many compounds such as bioactive peptides, phytosterols, fibers, fatty acids, and vitamins have the ability to regulate metabolic processes in human body. *Kevin Pfeifer et al. (2020),* summarized many commercially available products of archaeal cell factories are bacterioruberin, squalene, bacteriorhodopsin and diether-/tetraether-lipids, all of which are produced utilizing halophiles. Owing to bioactive metabolites and previously described seaweed properties, merging up two broad groups in a systematic fashion can help build up even more productive products in better processing setup.

Marta Ferreira, et al. (2022), carried out solid state fermentation using seaweed as a substrate to produce xylanase and β-glucosidase using *Aspergillus ibericus* and *A. niger*. They received a comparable cellulase activity using *Gracilaria sp.* Similarly, there are many other pieces of research on seaweed assisted fermentation for high-value product productions.

Fermentation of seaweed biomass causes changes in their macromolecule composition. It reduces dietary fibers in macroalgae (*Maiorano G, et al. 2022). Fook Yee Chye et al. (2017)* provides an insight into various products like anticoagulants, anticancer and antimicrobial agents produced by seaweed fermentation by microbes. Industrial fermentation of seaweed using bacteria provide many functional compounds and food delicacies with health benefits.

There are many researches focused on using edible seaweed, as they are easy to access and safe to handle. *Annette Bruhn et al. (2019)*, have worked on using lactic acid bacteria to ferment sugar kelp (*Saccharina latissima*) to produce a more consumer's acceptable flavour of sugar kelp. They found that heat treatment and fermentation within 40 hours at pH 4.5 produced the edible with better visuals, smell and with milder taste.

Catalina Landeta Salgado et al. (2021), worked on producing mycoprotein and hydrophobin-like proteins by submerged fermentation from *Paradendryphiella salina*, a marine fungi using *Ulva* as a substrate. They could obtain an appreciable total protein concentration of around 48%.

Therefore, the above search proves vast scope of microbial fermentation of seaweed to obtain many value-added products of commercial importance. Despite the above-mentioned enzymes and other products in being prepared using seaweed fermentation, there is limited literature on use of seaweed as substrate for production of certain extracellular enzymes such as pullulanase. This enzyme have been produced commercially using bacterial cultures of *Klebsiella (Takashi Kuroiwa et al. 2005) Bacillus, Streptomyce*s, etc. as well as fungi like *Aspergillus flavus*.

Pullulanase has a global market in food and beverage industry as it actions by breaking up α-1, 6 glucosidic linkages that are present in oligosaccharides like starch, amylopectin, pullulan to form simpler sugars like maltotriose (*Bindu Naik et al. 2023).*

Further, *Bindu Naik et al. (2023)* mentions various substrate sources like starch and other agricultural waste used for pullulanase production. *Piotr Tomasik and Derek Horton, (2012)*, suggest yield to be only 12.8% when starch is used as a substrate for pullulanase production. This paves way to further explore seaweed as a source for pullulanase production. Thus, the current study initiates a possibility of using seaweed hydrolysates and saltpan cultures for pullulanase production.

METHODOLOGY

"The method of scientific investigation is nothing but the expression of the necessary mode of working of the human kind."

-Thomas Henry Huxley

Chapter 3

METHODOLOGY

3.1 Sampling for Halophiles in Saltpan

Soil, salt and water samples were collected from Curca Saltpan, Maina, Goa (Lat: 73.883254°; Long: 15.459017°) to isolate halophiles. Field parameters including temperature, and TDS were recorded using Konvio TDS Meter. pH and salinity of water sample was recorded using KONVIO NEER Digital pH Meter and Brix refractometer respectively.

Data about Curca's temperature, wind speed, humidity and air pressure on 30th November 2023 between 12 noon to 18:00 hours was collected using AccuWheather App (https://www.accuweather.com/). The sampling was performed in following manner:

i. Soil sample was collected by scooping out surface soil till around 3 cm depth at different areas within the saltpan. The soil sample was packed in zip lock bag and kept at till further use.

ii. Salt sample was collected by break opening the salt heap and kept in zip lock bag at 25 ± 2°C till further use.

iii. Water samples from two different sites were collected using plastic bottles, sealed and kept at 25 ± 2°C till further use.

3.2 Screening and Isolation of Halophilic Cultures from Saltpan Samples

Soil samples were serially diluted using 15% saline and then spread plated on Halopiger Agar Media to selectively obtain growth of halophiles. Likewise, 10 grams of salt sample was mixed in 10 ml of autoclaved distilled water, serially diluted and then spread plated on Halopiger Agar Media under sterile conditions in Laminar Air Flow (LAF) (MSet HLF5472). Henceforth, sterility was mentioned in all the experiments wherever required.

For water sample, the protocol was followed as mentioned in literature (*Aparna Singh and Anil Kumar Signh, 2018)*. Briefly, 15 ml of water sample was first passed through 0.45 µm cellulose filter. The filter was inverted and placed on to the media plate.

Colony in (cfu/ml) = No. of colonies in plate × dilution factor/ volume of sample plated

All master plates were incubated at 25 ± 2°C for a week, colony count was recorded (using following formula) and then individual colonies were streaked to obtain isolates. The single colonies were picked up using nichrome loop and streaked on Halopiger agar plates using quadrant streak method.

Later, the pure isolates were numbered, grown on Halopiger media slants and stored at 4°C for further use. Glycerol stocks were also prepared and stored at -80°C.

3.3 **Colony Morphology and Characteristics**

The isolated cultures numbered as GUPM 1 to 25 were observed for colony morphology characters on Halopiger agar pates which include shape, size, elevation, margin, surface, opacity, colouration and consistency, followed by observing Gram characters.

Further, preliminary biochemical characterizations of cultures were done which include sugar utilization tests, starch assay, casein assay, skim milk assay, cellulose assay, salt tolerance test and antibiotic sensitivity assay. For all these biochemical tests, the culture inoculum was prepared by first growing the culture in Halopiger broth for 72 hours. Then 2 ml of broth was centrifuged (LAB-i-FUGE c series) and pellet was re-suspended in 15% saline. This step avoids false positive results that might have arose by growing of cultures in leftover Halopiger broth.

3.3.1 **Size Measurements**

The individual colonies were streaked on Halopiger agar using quandrant streak and five isolated colonies were selected for measuring size. The average colony size was recorded.

3.3.2 Gram Staining

The gram staining was performed *(Ann C. Smith and Marise A. Hussey, 2005)* with alteration in heat-fixing step. The colony from the plate was touched by sterile loop. Then a smear was made over a clean glass slide. As per protocol suggested by (*H.P Dussault, 1955)*, the smear was fixed by adding few drops of 5% acetic acid and allowing it to dry naturally. Acetic acid helps in fixing cells without affecting their morphology and also help remove salt that might hinder with further staining. This slide was then flooded with 2% crystal violet for one minute. The stain was washed gently with distilled water. A few drops of Gram's iodine were added and kept for 1 minute followed by adding of decolourizer and keeping for 30 seconds. Counterstain safranin of 0.25% was added to the slide and kept for 1 minute. Using a gentle stream of distilled water, the slide was washed. The slide was then kept in air to dry completely and were observed with light microscope (Lawrence & Mayo XSZ-N107T), under oil immersion magnification.

3.3.3 Biochemical Tests

A] Sugar Utilization a) Glucose b) Galactose c) Maltose d) Sucrose

The sugar utilization test was performed as mentioned in protocol by (*Karen Reiner, 2012)*, with slight modification with respect to increased NaCl content to maintain 25% salinity. Four different carbohydrate sources were used namely glucose, galactose, maltose and sucrose. The method included wet sterilization (Euitron Autoclave SLEFA) of tubes along with Durham tubes placed inside.

Phenol red carbohydrate broth (10 ml) was added to each tube. One ml of inoculum was inoculated in carbohydrate broth. The tubes were incubated at 25 ± 2°C and examined for colour change after every 24 hours for three days. A colour changes from pink to orange-yellow indicate uptake of particular carbohydrate by the organism.

Presence of bubble on Durham tube indicate production of gas during carbohydrate fermentation. However, no colour change refers negative results, which were confirmed by observing no colour change over a week of incubation.

B] Motility Test

The motility test for cultures were performed using stab method and further confirmed using hanging drop method. Briefly, 0.8% soft agar of Halopiger media was prepared and poured to make 10 ml stabs. Using sterile nicrome stab, the culture was touched and stabbed through the agar. The tubes were incubated for 72 hours at 25 ± 2℃ and then observed for zig-zag movement along the line of stabbing to indicate motility.

The positive cultures were then tested for motility by hanging drop method as mentioned by (*Ashabil Aygan & Arikan Burhan, 2007)*. In this method 5 µL of culture from broth was placed on cover slip and the edges were sealed with wax. A clean cavity slide was placed over the drop and inverted gently such that drop hangs in the space between cover slip and cavity. The slide was observed first under 10 X and then 40 X magnification to observe for culture movement.

C] Amylase Production Test

The starch assay was performed as per protocol mentioned by *Archana Lal and Naowarat Cheeptham, (2012)* in American Society of Microbiology. The starch assay media was prepared and salt concentration was adjusted to 25%. The media was poured in 15 cm petri plates and 25 spots were marked roughly at 1.5 cm spacing.

The culture inoculums (2 µL) were inoculated at each spot. The plates were incubated at 25 ± 2°C for 72 hours. The zone of clearance was observed for each colony and measured using Gram's iodine. The zone and colony size were compared to find percent clearance based on the following formula:

$$\text{Clearance \%} = \text{Zone of clearance} - \text{colony size} \div \text{colony size} \times 100$$

D] Caseinase Production Test

The caseinase production was estimated used two types of plates i.e. casein agar plates containing 1% casein and skim milk medium plate containing 1% skim milk. The plates were prepared at 25% salt concentration and poured in 15 ml petri plates. At the plate bottom, 25 spots were marked roughly at 1.5 cm spacing. A culture sample (2 µL) was inoculated at each spots. The plates were incubated at 25 ± 2°C for 72 hours. The zone of clearance were observed after overlaying with 1% Coomassie brilliant blue and the zone size was measured. The dye stained spots where casein/skim milk must have been degraded into smaller peptides. And rest plate remained unstained.

E] Cellulase Production Test

The Carboxy methyl cellulose (CMC) media plates were prepared in 25% saline as per the protocol mentioned in *Osaka Protocols (2010)*. Briefly, 2 µL of culture inoculums were inoculated on CMC plates and incubated at 25 ± 2°C for 72 hours. Then, 0.1% of Congo red solution was flooded over the plate and allowed to remain for 30 minutes. After that, the Congo red solution was discarded and plates were washed with distilled water.

The plates were flooded with 1M NaCl solution, and washed with distilled water after 5 minutes of incubation. Finally, 5% acetic acid was flooded and left for 5 minutes to observe for zone of clearance.

F] Salt Tolerance Assay

The salt tolerance assay was performed by preparing Halopiger media at different concentration starting from 0- 35% crude salt. Similar to other assays, the 2 µL of inoculum was inoculated in different salinity plates. These plates were incubated at 25 ± 2°C for 72 hours and observed for growth.

G] Antibiotic Sensitivity Assay

The antibiotic sensitivity assay was performed similar to as mentioned in protocol by *Tendencia, E. A. (2004).* The Halopiger plates were prepared, and cultures were grown for 72 hours in Halopiger broth. Using a sterile cotton swab, the culture containing broth was evenly spread over the Halopiger plate.

Five different antibiotic containing discs namely Penicillin, Streptomycin, Kanamycin, Chloramphenicol, and Metronidazole were aseptically placed over the culture-swabbed plates using forceps. The plates were incubated at 25 ± 2°C for 72 hours and observed and measured for zone of clearance against matt growth of culture.

H] Oxidase Test

The oxidase test was performed as per the direct plate protocol described by *Shields, P. & Cathcart, L., (2010)*. Briefly, Gordon and McLeod Reagent was freshly prepared using a solution of 1% dimethyl-p-phenylenediamine dihydrochloride in water. A drop of this reagent was placed over a spotted culture grown in Halopiger agar plate. The spot was observed for colour change to black after 60 minutes.

I] Catalase Test

The catalase test was performed based on the protocol described by *Karen Reiner, (2010).* The method involved use of taking a small inoculum of culture using a sterile toothpick and preparing a smear over a clean glass slide. Over the smear, 3% Hydrogen Peroxide (H_2O_2) was placed dropwise. A negative control was maintained by just placing the drop of H_2O_2 over slide without culture. Presence of bubbles or effervescence within 10 seconds indicate positive result.

J] Gas Production Test

The gas evolution method was performed to determine if culture produce highly gaseous products during general metabolism while growing in Halopiger broth.

The method included wet sterilization of tubes along with Durham tubes placed inside. Halopiger broth (10 ml) was added to each tube followed by inoculation of the loopful of culture under sterile conditions. The tubes were incubated at 25 ± 2°C and examined after every 24 hours for three days for presence of gas bubble in Durham tube.

Presence of bubble on Durham tube indicate production of gas. However, no bubble in Durham tube refers negative results, which were confirmed by examining tubes over a week of incubation.

3.4 Screening of Halophiles for Pullulanase Activity

The pullulanase activity was estimated of the cultures using two methods.

a) First a preliminary screening was performed using Pullulan agar media as mentioned by *Waleed M. et al. (2015)*. The cultures were first grown for 72 hours in Halopiger broth and then the broth was centrifuged at 10,000 rpm for 10 minutes and pellet was suspended in 15% saline. A sample of 2 µL was inoculated in Pullulan Agar plates. The overall clearance percentage was estimated using the formula:

Clearance % =Zone size -colony size ÷colony size ×100

b) Pullulan broth containing 1% pullulan as carbon source, along with KH_2PO_4, K_2HPO_4 as buffering agents and Ammonium chloride as nitrogen source was made and selected cultures from pullulan plate assay were inoculated in this broth. The broth was kept in 25 ± 2°C shaker (Biotechniques India R5/01) at 120 rpm for 72 hours. The pullulanase activity was measured using a DNSA reducing sugar assay method as mentioned by *N. Prabhu et al. (2017)*. Briefly, the incubated pullulan broth with culture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was used as crude pullulanase extract. Pullulanase extract of 1ml was mixed with 1ml of pullulan buffer.

The reaction was kept at water bath (LABQUEST Borosil WBC020) maintained at 40°C for 30 minutes. Post-30 minute incubation, 2 ml DNSA reagent was added. The tubes were kept in boiling water bath (Bio-techniques India PP1 UniX96) for 10 minutes and then immediately cooled by placing in ice. The spectrophotometric readings were taken at 540 nm (Shimadzu UV-Vis spec mini 1240) and pullulanase activity was estimated as per the standard (appendix no .IV).

"One unit of pullulanase activity can be defined by relating the amount of pullulanase enzyme required to produce 1 µ mol of reducing sugar per minute" (Yu Zhang et al. 2020). This refers that, the sugar concentration estimated using DNSA test was then divided by incubation time to get pullulanase activity. From these, best three cultures were selected and sent for sequencing.

3.5 **Sampling of Seaweed**

The seaweed sampling was performed during the low tide from the rocky shores of Vagator (Lat: 15.599919; Long: 73.734138) and Anjuana beach (Lat: 15.572201; Long: 73.721462). Water tide level, water temperature, conductivity, TDS, Water salinity, water pH, wind speed, atmospheric temperature, and humidity were recorded as mentioned in Tide chart app (https://www.tideschart.com/#google_vignette), TDS EC meter, refractometer, pH Meter, and AccuWheather App respectively.

Seaweed were collected, washed thoroughly in filtered sea water to remove shells and other sand particles. The seaweed were spin dried using 200 micron mesh bag and drier (DMR Spinner DMR50-50A) to remove excess water.

3.6 Processing of Seaweed Samples

The seaweed samples were identified tentatively based on their morphology using seaweed identification manual *(V.K. Dhargalkar and Devanand Kavlekar 2004)*. The seaweed were weighed (Shimadzu UniBloc ATX224) using pre-weighed trays.

The total weight was recorded and seaweed biomass was estimated by subtracting weight of tray from total weight. And then kept for drying at 50°C (Kumar industries Oven 45X45X45).

The seaweed were tossed between intervals to allow uniform heat distribution. Once the seaweed became crisper, the weight for monitored (using Weinsar Weigh Scales Limited PGB 3010) for every 30 minutes. The final total solid values were recorded if weight was found consistent for two consecutive readings. The wet and dry weights were compared and dry biomass conversion was calculated using the following formula:

Dry Biomass (%) = Dry Weight of Seaweed ÷ Wet Weight of Seaweed

Likewise, moisture content was analyzed using the formula:

Moisture Content (%) =Wet weight - dry weight ÷ wet weight ×100

The dried seaweed was powdered and sieved through 150 micron sieve. The powder was collected and stored in air tight containers till further use.

3.7 Characterization of Seaweed Biomass

The powdered seaweed biomass was characterized for few basic parameters like acid/alkaline nature, conductivity, amount of reducing sugar, amount of total carbohydrate, and protein quantification. For all the mentioned parameter estimations, firstly seaweed water extracts stock were prepared using 10 mg/ml concentration.

3.7.1 Acid/Alkaline Nature and Conductivity

Seaweed powder (100 mg) was added in 10 ml distilled water and mixed thoroughly. The pH paper were dipped in stock seaweed extract and pH was recorded upon colour change. The TDS EC Meter was dipped in stock seaweed extract and conductivity was recorded in µs/cm.

3.7.2 Biomass Characterization

The biomass samples were prepared for analysis by taking 5 mg of dried powder in 1ml of distilled water. The mixture was placed in close capped vials and five zirconium beads were added to each vial. The samples were homogenized using bead beating (BeatBug Microtube Homgenizer) for four cycles of 30 seconds homogenization at 2800 rpm followed a break of 30 seconds. The vials were centrifuged at 10,000 rpm for 2 minutes and the supernatants of respective seaweed biomass were stored at 4°C. Further, these seaweed extracts were used for estimation of reducing sugars, total carbohydrate and total proteins.

A] DNSA- Reducing Sugar estimation

The reducing sugar assay was performed by taking 1ml of extract and mixing it with 1 ml of DNSA reagent. The tubes were kept in 100°C boiling water bath for 10 minutes. The tubes were immediately cooled by dipping in ice to stop further reaction. The absorbance was taken at 540 nm using spectrophotometer. The sugar concentration was estimated by extrapolating readings against a standard DNSA graph with glucose (2 mg/ml). The total reducing sugar was measured in percentage of seaweed biomass (g/100g).

B] Anthrone Test- Total Carbohydrate Estimation

The Anthrone test was performed as per the protocol mentioned in *David T. Plummer, (1990)*. The Anthrone Reagent was first prepared kept in amber coloured reagent bottle at 4°C (Cellfrost Refrigerator) for 2 hours to make it cooler. One mililitres of seaweed extract was placed in reaction tube, following which, 5 ml of Anthrone reagent was added using glass pipette in the fume hood. The tubes were covered with cotton plug and placed in 100°C boiling water bath for 10 minutes. The tubes were removed and immediately placed in ice to stop further reaction.

The absorbance was taken using Spectrophotometer set at 620 nm. The total carbohydrate concentration was estimated by extrapolating readings against a standard Anthrone-total carbohydrate graph with glucose (200 µg/ml). The total carbohydrate was measured in percentage of seaweed biomass (g/100g).

C] Folin Lowry Method- Protein Estimation

The Folin Lowry method was performed as per standard protocol as mentioned in *GBioSciences*. The seaweed extract (1 ml) was mixed with 5 ml of copper reagent in tube and reaction was kept in dark for 10 minutes. Further, 0.5 ml of Folin Ciocalteau Reagent was added to tubes and incubated for 30 minutes.

The absorbance was immediately recorded using spectrophotometer set at 600 nm. The total protein concentration was estimated by extrapolating readings against a standard Protein graph with BSA (1 mg/ml). The total protein was measured in percentage of seaweed biomass (g/100g).

D] Vanillin Phosphoric Acid Method- Lipid Estimation

The vanillin extraction method was performed with slight modifications for quantitatively analyzing the lipid content present in the seaweed biomass. The protocol was performed as per the method described by *Emile Van Handel, (1985).*

Briefly, 25 mg of seaweed powder was mixed with 0.5 ml of 2:1 solvent of chloroform: methanol. The contents were heated in water bath at 60˚C till solvent evaporate completely. Then 0.2 ml of concentrated sulphuric acid was added and again heated for 10 minutes. Further, 5 ml of Vanillin phosphoric acid reagent was added to tubes and kept in dark for 10 minutes.

The readings were taken spectrometrically at 525 nm, against a standard graph prepared using 1 mg/ml of olive oil solution in chloroform. The total lipid content was measured in percentage of seaweed biomass (g/100g).

E] Gravimetric Analysis for Total Solid (TS), Total Volatile Solid (TVS) and Ash Content

The total solid, total volatile solid and Ash content was gravimetrically analyzed based on the standard protocols mentioned by *Abdul Razaque Sahito et al. (2013).* Crucibles were pre-heated for one hour and then cooled in desiccator to remove any traces of moisture. Seaweed biomass (0.5 gram) were weighed and transferred to crucibles and labelled with pencil. The crucibles with the biomass were weighed and recorded. Then, the crucible containing samples were placed in oven (i-therm AI-7981) set at 105˚C for 1 hour. Thereafter, crucibles were cooled to 25 ± 2℃ in desiccator and weighed again. The difference between initial and final weights was recorded and total solids (TS) was calculated based on the formula:

Total Solids (%) = W3 – W1 / W2 – W1 X 100

Key:

- W1 is the mass of the empty crucible
- W2 is mass of crucible with sample (0.5 g seaweed powder)
- W3 is the mass of crucible with sample after removing from 105°C

After weighing, the samples were kept in muffle furnace (Pathak Electrical Work PEW-202, Serial No. 792) at 550˚C again for one hour. The furnace was allowed to cool down overnight, then samples were removed and placed in desiccator. The crucibles with samples were weighed again, and ash content was recorded based on the formula:

Ash Content (%) = W4 – W1 / W3 – W1 X100

Key: W4 is mass of crucible with sample after removing from 550˚C

The total volatile solids (TVS) was calculated by subtracting the value of Ash content from total solids, and recorded in percentage.

Total Solids (TS) = Total Volatile Solids (TVS) – Fixed Solid or Ash
Therefore: TVS = TS- Ash

Moisture content was calculated using the following formula.

Moisture Content (%) = 100 – Total Solids

E] CHNS Analysis

The prepared seaweed powder was used for Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) analysis using Vario MICRO elemental analyser (Serial No. 15095077). Oxygen was calculated by adding carbon, hydrogen, nitrogen and sulphur from CHNS analysis and ash content value obtained from gravimetric analysis, and subtracting by 100%.

Oxygen % = 100 - (Carbon + Hydrogen + Nitrogen + Sulphur + Moisture Content + Ash)

The overall biomass composition was then represented in the form of pie chart by taking percentage values for carbon, hydrogen, sulphur, moisture content, ash content, and oxygen respectively.

3.8 Preparation of Seaweed Hydrolysates

The acid and alkaline seaweed hydrolysates were made by adding 5% seaweed powder in 0.4N HCl/ 0.4 N NaOH respectively. The suspension was than autoclaved (Autoclave-Renuka Enterprises Serial No. 1075.2K23.03.050) at 121°C, 15 psi, 20 minutes. The autoclaved hydrolysate was than centrifuged at 8000 rpm for 2 minutes using centrifuge (Thermo Scientific SORVALL S78R) and the supernatant was collected and stored at 4°C (Haier HRF 33-4P/2019) for further use.

3.9 Characterization of Hydrolysates

Similar to biomass characterization, the prepared hydrolysates were also analyzed for parameters including pH, salinity, reducing sugar content (by DNSA method), total carbohydrate content (by Anthrone Method), protein content (by Folin Lowry Method) and lipid content (Vanillin-phosphoric acid method).

However, except for pH and salinity which were estimated using pH meter (Eutech instruments pH 700) and refractometer, the rest parameters were analysed after neutralizing the pH to 7 using concentrated HCl/10M NaOH. For further usage of hydrolysate for culture cultivation salinity was set to 25% by adding NaCl.

3.10 Estimation of Pullulanase Production using Hydrolysates

Hydrolysates (5 ml) were dispensed in autoclaved tubes. The halophilic cultures stored after screening for pullulanase where grown in Halopiger broth for 72 hours. The broth was centrifuged at 10,000 rpm 10 minutes and the pellet was re-suspended in 15% saline. Then, 500 µL of saline suspended culture was inoculated in hydrolysates. The tubes were kept in shaking condition (120 rpms) at 25 ± 2℃ for 72 hours. Additionally, tubes containing 5 ml of 1% pullulan solution in 25% saline was inoculated with cultures as positive control for pullulanase production. Also, tubes with 3% starch solution and 3% cellulose solution with 25% salinity were also inoculated with cultures, as starch and cellulose are commercial substrates for pullulanase production.

By the end of 72 hours of incubation, the contents were transferred to eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes centrifuge. One millilitre of supernatant was transferred to new set of tubes and 1ml of 1% pullulan broth was mixed. The reaction was kept at 40°C water bath for 30 minutes and then 2 ml of DNSA reagent was added to the reaction tubes. Further, the tubes were kept in boiling water bath for 10 minutes and immediately cooled by dipping in ice.

The spectrophotometric readings were taken at 540 nm and pullulanase activity was estimated as per the standard method mentioned previously during screening. Hydrolysate supernatants post fermentation which exhibited highest pullulanase activity was selected for each culture and used for further optimization.

3.11 pH and Temperature Optimization

The best hydrolysate for each respective cultures were selected that supported better growth of culture. Next, to further optimize the conditions which support more growth of cultures in hydrolysate, the pH and temperature optimization was performed.

a) **pH optimization**

The selected hydrolysates for each culture were adjusted to varying pH ranging from 5-9 ranging a gap of 0.5 i.e. 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. In each tube 10 ml of pH adjusted hydrolysates were added. The cultures were first grown in Halopiger broth for 72 hours. The broth was centrifuged at 10,000 rpm 10 minutes and the pellet was re-suspended in 15% saline. One millilitre of saline suspended culture was inoculated in respective hydrolysates. The tubes were kept in shaking condition (120 rpms) at 25 ± 2°C for 72 hours. During the incubation, 750 µL of sample was removed after every 6 hour and subsequent 18 hour till 72 hours. The samples were used to measure growth by taking absorbance at 600 nm. The measurement for growth is based on wavelength selection as mentioned by *Krishnamurthi VR et al. (2021)*. The growth rates of cultures at different pH were recorded and plotted on graph. The best pH showing higher culture growth was selected for temperature optimization.

b) **Temperature Optimization**

The optimum pH conditions observed in pH optimization experiment were used for temperature optimization study. Hydrolysate (15 ml) was added to sterile tubes. The cultures were first grown in Halopiger broth for 72 hours. The broth was centrifuged at 10,000 rpm for 10 minutes and the pellet was re-suspended in 15% saline. Further, 1.5 ml of saline suspended culture was inoculated in respective hydrolysates. These tubes were kept in shaking conditions at 120 rpm in incubator shakers set at 25°C, 30°C, 37°C and 45°C respectively (Biotechniques India R5/01, Remi CIS-24 plus, Rivotek- incubator

shaker, Hally instruments Shaking Incubator respectively). A sample of 750 µL was removed at an interval gap of 3 hours, 3 hours and 18 hours till 72 hours. This sample was used to measure growth by taking absorbance at 600 nm using spectrophotometer. The growth rates of cultures at different temperature in respective pH were recorded and plotted on graph. The highest growth observed for the cultures at respective temperature and pH values were considered as optimum value for further analysis.

3.12 Growth Curve of Cultures in Hydrolysates

The hydrolysate pH was set at the optimium pH observed. In 250 ml flasks, 100 ml of hydrolysate was added in flask. The cultures were first grown in Halopiger broth for 72 hours. The broth was centrifuged at 9,500 rpm 12 minutes and pellet was re-suspended in 15% saline. Then, 10 ml of saline suspended culture was inoculated in respective hydrolysates. The inoculated hydrolysate were kept in shaking for at respective optimum temperatures for 72 hours. After inoculation 2 ml of sample was removed every two hours for 10 hours and then after a lag of 14 hours in a day. From this 1 ml of sample was used for estimation of growth rate by taking absorbance at 600 nm.

3.13 Estimation of Highest Pullulanase Activity During Culture's Growth Period

One millilitre sample collected during growth curve study was used to estimate pullulanase activity. The procedure involved was same as previously mentioned in section 3.10 wherein sample was centrifuged, and supernatant was allowed to react with 1% pullulan buffer for 30 minutes at 40 °C followed by DNSA-reducing sugar assay.

3.14 Fold Purification of Pullulanase

The seaweed hydrolysate showing the best growth of culture were used for fold purification study. The cultures were grown in 50 ml of their respective hydrolysates for 72 hours at their respective optimum pH and temperature. They were centrifuged (Thermo Scientific SORVALL lynx 4000) at 9500 rpm for 12 minutes and supernatant was used for analysis.

Then, 25 ml of this supernatant was further removed for ammonium salt precipitation. The salting out was done using single fraction for 80% saturation. The content was centrifuged at 8000 rpm for 5 minutes at 4°C and pellet was re-suspended in 10 ml of Phosphate buffer saline (PBS). Further, 5 ml of this sample was than dialyzed in phosphate buffer (pH 7.0) overnight using 50 kDa cellulose membrane by keeping in magnetic stirrer (Remi Equipment 5MLH) for constant mixing.

The protein content and pullulanase activity was recorded for crude sample, ammonium salt precipitated sample and dialyzed sample using Folin Lowry and Pullulan reaction following DNSA respectively. The calculations for specific activity, yield and fold purification were performed as per the protocol suggested by *M.A. Islam et al. (2009).*

The following are the formulas used for the calculation.

Total Protein (mg) = Protein Obtained in mg/ml X Sample Volume (ml)
Total Activity (U) = Enzyme Activity (µmol/min) X Sample Volume (ml)
Specific Activity (U/mg) = Total Activity (U) ÷ Total Protein (mg)
Yield % = Total Activity (U) ÷ Crude Sample's Total Activity (U) X 100
Fold Purification = Specific Activity (U/mg) ÷ Crude Sample's Specific Activity (U/mg)

3.15 Molecular Weight Analysis by SDS-PAGE

The extracellular protein profile of cultures were analyzed using SDS-PAGE. The cultures were grown in Halopiger broth and 1% pullulan broth at 30°C for 72 hours and then centrifuged at 9500 rpm for 12 minutes. The supernatant was collected and loaded in SDS-PAGE. Likewise, seaweed hydrolysate, supernatant of culture grown in seaweed hydrolysate, its ammonium salt precipitation fraction (dissolved in PBS) of the culture supernatant grown in seaweed hydrolysate and dialyzed samples were also loaded in the SDS-PAGE gel.

The gel was allowed to run for around two hours at 100 Volts (BioEra- BE/FPP/1209). The bands were obtained after staining overnight by placing gel in gel rocker (Gennei Gel Rocker 100) followed by de-staining.

The bands were compared with a high range molecular weight protein marker (99625(SRL)) of 14-220 kDa ran along with samples. After the de-staining, distance of bands from the well were measured in centimetres (cm) and graph was plotted for marker bands vs log10 of molecular weight. Based on the standard graph, the values of molecular weight of samples were recorded.

3.16 Estimation of Thermal Stability of Extracted Enzyme

The pullulanase produced by the culture using seaweed hydrolysate was further dialyzed and checked for thermal stability by placing 1 ml of the enzyme at different temperatures from 30˚C, 40˚C, 50˚C up to 100˚C for a constant time of 1 hour. The samples were further analyzed for activity using same protocol earlier mentioned in section 3.10 i.e. reaction of enzyme with 1% pullulan at 40˚C for 30 minutes followed by DNSA.

ANALYSIS AND CONCLUSION

“Every choice you make has an end result.”

- Zig Ziglar

Chapter 4

ANALYSIS AND CONCLUSION

4.1 Sampling for Halophiles in Saltpan

The following field parameters were recorded at time of sampling of soil, salt and water samples. Fig. 1 shows samples collected from Curca Saltpan, Maina, Goa (Lat: 73.883254°; Long: 15.459017°) to isolate halophiles. Fig. 2 provides an on-field photo of site at time of sample collection.

i. Water Temperature: 33°C

ii. TDS: 3723 ppm

iii. Water pH: 7.8

iv. Water Salinity: 35 ppt

v. Atmospheric temperature: 32°C

vi. Wind Speed: 5 Km/Hour North-East

vii. Humidity: 66%

viii. Air Pressure: 1009 Mbar

Fig. 1: Soil, Salt and Water Samples Collected from Saltpan

Fig. 2: Sampling Site for Halophile Culture: Curca Saltpan, Goa

4.2 Screening and Isolation of Halophilic Cultures from Saltpan Samples

A total of 25 colonies could be picked and purified from the Halopiger Agar plates, of which eight colonies were from water samples (GUPM1-8), nine colonies were from salt samples (GUPM9-17), and another eight colonies were from soil samples (GUPM18-25). Fig. 3 shows one set of master plates with cultures obtained salt, soil and water samples. The master plates of water samples had matt growth around the edges of the filters with very few isolated colonies. Thus, cfu/ml couldn't be estimated. The colony count for soil and salt samples was 5800 cfu/ml and 2900 cfu/ml, respectively.

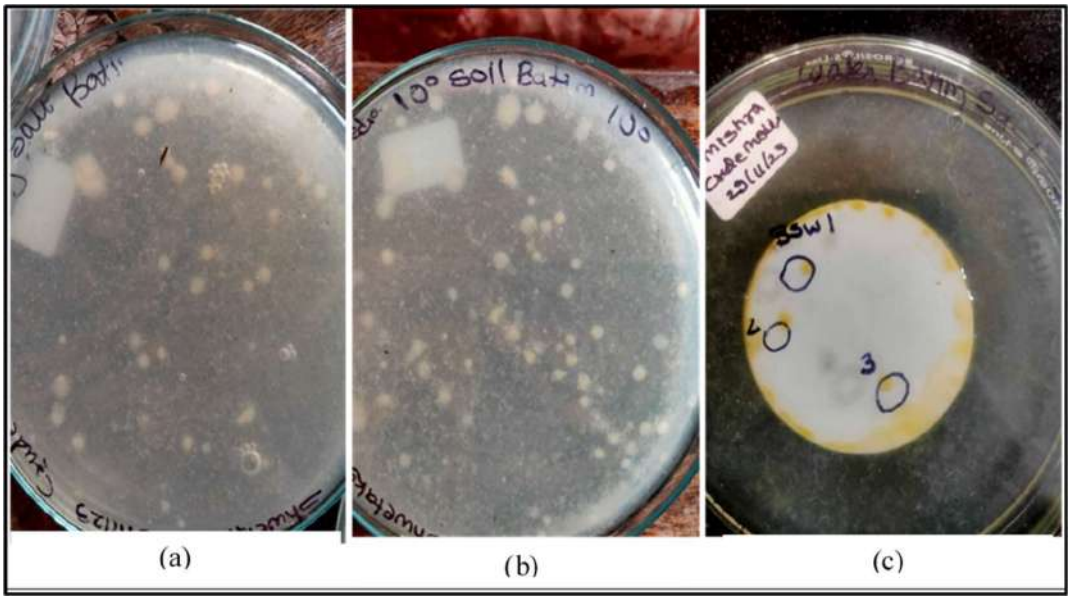

Fig. 3: Master Colony Plates of Halophilic Cultures

(a) Master culture plate for salt sample
(b) Master culture plate for soil sample
(c) Master culture plate for water sample

4.3 Colony Morphology and Characteristics

The isolated cultures numbered as GUPM1 to 25 (as shown in Fig. 4) were observed for colony morphology characters on Halopiger agar pates which include shape, size, elevation, margin, surface, opacity, colouration and consistency. Details of the colony characteristics for each culture are as shown in Table-1 below:

Table-1: Colony Morphology of Isolated Colonies (GUPM1-25)

Culture Name	Size (mm)	Shape	Elevation	Margin	Surface	Opacity	Pigment	Consistency
GUPM1	2	Round	Flat	Filament	smooth	translucent	greyish white	Buttery
GUPM2	2	Round	Flat	Entire	smooth	translucent	greyish white	Buttery
GUPM3	2	Irregular	Raised	Filiform	rough	opaque	off white	mucoid
GUPM4	2	Irregular	Flat	Filiform	smooth	opaque	off white	Viscid
GUPM5	3	Irregular	Raised	Entire	rough	translucent	buff	Oily
GUPM6	2	Round	Pulvinate	Filiform	wrinkled	translucent	buff	viscid
GUPM7	3	Irregular	Raised	undulate	rough	opaque	Parmesan	buttery
GUPM8	1	Filament	cratiform	Filiform	rough	opaque	off white	viscid
GUPM9	2	Round	Convex	Erose	wrinkled	translucent	buff	buttery
GUPM10	1	Irregular	Umbonate	Entire	smooth	opaque	white	watery
GUPM11	4	Dumble	Flat	Entire	smooth	opaque	white	watery
GUPM12	2	Spindle	Convex	Entire	smooth	opaque	white	watery
GUPM13	2	Irregular	Pulvinate	Entire	smooth	opaque	off white	viscid
GUPM14	1	Round	Convex	Entire	smooth	opaque	cream	mucoid
GUPM15	2	Irregular	Pulvinate	Entire	smooth	opaque	white	mucoid
GUPM16	1	Irregular	Flat	undulate	rough	opaque	cream	mucoid
GUPM17	2	Irregular	Raised	undulate	smooth	opaque	white	watery
GUPM18	2	Dumble	Umbonate	Filiform	smooth	opaque	Parmesan	Viscid
GUPM19	1	Round	Flat	Entire	rough	translucent	buff	Buttery
GUPM20	2	Round	cratiform	Filiform	rough	translucent	buff	Buttery
GUPM21	2	Irregular	Flat	Entire	rough	translucent	buff	Buttery
GUPM22	2	Irregular	Pulvinate	Filiform	smooth	opaque	cream	Watery
GUPM23	2	Round	Raised	Entire	rough	translucent	mustard	Viscid
GUPM24	2	Irregular	Flat	Erose	rough	translucent	Parmesan	oily
GUPM25	2	Irregular	Flat	Erose	rough	translucent	Parmesan	Oily

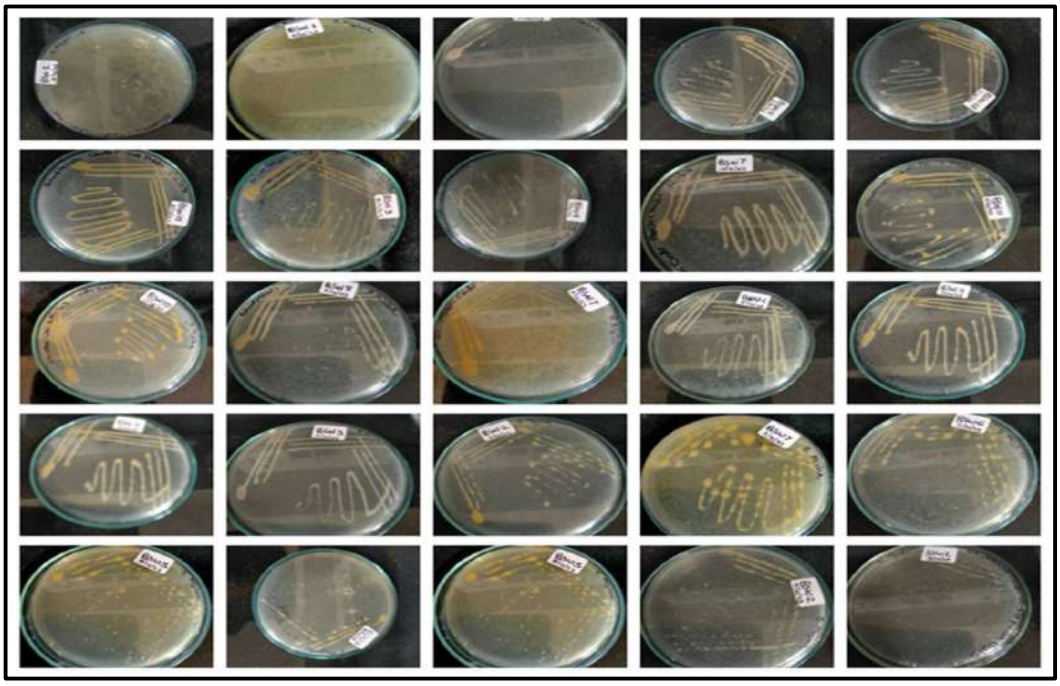

Fig. 4: Quadrant Streaked Isolated Cultures (GUPM1-25)

4.3.2 Gram Staining

Upon performing Gram staining morphology of cultures was recorded as mentioned in the below Table-2. The results revealed that 19 cultures showed Gram negative characters while six cultures had Gram positive nature. Fig. 5 shows Gram staining performed for few selected cultures. Since, most cultures are Gram negative, this can be correlated to their higher resistance to survive in harsh conditions of salt and temperature. *Zeinab Breijyeh et al. (2020)* also suggest higher resistance of Gram negative cultures towards salinity.

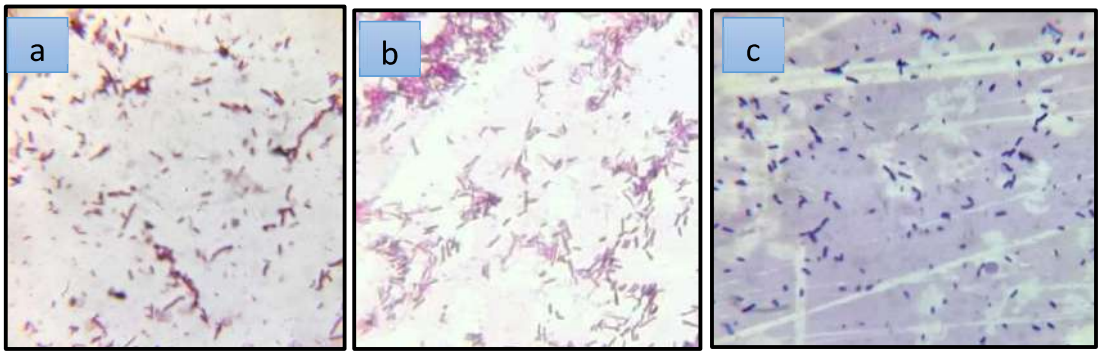

Fig.5: Gram Staining of Cultures

a) GUPM2 b) GUPM5 c) GUPM22

Table-2: Gram Character and Shape of Cultures

Culture	Gram Stain
GUPM1	Pink thick rods
GUPM2	Pink long rods
GUPM3	Pink rods
GUPM4	Pink rods
GUPM5	Pink rods
GUPM6	Pink rods
GUPM7	Pink rods
GUPM8	Pink rods
GUPM9	Purple rods
GUPM10	Pink short rods
GUPM11	Pink thick short rods
GUPM12	Purple short rods
GUPM13	Pink short rods
GUPM14	Purple short rods
GUPM15	Pink short rods
GUPM16	Purple short rods
GUPM17	Purple rods
GUPM18	Pink cocci forming clusters
GUPM19	Pink rods
GUPM20	Pink curved cell
GUPM21	Pink rods
GUPM22	Pink short rods
GUPM23	Pink rods
GUPM24	Pink rods forming chains
GUPM25	Purple rods arrange in V shape

4.3.3 Biochemical Tests

A] Sugar utilization a) Glucose b) Galactose c) Maltose d) Sucrose

The sugar utilization test was performed using four different sugar sources. Table-3 indicates utilization (+) or non-utilization (-) of sugar by a culture along with production of bubble (Y) or no production of bubble (N) in the Durham tube. Sugar utilization of culture with bubble formation is an indicative of culture being able to carry out fermentation.

Table-3: Qualitative Data for Various Sugar Utilizations by Cultures

Culture Name	Glucose Utilization	Sucrose Utilization	Maltose Utilization	Galactose Utilization
GUPM1	-	-	-	-
GUPM2	+ (Y)	+ (Y)	+ (N)	+ (Y)
GUPM3	+ (N)	-	-	-
GUPM4	-	-	+ (Y)	-
GUPM5	-	-	+ (Y)	+ (Y)
GUPM6	+ (Y)	+ (N)	+ (N)	+ (N)
GUPM7	-	-	-	-
GUPM8	-	-	-	-
GUPM9	+ (Y)	-	+ (Y)	-
GUPM10	-	-	-	-
GUPM11	-	-	+ (Y)	-
GUPM12	-	-	-	-
GUPM13	-	-	-	-
GUPM14	-	-	-	-
GUPM15	-	-	-	-
GUPM16	+ (Y)	+ (Y)	+ (Y)	+ (Y)
GUPM17	-	+ (N)	-	-
GUPM18	-	-	-	-
GUPM19	-	-	-	-
GUPM20	-	-	-	-
GUPM21	+ (Y)	+ (Y)	+ (N)	+ (Y)
GUPM22	-	-	-	-
GUPM23	-	-	+ (Y)	-
GUPM24	-	+ (Y)	+ (Y)	+ (N)
GUPM25	-	-	-	-

B] Motility Test

After performing motility test using stab and cover slip method, the results were recorded and observed details are recorded as mentioned in Table-4. A total of 17 cultures showed motility while eight cultures didn't showed any motility. The reason for most culture's motility may lie on their environmental influence. As suggested by *Anne E. Mattingly et al. (2018)* many cultures in environment may be found motile so that they can move to thrive to suitable location around for their survival.

Table-4: Motility Analysis of Cultures

Culture	Motility
GUPM1	+
GUPM2	+
GUPM3	+
GUPM4	+
GUPM5	+
GUPM6	+
GUPM7	+
GUPM8	-
GUPM9	+
GUPM10	+
GUPM11	-
GUPM12	-
GUPM13	+
GUPM14	+
GUPM15	+
GUPM16	-
GUPM17	-
GUPM18	+
GUPM19	+
GUPM20	-
GUPM21	+
GUPM22	+
GUPM23	-
GUPM24	+
GUPM25	-

(Key: motile (+) or non-motile (-))

C] Amylase Production Test

The starch assay was performed and zone of clearance percentage of each culture was observed and recorded. Table-5 depicts culture with respective zone of clearance percentage. Likewise, Fig. 6 shows a comparison amylase activity among 25 cultures based on zone of clearance. It can be observed that 19 cultures showed positive results for amylase production, of which only two cultures i.e. GUPM9 showed clearance zone of 75%.While, GUPM14 and GUPM19 showed clearance of 40%.

Table-5: Amylase Production Analysis from Cultures via Starch Assay

Culture	Colony Size (in mm)	Zone of Clearance (in mm)	Clearance %
GUPM1	5 ± 0.33	5 ± 0.33	14.29
GUPM2	3 ± 0.00	4 ± 0.33	22.22
GUPM3	4 ± 0.58	4 ± 0.58	0.00
GUPM4	5 ± 0.33	6 ± 0.00	28.57
GUPM5	3 ± 0.33	4 ± 0.33	30.00
GUPM6	3 ± 0.67	4 ± 0.33	10.00
GUPM7	4 ± 0.88	5 ± 0.67	7.69
GUPM8	4 ± 0.67	5 ± 0.33	7.69
GUPM9	3 ± 0.33	5 ± 0.33	75.00
GUPM10	7 ± 0.88	6 ± 0.67	10.00
GUPM11	6 ± 0.33	7 ± 0.58	10.53
GUPM12	7 ± 0.33	9 ± 0.33	30.00
GUPM13	5 ± 0.88	6 ± 0.67	35.71
GUPM14	4 ± 0.33	5 ± 0.33	27.27
GUPM15	3 ± 0.67	3 ± 0.33	25.00
GUPM16	4 ± 0.88	4 ± 0.33	0.00
GUPM17	8 ± 1.20	11 ± 0.88	28.00
GUPM18	2 ± 0.33	2 ± 0.33	0.00
GUPM19	1 ± 0.58	2 ± 0.00	40.00
GUPM20	3 ± 0.33	3 ± 0.33	0.00
GUPM21	1 ± 0.67	0 ± 0.00	0.00
GUPM22	4 ± 0.33	3 ± 0.33	30.77
GUPM23	4 ± 0.58	5 ± 0.58	25.00
GUPM24	6 ± 0.33	5 ± 0.33	0.00
GUPM25	2 ± 1.00	2 ± 0.33	16.67

* Values after ± are standard error values.

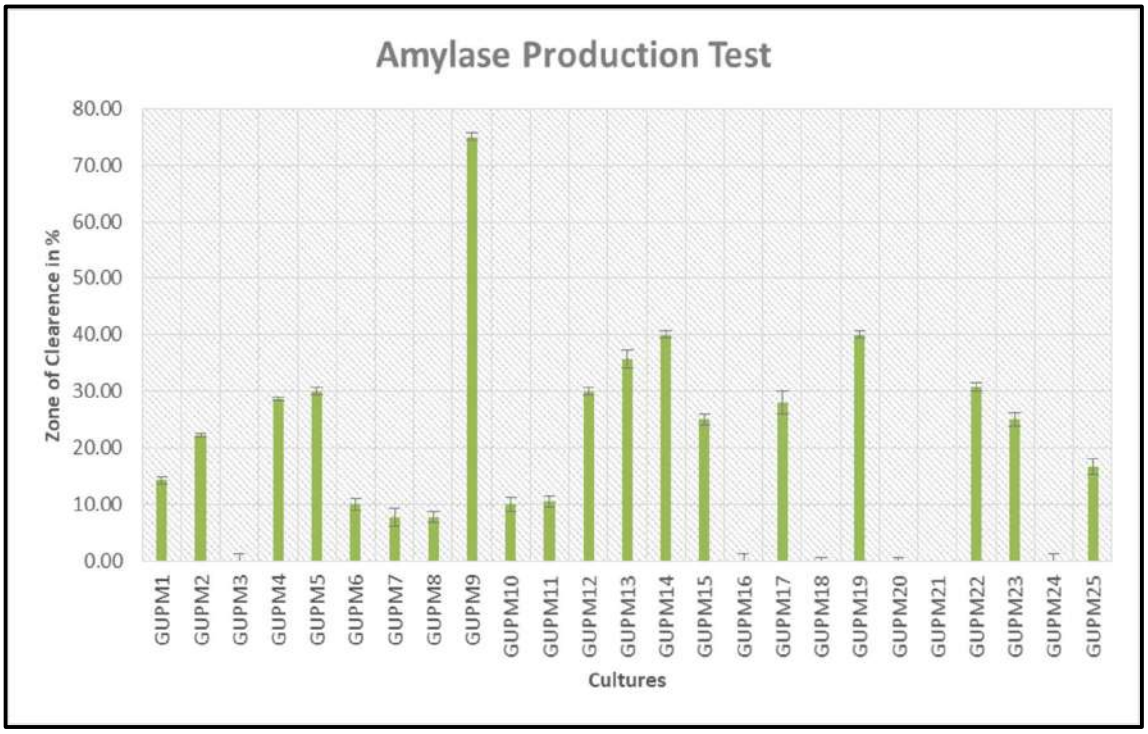

Fig. 6: Comparative Analysis for Amylase Activity in Cultures by Amylase Production Test

D] **Caseinase Production Test**

The casein assay was performed and zone of clearance percentage of each culture was observed and recorded. Table-6 shows culture with respective zone of clearance percentage. It can be inferred that almost all cultures showed some proteolytic activity against casein or skim milk agar, suggesting protease activity. Further, Fig. 7 and Fig. 8 show caseinase enzyme activity of different cultures at Casein and Skim milk agar plates respectively. As suggested in literature several halophilic cultures are known to have high proteolytic activity. *Manikandan P et al. (2018)* have also worked on protease producing cultures from saltpan of Tamil Nadu. They identified culture that produce stable protease at pH 8 at 40°C. Thus, such proteolytic activity can be beneficial in pharmaceutical applications.

Table-6: Zone of Clearance % in Casein and Skim Milk Agar (in mm)

Culture	Zone of Clearance % in Casein agar	Zone of Clearance % in Skim Milk agar
GUPM1	0.00	50.00
GUPM2	42.86	61.54
GUPM3	50.00	44.44
GUPM4	57.14	30.77
GUPM5	57.14	62.50
GUPM6	50.00	46.15
GUPM7	37.50	41.18
GUPM8	30.00	0.00
GUPM9	22.22	62.50
GUPM10	50.00	18.18
GUPM11	27.27	42.86
GUPM12	22.22	40.00
GUPM13	15.38	7.69
GUPM14	42.86	36.36
GUPM15	60.00	63.64
GUPM16	28.57	87.50
GUPM17	25.00	36.36
GUPM18	42.86	7.69
GUPM19	28.57	0.00
GUPM20	33.33	71.43
GUPM21	57.14	0.00
GUPM22	62.50	38.46
GUPM23	42.86	70.00
GUPM 24	42.86	85.71
GUPM 25	40.00	37.50

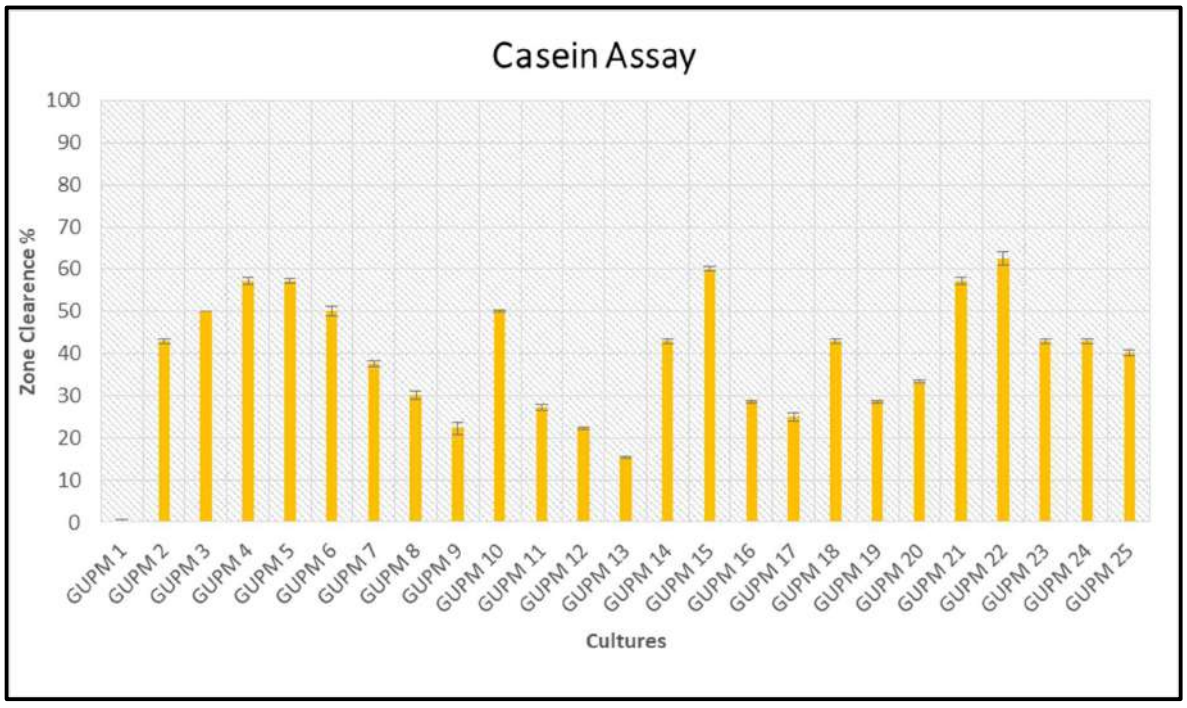

Fig. 7: Comparative Analysis for Caseinase Activity in Cultures Using Casein Assay

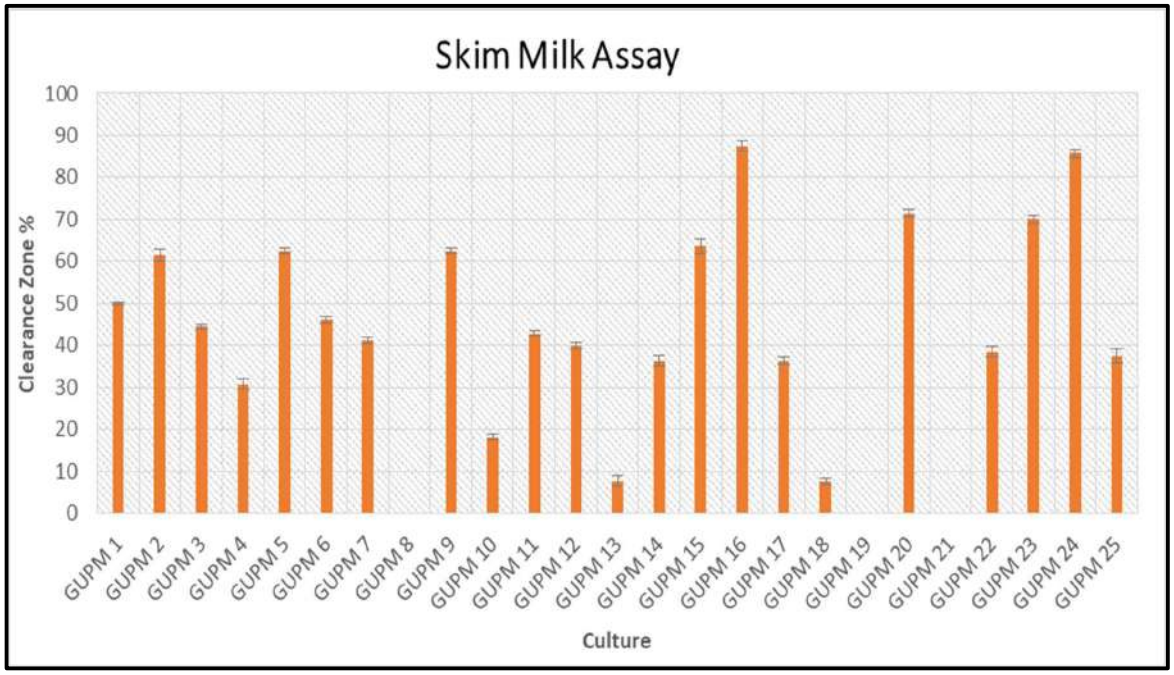

Fig. 8: Comparative Analysis for Caseinase Activity in Cultures Using Skim Milk Agar

E] Cellulase Production Test

The Carboxymethyl cellulose assay (CMC Assay) was performed and zone of clearance percentage of each culture was observed and recorded. Table-7 depicts culture with respective clearance percentage. Likewise, Fig. 9 show cellulose enzyme activity of different cultures at CMC agar. The CMC results showed that except GUPM15 and GUPM21 all cultures do produce cellulase, with culture GUPM2 showing highest production with 70% zone of clearance.

Table-7: Cellulase Activity Analysis from Cultures via CMC Assay

Culture	Colony Size (in mm)	Zone of Clearance (in mm)	Clearance %
GUPM1	2. ± 1.2	3 ± 1.3	14.29
GUPM2	3 ± 0.9	6 ± 0.7	70.00
GUPM3	6 ± 0.3	7 ± 0.3	17.65
GUPM4	7 ± 0.3	8 ± 0.3	4.55
GUPM5	6 ± 0.7	9 ± 0.9	36.84
GUPM6	6 ± 0.3	7 ± 0.3	35.29
GUPM7	9 ± 0.7	10 ± 0.7	11.54
GUPM8	6 ± 0.3	6 ± 0.0	5.88
GUPM9	4 ± 0.3	5 ± 0.3	45.45
GUPM10	6 ± 0.3	7 ± 0.3	15.79
GUPM11	7 ± 0.7	7 ± 0.7	10.00
GUPM12	6 ± 0.9	7 ± 0.6	10.53
GUPM13	6 ± 0.3	7 ± 0.3	15.79
GUPM14	6 ± 0.7	7 ± 0.3	5.26
GUPM15	8 ± 0.3	7 ± 0.3	0.00
GUPM16	8 ± 0.3	10 ± 0.3	26.09
GUPM17	6 ± 0.3	7 ± 0.0	10.53
GUPM18	6 ± 0.3	9 ± 0.3	36.84
GUPM19	3 ± 0.6	3 ± 0.3	11.11
GUPM20	4 ± 0.7	5 ± 0.7	27.27
GUPM21	3 ± 0.6	3 ± 0.6	0.00
GUPM22	10 ± 0.3	9 ± 0.3	0.00
GUPM23	5 ± 0.3	8 ± 0.3	43.75
GUPM24	6 ± 0.3	7 ± 0.3	29.41
GUPM25	3 ± 0.7	5 ± 0.3	40.00

* Values after ± are standard error values.

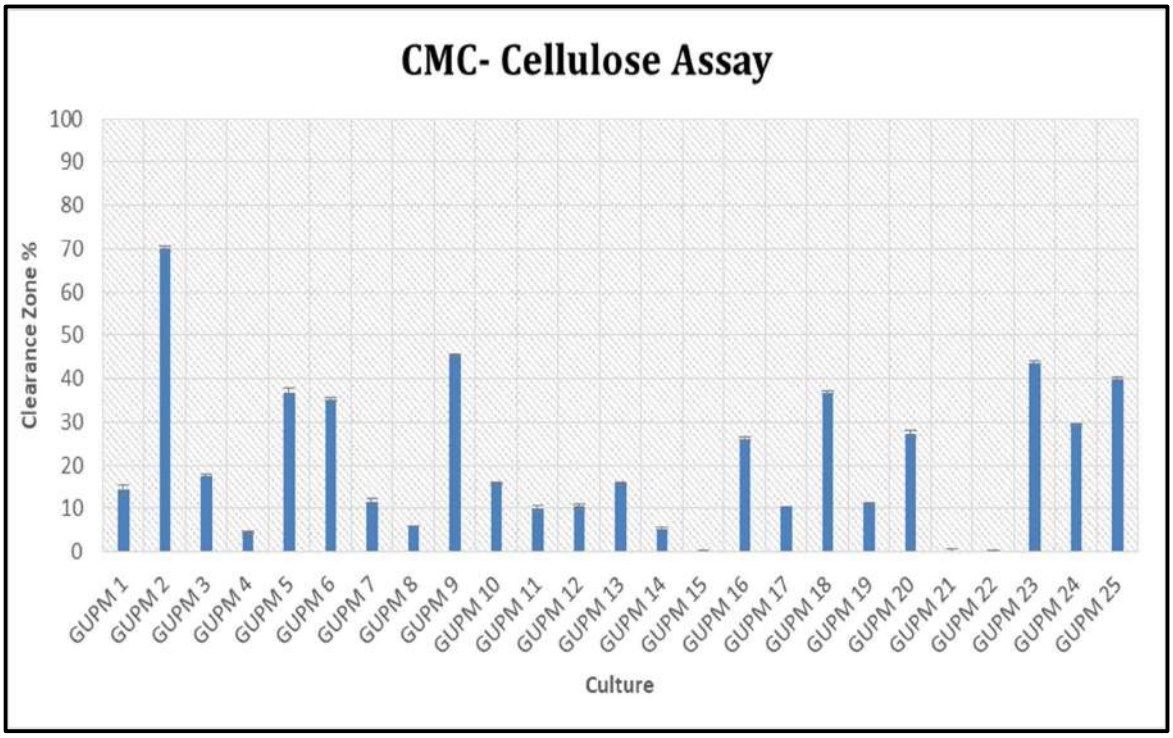

Fig. 9: Comparative Analysis for Cellulase Activity in Cultures Using CMC Assay

F] Salt Tolerance Assay

The salt tolerance assay is a qualitative approach that suggest whether cultures can grow in salt concentration of 0 to 35 percentage or more. The following table shows growth of cultures in different salt concentration. As seen from Table-8, the ability of all cultures to grow at salinity range of 0 to 35% suggest their adaptation to high range of salt concentration. Since, the cultures can also grow without salt, they can be suspected to be halotolerant in nature. However, minor variation could be observed with respect to time taken by cultures to grow and form colony, depending on salt concentration. It was observed that cultures grew fastest in 15% to 30% salinity i.e. small colonies were visible after 48 hours. However, colony inoculated in 0-10% salt and 35% salt were visible only after 72 hours of incubation.

Table-8: Qualitative Salt Tolerance Assay to Assess the Growth of the Culture in Various Salt Concentrations

Salt %	0%	5%	10%	15%	20%	25%	30%	35%
GUPM1	+	+	+	+	+	+	+	+
GUPM2	+	+	+	+	+	+	+	+
GUPM3	+	+	+	+	+	+	+	+
GUPM4	+	+	+	+	+	+	+	+
GUPM5	+	+	+	+	+	+	+	+
GUPM6	+	+	+	+	+	+	+	+
GUPM7	+	+	+	+	+	+	+	+
GUPM8	+	+	+	+	+	+	+	+
GUPM9	+	+	+	+	+	+	+	+
GUPM10	+	+	+	+	+	+	+	+
GUPM11	+	+	+	+	+	+	+	+
GUPM12	+	+	+	+	+	+	+	+
GUPM13	+	+	+	+	+	+	+	+
GUPM14	+	+	+	+	+	+	+	+
GUPM15	+	+	+	+	+	+	+	+
GUPM16	+	+	+	+	+	+	+	+
GUPM17	+	+	+	+	+	+	+	+
GUPM18	+	+	+	+	+	+	+	+
GUPM19	+	+	+	+	+	+	+	+
GUPM20	+	+	+	+	+	+	+	+
GUPM21	+	+	+	+	+	+	+	+
GUPM22	+	+	+	+	+	+	+	+
GUPM23	+	+	+	+	+	+	+	+
GUPM24	+	+	+	+	+	+	+	+
GUPM25	+	+	+	+	+	+	+	+

Key: + indicate growth, - indicate no growth

G] Antibiotic Sensitivity Assay

The standard Antibiotic discs were placed over swabbed cultures on Halopiger plates and Table-9 represent data for recorded clearance zones in mm, after 72 hours of incubation. Also, Fig. 10 shows a comparative analysis of cultures' sensitivity to different antibiotics. Cultures GUPM2, GUPM8, GUPM11, GUPM19, GUPM20, GUPM21, GUPM22, GUPM23, and GUPM25 did not show sensitivity to any of the antibiotics. While other cultures showed high sensitivity to Penicillin and Chloramphenicol.

Table-9: Zones of Clearance (in mm) by Cultures for Antibiotic Sensitivity Test

Cultures	Streptomycin (25 mcg)	Chloramphenicol (25 mcg)	Kanamycin (5 mcg)	Penicillin (2 mcg)	Metronidazole (4 mcg)
GUPM1	5 ± 0.0	47 ± 1.0	0	0	5.5 ±0.5
GUPM3	0	48.5 ±0.5	0	0	0
GUPM4	0	27 ±1.0	0	0	0
GUPM5	10.5 ± 0.5	42 ±1.0	6.5 ± 1.5	36.5 ±1.5	6.5 ±0.5
GUPM6	0	49 ±1.0	0	60 ±1.0	0
GUPM7	10 ± 2.0	50.5 ±0.5	0	36.5 ±1.5	0
GUPM9	6.5 ± 0.5	45 ±0.0	0	55 ±1.0	0
GUPM10	0	27 ±2.0	0	0	0
GUPM12	0	44.5 ±2.5	0	0	0
GUPM13	5.5 ± 0.5	22.5 ±1.5	0	0	0
GUPM14	0	23 ±2.0	0	0	0
GUPM15	0	20.5 ±1.5	0	0	0
GUPM16	0	61 ±1.0	0	21.5 ±0.5	0
GUPM17	0	13.5 ±1.5	0	0	0
GUPM18	0	49 ±1.0	0	17 ±2.0	0
GUPM24	5 ± 0.0	39.5 ±1.5	7.5	54 ±1.0	0

* Values after ± are standard error values.

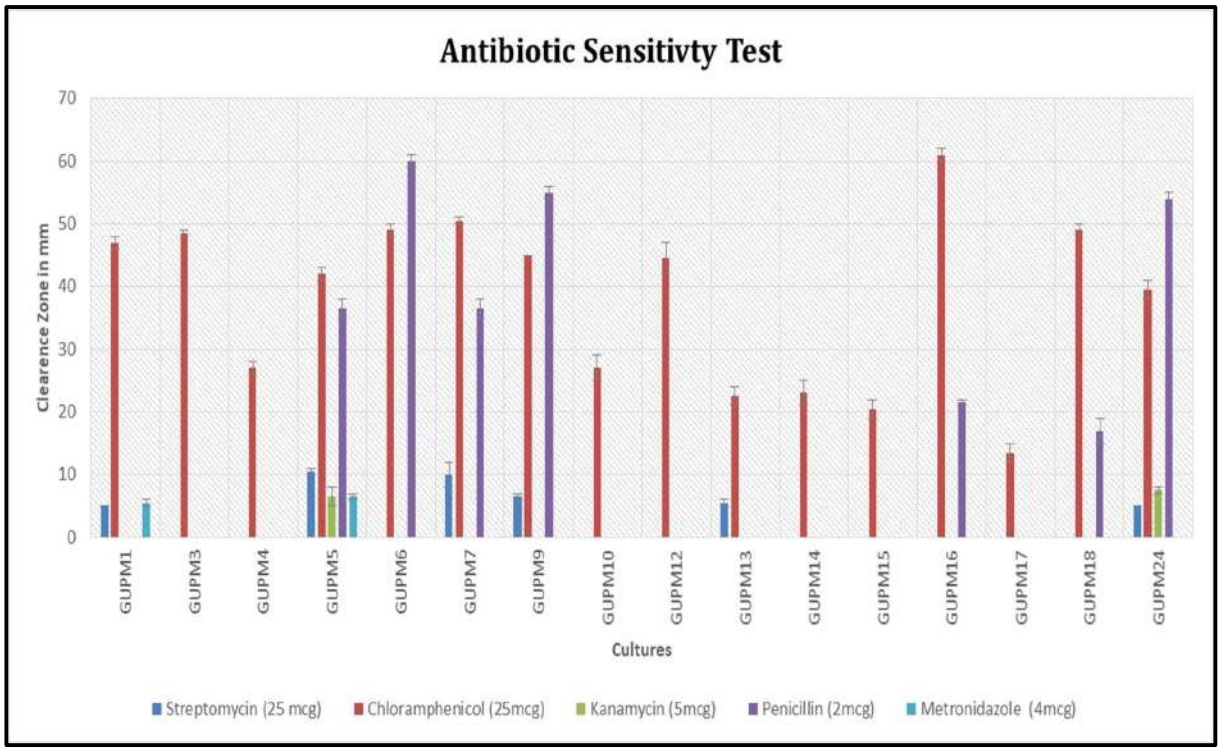

Fig. 10: Antibiotic Sensitivity of Analysis Cultures for Different Antibiotics

H] Oxidase Test

The colonies were observed for colour change as shown in Fig. 11. Table-10 depicts the whether the culture is positive (+) or negative (-) for oxidase production. It is evident from Table-10 that 14 cultures showed positive results for oxidase, while rest were negative. The oxidase positive cultures are known to have cytochrome c as the final electron acceptor that converts oxygen to water at the terminal chain during respiration process *(Flavia Fontanesi et al. 2008)*. However, since many cultures also show negative results, it suggest that they are either facultative anaerobes or have a different enzyme other than cytochrome c to accept the final electron in the electron transport chain.

Table-10: Oxidase Production Analysis

Culture	Oxidase
GUPM1	+
GUPM2	+
GUPM3	-
GUPM4	+
GUPM5	+
GUPM6	+
GUPM7	-
GUPM8	-
GUPM9	+
GUPM10	-
GUPM11	-
GUPM12	-
GUPM13	-
GUPM14	-
GUPM15	-
GUPM16	-
GUPM17	-
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GUPM21	+
GUPM22	+
GUPM23	+
GUPM24	+
GUPM25	+

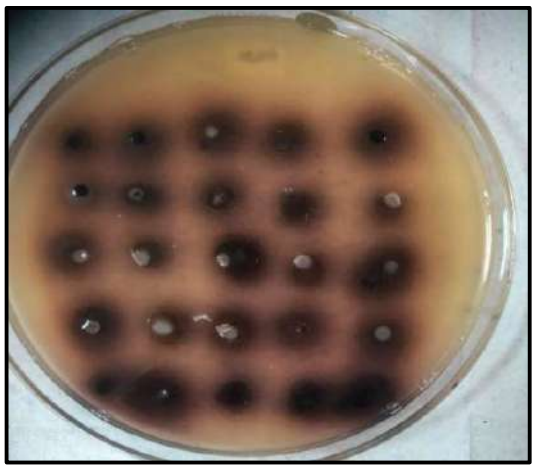


Fig. 11: Halopiger Agar Plate used for Oxidase Test, showing black colour colonies, indicating oxidase positive. White colour colonies are indicative of oxidase negative nature.

Key: (+) indicate oxidase production;

(-) indicate oxidase absence

I] Catalase Test

Effervescence, upon addition of 3% hydrogen peroxide, was observed on slides and compared against negative control slide. Table-11 depicts the whether the culture is positive (+) or negative (-) for catalase production. The results revealed that 14 cultures show positive for catalase production, suggesting the cultures are aerobics, use oxygen and also have catalase gene that protect them from toxic products that may be produced during respiration. Catalase negative cultures lack catalase producing genes, thus may be found useful for fermentation processes.

Table-11: Catalase Production Analysis

Culture	Catalase
GUPM1	+
GUPM2	+
GUPM3	+
GUPM4	+
GUPM5	+
GUPM6	+
GUPM7	+
GUPM8	-
GUPM9	+
GUPM10	-
GUPM11	-
GUPM12	-
GUPM13	+
GUPM14	-
GUPM15	+
GUPM16	+
GUPM17	-
GUPM18	+
GUPM19	+
GUPM20	+
GUPM21	-
GUPM22	-
GUPM23	-
GUPM24	-
GUPM25	-

Key: (+) indicate catalase production; (-) indicate catalase absence

J] Gas Production Test

The effervescence was observed on tubes and bubble formation was checked in tubes. Table-12 depicts whether the culture is positive (+) or negative (-) for gas production. Only, six out of 25 cultures showed gas production, suggesting that these cultures possibly produce high amounts of either carbon dioxide, hydrogen or methane.

Table-12: Gas Production Analysis

Culture	Gas Evolution
GUPM1	+
GUPM2	-
GUPM3	-
GUPM4	-
GUPM5	-
GUPM6	-
GUPM7	+
GUPM8	+
GUPM9	-
GUPM10	-
GUPM11	-
GUPM12	-
GUPM13	-
GUPM14	-
GUPM15	+
GUPM16	-
GUPM17	-
GUPM18	-
GUPM19	-
GUPM20	+
GUPM21	-
GUPM22	-
GUPM23	+
GUPM24	-
GUPM25	+

Key: (+) indicate gas production; (-) indicate no production of gas

4.4 Screening of Halophiles for Pullulanase Activity

a) Estimation of Pullulanase Activity Using Pullulan Agar

The pullulan plates after inoculation with cultures and incubation for 72 hours, zones of clearance were expected as mentioned in the literature, however, zones could not be identified with bare eyes. Flooding the plates with Gram's iodine was tried, and zones of clearance were observed and measured. The Table-13 shows zone of clearance percent of cultures in pullulan agar. Cultures GUPM2, GUPM4, GUPM5, GUPM11, GUPM16 and GUPM22 showed good pullulanase activity with above 40% clearance zone.

Table-13: Zone of clearance measured on Pullulan Agar plates in Pullulanase Production Assay

Culture	Colony Size (in mm)	Zone of Clearance (in mm)	Clearance %
GUPM1	5.3 ± 0.3	7.0 ± 0.6	31.25
GUPM2	3.7 ± 0.3	7.0 ± 1.0	90.91
GUPM3	4.7 ± 0.3	5.0 ± 0.0	7.14
GUPM4	5.3 ± 0.3	7.7 ± 0.3	43.75
GUPM5	6.0 ± 0.0	9.7 ± 0.3	61.17
GUPM6	5.7 ± 0.3	5.7 ± 6.3	0.00
GUPM7	4.3 ± 0.3	5.3 ± 0.3	23.08
GUPM8	3.7 ± 0.3	4.7 ± 0.3	27.36
GUPM9	5.0 ± 0.0	6.7 ± 0.3	33.33
GUPM10	6.0 ± 1.2	6.0 ± 1.2	0.00
GUPM11	3.0 ± 0.6	4.7 ± 0.7	55.56
GUPM12	4.7 ± 0.3	5.3 ± 0.7	14.29
GUPM13	5.3 ± 0.3	6.0 ± 0.0	12.50
GUPM14	5.3 ± 0.7	5.7 ± 0.3	6.25
GUPM15	5.0 ± 0.6	5.3 ± 0.3	6.67
GUPM16	4.7 ± 0.3	6.7 ± 0.3	42.86
GUPM17	6.7 ± 1.2	7.7 ± 0.9	15.00
GUPM18	0.0 ± 0.0	0.0 ± 0.0	0.00
GUPM19	1.3 ± 0.7	1.7 ± 0.9	25.00
GUPM20	1.3 ± 0.7	1.3 ± 0.7	0.00
GUPM21	0.0 ± 0.0	0.0 ± 0.0	0.00
GUPM22	3.7 ± 0.3	7.0 ± 0.6	90.91
GUPM23	5.0 ± 0.0	6.3 ± 0. 3	26.67
GUPM24	5.0 ± 0.6	5.7 ± 0.3	13.33
GUPM25	4.0 ± 0.0	4.0 ± 0.0	0.00

* Values after ± are standard error values.

From the results of pullulan agar assay, it was confirmed that isolated halopihilic cultures do have the potential to produce pullulanse. Fig. 12 also gives a comparative view extend of pullulanase production based on zone of clearance. Thus, further screening can be performed for better enzyme production.

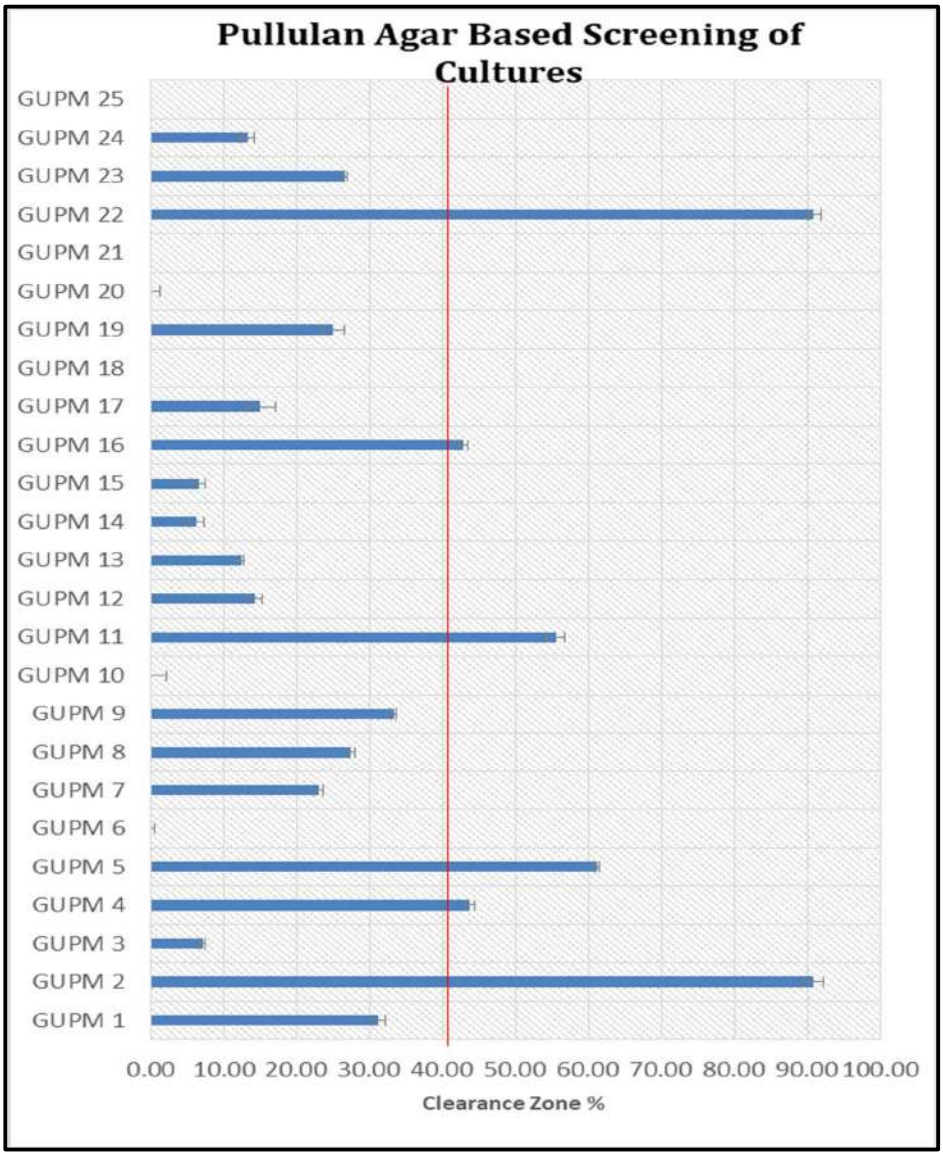


Fig. 12: Screening of Cultures for Pullulanase Production on Pullulan Agar

b) Estimation of Pullulanase Activity Using Pullulan Broth

Table-14 shows the enzyme concentration and enzyme activity of each culture in pullulan broth. Herein the enzyme concentration was estimated based on standard graph prepared on performing DNSA with glucose stock of 500 µg/ml. Further, the enzyme activity is calculated by dividing the enzyme concentration by 30, as 30 minutes is the total reaction time kept for reaction between pullulanase containing supernatant with pullulan.

Table-14: Pullulanase Activity Analysis of Cultures using Pullulan Broth and DNSA Assay

Culture	Pullulanse Enzyme Concentration (in µg/ml)	Enzyme Acitivity (IU) or 1 µmol/min
GUPM1	91.41 ± 0.002	3.05
GUPM2	364.10 ± 0.003	12.18
GUPM3	45.26 ± 0.001	1.51
GUPM4	136.41 ± 0.002	4.55
GUPM5	351.41 ± 0.002	11.71
GUPM6	37.70 ± 0.001	1.26
GUPM7	68.97 ± 0.002	2.30
GUPM8	73.59 ± 0.001	2.45
GUPM9	84.61 ± 0.001	2.82
GUPM10	45.00 ± 0.003	1.50
GUPM11	334.74 ± 0.002	11.16
GUPM12	42.95 ± 0.001	1.43
GUPM13	41.67 ± 0.002	1.39
GUPM14	38.21 ± 0.002	1.27
GUPM15	40.64 ± 0.002	1.35
GUPM16	149.62 ± 0.001	4.99
GUPM17	55.13 ± 0.001	1.84
GUPM18	48.33 ± 0.001	1.61
GUPM19	80.38 ± 0.003	2.68
GUPM20	26.03 ± 0.002	0.87
GUPM21	12.82 ± 0.003	0.43
GUPM22	370.77 ± 0.004	12.36
GUPM23	85.00 ± 0.002	2.83
GUPM24	56.80 ± 0.003	1.90
GUPM25	42.05 ± 0.002	1.40

* Values after ± are standard error values.

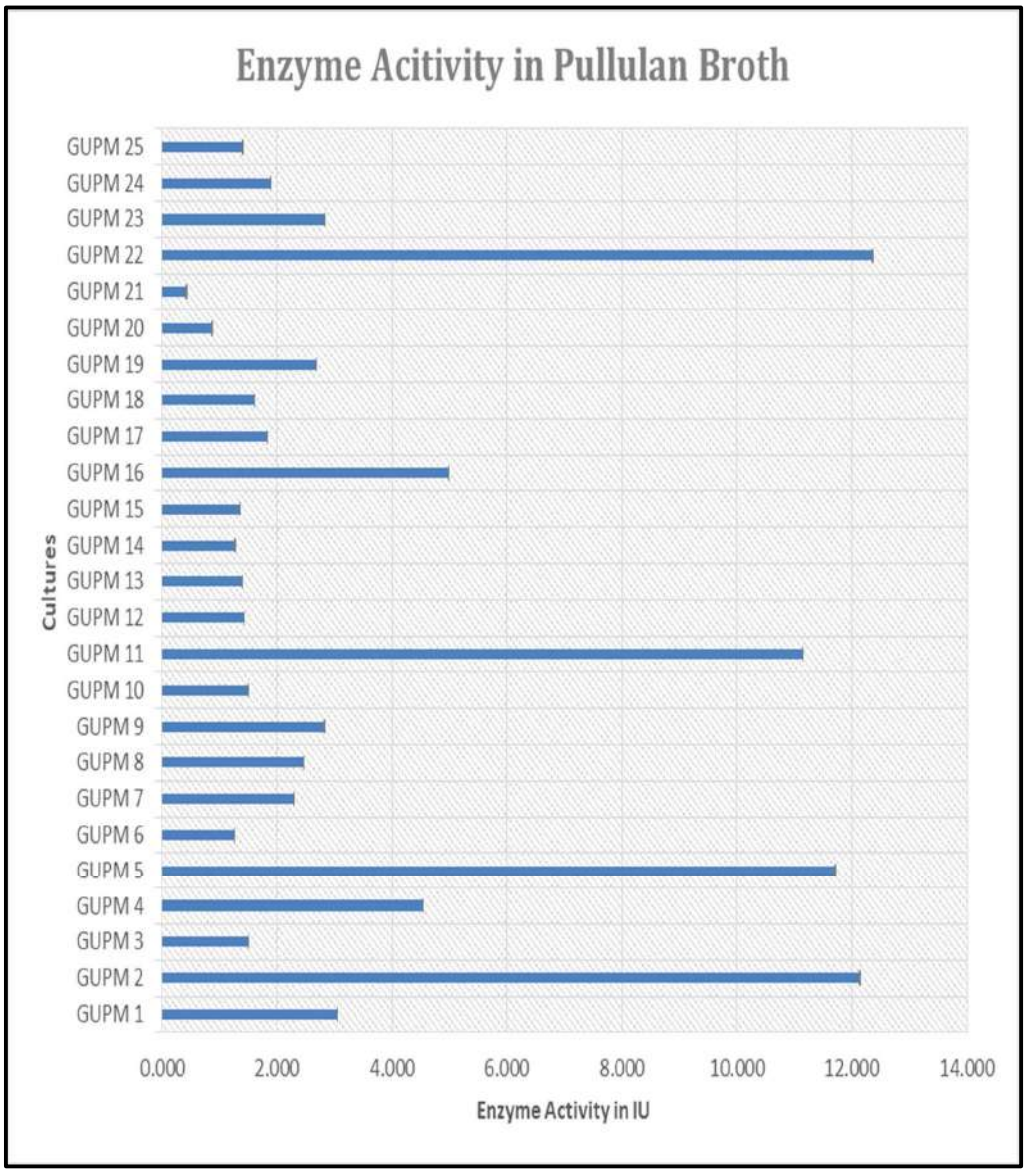


Fig. 13: Screening of Cultures for Pullulanase Activity using Pullulan Broth

Based on pullulanse activity observed in Fig. 13 and overall results obtained from two method of screening, three cultures i.e. GUPM2, GUPM5 and GUPM22 were selected for highest pullulanase activity. Thus, these three cultures were selected for further analysis.

4.5 Sampling of Seaweed

Table-15 summarizes the parameters that were recorded on the field i.e. as on 28th October 2023, 3rd December 2023 and 25th December 2023. Further, Fig. 14 provides a site view to the beach locations at the time of sampling. A total of six different varieties of seaweed were collected and further processed.

Table-15: Parameters Recorded on Field at the Time of Seaweed Sampling

Sr. No.	Parameter	28/10/23 (Vagator)	3/12/23 (Anjuna)	25/12/23 (Vagator)
i.	Water tide level	0.41 metres	1.01 metres	0.56 metres
ii.	Water temperature	28.3°C	29.1°C	27.4°C
iii.	TDS	29,546 ppm	30,002 ppm	29,764 ppm
iv.	Water salinity	35 ppt	35 ppt	35 ppt
v.	Water pH	8.2	8.3	8.2
vi.	Wind speed	13 km/hr SW	22 km/hr NW	12.6 km/hr NW
vii.	Atmospheric temperature	31.4°C	31°C	29°C
viii.	Humidity	86%	69%	71%

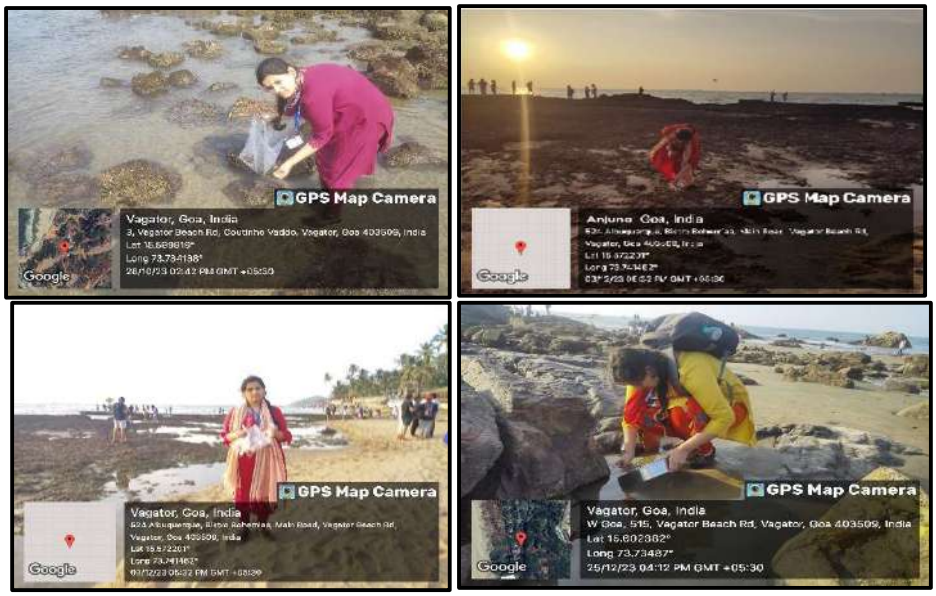


Fig.14: Seaweed Collection from Vagator and Anjuna Beach

4.6 Processing of Seaweed Samples

Based on basic morphological characters and comparison with reference manual, seaweeds were identified as belonging to one Chlorophyceae i.e. *Ulva*, two Rhodophyceae i.e. *Gracilaria* and *Solieria* and three Phaeophyceae i.e *Fucus, Sargassum*, and *Padina* respectively. Fig. 15 shows various seaweed collected from sampling site. These seaweed were processed and Table-16 shows wet to dry mass conversion of the seaweed samples.

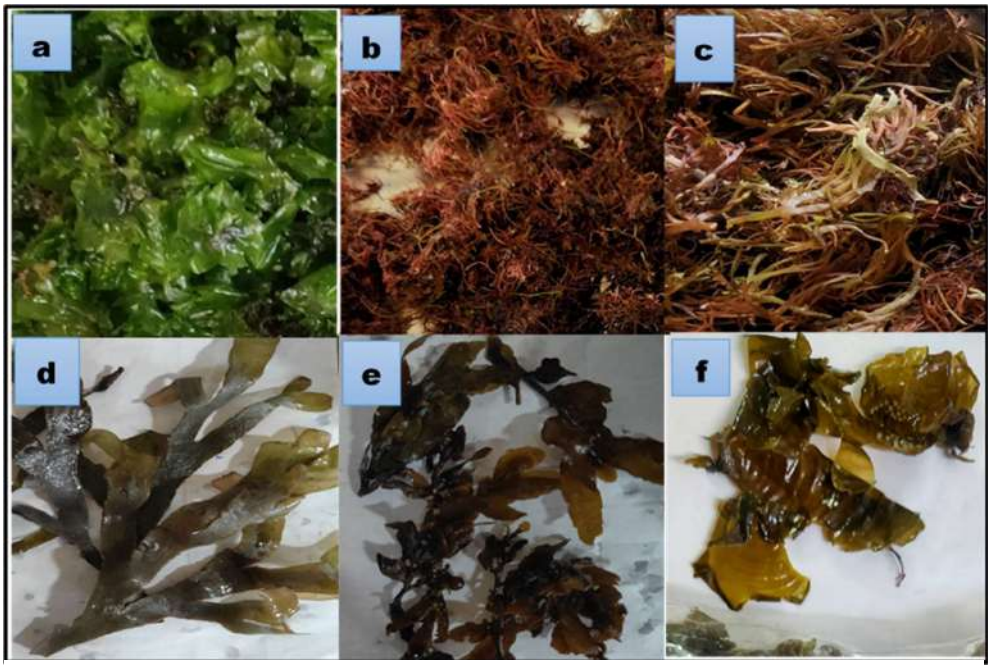


Fig. 15: Seaweed Samples Identified by using the Manual *(V.K. Dhargalkar and Devanand Kavlekar 2004)*

(a) Ulva sp.
(b) Gracilaria sp.
(c) Solieria sp.
(d) Fucus sp.
(e) Sargassum sp.
(f) Padina sp.

Table-16: Wet to Dry Mass Conversion

No.	Seaweed	Wet Weight (in gram)	Dry Weight (in gram)	Conversion %
1.	*Ulva*	276.94	27.32	9.86
2.	*Gracilaria*	104.18	12.68	12.17
3.	*Solieria*	74.24	7.45	10.04
4.	*Fucus*	719.44	113.08	15.72
5.	*Sargassum*	607.68	94.81	15.60
6.	*Padina*	261.58	33.91	12.96

4.7 Characterization of Seaweed Biomass

4.7.1 Acid/Alkaline nature and Conductivity

After adding 10mg seaweed powder in water, and upon checking the pH, it was observed that pH ranged from 5-6 for all the seaweed samples. Likewise, the conductivity values were poor below 1 µs/cm suggesting less release of free ions during powder formation. Table-17 provides the data recorded while performing pH and conductivity experiment.

Table-17: Acid/Alkaline Nature and Conductivity of Seaweed Water Extract

Sr. No.	Seaweed	pH	EC in µs/cm when 10mg biomass in 1ml DW
1	*Ulva*	6.0	0.175
2	*Gracilaria*	6.0	0.088
3	*Solieria*	6.0	0.202
4	*Fucus*	5.5	0.194
5	*Sargassum*	6.5	0.138
6	*Padina*	6.0	0.121

4.7.2 Biomass Characterization

A] DNSA- Reducing Sugar Estimation

Upon estimation of reducing sugar, results were observed as mentioned in Table-18. The reducing sugar ranged between 8-15% among all seaweed biomass, with highest being in *Solieria sp*. and least in *Fucus* sp.

B] Anthrone Test- Total Carbohydrate estimation

The total carbohydrate was estimated using Anthrone method and the following results were obtained as mentioned in Table-18.

The carbohydrate ranged between 25-37% among all seaweed biomass, with highest being similar to that of reducing sugar i.e. in *Solieria sp.* and least in *Padina* sp. The carbohydrate content in *Ulva, Solieria, Fucus, Sargassum* and *Padina* are similar to that reported in literature (*Khouloud M. Barakat et al. (2022); Ana Peñuela et al. (2018); Marcelo D. Catarino et al. (2018); Aurora Silva et al. (2023); Asmaa Maghawri et al. (2023)).*

However, carbohydrate content in *Gracilaria* was poor than mentioned in *P. Radha (2018).* One possible reason for low carbohydrate content could be the harvest before its complete growth.

C] Folin Lowry Method- Protein Estimation

The total protein value obtained upon analysis by Folin Lowry method are mentioned in Table-18. The protein ranged between 16 to 45% among all seaweed biomass, with highest being in *Solieria sp*. and lowest being in *Fucus* sp. The *Ulva* protein content is much higher than mentioned by *Khouloud M. Barakat et al. (2022)* of 12.66%. However, it is close 30% as to that mentioned *Ronan O' Brien et al. (2022).*

Likewise protein content of *Gracilaria, Solieria, Fucus, Sargassum* and *Padina* are also similar in percentage compared to values mentioned in literature. *(Abdullah Rasyid et al. (2019); Kevin Hardouin et al. (2014); Susan Løvstad Holdt and Stefan Kraan (2011); Adriana M. Bonilla Loaiza et al. (2022) and Yuliana Salosso et al. (2020)).*

D] Lipid Estimation

The total lipid using Vanillin Phosphoric acid method showed that lipid content ranged between 3-15% and results can be seen in Table-18. The highest lipid content was present in *Sargassum* sp. and lowest in *Ulva* sp. In literature as well, it is mentioned that *Ulva* contain very low lipid ranging between 0.3- 3.4%. *(João P. Monteiro et al. 2022).*

The red seaweed usually have lipid range of 1.1% to 6.2%, as mentioned by *(Vanessa Gressler et al. 2010).* The lipid values of *Gracilaria* and *Solieria* also came in this range. Likewise, *Fucus* lipid content is reported to be around 0.4-5% *(Marcelo D. Catarino et al. 2018)* and result also show 4.4% lipid content in *Fucus*. The brown seaweed have lipid range of 10-20% *(Kazuo Miyashita et al. 2013).* However, *Sargassum and Padina* show lesser lipid content then mentioned in literature.

Table-18: Reducing Sugar, Total carbohydrate, Total Protein and Total Lipid Content of Seaweed Biomass

Sr. No.	Seaweed Biomass	Reducing Sugar Content in % (g/100g)	Carbohydrate Content in % (g/100g)	Protein Content in % (g/100g)	Lipid Content in % (g/100g)
1	*Ulva*	12.01 ± 0.013	29.31 ± 0.002	31.16 ± 0.003	3.91 ± 0.002
2	*Gracilaria*	13.95 ± 0.001	36.41 ± 0.007	28.28 ± 0.002	5.91 ± 0.001
3	*Solieria*	14.86 ± 0.001	33.93 ± 0.001	33.86 ± 0.001	4.87 ± 0.001
4	*Fucus*	8.91 ± 0.001	29.76 ± 0.001	16.54 ± 0.002	4.39 ± 0.002
5	*Sargassum*	12.05 ± 0.019	26.01 ± 0.001	21.30 ± 0.001	8.47 ± 0.001
6	*Padina*	12.28± 0.002	29.33± 0.001	20.46 ± 0.001	5.35 ± 0.002

* Values after ± are standard error values.

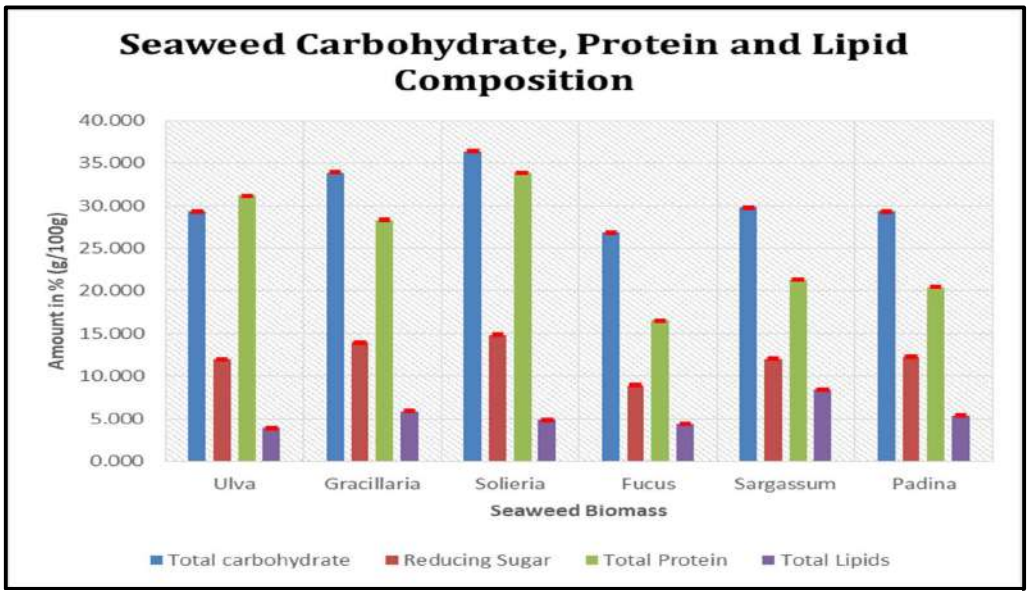


Fig. 16: Seaweed Biomass composition: Carbohydrate, Reducing Sugar, Protein and Lipid

E] Gravimetric Metric Analysis: TS, TVS and Ash Content Analysis

The following Table-19 represent the data obtained upon gravimetric analysis of the seaweed powdered biomass. The highest total solid was found in *Sargassum*, while highest total volatile solid and ash content were found in *Gracilaria* and *Fucus* respectively.

Table-19: TS, TVS, Ash and Moisture Content of Seaweed Biomass

No.	Seaweed	Total Solid (TS) in %	Total Volatile Solid (TVS) in %	Ash Content (%)	Moisture Content (%)
1	*Ulva*	84.39 ± 0.057	43.92 ± 0.1576	40.46 ± 0.101	15.62 ± 0.057
2	*Gracilaria*	87.26 ± 0.847	61.81 ± 0.0958	25.45 ± 0.752	12.74 ± 0.847
3	*Solieria*	87.93 ± 0.559	75.99 ± 0.5211	11.94 ± 0.039	12.07 ± 0.559
4	*Fucus*	87.13 ± 0.063	40.09 ± 0.2077	47.04 ± 0.155	12.87 ± 0.063
5	*Sargassum*	90.15 ± 0.140	60.82 ± 0.1273	29.33 ± 0.051	09.84 ± 0.140
6	*Padina*	89.06 ± 0.036	50.01 ± 0.1583	39.46 ± 0.179	10.94 ± 0.036

* Values after ± are standard error values.

F] CHNS Analysis

The following Table-20 represent the data obtained upon CHNS analysis of the seaweed powdered biomass. The *Solieria* showed a significant high carbon content of around 34% as well as highest hydrogen content of around 6%. This also infer that *Solieria* must have high carbohydrate content than rest, which is true as per Anthrone method. *Gracilaria* had the highest nitrogen content followed by *Solieria.* Nitrogen containing compounds are known for production of agents and polymer production. Since both are red seaweed, this provides their potent properties as chain transfer agents *(Liliana Krotz and Guido Giazzi, 2017).* Likewise, seaweed *Ulva, Gracilaria, Padina* and *Solieria,* also have high sulphur content above 2%. As suggested by *Fethi Mensi et al. (2022)*, presence of high sulphur would protect them from oxidative damages and thus make them a potent source for antioxidant agents.

Table-20: CHNS/O Data for Seaweed Biomass

No.	Sample Biomass	Carbon %	Hydrogen %	Nitrogen %	Sulphur %	Oxygen %
1	*Ulva*	18.56	5.06	2.00	2.83	31.09
2	*Gracilaria*	26.81	5.58	2.93	2.37	36.87
3	*Solieria*	33.84	5.92	2.76	2.02	43.53
4	*Fucus*	23.45	5.03	2.29	1.00	21.19
5	*Sargassum*	22.87	5.38	1.67	1.00	39.75
6	*Padina*	25.30	4.47	2.32	2.26	26.60

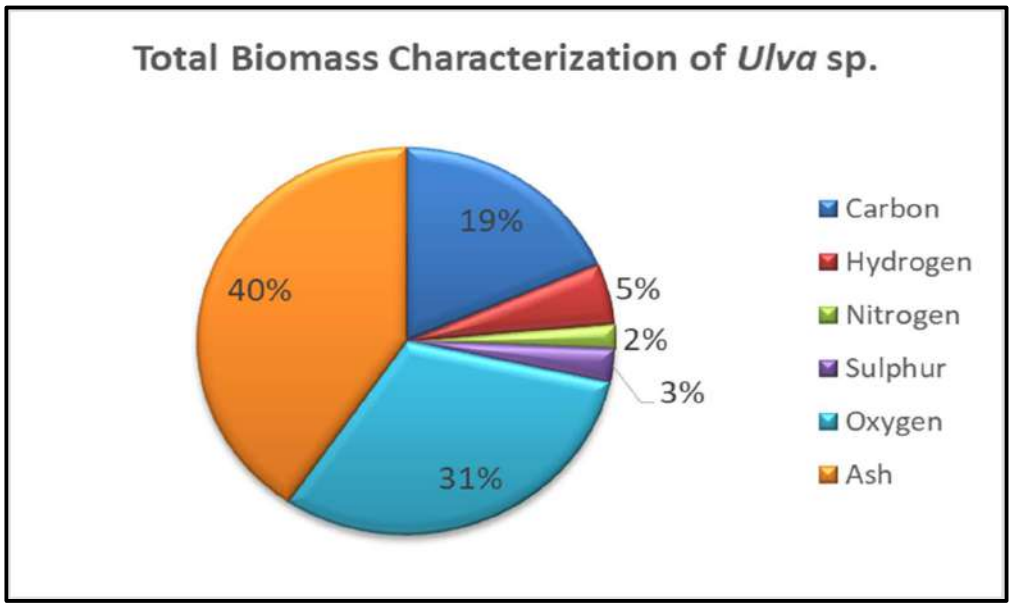


Fig. 17: Total Biomass Characterization of *Ulva* sp.

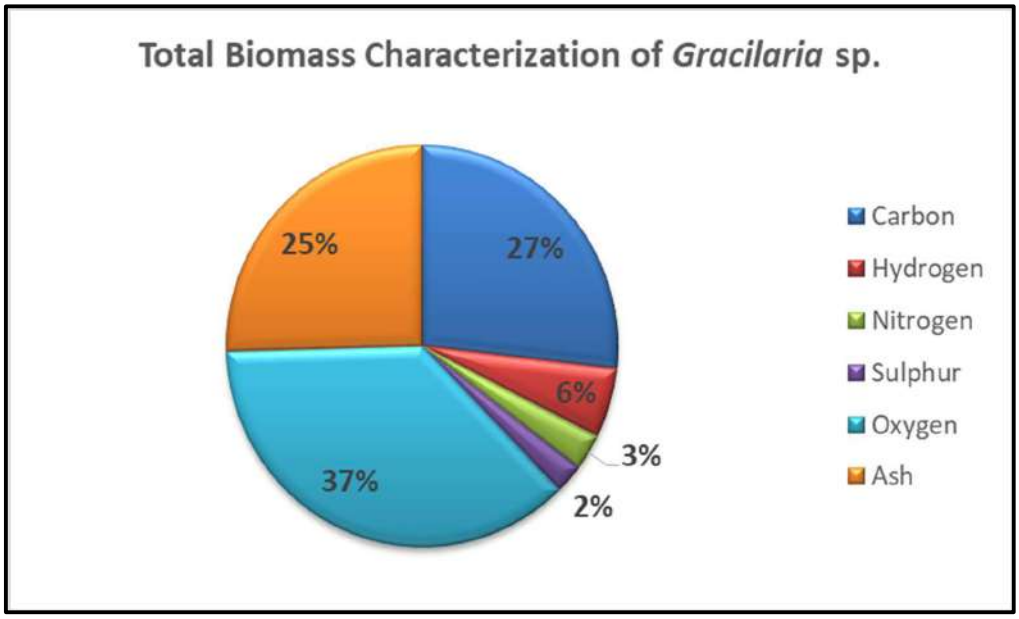


Fig. 18: Total Biomass Characterization of *Gracilaria* sp.

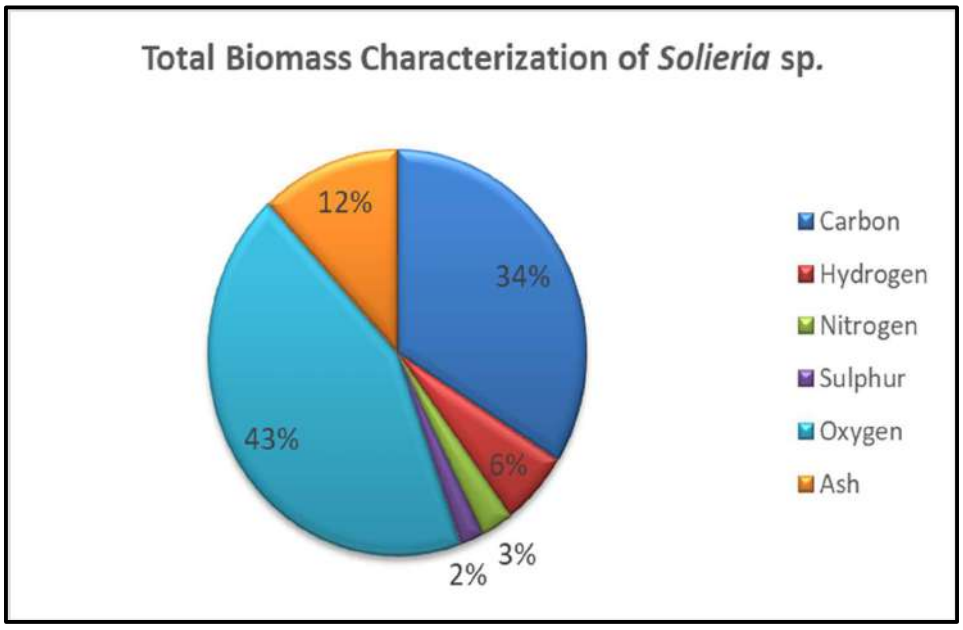


Fig. 19: Total Biomass Characterization of *Solieria* sp.

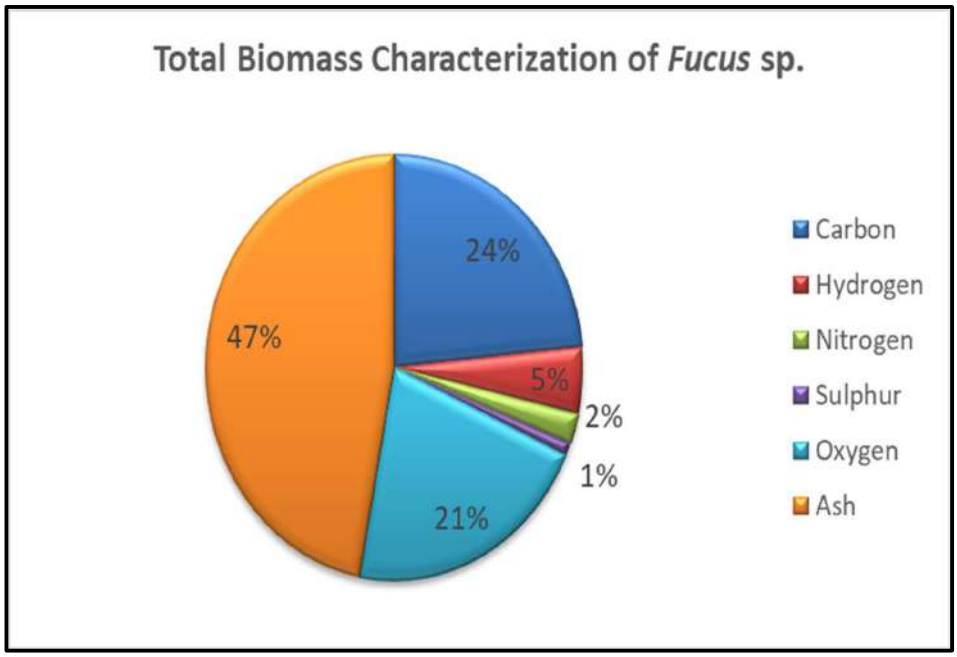


Fig. 20: Total Biomass Characterization of *Fucus* sp.

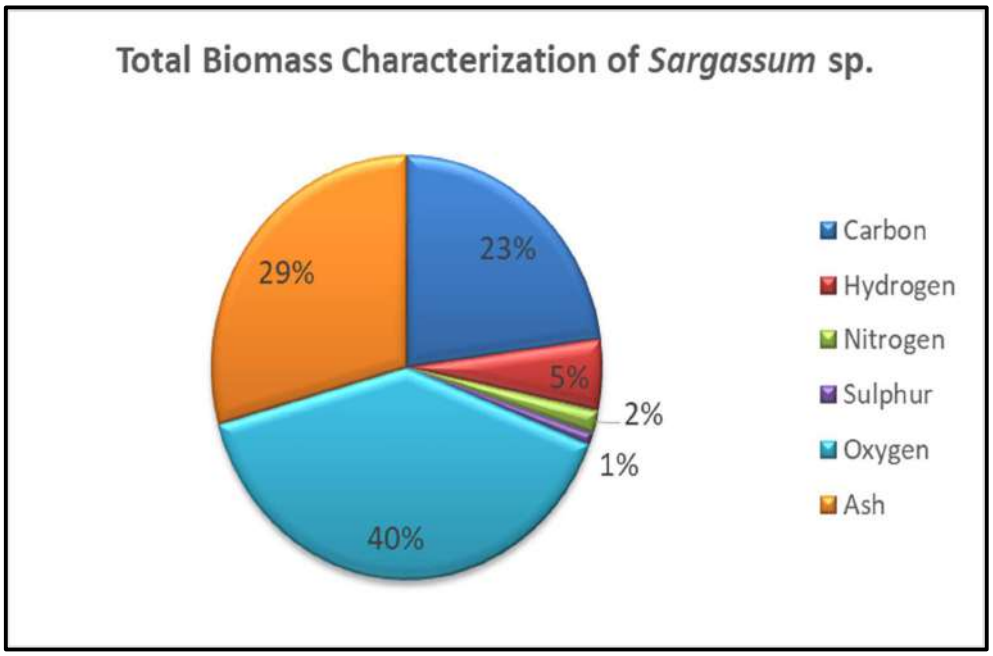


Fig. 21: Total Biomass Characterization of *Sargassum* sp.

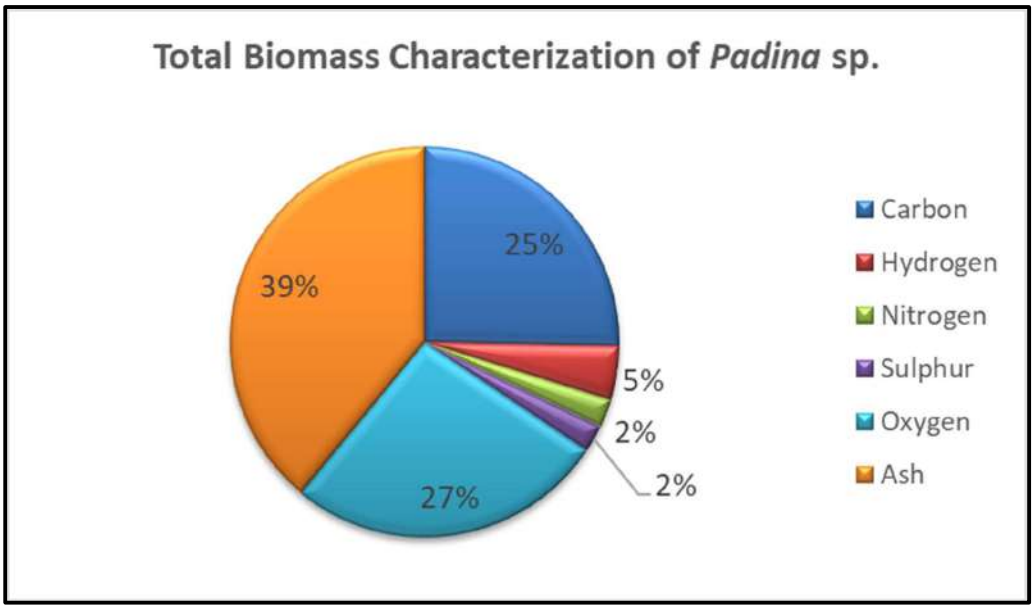


Fig. 22: Total Biomass Characterization of *Padina* sp.

4.8 Preparation of Seaweed Hydrolysates

The hydrolysates were prepared using 5% of seaweed biomass (dry weight basis). A total of 12 hydrolysate were made i.e. six acid hydrolysates and six alkaline hydrolysates of seaweed samples. Fig. 23 shows pictures of the prepared hydrolysate which were then stored for further use.

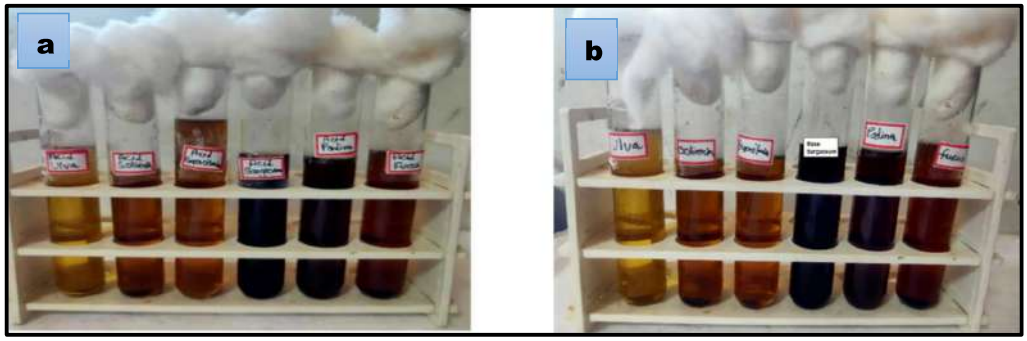


Fig. 23: Seaweed Hydrolysate Prepared from Six Seaweed
(a) Acid Hydrolysates (b) Alkaline Hydrolysates

4.9 Characterization of Hydrolysates

Similar to characterization of seaweed biomass, the hydrolysates were also characterized on similar parameters. The Table-21 and Table-22 provide data on results obtained upon pH, salinity, reducing sugar, total carbohydrate and protein of acid hydrolysates and alkaline hydrolysates respectively.

As a whole, mostly acid hydrolysate sequestered more sugars and carbohydrates than alkaline hydrolysates. While, protein was more in alkaline hydrolysate compared to acid hydrolysates. However, compared to a hypothetical yield wherein most sugar and proteins of the 5% biomass would have dissolved in hydrolysates, the values observed were very low i.e., around 10% of total content in each case. Fig. 24 shows a comparative analysis of reducing sugars, total carbohydrates, proteins and lipids present in acid and alkaline hydrolysates.

Table-21: Characterization of Acid Hydrolysates of Seaweed

No.	Acid Hydrolysate of	pH	Salinity (ppt)	Total Carbohydrate (mg/L)	Reducing Sugar (mg/L)	Total Protein (mg/L)	Total Lipid (mg/L)
1	*Ulva*	0.52	25	126.32 ± 0.001	87.51 ± 0.015	63.35 ± 0.001	37.53 ± 0.002
2	*Gracilaria*	0.66	40	200.70 ± 0.001	75.50 ±0.001	83.64 ±0.002	87.05 ± 0.002
3	*Solieria*	0.45	35	173.68 ± 0.001	75.02 ± 0.001	52.36 ±0.002	84.65 ± 0.001
4	*Fucus*	1.02	25	120.00 ± 0.002	68.45 ± 0.001	94.10 ±0.001	41.53 ± 0.002
5	*Sargassum*	0.38	30	193.61 ± 0.002	48.65 ± 0.001	105.04 ±0.001	61.50 ± 0.002
6	*Padina*	0.63	25	83.20 ± 0.001	63.61 ± 0.002	73.40 ±0.001	31.15 ± 0.001

* Values after ± are standard error values.

Table-22: Characterization of Alkaline Hydrolysates of Seaweed

No.	Alkaline Hydrolysate of	pH	Salinity (ppt)	Total Carbohydrate (mg/L)	Reducing Sugar (mg/L)	Total Protein (mg/L)	Total Lipid (mg/L)
1	*Ulva*	11.26	25	106.04 ± 0.004	22.00 ± 0.001	158.38 ± 0.002	31.94 ± 0.003
2	*Gracilaria*	12.03	20	75.90 ± 0.003	51.66 ± 0.001	209.11 ± 0.001	70.28 ± 0.003
3	*Solieria*	11.97	25	83.72 ± 0.001	35.71 ± 0.003	130.90± 0.001	69.48 ± 0.002
4	*Fucus*	12.52	30	98.23 ± 0.003	54.38 ± 0.001	235.25 ± 0.001	35.94 ± 0.001
5	*Sargassum*	11.04	35	130.10 ± 0.001	92.68 ± 0.001	262.60 ± 0.002	58.30 ± 0.001
6	*Padina*	11.64	35	121.29 ± 0.002	61.73 ± 0.001	183.51 ± 0.001	23.96 ± 0.002

* Values after ± are standard error values.

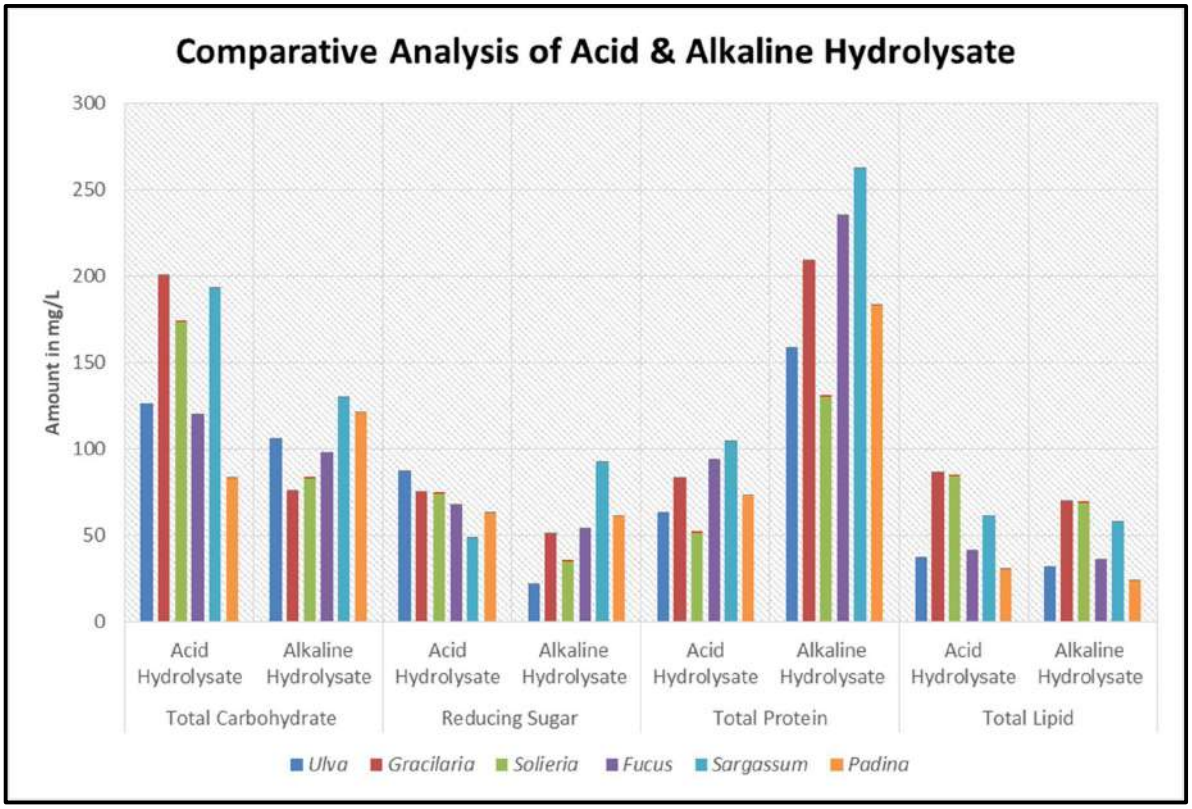


Fig. 24: Comparative Analysis of Total Carbohydrate, Reducing Sugar, Protein and Lipids Present in Acid/Alkaline Hydrolysate of Seaweed.

4.10 Estimation of Pullulanase Production Using Hydrolysates

The highest pullulanase producing cultures GUPM2, GUPM5 and GUPM22, as screened in 4.4 were inoculated in various hydrolsates. The pullulanase production by the three cultures in the hydrolysate were again recorded using DNSA method, as shown in Fig. 25, Fig. 26 and Fig. 27.

The pullulanase activity of each culture measured using different hydrolysated and three carbon substrate i.e. 3% starch, 3% cellulose and 1% pullulan are show in Table-23, Table-24 and Table-25.

Table-23: Pullulanase Estimation by GUPM2 in Different Substrates

Sr. No.	Hydrolysate/substrate for GUPM2	Pullulanse Enzyme Concentration (in µg/ml)	Enzyme Activity (IU) or 1 µmol/min
1	Acid *Ulva*	32.56	1.09
2	Acid *Gracilaria*	26.67	0.89
3	Acid *Solieria*	52.05	1.74
4	Acid *Fucus*	30.51	1.02
5	Acid *Sargassum*	127.69	4.26
6	Acid *Padina*	108.08	3.60
7	Alkaline *Ulva*	100.51	3.35
8	Alkaline *Gracilaria*	97.95	3.27
9	Alkaline *Solieria*	42.05	1.40
10	Alkaline *Fucus*	83.97	2.80
11	Alkaline *Sargassum*	31.03	1.03
12	Alkaline *Padina*	95.77	3.19
13	Starch (3%)	163.97	5.47
14	Cellulose (3%)	219.23	7.31
15	Pullulan (1%)	337.44	11.25

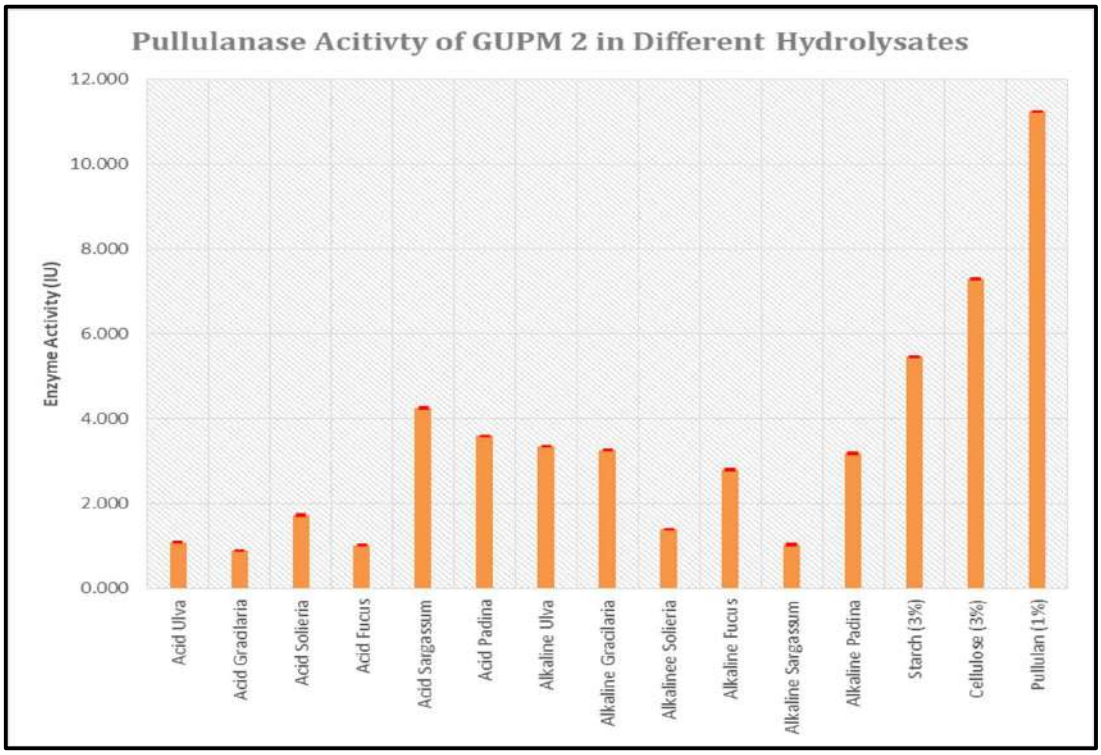


Fig. 25: Comparative Analysis of Pullulanase Activity of GUPM2 in Different Substrates

Table-24: Pullulanase Estimation by GUPM5 in Different Substrates

Sr. No.	Hydrolysate/substrate for GUPM5	Pullulanse Enzyme Concentration (in µg/ml)	Enzyme Activity (IU) or 1 µmol/min
1	Acid *Ulva*	47.05	1.57
2	Acid *Gracilaria*	55.77	1.86
3	Acid *Solieria*	82.95	2.77
4	Acid *Fucus*	22.70	0.76
5	Acid *Sargassum*	48.97	1.63
6	Acid *Padina*	67.05	2.24
7	Alkaline *Ulva*	96.67	3.22
8	Alkaline *Gracilaria*	112.56	3.75
9	Alkaline *Solieria*	65.39	2.18
10	Alkaline *Fucus*	124.74	4.16
11	Alkaline *Sargassum*	48.21	1.61
12	Alkaline *Padina*	92.05	3.07
13	Starch (3%)	150.64	5.02
14	Cellulose (3%)	168.08	5.60
15	Pullulan (1%)	319.49	10.65

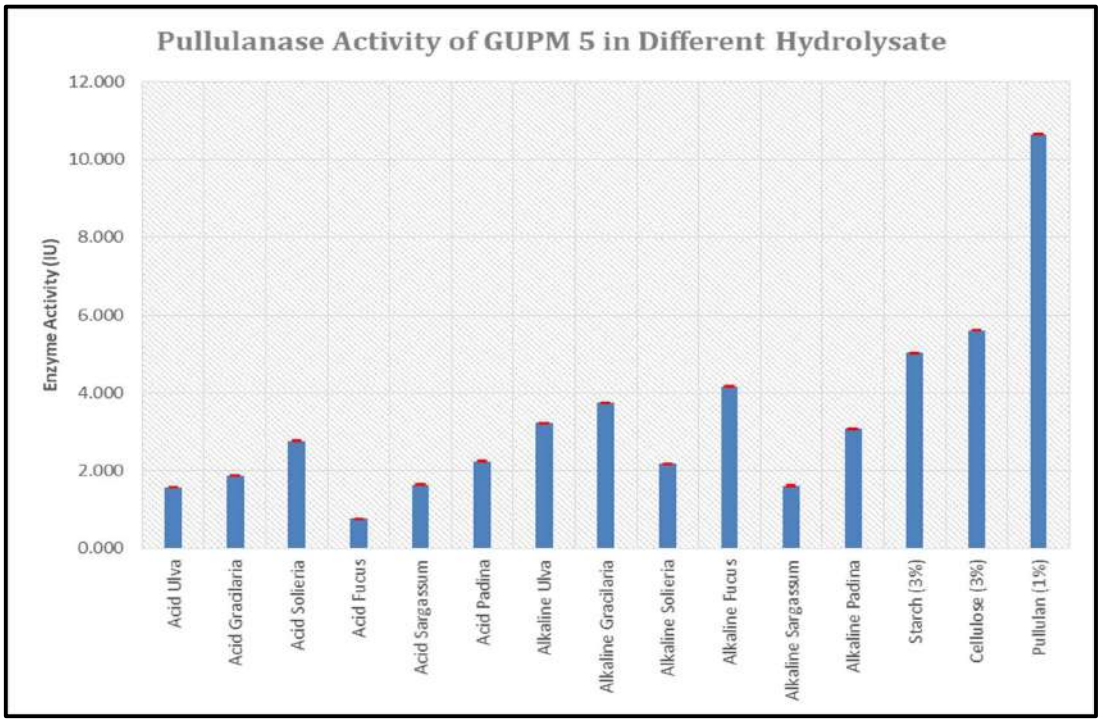


Fig. 26: Comparative Analysis of Pullulanase Activity of GUPM5 in Different Substrates

Table-25: Pullulanase Estimation by GUPM22 in Different Substrates

Sr. No.	Hydrolysate/substrate for GUPM22	Pullulanse Enzyme Concentration (in µg/ml)	Enzyme Activity (IU) or 1 µmol/min
1	Acid *Ulva*	39.49	1.32
2	Acid *Gracilaria*	14.23	0.47
3	Acid *Solieria*	47.69	1.59
4	Acid *Fucus*	42.82	1.43
5	Acid *Sargassum*	51.03	1.70
6	Acid *Padina*	116.54	3.89
7	Alkaline *Ulva*	84.23	2.81
8	Alkaline *Gracilaria*	115.26	3.84
9	Alkaline *Solieria*	68.21	2.27
10	Alkaline *Fucus*	171.67	5.72
11	Alkaline *Sargassum*	40.90	1.36
12	Alkaline *Padina*	88.59	2.95
13	Starch (3%)	189.10	6.30
14	Cellulose (3%)	168.08	5.60
15	Pullulan (1%)	354.49	11.82

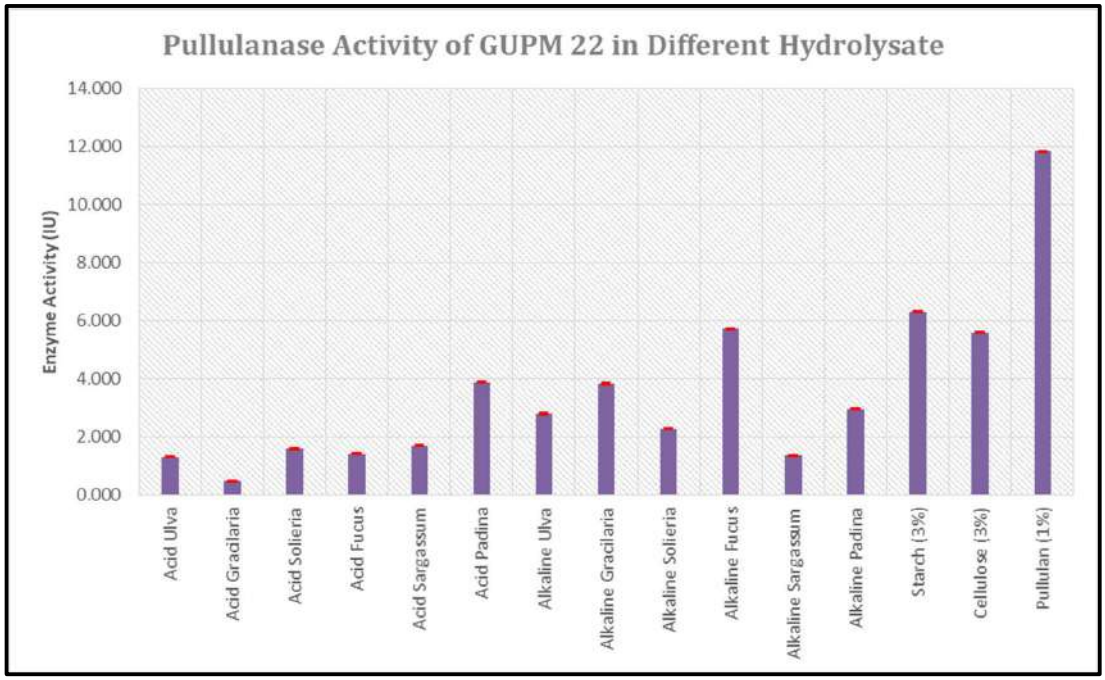


Fig. 27: Comparative Analysis of Pullulanase Activity of GUPM22 in Different Substrates

Based on the estimation of pullulanase activity of selected cultures in different hydrolysates, it was recorded that GUPM2 showed highest pullulanase activity in acid hydrolysate of *Sargassum* while GUPM5 and GUPM22 showed highest pullulanase activity in alkaline hydrolysate of *Fucus*. The activity is also comparable to commercial substrates used for pullulanase production i.e. 3% cellulose and 3% starch.

Thus, further optimization was performed for each culture using hydrolysate in which highest pullulanase activity was recorded i.e. acid hydrolysate of *Sargassum* and alkaline hydrolysate of *Fucus* for GUPM2, GUPM5 and GUPM22 respectively.

4.11 pH and Temperature Optimization

a) **pH optimization**

The graph at Fig. 28 shows comparison of growth of GUPM2 in acid hydrolysate of *Sargassum* set at different pH. It suggest that cultures grows best at pH 7.5. A similar analysis for pH optimization was done for GUPM5 and GUPM22 using alkaline *Fucus* hydrolysate set at different pH. The graphs at Fig. 29 and Fig. 30 also shows comparison of growth of GUPM5 and GUPM22 at varying pH of hydrolysate respectively. The results suggested that GUPM5 grows best at pH 7, while GUPM22 grows best pH of 7.5.

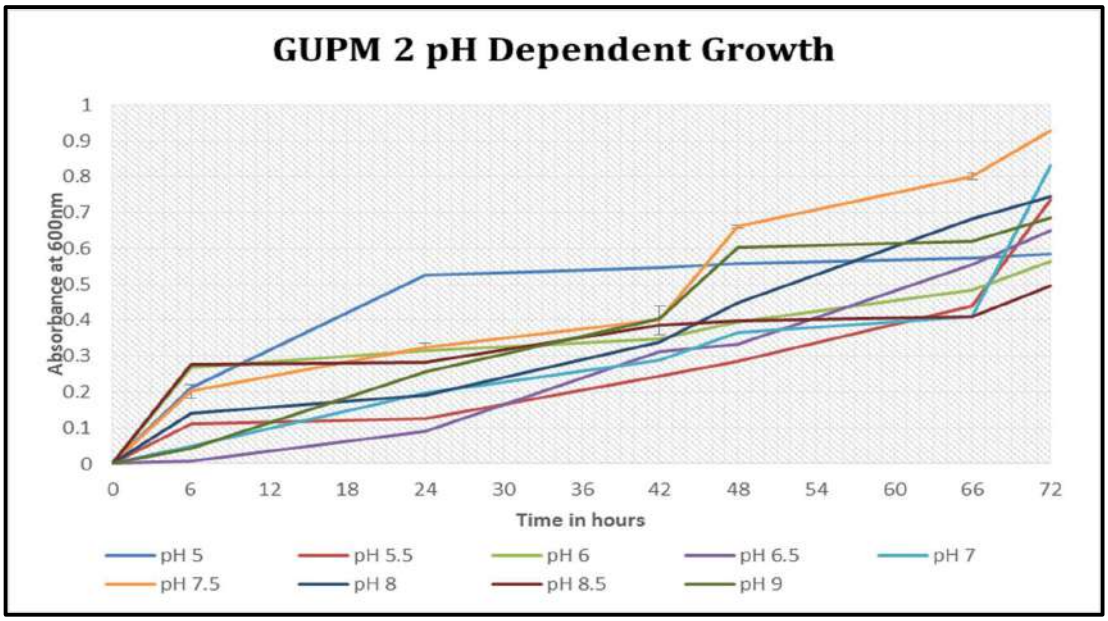


Fig. 28: Growth Curve of GUPM2 at Varying pH of Acid Hydrolysate of *Sargassum*

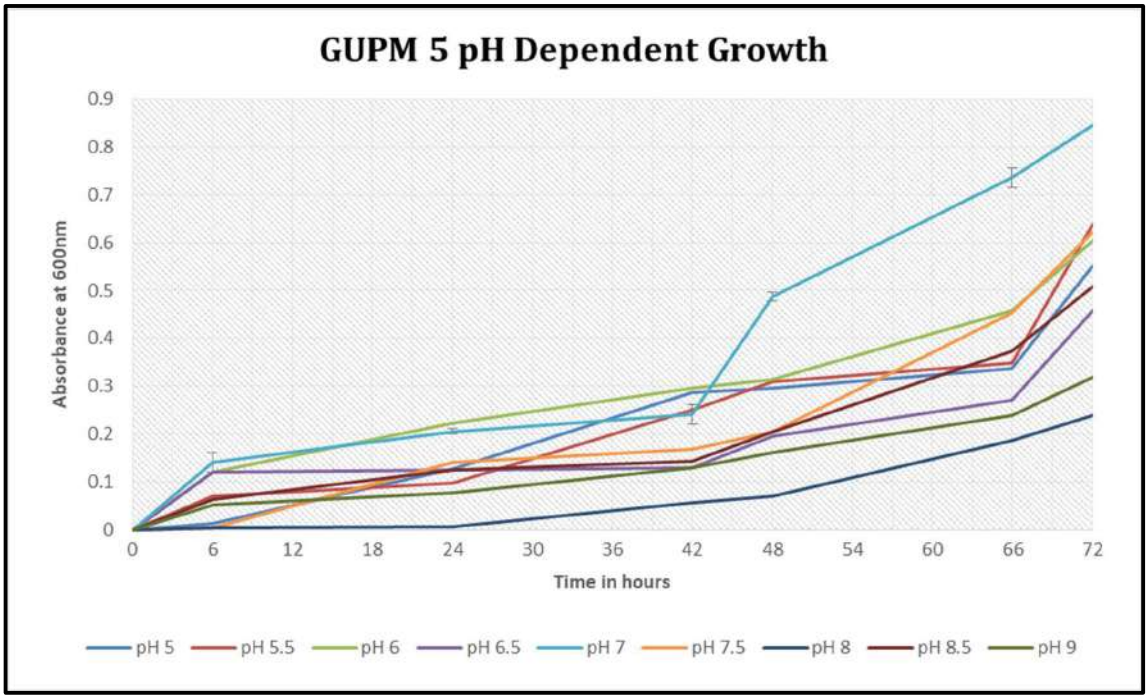


Fig. 29: Growth Curve of GUPM5 at Varying pH of Alkaline Hydrolysate of *Fucus*

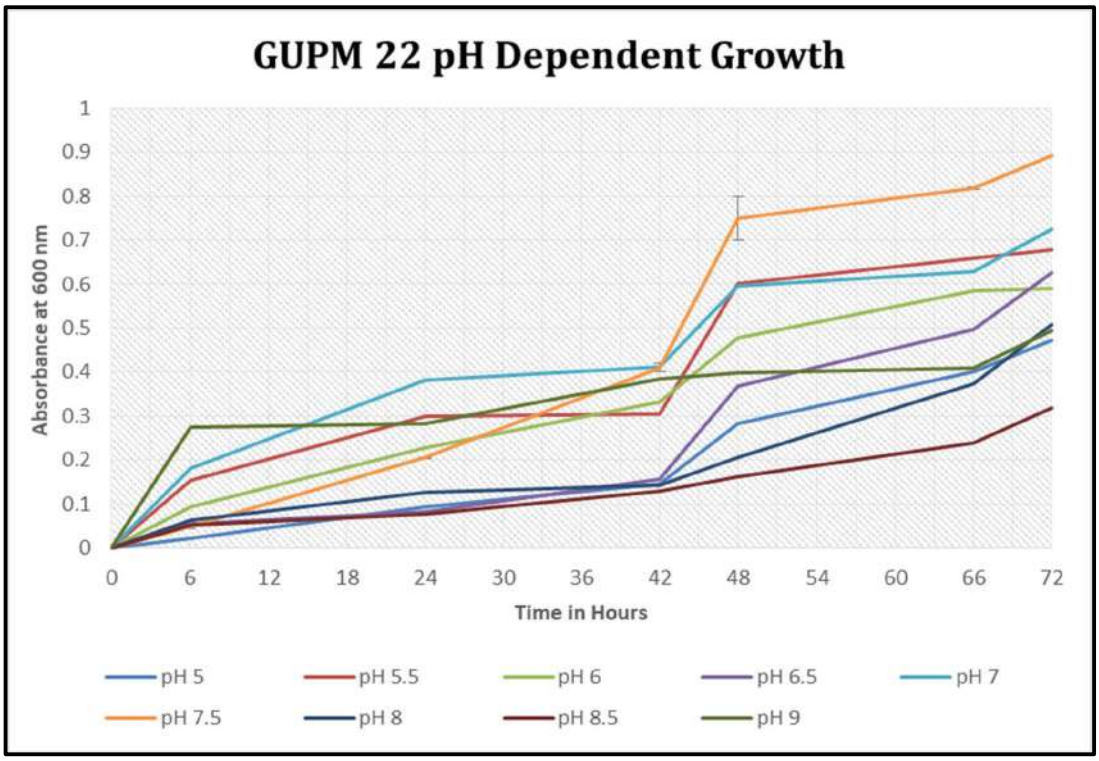


Fig. 30: Growth Curve of GUPM22 at Varying pH of Alkaline Hydrolysate of *Fucus*

b) **Temperature Optimization**

After the pH optimization, the hydrolysates, adjusted with optimum pH, were inoculated with respective cultures and kept at different temperature to check for growth of cultures by measuring growth rate at 600 nm. The Fig. 31 and Fig. 33 show growth of culture GUPM2 and GUPM22 in *Sargassum*'s acid hydrolysate and *Fucus*'s alkaline hydrolysate of set pH 7.5. The Fig. 32 shows growth curve for culture GUPM22 in *Fucus*'s alkaline hydrolysate set at pH 7. The growth rate suggest that GUPM2 grows best at 30°C while other two cultures i.e. GUPM5 and GUPM22 grows better at 37°C.

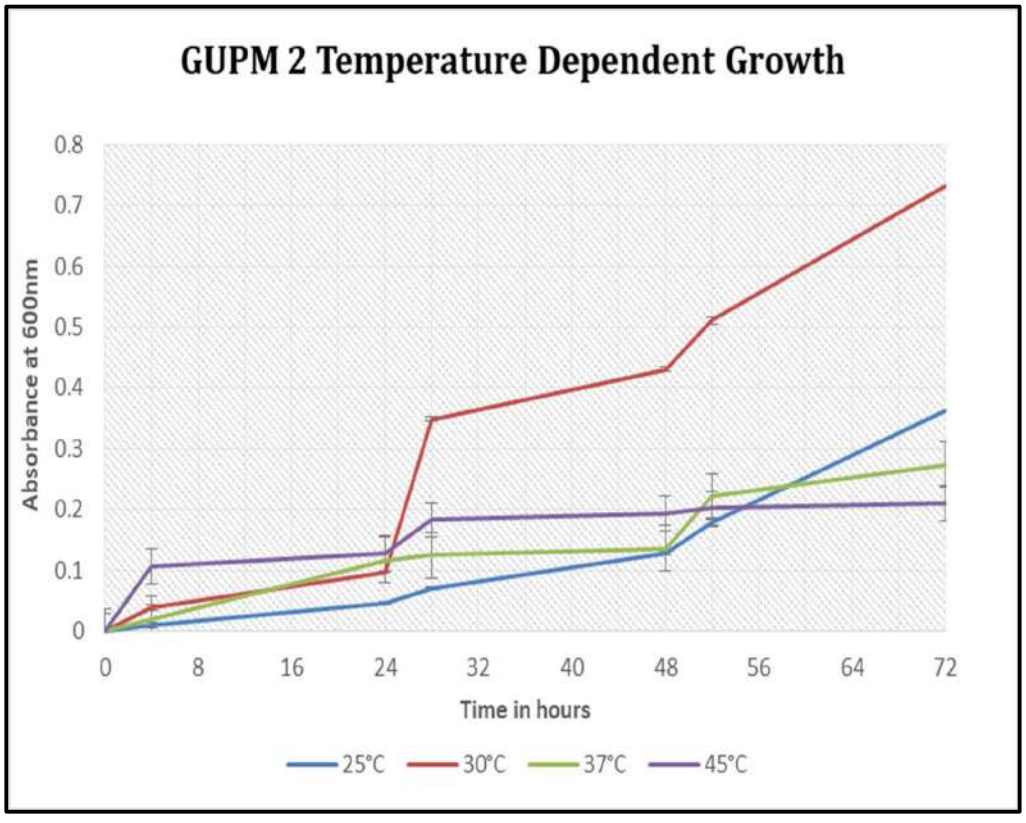


Fig. 31: Growth Curve of GUPM2 at Varying Temperatures of Acid Hydrolysate of *Sargassum* (pH 7.5)

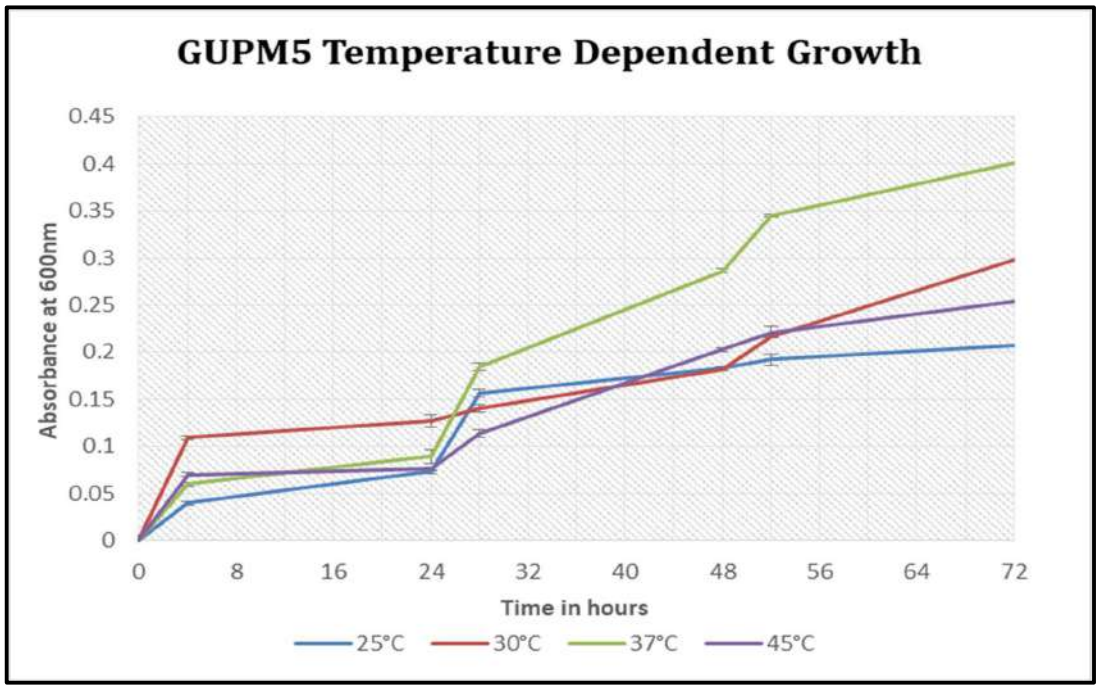


Fig.32: Growth Curve of GUPM5 at Varying Temperatures of Alkaline Hydrolysate of *Fucus* (pH 7.0)

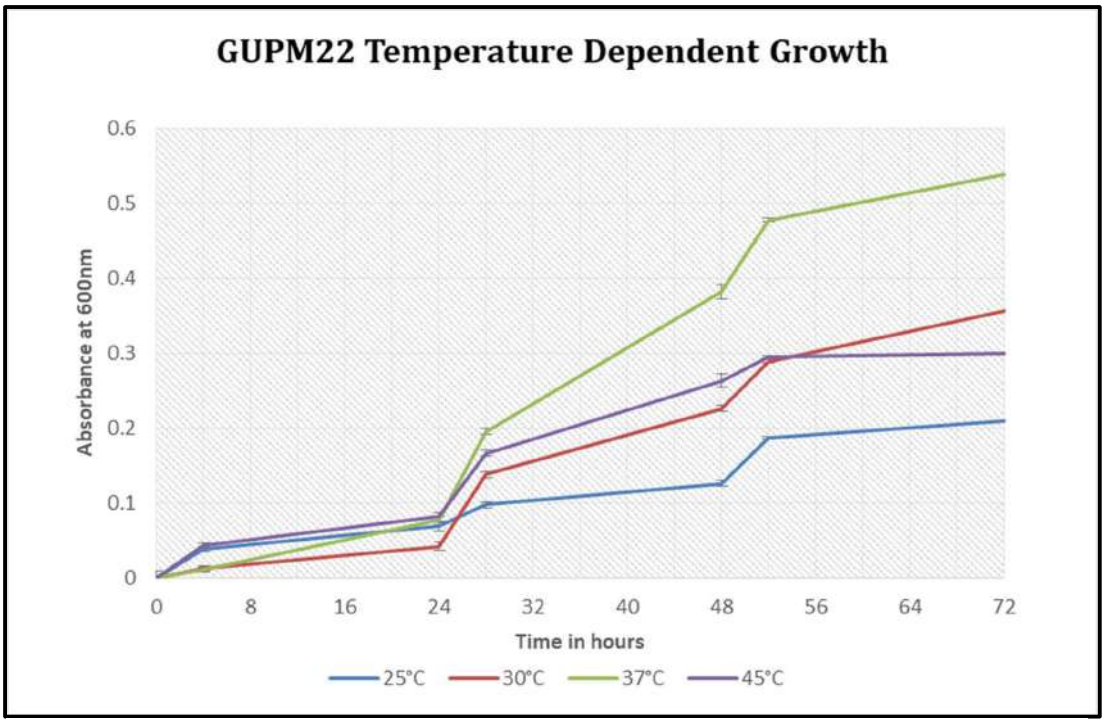


Fig. 33: Growth Curve of GUPM22 at Varying Temperatures of Alkaline Hydrolysate of *Fucus* (pH 7.5)

4.12 Growth Curve of Cultures in Hydrolysate

The cultures GUPM2, GUPM5 and GUPM22 were again grown in their respective hydrolysate at optimized conditions. The Fig. 34 represent the growth of these cultures. The experiment provided a confirmatory data that they grow at optimized pH and temperature parameters in the hydrolysates.

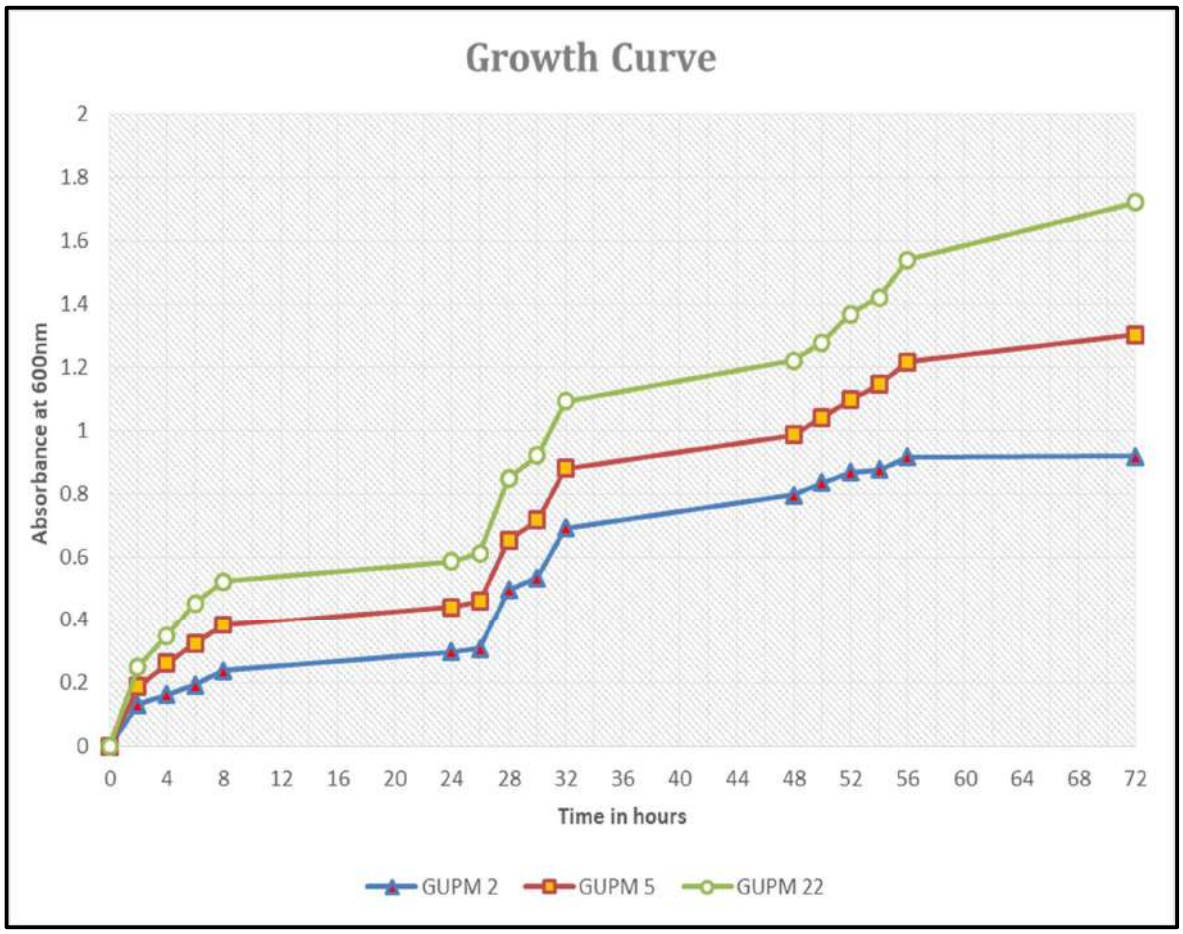


Fig. 34: Growth Curve of GUPM2, GUPM5 and GUPM22 in Hydrolsates in their Respective Optimized Temperature and pH Parameters.

4.13 Estimation of Highest Pullulanase Activity During Culture's Growth Period

The pullulanase activity of the cultures were measured along with the growth curve using the reducing sugar assay. It was observed that the pullulanase activity increased with increasing growth of cultures. The highest pullulanase activity for all cultures were recorded at 72 hours of incubation.

The highest enzyme activity was found to be 5.113 IU, 4.603 IU and 6.564 IU for cultures GUPM2, GUPM5 and GUPM22 respectively. The following graph represent pullulanase activity of cultures in a time interval of 72 hours, however as shown by graph in Fig. 35, the pullulanase activity of GUPM2 and GUPM22 might still be increasing after 72 hours of incubation.

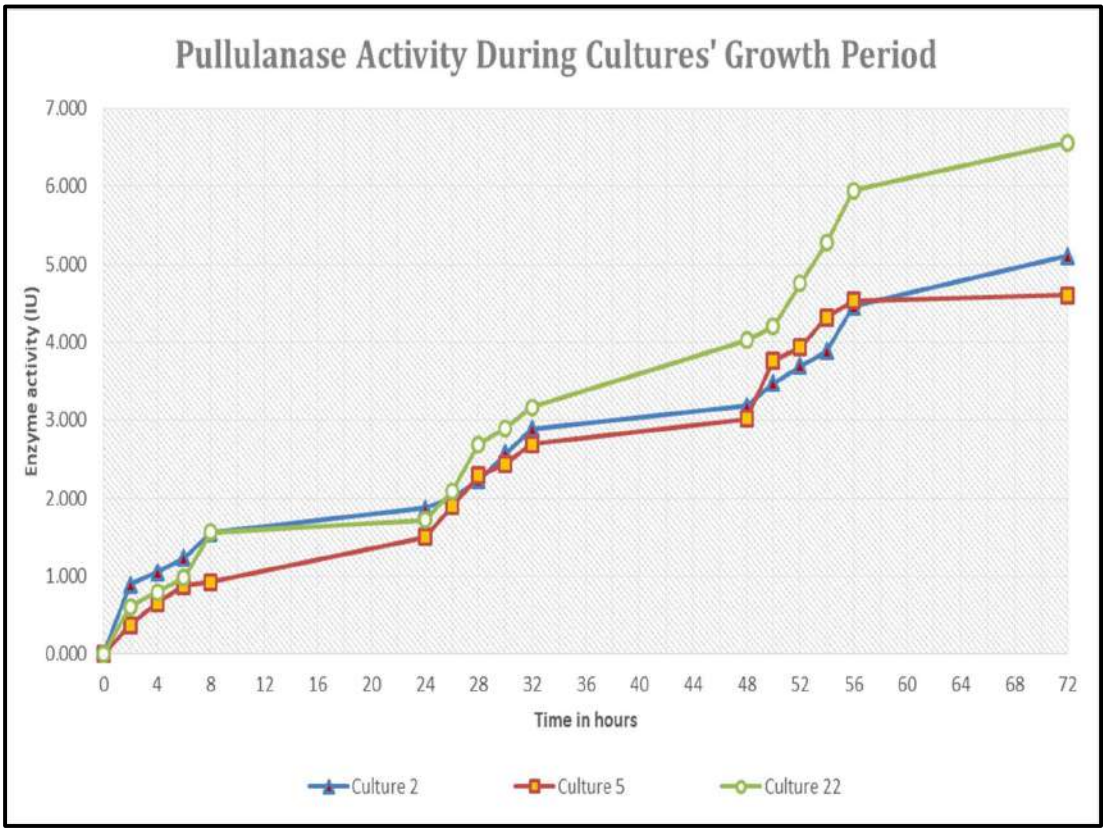


Fig. 35: Comparison of Pullulanase Activity between GUPM2, GUPM5 and GUPM22 During their Growth Period

It is noteworthy that optimization of pH and temperature for cultures have increased pullulanase production from the cultures compared to those obtained while analyzing in section 4.10. Table-26 and Fig.36 highlights a comparative analysis of pullulanase production by cultures before and after optimization along with pullulanase activity using standard substrates of 3% starch, 3% cellulose and 1% pullulan at 30°C, pH 7.5.

Table-26: Pullulanase Activity Comparison Among Cultures Before and After Optimization and with Standard Substrates

Pullulanase Activity in IU	GUPM2	GUPM5	GUPM22
Before Optimization	4.26 ± 0.001	4.16 ± 0.001	5.72 ± 0.002
After Optimization	5.11 ± 0.002	4.60 ± 0.002	6.56 ± 0.002
In 3% starch	5.47 ± 0.001	5.02 ± 0.001	6.30 ± 0.001
In 3% cellulose	7.31± 0.002	5.60 ± 0.001	5.60 ± 0.001
In 1% pullulan	11.25± 0.002	10.65 ± 0.001	11.82 ± 0.001

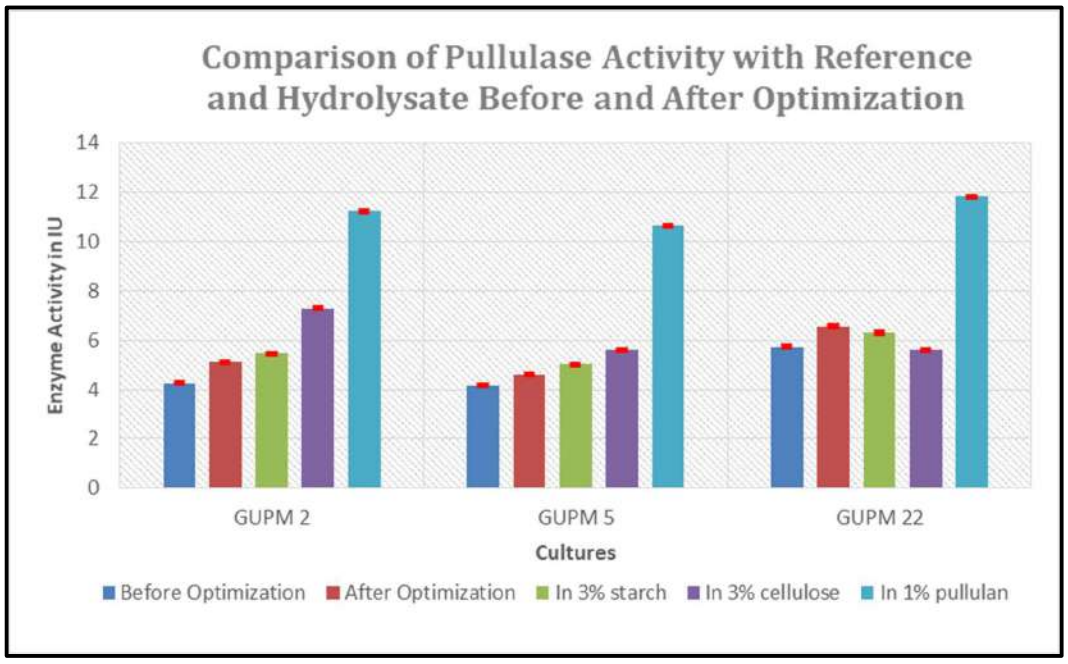


Fig. 36: Comparison of Pullulanase Activity with Standard Substrates and Hydrolysates Before and After Optimization

4.14 Fold Purification of Pullulanase

The fold purification was performed by Ammonium salt precipitation followed by overnight dialysis. Further, the data for protein content and pullulanase activity was recorded and calculations were performed.

Table-27 shows the overview of activity, specific activity, yield % and fold purification of pullulanase for each culture. It can be observed that dialysis provide nearly two fold purification to protein in acid hydrolysate of *Sargassum*. Likewise, dialysis provided nearly 5 X purification to protein in alkaline hydrolysate of *Fucus*.

Table-27: Downstream Steps for Fold Purification of Pullulanase

Culture	Down-stream Step	Sample Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Enzyme Activity in µmol/min	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold Purification
GUMP2	Crude	25	0.128	3.207	5.115	127.867	39.870	100.00	1.000
GUPM2	Precipitated Sample in PBS	5	0.116	0.579	5.312	26.560	45.866	20.771	1.150
GUPM2	Dialyzed	5	0.069	0.344	5.684	28.419	82.654	22.225	2.073
GUPM5	Crude	25	0.258	6.454	4.547	113.675	17.612	100	1.000
GUPM5	Precipitated Sample in PBS	5	0.232	1.160	5.184	25.919	22.344	22.801	1.269
GUPM5	Dialyzed	5	0.064	0.320	5.359	26.795	83.734	23.571	4.754
GUPM22	Crude	25	0.255	6.384	6.585	164.637	25.789	100	1.000
GUPM22	Precipitated Sample in PBS	5	0.236	1.180	6.714	33.568	28.441	20.389	1.103
GUPM22	Dialyzed	5	0.055	0.277	7.440	37.201	134.069	22.596	5.199

4.15 Molecular Weight Analysis by SDS-PAGE

The molecular weight estimation was performed using SDS-PAGE method. Herein, it was observed that acid hydrolysate of *Sargassum* had three protein bands corresponding to molecular weight of 148.71 kDa, 46.51 kDa and 20.99 kDa. Similarly, alkaline hydrolysate of *Fucus* also showed three bands corresponding to molecular weight of 142.81 kDa, 74.49 kDa and 31.28 kDa. Fig. 37 shows the labelled SDS-PAGE gel images which marks occurrence of these bands.

Culture supernatant from Halopiger broth showed two, five and two bands prominent bands for cultures GUPM2, GUPM5 and GUPM22 respectively. Table-28 shows the bands obtained by cultures in different samples and highlights pullulanase molecular weight. Though, standard pullulanase was not run during the SDS-PAGE, but literature suggest its molecular weight to be between 40-120 kDa (*Piotr Tomasik and Derek Horton, 2012)*.

High intensity bands, corresponding to pullulanase, in SDS-PAGE was expected in the culture grown in pullulan broth. The pullulanase band can be roughly identified based on its occurrence in all samples i.e. pullulan broth, crude, ammonium salt precipitate and dialyzed sample. It observation suggest that GUPM2 probably produce pullulanase of 59.40 kDa, while GUPM5 and GUPM22 produce pullulanase of 75.75 and 103.14 kDa respectively. Presence of more than one band in pullulan broth samples in GUPM5 and GUPM22 also indicate that cultures might produce more than one form of pullulanase when given pullulan substrate.

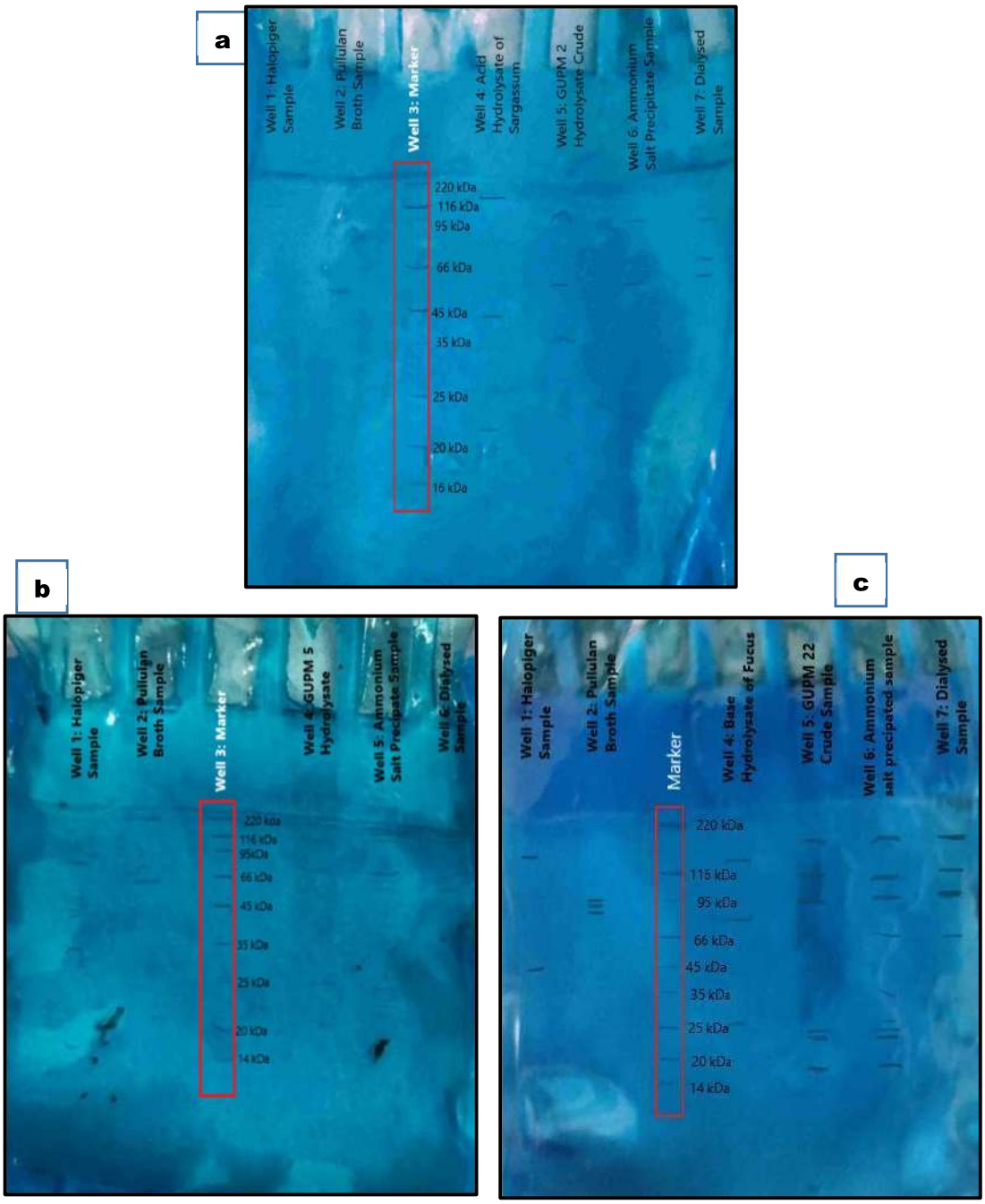


Fig. 37: SDS PAGE Gel Profile

(a) Samples of Culture GUPM2
(b) Samples of Culture GUPM5
(c) Samples of Culture GUPM22

Table-28: Molecular Weight (in kDa) of Protein Bands Obtained in SDS-PAGE

Molecular Weight of Protein Bands in SDS-PAGE					
	(1) Supernatant of the culture grown in Halopiger broth	(2) Supernatant of the culture grown in Pullulan Broth	(3) Supernatant of culture grown in hydrolysate (crude sample)	(4) Ammonium salt precipitate of crude sample (3) suspended in PBS	(5) Sample (4) after Dialysis
GUPM2	123.78	59.40	116.43	103.02	103.02
	67.13		59.40	59.40	67.13
			38.71		59.40
			28.51		
GUPM5	82.66	166.15	127.88	166.15	152.26
	48.97	75.75	75.75	152.26	139.54
	44.87	63.62	18.75	139.54	90.20
	41.12			90.20	75.75
	29.01			82.66	
				75.75	
				18.75	
GUPM22	159.17	103.14	177.41	177.41	177.41
	53.80	92.54	128.13	128.13	128.13
		83.02	103.14	103.14	103.14
				59.96	
				48.27	
				34.86	
				28.06	
				25.18	
				18.18	

4.16 Thermal Stability Analysis of Pullulanase

The thermal stability analysis was performed for the dialyzed fraction containing crude pullulanase. Fig. 38 provides graphical view to thermal stability of cultures at varying temperatures. It was found that dialysate fraction from GUPM2 and GUPM5 were stable up to 50°C for pullulanase activity, while GUPM22 showed stability up to 60°C. However, significant reduction in enzyme activity was observed in all the samples with increasing temperatures.

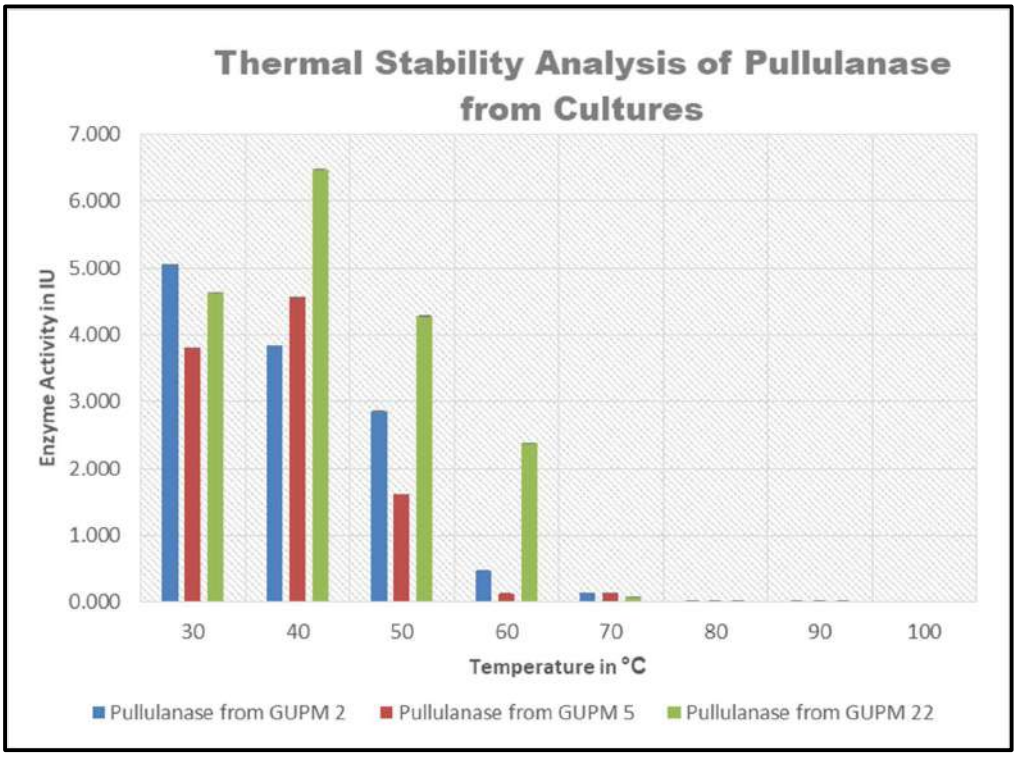


Fig. 38: Thermal Stability Analysis of Pullulanase Produced from Cultures in Respective Hydrolysates

REFERENCES

“The most important function of a bibliographic entry is to help the reader obtain a copy of the cited work.”

-Dariel J. Bernstein

5. References

1. Abdullah Rasyid, Ardi Ardiansyah and Ratih Pangestuti. (2019). Nutrient Composition of Dried Seaweed *Gracilaria gracilis. ILMU KELAUTAN: Indonesian Journal of Marine Sciences*. Volume 24(1):1-6. ISSN: 0853-7291.
2. Albert Pessarrodona, Jorge Assis, Karen Filbee-Dexter, Michael T. Burrows, Jean-Pierre Gattuso, Carlos M. Duarte, Dorte Krause-Jensen, Pippa J. Moore, Dan A. Smale, And Thomas Wernberg. (2022). Global Seaweed Productivity. *Science Advances*. Volume 8(37). DOI:10.1126/sciadv.abn2465
3. Ana-Marija Cikoš, Drago Šubarić, Marin Roje, Jurislav Babić, Igor Jerković, and Stela Jokić. (2022). Recent Advances on Macroalgal Pigments and Their Biological Activities. *Algal Research*. Volume 65. DOI: https://doi.org/10.1016/j.algal.2022.102748
4. Annette Bruhn, Gunhild Brynning, Aase Johansen, Mads Sarauw Lindegaard, Helle Hagen Sveigaard, Betina Aarup, Lene Fonager, Lene Lykke Andersen, Michael Bo Rasmussen, Martin Mørk Larsen, Dieter Elsser-Gravesen and Margrethe Elise Børsting. (2019). Fermentation of sugar kelp (*Saccharina latissima*)—effects on sensory properties, and content of minerals and metals. *Journal of Applied Phycology*. Volume 31, pages 3175–3187. DOI: https://doi.org/10.1007/s10811-019-01827-4
5. Asmaa Maghawri, Samar S. Marzouk, Heba M. Ezz El-Din and Mai Nashaat. (2023). Effect of brown algae *Padina pavonica* as a dietary supplement on growth performance and health status of cultured *Oreochromis niloticus*. *Egyptian Journal of Aquatic Research*. Volume 49 (3), Pages 379-385. DOI: 10.1016/j.ejar.2023.03.001

6. Aygan, Ashabil and Arikan, Burhan. (2007). An Overview on Bacterial Motility Detection. *International Journal of Agriculture and Biology*. Volume 9, Pages 193-196.
7. Aytul Hamzalioglu and Vural GökmenVural. (2016). Role of bioactive carbonyl compounds on the conversion of asparagine into acrylamide during heating. *European Food Research and Technology*. Volume 235 (06). DOI: 10.1007/s00217-012-1839-z.
8. Bleakley, S., and Hayes, M. (2017). Algal Proteins: Extraction, Application, and Challenges Concerning Production. *Foods (Basel, Switzerland)*, Volume 6(5), 33. DOI: https://doi.org/10.3390/foods6050033
9. Bonilla Loaiza AM, Rodríguez-Jasso RM, Belmares R, López-Badillo CM, Araújo RG, Aguilar CN, Chávez ML ,and Aguilar MA, Ruiz HA. (2022). Fungal Proteins from *Sargassum* spp. using Solid-State Fermentation as a Green Bioprocess Strategy. *Molecules*. Volume 27 (12): 3887. DOI: https://doi.org/10.3390/molecules27123887
10. Breijyeh, Z., Jubeh, B., and Karaman, R. (2020). Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. *Molecules (Basel, Switzerland),* Volume 25(6), 1340. DOI: https://doi.org/10.3390/molecules25061340
11. Catarino MD, Silva AMS, and Cardoso SM. Phycochemical Constituents and Biological Activities of *Fucus* spp. (2018). *Marine Drugs*. Volume 16(8):249. DOI: https://doi.org/10.3390/md16080249. PMID: 30060505; PMCID: PMC6117670.

12. Christopher R.K. Glasson, Ian M. Sims, Susan M. Carnachan, Rocky de Nys, and Marie Magnusson. (2017). A Cascading Biorefinery Process Targeting Sulfated Polysaccharides (Ulvan) from *Ulva Ohnoi. Algal Research*, Volume: 27, page: 383-391, DOI: https://doi.org/10.1016/j.algal.2017.07.001

13. Chye, F.Y., Ooi, P.W., Ng, S.Y., and Sulaiman, M.R. (2017). Fermentation-Derived Bioactive Components from Seaweeds: Functional Properties and Potential Applications. *Journal of Aquatic Food Product Technology*, Volume 27(2), Pages: 144 - 164. DOI: 10.1080/10498850.2017.1412375.

14. David T. Plummer (1990). An Introduction to Practical Biochemistry, 179, IIIrd Edition. McGraw-Hill, London, ©1987

15. Dhargalkar, V.K and Kavelekar D. (2004) Seaweeds: A Field Manual. National Institute of Oceanography, Dona Paula, Goa.

16. Dussault, H P. (1995). An improved technique for staining red halophilic bacteria. *Journal of bacteriology*. Volume. 70,(4), Pages: 484-485. DOI::10.1128/jb.70.4.484-485.1955

17. El-Beltagi, H. S., Mohamed, A. A., Mohamed, H. I., Ramadan, K. M. A., Barqawi, A. A., and Mansour, A. T. (2022). Phytochemical and Potential Properties of Seaweeds and Their Recent Applications: A Review. *Marine drugs*, Volume 20(6), 342. DOI: https://doi.org/10.3390/md20060342

18. Ferreira, M., Salgado, J. M., Fernandes, H., Peres, H., and Belo, I. (2022). Potential of Red, Green and Brown Seaweeds as Substrates for Solid State Fermentation to Increase Their Nutritional Value and to Produce Enzymes. *Foods (Basel, Switzerland)*, Volume: 11(23), 3864. DOI: https://doi.org/10.3390/foods11233864

19. Fontanesi, F., Soto, I. C., and Barrientos, A. (2008). Cytochrome c oxidase biogenesis: new levels of regulation. *IUBMB life*, Volume 60(9), Pages: 557–568. DOI: https://doi.org/10.1002/iub.86 PMID: 18465791; PMCID: PMC2630494.

20. G-biosciences. 'Assay for protein quantification' Catalog BE402 1-800-628-7730.

21. Gomes L, Monteiro P, Cotas J, Gonçalves AMM, Fernandes C, Gonçalves T, and Pereira L. (2022). Seaweeds' pigments and phenolic compounds with antimicrobial potential. *Biomolecular Concepts*. Volume: 13(1), Pages: 89-102.DOI: 10.1515/bmc-2022-0003. PMID: 35247041.

22. Gomez-Zavaglia, A., Prieto Lage, M. A., Jimenez-Lopez, C., Mejuto, J. C., and Simal-Gandara, J. (2019). The Potential of Seaweeds as a Source of Functional Ingredients of Prebiotic and Antioxidant Value. *Antioxidants (Basel, Switzerland)*, Volume: 8(9), 406. DOI: https://doi.org/10.3390/antiox8090406

23. José Alberto Herrera Barragán, Giuseppe Olivieri, Iulian Boboescu, Michel Eppink, Rene Wijffels and Antoinette Kazbar. (2022). Enzyme assisted extraction for seaweed multiproduct biorefinery: A techno-economic analysis. *Frontiers in Marine Science.* Volume 9, DOI: https://doi.org/10.3389/fmars.2022.948086

24. Kaliaperumal, N. (2003). Products from Seaweeds. *SDMRI Res Publ. 3*.

25. Karen Reiner (2012) 'carbohydrate utilization test'. American Society of Microbiology

26. Kazuo Miyashita, Nana Mikami and Masashi Hosokawa. (2013). Chemical and nutritional characteristics of brown seaweed lipids: A review. *Journal of Functional Foods*. Volume 5 (4), Pages: 1507-1517. DOI: https://doi.org/10.1016/j.jff.2013.09.019.

27. Kevin Hardouin, Gilles Bedoux, Anne-Sophie Burlot, Pi Nyvall-Collén, and Nathalie Bourgougnon.(2014). Enzymatic Recovery of Metabolites from Seaweeds: Potential Applications. *Advances in Botanical Research*, Volume 71, Pages 279-320. DOI: https://doi.org/10.1016/B978-0-12-408062-1.00010-X.

28. Khouloud M. Barakat, Mona M. Ismail, Hala E. Abou El Hassayeb, Nermeen A. El Sersy and Mostafa E. Elshobary. (2022). Chemical characterization and biological activities of ulvan extracted from *Ulva fasciata* (Chlorophyta). *Rendiconti Lincei. Scienze Fisiche e Naturali.* Volume 33, pages 829–841. DOI: https://doi.org/10.1007/s12210-022-01103-7

29. Kim S, Choi SK, Van S, Kim ST, Kang YH, and Park SR. (2022). Geographic Differentiation of Morphological Characteristics in the Brown Seaweed *Sargassum thunbergii* along the Korean Coast: A Response to Local Environmental Conditions. *Journal of Marine Science and Engineering*. Volume 10(4):549. DOI: https://doi.org/10.3390/jmse10040549

30. Krishnamurthi, V. R., Niyonshuti, I. I., Chen, J., and Wang, Y. (2021). A new analysis method for evaluating bacterial growth with microplate readers. *PloS one*, Volume: 16(1), DOI: https://doi.org/10.1371/journal.pone.0245205

31. Kumar, V., Naik, B., Choudhary, M., Kumar, A., and Khanduri, N. (2022). Agro-waste as a substrate for the production of pullulanase by *Penicillium viridicatum* under solid-state fermentation. *Scientific Reports*, Volume: 12(1), Pages: 1-9. DOI: https://doi.org/10.1038/s41598-022-16854-4

32. Kumar, Y., Tarafdar, A., and Badgujar, P. C. (2021). Seaweed as a source of natural antioxidants: therapeutic activity and food applications. *Journal of Food Quality*, Pages: 1–17.DOI: https://doi.org/10.1155/2021/5753391

33. Lal, Archana and Cheeptham, Naowarat. (2012). ASM ATLAS Protocol: Starch Agar.

34. Landeta-Salgado, C., Cicatiello, P., Stanzione, I., Medina, D. J. I., Mora, I. B., Gómez, C. A., and Lienqueo, M. E. (2021). The growth of marine fungi on seaweed polysaccharides produces cerato-platanin and hydrophobin self-assembling proteins. *Microbiological Research*, Volume: 251, DOI: https://doi.org/10.1016/j.micres.2021.126835

35. Liliana Krotz and Guido Giazzi. Thermo Fisher Scientific, Milan Italy. Elemental Analysis: CHNS/O characterization of polymers and plastics. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-42230-oea-chnso-polymers-plastics-an42230-en.pdf

36. Lomartire, S., and Gonçalves, A. M. M. (2022). An Overview of Potential Seaweed-Derived Bioactive Compounds for Pharmaceutical Applications. *Marine drugs*, Volume: 20(2), 141. DOI: https://doi.org/10.3390/md20020141

37. Lorbeer, A. J., Charoensiddhi, S., Lahnstein, J., Lars, C., Franco, C. M. M., Bulone, V., and Zhang, W. (2016). Sequential extraction and characterization of fucoidans and alginates from *Ecklonia radiata*, *Macrocystis pyrifera, Durvillaea potatorum*, and *Seirococcus axillaris*. *Journal of Applied Phycology*, Volume: 29(3), Pages: 1515–1526. DOI: https://doi.org/10.1007/s10811-016-0990-5

38. M.A. Islam, F. Parveen, K. Hossain, S. Khatun, Md. R. Karim, G.S. Kim, N. Absar and Md. S. Haque. (2009). Purification and Biochemical Characterization of Lipase from the Dorsal Part of Cirrhinus Reba. Thai Journal of Agricultural Science. Volume 42 (2), Page: 71-80.

39. Maiorano, G., Ramires, F. A., Durante, M., Palamà, I. E., Blando, F., De Rinaldis, G., Perbellini, E., Patruno, V., Gadaleta Caldarola, C., Vitucci, S., Mita, G., and Bleve, G. (2021). The Controlled Semi-Solid Fermentation of Seaweeds as a Strategy for Their Stabilization and New Food Applications. *Foods,* Volume: 11(18), 2811. DOI: https://doi.org/10.3390/foods11182811

40. Manikandan, P, Gnanasekaran A and Senthilkumar P.K. (2018). Screening and characterization of protease producing halophilic bacteria from saltpan area vedaranyam, Tamil Nadu. *International Journal of Pharmacy and Biological Sciences*. ISSN: 2321-3272.

41. Maryam Kokabi, Morteza Yousefzadi, Marzieh Razaghi and Mohamad Amin Feghhi. (2016). Zonation patterns, composition and diversity of macroalgal communities in the eastern coasts of Qeshm Island, Persian Gulf, Iran. *Marine Biodiversity Records*. DOI: 10.1186/s41200-016-0096-4

42. Mattingly, A. E., Weaver, A. A., Dimkovikj, A., and Shrout, J. D. (2018). Assessing Travel Conditions: Environmental and Host Influences On Bacterial Surface Motility. *Journal of bacteriology*, Volume: 200(11), DOI: https://doi.org/10.1128/JB.00014-18 , PMID: 29555698; PMCID: PMC5952383.

43. McHugh, Dennis J. (2003). A Guide to the Seaweed Industry. Italy, Food and Agriculture Organization of the United Nations.

44. Md. Shirajul Islam Sarkar, Md. Kamal, Mehedi Hasan, Md. Ismail Hossain, Fatema Hoque Shikha, and Md. Golam Rasul (2017). Manufacture of different value-added seaweed products and their acceptance to consumers. *Asian Journal of Medical and Biological Research* Volume: 2, Page: 639-645. DOI: 10.3329/ajmbr.v2i4.31009

45. Mensi, F., Ben Ghedifa, A., and Rajhi, H. (2022). Effects of seawater sulfur starvation and enrichment on *Gracilaria gracilis* growth and biochemical composition. *Scientific Reports*, Volume: 12(1), Pages: 1-12. DOI: https://doi.org/10.1038/s41598-022-15303-6

46. Monteiro P, Lomartire S, Cotas J, Pacheco D, Marques JC, Pereira L, and Gonçalves AMM. (2021). Seaweeds as a Fermentation Substrate: A Challenge for the Food Processing Industry. *Processes.* Volume: 9(11):1953. DOI: https://doi.org/10.3390/pr9111953

47. Mrigya Bansal, Amrita Poonia, Sujohn R. Paulson Kolluri, and Vasundhara. (2023). Introduction on Bioactive Compounds, Sources and their Potential Applications Springer Publishers. DOI: 10.1007/978-981-19-2366-1

48. Naik, B., Kumar, V., Goyal, S., Tripathi, A. D., Mishra, S., Saris, P. E. J., Kumar, A., Rizwanuddin, S., Kumar, V., and Rustagi, S. (2023). Pullulanase: unleashing the power of enzyme with a promising future in the food industry. *Frontiers in Bioengineering and Biotechnology,* Volume: 11. DOI: https://doi.org/10.3389/fbioe.2023.1139611

49. O' Brien, R., Hayes, M., Sheldrake, G., Tiwari, B., and Walsh, P. (2022). Macroalgal Proteins: A Review. *Foods (Basel, Switzerland),* Volume: 11(4), 571. DOI: https://doi.org/10.3390/foods11040571

50. Osaka Protocols (2010), 'CMC-plates with Congo red assay'

51. Pati, M. P., S. D. Sharma, L. Nayak, and C. R. Panda. (2016). Uses Of Seaweed And Its Application To Human Welfare: A Review. *International Journal of Pharmacy and Pharmaceutical Sciences,* Volume: 8 (10), Pages: 12-20, DOI: 10.22159/ijpps.2016v8i10.12740.

52. Pechsiri, J. S., Thomas, J., Risén, E., Ribeiro, M. S., Malmström, M., Nylund, G. M., Jansson, A., Welander, U., Pavia, H., and Gröndahl, F. (2016). Energy performance and greenhouse gas emissions of kelp cultivation for biogas and fertilizer recovery in Sweden. *Science of the Total Environment*, Volume: 573, Pages: 347–355. DOI: https://doi.org/10.1016/j.scitotenv.2016.07.220

53. Peñalver R, Lorenzo JM, Ros G, Amarowicz R, Pateiro M, and Nieto G. (2020). Seaweeds as a Functional Ingredient for a Healthy Diet. *Marine Drugs*, Volume: 18(6):301. DOI: https://doi.org/10.3390/md18060301

54. Peñuela A, Robledo D, Bourgougnon N, Bedoux G, Hernández-Núñez E, and Freile-Pelegrín Y. (2018). Environmentally Friendly Valorization of *Solieria filiformis* (Gigartinales, Rhodophyta) from IMTA Using a Biorefinery Concept. *Marine Drugs*, Volume: 16(12):487. DOI: https://doi.org/10.3390/md16120487

55. Pereira, Leonel, and Alan T Critchley. (2020). The COVID 19 novel coronavirus pandemic 2020: seaweeds to the rescue? Why does substantial, supporting research about the antiviral properties of seaweed polysaccharides seem to go unrecognized by the pharmaceutical community in these desperate times?.*Journal of applied phycology* Volume: 32,3 Pages: 1875-1877, DOI:10.1007/s10811-020-02143-y

56. Pfeifer, K., Ergal, İ., Koller, M., Basen, M., Schuster, B., and Rittmann, S. K. M. (2021). Archaea Biotechnology. *Biotechnology Advances*, Volume: 47, 107668. DOI: https://doi.org/10.1016/j.biotechadv.2020.107668

57. Prabhu, N., Maheswari, R., Singh, M. V. P., Karunakaran, S., Kaliappan, C., and Gajendran, T. (2018). Production and purification of extracellular pullulanase by *Klebsilla aerogenes* NCIM 2239. *African Journal of Biotechnology*, Volume: 17(14), Pages: 486–494. DOI: https://doi.org/10.5897/ajb2017.15915

58. Radha, P. (2018). Proximate Analysis and Mineral Composition of Seaweeds of Manamelkudi Coast, Pudukkottai District, India. International .Journal of .Current Microbiology and Applied .Sciences. Volume:7(08), Pages:3121-3128. DOI: https://doi.org/10.20546/ijcmas.2018.708.333

59. Reiner, Karen. (2010). Catalase Test Protocol. American Society of Microbiology.

60. Robinson P. K. (2015). Enzymes: principles and biotechnological applications. *Essays in biochemistry*, Volume: 59, Pages: 1–41. DOI: https://doi.org/10.1042/bse0590001

61. Ruangpan, L., and Tendencia, E. A. (2004). Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment. *Tigbauan, Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center*. http://hdl.handle.net/10862/1615

62. Sahito, Abdul Razaque & Mahar, Rasool & Siddiqui, Zuhaib and Brohi, Khan. (2013). Estimating Calorific Values Of Lignocellulosic Biomass From Volatile And Fixed Solids. *International Journal of Biomass & Renewables*. Volume 2. DOI: 10.61762/ijbrvol2iss1art13847.

63. Salehi, B., Sharifi-Rad, J., Seca, A. M. L., Pinto, D. C. G. A., Michalak, I., Trincone, A., Mishra, A. P., Nigam, M., Zam, W., and Martins, N. (2019). Current Trends on Seaweeds: Looking at Chemical Composition, Phytopharmacology, and Cosmetic Applications. *Molecules (Basel, Switzerland)*, Volume: 24(22), 4182. DOI: https://doi.org/10.3390/molecules24224182

64. Shields, Patricia and Laura A. Cathcart. (2010) Oxidase Test Protocol. American Society of Microbiology.

65. Shrivastava, S. (2022). Industrial applications of glycoside hydrolases. www.academia.edu. https://www.academia.edu/78563262/Industrial_Applications_of_Glycoside_Hydrolases

66. Silva A, Soares C, Carpena M, Oliveira PG, Echave J, Chamorro F, Donn P, Mansour SS, Barroso MF, and Prieto MA. (2023) Assessment of Nutritional Profile of *Sargassum muticum* Alga from the Spanish Coastline. *Biology and Life Sciences Forum*. Volume: 26(1):94. DOI: https://doi.org/10.3390/Foods2023-15028

67. Singh, Aparna, and Anil Kumar Singh. (2018) Isolation, characterization and exploring biotechnological potential of halophilic archaea from salterns of western India. *3 Biotech*, Volume: 8,(1) DOI: 10.1007/s13205-017-1072-3

68. Singh, Ravindra Pal, and C R K Reddy. (2014) Seaweed-microbial interactions: key functions of seaweed-associated bacteria. *FEMS Microbiology Ecology* Volume: 88 (2), Page: 213-30. DOI:10.1111/1574-6941.12297

69. Smith, Ann C. and Marise A. Hussey. (2005). Gram Stain Protocols. American Society of Microbiology.

70. Starko, S., Mansfield, S. D., and Martone, P. T. (2018). Cell wall chemistry and tissue structure underlie shifts in material properties of a perennial kelp. *European Journal of Phycology*, Volume: 53(3), Pages: 307–317. DOI: https://doi.org/10.1080/09670262.2018.1449013

71. Susan Løvstad Holdt and Stefan Kraan (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology* Volume: 23(3), Page: 543-597. DOI: 10.1007/s10811-010-9632-5

72. Takashi Kuroiwa, Hiroyuki Shoda, Sosaku Ichikawa, Seigo Sato and Sukekuni Mukataka. (2005). Immobilization and stabilization of pullulanase from *Klebsiella pneumoniae* by a multipoint attachment method using activated agar gel supports. Process Biochemistry. Volume: 40(8), Pages: 2637-2642. DOI: 10.1016/j.procbio.2004.10.002.

73. Teodoro, A. J. (2019). Bioactive compounds of food: Their role in the prevention and treatment of diseases. *Oxidative Medicine and Cellular Longevity*, Pages: 1–4. DOI: https://doi.org/10.1155/2019/3765986

74. Tomasik, P., & Horton, D. (2012). Enzymatic conversions of starch. *Advances in carbohydrate chemistry and biochemistry*, Volume 68, Pages: 59–436. DOI: https://doi.org/10.1016/B978-0-12-396523-3.00001-4

75. Van Handel E. (1985). Rapid determination of total lipids in mosquitoes. *Journal of the American Mosquito Control Association*, Volume 1(3), Pages: 302–304.

76. Vanessa Gressler, Nair Sumie Yokoya, Mutue Toyota Fujii, Pio Colepicolo, Jorge Mancini Filho, Rosangela Pavan Torres, and Ernani Pinto. (2010) Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. *Food Chemistry*, Volume 120 (2). Pages 585-590. DOI: https://doi.org/10.1016/j.foodchem.2009.10.028.

77. Waleed, M.,Faiza, A. A. and Nizar, I. (2015). Production optimization of pullulanase enzyme produced by *Bacillus cereus* isolated from Syrian sources. *International Food Research Journal*. Volume: 22, Pages:1824-1830.

78. World Bank Group. (2023, August 16). New farmed seaweed markets could reach $11.8 billion by 2030. World Bank. https://www.worldbank.org/en/news/press-release/2023/08/16/new-farmed-seaweed-markets-could-reach-11-8-billion-by-2030

79. Yuliana Salosso, Siti Aisiah, Lumban Nauli Lumban Toruan, and Wesly Pasaribu. (2020) Nutrient Content, Active Compound and Antibacterial Activity of *Padina australis* against *Aeromonas hydropilla*.. *Pharmacognosy Journal* Volume: 12(4): Pages: 771-776 DOI: 10.5530/pj.2020.12.110

80. Zhang, Y., Nie, Y., Zhou, X., Bi, J., and Xu, Y. (2020). Enhancement of pullulanase production from recombinant *Bacillus subtilis* by optimization of feeding strategy and fermentation conditions. *AMB Express*, Volume 10(1), Pages: 1-9. DOI: https://doi.org/10.1186/s13568-020-0948-5

81. Zollmann, M., Robin, A., Prabhu, M., Polikovsky, M., Gillis, A., Greiserman, S., and Golberg, A. (2019). Green technology in green macroalgal biorefineries. *Phycologia,* Volume: 58(5), Pages: 516–534. DOI: https://doi.org/10.1080/00318884.2019.1640516

APPENDIX

“No data is clean, but most is useful”

-Dean Abbott

Appendix I: Instruments Used

No.	Instrument	Details
1.	Autoclave	Renuka Enterprise, 1075.2K23.03.50
2.	Autoclave	Equitron Autoclave SLEFA
3.	Centrifuge (Sample Size over 2ml and above 8000rpm)	Thermo Scientific SORVALL lynx 4000
4.	Centrifuge (Sample size over 2ml and upto 8000rpm)	Thermo Scientifics SORVALL S78R
5.	Centrifuge (Sample Size upto 2ml)	LAB-i-FUGE c series
6.	Compound Microscope	Lawrence & Mayo XSZ-N107T
7.	Fridge	CellFrost R
8.	Fridge	Haier HRF-3304P/2019
9.	Fumehood	TEL AFA1000
10.	Gel Rocker	Genei Gel Rocker-100
11.	Ice Crusher	Scotman MF-30
12.	Incubator Shaker (set at 30°C)	Remi CIS-24 plus
13.	Incubator Shaker (set at 37°C)	Rivotek-incubator shaker
14.	Incubator shaker (set at 45°C)	Hally Insturment Shaking Incubator
15.	Laminar Air Flow	MSet ModelNo. HFL 5472
16.	Light Table	Bromma LKB-2017 MacroRead Light table
17.	Magnetic Stirrer	Remi Equipments 5MLH
18.	Muffle Furnace	Pathak Electrical Works PEW-202 Serial No. 792
19.	Oven (over 50°C upto 110°C)	i-Therm AI 7981
20.	Oven (upto 50°C)	Kumar Industries oven 45X45X45
21.	pH meter	Konvio Neer Digital pH meter
22.	pH meter	Eutech instruments pH 700
23.	Power Supply for SDS-PAGE	Bio-Era BE/FPP/120g
24.	Refractometer	BRIX
25.	Room temperature Shaker	Bio-techniques India R5/01
26.	Spectrophotometer	Shimadzu UV-Vis Spec Mini 1240
27.	TDS, EC and water temperature meter	Konvio Neer Digital Meters
28.	Wash Spin	DMR Spinner DMR 50-50A
29.	Water Bath	LABQUEST Borosil WBC020
30.	Water Bath (for 100°C)	Bio-techniques India PP1 UniX96
31.	Weighing Balance	Weinsar Weigh Scales Limited PGB 3010
32.	Weighing Balance (up to 220gram)	Shimadzu Uni Bloc ATX224

Appendix II: Chemical List

No.	Chemical Name	Brand	Details
1	Acetic Acid	SRL	Ref:93602;Batch No. 4346447
2	Acetone	HiMedia	A5025-2.5L;LOT RM/042/17
3	Acrylamide	HiMedia	GRM305-500G; LOT: 0000371836
4	Agar Powder	SRL	SRL19661 Batch No.: 6862917
5	Ammonium chloride	HiMedia	RM 717-500G; LOT 00000039586
6	Ammonium oxalate	SRL	Ref:0149172;Batch No.: T-837950
7	Ammonium Persulphate	SRL	Ref: 28575; Batch No.: 8218792
8	Ammonium Sulphate	HiMedia	GRM127T-500G; LOT: 0000237398
9	Anthrone	HiMedia	RM314; 2-313
10	Beef Extract	SRL	Ref: 60856; Batch No.:6396609
11	Beta-mercaptoethanol	HiMedia	MB041-500ml; LOT: 0000116439
12	Bisacrylamide	HiMedia	MB005-100G; LOT: 0000153331
13	Bromophenol Blue	SRL	Ref: 11458; Batch No: 4300755
14	BSA	HiMedia	MB083-100G; LOT: 0000109898
15	Carboxy Methyl Cellulose	HiMedia	RM9354-500G; LOT 0000071000
16	Casein	HiMedia	RM087; LOT 4-2628
17	Chloroform	SRL	Ref: 84155; Batch No.: 5608686
18	Comassie Brilliant Blue	HiMedia	RM344-25G; LOT 0000063903
19	Congo Red Solution	HiMedia	GRM927-25G; LOT 0000416244
20	Copper Sulphate Pentahydrate	HiMedia	RM677; 6-032
21	Crystal Violet	HiMedia	LOT 000008673
22	Dimethyl-p-penylenediamine	HiMedia	RM6939-25G
23	Dipotassium phosphate Anhydrous	HiMedia	GRM168-500G; LOT 00000229664
24	Disodium hydrogen phosphate dihydrate	HiMedia	BB126-500G; LOT 0000116749
25	DNSA powder	HiMedia	GRM1582-25G; LOT 0000176655
26	Ethanol	Parisil	Parisil 2GC Gemini Assocaites
27	Ethylenediaminetetraacetic acid (EDTA)	SRL	Ref: 054959
28	Folin Ciocalteu's Reagent	SRL	Ref: 39520; Batch No.: 3267521
29	Galactose	HiMedia	RM101-25G; LOT 0000006191
30	Glacial Acetic Acid	SRL	Ref: 93602; Batch No.: 4346447
31	Glucose	HiMedia	GRM077-500G; LOT 0000375111
32	Glycerol	Merck	Batch No.: AI2 A52242
33	Glysine	SRL	Ref: 074933; Batch No.: T8341419
34	Hydrochloric Acid	HiMedia	ASG04-2.5L
35	Hydrogen Peroxide	MOLychem	Product Code:23570; MCMT-3662

36	Iodine Crystalline	HiMedia	GRM-1065-100G; LOT 000037892
37	Magnesium Sulphate heptahydrate	SRL	Ref:83890;Batch No.: 3512799
38	Maltose	HiMedia	LOT: 0000927953
39	Methanol	HiMedia	AS061-2.5L; LOT: MET/200601
40	N,N,N′,N′ - Tetramethylethylenediamine (TEMED)	SRL	Ref: 84666; Batch No.: 8230839
41	Ortho Phosphoric Acid	HiMedia	AS011-500ML; LOT: RM/007/21
42	Peptone	HiMedia	RM001-500G; LOT 0000357760
43	Phenol	HiMedia	MB079-100G; LOT 0000326687
44	Phenol Red	HiMedia	Ref 8-0259
45	Potassium Chloride	HiMedia	GRM 698-500G; LOT 0000384295
46	Potassium Dihydrogen Phosphate Anhydrous	HiMedia	RM3943-500G;LOT 000005030
47	Potassium Iodide	HiMedia	GRM10692-250G; LOT 0000360906
48	Potassium Sodium Tartarate	HiMedia	GRM1046-500G; LOT 0000345488
49	Pullulan	TCI	P0978, Pullulan 25Gm
50	Safranin	HiMedia	GRM1315-25G; LOT 0000275890
51	Skim Milk Powder	SRL	Ref: 28582; Batch No.: 9805743
52	Sodium Carbonate Anhydrous	HiMedia	RM851-500G; LOT: 0000141003
53	Sodium Chloride	SRL	Batch No.: 6893810
54	Sodium dihydrogen phosphate monohydrate	HiMedia	RM6382-500G; LOT 0000083283
55	Sodium Hydroxide	HiMedia	TC460-500G; LOT 0000389398
56	Sodium-Lauryl-Sulphate	SRL	Ref: 54468; Batch No: 1412837
57	Starch Soluble	SRL	Ref: 64698;Batch No.: 8340462
58	Sucrose	HiMedia	GKM3063-500G; LOT 0000393293
59	Sulphuric Acid	HiMedia	AS016-2.5L; LOT: RM/030/23
60	Tris-Cl	SRL	Ref: 1185-53-1; Batch No.: 1051537
61	Tri-Sodium Citrate	HiMedia	RM 3953-500G; LOT 0000128651
62	Tryptone	SRL	Ref: 32007; Batch No. 5651867
63	Vanillin	HiMedia	RM616-100G; LOT: 0000003444
64	Yeast Extract	HiMedia	M456-500G; LOT 0000051656

Appendix III: Chemical Preparations and Media Composition

1. **15% saline –** 15grams of NaCl was added to distilled water to make up the volume to 100ml. The solution is mixed and autoclaved.
2. **Halopiger Media**: The following components (except agar) are added to make the media and final volume is made up to 1 litre using distilled water. The pH is set to 7.2. The agar is added and media is autoclaved at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in g/L
1.	NaCl (Crude Salt)	250
2.	Potassium Chloride	2
3.	Magnesium Sulphate	20
4.	Tri-Sodium Citrate	3
5.	Yeast Extract	10
6.	Agar (For Solid Media Only)	20

3. **Phenol Red Carbohydrate Broth**: The following components are added to make the media and final volume is made up to one litre using distilled water. The pH is set to 7.2. The solution is mixed and autoclaved.

No.	Chemical	Concentration in g/L
1.	Peptone	10
2.	Sodium chloride (NaCl)	250
3.	Beef extract	1
4.	Phenol red 0.018 g	0.018
5.	Carbohydrate (Either Glucose/Galactose/ Maltose/Sucrose)	10

4. **5% Acetic Acid**: 5 ml of acetic acid was added to distilled water to make up the volume to 100ml.

5. **2% Crystal Violet**: 2g of crystal violet in 20 ml of 95% ethanol and mix with 80ml of 1% aqueous solution of ammonium oxalate. The solution was filtered using Watmann filter paper to remove any crystal.
6. **Gram's Iodine:** In a motor and pestle grind 1g iodine crystal with 2g potassium iodide. Constantly mix using small portions of distilled water. Make up the volume to 300ml using DW and store in amber coloured bottle.
7. **Gram's Decolourizer**: Mix acetone and absolute ethanol in 1:1 ratio.
8. **0.25% safranin:** 1.25g of safranin in 38ml of absolute ethanol and 2ml of distilled water to make up the volume to 40ml.
9. **Starch Agar for Assay**: The following components (except agar) are added to make the media and final volume is made up to one litre using distilled water. The pH is set to 7.5. The agar is added and media is autoclaved at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in g/L
1.	NaCl (Crude Salt)	250
2.	Beef Extract	3
3.	Soluble Starch	10
4.	Agar	12

10. **Casein Agar for Assay:** 250 grams of NaCl and 10 grams of casein is added to make final volume of 1L using distilled water. The pH is set to 7.4. Then 18 agar is added and media is autoclaved at 15 psi, 15 minutes at 121°C.
11. **1% Coomassie brilliant blue**: 0.25g of Coomassie Brilliant Blue dye was mixed in 45% methanol and 10% glacial acetic acid.
12. **Skim milk Agar for assay**: 250 grams of NaCl and 10 grams of skim milk powder is added to make final volume of 1L using distilled water. The pH is set to 7.4. Then 18 agar is added and media is autoclaved using cooker for 3 whistles, 3minutes.

13. **CMC Media for assay**: The Luria–Bertani borth was prepared to which 1% CMC powder was added and final volume is made up to one litre using distilled water. The pH is set to 7.0. The 2% agar is added and media is autoclaved at 15 psi, 15 minutes at 121°C.
14. **0.1% Congo red Solution:** 0.1 g of Congo red dye with 90 ml of distilled water. Then, add 10 ml of 95% ethanol was added and mixed.
15. **1M NaCl:** Weigh 5.84g of NaCl to make up the volume to 100ml distilled water.
16. **1% Gordon Reagent:** 1 ml of dimethyl-p-phenylenediamine dihydrochloride was added to distilled water to make up the volume to 100ml. Store in Amber coloured bottle.
17. **Pullulan Media:** Mix 250g of crude salt in 800ml of distilled water. Filter the solution using whatman filter paper. In the solution add the following components (except agar) are added to make the media and final volume is made up to 1 litre using distilled water. The pH is set to 7.5. The agar is added and media is autoclaved at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in g/L
1.	Pullulan	10
2	Magnesium Sulphate	0.1
3	Potassium Dihydrogen Phosphate	0.12
4	Dipotassium hydrogen phosphate	0.17
5	Ammonium chloride	0.2
6	Agar (For Solid Media Only)	15

18. **DNSA Reagent**: The reagent is prepared in batches by making two solutions:
    a) Solution A: Dissolve 2g DNSA powder in 40ml of 2N NaOH
    b) Solution B: Under constant stirring using magnetic stirrer, add 60g of potassium- sodium tartrate in 100ml DW.

    Mix Solution A and B and makeup the volume to 200ml using distilled water.

19. **Phosphate Buffer (1M):** Dissolve 95 g of potassium phosphate monobasic and 52.5 g of potassium phosphate dibasic to 800 mL of distilled water and make up the volume to 1litre. Adjust the pH to 7, autoclave and store in reagent bottle.
20. **Pullulan Buffer:** Dissolve 1 g of pullulan in 0.02 M potassium phosphate buffer.
21. **Anthrone Reagent:** 0.2g of Anthrone is added to 100ml of cooled concentrated sulphuric acid and stored in amber coloured bottle.
22. **Copper Reagent for Protein Estimation:** The reagent is prepared in batches by making two solutions:
    a) Solution A: 2% sodium carbonate in 0.1N sodium hydroxide.
    b) Solution B: 0.5% copper sulphate in 1% potassium sodium tartrate.

    The reagent is prepared freshly by mixing Solution A and B in the ratio 50:1.
23. **Vanillin Phosphoric Acid Reagent:** Dissolve 600mg of vanillin in 100ml of warm DW. Then, add 400ml of 85% ortho-phosphoric acid. Mix and store in amber coloured bottle.
24. **3% Starch Solution:** Dissolve 12.5 g of crude salt in 40ml of DW and filter the solution using whatman filter paper. Add the following components in the solution and make up the volume to 50ml using DW and then autoclaved at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in mg
1.	Starch	1500
2	Magnesium Sulphate	15
3	Potassium Dihydrogen Phosphate	6
4	Dipotassium hydrogen phosphate	0.85
5	Ammonium chloride	10

25. **3% Cellulose Solution**: Dissolve 12.5 g of crude salt in 40ml of DW and filter the solution using whatman filter paper. Add the following components in the solution and make up the volume to 50ml using DW and then autoclaved at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in mg
1.	Cellulose	1500
2	Magnesium Sulphate	15
3	Potassium Dihydrogen Phosphate	6
4	Dipotassium hydrogen phosphate	0.85
5	Ammonium chloride	10

26. **Phosphate Buffer Saline:** Mix the following mentioned ingridents in 800 ml of distilled water. Make up the volume to 1 L and adjust pH to 7.0. Autoclave at 15 psi, 15 minutes at 121°C.

No.	Chemical	Concentration in g/L
1.	NaCl	8.01
2.	Disodium-hydrogen phosphate	1.44
3.	Potassium Chloride	0.2
4.	Potassium dihydrogen phosphate	0.27

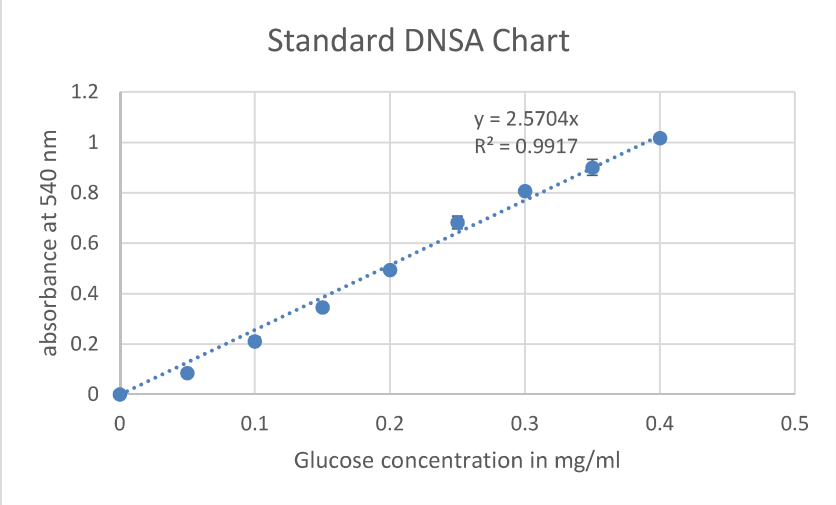
27. **Acrylamide-Bisacrylamide Stock Solution (30%):** Dissolve 29g of acrylamide and 1g of bisacrylamide in 100ml of DW. Mix and store in amber coloured bottle.
28. **Stacking Gel Buffer (pH 6.8):** Dissolve 12.14g of Tris-Cl in 100ml DW (1M Tris-Cl) and adjust the pH to 6.8.
29. **Resolving Gel Buffer (pH 8.8):** Dissolve 18.75g of Tris-Cl in 100ml DW (1.5M Tris-Cl) and adjust the pH to 8.8.
30. **10% Ammonium per sulphate (APS):** Dissolve 10g APS in 100ml DW.
31. **10% Sodium laurel sulphate (SLS):** Dissolve 10g SLS in 100ml DW.

32. **Running Buffer (1X):** Dissolve 30.3g of Tris-Cl, 14.4g glycine and 1g SLS in 1L DW and adjust pH to 8.3.
33. **10% Glycerol Stock:** Add 1ml of glycerol in 9ml of DW.
34. **Sample Loading Buffer (pH 6.8):** Take 1ml of stacking gel buffer. To this, add 280µL β-mercaptoethanol, 1g SLS, 0.1g bromophenol blue and 2ml of 10% glycerol stock. Make up the volume to 100ml using DW and adjust the pH to 6.8.
35. **Staining Solution**: Prepare a solution of 500ml by mixing 225ml methanol, 225ml DW and 50ml of Glacial Acetic Acid. Then mix 1.25g of Coomassie brilliant blue.
36. **Destaining Solution**: Prepare a solution of 500ml by mixing 225ml methanol, 225ml DW and 50ml of Glacial Acetic Acid.

Appendix IV: Standard Graphs

1. Estimation of Reducing Sugars Using DNSA method

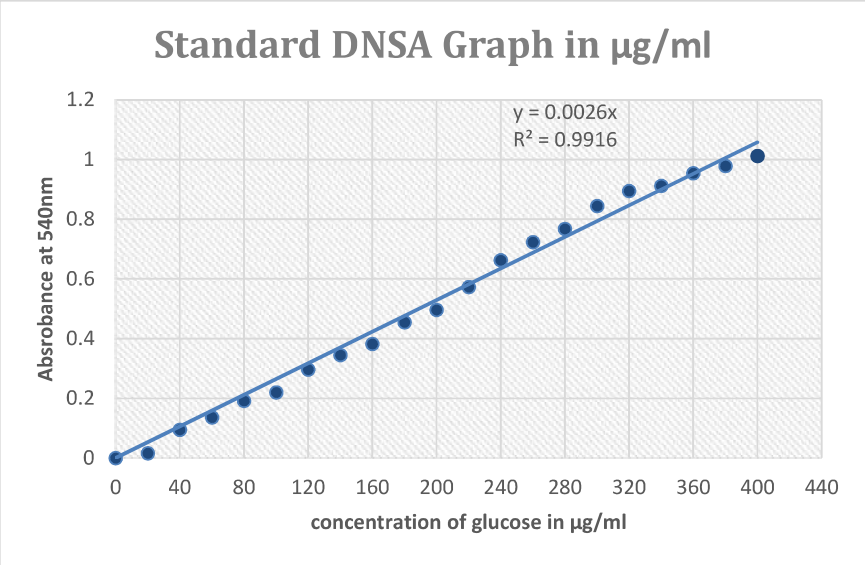
The DNSA standard graph was prepared using Glucose standard at 1mg/ml concentration.



Sr. No.	Glucose Concentration in mg/ml	Average Absorbance at 540nm	Standard Error
1	0	0.00	0.000
2	0.05	0.09	0.004
3	0.1	0.21	0.016
4	0.15	0.35	0.011
5	0.2	0.49	0.006
6	0.25	0.68	0.024
7	0.3	0.81	0.005
8	0.35	0.90	0.032
9	0.4	1.02	0.013

2. Estimation of Reducing Sugars Using DNSA Method

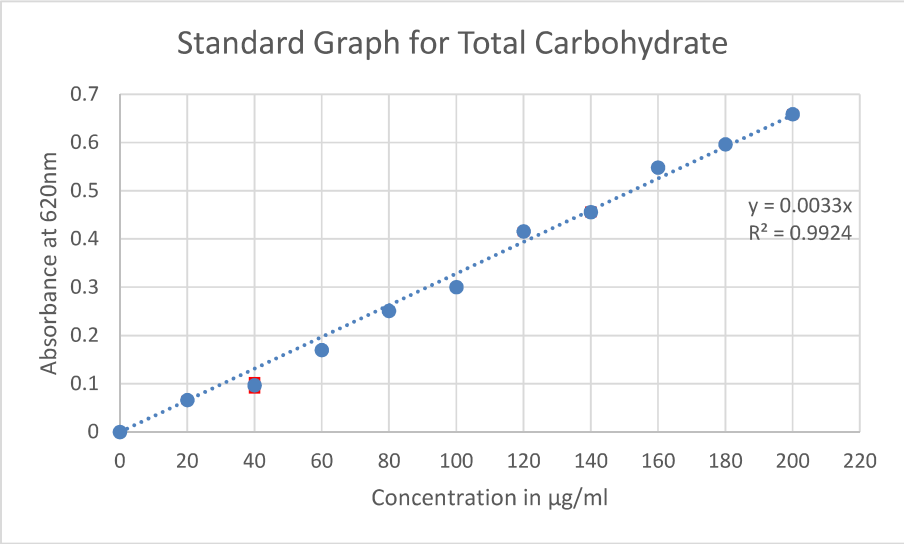
The DNSA standard graph was prepared using Glucose stock at concentration of 500µg/ml.



Sr. No.	Concentration of Glucose in µg/ml	Average Absorbance at 540nm	Standard Error
1	0	0	0.001
2	20	0.017	0.002
3	40	0.096	0.002
4	60	0.137	0.001
5	80	0.192	0.001
6	100	0.220	0.002
7	120	0.297	0.003
8	140	0.345	0.001
9	160	0.382	0.003
10	180	0.455	0.002
11	200	0.496	0.001
12	220	0.573	0.002
13	240	0.663	0.002
14	260	0.724	0.001
15	280	0.768	0.002
16	300	0.845	0.003
17	320	0.895	0.001
18	340	0.912	0.001
19	360	0.954	0.001
20	380	0.979	0.006
21	400	1.011	0.001

3. Estimation of Total Carbohydrate Using Anthrone Method

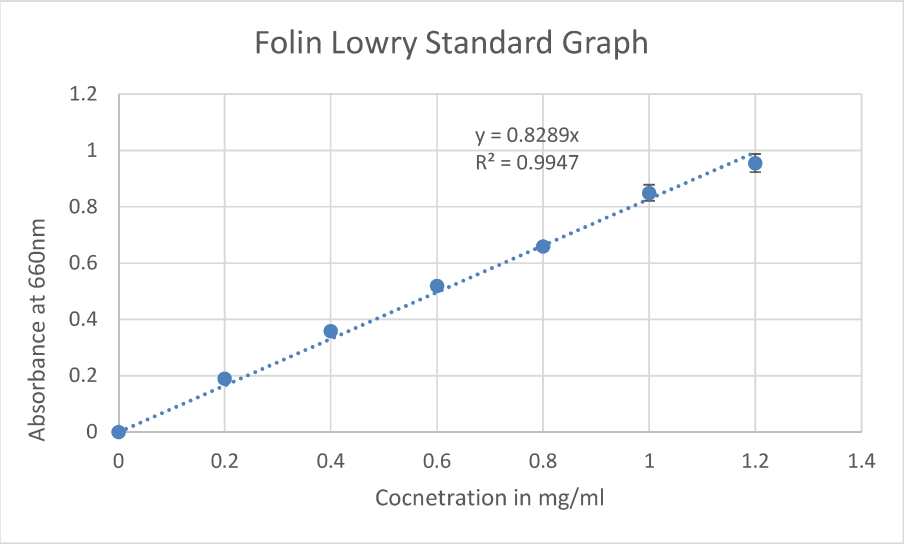
The total carbohydrate was measured using Anthrone Reagent and standard of glucose was prepared at 200µg/ml concentration.



Sr. No.	Concentration in µg/ml	Average Absorbance at 620nm	Standard Error
1	0	0.00	0.000
2	20	0.07	0.002
3	40	0.10	0.011
4	60	0.17	0.001
5	80	0.25	0.001
6	100	0.30	0.001
7	120	0.42	0.003
8	140	0.46	0.005
9	160	0.55	0.001
10	180	0.60	0.003
11	200	0.66	0.004

4. Folin & Ciocalteu's Method for Protein Estimation

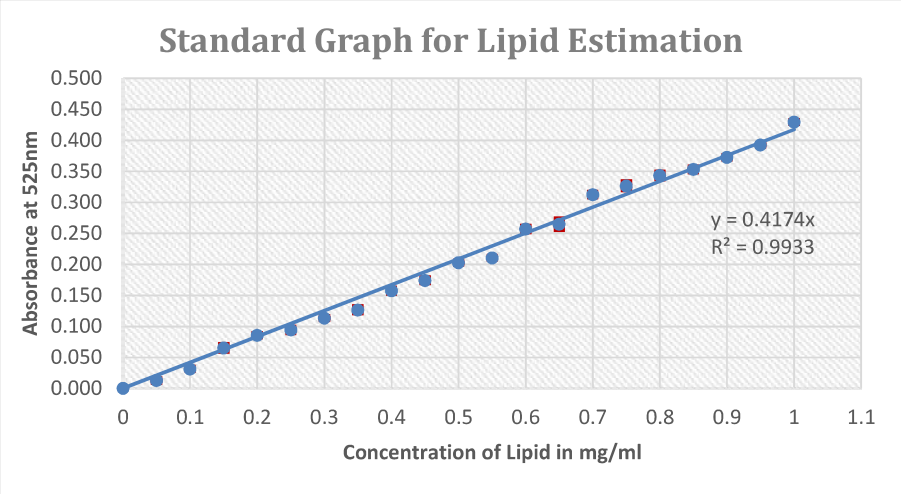
The protein estimation was performed using Folin Lowry method using Bovine Serum Albumin (BSA) in 2mg/ml concentration.



Sr. No	Concentration in mg/ml	Average Absorbance at 660nm	Standard Error
1	Blank	0.00	0.000
2	0.2	0.19	0.001
3	0.4	0.36	0.001
4	0.6	0.52	0.001
5	0.8	0.66	0.002
6	1.0	0.85	0.001
7	1.2	0.96	0.001

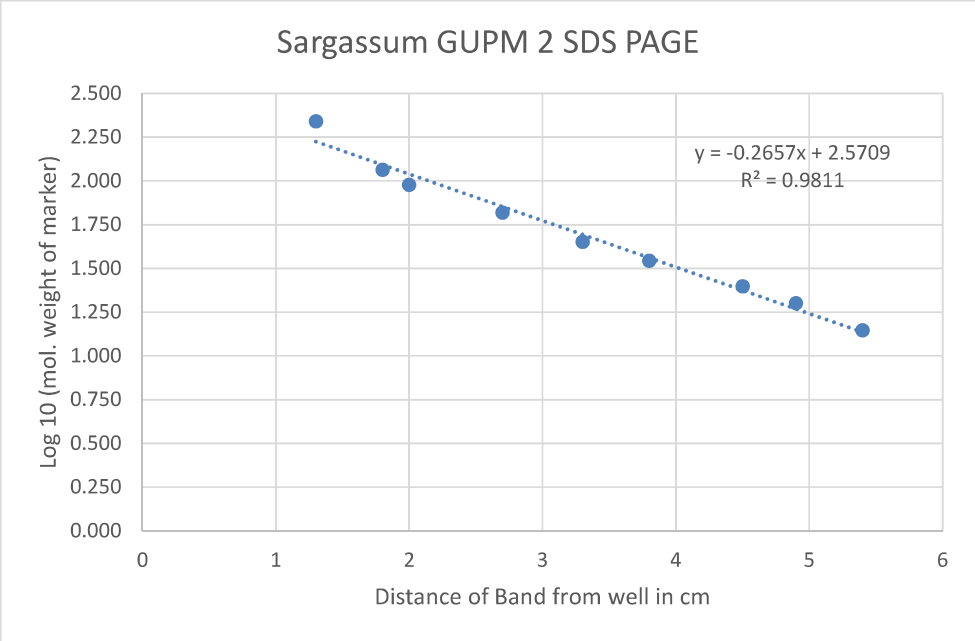
5. Vanillin Phosphoric Acid method for Lipid Estimation

The lipid estimation standard was prepared using olive oil at 1mg/ml concentration, by dissolving 100mg oil in 100ml chloroform.



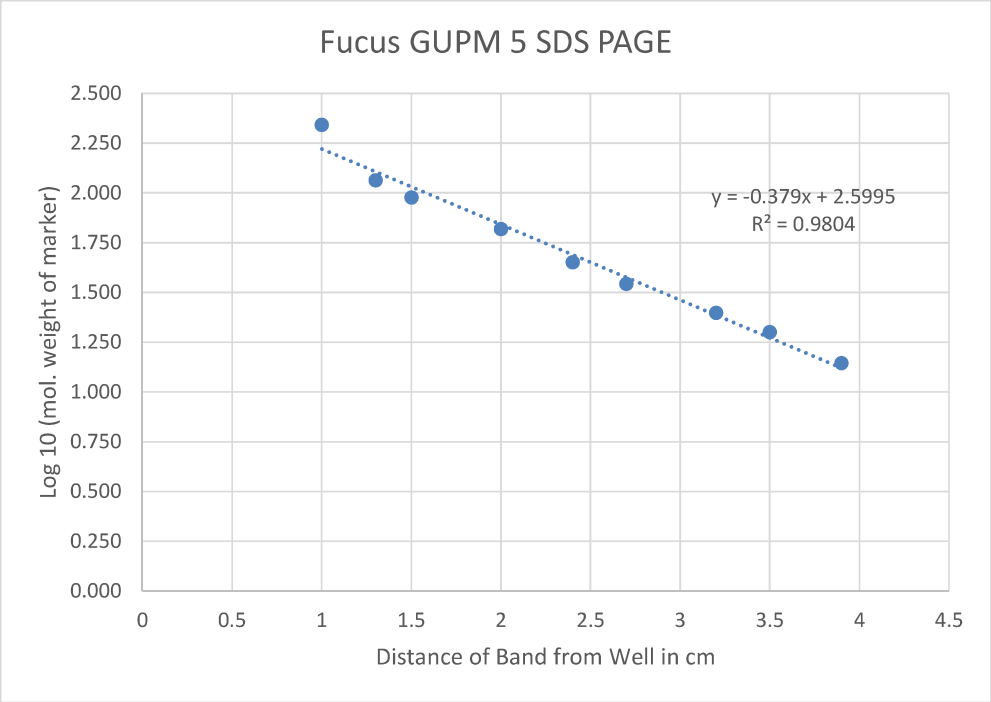
Sr. No.	Concentration in mg/ml	Average of Absorbance at 525nm	Standard Error
1	0	0.000	0.000
2	0.05	0.013	0.001
3	0.1	0.031	0.001
4	0.15	0.065	0.003
5	0.2	0.086	0.001
6	0.25	0.094	0.002
7	0.3	0.113	0.001
8	0.35	0.127	0.002
9	0.4	0.158	0.002
10	0.45	0.174	0.002
11	0.5	0.203	0.001
12	0.55	0.211	0.001
13	0.6	0.257	0.002
14	0.65	0.265	0.005
15	0.7	0.313	0.002
16	0.75	0.327	0.003
17	0.8	0.344	0.003
18	0.85	0.353	0.002
19	0.9	0.373	0.001
20	0.95	0.393	0.001
21	1	0.430	0.001

6. **Standard Graph for Marker Bands vs log_{10} of molecular weight for SDS PAGE Set I** (Containing Protein Samples of GUPM 2 and Acid hydrolysate and Sargassum)



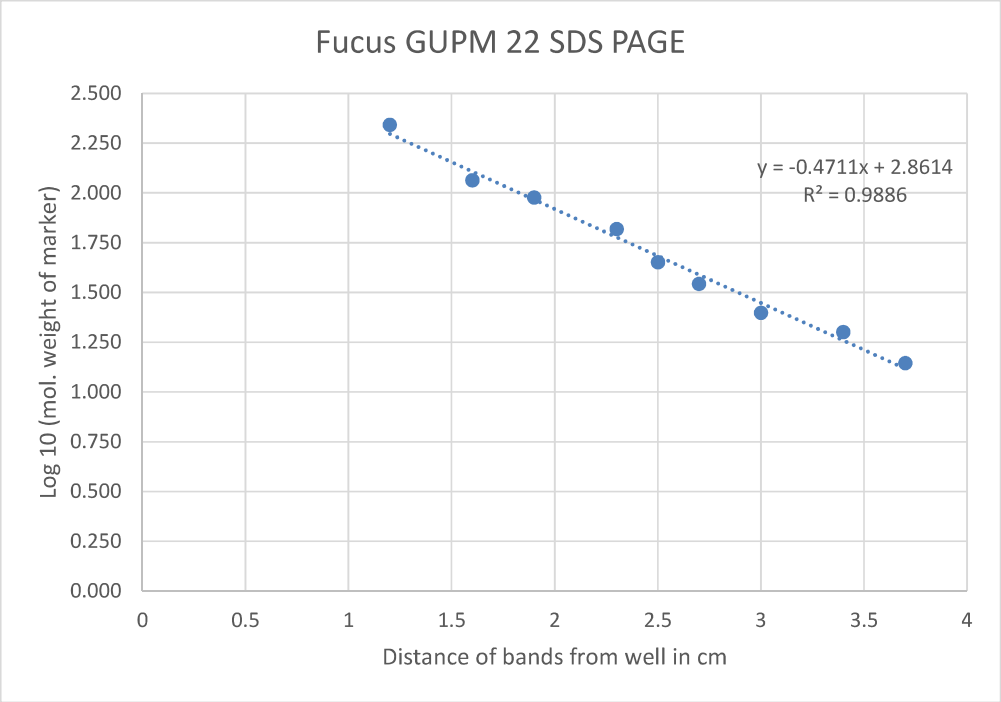
No.	Distance of Band from well in cm (x-axis)	Molecular Weight of band (kDa)	Log_{10} of Molecular Weight (y-axis)
1	1.3	220	2.342
2	1.8	116	2.064
3	2	95	1.978
4	2.7	66	1.820
5	3.3	45	1.653
6	3.8	35	1.544
7	4.5	25	1.398
8	4.9	20	1.301
9	5.4	14	1.146

7. **Standard Graph for Marker Bands vs $\log_{10}$ of molecular weight for SDS PAGE Set II** (Containing Protein Samples of GUPM 5 and Base hydrolysate and Fucus)



No.	Distance of Band from well in cm (x-axis)	Molecular Weight of band (kDa)	Log_{10} of Molecular Weight (y-axis)
1	1	220	2.342
2	1.3	116	2.064
3	1.5	95	1.978
4	2	66	1.820
5	2.4	45	1.653
6	2.7	35	1.544
7	3.2	25	1.398
8	3.5	20	1.301
9	3.9	14	1.146

8. **Standard Graph for Marker Bands vs log_{10} of molecular weight for SDS PAGE Set III** (Containing Protein Samples of GUPM 22 and Base hydrolysate and Fucus)



No.	Distance of Band from well in cm (x-axis)	Molecular Weight of band (kDa)	Log_{10} of Molecular Weight (y-axis)
1	1.2	220	2.342
2	1.6	116	2.064
3	1.9	95	1.978
4	2.3	66	1.820
5	2.5	45	1.653
6	2.7	35	1.544
7	3	25	1.398
8	3.4	20	1.301
9	3.7	14	1.146