Screening of seaweed associated bacteria for industrially important enzymes and its applications

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By

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Screening of seaweed associated bacteria for industrially important enzymes and its applications**" is based on the results of investigations carried out by me in the Department of Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Dr. Lata Gawade and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Date: 1/5/2023

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation "**Screening of seaweed associated bacteria for industrially important enzymes and its applications**" is a bonafide work carried out by **Ms. Prachi Ramesh Vishwakarma** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Microbiology at the School of Biological Sciences and Biotechnology, Goa University.

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AIMS AND OBJECTIVES

AIM:

To screen bacteria associated to the seaweeds for the industrially important enzymes (protease, cellulase, amylase, xylanase and agarase) and understand their potential in degradation of Dairy and seaweed waste**.**

OBJECTIVES:

Objectives of this study include:

- Collection and identification of different seaweed sample from intertidal region of the Goa coast.
- Isolation of seaweed associated bacteria
- Screening of the bacterial epiphytes for the production of amylase, xylanase, cellulase, agarase and protease.
- Degradation of seaweed waste using bacterial epiphytes
- Degradation of dairy waste using the bacterial isolates
- Identification of the bacterial isolates using biochemical tests
- Estimation of protein content in seaweeds.

INTRODUCTION

Introduction

The ocean harbors a great variety of marine organisms that vary in their adaptations and physiology. Both, macro- and micro- algae have vast diversity of sizes and forms and they constitute about 30,000 to 1 million different species or even more. Seaweeds are the marine macro algae that are primary photosynthetic non-flowering macrophytes (Suresh Kumar, 2018) which are mainly present on the coastline and represent a substantial part of the oceanic biomass (approximate 25,000–30,000 species) (Menaa et al., 2020).

They are evolutionary diverse and they do not represent one taxonomic entity in contrast to tracheophyte (vascular plants). Indeed, based on the molecular phylogeny and systematics, they may belong to the following four kingdoms: Plantae (e.g., rhodophytes and chlorophytes), Protozoa (e.g.-Protista), Chromista (e.g., phaeophytes—including diatoms and dinoflagellates), and Eubacteria (cyanophytes or blue- green algae). According to the International Code of Botanical Nomenclature (ICBN), the algae have been classified into 11 divisions, i.e., Euglenophycophyta (Euglenoids), Phaeophycophyta (brown algae) currently included in Ochrophyta (brown and golden-brown algae), Cryptophycophyta, Cyanophycophyta (blue-green algae), Charophyta (stoneworts), Bacillariophycophyta (diatoms), Chlorophycophyta (green algae), Pyrrophycophyta, Xanthophycophyta (yellow-green algae), Rhodophycophyta (red algae), Chrysophycophyta (golden algae). Depending upon the chemical composition like the type of pigments present, the seaweeds are divided into green algae, red algae and brown algae. As a result, pigments present in green seaweeds include chlorophyll a and b and xanthophylls, pigments present in red seaweeds include xanthophylls, allophycocyanin, phycoerythrin and chlorophyll a, and pigments present in brown seaweeds include chlorophyll a and c and fucoxanthin. (Menaa et al., 2020).

Recent attempts for long term tank cultivation of *Gracilaria cornea* using flue gases from power plant containing 12-15% CO_2 with addition of N and P, causing enhancement of algal growth that proves the ability of CO_2 bioremediation of seaweed cultures (Suárez-Álvarez et al., 2012). An important biomechanism in seaweeds to fix the carbon can be used to diminish the increment of atmospheric CO_2 and hence alleviate the trend toward global warming. These seaweeds are consumed by herbivores, whose feces sink and remain at the bottom for a while. Exudation as

dissolved organic matter could be of a critical loss. Hence, some of the seaweed carbon returns to the water column which can be either recaptured when photosynthesis takes place or eventually returned to the atmosphere. However, a significant part of the seaweed carbon can be sequestered for a long period of time, perhaps centuries on the sea floor depending on the location currents, etc (Mitra et al., 2014).

The understanding about the chemical content of the seaweeds is crucial for both the assessment of the nutritional importance to marine vertebrate or invertebrate herbivores, for the carbohydrate, protein and lipid evaluation for its application in commercial or for consumption by humans. In many parts of the world the seaweeds are used as fertilizer and food source. Hence the study of seaweeds chemical composition is very essential to understand their nutritive importance (Parthiban C et al., 2013). Due to high nutritional content, the seaweeds are used as feed and food by the coastal populations. It is crucial to bring up that the chemical content of the seaweed vary based on the environmental factors such as ocean acidification, geographical habitat, salinity, light intensity, water temperature, seasonal variation and nutrient concentration (Mitra et al., 2014).

Differences in the hydrodynamic forces have significant effect on the distribution of macroalgae, epiphytic fauna, algae–microbiota interactions, abundance and algal diversity. The macroalgal beds that are submerged boost the biodiversity, supporting food webs by supplying, food, habitats, reproductive refugia to variety of organisms like seabirds, invertebrates, mammals, fish, invertebrates (Menaa et al., 2020). The seaweed surface is a suitable for the multiplication of epiphytic bacteria and the development of microbial biofilms by providing appropriate substratum for the microbes to settle and also produces diverse organic substances that act as nutrients for their growth.(Singh & Reddy, 2014). The marine ecosystems are depended on the various types of interactions with the organisms that may be parasitic, competitive, mutualistic, symbiotic or parasitic. The surfaces of the seaweeds are covered by an organic layer, because of adsorption of inorganic and organic molecules, therefore allowing them to maintain a great diversity of the associated microorganisms like bacteria, diatoms, fungi and other single celled organisms, larvae and spores of invertebrates present in the marine habitat. (Menaa et al., 2020) and among these microbial communities, bacteria are the most extensive and can be present either on the seaweed surface or in the cell cytosol (Singh & Reddy, 2014).

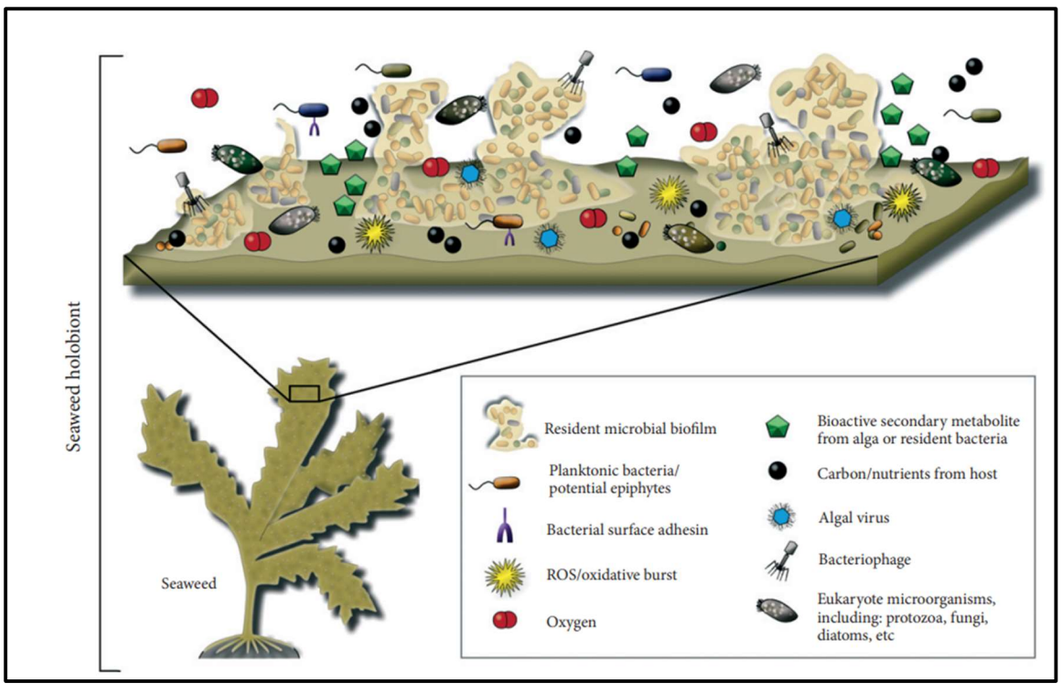


Figure 1: The seaweed holobiont and the components predicted to affect bacterial colonization on seaweed hosts(Egan et al., 2013)

Algae or seaweeds are used to produce biofuels biomass oil or biodiesel, thereby creating a new technology for generation of bio-energy for biodiesel industry this will help in creating stable, healthy, new and sustainable development for bio-fuel industry. However, a large amount of agal waste is generated due to the production of bio-fuel. This waste is consisting of organic matter and nutrients hence if discharged in water bodies it way led to red tide and eutrophication. Hence treatment of seaweed is important step before its discharge in the environment (Zhao & Zhang, 2018). In this study, microorganisms producing enzymes amylase, agarase and protease are used in the degradation of seaweed waste that is generated.

Huge quantity of proteinaceous wastes is generated annually that could be estimated in tons. They are produced from the industries worldwide as residues. However, the cost of waste treatment or disposal have increased. Because of the legislation that is being imposed, and also because of its cost effectiveness, other technologies like use of microorganisms have become more focused for the waste treatment (Sharma, 2010; Gareth et al. 2003). The dairy processing industries produce whey containing proteaceous waste with 12.1 -17.9% protein content. Proteases are the class of enzymes that catalyse the hydrolysis of peptide bonds in the polypeptide chains. Microorganism, commonly bacteria and fungi are most common source of these enzymes. The biotreatment method

using microorganisms was constructed to understand the potential of proteolytic bacteria to carry out hydrolysis of proteinaceous waste produced by the dairy industry. (Sharma, 2010).

Industrially important enzymes from bacteria

Enzymes are commonly referred as “biocatalysts”. They are biomolecules produced by all living beings. The recent shift from using of hazardous chemicals to using green chemistry methods in the industrial sector has led to an increase in demand for enzymes (Ejaz et al., 2021).

Industrially important enzymes are those enzymes that are used to facilitate various industrial processes and product production. Enzymes have found application in various industries, these includes food, textile, paper, detergent, leather chemical and animal-feed industries. The new and emerging application are resulting in an increasing demand for industrial enzymes. Currently the most used industrial enzymes are hydrolases, including proteases and lipases, which are the most dominant enzyme type and are highly used in the dairy, detergent and chemical industries. The second largest group includes carbohydrases, essentially amylases and cellulases (Zhu et al., 2011).

The first large scale application of enzymes obtained from microbes namely, proteinases was seen in detergent industry. Lipases were introduced in late 1980s to hydrolyse fats into glycerol and fatty acids. Cellulases were used for the removal of cotton-based cellulose and amylases were used to remove starch-based stains. The textile industry is extending its interests on enzymology and other industries are using hydrolytic enzymes for product manufacture (Williams, 2005).

Due to their biochemical and physiological properties, ease of cell manipulation and simplistic culture conditions, the most common commercial enzymes sources are microorganisms (Rodarte et al., 2011).

The macroalgal cell walls are rich carbon constituents, this is one of the factors that is likely to be significant for colonization of bacteria. The cell wall components of the macroalgae may act as a nutrient rich source for the bacteria that are capable of utilizing these biopolymers (Egan et al., 2013).

The cell wall of red algae is primarily composed of agar, which serves a source of energy and carbon to microbes (Khalifa & Aldayel, 2019). The cell wall microfibrils of the order Bryopsidales s consist of either 1,4-β-D-mannans or 1,3-β-D-xylans, with complete absence of cellulose. The 1,3;1,4-β-D-xylans and 1,3-β-D-xylans are two main types of xylan reported in red algae(Hsieh & Harris, 2019). The members of family Chlorophyceae have wide range of cellulose-pectin complexes walls (Baghel et al., 2021). In various species of seaweeds srach is the storage polysaccharide like in Cladophra it is present in chloroplast, and it is present in the cytoplasmic granules of some red algae (Prabhu et al., 2019). Goecke et al. (2010) has given an overview of specific enzymatic activities that has been detected in the relevant marine bacteria that degrade macroalgal cell walls (Egan et al., 2013). Although, bacterial degradation of tissue of macroalgae is detrimental to the host, it is very essential for the global carbon and nutrient cycling (Egan et al., 2013)..

Some of the enzymes that are produced by the seaweed associated bacteria are:

- **Amylase**

One of the most famous industrial enzymes are amylases. α-Amylases play an important role in carbohydrate metabolism. It has great application in many industrial processes like paper, fermentation, pharmaceutical, food, textile and detergent industries. Amylases are the enzymes that breakdown starch into sugars (Zhu et al., 2011).

Starch is a polysaccharide consisting of glucose unit linked to another one via a glycosidic linkage. There are two types of polymers of glucose present in starch namely amylose and amylopectin. The two polymers differ in their structures and properties. Amylose consists of about 6000 units of glucose linked through α-1,4 glycosidic bonds and it is a linear polymer. While, amylopectin consists of α-1,4 glycosidic linked to linear chains consisting of 10–60 glucose units and α-1,6 bonded to side chains consisting of 15–45 glucose units (Monteiro De Souza, 2010).

They belong to the group of hydrolases that act on act on α-1,4-glycosidic bonds. Amylases that are of microbial origin are divided into endo- and exo-acting enzymes. Endo-amylases (α-amylases, EC 3.2.1.1) are the enzymes that produce different chain lengths of malto-oligosaccharides by hydrolyzing internal α-1,4 bonds and α-1,6 linkages in the amylopectin and glycogen. Whereas exo-amylases are the enzymes that include β-amylases and glucoamylases. The cleavage of α-1,4-glycosidic bonds from the nonreducing ends in starch to give maltose is catalysed by the enzyme β-Amylases (EC 3.2.1.2). The breakdown of α-1,4 and α-1,6 glucosidic linkages to produce β-D-glucose from the nonreducing ends of starch and related poly- and oligosaccharides with lower rate for α-1,6 cleavage is catalysed by Glucoamylases (EC 3.2.1.3). The enzyme has been isolated from different sources including animals, plants and microbes. The amylases that are produced by the microorganisms have diverse industrial uses as they possess greater stability than that of those obtained from plant and animal origin. The enzymes obtained from bacterial and fungal sources have crucial application in the industrial sector (Zhu et al., 2011).

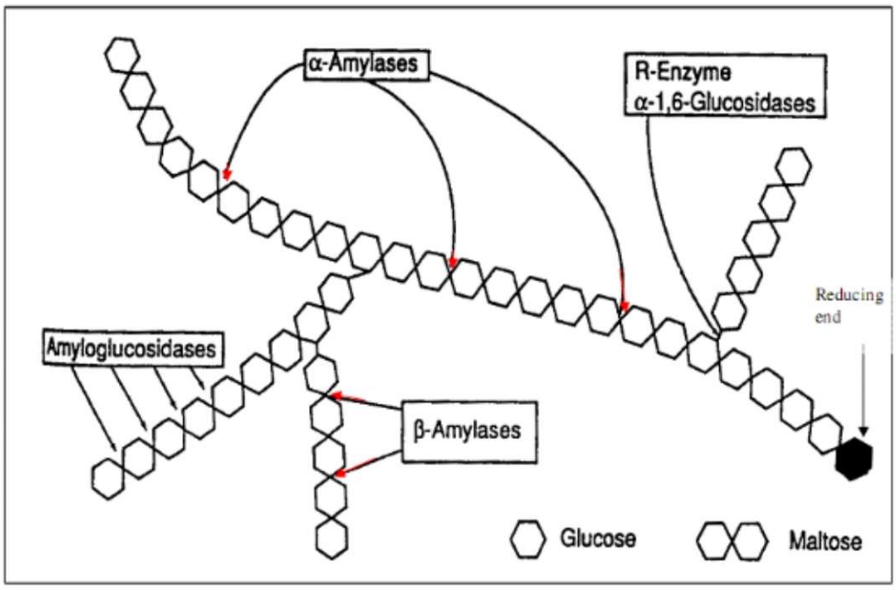


Figure 2: mechanism of action of α and β- amylases [1].

- **Cellulase**

The most abundant biomass on the Earth is cellulose. The cell-wall of most of the plants constitutes of cellulose and it forms the structural framework of the cell wall. Enzyme cellulase have the potential to degrade cellulose (Sethi et al., 2013).

Cellulase, since a couple of decades has gained wide recognition as an industrially essential enzyme with vast applications. The glucose produced in the process of saccharification by using cellulase can be converted into bioethanol -like biofuel by using ethanologenic microbes (Bhardwaj et al., 2021; Nguyen et al., 2018). Cellulases are most commonly used in the textile industries for softening of cotton, in colour care detergents; in food and paper industries. Cellulase can be produced by many microorganisms, mainly fungi and bacteria. Due to higher growth rate of bacteria as compared to that of the fungi, bacteria have a good potential for the production of cellulase (Sethi et al., 2013).

Cellulases are classified into three categories: endo-1,4-β-D-glucanase (endoglucanase), β-glucosidase and exo-1,4-β-D-glucanase (exoglucanase). The function of these enzymes are:

Exoglucanase: it forms glucose or cellobiose as the major end product by hydrolyzing non-reducing ends of crystalline cellulose.

Endoglucanase: it carries hydrolysis of the internal sites of oligosaccharides present in amorphous cellulose, carboxymethyl cellulose and cellooligosaccharides.

β-glucosidase: it catalyses hydrolysis of cellobiose and cellodextrin at the non-reducing end.

A synergetic action of the above mentioned three enzymes is required for accomplishing the effective degradation of cellulose (Ejaz et al., 2021).

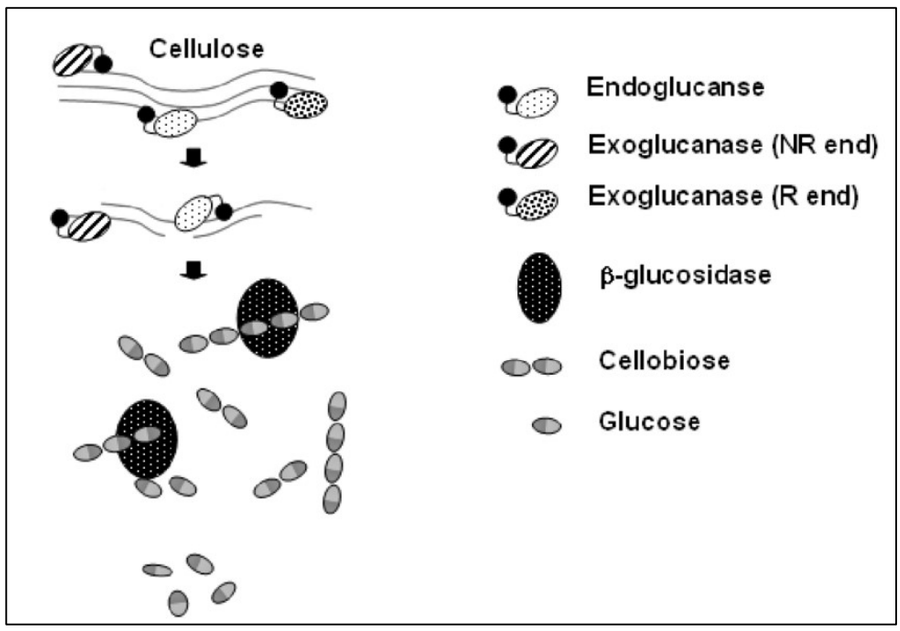


Figure 3: mechanism of action of enzyme cellulase [2]

- **Xylanase**

Xylan is a substrate for xylanase enzyme. It is a structural polysaccharide present in the plant cell walls and they constitute about 30% of the dry weight. Apart from the terrestrial plants, it is also synthesized by the marine algae having different chemical structure is based on a β-1,3- D- xylosyl back bone (Bajpai, 2014).

There are different types of xylanases present which are widespread in actinomyces, fungi and bacteria, these include acetyl xylan esterase (EC 3.1.1.72), β-1,4-endoxylanase (endo-1,4-β-xylanase, EC 3.2.1.8), α-L-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55), β-xylosidase (xylan 1,4-β-xylosidase, EC 3.2.1.37), α-glucuronidase (α-glucosiduronase, EC 3.2.1.139) and phenolic acid (ferulic and p-coumaric acid) esterase (EC 3.1.1.73). Endo-β-1,4-xylanases produce xylooligosaccharides that can be later converted into xylose (by β-xylosidase) thus catalyzing the breakdown xylan backbone (Zhu et al., 2011).

The enzymatic degradation of xylan initially requires the elimination of the side chains and then hydrolysis of xylan backbone. Therefore, the side chains are eliminated by the enzymes called Carbohydrate Esterases. Endo-β-1,4-xylanases attack the backbone of plant and seaweed xylan

and are endo-acting glycoside hydrolase while endo-β-1,3-xylanases attack the β-1,3-linked xylose present in seaweed (Nguyen et al., 2018). The xylanases obtained from microorganism have exhibited great potential in the essential industrial processes, such as pulp and paper, food and feed industries. Xylanases are being greatly applied in the extracting cellulose from pulps in order to produce cellophane, rayon and other chemicals which include esters of cellulose like butyrates, nitrates, acetate and propionates, and ethers of cellulose like methyl and ethyl and carboxymethyl cellulose. They are also used in food industries to enhance dough-handling properties, in order to improve the quality of the bread and extend the shelf-life by decreasing the rate of staling (Zhu et al., 2011).

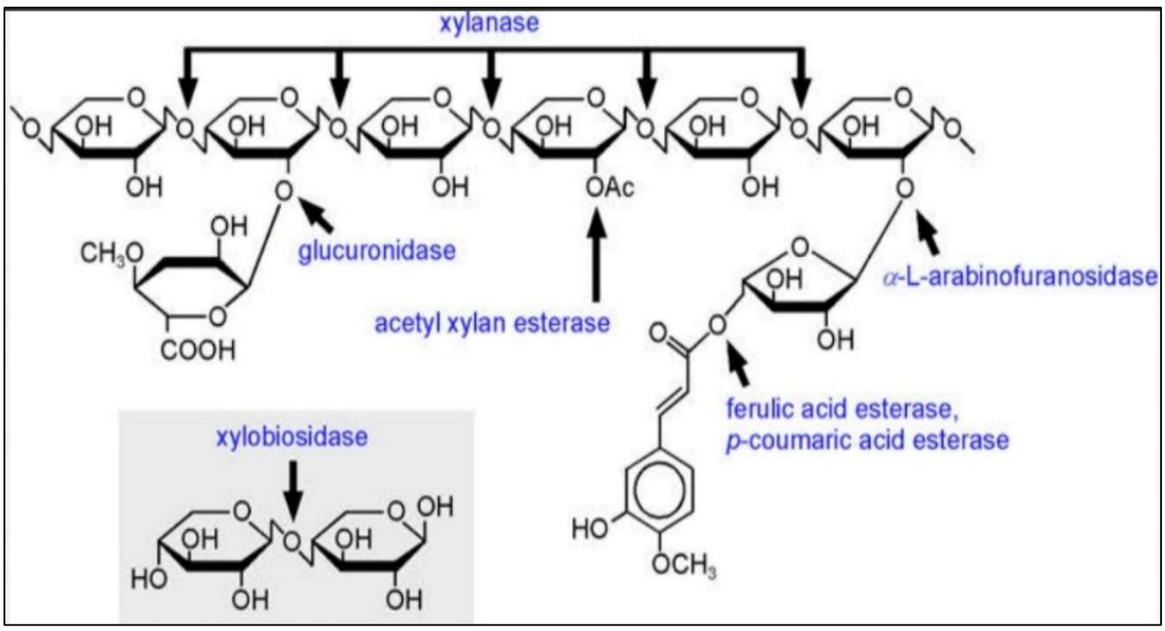


Figure 4: Mechanism of action of xylanase [3].

- **Agarase**

Agarase catalyses the hydrolysis of agar. Due to the gelling and stabilizing properties of agar, agar has large variety of applications. It is mainly used solidifying agent in microbiological media as it is difficult for microbes to degrade it and also because it forms stable, clear and firm gels. Agar is also used as a food additive, used in jelly sweets, glazes, icings, processed cheese and marshmallows. Agar is made up of a mixture of two polysaccharides namely agarose and agaropectin. The basic structure of agarose consists of repetitive units of β-D-galactose and 3,6-anhydro-α-L-galactose low content of sulfate esters. The disaccharide-repeating units agaropectin and agarose are same with some hydroxyl groups of 3,6-anhydro-α-L-galactose residues replaced by sulfoxy or methoxy and pyryvate residues. Agarose has molecular mass higher than that of agaropectin. The molecular mass of agarose is more than100,000 Daltons and has low content of suphate. While molecular mass of agaropectin is 20,000 Daltons, with high sulphate content. Based on the pattern of cleavage, agarase are divided into two groups that is α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81).α-Agarases hydrolyze α-1,3 linkages to yield agarooligosaccharides of series associated to agarobiose ,while hydrolysis of β-1,4 linkages is carried out by β-agarases to produce neoagarooligosaccharides of series associated to neoagarobiose (Fu & Kim, 2010).

α-glycosidic linkages of agarose is hydrolyzed by agarases, leading in the agarooligosaccharides (AOs) production with 3,6-anhydro-α-L-galactose (AHG) at the reducing end, while β-agarases break the β-glycosidic linkages of agarose to produce neoagarooligosaccharides (NAOs) with G at the reducing end. Acid treatment can be used to cleave the α-Glycosidic linkages. Exogenous activity of α-Neoagarooligosaccharide hydrolases (α-NAOSH) can cleave α-1,3 linkage producing AHG from NAOs. Similarly, α-NAOSH or α-neoagarobiose hydrolases (α-NABH) can degrade neoagarobiose (NA2) into AHG and G. A general transport system and catabolic pathway that is present in many bacteria uses the released G but the breakdown of AHG requires two specific enzymes, 3,6- anhydrogalactonate cycloisomerase and 3,6-anhydro-α-L-galactose dehydrogenase that have been identified only in agar degrading bacteria. hence bacteria that can degrade agar by using these enzymes can use AHG as the sole carbon source. (Park et al., 2020).

Many agarases have been obtained from diverse groups of bacteria present in marine sediments, fresh water, marine algae, seawater, soil and other habitats. The large variety of microorganisms

are isolated from different habitats have reported to have agarase activity. These include *Acinetobacter sp., Thalassomonas sp., Bacillus sp., Pseudomonas sp. Vibrio sp. Alteromonas sp,* etc (Fu & Kim, 2010).

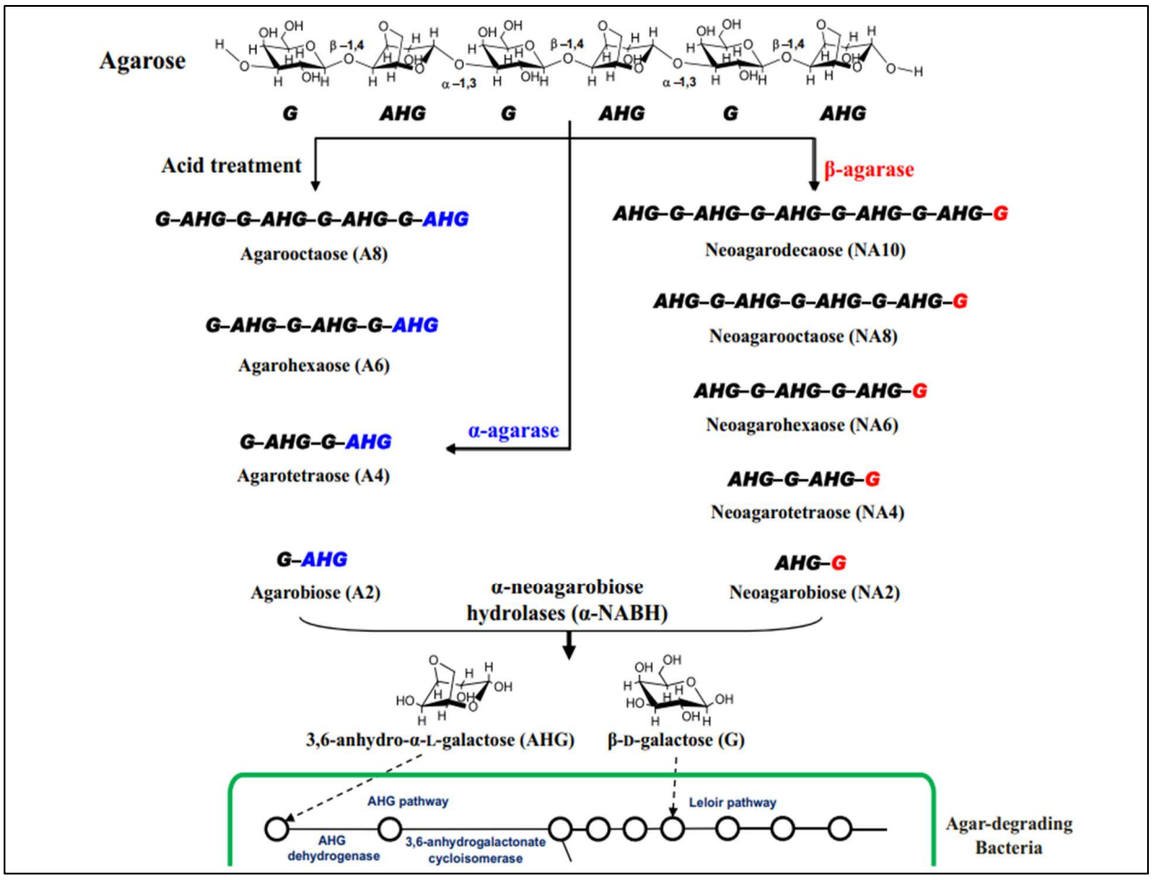


Figure 5: Agar hydrolysis by agarases (Park et al., 2020).

- **Protease**

Proteases are the most significant among the enzymes produced by the microorganisms and they constitute about 60% of the overall industrial enzyme market (Rodarte et al., 2011). Proteases are among one of the three enzyme groups whose global market is rapidly expanding annually (Razzaq et al., 2019). These proteases have found application in pharmaceuticals, food processing, leather processing, x-ray film industry in silver retrieval and as detergent additives (Rodarte et al., 2011)

Proteases are present naturally in all the organisms and are important for cell differentiation and growth and exhibit different functions that involve significant applications in the field of biotechnological. An excellent source of enzymes are microorganisms. Proteases carry out proteolysis in the polypeptide chains by hydrolyzing the peptide bonds. (Sharma, 2010). High yield, mear space requirement, economical and less time consumption, have made proteases

obtained from microbial origin suitable for industrial and biotechnological application. Among the proteases of microbial origin, alkaline proteases belong to the highest enzyme market. Proteases produced by the microorganisms have many applications in various industries: in the food industries, for production by cheese from milk by hydrolysing kappa casein to inhibit the coagulation by stabilizing the formation of micelle and for quick dough preparation by partial hydrolysis of gluten; in waste management; in leather industry, alkaline protease is used due to its keratinolytic and elastolytic activity; in detergent industry, proteases are important component in dentures, contact lense and detergents used in household laundry; in photographic industry, the proteases obtained from *B. subtilis* have been successfully used recover silver from the x-ray films and in medical field, it is used in an important constituent in ointments and some are also used in the anticancer drug therapy (Razzaq et al., 2019).

Proteases are subdivided into two major groups based on their site of hydrolysis i.e., exopeptidases and endopeptidases.

Exopeptidases catalyze the hydrolysis of the bond close to carboxy or amino termini of the polypeptide chains. Depending on their site of action exopeptidases are classified into Aminopeptidases and carboxypeptidases. Aminopeptidases release a single amino acid, di- or tri-peptide residues by acting on the free N terminus of the substrate. Carboxypeptidases release a single amino acid, di- or tri- peptide residues by acting on the polypeptide chain at the C-terminals. Depending on the nature of enzyme active sites, carboxypeptidases are grouped into metallocarboxypeptidases, serine carboxypeptidases and cysteine carboxypeptidases.

Endopeptidases attack the peptide bonds present at the inner regions, away from the C and N termini in a polypeptide chain. The endopeptidases are further divided into four groups depending on their mechanism of catalysis (Rao et al., 1998).

(i) Serine proteases: the active site of serine protease consists of serine group. They found in bacteria, eukaryotes, viruses and bacteria. Hence proving that it is essential for the organisms. They are further grouped into twenty families depending on their structural similarities. The optimal pH for the enzyme is between pH 7 and 11 (Rao et al., 1998).

(ii) aspartic proteases: They are acidic or Aspartic acid proteases. They are endopeptidases and for their catalytic activity they depend on aspartic acid residues. Milk clotting ability has been seen in some of these proteases (Rao et al., 1998).

(iii) cysteine proteases: it is also known as thiol proteases. It is found in both eukaryotes and prokaryotes. They are endoproteases and at the catalytic site a cysteine residue is present. They have an optimal activity at neutral pH (Rao et al., 1998).

(iv) metalloproteases: they have divalent metal ion at their catalytic site for their activity. metalloproteases are sub-divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II depending on their mode of action. They are inhibited by chelating agents like EDTA (Rao et al., 1998).

Protease	Mode of action[a]	EC no.
Exopeptidases		
Aminopeptidases	●↓-○-○-○-○---	3.4.11
Dipeptidyl peptidase	●-●↓-○-○-○---	3.4.14
Tripeptidyl peptidase	●-●-●↓-○-○---	3.4.14
Carboxypeptidase	---○-○-○-○-○↓-●	3.4.16–3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase	---○-○-○-○↓-●-●	3.4.15
Dipeptidases	●↓-●	3.4.13
Omega peptidases	*-●↓-○-○---	3.4.19
	---○-○-○↓-●-*	3.4.19
Endopeptidases	----○-○-○↓-○-○-○---	3.4.21–3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

[a] Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.

Figure 6: types of proteases (Rao et al., 1998).

MATERIALS AND METHODS

Materials and method

- **Seaweed sampling and collection**

  The seaweed samples were collected randomly from the intertidal zones (Kumar et al., 2011) from Bambolim beach, Cacra beach and Ajuna beach in Goa.

  The samples were collected during the low tide in a zip-lock bag. They were rinsed with autoclaved sea water to remove any attached debris. (Vijayalakshmi et al., 2008).

- **Protein estimation in seaweed**

  About 500mg of the sample tissue was weighed and homogenized in 5 ml of phosphate buffer saline followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected for the estimation of protein. 1 ml of the sample supernatant was used for the estimation of protein by Folin-lowry method and absorbance was checked at 660nm.

- **Isolation of seaweed associated epiphytic bacteria**

  Bacteria associated with the surface of the seaweed were isolated by placing it in 10ml of the autoclaved seawater, followed by vertexing it for 5 mins (Kumar et al., 2011) and then serially diluting to about 10^{-6} dilution. 0.1 ml aliquots were spread plated on Zobell Marine agar in triplicate. The inoculated Zobell Marine agar plates were incubated at room temperature (28 ± 2^{o} C) for 48 hours (Vijayalakshmi et al., 2008). Individual bacterial colonies obtained after incubation were further streaked on Zobell Marine agar plates to attain single colonies(Singh et al., 2011). Isolates were sub-cultured routinely and maintained on Zobell marine agar at room temperature.

- **Screening for the presence of different enzymes:**
- **Screening for production of Amylase**

  Production of amylase by the seaweed associated bacteria was determined by spot inoculating the isolates on the artificial seawater-based starch agar plates (2% starch) and incubated at

room temperature (28 ± 2° C) for 48 hours. Zone of hydrolysis was checked by flooding the plates with iodine solution (Naik *et al.*, 2019).

- **Screening for production of Xylanase**

  For determining the production of xylanase by the bacterial isolates, the isolates were spot inoculated on the artificial seawater-based agar plates supplemented with 1% xylan and incubated at room temperature (28 ± 2° C) for 48 hours.

  The plates were then flooded with 1% Congo red dye. After 5 min, stain was removed and washed with distilled water. Yellow opaque area appeared around the colonies indicating degradation of xylan against a reddish colour for undegraded (R. Sethi *et al*., 2013).

- **Screening for production of Agarase**

  The bacterial isolates were spot inoculated on artificial seawater-based agar medium supplemented with 2% agar as sole source of carbon and incubated for 24–48 h at room temperature (28 ± 2° C) for 24 –48 hours. the plates were flooded with Lugol's iodine and the isolates showing the zone of clearance around the colony and depression in agar medium were selected as agarase-producing bacteria (Naik *et al*., 2019).

- **Screening for production of Cellulase**

  For determining the production of cellulase by the bacterial isolates, the isolates were spot inoculated on the artificial sea water-based agar plates supplemented with 2% CMC and incubated at room temperature (28 ± 2° C) for 48 hours.

  The plates were then flooded with 1% congo red dye. After 5 min, stain was removed and washed with distilled water. Yellow opaque area appeared around the colonies indicating degradation of cellulase against a reddish colour for undegraded (R. Sethi *et al.*, 2013).

- **Screening for production of Protease**

  For screening, the bacterial isolates were spot inoculated on the skim milk agar plates and incubated at room temperature (28 ± 2° C) for 48 hours. The zone of clearance around the colonies indicated the positive results for protease production (Jayadev & Jayadev, 2016)

- **Degradation of seaweed waste**

  2% ground powder of S*argassum* was added in Seawater-based broth and no other carbon source was added to the medium. The bacterial culture was inoculated and incubated at 48 hours with constant shaking at 150 rpm at room temperature (28 ± 2^{o} C). After incubation the 5ml of the broth was centrifuged at 10,000 rpm and the supernatant was collected and analysed by 3,5-dinitrosalicylic acid (DNSA) method to measure the release of reducing sugar from algal waste at 540 nm, with glucose as a standard (Naik *et al.,* 2019).

- **Dairy waste degradation by using seaweed associated bacteria**

  Determination of proteolytic activity: For determining, the efficacy to digest the protein by the bacterial isolates, proteinaceous wastes generated by the dairy industry was used as the substrate. A 10% solution of the proteinaceous substrate was prepared in the distilled water and sterilized. 2ml inoculum of the each of the isolates was inoculated in 20 ml of the proteinaceous substrate solution prepared. Incubation of the inoculated solution was done at room temperature (28 ± 2^{o} C) for 24 hours (Sharma, 2010).

  Total protein assay: eight isolates of bacteria were inoculated in the respective substrates solution to determine their proteolytic activity. An uninoculated sample was used as a control each time. The inoculated samples were centrifuged and the supernatant was collected to estimate for the total protein content by Folin and Lowry method. (Sharma, 2010)

  The protease activity was determined by using Folin-Lowry method. Casein can be hydrolyzed by using protease to give amino acids containing phenolic groups, and under alkaline condition it produces tungsten blue which has an absorption at 660 nm. Different concentrations of tyrosine were used to make a standard curve and based on the standard curve the protease activity was calculated (Wang *et al*., 2022).

Biochemical tests for identification

- **Gram staining**

  On a clean glass slide, a bacterial smear of 18 hours old isolates was made, followed by air drying and heat fixing. It was flooded with Gram's crystal violet for 1 minute, followed by draining and flooding it with Gram's iodine for 1 minute. Slide was drained and decolorization was done using 95% ethanol for 30 seconds. Slide was rinsed with water and counter stained with saffranine for 30 seconds. Slide was washed with water, air dried and observed under oil immersion lens**.**

- **Sugar fermentation**

  18 hours old isolates were inoculated in peptone water-based medium containing inverted Durham's tube, phenol red as indicator and 0.5% sugars (lactose, glucose, maltose and sucrose) and incubated at room temperature (28^{o} C±2) for 24 hours. Sugar fermentation is indicated by color change of phenol red medium from red to yellow and presence gas bubbles in the Durham's tube.

- **Catalase test**

  On a clean glass slide, suspension of 18 hours old bacterial isolates was prepared in a drop of saline and a drop hydrogen peroxide was added. Production of effervescence indicated a positive result.

- **Motility test**

  The nutrient agar butt consisting of 0.4% agar were stabbed with 18 hours bacterial isolates and incubated at room temperature (28^{o}C±2). Growth throughout the tube indicated positive test.

- **Methyl red (MR) and Voges-Proskaeur (VP) test**

  In glucose phosphate broth medium, a loop full of 24 hours old isolates were inoculated and incubated at room temperature (28^{o}C±2) for 24 hours, after the incubation, in two test tubes equal amount of culture broth was distributed and labeled as MR and VP based on the test.

For MR test, 2 to 3 drops of methyl red reagent are added. A positive result was indicated by re coloration of the broth.

For VP test, 2 drops of Omeara's reagent are added, mixed and was kept for 30 minutes. Positive tests are indicated by pink coloration**.**

- **Indole tests**

  The bacterial isolate was inoculated in the tryptone broth and incubated for 24 hours at room temperature. 3-4 drops of Kovac's reagent are added to it. A positive result was indicated by ring formation**.**

- **Hugh leifson test**

  5 ml of Hugh leifson medium was added in two test tubes and sterilized. The bacterial isolate was stab inoculated and one of the tubes was overlayed with liquid paraffin oil and the tubes were incubated at room temperature ($28^{o}C\pm2$) for 24 hours. Growth in both the tubes that the isolate is facultative anaerobe. Growth in the tube containing liquid paraffin indicates anaerobe and growth in the tube without liquid paraffin indicates aerobe.

RESULTS AND DISCUSSION

Sample collection

To study the association of the bacteria with the seaweeds, the seaweed samples were collected from the intertidal regions of the Goa. The sampling sites chosen for the collection of seaweed samples included Bambolim, Anjuna and Cacra. A total of 13 different seaweeds were collected and identified following Jha et.al., 2009 and Manisseri et.al., 2012.

Seaweed samples collected from the Bambolim beach:

Figure 7: *Ulva intestinalis*

Figure 8: *Ulva lactuca*

Seaweed samples collected from the Anjuna beach:

Figure 9: *Gracilaria sp.*

Figure 10: *Dictyopteris acrostichoides*

Figure 11: *Amphiroa sp.*

Figure 12: *Acrosiphonia orientalis*

Fig.13: *Sphacelaria sp.*

Seaweed samples collected from the Cacra beach:

Figure 14: *Scinaia sp.*

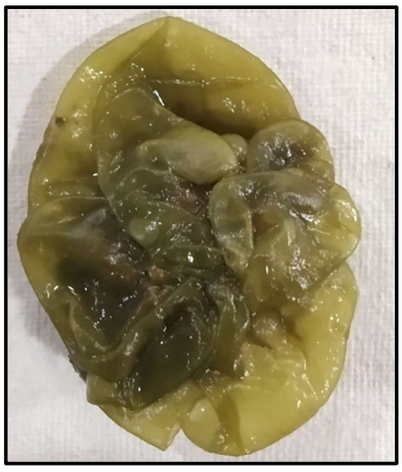


Figure 15: *Colpomenia sinuosa*

Figure 16: *Padina tetrastromatica*

Figure 17: *Padina boergesenii*

Figure 18: *Sargassum sp.*

Seaweed sample acquired from southern coast of Karnataka

Figure 19: *Gracilaria foliifera*

Isolation of seaweed associated bacteria

The seaweed samples were washed to remove loosely associated bacteria and the plating media used was Zobel marine agar. About 65 bacterial isolates were obtained from the 13 different seaweeds of the sampling sites: Bambolim, Anjuna and Cacra in Goa, and southern coast of Karnataka. The colony characters of all the colonies were recorded and the colonies were purified by streaking. These isolates were screened for the industrially important enzymes (protease, cellulase, amylase, xylanase and agarase) and checked their potential in degradation of Dairy and seaweed waste**.** (Letter "S" denotes the sample and letter "C" denotes the colony, e.g., S1C1 it refers to the seaweed sample 1 and its colony no.1).

Table1: Number of bacterial isolates obtained from each seaweed.

Sample no.	Names of the seaweed	No. of bacterial colonies isolated
S1	*Ulva intestinalis*	8
S2	*Sargassum sp*	5
S3	*Sphacelaria sp.*	2
S4	*Padina tetrastromatica*	7
S5	*Ulva lactuca*	7
S6	*Padina boergesenii*	6
S7	*Acrosiphonia orientalis*	4
S8	*Gracilaria sp.*	3
S9	*Colpomenia sinuosa*	4
S10	*Dictyopteris acrostichoides*	2
S11	*Gracilaria foliifera*	7
S12	*Amphiroa sp*	5
S13	*Scinaia sp.*	5

Protein estimation in seaweed

The estimation of protein in seaweeds was carried out by using Folin-Lowry method, using Bovine serum Albumin was used as a standard.

Table2: Estimation of protein in seaweed.

Sr no	Names of the seaweed	Concentration of Protein in µg/ml
1	*Ulva intestinalis*	157.6
2	*Sargassum sp*	178.4
3	*Sphacelaria sp.*	127.1
4	*Padina tetrastromatica*	305.8
5	*Ulva lactuca*	7.17
6	*Acrosiphonia orientalis*	372.8
7	*Gracilaria sp.*	196.7
8	*Colpomenia sinuosa*	86.7
9	*Dictyopteris acrostichoides*	82.3
10	*Gracilaria foliifera*	113.2
11	*Amphiroa sp*	238.0

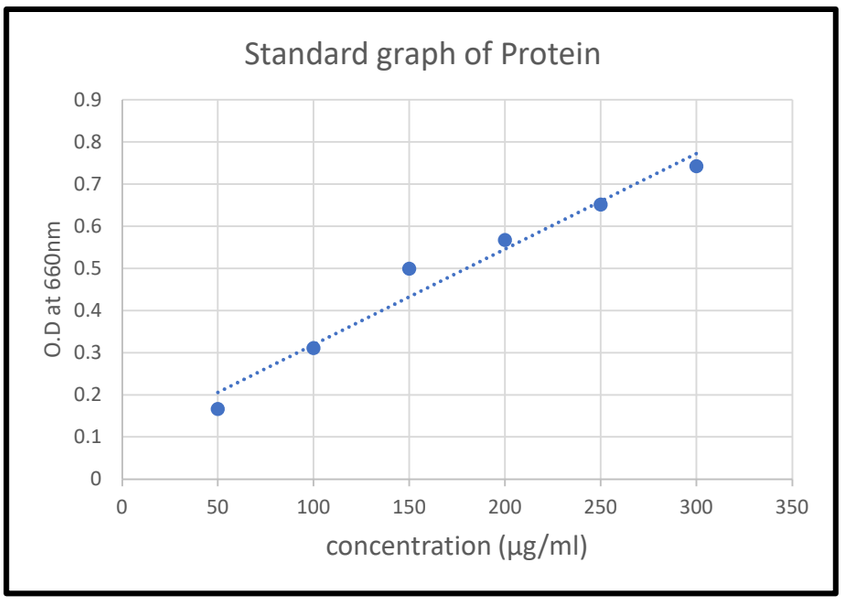


Figure 20: standard curve for protein.

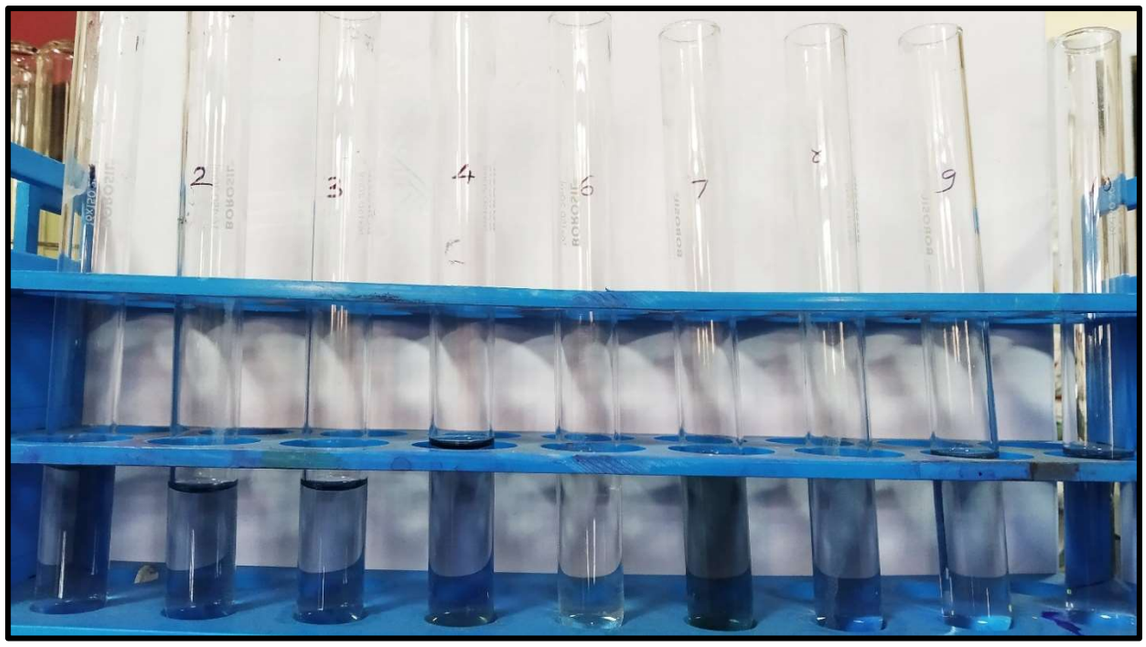


Figure 21: Estimation of protein content in the different seaweeds by Folin-Lowry method and determining its absorbance at 660nm.

Screening for the presence of enzymes

1. **Screening of the bacterial isolates for production of Amylase**

Sixty- five colonies were spot inoculated to check for the production of amylase on starch agar plates. Sixteen out of fifty-nine bacterial colonies showed zone of clearance around them after the addition of iodine, indicating positive result for the production of amylase. These colonies were S1C6, S1C8, S2C2, S4C3, S4C4, S5C7, S6C1, S6C6, S7C1, S8C3, S10C1, S12C2, S12C3, S12C5, S13C3 and S13C4. Of these sixteen colonies, S4C3, S10C1 and S12C3 isolates showed the highest zone of clearance. The starch present in the medium is utilized as substrate by the bacteria, by the production of amylase enzyme. Amylase hydrolyses starch into sugars. The zone of clearance indicates the hydrolysis of starch into sugars which does not bind to iodine and appears as a clear zone around the bacterial growth.

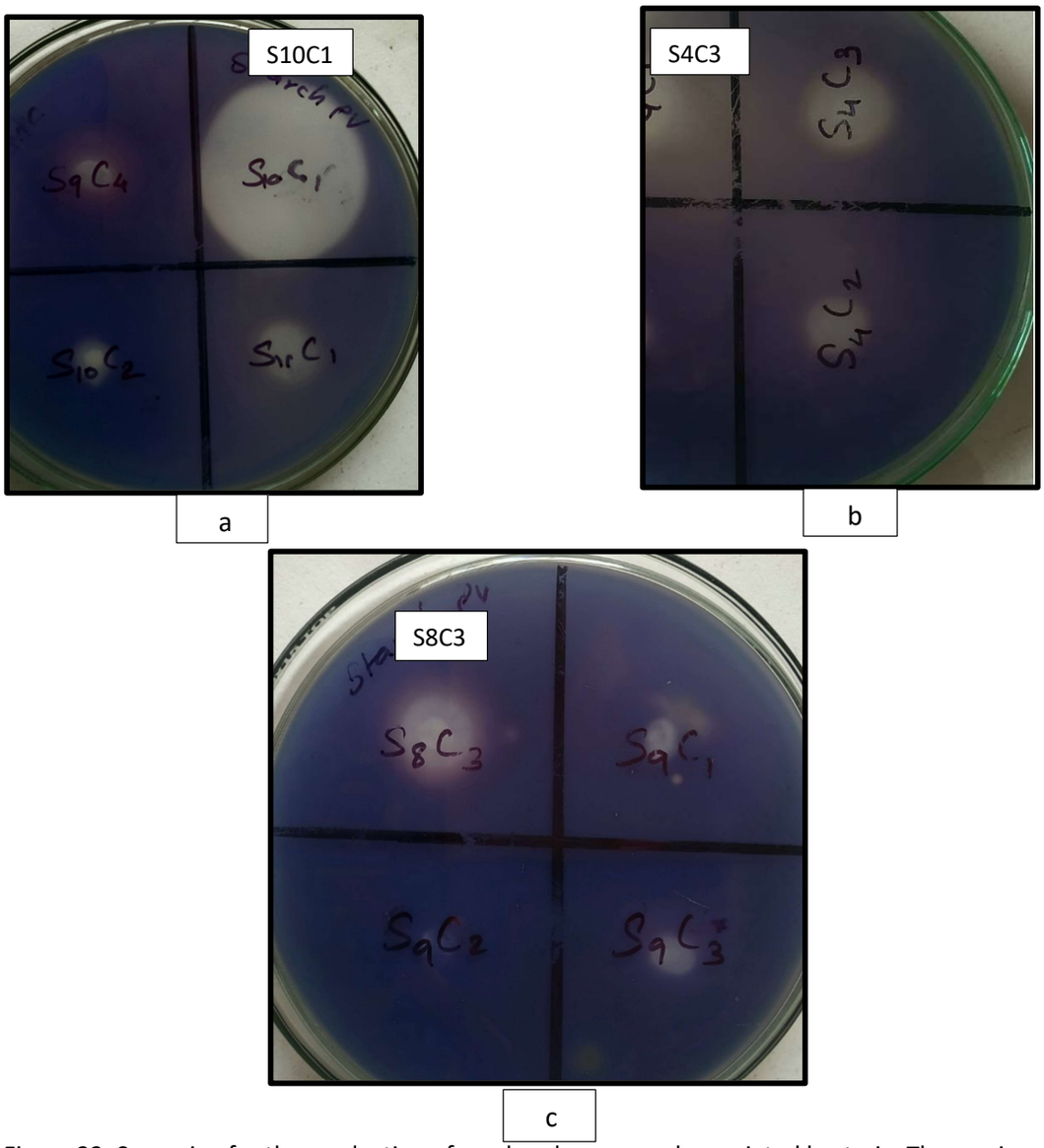
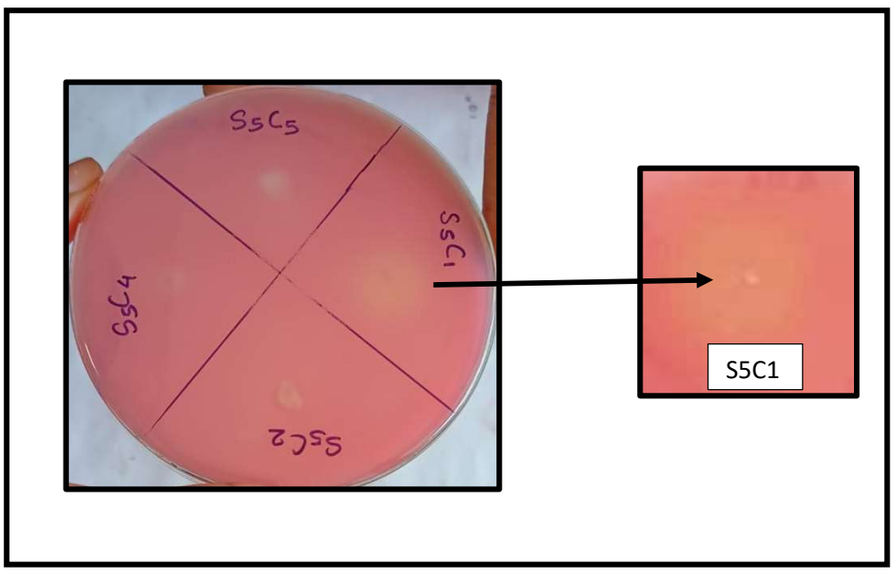


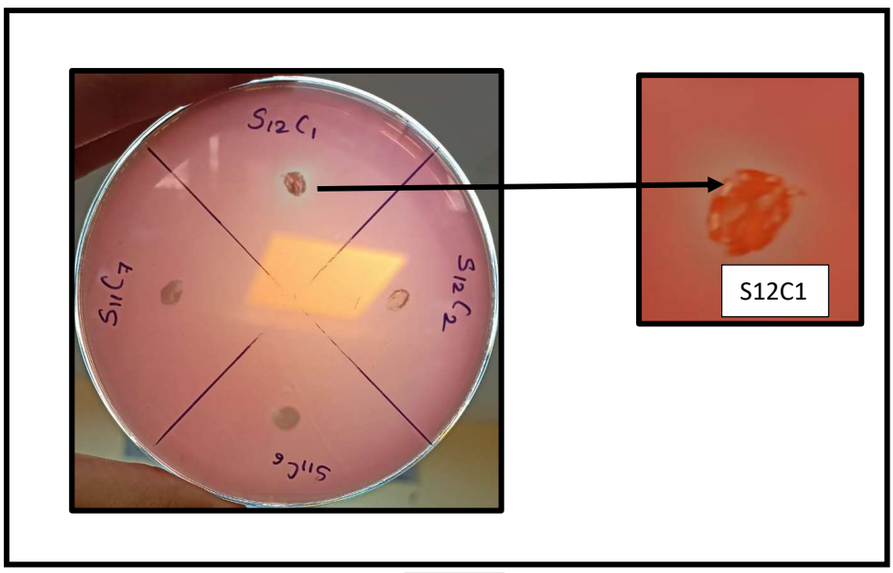
Figure 22: Screening for the production of amylase by seaweed associated bacteria. The maximum zone of clearance was observed in the bacterial isolates (a) S10C1 (b)S4C3 and (c)S8C3 after 48 hours of incubation at room temperature and flooding with Lugol"s iodine.

2. **Screening of the bacterial isolates for production of Xylanase**

Bacterial colonies were spot inoculated artificial seawater-based media plates and incubated room temperature for 48 hours the media supplemented with xylan as the sole source of carbon to check for the production of enzyme xylanase. Two out of fifty-nine colonies showed yellow opaque area around the colonies indicating the production of xylanase enzyme. The two colonies that showed positive results were S5C1 and S12C2 isolates. Colonies producing xylanase hydrolyse the xylan present in the media and produce a yellow opaque zone that is observed after flooding the plates with congo red dye.



a



b

Figure 23: Production of enzyme Xylanase by seaweed associated bacteria. The yellow opaque zones were observed in (a) S5C1 and (b) S12C1 indicating positive result for the production of the enzyme xylanse.

3. Screening of the bacterial isolates for production of Agarase

The isolates were spot inoculated on artificial seawater-based agar medium supplemented with 2% agar and incubated room temperature for 48 hours to check for the production of agarase by the bacteria. The isolates that showed zone of clearance after flooding the plates with Lugol's iodine were S1C2, S1C4, S4C3, S11C1, S12C2, S12C3, S12C5, S13C3 and S13C3. The colonies that showed the good depressions and zone of clearance were S1C4, S4C3, S12C3 and S13C3. Zone of clearance observed after the plates were flooded with iodine indicated that the agar in the medium was hydrolysed by the bacteria by production of enzyme agarase.

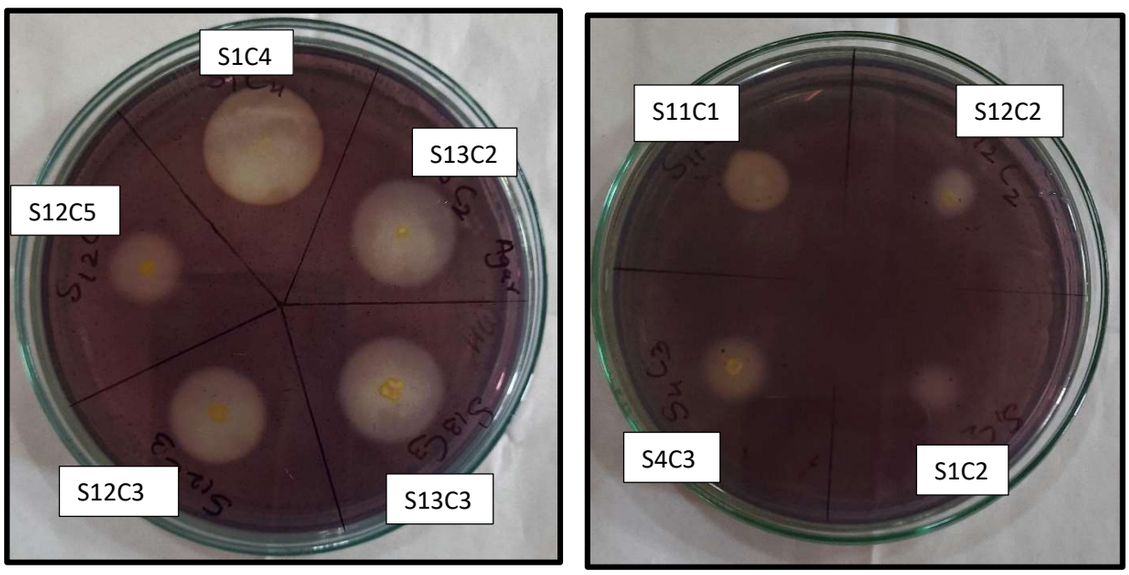


Figure 24: The production of amylase by seaweed associated bacteria. Zone of clearance was observed after flooding thee plates with Lugol's iodine. Bacterial isolates showing depressions and zone of clearance in the agar medium had a great potential in the breakdown of agar. S1C4, S4C3, S12C3 and S13C3 seaweed associated bacterial isolates showed depressions in the agar medium plates and zone of clearance after flooding the plates with Lugol's iodine.

Figure 25: bacterial isolate S4C3 showed the maximum depression in the agar medium after 48 hours of incubation at room temperature indicating its potential in agar degradation.

4. Screening of the bacterial isolates for production of Cellulase

The bacterial isolates were spot inoculated on the artificial seawater agar supplemented with CMC. All the isolates were negative for the production of cellulase as no yellow opaque region was observed around the colonies after flood the plates with Congo red dye.

Figure 26: no yellow opaque zone around the colonies was seen artificial seawater agar supplemented with CMC on after flooding the plates with Congo red dye.

5. **Screening of the bacterial isolates for production of Protease**

The bacterial isolates were spot inoculated on the skim milk agar plates and incubated at room temperature for 48 hours for determining the proteolytic activity of the bacteria. Six bacterial isolates showed best results for production of protease. Isolates showing the highest zone of clearance were S2C3, S4C2, S5C1, S6C1, S11C3 and S13C1. The zone of clearance is observed when the bacteria is able to carry out casein hydrolysis.

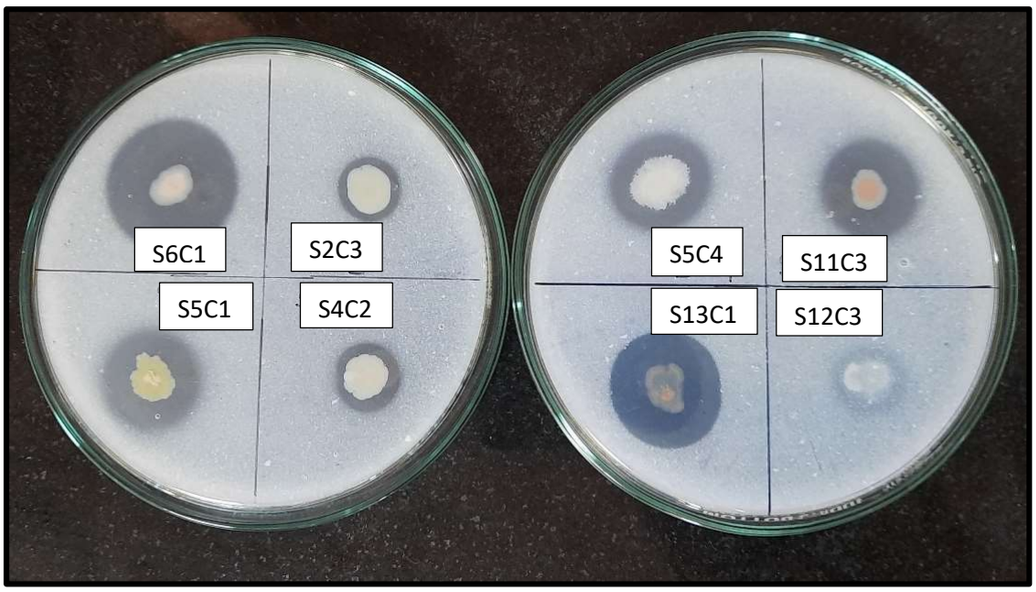


Figure 27: Production of protease by seaweed associated bacteria isolates. Zone of clearance observed on the skim milk agar plates spot inoculated by seaweed associated bacterial isolates.

6. Degradation of seaweed waste

Four bacterial isolates were selected to determine their potential for the degradation of seaweed wastes. These isolates showed a positive result in screening for the production of amylase, agarase and protease. The isolates that were selected are S1C4, S4C3, S12C3 and S13C3. The four bacterial isolates were spot inoculated on artificial seawater-based agar plates containing 2% dried seaweed powder as the carbon source. the plates were incubated at room temperature for 48 hours. The bacterial isolates were also inoculated in on artificial seawater-based broth containing 2% dried seaweed powder as sole source of carbon. The utilization of the seaweed powder by the bacterial isolates was determined by analyzing release of the reducing sugar by using DNSA method. The reducing sugar released after 48 hours by inoculating isolates S1C4, S4C3, S12C3 and S13C3 was 29.47, 102.18, 18.28 and 53.87 µg/ml respectively.

Figure 28: degradation of seaweed by bacterial isolates S1C4, S4C3, S12C3 and S13C3.

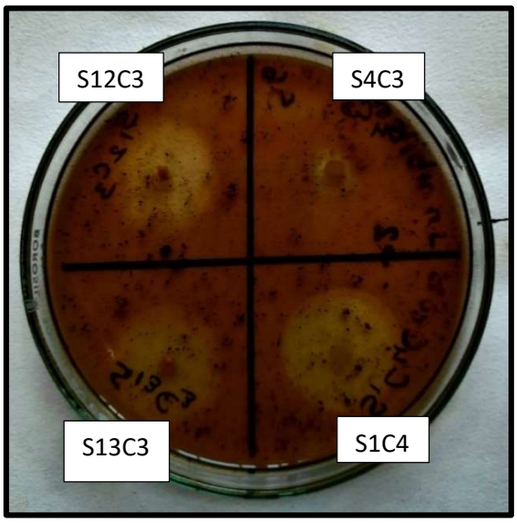


Figure 29: spot inoculation of bacterial isolates S1C4, S4C3, S12C3 and S13C3 on seawater-based media containing 2% dried seaweed powder as the carbon source to determine their potential in seaweed degradation.

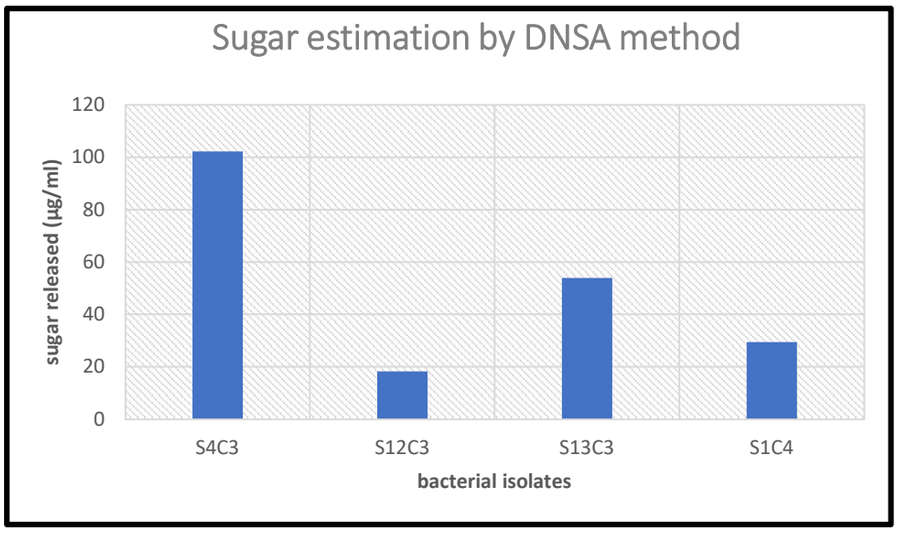


Figure 30: Estimation of reducing sugar released by bacterial isolates S4C3, S12C3, S13C3 and S14C4 after inoculation in artificial seawater broth containing 2% dried seaweed powder as sole source of carbon.

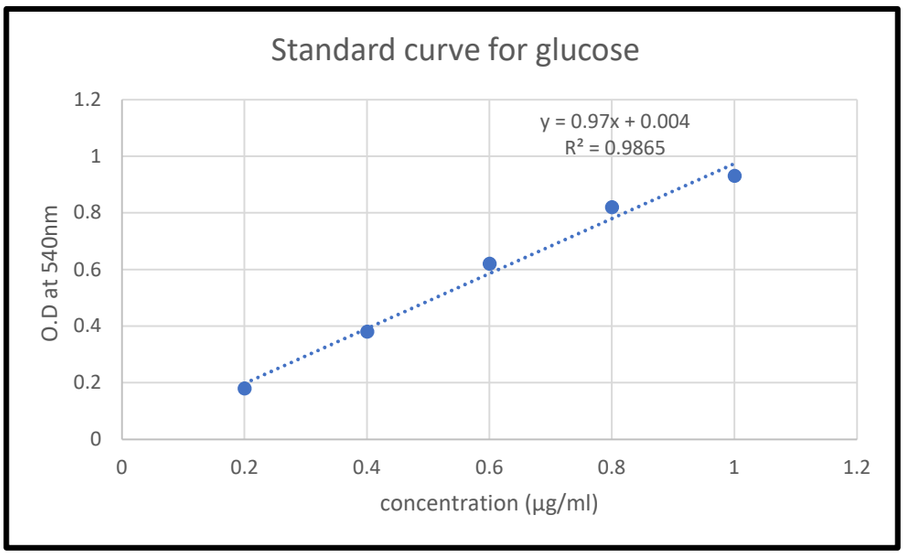


Figure 31: Standard curve for glucose.

7. Degradation of dairy effluent

Bacterial isolates S2C3, S4C2, S5C1, S5C4, S6C1, S11C3 and S13C1 were selected for determining their ability dairy waste degradation. These bacterial isolates showed positive results in the secondary screening for the production of protease enzyme. The total protein assay of the supernatant before and after the inoculation of bacterial isolates in the dairy effluent was carried out by using by Folin -Lowry method in which each time an uninoculated sample was used as a control. The test samples were centrifuged and supernatant was used to estimate protein by Folin-Lowry method. Maximum absorbance was seen in bacterial isolates S6C1, S5C1, S5C4 and S13C1 with an increase in the concentration of 12.5, 10.8, 12.1 and 15.5 µg/ml. An increase in the protein concentration was observed from the initial concentration depicts that the protein present in the waste was utilized by the bacterial isolates and it was degraded into amino acids.

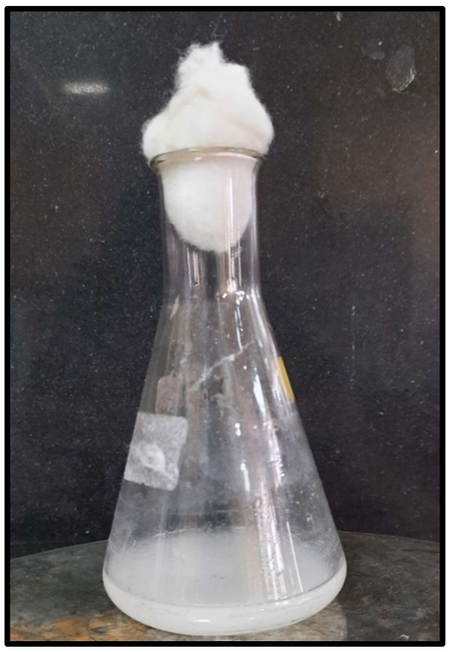


Figure 32: Dairy effluent acquired from Goa Dairy milk processing plant, Ponda- Goa to study proteolysis by protease producing bacterial isolates S2C3, S4C2, S5C1, S5C4, S6C1, S11C3 and S13C1 isolated from Sargassum sp., *Padina tetrastromatica, Ulva lactuca, Padina boergesenii, Gracilaria foliifera* and *Scinaia sp respectively.*

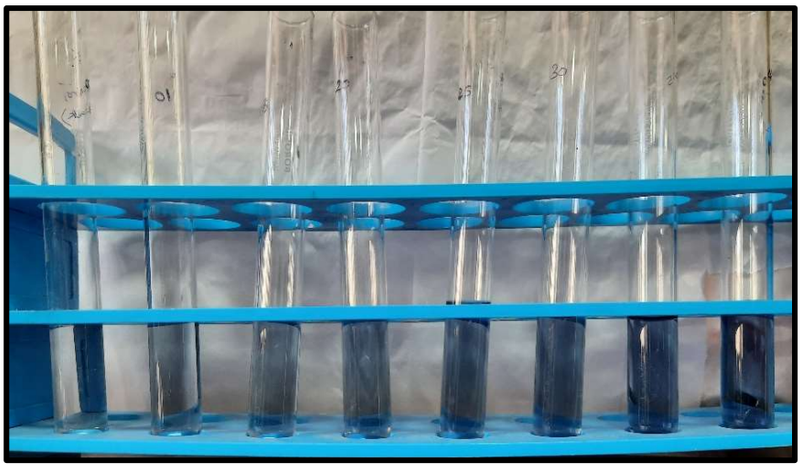


Figure 33: Estimation by folin-lowry method

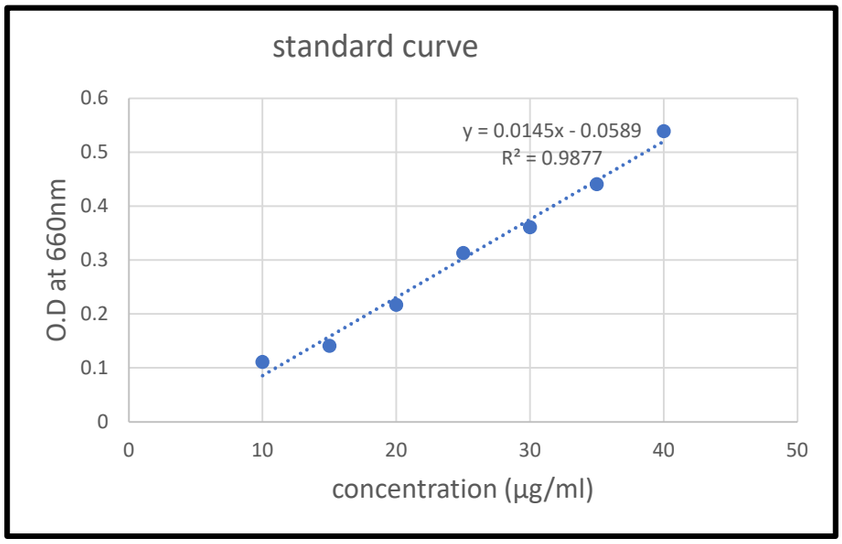


Figure 34: Stand curve for tyrosine

Table 3: Degradation of dairy waste by proteolytic bacteria

Bacterial Isolates	Absorbance at 660 nm	Increase in protein concentration (µg/ml)
S6C1	0.121	12.5
S2C3	0.05	7.7
S5C1	0.096	10.8
S4C2	0.067	8.8
S5C4	0.115	12.1
S11C3	0.059	8.3
S13C1	0.165	15.5

Biochemical tests results

Table 4: colony characteristics

(a) Bacterial isolates used for screening of enzyme.

	Amylase producing bacteria			**Xylanase producing bacteria**	
	S10C1	**S4C3**	**S8C3**	**S5C1**	**S12C2**
Shape	circular	circular	circular	circular	circular
Size	pinpoint	0.4cm	0.1cm	0.4cm	0.3cm
Surface	smooth	smooth	smooth	smooth	smooth
Colour	pale white	pale yellow	white	light yellow	white
Opacity	opaque	opaque	opaque	opaque	opaque
Elevation	convex	crateriform	convex	convex	convex
Margin	entire	entire	entire	even	entire
Consistency	butyrous	slimy	viscid	slimy	slimy
Gram character	Gram negative rods	Gram negative cocci	Gram positive cocci	Gram negative rods	Gram negative rods
Citrate test	+	-	-	-	-
Indole test	-	-	-	-	-
Methyl red test	-	-	-	+	-
Voges-Proskaeur test	-	-	-	-	-
Catalase test	+	-	-	+	+
Motility test	-	+	-	-	+
Sugar fermentation test					
Sucrose	-	-	-	-	-
Lactose	-	-	-	-	-
Glucose	+	+	+	+	+
Maltose	-	-	+	-	+
Hugh leifson					
Aerobic	+	-	+	+	-
anaerobic	-	+	+	+	+

For sugar fermentation tests:

- : negative

\+ : Production of acid

++ : Production of acid and gas

(b) Bacterial isolates used for screening of enzyme.

	Bacteria producing agarase			
	S1C4	**S4C3**	**S12C3**	**S13C3**
Shape	irregular	circular	circular	circular
Size	0.5cm	0.4cm	0.3cm	0.3cm
Surface	wrinkle	smooth	smooth	smooth
Colour	creamy white	pale yellow	yellow	white
Opacity	opaque	opaque	opaque	opaque
Elevation	flat	crateriform	convex	flat
Margin	filamentous	entire	entire	entire
Consistency	Slimy	slimy	Slimy	slimy
Gram character	Gram positive rods	Gram negative cocci	Gram positive cocci	Gram positive cocci
Citrate test	+	-	-	-
Indole test	-	-	-	-
Methyl red test	-	-	-	-
Voges-Proskaeur test	-	-	-	-
Catalase test	+	-	+	+
Motility test	-	+	+	-
Sugar fermentation test				
Sucrose	-	-	-	-
Lactose	-	-	-	-
Glucose	-	+	-	+
Maltose	-	-	-	+
Hugh leifson				
Aerobic	-	-	+	+
anaerobic	-	+	-	+

For sugar fermentation tests:

- : negative

\+ : Production of acid

++ : Production of acid and gas

(c) Bacterial isolates used for screening of enzyme.

	Bacteria producing protease						
	S2C3	S4C2	S5C1	S5C4	S6C1	S11C3	S13C1
Shape	circular	circular	irregular	circular	circular	circular	circular
Size	0.5 cm	0.3cm	0.5 cm	0.2cm	0.3cm	0.3cm	0.2cm
Surface	smooth	smooth	smooth	dull	smooth	smooth	smooth
Colour	pale yellow	white	pale yellow	cream	brown	pale white	orange
Opacity	opaque	opaque	opaque	opaque	opaque	opaque	opaque
Elevation	convex	crateriform	flat	convex	convex	convex	convex
Margin	entire	entire	entire	even	entire	entire	entire
Consistency	butyrous	dry	dry	slimy	slimy	slimy	slimy
Gram character	Gram negative cocci	Gram positive cocci	Gram negative rods	Gram positive rods	Gram positive rods	Gram positive cocci	Gram positive rods
Citrate test	-	-	-	-	-	+	-
Indole test	-	-	-	-	-	-	-
Methyl red test	-	-	+	+	-	-	-
Voges-Proskaeur test	-	-	-	-	-	-	-
Catalase test	+	+	+	-	+	+	-
Motility test	-	-	-	+	+	+	-
Sugar fermentation test							
Sucrose	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Glucose	-	-	+	+	+	-	-
Maltose	-	-	-	+	-	+	+
Hugh leifson		**-**					
Aerobic	-	-	+	+	-	-	-
anaerobic	-	-	+	-	-	-	-

For sugar fermentation tests:

- : negative

+ : Production of acid

++ : Production of acid and gas

Gram staining results:

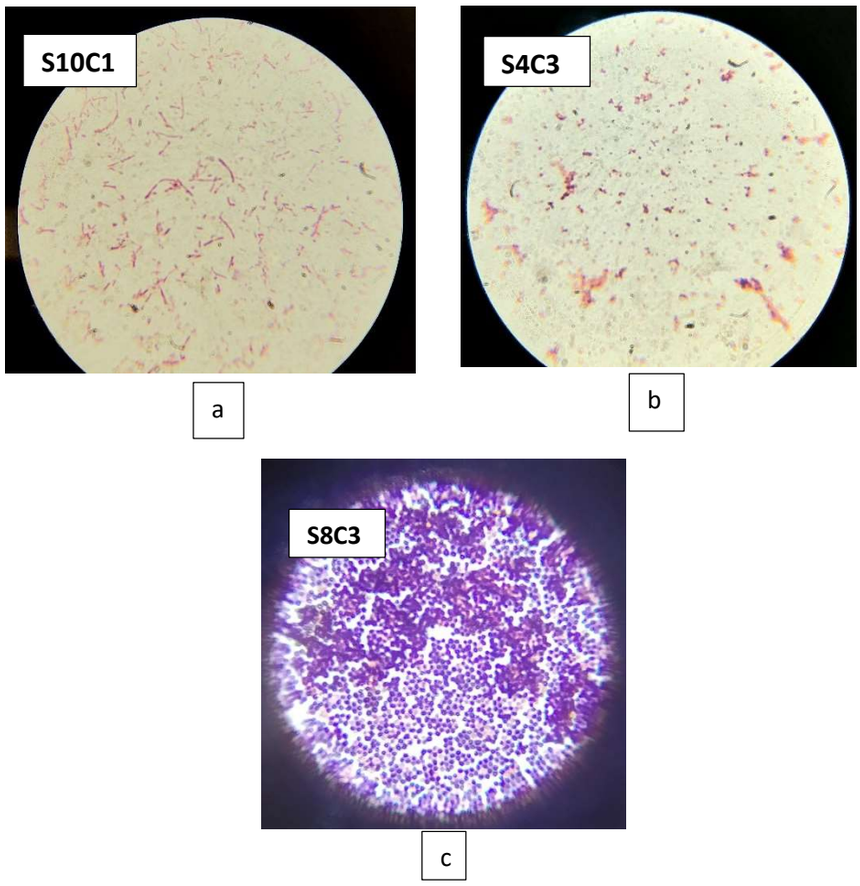


Figure 35: Gram staining of amylase producing bacteria (a) Gram negative rods (b) Gram negative cocci (c) gram positive cocci. S10C1 was tentatively identified as *staphylococcus sp*. And S4C3, S8C3 were tentatively identified as *streptococcus sp.*

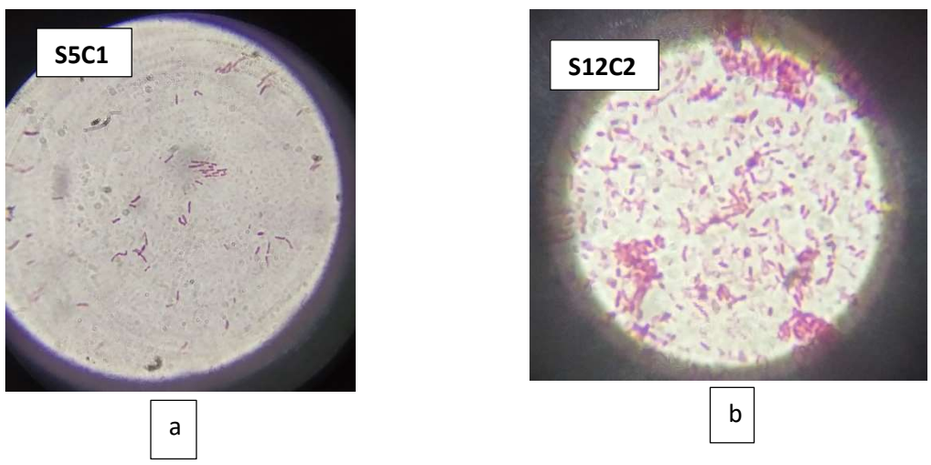


Figure 36: Gram staining of xylanase producing bacteria (a) Gram negative rods (b) Gram negative rods. S5C1 and S12C2 were tentatively identified as *Staphylococcus sp*. and *Vibrio sp*.

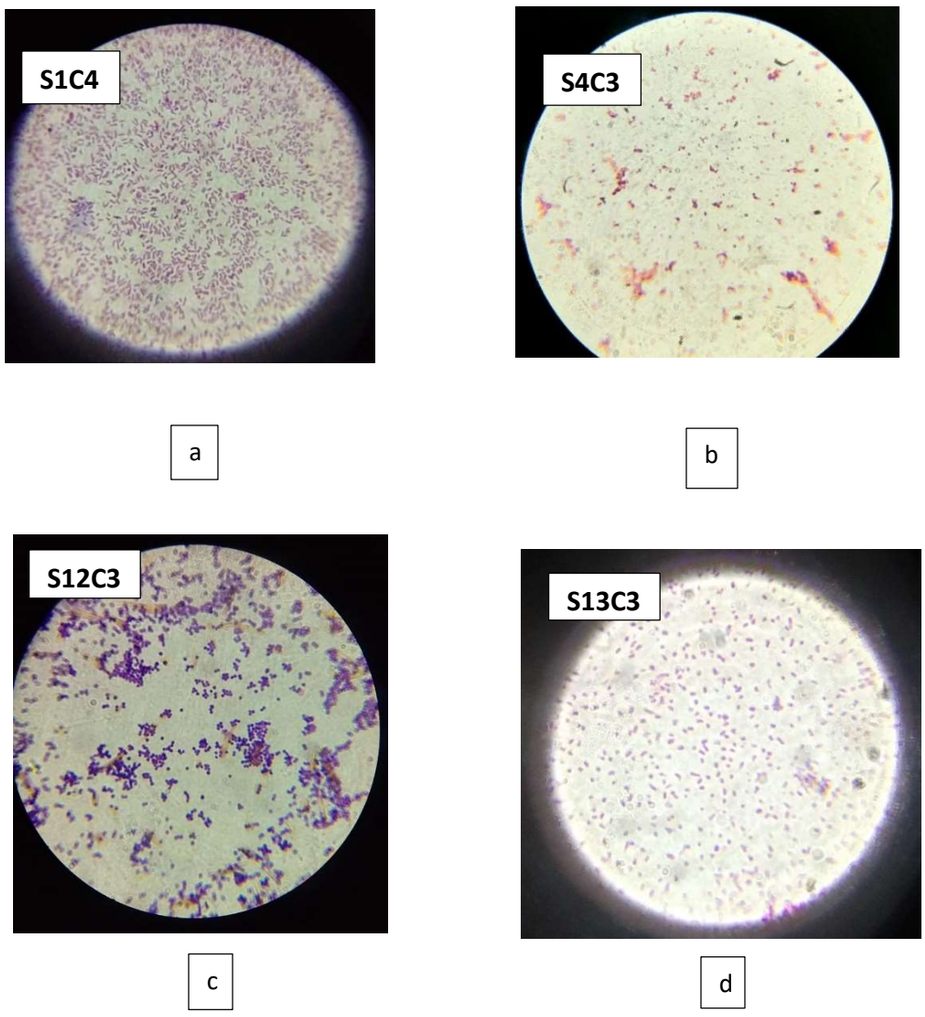


Figure 37: Gram staining of agarase producing bacteria (a) Gram positive rods (b) Gram negative cocci (c) Gram positive cocci (d) Gram positive cocci. S1C4, S4C3, S13C3 were tentatively identified as *Micrococcus sp.*, *Streptococcus sp*., and *Staphylococcus sp.*

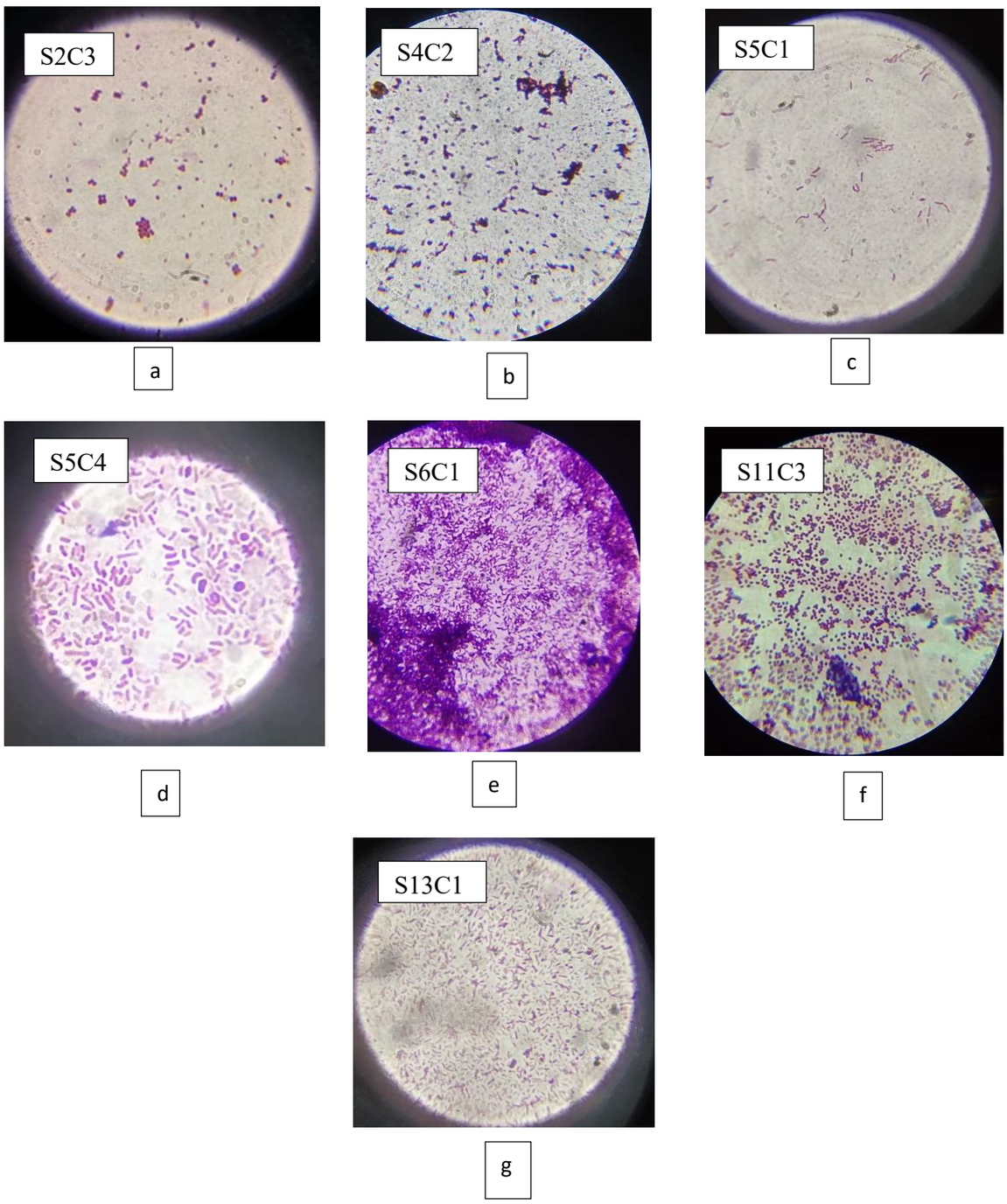


Figure 38: Gram staining of protease producing bacteria (a) Gram negative cocci (b) Gram positive cocci (c) Gram negative rods (d) Gram positive rods (e) Gram positive rods (f) Gram positive cocci (g) Gram positive rods. S4C2 and S5C1 were tentatively identified as *Pseudomonas sp.* and *staphylococcus sp.*

SUMMARY

Summary

In this study, the seaweed samples were collected from the rocky intertidal regions. Thirteen different types of seaweed were collected, this includes: *Ulva intestinalis, Sargassum sp, Sphacelaria sp., Padina tetrastromatica, Ulva lactuca, Padina boergesenii, Acrosiphonia orientalis, Gracilaria sp., Colpomenia sinuosa, Dictyopteris acrostichoides, Gracilaria foliifera, Amphiroa sp and Scinaia sp.* The protein concentration in the seaweeds were estimated by Folin-Lowry method. The concentration of protein was highest in *Padina tetrastromatica and Acrosiphonia orientalis that is* 305.8 and 372.8 µg/ml respectively. The bacteria associated with the seaweeds were isolated and screened for the production of enzymes like amylase, xylanase, cellulase, agarase and protease which have a good demand in various industries. Screening for the production of enzymes was performed on sixty-five bacterial isolates that were associated with the seaweeds. S4C3, S10C1 and S12C3 isolates showed the highest zone of clearance on the starch agar plates indicating that these bacterial isolates have a great potential for the production of amylase enzymes and could be used in amylase production at commercial level. Bacterial isolate S5C1 and S12C2 showed positive results for the production of xylanase. S1C4, S4C3, S12C3 and S13C3 colonies showed the zone of clearance and the maximum depression formed on the media contain agar as the sole source of carbon. S1C4, S4C3, S12C3 and S13C3 were used to determine the seaweed waste degradation. The isolate S4C3 had the maximum concentration of released sugar indicating its potential degradation of seaweed. Bacterial isolates S2C3, S4C2, S5C1, S5C4, S6C1, S11C3and S13C1 were selected for the degradation of dairy waste. Among these, maximum absorbance was seen in bacterial isolates S6C1, S5C1, S5C4 and S13C1 with an increase in the concentration of 12.5, 10.8, 12.1 and 15.5 µg/ml. S10C1, S8C3, S13C3 and S5C1 were tentatively identified as *Staphylococcus sp*. S1C4, S4C3, S12C2 and S4C2 were tentatively identified as *Micrococcus sp., Streptococcus sp., Vibrio sp*., *and Pseudomonas sp.* Data acquired from the biochemical tests was insufficient for the tentative identification of other bacterial isolates.

Conclusion

The coast of Goa harbors a vast variety of seaweed species that are rich in the nutritional content and they host diverse groups of microorganisms. The concentration of protein was highest in *Padina tetrastromatica and Acrosiphonia orientalis that is* 305.8 and 372.8 µg/ml respectively. In many parts of the world the seaweeds are used as fertilizer and food source. Hence the study of seaweeds chemical composition is very essential to understand their nutritive importance. The bacteria associated with the seaweeds showed good potential for the production of enzymes like amylase, xylanase, cellulase, agarase and protease which have a good demand in various industries. Screening for the production of enzymes was performed on sixty-five bacterial isolates that were associated with the seaweeds. S4C3, S10C1 and S12C3 bacterial isolates that were associated to seaweeds *Padina tetrastromatica, Dictyopteris acrostichoides* and *Amphiroa sp* respectively indicated production of amylase enzymes. Bacterial isolate S5C1 and S12C2 that were isolated from the seaweed *Ulva lactuca and Amphiroa sp* respectively showed positive results for the production of xylanase. Agarase production was seen in S1C4, S4C3, S12C3 and S13C3 colonies that were isolated from seaweeds *Ulva intestinalis, Padina tetrastromatica, Amphiroa sp* and *Scinaia sp* respectively. The isolate S4C3 had the maximum concentration of released sugar indicating its potential degradation of seaweed. Bacterial isolates S6C1 isolated from *Padina boergesenii*, S5C1and S5C4 were isolated from *Ulva lactuca* and S13C1 isolated from *Scinaia sp* can be used for the degradation of dairy waste. Therefore, it can be concluded that the bacteria associated with the marine seaweeds have a great potential for the production of various industrially important enzymes and also, they can be used in the process of bioremediation.

FUTURE PROSPECTS

Future Prospects

- Carbohydrate and lipid contents of different seaweeds can be estimated and compared.
- Antibacterial activity of different seaweed extracts on the pathogenic bacteria can be determined.
- Seaweeds and associated epiphytic bacteria can be screened for omega-3 fatty acids.
- Fungi associated with different seaweeds can be isolated and studied.

APPENDIX

Appendix 1

1) Solid media

- **Zobell marine agar**

Composition	g/L
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000
pH (at 25°C)	7.6±0.2

55.25 g is suspended in 1000 ml distilled water. Dissolve the media completely by heating.

Autoclave at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour in sterile Petri plates.

- **Zobell marine broth**

Composition	g/L
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
pH (at 25°C)	7.6±0.2

40.25 g is suspended in 1000 ml distilled water. Dissolve the media by heating. Autoclave at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

- **Seawater based agar**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670
Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660
Sodium bicarbonate	0.180
Agar	20.00
Final pH (at 25°C)	7.5±0.5

Suspend in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in sterile Petri plates.

- **Artificial seawater-based starch agar plates (2% starch)**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670
Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660
Sodium bicarbonate	0.180
Agar	20.00
Starch	20.00
Final pH (at 25°C)	7.5±0.5

Suspend in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in sterile Petri plates.

- **Artificial seawater-based starch agar plates (1% xylan)**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670
Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660
Sodium bicarbonate	0.180
Agar	20.00
Xylan	10.00
Final pH (at 25°C)	7.5±0.5

Suspend in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in sterile Petri plates.

- **artificial seawater-based with 2% agar**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670
Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660

Sodium bicarbonate	0.180
Agar	20.00
Final pH (at 25°C)	7.5±0.5

Suspend in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in sterile Petri plates.

- **artificial seawater-based with 2% CMC (catrboxymethyl cellulose)**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670
Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660
Sodium bicarbonate	0.180
Agar	20.00
CMC	20.00
Final pH (at 25°C)	7.5±0.5

Suspend in 1000 ml distilled water. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour in sterile Petri plates.

- **Skim milk agar**

Composition	g/L
Sodium chloride	24.600
Potassium chloride	0.670

Calcium chloride 2H2O	1.360
Magnesium sulphate 7H2O	6.290
Magnesium chloride 6H2O	4.660
Sodium bicarbonate	0.180
Agar	20.00
Skim milk	20 ml
Final pH (at 25°C)	7.5±0.5

Appendix 2

- **1% congo red solution**

Congo red	10g
Distilled water	1000ml

- **1M NaCl solution**

NaCl	55.8g
Distilled water	1000ml

- **Lugol's iodine**

KI	10 g
I_2	50g
Glacial acetic acid	100ml

Make up the total volume upto 1000ml

- 3**,5-Dinitrosalicylic acid (DNSA) reagent**

NaOH	2g
Crystalline phenol	0.2g
DNSA	0.2g
Distilled water	100g

- **Sodium potassium tartarate solution**

  Sodium potassium tartarate 33g

  Distilled water 100ml

- **Folin -lowry reagents**

  Reagent A: 2% Na_2CO_3 in the 0.1M NaOH

  Add 0.4g NaOH in 100ml distilled water. Then add 2g Na_2CO_3 to it and mix well.

  Reagent B: 0.5% copper sulphate in 1% sodium potassium tartarate

  Add 0.1g sodium potassium tartarate in 10ml distilled water. Dissolve and add 0.05g of copper sulphate in the prepared solution.

  Reagent C: Alkaline copper solution

  Mix 50 ml reagent A and 1 ml of reagent B.

  Folin ciocalteu: 1ml of folin ciocalteu reagent in 1 ml distilled water.

- **(10X) 100ml Phosphate buffered saline**

  Na_2HPO_4 1.78g

  KH_2PO_4 0.24g

  NaCl 8g

  KCl 0.2g

  Adjust the volume to 100ml. to prepare 1X PBS take 10ml from 10X and add 90 ml of Milli-Q water.

Biochemical tests

- **Saline (0.85%)**

  NaCl 0.85g

  Distilled water 100ml

- **Methyl red (for MR test)**

Methyl red	0.2g
Ethanol	600ml
Distilled water	400ml

- **Peptone water (sugar fermentation)**

Composition	g/L
Peptic digest of animal tissue	10
NaCl	5
1% phenol red	10ml
Final pH	7.2±0.2

- **Motility agar**

Composition	g/L
Nutrient broth	100ml
pH	7.4
agar	0.4

- **Glucose phosphate broth (MR-VP)**

Composition	g/L
Peptone	7
Dipotassium phosphate	5
Dextrose	5
pH	7.4±0.2

- **Tryptone broth (indole test)**

Composition	g/L
Tryptone	1
NaCl	0.5
Distilled water	100ml

pH	7.2±0.2

- **Hugh leifson medium**

Composition	g/L
Peptone	2
Sodium chloride	5
Dipotassium phosphate	0.3
Glucose	10
Bromothymol blue	0.03
Agar	3
pH	7.1±0.2

20.33 g were suspended in 1000ml distilled water. Heat to boil to dissolve the medium completely. Dispense into test tubes and sterilize by autoclaving at 15lbs for 20 minutes. Cool.

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