Isolation of Seaweed Associated Bacteria for Potential Enzymes and Application of Agarase Enzyme in Valorisation of Agar Waste

A Dissertation Report for

Course code and Course Title: MID Dissertation

Credits: 8

Submitted in partial fulfilment of Master's Degree

in MSc Microbiology

by

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled "Isolation of Seaweed Associated Bacteria for Potential Enzymes and Application of Agarase Enzyme in Valorisation of Agar Waste'' is based on the results of investigations carried out by me in the Microbiology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Mentorship of Dr. Lakshangy Charya 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 in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Isolation of Seaweed Associated Bacteria for Potential Enzymes and Application of Agarase Enzyme in Valorisation of Agar Waste"** is a bonafide work carried out by **Ms. Nikita Feliciana Mendes** under my mentorship in partial fulfilment of the requirements for the award of the degree of **Master's of Science** in the Discipline Microbiology at the, School of Biological Sciences and Biotechnology, Goa University.

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ACKNOWLEDGEMENT

Foremost, I greatly acknowledge the precious blessings that I have received from the Almighty God. I am thankful to my parents for being a major support in this study

The completion of this work would not have been possible without the expertise of my dissertation guide, Dr. Lakshangy Charya, Program Director, Microbiology Discipline, SBSB, Goa University, who guided and supported me throughout the study. Your teachings and advice are truly priceless.

I sincerely thank Dr. Vijaya Kerkar, Professor of Botany and Dr. Megnath Prabhu, Program Director, Biotechnology Discipline, SBSB Goa University for their help and support in crucial parts of my study. My sincere thanks to Dr. Savita Kerkar, Dean of SBSB, for her thoughtfulness and support during the study.

I express my sincere thanks to all the faculty members of Microbiology Discipline; Professor Sandeep Garg, Dr. Milind Naik, Dr. Bhakti Salgaoncar, Dr. Lata Gawde, Dr. Judith Noronha and Dr. Trupti Asolkar for their help and support in this period of study.

My special thanks to all the non-teaching and office staff of Microbiology Discipline for their endless support and generous help at every stage of my work.

I am thankful to the research fellows for their help, advice and guidance during my study. A big thankyou to all my friends for supporting me and motivating me throughout the journey.

LISI OF ABBREVIATIONS

AHL	N-acyl homoserine lactone
ASW	Artificial Seawater
BSS	Basal Salt Solution
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic Acid
DNSA	Dinitrosalycyclic acid
IU	International Units
KOH	Potassium hydroxide
MR-VP	Methyl Red Vogues Proskauer
NA	Nutrient Agar
NB	Nutrient Broth
NaCl	Sodium Chloride
[NaCl]	Concentration of Sodium Chloride
PET	Polyethylene Terephthalate
PSU	Practical Salinity Unit
ZMA	Zobelle Marine Agar
ZMB	Zobelle Marine Broth

STANDARD ABBREVIATIONS

%	Percentage
µ	Micro
g	Gram
hrs.	hours
L	Liter
M	Molar
mg	Mili-gram
min	Minutes
ml	Mili-liter
mm	Millimeter
ºC	Degree Celsius
R. T	Room Temperature
U	Units
α	Alpha
β	Beta

INDEX

1. INTRODUCTION AND LITERATURE SURVEY

1.1Introduction

Seaweeds are a diverse group of macro-algae, and are classified into mainly red, green and brown seaweeds. They are mainly found in the marine environments such as seas and oceans. They are factories of several structural and reserve polymers such as carbohydrates, proteins, lipids. Hence are gaining importance as food in many countries (Mišurcová, 2011)

As seaweeds are a source of variety of nutrients, they harbor a consortium of organisms including micro-organisms such as bacteria, fungi, algae, protozoa, spores and also larvae of marine invertebrates. Bacteria forms a predominant group that is associated with seaweeds. Their symbiotic association is mutually benefitting each other. Bacteria take the carbon and other nutrients from seaweeds and in return play an important role in growth and morphogenesis of seaweeds (Singh & Reddy, 2014; Juhmani et al., 2020))

A material which is unwanted, unusable and is discarded after primary use, is defective and is termed as waste. There are different types of wastes which we generate and most of it ends up in the environment, as non-biodegradable and causing harm to it. The alarming rate of increasing amount of waste has a large impact on the environment which leads to issues such as pollution, littering, loss of biodiversity, etc.

In any science laboratory a large amount of waste is generated while working. It can be physical, biological and/or chemical waste. The proper disposal of this waste is very important to avoid hazards. Different wastes have different methods of disposal. Improper disposal can be harmful to the environment, the plants, animals and even to humans.

Agar-agar is used in the Microbiology laboratory as a solidifying agent for the preparation of media. After cultivation of microorganisms the used media is decontaminated and then discarded. The discarded media goes into the environment, further the nutrients present in it may find its way in the water bodies, enriching the nutrient content of water bodies. This may lead to eutrophication of water bodies and boost the growth of plants and harmful alga. Also, after decontamination, the properties of agar get wasted.

Valorization of waste in simple terms means, adding worth to the material which we usually regard as waste and discard. It is the method of converting them into more useful products such as

chemicals or sources of energy by recycling, reusing or composting them. This topic is of major importance now, because it can help to overcome the global problems such as depletion of natural resources and non-renewable sources due to their continuous utilization.

The seaweed associated bacteria produce a variety of enzymes to breakdown the polymers of the seaweeds ,such as agarase, amylase, cellulase, protease, esterase, lipase, chitinase, alginate lyase, carrageenase, pectinase, pullulan hydrolase, β- glucanase, laminarase and hence become industrially important(Jonnadula & Ghadi, 2011 ;Naik et al., 2019).

These bacteria producing different enzymes can be harnessed for different purposes and one of it could be valorization of waste. This waste includes the one which is made up of polymers which can be degraded by the bacteria, such as protein containing dairy waste, starch containing potato peel waste, agar waste, cellulose and xylan containing wood waste(Veerakumar & Manian, 2022).

Literature Survey

1.2 Seaweeds

Algae are the unicellular, eukaryotic, photosynthetic organisms present in environments such as rivers, lakes, ponds, oceans. Based upon their morphological characteristics, they can be divided into two groups; Macro-algae and microalgae. Macroalgae includes the seaweeds which consists of three main categories , the red algae belonging to Rhodophyceae, the green algae belonging to Chlorophyceae and the brown alga belonging to Phaeophyceae (Pradhan et al., 2022). The classification is mainly based on the pigment they produce, the polysaccharides it reserves and their cell wall nature (Salehi et al., 2019) as described in the Table 1.1.

Seaweeds lacks the true root, stem and leaf structures. The entire body of seaweed is called a thallus. The leaf-like flattened structure is called the lamina, stem-like structure is the stipe. The air bladder or the float, is an air-filled structure which helps the seaweeds to float in water and it consists of root like structures called holdfast which anchors it to the surface, its primary role is attachment rather than acquiring nutrients *(*Encyclopedia Britannica, March 2023) (Figure 1.1).

In the oceans, seaweeds are the primary producers supporting the marine biodiversity on a large scale and are well known environmental stress indicators such temperature stress, desiccation, competition with coastal fauna which will widely affect the distribution of seaweeds(Juhmani et al., 2020)

They are also a reserve and producer of various secondary metabolites like gums and resins which have industrial importance and are a very rich source of proteins , carbohydrates , fatty acids hence are consumed as food in Asian countries (Salehi et al., 2019). The nutrient composition of different seaweeds is given in Table 1.2 (Mathew et al., 2018), (Mišurcová, 2011)

Table 1.1: Classification of Seaweeds

Criteria	Chlorophyceae	Rhodophyceae	Phaeophyceae
PIGMENT			
1.Cholorophyll	Chl a, b	Chl a	Chl a, c
2.Carotenoids	beta-carotene, lutein violaxanthin, zeaxanthin neoxanthin	beta-carotene, lutein zeaxanthin	beta-carotene, fucoxanthin violaxanthin
3.Phycobiliproteins	-	Phycocyanin, Phycoerythrin	-
POLYSACCHARIDES			
1.Storage	Starch	Floridean starch	Laminarin
2.Structural (Cell wall)	Ulvan, Xylan, Mannan Cellulose	Carrageenan, Agar Xylan, Mannan Cellulose	Alginate, Fucoidan Cellulose
Examples	*Ulva intestinalis, Ulva linza, Ulva rigida, Codium bursa, Cladophora rupestri, Parsiolla carophyla*	*Chondrus crispus, Gelidium pusillum, Gracilaria gracillu,s Palmaria palmata, Rhodochorton purpureum*	*Fucus serratus , Laminaria hyperborean, Laminaria digitata, Padina pavonia Petalonia fascia*

Fig.1.1: Structure of seaweed (Source: Encyclopedia Britannica, March 2023)

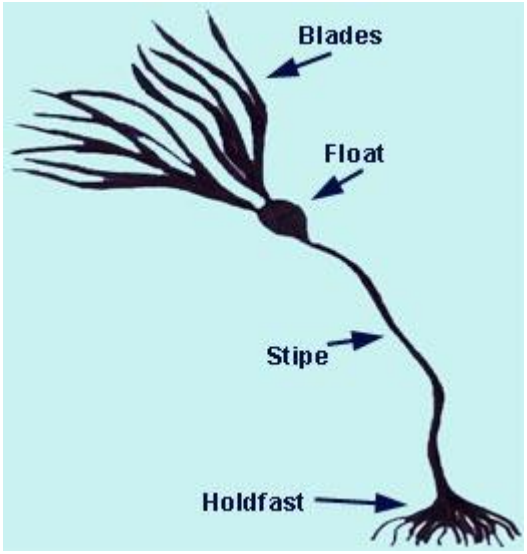


Table 1.2: Nutrient composition of different seaweeds

Nutrient Composition		Chlorophyceae	Rhodophyceae	Phaeophyceae
1.Proteins		10-30%	15-20%	5-16%
2. Lipids		alpha-linolenic acid, cholesterol, methylene cholesterol and β-sitosterol	eicosapentanoic acid, docosahexaenoic acid, desmosterol, cholesterol, sitosterol, fucosterol and chalinasterol	eicosapentanoic acid, docosahexaenoic acid, fucosterol, desmosterol,
3.Minerals		Calcium, iodine, iron, potassium, phosphorus, manganese		
4.Vitamins	**Water - soluble**	Vitamin B12, Vitamin C	Vitamin B, Riboflavin, Thiamine	Vitamin B Riboflavin, Thiamine, Vitamin C,
	Fat soluble	Vitamin E, β-carotene, lutein, violaxanthin zeaxanthin neoxanthin	Vitamin E, β-carotene, lutein, zeaxanthin	β-carotene, fucoxanthin, violaxanthin
5.Dietary fibers	**Water-soluble**	Starch in the chloroplast	Floridean starch granules outside plastids	Laminarin as oil Droplets
	Water-Insoluble	Ulvan, Xylan Mannan, Cellulose	Carrageenan, Agar, Xylan Mannan, Cellulose	Alginate, Fucoidan Cellulose

1.3 Chemical composition of seaweed polymeric substances

Proteins

The concentration of protein in seaweeds differs depending upon the species and environmental conditions. Reports suggest that the protein concentration is high in green and red seaweeds and the average value goes up to 10 – 30% of the dry weight. In *Palmaria palmata* and *Porphyra tenera* the red seaweeds, proteins range from 35 to 48% of dry weight and in brown seaweed, the protein content is generally low between 1-5 %. In concern with consumption of seaweeds as food, the three amino acids alanine, glutamic acid, and glycine are responsible for addition of taste to the

seaweeds. Photosynthetic pigment proteins such us phycobiliproteins mainly from seaweeds have antioxidant activities. Other proteins extracted from seaweeds are known to be bioactive compounds having many medical properties (Mišurcová, 2011).

Carbohydrates

These are the main chemical components of seaweeds which act like signatures for their identification and classification. They are mostly polysaccharides, branched or linear or monosaccharide with alcohol groups or sulfated or non-sulfated. The polysaccharide content makes the seaweeds of high commercial value(Salehi et al., 2019)

a. Agar

These are the structural polysaccharide present in the red algae. It is mainly found in the *Gelidium* and *Gracilaria* . It consists of two components, agarose and agaropectin, with agaropectin in abundance. Agarose is a polymer of the repeating unit [-d-galactose-β-(1→4)-3,6-anhydro-l-galactose-α-(1→3)-] and is mainly responsible for the agar gelling properties, while agaropectin is formed from the repeating units of [-d-galactoseβ-(1→4)-l-galactose-α-(1→3)-]. Due to its gelling properties , it is used as a food additive(Tawara et al., 2015)

b. Carrageenan

These are sulfated structural polysaccharides present in red seaweeds. They are mostly extracted from *Eucheuma* and *Kappaphycus.* They are linear chains of repeating sequence of [-β-d-galactose-(1→4)-3,6-anhydro-α-d-galactose-(1→3)-]. The presence of one sulfate group at C-4 position of β-d-galactose unit makes it kappa carrageenan and at C-2 position of 3,6-anhydro-α-d-galactose unit makes it iota carrageenan. As they can for thermo-reversible gels they are of high commercial value in pharmaceutical, cosmetic and food industries. Moreover , the red seaweeds are a source of other carbohydrates such as floridean starch , cellulose , xylan and mannan(El-Said & El-Sikaily, 2013)

c. Ulvan

These are the polysaccharides from green seaweeds and are sulfated. It consists of rhamnose and xylose sulfated with glucuronic acid and iduronic acids and glucose. The glycosidic linkages are α-l-Rhamnose and β-d-glucuronic acid (1→4), α-l-Rhamnose and α-l-iduronic acid (1→4). It is highly sulphated. Due to the presence of a rare sugar rhamnose, they are of commercial importance in food industry for the production of flavored compounds. Depending on their sulphation degree they also have antioxidant and anticoagulant properties (Gonzalez et.al.,1996).

d. Alginates

These are the structural polysaccharides present in brown seaweeds. These seaweeds are flexible due to the presence of mannuronic and glucuronic acids in alginates. They are non-sulphated rather each monomer has a terminal carboxylic group. In higher percentage these are present in, *Laminaria hyperborean, Saccharina japonica, Macrocystis pyrifera,* and *Laminaria digitata.* Because of their non-toxic gelling properties, they have applications in food industry as a food additive and also in pharmaceutical and cosmetic industries (Li et.at.,2021).

e. Fucoidan

These are structural polysaccharides present in brown seaweeds which are sulfated. There are mainly present in *Ascophyllum nodosum*, and *Undaria pinnatifida.* It consists of glycosidic linkages type I: L-Fucose (α-1→3) and type II: L-Fucose alternating (α-1→3 and α-1→4). Fucoidan makes the seaweed flexible which helps them to overcome strong oceans. It has anticoagulant, antimicrobial, and antitumor activities (Hakim et.al.,2020).

f. Laminarin

These are storage polysaccharide present in brown seaweeds. It consists of D-glucose, linked by (β-1→3) glycosidic linkages. These are mainly present in *Fucus spiralis* and in *Laminaria hyperborea*. Laminarin content in seaweeds is specifically high in winter season and as it a storage polysaccharide it is produced during the growth phase of the seaweed. It lacks gelling abilities but have antitumor and anticoagulant action (Li et.at.,2021).

1.4 Seaweeds of Goa

The state of Goa is well known for fishing and farming, it has a coastline of 120 km, which is segmented into beaches, creeks, bays, cliffs, reefs. There are seven estuaries, three bays, sandy and rocky beaches are also found. Along the Goan coast, the seaweeds occur in intertidal zone and beyond the tidal zone. Dhargalkar, et al in 1981 reported 74 seaweed species in Goa. As of now 146 species of marine algae have been reported in Goan coast which includes 41 species of green, 64 of red and 40 of brown algae. New 70 species of seaweeds have been recorded (Pereira and Almeida, 2018)

The most dominant seaweed found in Goa is the *Sargassum* along the coastline of Anjuna beach. Different seasons in Goa supports the widespread of different seaweeds. Red seaweeds such as *Porphyra* flourish during the months from October to December, whereas *Gelidium* grow from the months of November to April (Cordiela, 2020)

The importance of seaweeds is not known to the local Goans, so the fisherman just discards the seaweeds what comes in the fishing net along with the fish. As the global demand for seaweeds is increasing for various uses, it is important to spread awareness about seaweeds throughout Goa. Goan researchers aim to set up seaweed farming projects in Goa as they believe that seaweed farming can mitigate the loss of biodiversity and be a future source of food security.

1.5 Seaweed associated bacteria

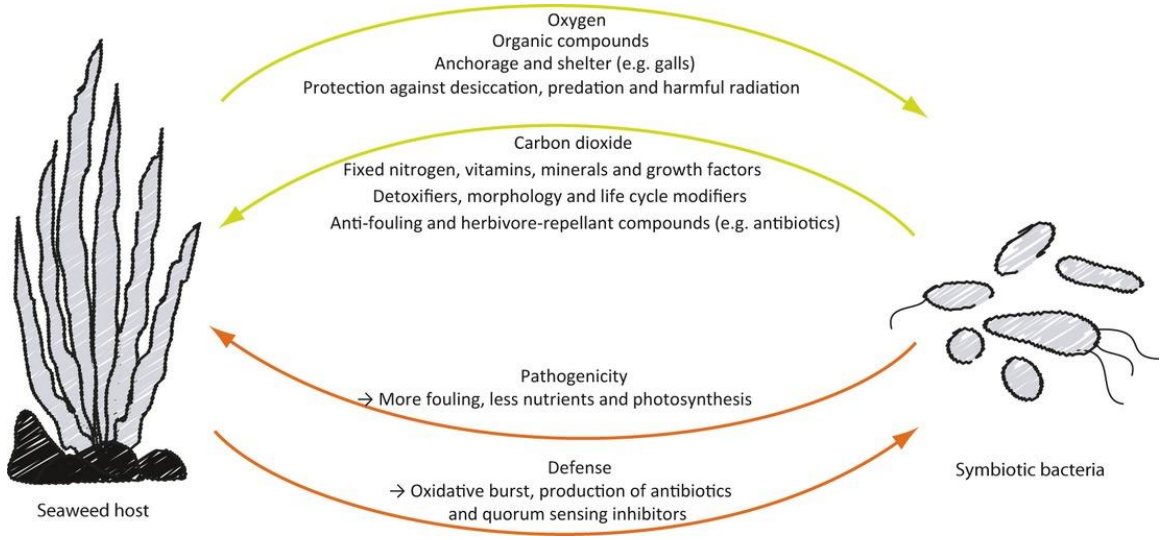
Seaweeds are the factories of various polymers and organic compounds and as the seaweed surface is in continuous exposure to the environment it becomes an attractive substratum for the settlement of microorganisms and formation of microbial biofilms. These surface will support the growth and development of the microbes (Juhmani et al., 2020).

The biofilms consist of consortium of microorganisms including the bacteria , fungi , protozoa , algae (Singh & Reddy, 2014). The bacteria can be epiphytic- on the surface or endophytic – inside the cytosol. The seaweed associated bacteria vary from species to species, most commonly found are *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria* and *Gammaproteobacteria* and representatives of the *Bacteroidetes* and *Planctomycetes*. (Meusnier et al.2001). This is a

symbiotic association wherein the bacteria as well the algal partner (the host) benefit from each other (Figure1.2). This association can be of different types such as mutualism; wherein both the partners benefit from each other, Commensalism ; wherein one partner benefits from the other partner which is not harmed nor benefitted , Parasitism : This is a negative relationship wherein one partner is benefitted and the other partner is harmed, some macro-algae produce compounds which can be harmful to the bacteria and also some bacteria can be parasitic to the algae(Menaa et al., 2020).

The bacterial community also differs due to different seasons and different stages of life cycle of the host (Laycock, 1974; Sakami, 1996; Singh, 2013). Different bacterial communities are present on different parts of the seaweed thallus. This is caused due to lack of vascular connections in the thallus which leads to inefficient translocation of resources. The parts of rhizoid are in association with other organisms and the substrate surrounding it which leads to differentiation of bacteria from other parts. Due to generation of water currents the long old phyloid moves in the water which causes mechanical stress leading to algal tissue damage making it more susceptible to bacterial decomposition and creating a new niche for more bacterial communities (Singh & Reddy, 2014).

Fig 1.2 Symbiotic association between seaweed and bacteria (Source: Holland et. al.,2012)



1.6 Benefits of seaweed-bacterial association

As seaweed are a good source of various polysaccharides , the bacteria utilize the agar, carrageenan, alginate, fucoidan, laminarin, cellulose, and pectin as carbon sources for its colonization(Menaa et al., 2020).

The normal morphological development and growth of seaweed is largely influenced by associated bacteria by providing CO2 , fixed nitrogen required for its photo-autotrophy, survival and growth(Menaa et al., 2020). The formation of biofilm by bacteria on seaweed surface provide a substrate for the settlement of other microorganisms and also eukaryotes such as phytoplankton, intertidal algae and larvae. It also enhances the algal zoospore settlement which is the initial event in the life cycle of seaweed and reports suggest that mixed microbial communities in a biofilm increase the zoospore settlement to a higher rate(Singh & Reddy, 2014).

The associated bacteria also produce plant growth promoting substances such as auxins, indole acetic acid, cytokinin and vitamins which help in the morphogenesis of the seaweeds. The bacteria also produce exopolysaccharides which help the marine bacteria and other organisms to remain aggregated enhancing the biofilm formation. Bacteria are involved in degradation of toxins from surrounding environments and protect the seaweeds against it.

Several Gram-negative bacteria are found to express signaling cascades Lux R and Lux I for the expression of N-acyl homoserine lactone (AHL). AHL is known to regulate to expression of genes involved in biofilm formation, antibacterial activity and motility etc. This bacteria in association with seaweeds produce AHL which have importance in zoospore settlement, this is mainly reported in *Ulva*. It is involved in carpospores settlement *Gracilaria* and *Acrochaetium* species (Singh & Reddy, 2014)

1.7 Enzymes produced by Seaweed Associated Bacteria

Seaweed associated bacteria produce a variety of industrially important enzymes such as amylase, agarase , cellulase, protease, xylanase, carrageenase , esterase , lipase ,beta-galactosidase , laminarase, pullulan hydrolase , alginate lyase , chitinase (Jonnadula & Ghadi, 2011) which enable them to act on and solubilize the polymers present in seaweeds, utilize it as a carbon source and hence contribute to biogeochemical cycling such as , carbon , nitrogen and sulphur cycle (Naik et

al., 2019).These enzymes from marine environment are more stable in having certain properties such tolerance to pH and salinity , thermo-stability, barophilicity and tolerance to extreme conditions . These properties arise from the conditions of the marine environment such as high salinity , low temperature and high pressure and high tides(Wijaya et al., 2021).

Enzymes obtained from marine sources perform multiple functions, act on different components and are used in a variety of applications. Proteases act on the proteins and make up 60% of the total enzymes, they have application in detergent, leather and in pharmaceutical for its anti-inflammatory functions(Parte et al., 2017). Lipases are the most important hydrolases specially in the detergent industry, they are used in the food flavoring, paper, cosmetic and organic synthesis. Esterases can be used to hydrolyze the polyethylene and degradation of plastics, esters synthesis and used in food industry. Many enzymes such as cellulase , xylanase , lipases , esterases are involved in the deinking process(Parte et al., 2017).

1.8 Application of enzymes in valorization of waste

The waste rich in proteins, carbohydrates, lipids can be hydrolyzed enzymatically to produce products of higher value. A proteinase , keratinase can hydrolyze keratin protein hence can be used for hydrolysis of feather and waste of poultry which is rich in keratin (Kumar et al., 2015). Degradation of starch-based waste by enzyme pullulan hydrolase isolated from microorganisms has been reported. It acts on the α-1,4- and α-1,6-glucosidic linkages of the starch under industrial conditions. It was reported that this enzyme can be used to degrade the food waste such as damaged corn grains, damaged wheat grains for the production of maltose and glucose syrups(Rashid et al., 2018). The Municipal solid waste contain 40-50% cellulose, hence the bacteria producing cellulase enzyme can used it for its growth , also a large amount of agricultural waste such as cassava peels , yarn peels , rice straw can be degraded using cellulase enzyme and can be converted into value-added products such as bio-ethanol and bio-fuel (Khan et al., 2016). Bacteria *Bacillus amyloliquefaciens* XR44A has been found to produce enzymes cellulase and xylanase which can be used in the saccharification of brewers spent grain and bioconversion of lignocelluloses to fermentable sugars (Amore et al., 2015). To overcome the increasing global plastic burden, micro-organisms can play an important role. Microbial enzymes such as, esterase and lipases can be used for degradation of synthetic polymers such as Polyethylene tetra phthalate (PET) As reported by

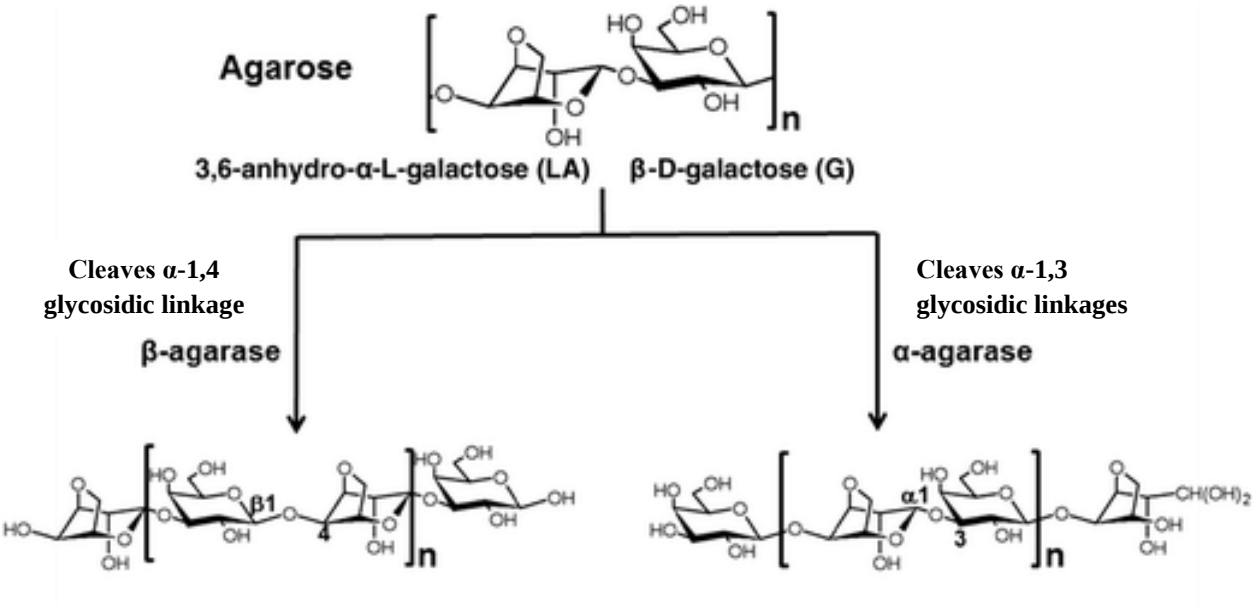
Tamoor et al. (2021) *Ideonella sakaiensis* 201-F6, *Bacillus* and *Escherichia* are reported to express enzyme PETases and can utilize PET. Several food waste and microbiological waste contain polymers like carrageenan and agar , such wastes can be degraded by the enzymes such as carrageenase and agarase produced by bacteria (Naik et al., 2019).

1.9 Agarase enzyme and its mode of action

Agarase is the enzyme involved in, the degradation of carbohydrate agar. The system name of agarase enzyme is agarose 4-glycanohydrolase. This can be extracted from many agarolytic bacteria which uses agar as the main source of carbon. This is the first enzyme used in the agar catabolic pathway. Agar structure is basically made up of β-D-galactose and 3,6-anhydro-α-L-galactose.

Depending upon the mode of action or the cleavage site there are two types of agarase : α-agarase and β-agarase, The former acts on the α-1,3 linkages of agarose and cleaves it to yield agaro-oligosaccharides i.e. anhydro-L-galactose at the reducing end and the latter identifies the β-1,4 linkages of agarose and cleaves to produce neoagaro-oligosaccharides of series related to neoagarobiose (Naik et al., 2019)(Figure1.3)

Fig 1.3 Mode action of α agarase and β agarase (Jung et. al., 2017)



1.10 Bacteria producing agarase

Agarase enzyme is produced by various bacteria to breakdown agar and utilize it as the sole source of carbon and energy and arise in different ecological niches. Hinojosa et. al. (2018) reported a bacterium from genera *Pseudoalteromonas f*ound from seaweeds. Bacteria *Ammoniibacillus agariperforans* was isolated from the compost had agarase activity (Sakai M et al, 2015). Agarolytic bacteria belonging to genera *Flammeovirga* was isolated from coastal sediments (Liu Y, et al,2019; Khalifa and Aldavel,2019).

Many agarolytic bacteria have been isolated from marine environments such as the marine animal samples, seaweeds and sponges from. It mainly includes the genera *Alterococcus*(Kim et al.,2012) *Alteromonas(Kirimura et al.,1999), Agarivorans*(Du et al.,2011*), Psuedoaltermonas*(Schroder D et al.,2003*), Microbulbifur*(Gonzalez et al.,1997), *Marinimicrobium*(Lim et al.,2006), *Simiduia*(Kim et al.,2012), *Persicitalea*(Yoon et al.,2007), *Vibrio*(Aoki et al.,1990*), Thalassomonas*(Jean et al., 2006) *Saccharophagus*(Kim et al.,20; Cheng et al., 2015). Moreover, *Microbulbifer agarilyticus*, *Psychromonas agarivorans*, and *Marinimicrobium agarilyticum* (Hosoya et al., 2009; Miyazaki et al., 2008; Lim et al., 2006) was isolated from marine sediment samples. A bacterium *Salegentibacter agarivorans* was recovered from a sponge species (Nedashkovskaya et al., 2006; Du et al., 2011). *Bacillus subtills* have been reported to produce beta-agarase extracellularly(Li et al., 2020). The marine organisms such as mollusks which live on seaweeds, thrive on agar, hence their digestive tract contains micro-organisms producing agarase in order to digest the agar , e.g.: from the gut of turban shell *Turbinidae batillus cornutus*(Fu & Kim, 2010)

1.11 Significance of agarase

By expressing agarase enzyme bacteria solubilizes agar and can use it as a carbon source. This helps it adapt to the environment by utilizing any of the waste products containing agar (Parro V, Mellado RP 1994). In the marine environment, seaweeds containing agar in their cell wall can be degraded by agarolytic bacteria and use it as its food. The digestive tract of many marine organisms contain symbiotic agarolytic bacteria for the digestion of agar(Fu & Kim, 2010)

A potential bacterial agarase should have the following characteristics such as, wide tolerance to pH and temperatures, high affinity to substrate and high activity of degradation of agarose. It has a vast application in research field specially in the recovery of DNA bands from low melting point agarose gels. β agarase enzyme has been used by the Takara Company for the production of kits for purification of DNA. It is used in the production of oligosaccharides agaro-oligosaccharides and neo-oligosaccharides. Wang J et al. (2004) has reported bacterial isolate *Vibrio QJH-12* produces agarase enzyme which can hydrolyze agar to form oligosaccharides which has the anti-oxidative activities in inhibiting lipid peroxidation, scavenging superoxide radical and hydroxyl radical. The oligosaccharides have a very important application in the cosmetics, pharmaceutical and food industries because of its many biophysical properties such as antioxidant , anti-inflammatory , anti-bacterial , skin whitening etc. which helps in production of products with improved biological properties. (Jahromi & Barzkar, 2018). Agarase can be used for the degradation of red seaweeds to release important bioactive compounds from them such as vitamins, fatty acids and carotenoids(L.M Morrice et al., 1983) It is used in the enzymatic hydrolysis of large seaweeds to attain single cells or protoplasts (Groleau D *et al*.,1977). These protoplasts help in the cytological and physiological studies (P.S Carlson et sl., 1973)

1.12 Utilization of galactose

Various conversion technologies can be used for the production of bio-ethanol which is the most promising fuel obtained from renewable sources like feedstock. It has gained major importance because it is non-toxic, biodegradable and renewable (S.H Mohd Azar et al.,2017. Bio-ethanol can be produced by yeast *Saccharomyces cerevisiae*, because it can tolerate high concentrations of alcohol and sugar, it is osmotolerant, produces high concentration of ethanol even with low level of by-products, it is easy to handle. (S.H Mohd Azar et al.,2017)

Galactose is the sugar produced by degradation of agar can be used to for ethanol production by yeast. However, ethanol production by glucose is more efficient than that by galactose. But it was reported by Kim et. al. (2014) that a yeast *Saccharomyces cerevisiae* KL7 isolated from soil was significant in production of ethanol by fermentation of galactose as compared to glucose with reduction in fermentation time and higher ethanol productivity. In *Saccharomyces cerevisiae*, the

transport of galactose from its plasma membrane to cytoplasm is catalyzed by enzyme galactose permease.

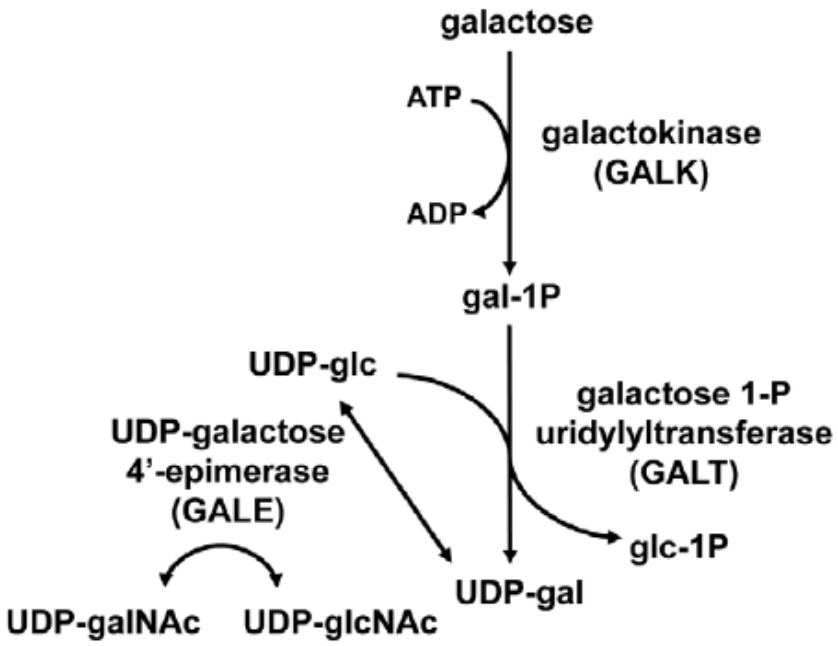
For production of ethanol by fermentation of galactose sugar first the Leloir pathway(Fig 1.3) is used by yeast, wherein it uses five enzymes for the metabolism of galactose to glucose-6-phosphate: galactose mutarotase , galactokinase galactose-1-phosphate uridyltransferase , UDP-galactose-4-epimerase and phosphoglucomutase. Further the yeast finally converts it to ethanol fermentation(Mohd Azhar & Abdulla, 2018).

Apart from conversion to ethanol by fermentation, galactose can also be utilized in the production of D-Tagatose which is a C-3 epimer of galactose. It can be produced chemically or enzymatically. The enzyme L-arabinose isomerase from microorganism (ex. *Thermotoga* species), is involved in conversion of galactose to D-Tagatose. It can be used in many dairy products , it has low caloric value hence many health benefits(Shintani, 2019).

Aureobasidium spp. secretes many hydrolytic enzymes involved in biomass degradation. It is involved in the conversion of waste products containing galactose to lipids. The large amount of agro-industrial waste containing galactose such as whey powder and soy molasses can be used to accumulate high content lipids by yeast *Aureobasidium namibiae*(Wang et al., 2021)

Galactose sugar can be converted into levulinic acid which is an important component of biofuels and biochemicals. This conversion is done using sulphuric acid as the catalyst at a temperature range of 140 to 200 ºC using initial galactose concentrations as 0.05 to1.110 mg and sulphuric acid concentration 0.05 to 1M(Martina et al., 2022).

Fig 1.4 the Leloir pathway of galactose metabolism in yeast (Sanders et. al., 2012)



1.13 Uses of decontaminated agar

Agar is an important component of the many industries carrying out microbiological experiments. After cultivation of micro-organisms, the agar is decontaminated to get rid of microorganisms and it is discarded. All the microorganisms growing on the solid media do not utilize all the nutrients from it , hence by discarding a large amount of nutrients also get wasted even the but the properties of agar get wasted. Hence, there is a need to recycle and reuse the agar for laboratory purposes. As reported, this can be done by physical treatment such as freezing and thawing followed by chemical treatment of acid-alkali, bleaching (Sharma V et al., 2020). The used agar can be recycled and reused as a biofertilizer in laboratory. This can be used for the in vitro propagation of banana (CV. Grand Naine, 2020).

Shaikh et. al. (2014) also reported that as the microorganisms do not utilize all the nutrients from the solid media in one growth, this media can be recycled by autoclaving for 30 minutes and used for the growth of same microorganisms for the second time. Their results suggested agar was the main component, which remain unaltered during recycling, the nutrient content decreased after recycling but was enough to support the growth of microorganisms for the second time.

2. AIMS & OBJECTIVES

2. Aims and objectives

An ample of waste is generated in the environment due to human activities and this also accounts the wastes from Microbiology laboratory. Agar-agar is an important component of Microbiology being used as solidifying agent in the media used for the microbial growth. After cultivation of microorganisms, for proper disposal of the microbial cultures growing on the media, the solid media is decontaminated, the agar is melted and disposed of. When a microorganism grows on a solid media, all the nutrients from the media are not utilized by it. So, the used agar contains a good amount of nutrients and by discarding the agar after use, we tend to waste these nutrients and also the properties of agar get wasted. This also makes way for the nutrients to enter the water bodies and increase the plant and algal growth causing eutrophication. A topic of keen interest would be the utilization of the used media as a way of valorization of waste. There are ways in which this can be done, by recycling the agar by physical and chemical methods and re-using it, utilizing it as a biofertilizer for plant growth and also reusing the nutritive value of the media by, sterilizing it and growing the same culture on it for a second time.

This study aimed to isolate a bacterium which can produce agarase enzyme and degrade the used agar. As seaweeds are a great source of many polysaccharides including agar in their cell walls, it harbors many micro-organisms which produce certain enzymes for the degradation of polysaccharides to use it as a carbon source, one of it is agarolytic bacteria, hence seaweeds were taken as the source for isolation of this bacteria. The degradation of used agar by agarolytic bacteria leads to production of galactose hence as a valorization of this agar waste it involves the utilization of galactose by yeast *Saccharomyces cerevisiae* for fermentation process for ethanol production.

This work had the following aims and objectives;

1. Isolation of seaweed associated bacteria from different seaweeds.
2. Screening of bacterial isolates for different enzymes.
3. Application of agarase enzyme produced by seaweed associated bacteria.
4. Valorization of agar waste for bio-ethanol production

3.METHODOLOGY

3.Methodoloy

3.1 Sample collection

Seaweed and water samples were collected from Vagator beach. The seaweed and water samples were collected in zip lock bags and bottles respectively. The water samples were checked for the physicochemical parameters such as salinity, pH and temperature. The seaweed samples were used for isolating seaweed associated bacteria.

3.2 Isolation of seaweed associated bacteria

The seaweed samples were rinsed using filter sterilized sea water and were made into pieces using sterile forceps. The enrichment for the isolation of seaweed associated bacteria was done in two ways (1) Direct plating wherein, the seaweed pieces were placed on Zobelle marine agar (ZMA), (2) Enrichment in broth, the seaweed pieces were inoculated in Zobelle Marine broth. The plates and flasks were incubated at 28ºC for 24 hours. After incubation, from the plate, the bacterial cultures growing at the edge of the seaweed were streaked on ZMA. From the cultures grown in flask serial dilutions were carried out up-to 10^{-4} and the 10^{-2}, 10^{-3}, 10^{-4} dilutions were spread plated on ZMA. Streaked and spread plates were incubated at room temperature for 24 hours. The methodology was taken from Tawara et. al, (2015) used for the isolation of seaweed associated bacteria.

3.3 Purification and maintenance

The colonies obtained were purified by streaking on ZMA and maintained.

3.4 Screening of enzymes

The purified bacterial isolates were screened for the following enzymes:

3.4.1 Screening of agarase enzyme

The ability of bacterial isolates to produce agarase was checked by spot inoculating the isolates on Artificial Seawater (ASW) Agar containing agar as the sole source of carbon. The plates were incubated at room temperature for 36-48 hours. After incubation the plates were flooded with 5% Lugol's Iodine and were observed for zones of clearance.

This methodology is taken from Naik et al., (2019), the best isolate was determined as the one showing maximum zone of clearance on addition of lugols iodine.

3.4.2 Screening of protease enzyme

The ability of bacterial isolates to produce protease was checked by spot inoculating the isolates on ASW agar containing 1% skimmed milk. The plates were incubated at room temperature for 24 hours. After incubation the plates were observed for zones of clearance. This methodology was taken from Sneha et al., (2014) for isolation of protease producing bacteria from marine environment.

3.4.3 Screening of amylase enzyme

The ability of bacterial isolates to produce amylase was checked by spot inoculating the isolates on ASW agar medium containing 1% soluble starch. The plates were incubated at room temperature for 24-48 hrs. After incubation the plates were flooded with 1 % Gram's Iodine and were observed for zones of clearance. This methodology was taken from Naik et. al. (2019), wherein amylase activity of seaweed associated bacteria was checked by growing the culture in seawater broth containing starch as the carbon source.

3.4.4 Screening of lipase enzyme

The ability of bacterial isolates to produce lipase was checked out by spot inoculating the isolates on ASW agar medium containing 1% Tween 80. The plates were incubated at room temperature for 24 hrs. After incubation, the plates were observed for white precipitate around the colonies. This methodology was taken from L. Ramnath et.al. (2017)

3.4.5 Screening of esterase enzyme

The ability of bacterial isolates to produce esterase was checked by spot inoculating the isolates on ASW agar medium containing 1% Tween 20. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for precipitate around the colonies. This methodology was taken from L. Ramnath et. al , (2017).

As explained by L. Ramnath et. al. (2017), Tween 80 consists of esters of oleic acid and hence is degraded by lipase and tween 20 consists of esters of lower chain fatty acids and is degraded by esterase enzyme. The principle is based on calcium precipitation, wherein the fatty acids released bind to the calcium present in the medium and forms an insoluble precipitate as observed around the colony.

3.4.6 Screening of alginate lyase enzyme

The ability of bacterial isolates to produce alginate lyase was checked by spot inoculating the isolates on sodium-alginate agar containing 0.5% sodium alginate. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for zones around the colonies. The methodology was taken from Wang et.al. (2017) , for the screening of alginate lyase producing bacteria

3.4.7 Screening of cellulase enzyme

The ability of bacterial isolates to produce cellulase was checked by spot inoculating the isolates on ASW agar medium containing 1% carboxy-methyl cellulose. The plates were incubated at room temperature for 24-48 hours. After incubation, the plates were flooded with 1% congo red for 15 minutes and then decolorized with 1% NaCl for 15 minutes. These steps were repeated three times to remove excess unbound congo red and the zones of clearance around the colonies were observed. This methodology was given by Naik et al (2019).

3.4.8 Screening of xylanase enzyme

The ability of bacterial isolates to produce xylanase was checked by spot inoculating the isolates on ASW agar medium containing 0.5% birchwood xylan. The plates were incubated at room temperature for 24-48 hours. After incubation, the plates were flooded with 0.4% congo red for 10 minutes followed by 1M NaCl for washing. Zones of clearance were observed. This methodology was given by A. Burlacu et al. (2016)

3.4.9 Screening of pectinase enzyme

The ability of bacterial isolates to produce pectinase was checked by spot inoculating the isolates on ASW agar medium containing 0.5% pectin. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for clear zones of hydrolysis around the colonies. This methodology was given by T. Fouzia et al. (2018).

3.4.10 Screening of carrageenase enzyme

The ability of bacterial isolates to produce carrageenase was checked by spot inoculation the isolates on ASW agar medium containing 5% carrageenan as the gelling agent. The plates were incubated at room temperature for 24-48 hours. After incubation, the plates were flooded with phenol red. The formation of yellow zones around the colonies is a positive result for carrageenase enzyme. This methodology is followed by Naik et. al. (2019) wherein it reports that the production of yellow color is due to the formation of acidic product formed due to carrageenan degradation which decreases the pH of the medium to acidic and is indicated by phenol red.

3.4.11 Screening of β-glucosidase enzyme

The ability of bacterial isolates to produce β-galactosidase was checked by spot inoculation the isolates on ASW agar medium containing 0.5% esculin and 1% ammonium iron citrate. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for formation of brown precipitate around the colonies. This methodology was taken from Veena et. al. (2009).

3.5 Biochemical characterization

The selected bacterial isolates were tested for the following biochemical characteristic

Gram character

Gram staining

Smear of bacterial isolates was prepared on clean grease free slides, air dried and heat fixed. The heat fixed smear was flooded with Gram's Crystal violet for 1 minute. The side was drained, washed with water and flooded with Gram's iodine for 1 minute. The slide was washed with 70% ethanol for 10-20 secs until the color of crystal violet comes out. The slide was flood with safranin for 1 min. It was then washed briefly, drained, air dried and observed under oil immersion lens. The Gram character and morphology of the bacterial isolates were noted down.

KOH test

A clean grease free slide is taken and a drop of 3 % KOH was placed on it, a 24hour old culture was picked using toothpick and gently mixed in 3% KOH solution. The toothpick was raised and lowered from the slide to check for string formation. String formation denotes the Gram character of bacteria is Gram negative and no string formation denotes Gram positive.

Motility test

A 24hour old culture was stabbed into nutrient agar stubs with 0.4 % agar and incubated at room temperature for 24 hours. The spread of the culture throughout the agar will indicate motile bacteria

Sugar fermentation test

5ml fermentation broths supplemented with 1% different sugars (glucose, lactose, sucrose, maltose, galactose) with inverted Durhams tube were inoculated with 24 hour old bacterial culture . The tubes were incubated at R.T for 24 hours. Red to yellow color change indicates acid production and a bubble in the Durham's tube indicates gas production.

Hugh Leifson's test

Hugh Leifson's media was prepared and dispensed in two test tubes, one tube was covered with mineral oil to create anaerobic conditions and one was open for aerobic condition .24hour old culture was stabbed in the stubs and the tubes were incubated at room temperature for 24 hours. The following color pattern was checked:

Open	Covered	Metabolism	Mode of growth
Acid(yellow)	Alkaline (green)	Oxidative	Strict aerobic
Acid(yellow)	Acid(yellow)	Fermentative	Facultative anaerobic (microaerophilic)
Alkaline (green)	Alkaline (green)	Non- saccharolytic	

Catalase test

A clean grease free slide was taken and a colony was applied to it, 3% hydrogen peroxide was added. Gas formation in the form of effervescence is observed. This indicated positive test.

Indole test

4ml tryptophan broth was prepared in test tube and was inoculated with a loop full of 24hour old culture. The tubes were incubated at room temperature for 24 hours. After incubation 0.5 ml Kovacs reagent was added to check for the formation of pink colored ring which indicates positive test.

Methyl red test

1ml MRVP broth was prepared in test tube and inoculated with a 24 hour old culture an tubes were incubated at R.T for 24 hours. 5 drops of Methyl red indicator was added to check the formation of red coloration which indicated positive results

Voges Proskauer test

2ml MRVP broth was prepared in test tubes and was inoculated with a 24hour old culture and incubated at R.T for 24 hrs. After incubation ,1 ml of O'Meara's reagent was added and mixed. After 10-15 minutes appearance of red color indicates a positive test

Citrate test

Simmons citrate agar slants were prepared and 24hour old culture was streaked on it and the slants were incubated at room temperature for 24 hrs. A color change from green to blue indicates positive test.

3.6 Determination of agarase activity of the selected bacterial isolate.

To confirm if the selected bacterial isolate utilizes agar as carbon source, it was inoculated in ASW broth containing 0.3 % agar as the carbon and incubated at 28 ºC at 120 rpm for 24 hours. The growth was determined by reading the absorbance at 600 nm. This methodology is taken from D. Saravanan et. al.(2015) to determine the agarase activity.

3.7 Enzyme extraction

The bacterial isolate was grown in Nutrient broth and incubated at 28 ºC for 18 hours. 2.5 ml of this was inoculated in 50 ml ASW broth supplemented with 0.3% agar and incubated at 28 ºC at 120 rpm for 72 hours. The grown culture was centrifuged at 4 at 6,000 rpm, at 4 ºC for 30 minutes. The supernatant was collected and analyzed for protein estimation by Folin Lowry's method and the protein concentration was determined by using the standard equation obtained by plotting the standard graph of BSA protein estimation as given in Annexure C. The supernatant was further used for agarase enzyme assay. This methodology is taken from D. Saravanan et al. (2015) for the extraction of agar enzyme from the agarolytic bacterial strain.

3.8 Enzyme Assay

For enzyme assay, 0.1% agar substrate was prepared**.** The supernatant was allowed to react with 0.1 % of agar substrate in the presence of Phosphate buffered saline (pH=7) at room temperature for 30 minutes. The volume of total reaction mixture was 25 ml. Substrate control and enzyme control were also kept at the same time. The reaction set-up is given in Table 1.1.

Enzyme activity was determined by measuring the production of galactose sugar which was determined by Dinitro salicylic acid method for estimation of reducing sugars. The concentration of sugar produced was determined by the standard equation obtained plotting a standard curve of galactose sugar which is given in Annexure C. This methodology is given by D. Saravanan et al. (2015) for checking the enzyme activity with 0.5% substrate

Table 1.1; Reaction set-up for agarase enzyme assay

	Supernatant (ml)	Buffer (ml)	Substrate (Agar) (ml)
Test	1	20	4
Enzyme control	1	24	-
Substrate control	-	21	4

3.9 Salt tolerance of agarase producing bacterial isolate

The importance of the agarase positive seaweed associated bacterial isolate was to be used in degradation of used agar from Microbiology laboratory, as the media can also contain salts, it was necessary to check its salt tolerating ability of the bacteria. The bacterial isolate was grown in ZMB with increasing concentrations of NaCl: 0%, 5%, 10%, 15%, 20%, 25 %, 30% and incubated at 28 ^{o}C at 120 rpm for 24 hours. The growth at each concentration was determined by reading the absorbance at 600 nm. A graph of NaCl concentration vs absorbance at 600nm was plotted.

Salt tolerance of a bacteria isolated from marine environment was checked by Khalifa and Ashraf (2019) by growing in Nutrient medium with NaCl concentrations up-to 15%, the cultures were incubated at 28 ºC for 3 days.

3.10 Application of Seaweed Associated Bacteria producing agarase.

Used ZMA was taken and decontaminated twice. The solid agar was taken and 20g was weighed distilled water was added and volume was made up to 100ml. It was melted by autoclaving to form a semi-solid suspension. This was done in two flasks. A 24hour old culture grown in ASW broth containing 0.3% agar was take and 5% of it was inoculated in one flask and 10% in the second flask. An uninoculated control was maintained. The flasks were incubated at R.T at 100 rpm for a period of five days. Each day 2 ml was withdrawn from both the flasks and was used for galactose sugar estimation by DNSA method. A bar graph was plotted of days vs galactose concentration to determine which day there was maximum sugar concentration and to compare the galactose sugar production by 5% and 10% cultures. Same procedure was followed for degradation of used Nutrient agar containing 5% salt concentration using 5% bacterial culture, pre-grown in ASW broth with 0.3% agar. A graph was plotted of days vs the concentration of galactose to determine and compare the trend of galactose production each day with the previous experiment. The growth behavior exhibited by an agarolytic bacteria isolated from seaweed when grown in the presence of both the agar and other nutrients was studied by Furuswa et. al. (2017). It also explains the utilization of galactose by the same bacteria.

3.11Valorization of agar waste.

With an aim to utilize the galactose sugar produced by degradation of used agar for the production of ethanol the galactose sugar had to be harvested on the day when there was maximum galactose production by the bacteria. The used ZMA agar inoculated with 5% bacterial culture was incubated at R.T at 100 rpm. After 2 days of growth the degraded agar was taken and filtered using double layered muslin cloth to get rid of the residual agar, the filtrate obtained was subjected to galactose estimation by DNSA method. The agar retained on the muslin cloth after filtration was taken and

its wet weight was taken, it was air dried for 48 hours to take the dry weight. The filtrate was collected and centrifuged at 8000 rpm for 10 minutes, the supernatant was collected and the galactose concentration was determined by DNSA method. The supernatant was sterilized by autoclaving. For ethanol production, 10% activated baker's yeast, *Saccharomyces cerevisiae* was inoculated in the sterile supernatant and incubated at room temperature under dark conditions. The flask was swirled twice every day. 4 ml of it was withdrawn every day for 5 days and centrifuged, the supernatant was collected and ethanol concentration was determined using potassium dichromate method for ethanol estimation using the standard equation obtained from Standard curve for ethanol given in annexure C. Also, the supernatant was analyzed for galactose estimation using DNSA method. A bar graph was plotted to depict the increase in ethanol concentration and decrease in galactose concentration over a period of 5 days

Utilization of galactose for ethanol production was studied by Kim et. al. (2014) by using newly isolated yeast *Saccharomyces cerevisiae* KL17. Similar methodology was used by Mohd S.H and Abdulla R. 2018 for the utilization of galactose sugar for bioethanol production by immobilized yeast *Saccharomyces cerevisiae*.

4.RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Seaweed sample collection

Seaweed samples were collected from Vagator beach, the sampling site is shown in figure 4.1 The physicochemical parameters were noted and are mentioned in the table 4.1. Six different types of seaweeds samples were collected and identified by visualizing their morphological characteristics with the help of Dr. Vijaya Kerkar, Professor of Botany, SBSB, Goa University The seaweed samples are shown in figure 4.2

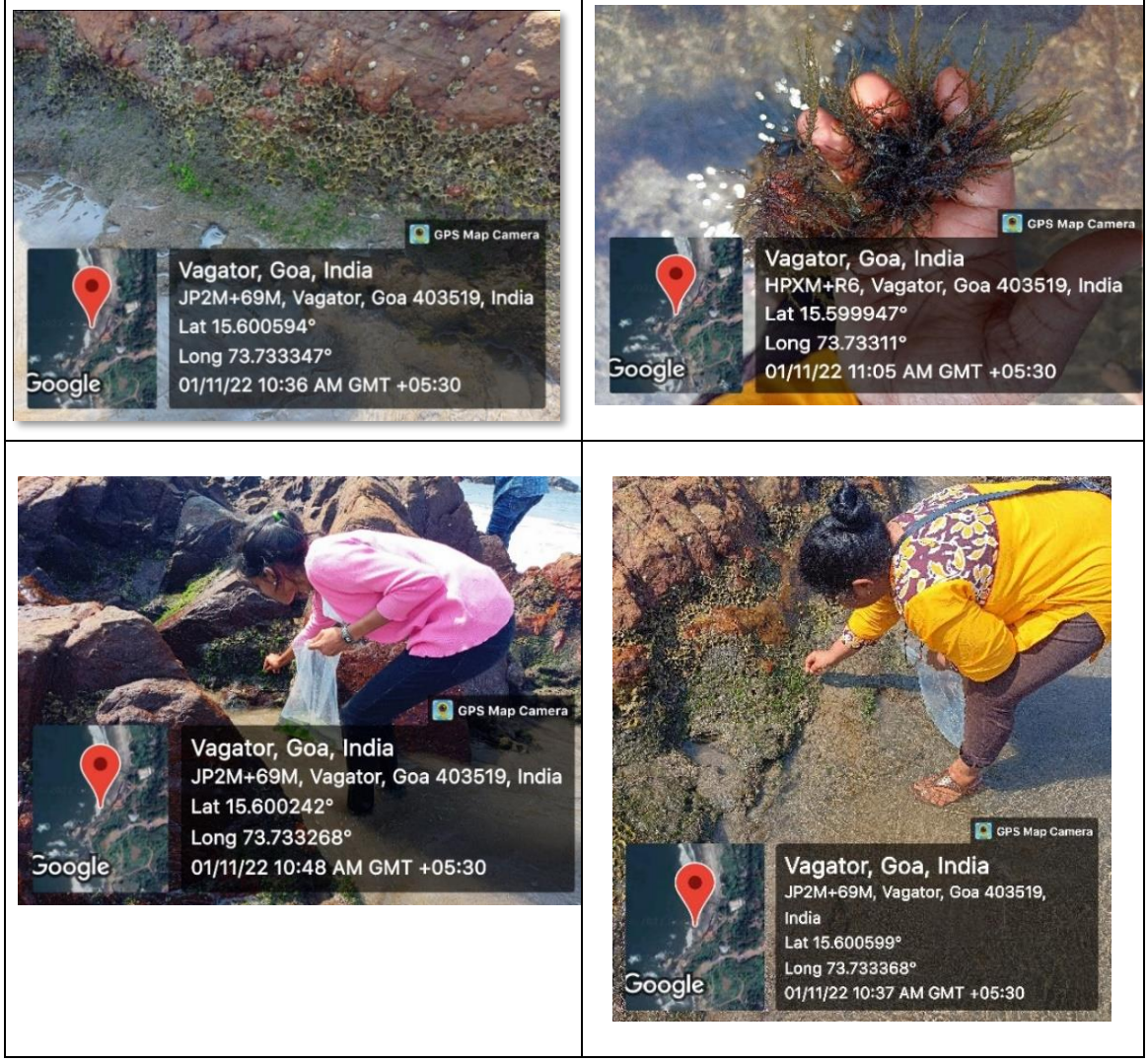


Fig 4.1: Seaweed sampling at Vagator beach

Table 4.1; Physicochemical parameters of water sample collected from the sampling site

Physicochemical parameters	Result
Salinity	3%
Ph	8.0
Temperature	31.1ºC

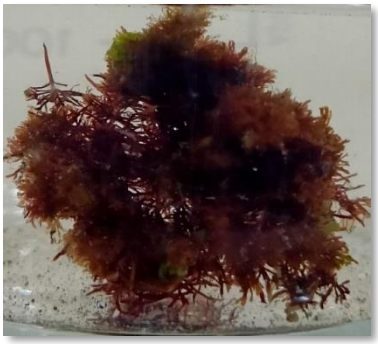
Chlorophyta	**Rhodophyta**	**Phaeophyta**
(a) 	**(c)** 	**(e)** 
(b) 	**(e)** 	**(f)** 

Fig 4.2: Seaweed samples collected and identified (a)*Ulva fasciata*; (b)*Chaetomorpha media;* (c)*Gelidium pusillum;* (d)*Gracilaria corticata;* (e)*Sargassum tenerrimum;* (f)*Padina tetrastromatica.*

4.2 Isolation of seaweed associated bacteria

Seaweed associated bacteria were isolated by direct platting and enrichment method (figure 4.3). A total of 27 morphologically distinct bacteria were isolated from seaweeds (figure 4.4 a, b, c, d, e, f.). The colony characteristics of the isolates are summarized in Table 4.2 a, b, c.

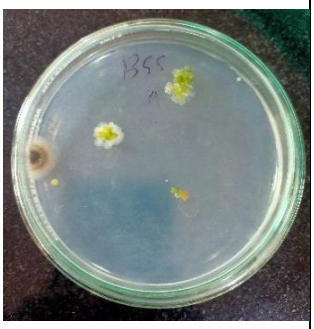
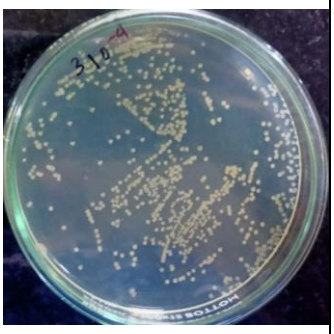
			
(a)	(b)	(c)	(d)

Fig 4.3: Isolation of Seaweed associated bacteria, (a) Enrichment in ZMB; (b)Enrichment by direct platting on ZMA; (C)Isolated colonies obtained on ZMA; (d)Purified bacterial colonies on ZMA

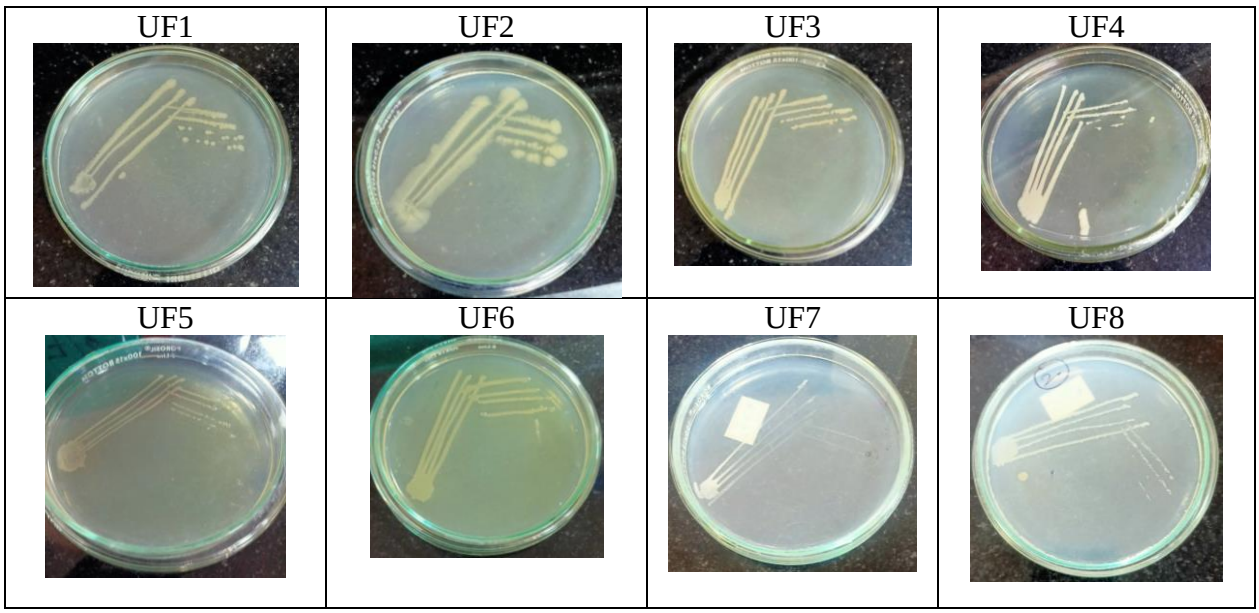


Fig 4.4a Bacterial isolates UF1 To UF8 obtained from seaweed *Ulva fasciata*

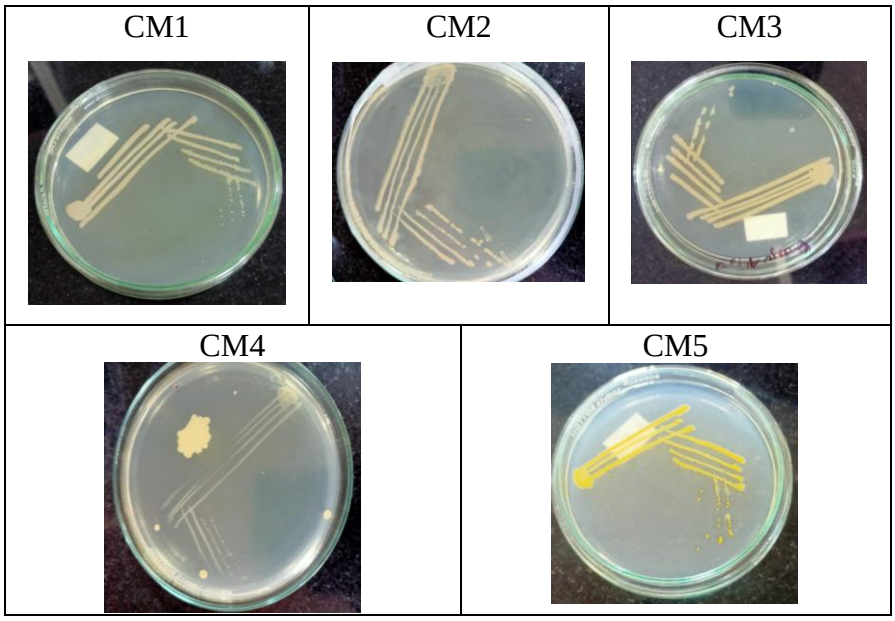


Fig 4.4b Bacterial isolates CM1 To CM5 obtained from seaweed *Chaetomorpha media*

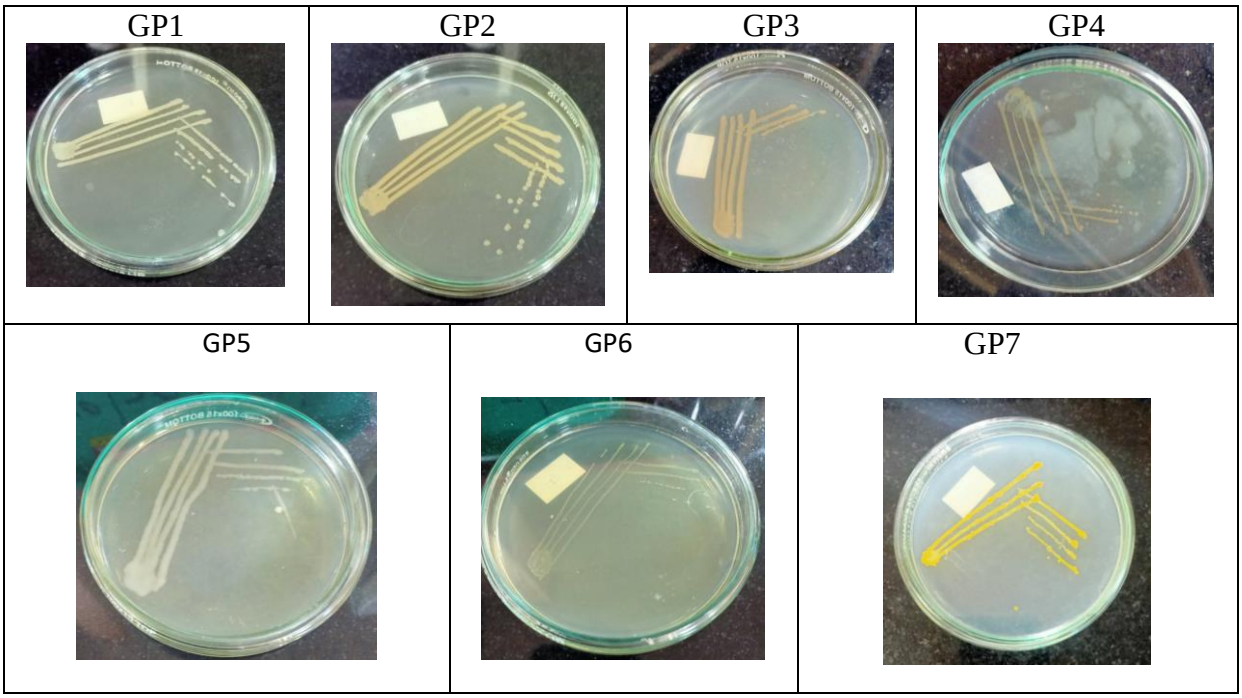


Fig 4.4c Bacterial isolates GP1 to GP7 obtained from seaweed *Gelidium pusillum*

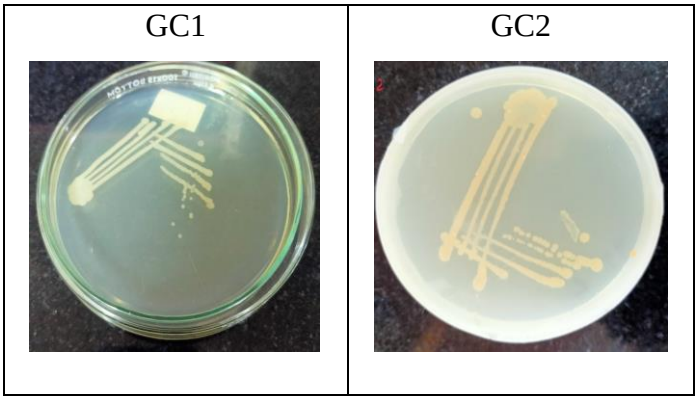


Fig 4.4d Bacterial isolates GC1 and GC2 obtained from seaweed *Gracilaria corticata*

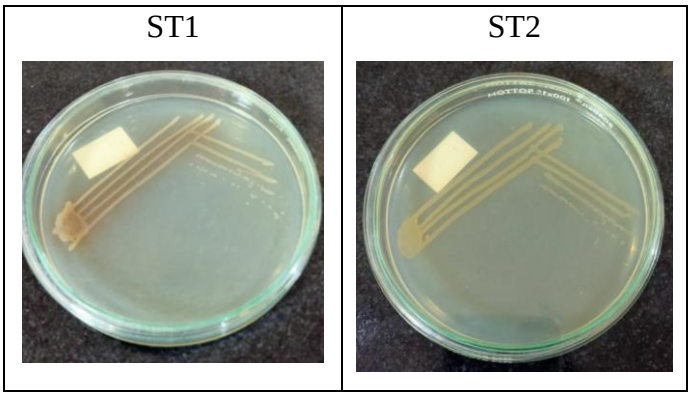


Fig 4.4e Bacterial isolates ST1 and ST2 obtained from seaweed *Sargassum tenerrimum*

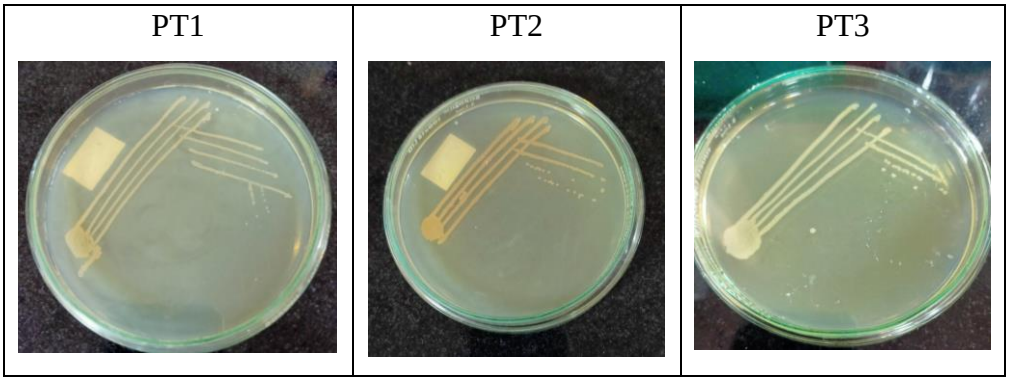


Fig 4.4f Bacterial isolates PT1,PT2,PT3 obtained from seaweed *Padina tetrastromatica.*

Table 4.2a: Colony characteristics of bacterial isolates UF1, UF2, UF3, UF4, UF5, UF6, UF7, UF8 and CM1 obtained from *Ulva fasciata* and *Chaetomorpha media* respectively.

CM1	UF8	UF7	UF6	UF5	UF4	UF3	UF2	UF1	Isolate
Circular	circular	circular	circular	circular	circular	circular	irregular	Circular	**Shape**
1mm	0.5 mm	0.5 mm	2mm	I mm	1mm	1mm	3mm	2 mm	**Size**
creme	white	white	yellow	Light orange	White	creme	Off-white	Of white	**Color**
smooth	rough	smooth	smooth	glistening	glistening	rough	Rough	smooth	**Surface**
opaque	opaque	translucent	opaque	opaque	opaque	opaque	Opaque	Opaque	**Opacity**
raised	flat	flat	flat	raised	raised	raised	flat	raised	**Elevation**
Entire	Wavy	Entire	Entire	entire	entire	entire	lobate	Entire	**Margin**
Butyrous	brittle	Brittle	butyrous	butyrous	slimy	butyrous	Brittle	butyrous	**Consistency**
ZMA	BSS	BSS	NA	NA	ZMA	ZMA	ZMA	ZMA	**Media**
24 hrs	24-48 hrs	24-48 hrs	24 hrs	24 hrs	24 hrs	24 hrs	24 hrs	24 hrs	**Incubation time**
Gram positive cocci	Gram positive short thin rods	Gram negative thin rods scattered	Gram positive cocci	Gram positive diplococci	Gram positive cocci	Gram positive rods	Gram positive cocci	Gram positive cocci, clustered	**Gram Character**

Table 4.2b; Colony characteristics of CM2, CM3, CM4, CM5 and GP1, GP2 ,GP3 , GP4 bacterial isolates obtained from *Chaetomorpha media* and *Gelidium pusillum* respectively.

GP4	GP3	GP2	GP1	CM5	CM4	CM3	CM2	Isolate
Circular	Circular	Circular	Circular	Circular	Circular	Circular	Irregular	**Shape**
1mm	2mm	3mm	2mm	1mm	0.5 mm	2mm	2mm	**Size**
yellow	Dark orange	Light orange	creme	Bright yellow	white	orange	creme	**Color**
opaque	smooth	glistening	smooth	rough	rough	glistening	rough	**Surface**
opaque	Opaque	opaque	opaque	opaque	opaque	opaque	opaque	**Opacity**
flat	flat	Convex	convex	convex	flat	convex	flat	**Elevation**
Entire	Entire	Entire	Entire	Entire	Entire	Entire	Wavy	**Margin**
brittle	butyrous	butyrous	slimy	slimy	brittle	brittle	slimy	**Consistency**
ZMA	ZMA	ZMA	ZMA	BSS	ZMA	ZMA	ZMA	**Media**
24hrs	24 hrs	24 hrs	24 hrs	24-48 hrs	24 hrs	24 hrs	24 hrs	**Incubation Time**
Gram positive cocci	Gram positive coccobacilli	Gram negative short rods	Gram negative short rods	Gram negative, pleomorphic	Gram positive thin rods , scattered	Gram positive cocci	Gram negative cocci scattered	**Gram Character**

Table 4.2c ; Colony characteristics of GP5, GP6 , GC1,GC2 and ST1,ST2 and PT1,PT2,PT23 bacterial isolate obtained from *Gelidium pusillum , Gracilaria corticata, Sargassum tenerrimum and Padina tetrastromatica* respectively

PT3	PT2	PT1	ST2	ST1	GC2	GC1	GP7	GP6	GP5	Isolate
Circular	Circula	Circular	Circular	Circula	Irregular	Circular	Circular	Circular	Irregular	**Shape**
1 mm	1 mm	0.5 mm	1.5 mm	1 mm	3mm	2mm	1mm	0.5	2mm	**Size**
creme	Dark	creme	Light	Dark	Dark	creme	Dark	white	creme	**Color**
smooth	Smoot	smooth	smooth	smooth	smooth	smooth	rough	rough	rough	**Surface**
opaque	opaque	translucent	Opaque	opaque	opaque	opaque	opaque	opaque	opaque	**Opacity**
raised	convex	Flat	flat	convex	raised	convex	raised	flat	flat	**Elevation**
entire	entire	entire	entire	entire	entire	entire	wavy	entire	wavy	**Margin**
slimy	Butyro us	brittle	butyrous	Butyro us	butyrous	Slimy	butyrous	brittle	firm	**Consistenc y**
NA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	BSS	NA	NA	**Media**
24 hrs	24 hrs	24 hrs	24 hrs	24 hrs	24 hrs	24 hrs	24-48 hrs	24 hrs	24 hrs	**Incubation time**
Gram positive cocci	Gram positiv e cocci	Gram positive diplococci	Gram negative pleomor phic	Gram positiv e cocci ,	Gram negative, pleomor phic	Gram positive pleomor phic	Gram positive branching	Gram negative, diplobaci lli	Gram positive, long thin rods	**Gram Character**

4.3 Screening of enzymes

The 27 isolates obtained from seaweed samples were screened for 11 different enzymes; agarase, protease, amylase, esterase, lipase, cellulose, xylanase, pectinase, carrageenase, alginate lyase and β-glucosidase. The isolates showing positive results for these enzymes are given in table 4.3.

Table 4.3: Bacterial isolates showing positive results for following enzymes

Culture	Agarase	Protease	Amylase	Esterase	Lipase
UF3	+	+	+	-	-
GP2	-	+	-	+	-
CM3	-	+	-	-	-
CM4	-	+	-	-	-
GP3	-	+	-	-	-
GP4	-	+	-	-	-
ST1	-	-	-	+	+
GC2	-	-	-	+	-
PT1	-	+	-	-	-

KEY +: Positive Enzyme Activity -: Negative Enzyme Activity

Out of 27 bacterial isolates obtained, isolate UF3 showed agarase activity. As reported by Naik et. al. (2019) the agarase activity was confirmed by flooding the plates with Lugol's iodine. Zones of clearance around the colonies confirmed the agarase activity as shown in figure 4.5(a)

Previous studies have shown that agarase producing bacteria can be obtained from various sources Suzuki et al., (2003) reported that bacterium *Bacillus sp. MK03* isolated from soil could produce β-agarase. Sakai M.et. al. (2015) isolated an agarase positive bacteria *Ammoniibacillus agariperforans* bacteria was isolated from the compost had agarase activity bacteria. Agarolytic bacteria belonging to genera *Flammeovirga* was isolated from coastal sediments by Liu Y, et. al. (2019).

Reports have been made on agarolytic bacteria isolated from seaweeds. Du et.al. (2011) isolated an agarase positive bacteria *Agarivorans gilvus* from seaweeds. A novel bacterial species belonging to genus *Gilvimarinus* was isolated from seaweeds by Cheng et. al. (2015) which could digest agar by producing agarase enzyme. Extracellular β agarase producing marine bacteria *Microbulbifer sp.BN3* was isolated from red seaweeds *Gracilaria and Gelidium* by Li et. al. (2020). Three agarolytic bacterial species were isolated from green seaweed *Ulva lactuca*, these were, *Vibrio brasiliensis*, DM1, *Bacillus subtilis* strain DM5 and *Psuedomonas aeruginosa* strain DM15 by Naik et.al. (2019). These bacteria also showed amylase and protease activity.

Bacterial isolate UF3 showed amylase activity, by showing clear zone around the colony on starch agar plate on addition of 1 % Gram's iodine as shown is figure 4.5(b). As reported, Javee et. al. (2019) isolated *Streptomyces sp.* SNAJSM6 from brown seaweed *Sargassum myriocystum* having maximum extracellular alpha amylase activity.

The bacterial isolates UF3, GP2, CM3, CM4, GP3, GP4, PT1 showed positive results for protease enzyme, by showing a zone of clearance on the skim milk agar as shown in figure 4.5.(c)-(f). As per the recent reports, Javee et. al. (2022) isolated a bacterium *Shewenella alga* with protease activity from the brown seaweed *Sargassum myriocystum.*

Bacterial isolates GP2, ST1, GC2 showed esterase activity, by showing precipitation around the colonies as shown in figure 4.5(g)-(h). As reported by Martina et. al. (2021) bacteria belonging to Gamma proteobacteria and Flavobacteria isolated from brown seaweed *Ascophyllum nodosum* showed esterase activity.

Bacterial isolate ST1 showed lipase activity by formation of precipitate around the colonies as shown in figure 4.5(i)

With our objective to check for agarase activity in seaweed associated bacteria, bacterial isolate UF3, isolated from the green seaweed *Ulva fasciata* was selected for further study.

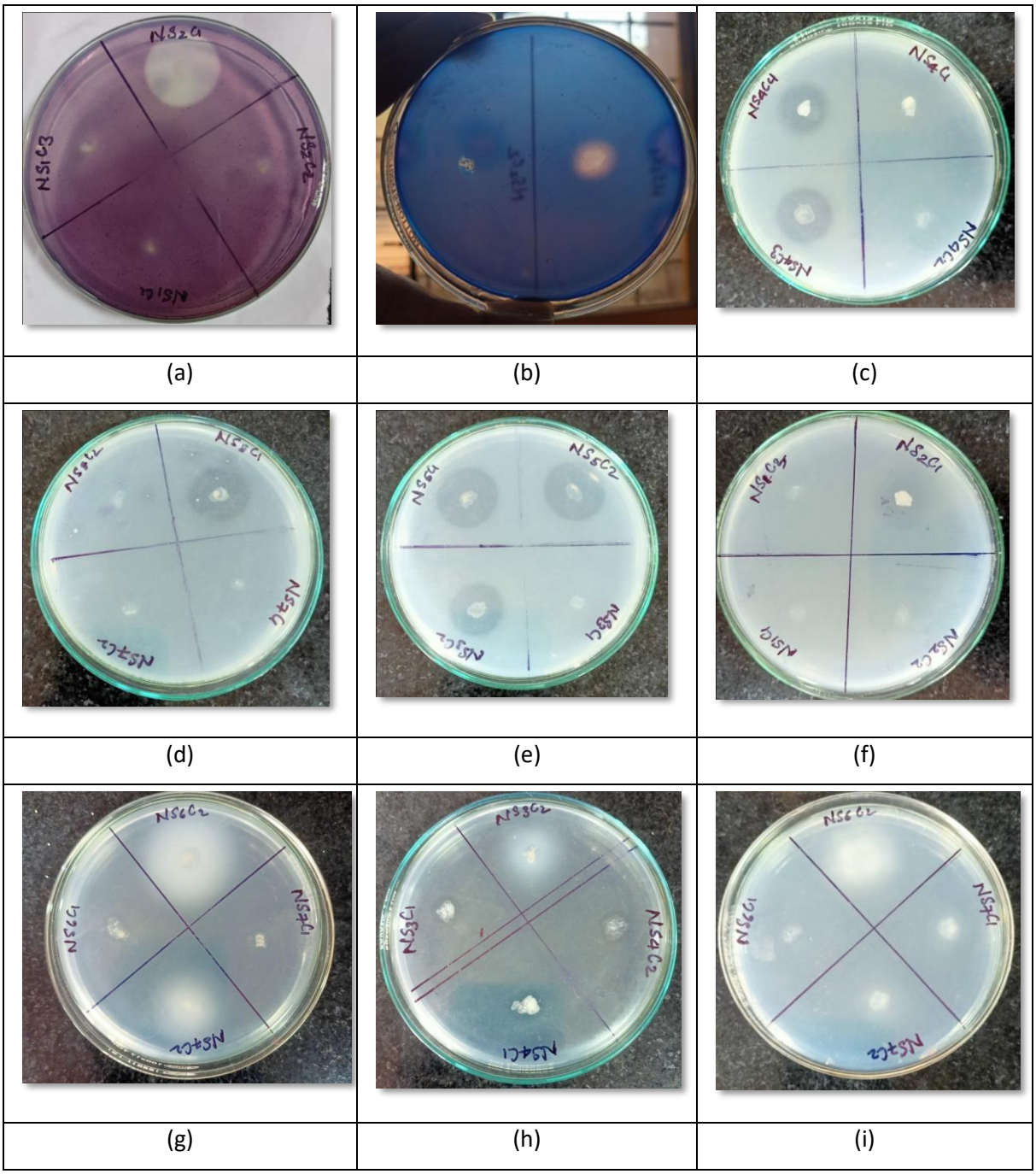
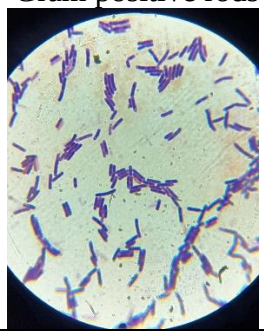


Fig 4.5 Seaweed associated bacterial isolates showing enzyme activity (a)Agarase enzyme; (b)Amylase enzyme; (c),(d),(e),(f)Protease enzyme; (g),(h)Esterase enzyme; (i)Lipase enzyme

4.4 Biochemical Identification

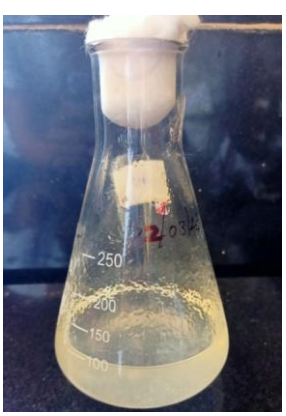
TESTS	Bacterial Isolate UF3
Gram character	Gram positive rods 
Motility	+
Catalase	+
Indole	-
Methyl red	+
Voges Proskauer	-
Citrate	+
Glucose	+
Lactose	-
Sucrose	+
Galactose	+
Maltose	-
Hugh-leifsons	Aerobic
Pigment	-

KEY: + : Positive Test - :Negative Test

The selected bacterial isolate UF3 having agarolytic activity as observed under the microscope by Gram staining method was Gram positive rods. Further biochemical tests were done, but the results were not sufficient to identify the bacteria even at the Genus level.

4.5 Determination of agarase activity of bacterial isolate UF3

Bacterial isolate when grown in seawater broth containing 0.3% agar as sole source of carbon for 24 hours showed turbidity (O.D at 600 nm = 1.4) as compared to the uninoculated control. Results are shown in figure 4.6. This implies that the bacterial culture utilizes the agar as the carbon source producing the enzyme agarase.



(a)

(b)

Fig 4.6 Bacterial isolate UF3 growing in seawater broth with 0.3% agar as sole source of carbon. (a)uninoculated Control; (b)24hour old grown culture showing turbidity

4.6 Crude enzyme extraction and enzyme assay

The cell free supernatant obtained from a 72hour grown UF3 bacterial culture was subjected to protein estimation and its protein concentration was determined to be 150.57 µg/ml.

Agarase enzyme assay was carried out using the cell free supernatant to determine the agarase activity The supernatant was allowed to react with 0.1 % agar in the presence of phosphate buffered saline, followed by sugar(galactose) estimation by DNSA method, as reported by D. Saravanan et.al. (2015). Agarase activity is determined as the amount of enzyme, producing 1 u/mol of galactose under assay conditions per minute The absorbance at 540 nm for the test, substrate control and enzyme control is given in Table 4.4.

Table 4.4; Absorbance at 540nm taken after enzyme assay and galactose estimation

Flask	O.D At 540 nm
Test	0.026
Enzyme control	0.002
Substrate control	0.003

The agarase enzyme activity was determined as per the calculations showed in Annexure C and it was found to be 44.16 IU. The earlier reports suggest the partial purification of the agarase enzyme was carried out by various techniques such as the column chromatography, anion exchange, ammonium sulphate purification. Suzuki et. al. (2003) reported 14.1U/ml enzyme activity of crude agarase enzyme preparation. Saravanan et.al. (2015). isolated an agarolytic bacteria from marine environment and carried out partial purification of cell free supernatant by ammonium sulphate precipitation and dialysis. The enzyme activity of partially purified enzyme was 29.7 U/ml. As reported by Li et.al. (2020) extracellular β- agarase was extracted from marine bacterium *Microbulbifer BN3*, after purification its enzyme activity was determined to be 100 U/mg.

4.7 Salt tolerance of bacterial isolate UF3

We were aiming to use bacterial isolate UF3 for the valorization of agar waste from the microbiology laboratory. The used media can also contain different concentrations of salts, hence it is necessary to study the salt tolerating ability of the bacterial isolate and ability to produce agarase and utilize agar for its growth. Therefore, the salt tolerance of the bacterial isolate was checked**.**

The bacterial isolate UF3 was grown in ZMB amended with varying sodium chloride concentrations i.e., from 5 to 30%, the results were plotted graphically in a bar graph shown in figure 4.7. From the graph it is evident that bacterial growth was maximum at 0% salt concentration and then it slightly decreased at 5%. Beyond 5% NaCl concentration there was no growth.

This makes it clear that bacteria can tolerate up-to 5% salt concentration and possibly degrade agar containing maximum 5% salt concentration.

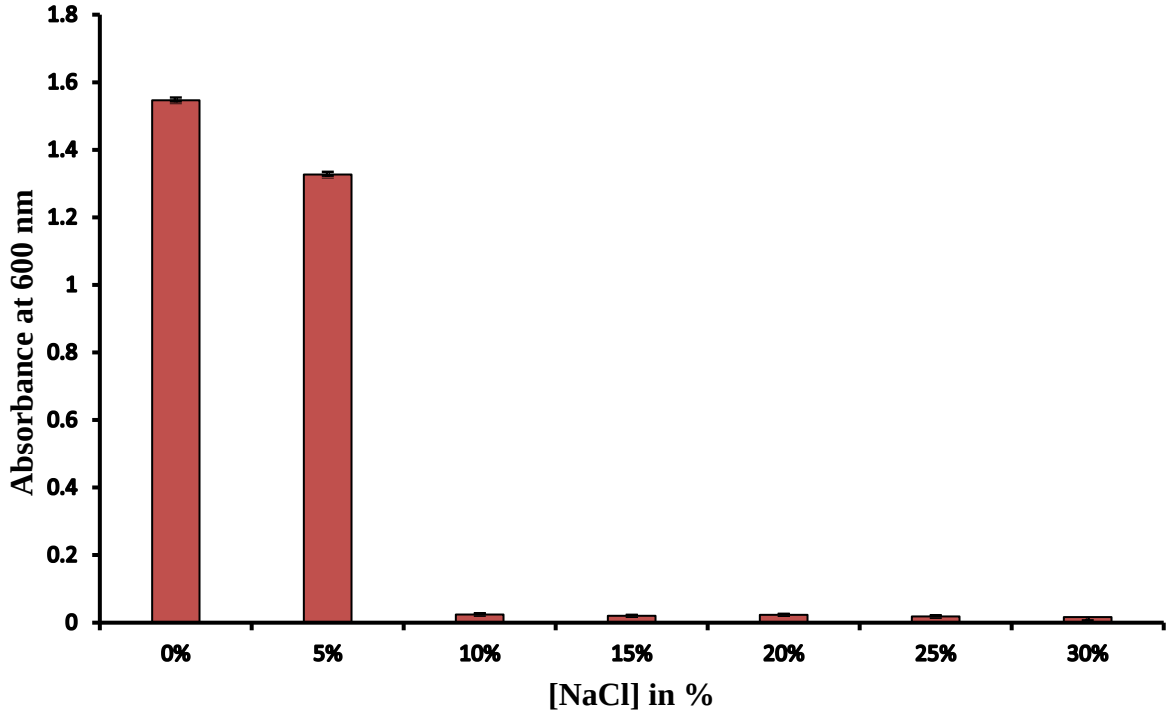


Fig 4.7: Graph showing salt tolerance of bacterial isolate UF3

As reported by Herlemann et al. (2011) salinity levels do not affect the seaweed associated marine environment and they are adapted to it. As reported by Saha et.al. (2020) *Bacillus sp., Vibrio sp., Chrysobacterium sp.* bacteria isolated from seaweed *Agarophyton vermicullophylum* could tolerate high salinity levels of 16.5 PSU, whereas, among the Gammaproteobacteria, *Psuedmonas,* could tolerate low salinity levels of 8.5 PSU.

4.8 Application of seaweed associated bacteria producing agarase enzyme

To check the ability of the bacterial isolate UF3 to degrade agar waste generated in microbiology laboratory, used ZMA agar was inoculated with 5% and 10% bacterial inoculum. The degradation of agar by the bacterial isolate producing agarase enzyme was determined by estimating the amount of galactose formed over a period of 5 days. The results obtained is presented graphically by bar graph in figure 4.8.

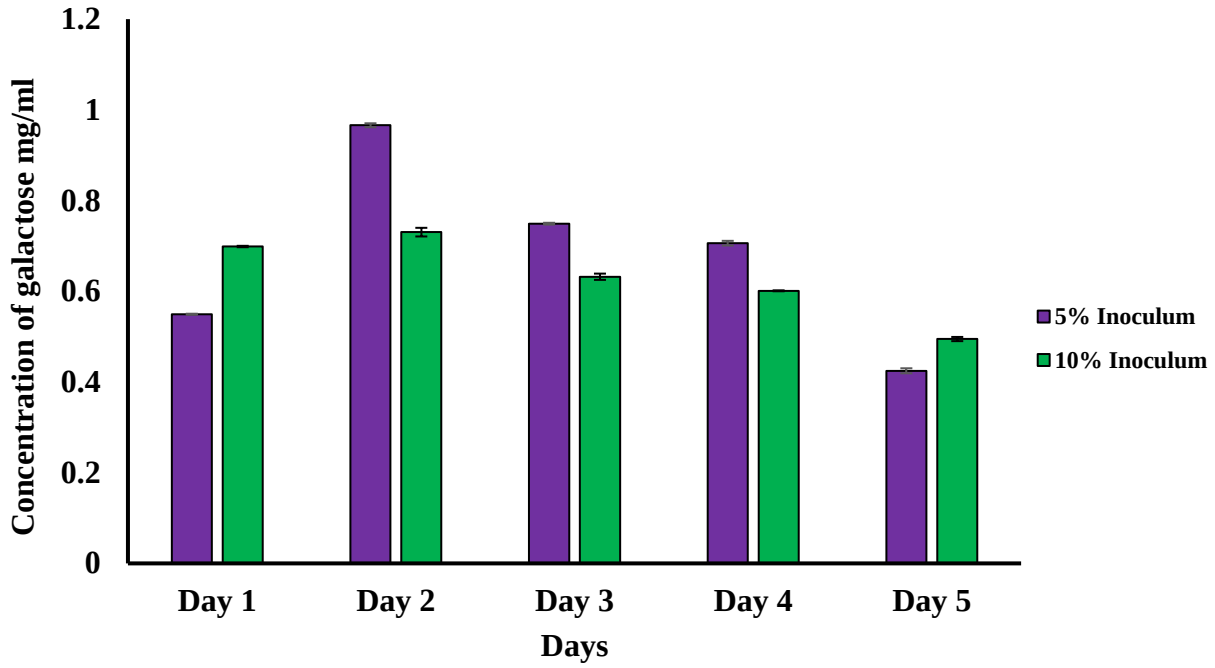


Fig 4.8: Graph showing the concentration of galactose formed over a period of 5 days

From the graph it is evident that, the 5% inoculum is better than 10% inoculum as galactose concentration is higher in 5% as compared to 10%. Also, in both the sets same trend was observed that the galactose production increased until day 2 and then decreased till day 5. This may be possibly because as the ZMA is a nutrient rich medium, the bacteria tend to utilize the nutrients and, as the nutrients are depleted, it utilizes the agar to produce galactose.

Hence, the production of galactose is increasing until day 2 and then the concentration of galactose decreases. The possible explanation to this is that the isolate itself utilizes galactose.

Similar findings were reported by Furusawa et. al. (2017) wherein they isolated a bacterium, *Persicobacter* sp. from the seaweed *Ulva,* with ability to produce agarase enzyme. When the bacterium was grown with tryptone and agar, it showed a diauxic growth curve, the isolate first utilized tryptone followed by agar. Further, it was found to express the genes involved in Leloir pathway of galactose metabolism i.e., *galK*, *galE,* and phosphoglucomutase genes in the second growth phase. This indicated that the bacterium is utilizing the galactose by hydrolyzing the agar and is exhibiting nutrient prioritization.

Though the galactose formed was more in the flasks with 5% inoculum as compared to 10%, visually, it was observed that the media flask with 10 % inoculum showed more clearance then the 5% flask for all the 5 days. From day 1 to day 5 the flask showed increased clearance as compared to the control flask. The agar clearance is seen in figure 4.9. Both the flasks containing 5% and 10% inoculum showed increased liquification from day 1 to day 5 as compared to the control flask.

Day	(a)	(b)	(c)
DAY 1			
DAY 2			
DAY 3			
DAY 4			
DAY 5			

Fig 4.9; Observation of clearance in agar from day 1 to day 5 and between 5% and 10% (a) Control flask with no culture; (b)Flask with 5% inoculated culture; (c)Flask with 10% inoculated culture

The same procedure was followed for used nutrient agar with 5% salt concentration, as it was observed that the isolate UF3 tolerated NaCl up-to 5%. Since the previous experiment showed that the 5% inoculum was better, in this set of experiment 5% inoculum was used. The results obtained were presented graphically as shown in figure 4.10.

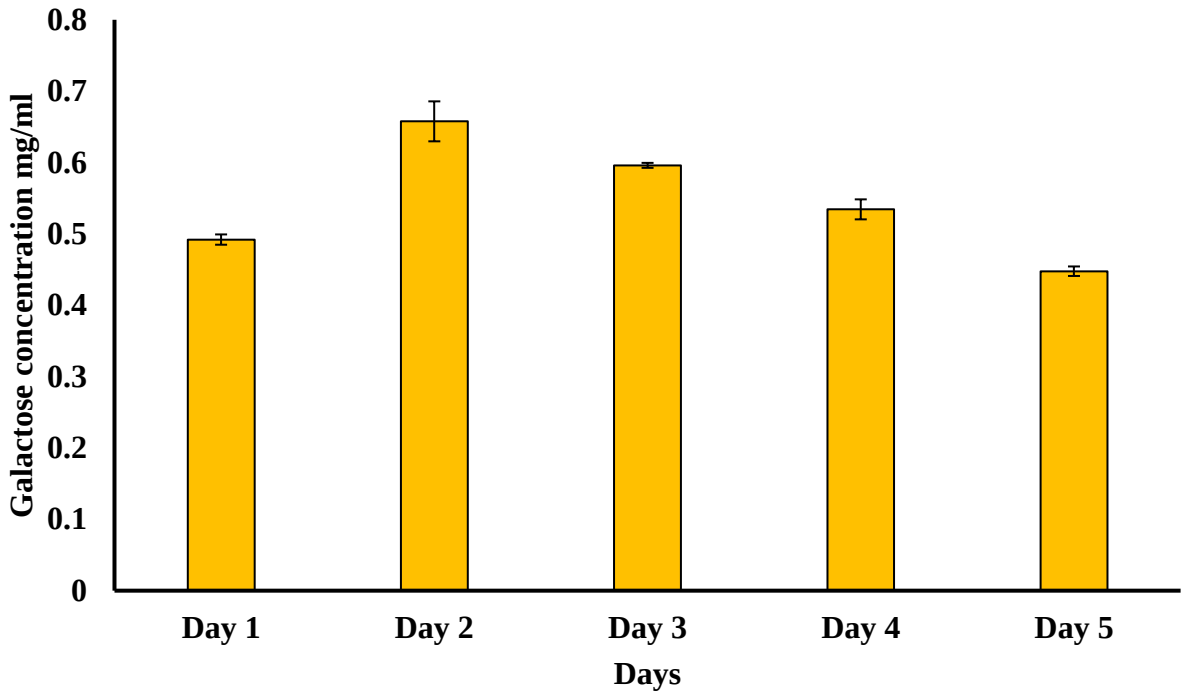


Fig 4.10: Graph showing galactose production by degradation of nutrient agar with 5% salt for 5 days

From the graph it is evident that the production of galactose followed the same trend but the sugar formed with 5% NaCl was comparatively lesser than without additional salt.

4.9 Valorization of the agar waste

In an attempt to utilize the galactose sugar for bio-ethanol production, the crude galactose extract was inoculated with the baker's yeast *Saccharomyces cerevisiae*. In an another experiment the galactose sugar was harvested by filtration on day 2 as the galactose production was maximum on second day. The concentration of galactose was found to be 0.11µg/ml and 0.913 µg/ ml in the uninoculated control and the inoculated medium, respectively. The agar retained on muslin cloth

for the uninoculated control and the inoculated medium is shown in figure 4.10 and their respective wet weight was determined to be 40.6 g and 7.2 g. After air drying for 24 hours the dry weight was 0.48 g and 0.38 g for the uninoculated control and the inoculated medium, respectively.

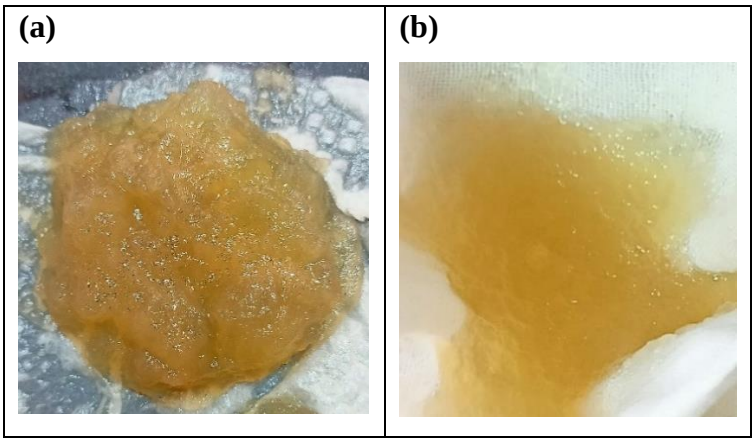


Fig: 4.10 Residual agar after filtration from(a)Uninoculated Control (b)Inoculated medium

After centrifugation, the galactose concentration of the supernatant was determined to be 0.823 µg/ml. After sterilizing the supernatant was inoculated with 10% activated baker's yeast Saccharomyces cerevisiae for ethanol production. The percentage of ethanol was determined each day for 5 days, also the residual galactose concentration was determined. The data is presented graphically as shown in figure 4.11 From the graph it is evident that as ethanol concentration increased each day and the galactose concentration decreases, this is due to utilization of galactose by yeast for production of ethanol.

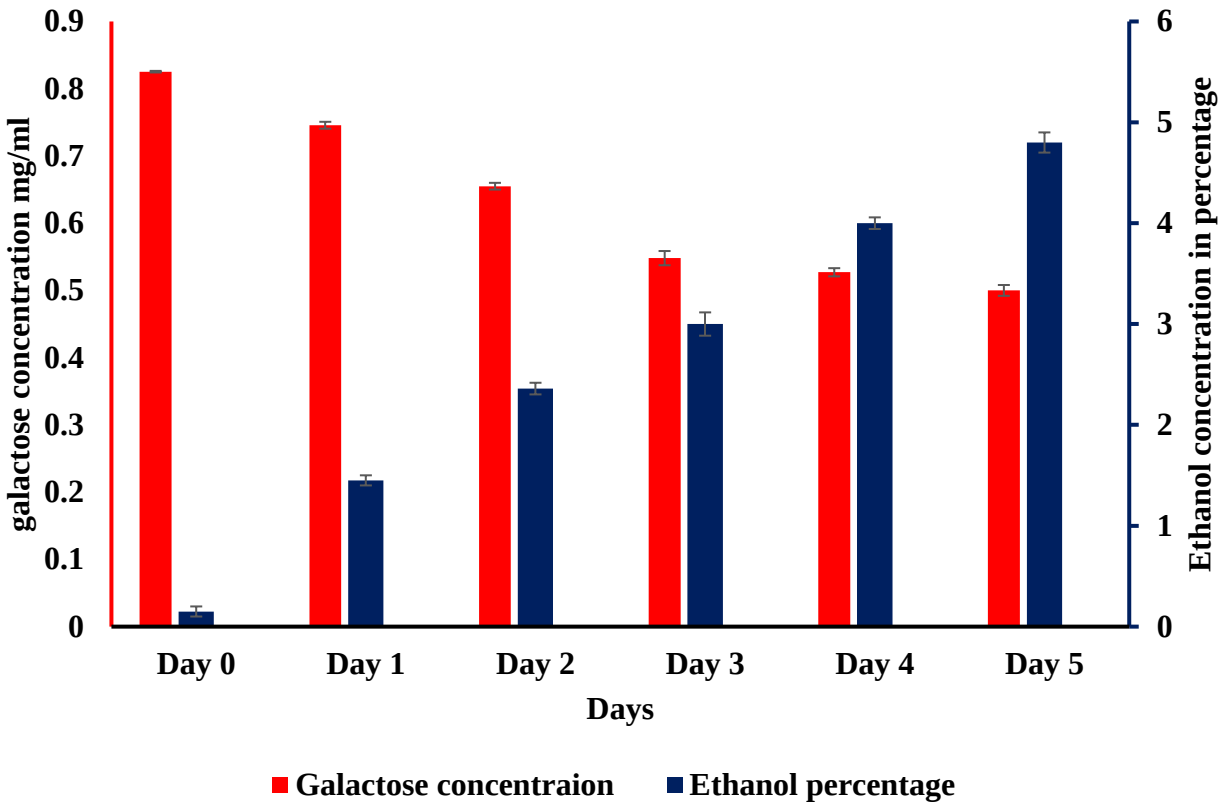


Fig 4.11 Production of ethanol from galactose sugar by *Saccharomyces cerevisiae* over a period of 5 days

Mohd et. al. (2018) reported that galactose metabolism by yeast *Saccharomyces cerevisiae* to glucose-6-phosphate by the Leloir pathway of galactose metabolism.Glucose-6-posphate can be fermented to ethanol. Kim et. al. (2014) isolated a wild type yeast *S. cerevisiae KL17* from soil which had the ability to ferment galactose and produce ethanol concentration of 3.46 g/L/h. The fermentation of galactose by this yeast was compared with that of glucose in terms of ethanol productivity and fermentation time. It was noted that the fermentation time was reduced with galactose as compared to glucose but the productivity of ethanol was higher. Hence, this set up was useful in production of ethanol from galactose by the degradation of used agar from the microbiology laboratory.

5.CONCLUSION

5.Conclusion

In the present study, six different seaweed samples were collected from Vagator beach and 27 morphologically distinct seaweed associated bacteria were isolated. These bacteria were screened for 11 different enzymes such as protease, agarase, amylase, esterase, lipase, carrageenase, alginate lyase, cellulase, pectinase, xylanase, β galactosidase. Out of all, 9 bacterial isolates were positive for five enzymes. Isolates UF3, GP2, CM3, CM4, GP3, GP4, PT1 showed protease activity, isolate UF3 also showed agarase activity and amylase activity, GP2, ST1, GC2 showed esterase activity and isolate ST1 showed lipase activity.

For further study, agarase producing isolate UF3 isolated from seaweed sample *Ulva fasciata* was used, this bacterium also showed protease and amylase activity. The bacterial isolate exhibited the ability to utilize agar from ASW broth and the agarase activity for degradation of 0.1% agar was determined to 44.16 IU. The bacterial isolate was able to tolerate 5% NaCl concentration.

As an application of the seaweed associated bacteria in degradation of used agar from microbiology laboratory, 5% and 10% bacterial inoculum was inoculated in ZMA agar and galactose production resulting from agar degradation was estimated for 5 days. It was observed that galactose concentration was maximum until day 2, further it decreased until day 5. Galactose concentration was found to be better by 5% bacterial inoculum.

As the bacteria could tolerate 5% salt, used NA agar containing 5% salt was inoculated with 5% bacterial culture and it was found to show similar trend of galactose production per day as without salt. The concentration of galactose produced was comparatively lesser

As an application of seaweed associated bacteria in valorization of agar waste. The galactose produced by agar degradation was directed towards alcohol production by fermentation process using baker's yeast *Saccharomyces cerevisiae,* the ethanol concentration and galactose concentration was checked for 5 days. It was found that the galactose sugar decreased and the ethanol concentration gradually increased until day 5.

Hence, this set up could be useful in production of ethanol from galactose by the degradation of used agar from the microbiology laboratory with the help of seaweed associated bacteria.

6.FUTURE PROSPECTS

6.Future prospects

In this study, seaweed associated bacteria were isolated and screened for different enzymes, out of which the bacterial isolate having protease, amylase, agarase, lipase, esterase enzyme activity was found. Bacterial isolate UF3, isolated from seaweed *Ulva fasciata* was selected for agarase activity and was used in the application in valorization of agar waste. It was used for degradation of used agar from microbiological laboratory for the production of galactose sugar and as a valorization of this waste agar, the produced galactose was directed for fermentation process by yeast *Saccharomyces cerevisiae* for the production of ethanol. The results obtained in this piece of research opened up a lot of scope for further study. Some of the future perspective could be:

- Utilizing the enzymes protease, agarase, amylase, esterase, lipases in valorization of wastes such as dairy waste, starch-based waste, plastic waste and oils.
- Development of a proper design for bio-ethanol production from renewable waste sources such agar waste
- Use of agarase in production of agar-oligosaccharides which have application in different areas such as food, nutraceutical, pharmaceutical and cosmetics.

7. SUMMARY

7.Summary

To summarize the research work carried out in this study

1. A total of six different seaweed samples were collect from Vagator beach and were identified and 27 morphologically distinct bacteria were isolated from it and purified. Enzyme screening was carried out for all the isolates for 11 different enzymes. Out of 9 bacterial isolates were positive for five enzymes. Isolates UF3, GP2, CM3, CM4, GP3, GP4, PT1 showed protease activity, isolate UF3 also showed agarase activity and amylase activity, GP2, ST1, GC2 showed esterase activity and isolate ST1 showed lipase activity.
2. The bacterial isolate UF3 having agarase activity was used for further study. It was grown in ASW broth, supplemented with 0.3% agar as the carbon source and its growth was determined by reading the absorbance at 600nm. The media showed turbidity(abs=1.4) as compared to the control, uninoculated broth. This confirmed that the bacterial isolate utilizes the agar for its growth.
3. Enzyme assay was carried out of the cell free supernatant which was obtained by centrifuging 72hour old culture and discarding the pellet. It was allowed to react with 0.1% agar substrate in the presence of Phosphate Buffered Saline (pH =7) at R.T for 30 minutes. Substrate control and enzyme control was kept. Galactose sugar produced by degradation of the agar was estimated by DNSA method and the enzyme activity was estimated to be 44.16 IU. Protein estimation of the cell free supernatant was carried out using the Folin-Lowrys method of protein estimation and was determined to be 150.57 µg/ml.
4. As we were aiming to use the bacterial isolate for the degradation of microbiological agar waste, and as the media can also contain salt hence it was necessary to check the salt tolerating ability of the bacterial isolate. It was determined by growing the culture in ZMB embedded with 5%, 10% ,15%, 20%, 25%, 30% NaCl concentration. It was determined that the bacterial culture can tolerate up to 5% NaCl concentration.

5. As an application of the agarolytic bacterial isolate in degradation of the used microbiological agar waste, the used ZMA agar was decontaminated twice and was inoculated with 5% and 10% of the bacterial culture and incubated at R.T at 100 rpm. The galactose sugar produced by degradation of agar was estimated over a period of 5 days using DNSA method. It was observed that the galactose concentration first increased until Day 2, after which it decreases until day 5. This might be possibly due to the bacterial isolate first utilizing nutrients from the media for its growth and later degrades agar by agarase to produce galactose therefore the galactose concentration increases. Further the galactose produced could be utilized by the bacteria hence the concentration of galactose decreases.
6. As the bacterial culture can tolerate up-to 5% NaCl concentration, used NA agar containing 5% salt was inoculated with 5% bacterial culture to check the degradation of agar and production of galactose. The production of galactose per day showed similar trend as with degradation of agar with no salt. It was observed that galactose production in this case was comparatively less.
7. As a part of using the bacteria for valorization of waste galactose sugar produced by the degradation of the agar was used for ethanol production. For harvesting maximum galactose sugar the used ZMA agar was inoculated with 5% bacterial culture and was incubated until Day 2, as it showed maximum galactose concentration on second day. The agar was filtered and centrifuged. The supernatant obtained was sterilized by autoclaving for 10 minutes. For ethanol production the supernatant was inoculated with 10% baker's yeast *Saccharomyces cerevisiae* to carry out the fermentation process and was incubated in dark conditions. The flask was swirled twice every day. To determine the amount of amount of alcohol produced ethanol concentration was determined by potassium dichromate method of alcohol estimation and galactose sugar estimation was also carried out for 5 days. It was determined that ethanol concentration gradually increased and the galactose concentration decreased until day 5.

8. ANNEXURE

ANNEXURE A: Composition of Media

Zobelle Marine Agar (ZMA)

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
Distilled water	1000ml
pH	7.2

(ZMB) Zobelle Marine Broth

Composition	g/L
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
Distilled water	1000ml
Final pH (at 25°C)	7.6±0.2

(BSS)Basal Salt Solution

Composition	g/L
$NaNO_3$	2g
$K2HPO_4$	0.5g
MgSo4.7H_2O.	0.2g

$CaCl_2.2H_2O$	0.02g
$MnSo_4.H_20$	0.02g
$FeSo_4.7H_2O$	0.02g
Agar	15g
Distilled water	1000ml
pH	7.2

NA (Nutrient Agar)

Composition	g/L
Peptone	50g
Beef extract	10g
NaCl	5g
Agar	15g
Distilled water	1000ml
pH	7.2

(ASW)Artificial seawater broth

Composition	g/L
NaCl	24.600
KCl	0.670
$CaCl_2.2H_2O$	1.360
$MgSo4.7H_2O.$	6.290
$MnCl_2.H_20$	4.66g
$NaHCO_3$	0.180g
Distilled water	1000ml

pH	7.5

Artificial sweater Agar

Composition	g/L
NaCl	24.600
KCl	0.670
$CaCl_2.2H_2O$	1.360
$MgSo4.7H_2O.$	6.290
$MnCl_2.H_20$	4.66g
$NaHCO_3$	0.180g
Agar	15g
pH	7.5
Distilled water	1000ml

Fermentation broth

Composition	g/L
Peptone	1g
NaCl	0.5g
Phenol red	0.01
Distilled water	1000
pH	7.4

(**MRVP) broth Methyl Red Vogues Proskauer**

Composition	g/L
Peptone	7g
Glucose	5g
K2HPo4	5g
Distilled water	1000
pH	6.9

Tryptophan broth

Composition	g/L
Peptone	10g
NaCl	5g
DL-Tryptophan	1g
MgSo4	2g
Distilled water	1000ml
pH	7.2

Simmons Citrate Agar

Composition	g/L
NaCl	5g
Sodium citrate	2g
Sodium dihydrogen phosphate	1g
Magnesium sulphate	2g
Bromothymol blue	0.08
Dipotassium phosphate	1g

Agar	20g
Distilled water	1000ml
pH	6.5-7.0

Hugh-Leifsons Media

Peptone	2g
NaCl	5g
Dipotassium phosphate	0.30
Bromothymol blue	0.05
Agar	2g
Distilled water	1000ml
pH	7

Nutrient broth

Composition	g/L
Peptone	50g
Beef extract	10g
NaCl	5g
Distilled water	1000ml
pH	7

Sodium Alginate Agar

Composition	g/L
Sodium alginate	5g
Ammonium sulphate	5g
Dipotassium phosphate	2g
Magnesium sulphate	1g
Agar	2g
Distilled water	1000ml
pH	7.2

ANNEXURE B: Composition of Reagents

Saline

Composition	g/L
NaCl	8.5g
Distilled water	1000

Kovac's reagent

Composition	g/100ml
Conc.HCl	25ml
Amyl alcohol	75ml
Para dimethyl amino benzaldehyde	5g

Methyl red reagent

Composition	g/500ml
Methyl red	0.1g
Ethyl alcohol	300ml
Make volume up to 500 ml	

O'Meara's reagent

Composition	g/100ml
Potassium hydroxide	40g
Creatine	0.3g
Distilled water	100ml

3% KOH

Composition	g/100ml
Potassium hydroxide	3g
Distilled water	100ml

(PBS) Phosphate Buffered Saline

Composition	g/L
NaCl	4g
KCl	0.1g
Na2HPO4	0.72g
KH2PO4	0.12
Autoclaved distilled water	Up to 500ml
pH	7.2

Lugol's Iodine

Composition	g/100ml
Potassium iodide	10g
Iodine	5g
Distilled water	100ml

Gram's Iodine

Composition	g/300ml
Potassium Iodide	2g
Iodine	1g
Distilled water	300ml

1% Congo Red

Composition	g/300
Congo red	1g
Distilled water	50ml
Ethanol	50ml

1 M NaCL

Composition	g/L
NaCl	58.44g
Distilled water	1000ml

ANNEXURE C: Methods for Quantitative estimation

C.1 Folin Lowrys method for protein estimation

Reagents

Reagent A (2%Na2CO3 in 0.1N NaOH):

NaOH 0.4g

Distilled water 100ml

Dissolve 2g Na2CO3 in an aliquot of 0.1N NaOH solution and make volume to 100 ml with 0.1N NaOH

Reagent B (0.5% CuSO4 in1% sodium potassium tartarate)

1% sodium potassium tartarate

Sodium potassium tartarate 1g

Distilled water 100

Dissolve 0.5g CuSO4 in 1% sodium potassium tartarate solution and make up the volume to 100 ml

Reagent C

Reagent A 50ml

Reagent B 1ml

Prepare fresh reagent C by mixing reagent A and Reagent B in the ratio of 50:1 prior to use

Reagent D (Folin and Ciocalteau's phenol reagent)

Commercially available reagent diluted with equal volume of distilled water on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids

Standard Bovine Serum Albumin solution(200µg/ml)

BSA standard stock

BSA	0.2g
Distilled water	100ml

BSA working stock

BSA	1ml
Distilled water	9ml

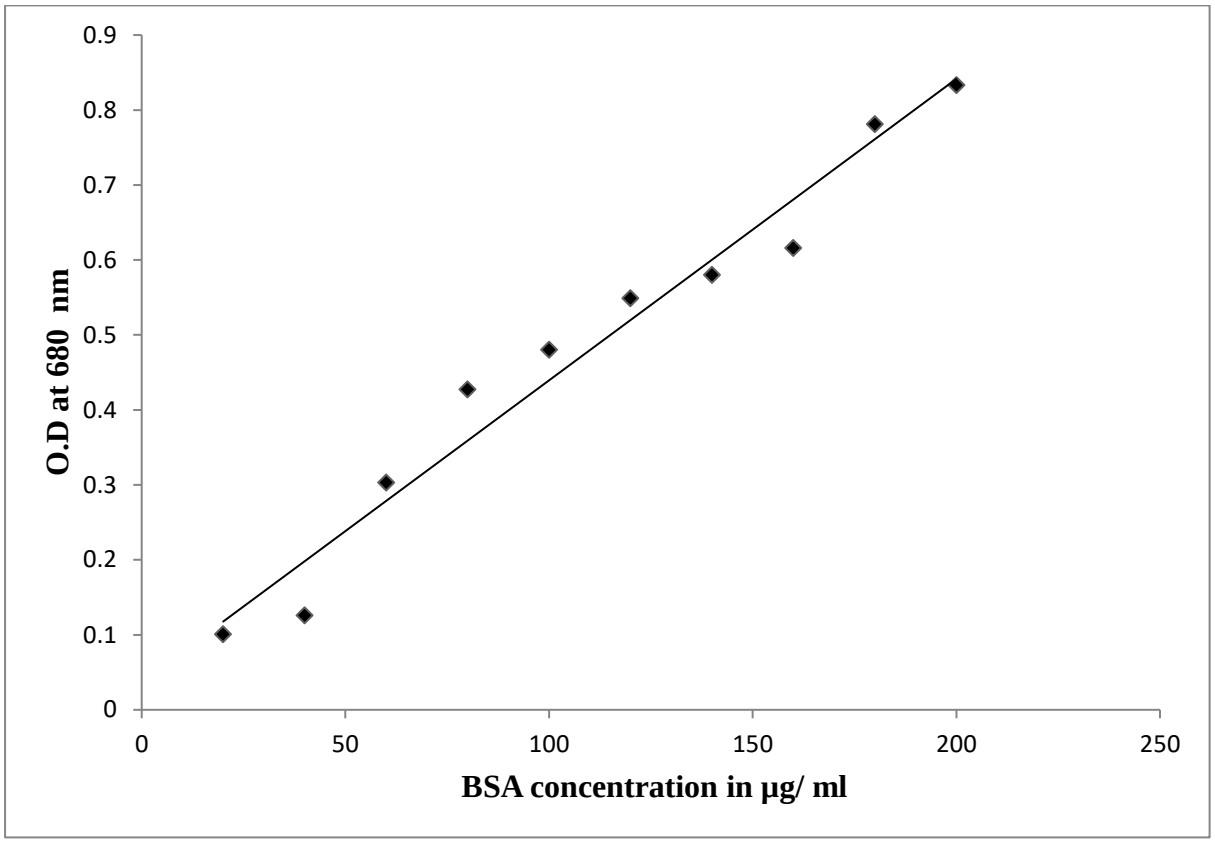
BSA working stock is prepared by mixing 1 ml of BSA standard in 9ml of distilled water

Procedure

To 1ml of sample ,5ml Reagent C was added and mixed. 0.5 ml of Reagent D was then added and tubes were kept standing in dark for 30 minutes. Absorbance was measured at 680nm against reagent blank. Standard calibration curve was prepared using bovine serum albumin as standard (20-200µ/ml. Absorption values were converted into protein by using the following equation

$$[\text{Protein}]=(\text{Absorption}-0.037)/0.004) \qquad R^2=0.966$$

This is a linear regression equation of standard calibration curve prepared using different concentrations of BSA (20-200µg/ml)


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D at 680 nm
0
50
100
150
200
250
BSA concentration in µg/ ml

C.2 Dinitrosalycyclic acid (DNSA) method for galactose estimation

Reagents:

Dinitrosalycyclic acid solution

4% NaOH Solution was prepared by dissolving 4g in 100ml distilled water. To this 0.4g of phenol and 0.4g pf DNSA is added with constant stirring until the ingredients are completely dissolved. 40 g of potassium sodium tartarate is added and mixed. 0.05g of sodium sulphite is added just before use and mixed thoroughly.

Standard stock of galactose (0.6 mg/ml)

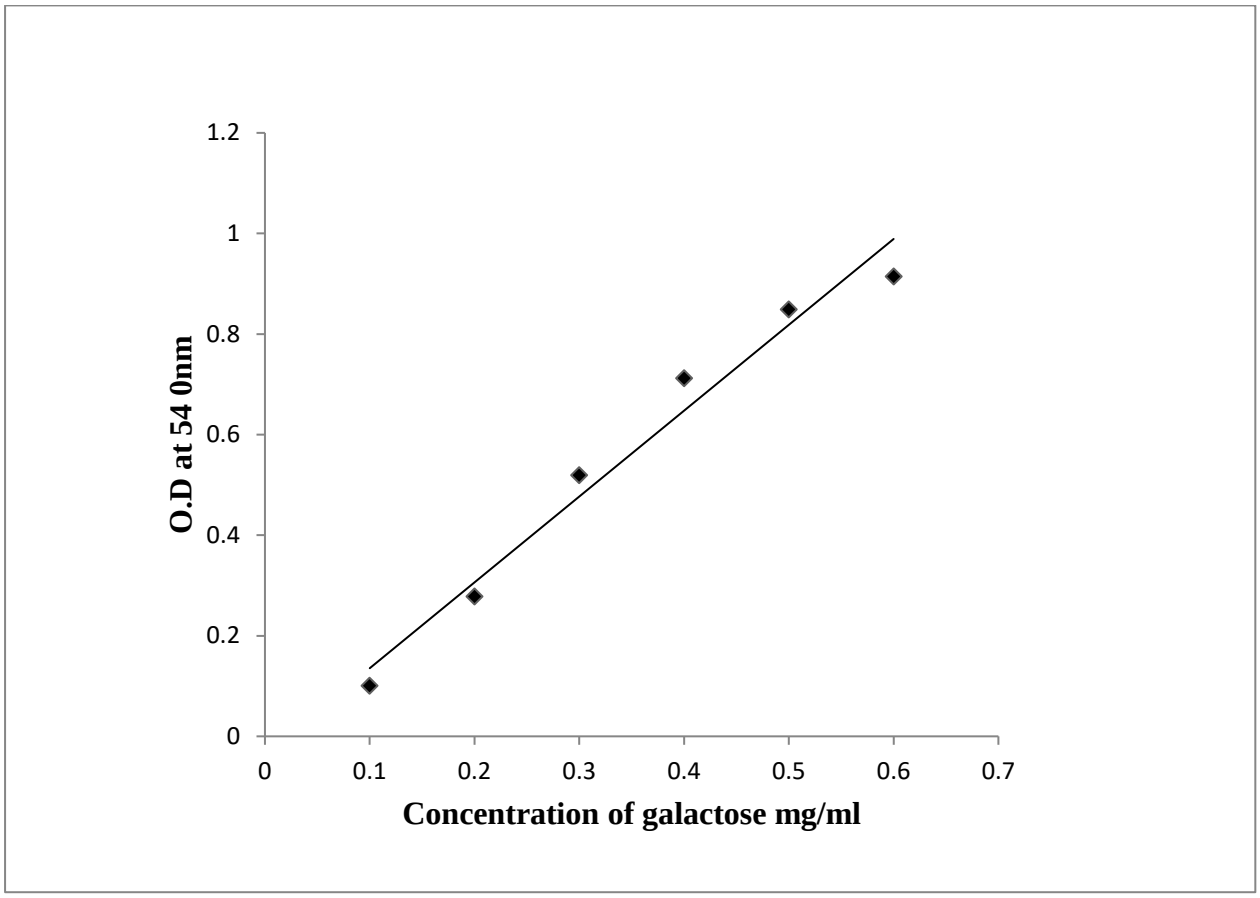
Standard stock is prepared by adding 0.006g of galactose in 10 ml distilled water.

Procedure

1 ml of sample is taken and to it 1ml of DNSA reagent is added. The tubes are incubated in boiling water bath for 10 minutes. After this, the tubes are cooled and 10 ml distilled water is added and mixed thoroughly. Absorbance is taken at 540 nm against reagent blank. Standard calibration curve was prepared using galactose as standard (0.1-0.6mg/ml). Absorption values were converted to galactose concentration using the following equation.

$$[\text{Galactose}] = (\text{Absorption} + 0.353)/1.071 \qquad R^2 = 0.972$$

This is a linear regression equation of standard calibration curve prepared using different concentrations of galactose (0.1-0.6mg/ml)


1.2
1
0.8
0.6
0.4
0.2
0
O.D at 54 0nm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Concentration of galactose mg/ml

C.3 Alcohol estimation by Potassium dichromate method

Reagents

Acidified Potassium dichromate

325 ml of conc. H_2SO_4 is taken and mixed with 400 ml of distilled water. To this 16.884g of Potassium dichromate is added and mixed. The volume is made up to 1L using distilled water

Standard stock of 20% ethanol

100% Ethanol	10ml
Distilled water	40ml

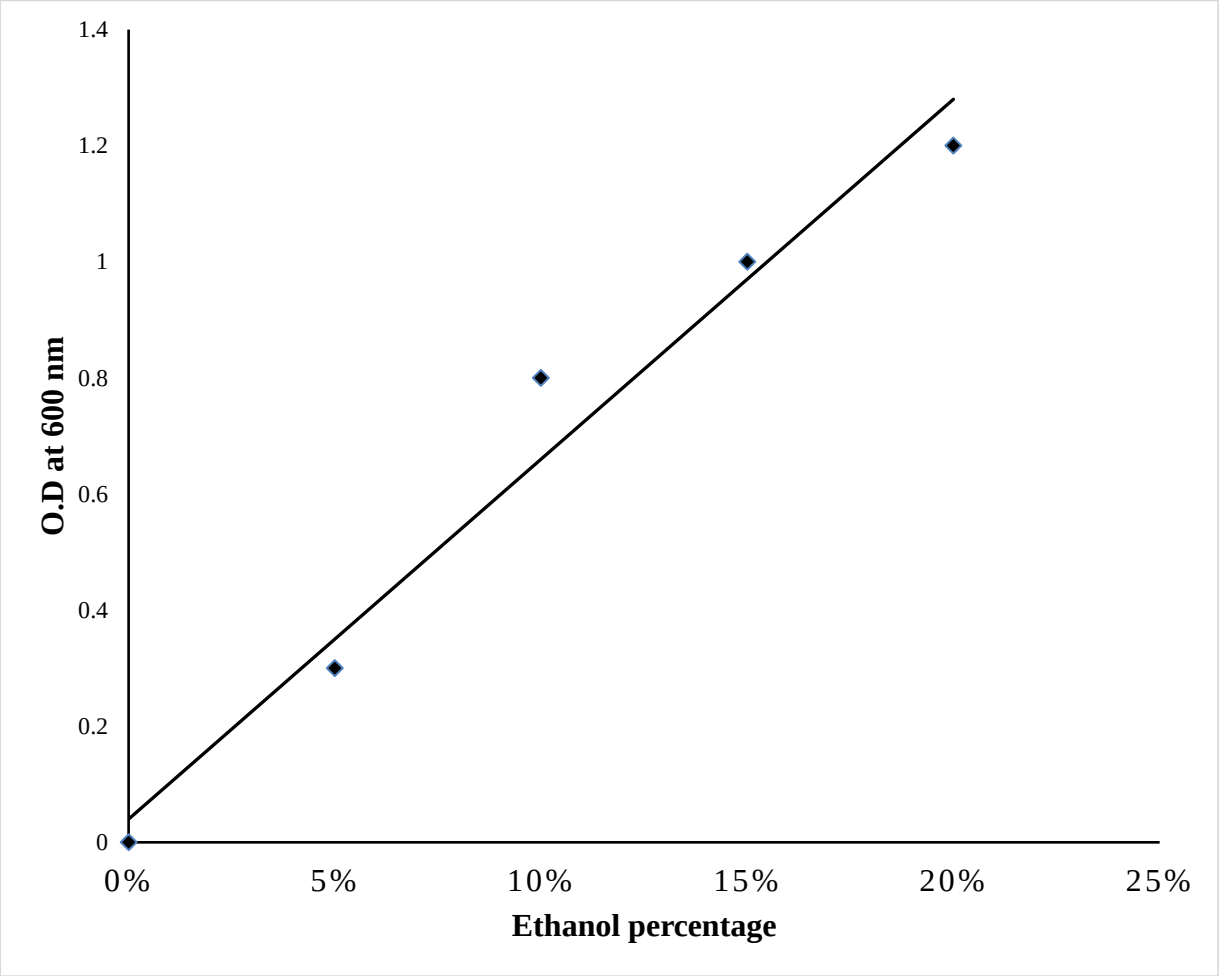
To prepare 20% stock of ethanol total 50ml, 10 ml of 100% ethanol stock was mixed in 40ml distilled water.

Procedure

1ml of sample is taken and to it 25ml of acidified potassium dichromate is added. The solution is heated at 60degree Celsius for 20 minutes. It is cooled and absorbance is taken at 600nm. Standard calibration curve was prepared using ethanol as standard (0-20%). Absorption values were converted into percentage of alcohol using the following standard equation.

$$\text{Alcohol\%} = (\text{Absorption} - 0.004)/6.2 \qquad R^2 = 0.968$$

This is a linear regression equation of standard calibration curve prepared using different percentage of ethanol (0-20%)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
O.D at 600 nm
0%
5%
10%
15%
20%
25%
Ethanol percentage

C.4 Determination of agarase enzyme activity

The galactose sugar produced after reaction of cell free supernatant with 0.1% agar for 30 minutes is 1325µg/ml.

Agarase activity = Amount of galactose produced (µg per ml) / reaction time(minutes)

=1325/30

=44.16 IU

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