Evaluating the bioactive potential of polysaccharides and oligosaccharides from the seaweeds of the Indian coast

A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE School of Earth, Ocean And Atmospheric Sciences

(Marine Science)

GOA UNIVERSITY

By

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December 2022

DECLARATION

As required under the University Ordinance OA-19A-1. I **Ashok Shivaji Jagtap** hereby declare that the thesis entitled **"Evaluating the bioactive potential of polysaccharides and oligosaccharides from the seaweeds of the Indian coast"** submitted to the Goa University, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in **School Of Earth, Ocean And Atmospheric Sciences (Marine Sciences)**, is a record of original and independent research work done by me under the supervision and guidance of **Dr. Cathrine S. Manohar**, Principal Scientist, Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, Goa and it has not been submitted for the award of any Degree/ Diploma/ Associateship/ Fellowship or other similar title of any candidate of any University. The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgement has been made wherever required.

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Ashok Shivaji Jagtap

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CERTIFICATE

This is to certify that the thesis, entitled "**Evaluating the bioactive potential of polysaccharides and oligosaccharides from the seaweeds of the Indian coast**", submitted to the Goa University, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Marine Science, is a record of original research work done by **Mr. Ashok Shivaji Jagtap**, under my supervision and guidance and the thesis or any part has not been previously submitted for the award of any Degree/ Diploma/ Associateship/ Fellowship or other similar title to any candidate of any University.

Signature of the Guide

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ACKNOWLEDGEMENT

This thesis becomes a reality with a kind support and help of many individuals. I would like to extend my sincere thanks to all of them.

I take this opportunity to express my sincere gratitude to my research supervisor, Dr. Cathrine S. Manohar, Principal Scientist, Biological Oceanography Division CSIR-National Institute of Oceanography, Dona Paula, Goa, India, for her valuable guidance, constant encouragement and support and constructive criticism during the entire Ph.D. research work. Her dedication and keen interest above all her overwhelming attitude to help her students had been mainly responsible for completing this work. Her timely advice, meticulous scrutiny, scholarly advice and scientific approach have helped me to great extent to accomplish research work.

I am grateful to Prof. Sunil Kumar Singh, Director, CSIR-NIO, Goa, for providing the necessary facilities during my tenure in the institute.

I am greatly thankful to Dr. C. U. Rivonkar, Professor, School of Earth, Ocean and Atmospheric Sciences, Goa University, for his constant support and valuable suggestions in research progress during DRC meetings. I am also thankful to Dr. Narsinh Thakur, Senior Principal Scientist, Chemical Oceanography Division, CSIR-NIO, Goa, for his encouragement and suggestions during DRC meetings. I am thankful to Dr. Manguesh Gauns, Senior Principal Scientist, Biological Oceanography Division, CSIR-NIO, Goa, for his suggestions and encouragement during SRF extension meetings.

I am extremely thankful to Dr. Kisam M. Kodam, Professor, Biochemistry Division, Chemistry Department, Savitribai Phule Pune University, Pune, for their guidance moral support and encouragement.

I am thankful to Dr. Surya Nandan Meena, Dr. Ravi Singh Baghel, Dr. Ashwini Chaudhari, Dr. Viresh Thamke, Dr. Savita Tapase, Dr. Vinod Nandre, Dr. Aditi Bagade, Dr. Vijay Markad, for their constant encouragement.

I am greatly thankful to Ms. Vasudha C Bhatawadekar, for her immense help in GC analysis and moral support received during entire research work. I also would like to thank my friend Mr. Nitin S Kadam for his kind support and assistance during analysis of samples and data generation.

I shall always remain indebted to the scientists from NIO, Dr. Mohandass C., Dr. Samir Damare, Dr. Supriya Tilvi, Dr. Mandar Nanjkar and Dr. Lidita Khandeparkar for their help and support.

I am thankful to Mr. Ram Murti Meena, Mr. Sudesh Morajkar, Ms. Teja Naik and Ms. Safia Khan for their help during analytical services.

I am extremely thankful to my friends and lab mates Ashutosh Parab, Ashwini Toraskar, Jovitha Lincy, Aparna Mol TA, Soumadipta, Deepak, Akash Surwase, Vijay Sankar, Rajyashree, Amarachi, Sumit, Uktika, Prantik Patra, Seleyeino Seleyi, Anushruti and Meenakshi Naik for their help and support.

I owe everything in this life to my parents and all my family members for their support and motivation during my study. I would like to thanks my friends Akshay Khalate, Ravi Sable, Vaibhav Shinde, Vishal Bandgar, Sachin Bhosale, Rajendra Ransing, Vruti Naik, Sujata and Laxman for their delightful cohabitation.

I am greatly thankful to Council of Scientific and Industrial Research for the JRF and SRF fellowship.

Finally, my sincere thanks and apologies are to everybody that supported me and my memory did not aid me to mention them.

Ashok Shivaji Jagtap

….DEDICATED

TO MY

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Abbreviations

AO: Agaro-oligosaccharide

AAE: Ascorbic acid equivalent

AOAC: Association of Official Agricultural Chemists

CAZy: Carbohydrate-Active enZYmes

CFU: colony-forming units

DNSA: Dinitrosalicylic acid

FOS: Fructooligosaccharides

GAE: Gallic acid equivalent

GH: Glycoside hydrolase

MAB: Macroalgae associated bacteria

MEX: Macroalgal extracts

MPS: Macroalgal polysaccharides

NAO: Neoagaro-oligosaccharide

PC: Polysaccharide content

PL: Polysaccharide lyase

POS: Porphyran oligosaccharides

PB1: Porphyranase from *Bacillus* sp.

TPC: Total phenolic content

ULB1: Ulvan lyase from *Bacillus* sp.

UOS: Ulvan oligosaccharides

α-GI: α-glucosidase inhibition

Chapter 1

Introduction

1.1 Structure and classification of Macroalgae or Seaweeds

The ocean covers nearly 70% of the earth's surface area and the marine habitats have tremendous biodiversity. More than half of the global carbon fixation through photosynthesis is reported from these habitats due to the growth of marine autotrophs, microalgae, macroalgae and seagrasses (Roleda and Hurd, 2019). Macroalgae or seaweeds are primitive, multicellular, aquatic non-vascular plants which play a vital role in the sustainable productivity of coastal, intertidal waters (Duarte et al., 2017). They grow predominantly along the coastal, rocky shores across all geographical locations, including the tropical, subtropical, temperate and polar regions where photosynthetic light and substratum are available (Pellizzari et al., 2017; Selvarajan et al., 2019; Hung et al., 2021). The complete structure of macroalgae is called a thallus and is composed of a holdfast, stipes and blades. Holdfast is a specialized structure at the base of macroalgae that plays an essential role as an anchor to the substratum or rocks. Stipe is a stem-like structure in some groups of macroalgae and blades provide the larger surface area for photosynthesis (**Figure 1.1**).

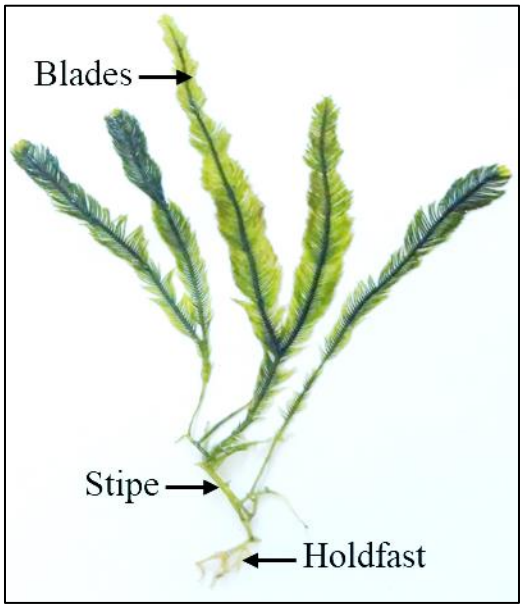


Figure 1.1. *Caulerpa sertulariodes* depicting the structure of marine macroalgae collected from the Goa coast.

Macroalgae or seaweeds are classified into three major groups, Rhodophyta (Red), Phaeophyta (Brown) and Chlorophyta (Green), based on their pigment and cell wall composition (Keith et al., 2014). Red macroalgae are phylogenetically the oldest group, with maximum diversity. This division includes the economically most important macroalgae such as *Kappaphycus*, *Eucheuma*, *Gracilaria*, *Gelidium* and *Porphyra* (Lim et al., 2017; Torres et al., 2019). Red macroalgae constitutes specific sulfated galactan of carrageenan, agar and porphyran as polysaccharides and phycobilliproteins such as phycocyanin, allophycocyanin and phycoerythrin (Aguilera et al., 2002; Chellamanimegalai et al., 2022). They are rich in complex sugars, polyphenol, protein, polyunsaturated fatty acid and minerals, due to which they are known for their plethora of biological activities and commercial applications (Sudhakar et al., 2015; Ismail et al., 2020).

Brown macroalgae or Phaeophyta are the second largest group of macroalgae broadly available from tropical (*Sargassum, Turbinaria, Dictyota, Padina*) to polar (Macrocystis or kelp including *Laminaria*) waters (Pellizzari et al., 2017). The brown color of macroalgae is due to the presence of xanthophyll (0.3%) composition (Pereira et al., 2021). These brown macroalgae contain pigments, cellulose, polyphenolic contents, sulfated polysaccharides such as alginate, laminarin, and fucoidan with enormous bioactive potential (Kadam et al., 2015; Baghel et al., 2020). *Laminaria* and *Undaria* are the major commercially cultivated seaweeds used for alginate production. *Sargassum* is the most common and wild harvested macroalgae for its specific polysaccharides such as alginate, fucoidan and laminarin (Mantri et al., 2020a).

Green macroalgae or chlorophytes are usually grown in rocky, shallow intertidal zones with low tidal fluctuations and high intensity of sunlight. Green macroalgae *Ulva lactuca*, *Caulerpa racemosa*, *Enteromorpha intestinalis* and *Chaetomorpha antennina* are studied and explored for their nutritional values (de Gaillande et al., 2017; Kumar et al., 2018; Mantri et al., 2020b;

Vinuganesh et al., 2022). They are rich in sulfated polysaccharide ulvan and non-essential amino acids and dietary fibers, which are utilized in dietary supplements (Wells et al., 2017; Mantri et al., 2020b). Ulvan is a polydisperse heteropolysaccharide with a rare composition of iduronic acid, glucuronic acid and sulfated rhamnose sugar moieties (Lahaye et al., 1999). Green macroalgae have a huge bioactive potential for direct consumption as a dietary supplement and functional food (Surget et al., 2017). Overall, macroalgae have unique biomolecules which include polyphenols, phlorotannins, steroids, polyunsaturated fatty acids, carotenoids, flavonoids, terpenoids, vitamins, minerals, pigment and unique polysaccharides, which can be used as active pharmaceutical ingredients. Exploring the potential of these biomolecules are in high demand in the healthcare sector which can promote the development of a Blue Economy.

1.2 Ecological importance of macroalgae

Macroalgae play a critical ecological role in photosynthesis through carbon fixation and can sequester higher carbon dioxide levels per unit area than microalgae and terrestrial plants (Mongin et al., 2016). Their potential to reduce carbon footprint is widely recognized as the crucial and most effective process for mitigating the harmful effects of climate change and reducing the impact due to ocean acidification and deoxygenation (Chung et al., 2011; Duarte et al., 2017). Due to their effective photosynthesis, they have high productivity rates, with an estimated gross primary productivity of around 1600 g $C/m^2/y$ (Hughes et al., 2012). Seaweed cultivation in the coastal areas does not require agricultural inputs such as fertilizer, pesticides, or irrigation and most importantly, no fertile land is required. These benefits of seaweed farming make them superior to the other photosynthetic organism that can be used for CO_2 sequestration. Large scale cultivation of macroalgae can also contribute to improving the economic status of the coastal communities,

fishermen and rural population by developing alternate employment opportunities. Hence seaweed cultivation can be the way forward, as it facilitates CO_2 sequestration and is a widely accepted measure for reducing the carbon footprint and provide good economic returns (Duarte et al., 2017; Hurd et al., 2022). The carbon fixation by macroalgae through photosynthesis is stored into novel and unique sugars or polysaccharides, which are reported to have wide applications in various pharmaceutical and nutraceutical industries.

1.3 Economic importance of macroalgae

The requirement for algal foods is rising around the world as they are progressively consumed for nutrition and health (Vellinga et al., 2022). Macroalgae is a proven source of metabolites, proteins, pigments, bioactive compounds and unique polysaccharides (Keating et al., 2014; Camus et al., 2019; Shannon and Ghannam, 2019; Gajaria and Mantri, 2021). They have a long history of being used as a food ingredient and a wide range of literature, from ancient texts to recent studies, also appertain their broad applications throughout history to treat various illnesses and disorders (Cian et al., 2015). Macroalgae are renewable bioresources and well-accepted marine vegetables globally with rich bioactive compounds, consumed regularly in Southeast Asian countries. In recent years, the utilization of marine macroalgae are expanding exponentially due to their various biomolecules, which serve as a natural resource for health benefits (Olasehinde et al., 2019; Venkatesan et al., 2019). Macroalgae have the edge over other plants as a source of bioactive molecules; they are devoid of lignin and are composed of unique structural and storage polysaccharides, which are not commonly present in terrestrial plants (Li et al., 2020). Macroalgal biomolecules are known to provide bio-protection and get enormous attention in the functional food and biomedicine industry (Baghel et al., 2014; Nunes et al., 2020; Sanjeewa et al., 2018).

Harnessing these biomolecules for healthcare applications can explore its potential for sustainable development of blue economy.

1.4 Scope of the study

Various studies show that regular consumption of macroalgae can reduce chronic diseases, including cancer, obesity and diabetes (Tanna and Mishra, 2018; Gajaria and Mantri, 2021). There is a vast diversity of macroalgae, with 7500 red, 2000 brown, and 1800 green macroalgal species reported worldwide (Keith et al., 2014). Macroalgal diversity from India is well studied and documents approximately 5000 macroalgal species along the 7500 km coastal stretch, including islands. Approximately 865 macroalgal species of, 442 red, 211 brown and 212 green macroalgae are recorded from Indian waters (Mantri et al., 2019). The maximum macroalgal diversity is reported from Tamil Nadu (302), followed by Gujarat (202), Maharashtra (159), Goa (75) and Lakshadweep (89) (Pereira and Almeida, 2014; Venkataraman and Wafar, 2005). Extensive research on the diversity, aquaculture practices, production of value-added products and extraction of bioactive molecules from different macroalgal groups are reported from the Indian sub-continent and its adjoining islands. Previous studies have reported the diversity and interactions of microbes in the macroalgal ecosystem from these regions (Singh and Reddy, 2014; Naik et al., 2019). They have also depicted that macroalgae provide a suitable substratum for settling microorganisms and release various organic substances that promote bacterial growth and biofilm formation (Singh et al., 2011; Selvarajan et al., 2019). However, the biotechnological potential of the macroalgae-associated bacteria for its enzymes to produce algal oligosaccharides is yet to be fully explored. The macroalgal polysaccharides are a significant component of macroalgae with an immense potential source of natural dietary fibers and hydrocolloids and are used in anticancer,

anticoagulant products, drug delivery and tissue engineering applications (Tanna and Mishra, 2019; Otero et al., 2021). However, their utility in nutraceutical and functional food industries is limited due to their high molecular weight, low biocompatibility and complex structure (Lovegrove et al., 2017; Gopinath et al., 2018). The enzymatic hydrolysis of these polysaccharides is gaining importance due to the specificity of enzymes to cleave or hydrolyze the glycosidic bonds to produce stereo-specific bioactive macroalgal oligosaccharides (Cheong al., 2018). The macroalgae-associated bacteria are the source of these specific polysaccharide-degrading enzymes, which can be explored for their potential to produce functional oligosaccharides.

In this study, I propose to evaluate the bioactive potential of macroalgal polysaccharides from the seaweeds of the Indian coast and study the enzymatic activities of macroalgae-associated bacteria for hydrolyzing complex polysaccharides into bioactive oligosaccharides for healthcare applications.

1.5 Research aims and objectives

Aim: Evaluating the bioactive potential of polysaccharides and enzymatically produced oligosaccharides for healthcare applications.

- Objective 1: Isolation of bacteria associated with green, red and brown algae collected from the major seaweed beds of India.
- Objective 2: Extraction and characterization of algal polysaccharides with nutraceutical potential.
- Objective 3: Production of algal oligosaccharides to evaluate its nutraceutical potential.

1.6 Sampling site for macroalgae collection

Macroalgal samples were collected from major seaweed beds along the Indian coast to extract seaweed polysaccharides and to isolate macroalgae-associated bacteria. The sampling stations included Okha and Porbandar along the Gujarat coast, Malvan and Kunkeshwar along the Maharashtra coast, Vagator, Dona Paula, Palolem from the Goa coast and Mandapam along the Tamil Nadu coast (**Figure 1.2**).

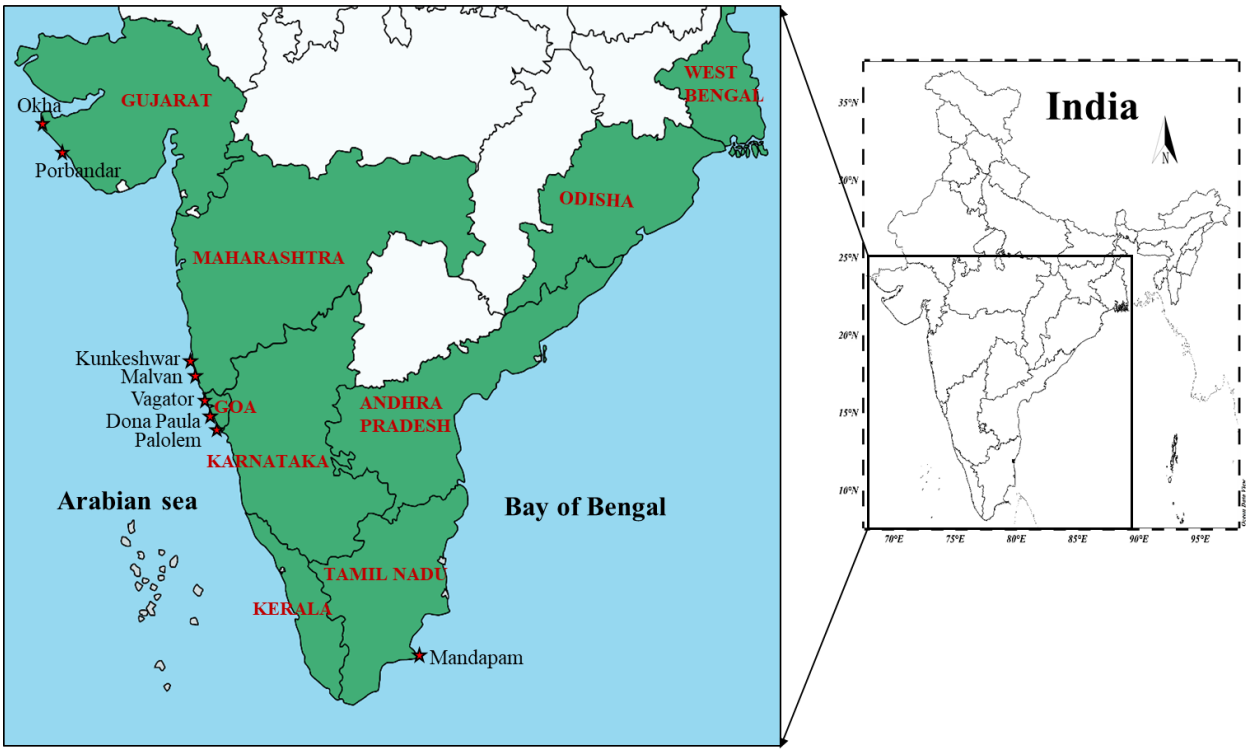


Figure 1.2 Sampling locations along Gujarat, Maharashtra, Goa and Tamil Nadu coast.

Chapter 2

Extraction and characterization of macroalgal polysaccharides for its bioactive potential

2.1 Introduction

Marine bioresource macroalgae or seaweed have the potential to treat a wide range of chronic ailments, including cancer, obesity, diabetes, and coronary heart disease. There is a wide range of studies, which appertain their broad applications as a food ingredient and for their therapeutic value (Cian et al., 2015; Gajaria and Mantri, 2021). Macroalgae are exposed to various environmental stresses such as wind currents, tidal fluctuations and temperature variations. Hence, they have developed an effective antioxidative defense system by synthesizing various biomolecules in their cell to counteract the effect of free radicals produced due to oxidative stress (Maharana et al., 2015; Michalak et al., 2020). These biomolecules can be harnessed to prevent oxidative stress. In an individual with a healthy physiological condition, reactive oxygen species are effectively eliminated by antioxidant enzymes produced by the defense system of the body. However, when there is excessive production of reactive oxygen species and it overwhelms the natural defense system of the body (Poljsak et al., 2013). This triggers various cellular pathways and leads to several physiological damages such as pancreatic β-cells dysfunction leading to the onset of diabetes, lipopolysaccharide-induced inflammatory diseases and immune diseases due to the inhibition of cytokine production (Wright et al., 2006; Sebastian et al., 2021; Sruthy and Philip, 2021). Several reports have revealed that macroalgal extracts and polysaccharides from various seaweed species exhibit potent free radical scavenging activity and can be used as antioxidants to prevent oxidative damage in living organisms (Rajauria et al., 2021; Vanavil et al., 2020; Wang et al., 2021). The antioxidant activity is very vital and as it can prevent a cascade of physiological stress, which leads to the progression of numerous chronic diseases. Most of the studies on polysaccharides have reported their applications in biomedicine as an anticoagulant, antiplatelet, immunomodulation, anti-inflammatory, antiviral, antidiabetic or antithrombic agents individually

(Adrien et al., 2019; Cui et al., 2019; da Silva Chagas et al., 2020; Koh et al., 2020; Bhardwaj et al., 2021). However, macroalgal dietary sugars or polysaccharides, which are one of the most biologically active molecules that exist in the marine environment, are not thoroughly investigated and characterized for a combination of bioactivities such as antioxidant, antiglycemic and prebiotic activities, which can be used in various functional food, nutraceutical and pharmaceutical applications.

Macroalgal biomass comprises about 50% of complex polysaccharides in its cell wall and it is the only source of industrially important unique polysaccharides, including agar, carrageenan, porphyran from red algae; alginate, fucoidan and laminarin from brown algae and ulvan from green algae (Venkatesan et al., 2016; Khora and Navya 2020). These macroalgal polysaccharides (MPS) are constituted of pentose sugars (xylose and arabinose), hexose sugars (galactose, glucose and mannose) and rare sugars (rhamnose, uronic acids), which are unique to macroalgae (Robin et al., 2017; Nguyen et al., 2020). MPS have been extensively used as gelling agents and emulsifiers. Off late, macroalgal sugars are in great demand as a source material for biofuel and bioplastic production and as a pharmaceutical ingredient (Baghel et al., 2020; Tanna and Mishra, 2019).

Studies on the bioactive potential of macroalgal extracts and polysaccharides extracted from the seaweeds of India have been conducted to evaluate their antioxidant and anticoagulant, antioxidant and antibacterial activities (Mohan et al., 2019; Palanisamy et al., 2017; Seedevi et al., 2017; Sudharsan et al., 2015; Vanavil et al., 2020). However, the potential of macroalgal extract and polysaccharides for their combined antioxidant, antiglycemic and prebiotic activities have not been explored. Hence, this chapter aims to extract macroalgal polysaccharides through aqueous extraction and characterize them using Fourier Transform Infrared spectroscopy (FTIR) and Gas Chromatography (GC) to determine their functional group and monosaccharide composition.

Evaluation of antioxidant and antiglycemic potential of polysaccharides was also carried out using the model radical compounds such as DPPH, ABTS, H_2O_2 and inhibition of the α-glucosidase enzyme activity respectively. The prebiotic activity of polysaccharides was also determined through the growth profiling of probiotic and enteric pathogenic bacteria. The results were also assessed to find the relationship between the chemical composition of aqueous extract and their bioactivity.

2.2 Materials and methods

2.2.1 Chemicals and reagents

The chemicals 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide (H_2O_2), ascorbic acid and gallic acid were procured from Sigma Aldrich, USA. Commercial α-glucosidase, protease and acarbose were obtained from Sisco Research Laboratory, India. Molecular grade 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and the standard sugars rhamnose, fucose, arabinose, xylose, glucose and galactose were bought from Himedia laboratories, India. All other reagents, solvents and acids were used of analytical grade.

2.2.2 Seaweed collection and processing

Macroalgal samples were collected during low tide in the post-monsoon season (September to December) from the selected locations Okha (22.28423N, 69.04223E), Porbandar (21.37596N,

Red Macroalgae	Brown Macroalgae	Green macroalgae
		
Acanthophora spicifera	*Padina tetrastromatica*	*Chaetomorpha antennina*
		
Gracilaria corticata	*Sargassum cinereum*	*Caulerpa racemosa*
		
Halymenia venusta	*Spatoglossum asperum*	*Caulerpa sertulariodes*
		
Hypnea muciformis	*Stoechospermum marginatum*	*Ulva taeniata*
		
Porphyra umbilicalis	*Sphacelaria rigidula*	*Ulva lactuca*

Figure. 2.1 Macroalgae selected for the study (Diameter of the coin is 20mm).

69.36227E), Kunkeshwar (16.20003N, 73.23286E), Malvan (16.063120N, 73.455749E), Vagator (15.35591N, 73.43577E), Anjuna (15.34051N, 73.44278E), Dona Paula (15.27095N, 73.48037E) and Palolem (15.0019N, 74.01338E) along the Indian coasts (**Figure 1.2).** Based on their biomass availability, the fifteen macroalgal species, five from each red, brown and green macroalgal group, were selected for this study (**Figure 2.1**). Macroalgal samples were rinsed with sterile seawater to remove the sand particles, epiphytes and seashells. The macroalgae were sun-dried at an ambient temperature of 28 ± 5 °C for 2 – 3 days, followed by further heat drying in a ventilated oven at 40 ± 2 °C for 12 h and finely ground using an electrical mixer-grinder into a fine powder and used for aqueous extract preparation and extraction of polysaccharides.

2.2.3 Macroalgal aqueous extracts preparation and macroalgal polysaccharides extraction

The aqueous extract was prepared by immersing 100 gm of macroalgal powder (1:40 w/v) in distilled water and subjected to a steam explosion at 121 °C and 15 psi pressure for 30 minutes. The extract was filtered through a 20-micron mesh and the supernatant was concentrated on a rotary evaporator to ¼th its volume and lyophilized in a freeze-dryer (Labconco, USA). The macroalgal aqueous extract (MEX) of the 15 different seaweeds was prepared and sequentially labeled MEX1 to MEX15 (**Table 2.1**). The aqueous extracts were treated overnight with chilled ethanol at 4 °C to precipitate polysaccharides. The precipitate was collected through centrifugation at 8000 rpm for 10 minutes and washed twice with ethanol, freeze-dried and used for further analysis. The macroalgal polysaccharide (MPS) from the fifteen different macroalgae were labeled as MPS1 - MPS15 (**Table 2.1**).

Table 2.1. List of macroalgae selected for the preparation of aqueous extract (MEX) and macroalgal polysaccharides (MPS).

Macroalgae	Collection site	Collection date	Extract Id	Polysaccharide Id
Red macroalgae				
Acanthophora spicifera (M.Vahl) Børgesen	Palolem, Goa	12/12/2018	MEX1	MPS1
Gracilaria corticata (J. Agardh) J. Agardh	Malvan, Maharashtra	02/01/2019	MEX2	MPS2
Halymenia venusta Børgesen	Okha, Gujarat	29/12/2018	MEX3	MPS3
Hypnea musciformis (Wulfen)	Malvan, Maharashtra	02/01/2019	MEX4	MPS4
Porphyra umbilicalis Kützing	Vagator, Goa	11/12/2018	MEX5	MPS5
Brown macroalgae				
Padina tetrastromatica Hauck	Anjuna, Goa	11/12/2018	MEX6	MPS6
Spatoglossum asperum J. Agardh	Palolem, Goa	12/12/2018	MEX7	MPS7
Sargassum cinereum J. Agardh	Dona Paula, Goa	11/12/2018	MEX8	MPS8
Stoechospermum polypodioides (J.V.Lamouroux)	Anjuna, Goa	11/12/2018	MEX9	MPS9
Sphacelaria rigidula Kützing	Dona Paula, Goa	11/12/2018	MEX10	MPS10
Green macroalgae				
Chaetomorpha antennina (Bory) Kützing	Vagator, Goa	11/12/2018	MEX11	MPS11
Caulerpa racemosa (Forsskål) J. Agardh	Okha, Gujarat	29/12/2018	MEX12	MPS12
Caulerpa sertularioides (S.G.Gmelin) M. Howe	Kunkeshwar, Maharashtra	02/01/2019	MEX13	MPS13
Ulva taeniata Setchell & N.L. Gardner	Porbandar, Gujarat	29/12/2018	MEX14	MPS14
Ulva lactuca Linnaeus	Vagator, Goa	11/12/2018	MEX15	MPS15

2.2.4 Biochemical analysis of macroalgal extracts and polysaccharides

The total sugar content in all the fifteen MEX and MPS was estimated following the Phenol-sulfuric acid method (Dubois et al., 1956) using galactose as the standard. Briefly, the MEX and MPS were mixed with 5% phenol, followed by the addition of 96% sulfuric acid and cooled for 10 min. The intensity of the colored complex was measured at 490 nm using a UV-visible spectrophotometer (Shimadzu, Japan). Similarly, reducing sugar was also determined following dinitrosalicylic acid method (Miller, 1959). For this, the MEX and MPS were mixed with 3, 5-dinitrosalicylic acid (DNSA) reagent, followed by incubation for 5 min in a boiling water bath to stop the reaction. Then the tubes were cooled and the absorbance of the colored complex formed was measured at 540 nm. The polysaccharide content (PC) was calculated by estimating the reducing and total sugar content using different concentrations of galactose and expressed as µg mg^{-1} dry weight of the MEX and MPS (**Appendix 2.1**). The total phenolic content (TPC) of the MEX and MPS was determined using the Folin–Ciocalteu method reported by Martins et al. (2013) with slight modifications. In brief, MEX and MPS were mixed with Folin–Ciocalteu reagent and incubated for 5 min at 25 °C to facilitate the reaction. Following this, Na_2CO_3 (7.5%) was added to eliminate the residual reagent and after incubation for 30 minutes, the absorbance was measured at 760 nm. The TPC content was estimated using different concentrations of gallic acid as standard (**Appendix 2.2**) and expressed as mg GAE g^{-1} dry weight of the MEX and MPS. The enzyme gravimetric technique (AOAC-991.43) was used to estimate the total amount of dietary fibre, as described by McCleary et al. (2015). Briefly, the MPS samples (1 gm) were accurately weighed in a glass tube with 1 ml ethanol and 35 ml of 100 mM sodium maleate buffer (pH 6.0) was added and the mixture was stirred at 150 rpm for 10 min to equilibrate at 37 °C. To the mixture, 5 ml of the enzyme solution containing pancreatic α-amylase (6 KU/5 ml) and amyloglucosidase (1.7

KU/5 ml) was added and incubated at 37 °C for exactly 4 h. After incubation, the reaction was terminated with 3 ml of Tris base (pH 11.0), followed by the addition of 0.1 ml protease solution at 60 °C and incubated for 30 min. The pH of the solution was adjusted to pH 4.3 using 2 M acetic acid and dietary fibers were precipitated with chilled 95% ethanol for 1 h. The residue was vacuum filtered and washed with 78% ethanol. The residue was dried overnight at 105 °C and the dietary fiber content was calculated from the residual mass.

The carbon and nitrogen contents in MPS were determined using CN elemental Analyzer (EURO 177 EA3000/EUROVECTOR) and calibrated using 2 - 3 mg BBOT (2, 5-Bis (5-tert-butyl-benzoxazol-oxazol-2-yl) thiophene) as standard. The neutral sugar content of each MPS was determined based on the $BaCl_2$-gelatin turbidity method (Dodgson and Price, 1962) and the sulfate content was also estimated through the resorcinol sulfuric acid method (Monsigny et al., 1988) using different concentrations of potassium sulfate as standard (**Appendix 2.3**). The protein and phenolic contents using different concentrations of bovine serum albumin were also determined to check the purity of MPS (**Appendix 2.4**).

2.2.5 Structural characterization of macroalgal polysaccharides

2.2.5.1 FTIR spectroscopy to analyze functional groups

The MPS powder was mixed with potassium bromide to record the Fourier Transform Infrared spectrum, to determine the functional groups in all the fifteen algal polysaccharides (MPS1 - MPS15). The spectrum was determined by recording 50 scans in the range of 4000 - 400 cm^{-1} wavenumber using the Shimadzu-8201PC spectrometer. The functional groups of MPS were elucidated based on the absorption peaks and compared with commercially available seaweed polysaccharides.

2.2.5.2 Gas chromatography to analyze monosaccharide composition

The monosaccharide composition of MPS was determined following the method of Cui et al., (2018) using gas chromatography. In brief, MPS (10 mg) was hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 4 h in sealed glass tubes. After complete hydrolysis, excess acid was removed by co-distillation with methanol using a rotary evaporator (four times wash). Pyridine (0.5 ml) and hydroxylamine hydrochloride (10 mg) were added to the samples and standard sugars (Rhamnose, Fucose, Glucose, Galactose, Xylose, and Arabinose) and the tubes were incubated at 90 °C for 30 minutes. It was followed by acetylation for 30 minutes at 90 °C using acetic anhydride (0.5 ml). The residual pyridine and acetic anhydride were removed using a dry nitrogen flush. These derivatized samples were thoroughly dried and dissolved in a known volume of chloroform and analysed by gas chromatography. The Shimadzu GC-2010 plus system with a capillary column WCOT fused with silica (film thickness of 0.2 µm, 25 m) and a flame ionization detector was used. The detector and injector temperature was maintained at 220 °C. Injections were carried out in splitless mode while the oven temperature was first set at 160 °C, kept for 2 minutes, ramped up to 220 °C at a rate of 5 °C min^{-1} and then maintained at this temperature for 10 minutes.

2.2.6 Antioxidant activity of the macroalgal extracts and polysaccharides

2.2.6.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of MEX and MPS was evaluated using the modified method of Cui et al., (2018). In brief, 0.2 mM DPPH was prepared in 99.5% ethanol and kept in the dark for 2 h to stabilize the radicals. After stabilization, the absorbance was adjusted to 1.00 ± 0.05 at 517 nm with 200 µl of ethanol and 800 µl of Tris-HCl buffer (pH 7.4) and a DPPH reagent was used for analysis. Briefly, 1 ml (0.2 mM) DPPH-methanol solution was mixed with different

concentrations of MEX and MPS (0.5 - 2 mg ml^{-1}). The reaction mixture was incubated at 25 °C for 30 minutes in the dark and the reduction in absorbance was measured at 517 nm. Ascorbic acid was used as a standard antioxidant compound.

2.2.6.2 ABTS radical scavenging activity

The ABTS radical scavenging activities of MEX and MPS were evaluated using an improved ABTS decolorization assay (Re et al., 1999). Briefly, ABTS was dissolved in water at 7 mM concentration with 2.45 mM potassium persulphate and the reaction mixture was incubated for 12 - 16 h to stabilize the ABTS cation radicals ($ABTS^{+\circ}$), as they react stoichiometrically at a 1:0.5 ratio, which leads to partial oxidation of ABTS and the formation of $ABTS^{+\circ}$ radicals. The ABTS solution was diluted using phosphate-buffered saline (pH 7.0) to adjust the absorbance to be less than 0.80 at 734 nm. For the assay, the ABTS radical solution was premixed with different concentrations of MEX and MPS solution (0.5 - 2 mg ml^{-1}) and the decrease in the absorbance was measured at 734 nm. Ascorbic acid was used as a standard antioxidant compound.

2.2.6.3 H_2O_2 radical scavenging activity

The potential of MEX and MPS to scavenge H_2O_2 radicals was determined following the method reported by Smirnoff and Cumbes (1989). For the assay, the reaction mixture consisted of different concentrations of MEX and MPS (0.5 - 2 mg ml^{-1}), 9 mM FeSO4, 9 mM salicylic acid and 9 mM H_2O_2, which was incubated for 10 minutes at 37 °C. The reduction in absorbance was recorded at 510 nm. The DPPH, ABTS and H_2O_2 radical scavenging activity (%) of MEX and MPS were determined and compared with ascorbic acid as a model compound using the below formula.

Scavenging activity (%) = (Abs. Control - Abs. Sample) / Abs. Control × 100

2.2.6.4 Total antioxidant capacity assay

The total antioxidant capacity of the MEX and MPS was determined using the reported method (Zayed et al., 2016). In a test tube, different concentrations of MEX and MPS (0.5 - 2 mg ml^{-1}) were mixed with Phosphomolybdate reagent (sulfuric acid (0.6 M), sodium phosphate (28 mM) and 4 mM ammonium molybdate) and the test tube was covered with aluminium foil and heated to 95 °C for one hour. After the reaction mixture had reached room temperature, the absorbance at 765 nm was measured. The data were reported as mg g^{-1} of gallic acid equivalents using gallic acid as the benchmark (GAE).

2.2.7 Antiglycemic activity of macroalgal extracts and algal polysaccharides

The antiglycemic activity of the MEX and MPS was evaluated through the inhibition of the α-glucosidase enzyme (Zaharudin et al., 2019). For this, the enzyme α-glucosidase (0.1 U ml^{-1}) was premixed with different concentrations of MEX (20 - 200 µg ml^{-1}) and MPS (500 - 1000 µg ml^{-1}) in 20 mM sodium phosphate buffer (pH 6.9). The reaction was incubated for 30 minutes at 37 °C in dark, followed by the addition of 3 mM p-nitrophenyl-α-D-glucopyranoside as substrate. Acarbose was used as a standard antidiabetic compound. The reaction was aborted by adding 0.1 M Na_2CO_3 and the absorbance was measured at 405 nm to estimate the % inhibition of α-glucosidase activity using the below formula.

Inhibition activity (%) = (Abs. Control - Abs. Sample) / Abs. Control × 100

2.2.8 Prebiotic activity of macroalgal polysaccharides

The prebiotic activity of MPS was determined based on their ability to promote the growth of two potential probiotic bacteria, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* and suppress the growth of enteric culture, *Escherichia coli* (DSM 1576). Fructooligosaccharides from chicory (FOS) were used as a commercial prebiotic compound. The growth was determined by culturing these isolates in a dextrose-free Man-Rogosa-Sharp medium supplemented with 1% MPS as the sole carbon source and measuring the absorbance at 600 nm at 0 and 24 h. The proliferation of probiotic and enteric cultures in the presence of MPS as the sole carbon source was measured by enumerating the number of colony-forming units (CFUs). To calculate the prebiotic score, 100 µl of starter and 24 h grown probiotic and enteric cultures were spread plated on basal Man-Rogosa-Sharp medium and incubated at 37°C for 24 h under anaerobic conditions in a RUSKINN CONCEPT 400M workstation. The growth of probiotic and enteric cultures was measured by enumerating the number of colony-forming units (CFUs).

The prebiotic activity score (PA) was calculated using the following equation.

$$\mathrm{PA} = [(\log \mathrm{Px}^{24} - \log \mathrm{Px}^{0}) \div (\log \mathrm{Pg}^{24} - \log \mathrm{Pg}^{0})] - [(\log \mathrm{Ex}^{24} - \log \mathrm{Ex}^{0}) \div (\log \mathrm{Eg}^{24} - \log \mathrm{Eg}^{0})]$$

Where Px^{24} and Px^{0} are the CFU measured of probiotic cultures in 1% MPS or FOS supplement at 24 h and 0 h, respectively. Pg^{24} and Pg^{0} are the CFU measured in 1% glucose supplement at 24 h and 0 h, respectively. Similarly, Ex^{24} and Ex^{0} and Eg^{24} and Eg^{0} are the CFU measure of enteric culture, *E.coli*. The CFU was enumerated by plating the culture broth on Man-Rogosa-Sharp media plates (Hu et al., 2006).

2.2.9 Cytotoxicity assay of potential macroalgal polysaccharides

The cytotoxicity analysis of those polysaccharides which showed high bioactivity was determined based on colorimetric tetrazolium MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Arunkumar et al., 2021). For this, the ability of the metabolically active cells to reduce yellow tetrazolium MTT through the action of mitochondrial dehydrogenase to produce NADH and NADPH were tested. This resulted in the formation of purple formazan which was directly related to the number of viable cells and quantified by spectrophotometer. For the assay, Neonatal Mouse Dermal fibroblast cell line (NMDF) were cultured in medium (DMEM + 10% FBS + 1X Antibiotic-Antimycotic solution) and the cells were homogeneously seeded in 96-well plates at a density of 8000 thousand cells per well after being counted with a hemocytometer, and they were then incubated in 5% CO_2 for 24 hours. Following the attachment of the cells, 100 µl of a polysaccharide sample was introduced to the wells at varying concentrations (50 - 250 µg ml^{-1}) and left to incubate for 24 hours. Control wells with cells and no additional medication were estimated while blank wells contained only DMEM. Under sterile conditions, MTT (5 mg ml^{-1}) was applied to each well and the plates were then incubated at 37 °C in the dark for 4 hours. After incubation, plates were centrifuged at 200 × g for 5 minutes. After carefully aspirating 50 µl of the supernatant without disturbing the pellet, the formazan crystals in it were dissolved in DMSO (Arunkumar et al., 2021). The absorbance was measured at 540 and 570 nm and the cytotoxicity of MPS8, MPS9 and MPS10 were calculated using the formula.

Viability (%) = (Sample absorbance / Control absorbance)*100

2.2.10 Statistical analysis

All experimental studies were carried out in triplicates (n=3) and mean values are expressed along with standard deviations. The tests of normality were performed using Shapiro-Wilk statistics and

the homogeneity of variance test was carried out using Levene statistics. Pearson's correlation was carried out to assess the relationship between the variables and the significance level was set as $P \leq 0.05$ using statistical tools.

2.3 Results

2.3.1 Biochemical composition of macroalgal extracts

The PC and TPC of fifteen MEX were determined and the results show that MEX14 and MEX15 of green alga *Ulva* sp. had the maximum PC of 83.68 and 80.2 mg g^{-1} respectively, followed by MEX1 and MEX4 of red algae, *Acanthophora* sp. and *Hypnea* sp. with 53.43 and 39.12 mg g^{-1} of PC respectively (**Figure 2.2a**). Maximum PC content was seen in MEX9 of brown algae *Stoechospermum* sp. with 35.68 mg g^{-1}. In all the other macroalgae, the PC was less than 30 mg g^{-1}. MEX10 of *Sphacelaria* sp. showed the highest TPC of 18.40 mg g^{-1} GAE among the macroalgae studied. It was followed by MEX1 and MEX9 of *Acanthophora* sp. and *Stoechospermum* sp. and with 16.68 and 15.30 mg g^{-1} GAE of TPC, respectively. The TPC in all the other macroalgae tested was less than 13 mg g^{-1} GAE (**Figure 2.2b**).

2.3.2 Antioxidant activity of macroalgal extracts

The efficiency of MEX for their ability to stabilize the free radicals was tested using model radicals DPPH, ABTS and H_2O_2. The MEX9 and MEX1 of *Stoechospermum* sp. and *Acanthophora spicifera* showed the highest DPPH radical scavenging activity of 98% and 93%, respectively, which was close to the model compound ascorbic acid. The MEX10 of *Sphacelaria* sp. also

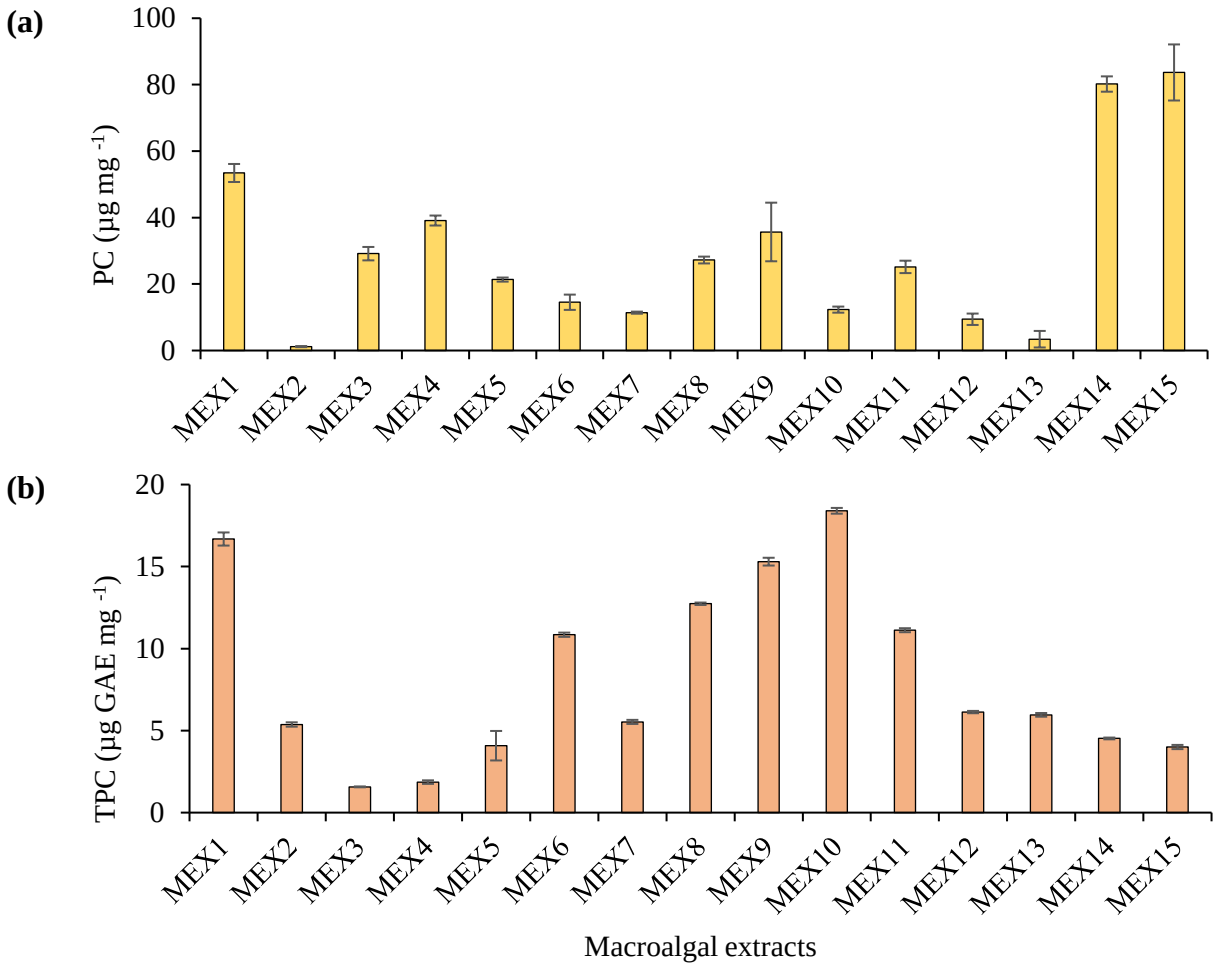


Figure 2.2 Polysaccharide content **(a)** and total phenolic content **(b)** of macroalgal extracts (MEX). The error bars denote standard deviations of the mean (n = 3).

showed about 68% DPPH radical scavenging activity (**Table 2.2**). Similarly, the highest ABTS radical scavenging activity was shown by MEX9 of brown algae *Stoechospermum* sp. with 65% scavenging activity, followed by MEX10 of *Sphacelaria* sp. with 36% ABTS radical scavenging. The brown algae MEX9 and MEX10 of *Stoechospermum* sp. and *Sphacelaria* sp. also showed up to 89% and 75% of H_2O_2 radical scavenging activity, respectively. The total antioxidant capacity assay was carried out to test the concentration-dependent antioxidant capacity of all the MEX. The

MEX1 of red macroalgae *Acanthophora* sp. showed the highest antioxidant capacity of 29.3 mg g^{-1} AAE, followed by MEX10 and MEX9 of brown macroalgae *Sphacelaria* sp. and *Stoechospermum* sp. with 28.6 mg g^{-1} AAE and 22.9 mg g^{-1} AAE respectively (**Table 2.2**).

Table 2.2 Antioxidant activities of macroalgal extracts at different concentrations.

	DPPH (%)				**ABTS (%)**				**H_2O_2 (%)**				**TAC (mg g^{-1} AAE)**			
	0.1	0.5	1.0	2.0	0.1	0.5	1.0	2.0	0.1	0.5	1.0	2.0	0.1	0.5	1.0	2.0
Sample ID	(mg ml^{-1})				(mg ml^{-1})				(mg ml^{-1})				(mg ml^{-1})			
MEX1	16	41	76	93	4	14	16	20	10	21	36	60	2.5	9.1	16.2	29.3
MEX2	4	6	14	19	1	2	4	7	13	20	24	43	0.4	1.4	1.3	3.2
MEX3	26	28	30	35	1	2	5	10	10	15	29	36	0.3	0.4	0.6	1.0
MEX4	23	24	23	26	1	2	6	10	12	16	23	30	0.1	0.6	0.7	1.4
MEX5	29	34	41	57	3	8	15	25	4	16	31	55	0.6	2.4	5.4	16.8
MEX6	10	17	27	50	4	4	11	23	9	24	42	70	1	1.2	2.3	3.1
MEX7	6	15	26	50	1	2	4	10	6	9	28	42	1	1.3	2.3	3
MEX8	11	24	37	60	2	11	30	36	8	24	41	70	1.2	1.4	2.5	3.2
MEX9	31	64	86	98	2	18	38	65	18	54	78	89	1.5	6.3	11.7	22.9
MEX10	20	26	52	68	2	11	30	36	12	24	44	75	2.0	8.5	15.5	28.6
MEX11	8	25	39	53	2	3	13	32	15	24	39	59	0.2	0.3	0.4	0.5
MEX12	5	14	24	39	1	3	4	10	8	16	25	37	1.2	1.3	2.5	3.1
MEX13	7	13	23	38	2	5	5	12	14	17	27	39	0.5	2.2	5.1	8.9
MEX14	20	30	35	47	2	4	7	12	11	22	37	55	0.3	1.6	2.3	4.5
MEX15	22	31	38	49	1	2	6	11	10	21	36	61	0.3	1.2	2.1	4.5

The IC_{50} values for each of the radicals were calculated at four different concentrations between 0.1 to 2 mg ml^{-1} of the MEX, along with their % radical scavenging activity (**Table 2.3**). The highest DPPH radical scavenging activity was shown by MEX9 of *Stoechospermum* sp. with IC_{50}

0.22 mg ml^{-1}, followed by MEX1 of *Acanthophora* sp. and *Sphacelaria* sp. having an IC_{50} value of 0.5 mg ml^{-1} and 0.9 mg ml^{-1} respectively. The ABTS radical scavenging activity of brown macroalgae *Stoechospermum* sp. (MEX9) and *Sphacelaria* sp. (MEX10) showed an IC_{50} value of 1.33 mg ml^{-1} and 3.9 mg ml^{-1}, respectively. In the H_2O_2 radical scavenging assay, MEX9 of *Stoechospermum* sp. showed the lowest IC_{50} value of 0.38 mg ml^{-1}, followed by MEX10 of *Sphacelaria* sp. (1.05 mg ml^{-1}) and MEX 1 of *Acanthophora* sp. (1.49 mg ml^{-1}).

Table 2.3 IC_{50} value of macroalgal extracts for free radical scavenging activity.

	Antioxidant activity		
	IC_{50} value (mg ml^{-1}) to scavenge free radicals		
Sample ID	**DPPH**	**ABTS**	**H_2O_2**
MEX1	0.5±0.41	4.43±0.47	1.49±0.15
MEX2	14.2±0.11	59.1±4.83	3.19±0.67
MEX3	3.7±0.20	15.93±7.06	3.73±0.29
MEX4	7.1±2.16	13.35±3.92	5.48±0.59
MEX5	1.6±0.54	3.62±0.11	1.71±0.10
MEX6	2.22±0.67	4.42±0.53	2.88±0.98
MEX7	1.97±0.40	10.89±0.53	2.82±0.07
MEX8	1.55±0.21	3.52±0.33	1.84±0.22
MEX9	0.9±0.16	3.90±0.25	1.05±0.11
MEX10	0.22±0.02	1.33±0.06	0.38±0.02
MEX11	1.6±0.19	4.36±0.22	1.50±0.18
MEX12	2.8±0.18	17.82±23.0	3.13±0.16
MEX13	2.72±0.28	8.57±1.07	2.65±0.45
MEX14	2.32±0.06	7.66±0.26	2.59±0.07
MEX15	2.1±0.22	8.61±0.76	2.73±0.19
Ascorbic acid	0.02±1.20	0.076±2.20	0.08±2.56

2.3.3 Antiglycemic activity of macroalgal extracts

Antiglycemic activity based on α-glucosidase inhibition assay was estimated for all the fifteen MEX at 0.5 mg ml^{-1} concentration. Among the macroalgae tested, only three MEX of *Acanthophora* sp. (MEX1), *Stoechospermum* sp. (MEX9) and *Sphacelaria* sp. (MEX10) showed positive results and were further tested for enzyme inhibition activity at different concentrations (0.005 to 0.2 mg ml^{-1}). Brown macroalgal extract of *Sphacelaria* sp. (MEX10) showed an excellent αGI activity of 91.84%, followed by *Acanthophora* sp. (MEX1) and *Stoechospermum* sp. (MEX9) with 66% and 56% inhibition, respectively (**Figure 2.3**).

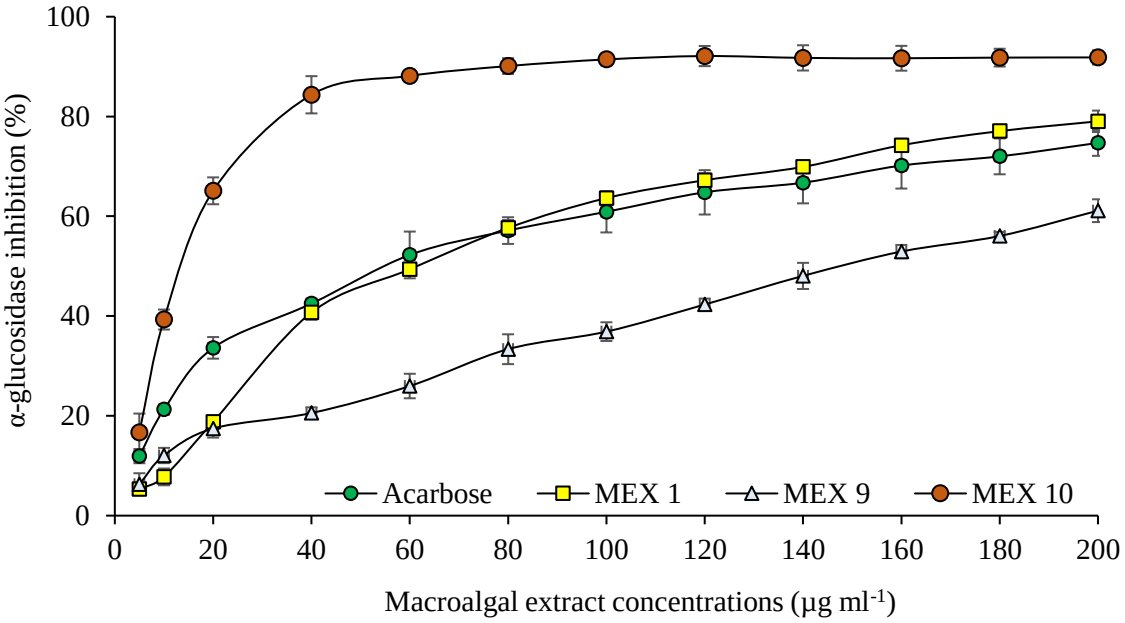


Figure 2.3 α-glucosidase inhibition activity of macroalgal extracts MEX1, MEX9, MEX10 compared with acarbose.

The highest αGI activity with the lowest IC_{50} value was shown by MEX10 of brown algae *Sphacelaria* sp. at 0.013 mg ml^{-1}, which was lower than positive control acarbose with an IC_{50} value of 0.054 mg ml^{-1}. The MEX1 and MEX9 of macroalgae *Acanthophora* sp. and

Stoechospermum sp. also showed good αGI activity with IC_{50} values of 0.062 mg ml^{-1} and 0.151 mg ml^{-1} respectively. Brown macroalgal extract of *Sphacelaria* sp. with a very low IC_{50} value (0.013 mg ml^{-1}) and high (91.84 %) αGI prove to have the highest αGI activity, surpassing the model compound acarbose which is widely used for diabetes control.

2.3.4 Biochemical composition of macroalgal polysaccharides

The total dietary fiber, polysaccharide, sulfate, neutral sugar, carbon and nitrogen content of all the MPS was determined (**Table 2.4**). Red macroalgal polysaccharides showed 35 to 79% of dietary fiber content. Brown macroalgal polysaccharides MPS6 and MPS7 reported 81% and 76% of dietary fiber content. The MPS8 and MPS10 showed about 66% dietary fiber and about 47 % only was recorded in MPS9. Green algal polysaccharides have a broad range of 35 - 87% dietary fiber content, with >80% found in MPS11, MPS14 and MPS15 (**Table 2.4**). The red macroalgal polysaccharides showed PC of 224 - 924 mg g^{-1} with the highest PC content recorded in MPS5. The brown MPS showed low PC in the range of 116 - 515 mg g^{-1} and the green MPS showed the highest PC of 599 to 815 mg g^{-1}. The sulfate group content was found in only a few macroalgal polysaccharides, MPS1, MPS2 and MPS3, with 7, 5 and 1% sulfate group content, respectively. The sulfate content of 5, 6 and 1% was recorded in brown algal polysaccharides MPS6, MPS7 and MPS10. Similarly, green macroalgae MPS14 and MPS15 also showed sulfation of 6 - 7% and others showed less than 2% sulfate content only (**Table 2.4**). The high neutral sugar content was found in red algal polysaccharides from 19 - 43% and the minimum neural sugar found in brown and green macroalgal polysaccharides ranges between 12 - 35%. Similarly, carbon and nitrogen were high in red macroalgal polysaccharides (10 - 32% C and 0.8 - 3.79% N).

Table 2.4 Biochemical composition in studied macroalgal polysaccharides.

Macroalgal polysaccharides	Polysaccharide content (mg g^{-1})	Total dietary fibre (%)	Sulphate group content (%)	Carbon content (%)	Nitrogen content (%)
MPS1	621±3.88	79±3.82	7±0.06	22.1±1.5	0.81±0.1
MPS2	224±0.42	65±1.48	5±0.19	10.9±0.5	0.88±0.1
MPS3	797±3.46	35±3.54	1±0.11	29.4±7.4	3.47±2.7
MPS4	628±1.20	69±0.49	ND	24.8±0.0	0.80±0.0
MPS5	927±1.97	43±6.90	ND	32.8±0.0	3.79±0.0
MPS6	130±0.14	81±0.49	5±0.17	9.9±0.1	0.57±0.0
MPS7	116±0.28	76±0.49	6±0.26	8.5±0.1	0.41±0.0
MPS8	228±0.35	66±0.99	ND	20.6±0.6	0.24±0.1
MPS9	515±1.13	47±6.15	ND	22.4±0.2	0.21±0.0
MPS 10	269±2.54	66±6.15	1±0.21	22.5±7.3	2.68±0.8
MPS 11	815±1.62	84±1.63	1±0.1	24.3±0.4	1.15±0.1
MPS 12	758±0.28	67±3.25	2±0.08	20.2±0.1	1.59±0.0
MPS 13	611±2.12	35±8.91	ND	19.7±0.3	3.40±0.0
MPS 14	599±0.14	82±1.48	7±0.11	23.5±0.0	1.03±0.0
MPS 15	782±0.42	87±1.56	6±0.08	22.2±0.1	1.10±0.1

ND- not detected

The green macroalgal polysaccharides showed a comparatively good amount of carbon and nitrogen between 20 - 25% C and 1 - 3.5% N. The low carbon and nitrogen content of 8.5 - 22.5% C and 0.2 - 2.68% N was recorded in brown macroalgal polysaccharides. The protein and phenolic

content in red, brown and green macroalgal polysaccharides were less than 20 mg g^{-1} and 11.79 mg g^{-1} GAE, respectively.

2.3.5 Structural characterization of macroalgal polysaccharides

2.3.5.1 Infrared spectroscopy

The IR spectrum of MPS was recorded using an IR spectrophotometer to evaluate the functional groups. The vibrations between 750 - 950 cm^{-1} (anomeric region) and 950 - 1200 cm^{-1} (sugar region) are important structural regions for the functional group characterization of macroalgal polysaccharides. In the IR spectrum, the broad peak between 3423 - 3443 cm^{-1} and small peaks at 2912 - 2939 cm^{-1} are assigned to O–H and C–H stretching vibrations, respectively. The peak between 1602 - 1643 cm^{-1} and 1408 - 1421 cm^{-1} is characteristic of carboxylate O–C–O asymmetric stretching and C-OH deformation vibration, respectively. The absorption peak between the wave number 1020 - 1093 cm^{-1} assigns C-O and C-C stretching vibrations due to the pyranose ring. The red algal polysaccharide's specific absorption was observed at 931 cm^{-1} which could be due to the C-O-C of 3, 6- anhydro-L/D-galactose. The peak at 830 cm^{-1} and 840 cm^{-1} is assigned for sulfation on C2 and C4 of galactose, respectively. The peak at 805 cm^{-1} is due to the sulfation on C2 of 3, 6-anhydro-L-galactose. The IR spectrum of agar and carrageenan was compared with the MPS and the MPS2 and MPS3 showed functional groups similar to carrageenan. The MPS1, MPS4 and MPS5 showed functional groups similar to agar (**Figure 2.4a, b**). The band at 1124 - 1136 cm^{-1} is assigned to the S=O stretching vibrations in the sulfate ester form. Peaks peculiar to brown algal polysaccharides were seen between 877 and 892 cm^{-1} and they were attributed to C-H deformation vibration of β-mannuronic acid residues. The band at 821 cm^{-1} and around 808 cm^{-1} could be characteristic of mannuronic acid and glucuronic acid residues.

The IR spectrum of alginate and laminarin was compared with the MPS and the MPS6, MPS7 and MPS8 showed functional groups similar to alginate (**Figure 2.5a)**. The MPS9 and MPS10 showed functional groups similar to laminarin (**Figure 2.5b**).

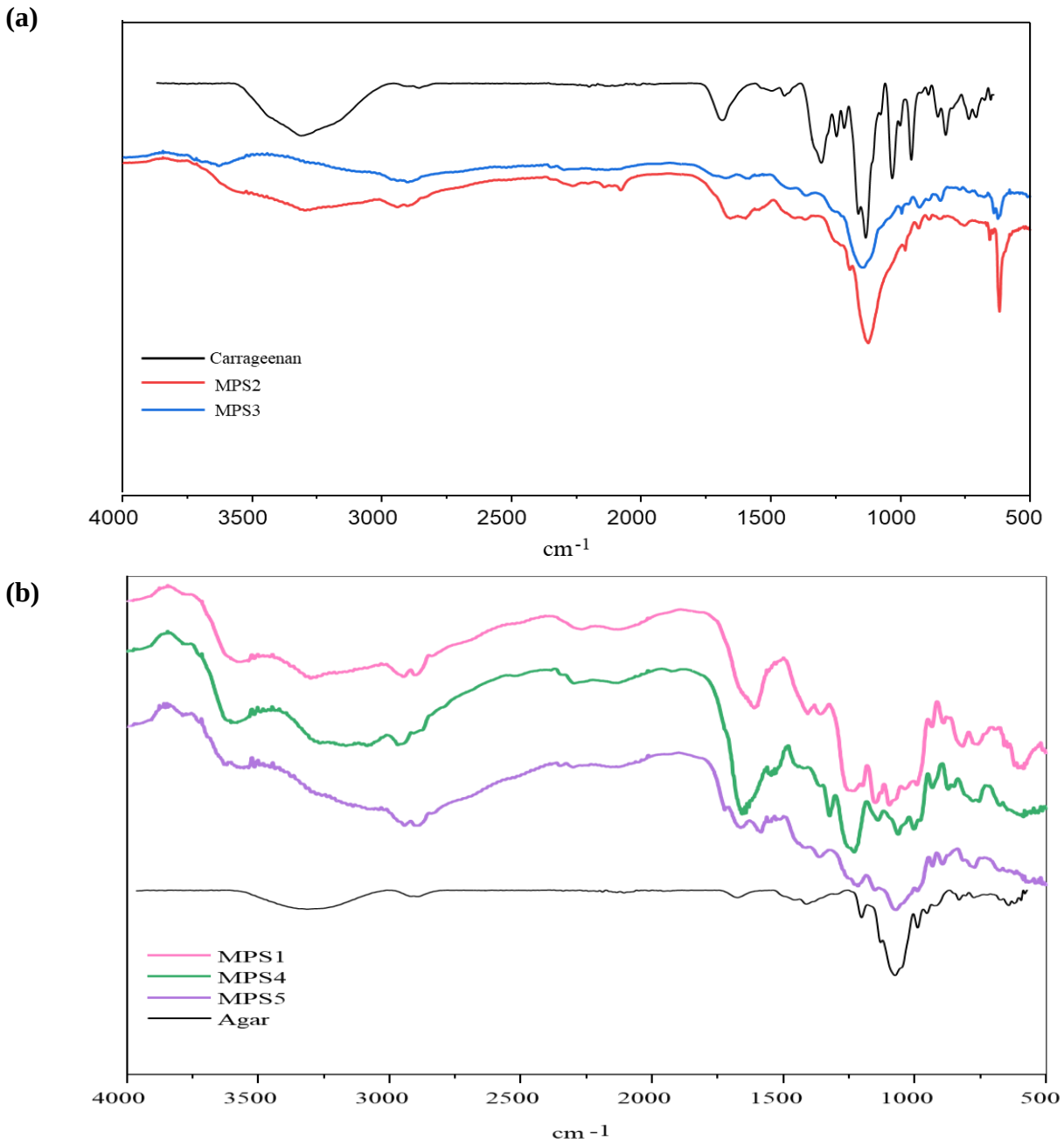


Figure 2.4 Fourier Transform Infrared spectrum of macroalgal polysaccharides of red macroalgae compared with carrageenan **(a)** and agar **(b)**.

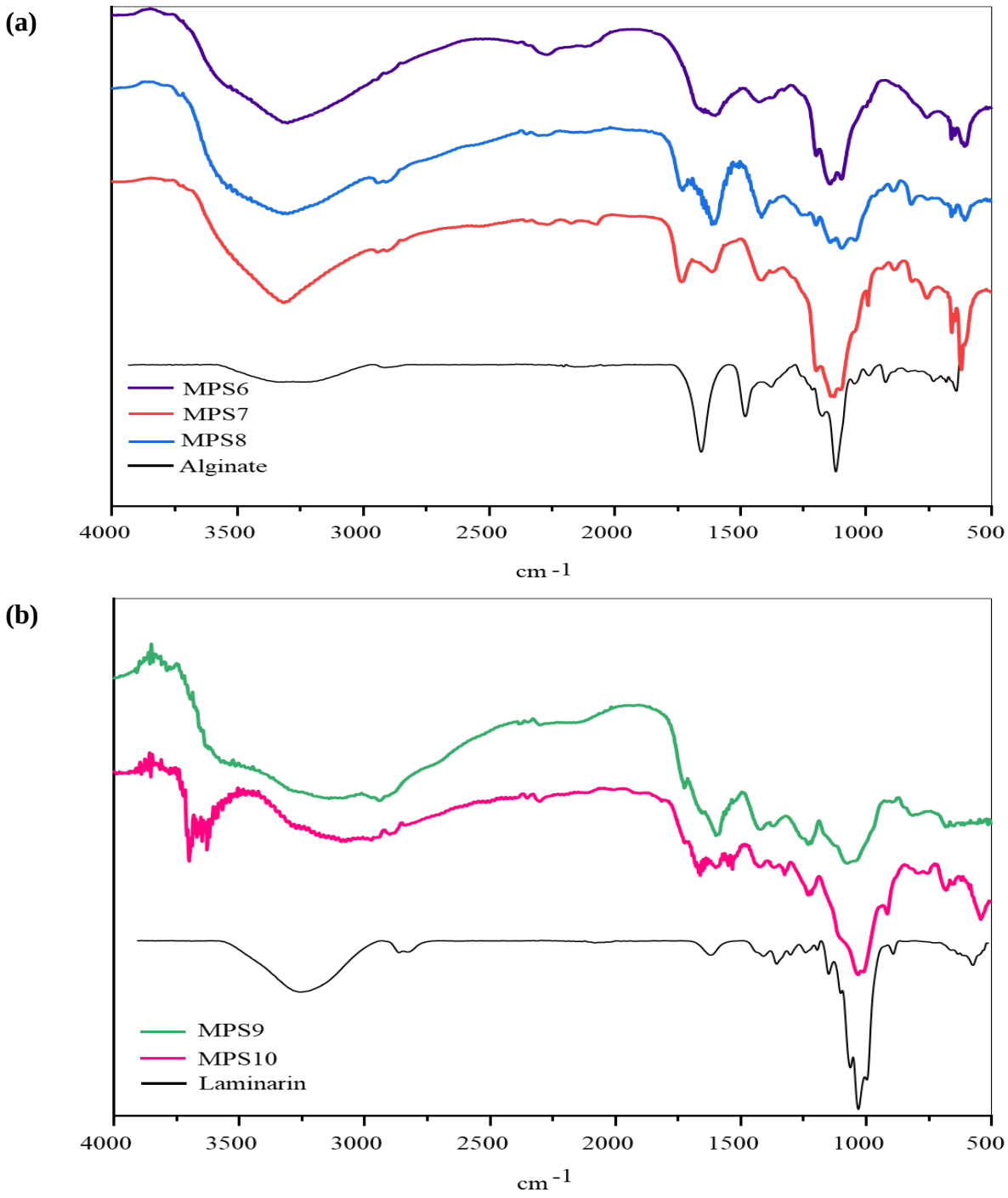


Figure 2.5 Fourier Transform Infrared spectrum of macroalgal polysaccharides of brown macroalgae compared with alginate **(a)** and laminarin **(b)**.

The peak at 846 - 848 cm^{-1} was assigned to C-O-S stretching vibration in ulvan polysaccharides and absorption at 1128 - 1148 cm^{-1} is attributed to the S=O stretching in the ulvan. The MPS from green macroalgae was compared with commercial ulvan and they showed a functional group similar to ulvan (**Figure 2.6**). All these characteristic peaks, O-H, C-H, O-C-O, C-OH, C-O, C-C, C-O-S and S=O, clearly aid in the identification of all the different polysaccharides from the three major macroalgal groups (**Table 2.5**).

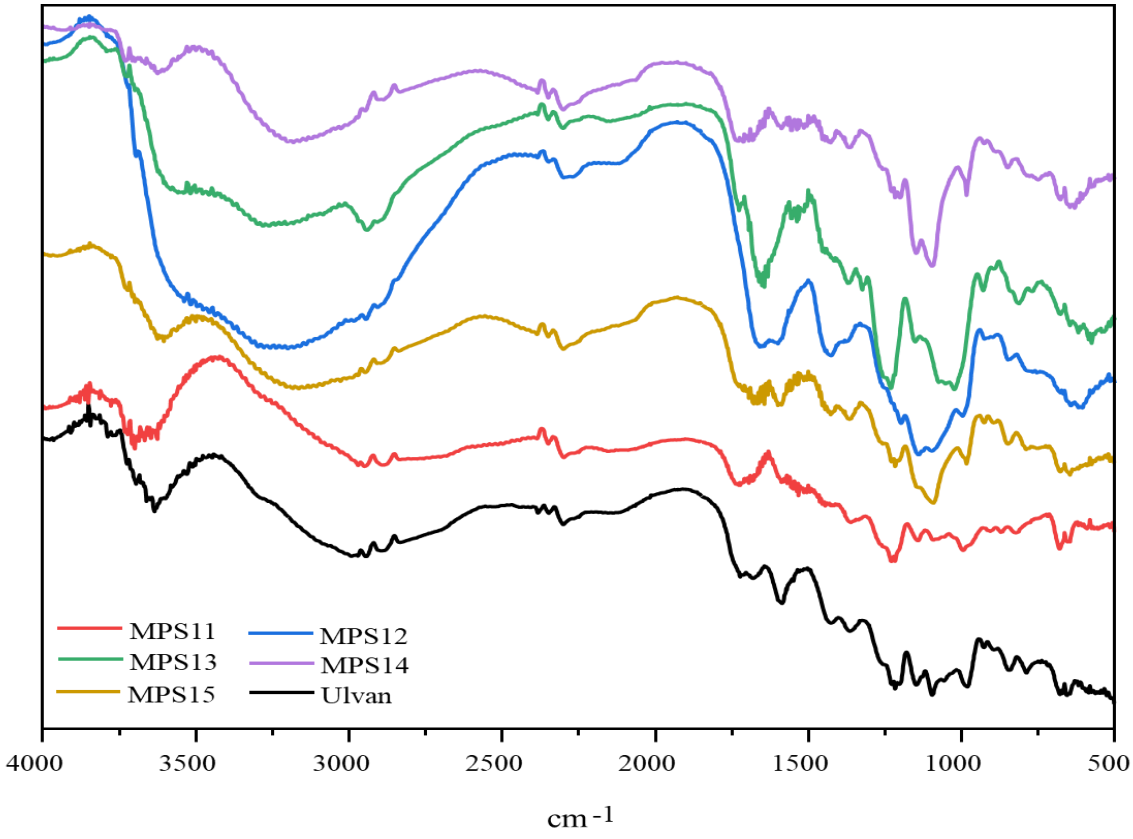


Figure 2.6 Fourier Transform Infrared spectrum of MPS from green macroalgae compared with commercial ulvan.

Table 2.5 A list of IR spectrum assignments specific to macroalgal polysaccharides

Wave number (cm^{-1})	Major peak characteristics
Specific to red macroalgal sugars	
845-850	C-O-S bending vibrations
890	Anomeric CH of β-galactose residue
930	C-O-C of 3,6-anhydro-L/D-galactose
1040-1065	Symmetric C-O vibrations with C-O-SO3 group
1225-1254	Asymmetric S=O stretching vibrations
2912-2950	C-H stretching vibrations
3400-3450	O-H stretching vibrations
Specific to brown macroalgal sugars	
820 & 840	C-O-S bending vibrations
931.8-935.3	C=O stretching vibrations in uronic acid
1030 & 1100	Glycosidic bond and pyranose ring stretching vibrations
1250	S=O stretching vibrations
1420 & 1600	COOH- stretching vibrations
2912-2950	C-H stretching vibrations
3400-3450	O-H stretching vibrations
Specific to green macroalgal sugars	
846-848	C-O-S stretching vibrations ulvan specific
980-1000	C=O stretching vibrations
1147-1256	S=O stretching ulvan specific
1610-1650	COOH- group-specific vibrations
2912-2940	C-H stretching vibrations
3400-3450	O-H stretching vibrations

2.3.5.2 Gas chromatography to analyze monosaccharide composition

The percentage of individual monosaccharides in algal polysaccharides was calculated from the peak areas and compared with the six standard sugars. The GC analysis of the acetylated monosaccharides from all fifteen polysaccharides revealed the primary sugar composition (**Table 2.6**). Xylose was detected in almost all algal polysaccharides as it is the major cell wall component in all plant species. The red macroalgae are mainly composed of sulfated galactan, such as agar and carrageenan. The GC analysis showed that the polysaccharides from red macroalgae are mainly composed of galactose (34 - 85%) residues along with xylose (1.2 - 45%) and glucose (0.6 – 5.9%) sugars (**Appendix 2.5**). Apart from these major sugars, rhamnose and arabinose sugars are also identified in some red algal polysaccharides. GC analysis of brown algal polysaccharides showed that MPS6 of *Padina* sp., MPS7 of *Spatoglossum* sp., MPS8 of *Sargassum* sp. and MPS9 of *Stoechospermum* sp. are mainly fucose (12 – 87%) and xylose (7.7 - 11%) residues (**Appendix 2.6**). MPS10 of *Sphacelaria* sp. showed the major composition of glucose (76%), which may be due to the presence of laminarin in macroalgae. The green macroalgal polysaccharides are mainly composed of sulfated rhamnose sugars and GC results showed the dominance of rhamnose (4.02 - 64.9%) sugar followed by xylose (7.4 - 26.7%) as the cell wall component (**Appendix 2.7**). The MPS12 showed the highest content of rhamnose, followed by MPS14 and MPS15 along with xylose, glucose and galactose. The highest arabinose content (68.7%) was recorded in MPS11 of *Chaetomorpha* sp. among all the fifteen MPS tested and minor concentrations of this sugar were present in MPS5 and MPS12 (**Table 2.6**). The monosaccharide composition analysis showed that the sugars are specific to the macroalgal genera.

Table 2.6 Monosaccharide composition (%) of macroalgal polysaccharides determined using gas chromatography.

Macroalgal polysaccharides	Monosaccharide composition (%)					
	Rhamnose	**Fucose**	**Arabinose**	**Xylose**	**Glucose**	**Galactose**
MPS1	ND	ND	ND	10.5	5.6	83.8
MPS2	9.3	ND	ND	45.6	5.9	34.9
MPS3	ND	ND	ND	34.9	0.6	65.0
MPS4	1.4	ND	1.4	1.2	4.0	85.9
MPS5	1.6	ND	2.4	26.4	4.2	63.0
MPS6	ND	87.8	ND	7.7	ND	ND
MPS7	ND	13.9	ND	10.1	ND	ND
MPS8	ND	65.6	ND	11.0	ND	ND
MPS9	ND	46.7	ND	10.3	ND	2.4
MPS10	ND	12.7	ND	9.5	76.0	ND
MPS11	4.4	ND	68.7	7.4	ND	9.3
MPS12	71.8	ND	2.0	9.2	3.4	2.3
MPS13	11.5	3.8	2.5	20.8	8.0	24.0
MPS14	64.9	ND	ND	26.7	3.7	ND
MPS15	50.0	ND	ND	25.7	12.0	5.4

ND=Not detected

2.3.6 Antioxidant activity of macroalgal polysaccharides

The potential of MPS to scavenge the free radicals was assessed and the results showed that MPS of red macroalgae MPS1 and MPS5 showed < 5% DPPH radical scavenging activity. The brown macroalgal polysaccharides MPS8 of *Sargassum* sp., MPS10 of *Sphacelaria* sp. and MPS9 of *Stoechospermum* sp. recorded 27.8, 27.6 and 11.3% DPPH radical scavenging activity at 1mg ml^{-1} concentration, respectively (**Figure 2.7a**). The MPS from green macroalgae did not exhibit DPPH scavenging activity, except MPS11 of *Chetomorpha* sp. with < 3% radical inhibition.

The ABTS radical scavenging analysis showed that the MPS5 of *Porphyra* sp. could scavenge 24.5% of ABTS radicals. However, the highest ABTS radical inhibition was recorded in MPS of brown macroalgae MPS6 of *Padina* sp., MPS8 of *Sargassum* sp. and MPS9 of *Stoechospermum* sp. with 74.1%, 58.6% and 89.2% inhibition of ABTS radicals at 1 mg ml^{-1} concentration (**Figure 2.7b**). The MPS from green macroalgae did not show any ABTS radical scavenging ability. The H_2O_2 radical scavenging assay results showed that the MPS of red macroalgae MPS4 of *Hypnea* sp. showed less than 2.5% inhibition of H_2O_2 radicals. The MPS of brown algae MPS8, MPS9 and MPS10 were able to scavenge 14.8%, 55.7% and 46.6% of H_2O_2 radicals at 1 mg ml^{-1} concentration. However, MPS from green macroalgae did not show any inhibition of H_2O_2 radicals (**Figure 2.7c**). All the investigated MPS exhibited different total antioxidant capacity levels, ranging from 0.23 to 2.86 µg mg^{-1} GAE (**Figure 2.7d**). The brown polysaccharide MPS8 (2.30 µg mg^{-1} GAE), MPS9 (2.47 µg mg^{-1} GAE) and MPS10 (2.86 µg mg^{-1} GAE) showed maximum antioxidant activity, MPS of red and green macroalgal polysaccharides showed very minimal of <1 µg mg^{-1} GAE activity at tested concentration.

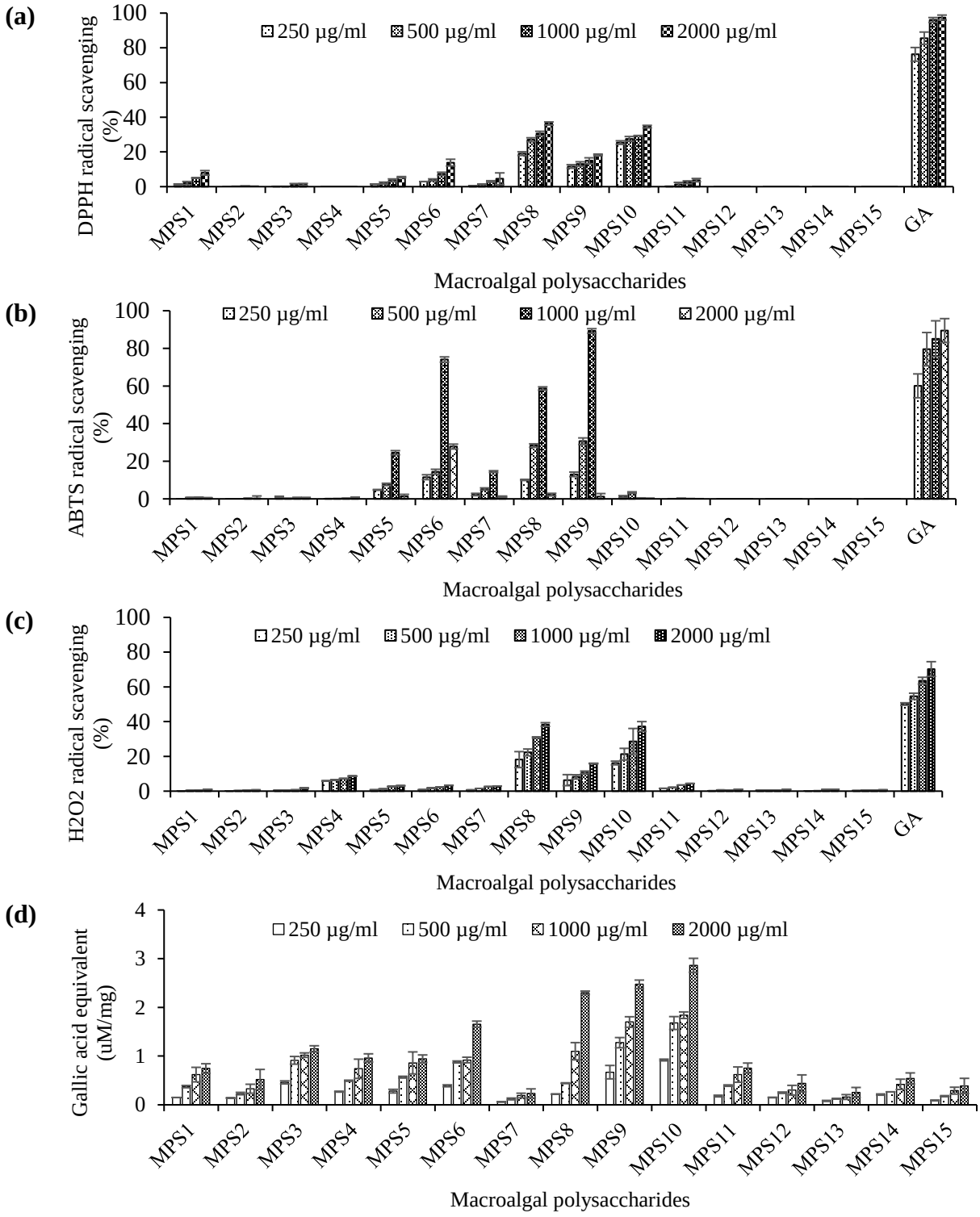


Figure 2.7 Antioxidant activities of algal polysaccharides using **(a)** DPPH, **(b)** ABTS, **(c)** H_2O_2 radical scavenging and **(d)** total antioxidant power activity. GA (gallic acid) is a positive control for antioxidant activity.

2.3.7 Antiglycemic activity of macroalgal polysaccharides

The antiglycemic potential of algal polysaccharides was studied for α-glucosidase inhibition (αGI) activity at 1 mg ml^{-1} concentration. Results showed that the MPS of red macroalgae MPS1 of *Acanthophora* sp., MPS2 of *Gracilaria* sp. and MPS3 of *Halymenia* sp. were able to inhibit > 15% α-glucosidase activity. The brown algal polysaccharides MPS6 of *Padina* sp., MPS8 of *Sargassum* sp., MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp., are shown to have good inhibitory potential against α-glucosidase with inhibition of 50-97% of enzyme activity. The highest αGI activity was shown by MPS10 of *Sphacelaria* sp. (97%), which was equivalent to the model antidiabetic drug acarbose (98%) (**Figure 2.8**). The MPS of green macroalgae MPS11 of *Chaetomorpha* sp. has also been shown to inhibit up to 30% of α-glucosidase enzyme activity.

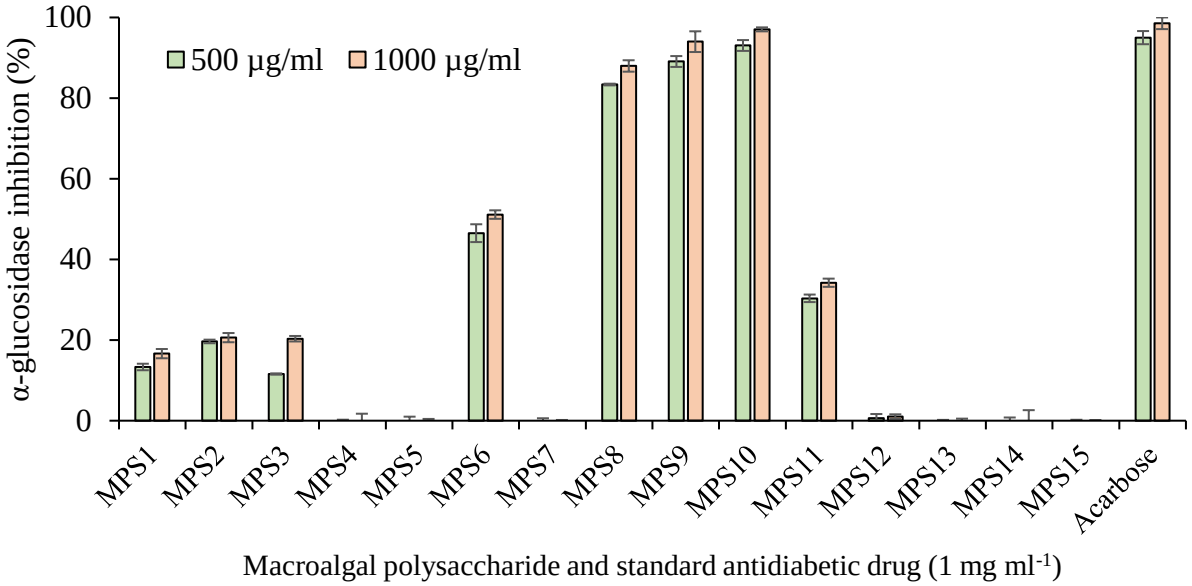


Figure 2.8 Antiglycemic activity of macroalgal polysaccharides compared with acarbose through inhibition of the α-glucosidase enzyme.

2.3.8 Prebiotic activities of macroalgal polysaccharides

The prebiotic potential of MPS was compared to commercial prebiotic FOS based on the growth profiling of probiotic bacteria, *L. acidophilus*, *L. bulgaricus* and enteric *E. coli* and their prebiotic activity score (**Figure 2.9a**). The results show that the growth of probiotic bacteria, *L. acidophilus* and *L. bulgaricus*, in the presence of 1% MPS10 of *Sphacelaria* sp. (1.27 and 1.18),

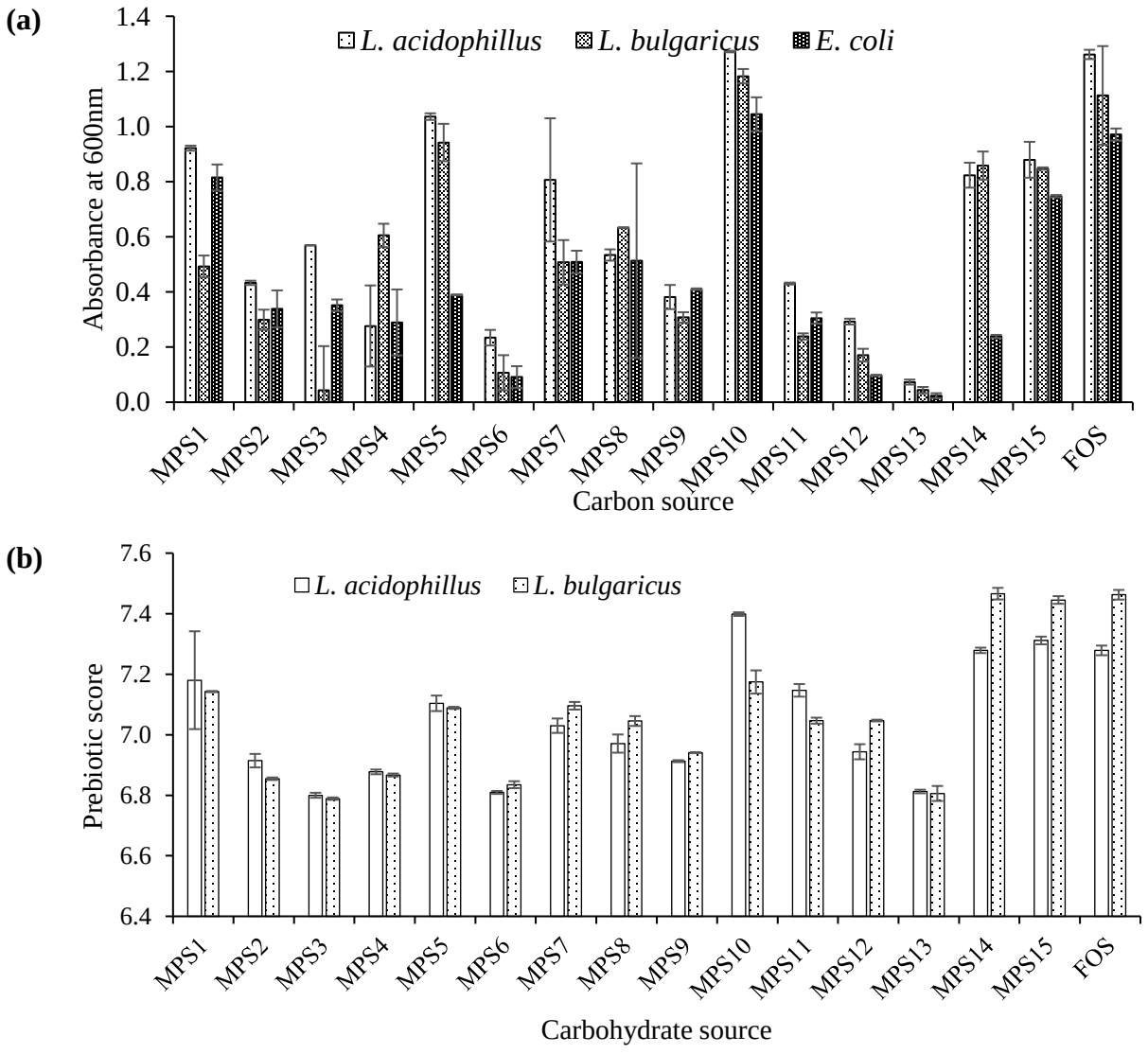


Figure 2.9 Prebiotic potential of MPS based on the (**a)** growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and (**b)** prebiotic activity score of probiotic bacteria in the presence of MPS and fructooligosaccharides.

was equivalent to the commercial FOS (1.26 and 1.11), respectively. For potential prebiotic activity, the MPS are tested for their ability to suppress the growth of enteric pathogenic strains of *E. coli.* The MPS5 of *Porphyra* sp. and MPS15 of *Ulva* sp. showed the proliferation of probiotic bacteria with the inhibition of the growth of enteric pathogenic strains of *E. coli* than FOS. The prebiotic score was also calculated based on the CFU numbers obtained from the growth of probiotic and enteric bacteria in the presence of MPS and FOS. The results show that the score of MPS10 of *Sphacelaria* sp. was 7.40 for *L. acidophilus*, which was higher than commercial prebiotic FOS with a prebiotic score of 7.28 (**Figure 2.9b**). Green macroalgal polysaccharides MPS14 and MPS15 of *Ulva* sp. showed good growth of probiotic cultures and prebiotic score equivalent to model prebiotic compound fructooligosaccharides.

2.3.9 Cytotoxicity analysis of selected macroalgal polysaccharides

The cell cytotoxicity analysis of macroalgal polysaccharides MPS8 of *Sargassum* sp., MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp. at various concentrations (50 – 200 µg ml^{-1}) on Neonatal Mouse Dermal fibroblast cell lines. The results show that as the increasing concentration of MPS8 of *Sargassum* sp. increased from 50 to 100 µg, there was an increment in cell survival and slight cell death at 150 – 200 µg ml^{-1} concentration (**Figure 2.10**). Conversely, the MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp. showed high survival (>100%) of Neonatal Mouse Dermal fibroblast cells at a tested concentration (50 – 200 µg ml^{-1}). MPS9 and MPS10 do not show cell cytotoxicity at such high concentrations. Hence, these polysaccharides with high bioactivity without any toxic effect on normal cell lines can be used further for various functional food and healthcare applications.

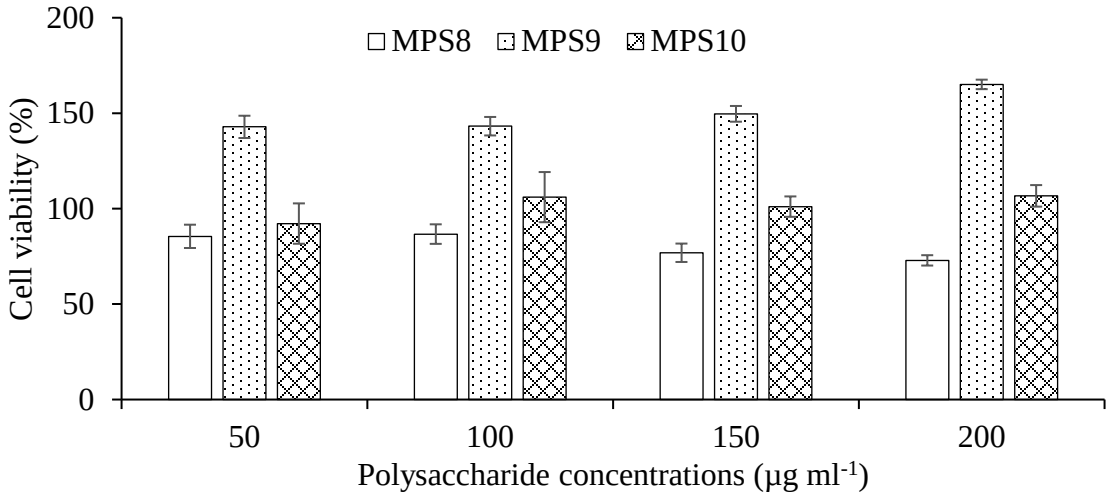


Figure 2.10 Cell survivability of Neonatal Mouse Dermal fibroblast cell lines in presence of macroalgal polysaccharides (MPS8, MPS9 and MPS10) tested using MTT assay.

2.4 Discussion

Macroalgae are the most generous reserve of marine natural products in the ocean. They are exposed to solar radiation, temperature and tidal fluctuations, leading to the generation of oxidative stress (Maharana et al., 2015). Hence, they have developed a strong defense system and produce various biomolecules such as pigments, fatty acids, polyphenols, sterols, carotenoids, dietary fibers and polysaccharides to adapt to harsh environmental conditions (Sharifuddin et al., 2015). Numerous studies have shown the antioxidant, anticoagulant, anti-inflammatory, immunostimulation and antiglycemic activities of solvent extracts / sulfated polysaccharides from red macroalgae of the order Gracilariales and Gelidiales from brown algae of order Fucales and Dictyotales and order Bryopsidales and Ulvales from green macroalgae (Imbs et al., 2015; Imjongjairak et al., 2016; Adrien et al., 2019; Bhardwaj et al., 2021; Jiang et al., 2019; da Silva Chagas et al., 2020; Je et al., 2021). Various species from these macroalgal groups are commonly

available in Indian waters. However, the bioactive potential of their aqueous extracts and polysaccharides has not been explored for functional food applications.

Antioxidant activities are extensively studied and reported in various macroalgal biomolecules such as pigments, sugars and phenolic compounds. Oxidative stress is caused by a high amount of free radicals and a concurrent decline in the oxidative defence mechanism. Oxidative stress deteriorates cell organelles and enzymes, increases lipid peroxidation, and develops insulin resistance (Mancini et al., 2018). Hence, macroalgal extracts and polysaccharides with radical scavenging activity could be a natural source to improve the health of individuals. Macroalgal biomolecules such as pigments, TPC and PC are largely studied for their antioxidant activity (Vasconcelos et al., 2019). Results from our study show that the extracts of brown macroalgae MEX9 of *Stoechospermum* sp., MEX10 of *Sphacelaria* sp. and MEX1 of the red macroalgae *Acanthophora* sp. showed the maximum radical scavenging and total antioxidant activity. Among these, MEX9 of *Stoechospermum* sp. shows the highest antioxidant activity and lowest IC_{50} value. MPS from all the studied macroalgae showed good dietary fiber content; this may be due to these polysaccharides' unique composition, making them unavailable for pancreatic enzymes. Especially the polysaccharides of green macroalgae, which are made up of rare sugar rhamnose, showed high dietary fiber content because the polysaccharides of rhamnose cannot be acted upon by salivary amylase or pepsin (Jiang et al., 2019; Koh et al., 2020). Studies from across the globe have tried to understand the efficiency of various algal antioxidant biomolecules for managing diabetes (Zaharudin et al., 2019). Since the reduction of oxidative stress and the antiglycemic and prebiotic activity can play a crucial role in controlling diabetes, the current emphasis of the research is to study and identify biomolecules that have a combination of antioxidant, antiglycemic and prebiotic activity. Few studies have specifically targeted the

antiglycemic activity of the phenolic compounds and MEX (Husni and Wijayanti, 2014; Pirian et al., 2017). Antiglycemic activity based on the inhibition of α-glucosidase, the major gastrointestinal enzyme, is considered one of the approaches for the prevention and treatment of diabetes. The α-glucosidase can be inhibited by several oral α-glucosidase inhibitors such as acarbose and voglibose (Liu and Ma, 2017). They have limited potency and common side effects like flatulence, bloating, abdominal pain or discomfort, abdominal fullness, and nausea (Dabhi et al., 2013). Hence there is a demand for natural α-glucosidase inhibitors and our results show that macroalgae have very good potential.

Results from this study show that the IC_{50} values for αGI activity of the three best macroalgae (MEX1 of *Acanthophora* sp., MEX9 of *Stoechospermum* sp. and MEX10 of *Sphacelaria* sp.) ranged from 0.013 to 0.151 mg ml^{-1}. The potential of these MEX to inhibit α-glucosidase activity is higher than earlier reports on *Halimeda* sp. with an IC_{50} value of 6.38 mg ml^{-1} (Chin et al., 2015); *Sargassum siliquosum* (0.57 mg ml^{-1}) and *Sargassum polycystum* (0.69 mg ml^{-1}) and *Undaria pinnatifida* with IC_{50} 0.080 mg ml^{-1} (Nagappan et al., 2017; Zaharudin et al., 2019). The MEX10 of brown algae *Sphacelaria* sp. even showed better αGI activity than the commercial antidiabetic drug acarbose at a low concentration. Previously, the brown macroalgae of *Fucus vesiculosus* showed to have the highest α-glucosidase inhibition activity at a low IC_{50} value (0.32 μg ml^{-1}) (Lordan et al., 2013). The three macroalgae MEX1 of *Acanthophora* sp., MEX9 of *Stoechospermum* sp. and MEX10 of *Sphacelaria* sp. are shown to have good antioxidant and antiglycemic activity. A significant correlation was observed between TPC and antioxidant and antiglycemic activity (**Appendix 2.8**). Earlier reports have shown that the radical scavenging activity of macroalgae to be mostly related to their TPC and PC content (Ganesan et al., 2011; Loradan et al., 2013; Kim et al., 2014; Makkar and Chakraborty 2017). Results from this study

shows that few of the macroalgae such as MEX1 of *Acanthophora* sp., MEX9 of *Stoechospermum* sp. and MEX10 of *Sphacelaria* sp. which are not well explored, showed very good activity much higher than the model compound tested (Jagtap et al., 2021).

The antioxidant potential of MPS showed that MPS8 of *Sargassum* sp. and MPS10 of *Sphacelaria* sp. were able to scavenge DPPH radicals with IC_{50} values of 6.24 mg ml^{-1} and 4.33 mg ml^{-1}, respectively. The ABTS radical scavenging activity for MPS8 of *Sargassum* sp. was 0.41 mg ml^{-1}, these IC_{50} value was higher than the previously reported polysaccharide fractions of *Sargassum tenerrimum* with IC_{50} values ranging from 2.044 - 4.548 mg ml^{-1} for DPPH and 0.122-0.261 mg ml^{-1} for ABTS (Mohan et al. 2019). This high DPPH and ABTS radical scavenging activity shown by Mohan et al. (2019) may be due to simultaneous purification procedures to purify the polysaccharides. Similar studies on polysaccharides from *Gracilaria corticata* showed high radical scavenging activity at low concentrations (73.01% at 160 µg ml^{-1} for DPPH and 74.5% at 125 µg ml^{-1} for ABTS) compared to the current study (Seedevi et al., 2017). Only a few studies are reported on the antiglycemic potential of algal polysaccharides (Jiang et al., 2019; Koh et al., 2020). In this chapter the MPS 8 of *Sargassum* sp., MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp. showed inhibition of α-glucosidase enzyme with IC_{50} value < 0.5 mg ml^{-1}. The previous study on the inhibition of α-glucosidase enzyme using sulfated polysaccharide isolated red macroalgae *Bangia fusco-purpurea* showed an IC_{50} value of 1.34 mg ml^{-1} (Jiang et al., 2019), which was higher than our study (IC_{50} value 0.4 mg ml^{-1} for MPS10 of *Sphacelaria* sp.). The brown algal polysaccharide MPS10 showed the highest growth and prebiotic activity score than all other polysaccharides, which was equivalent to FOS. Green macroalgal polysaccharides MPS14 and MPS15 of *Ulva* sp. showed good growth of probiotic cultures and prebiotic score equivalent to model prebiotic compound fructooligosaccharides.

The statistical analysis using two-tailed Pearson's correlation results showed that there is a high correlation between polysaccharide and sulfate content with the ABTS radicals scavenging activity and total antioxidant capacity ($p < 0.01$) (**Appendix 2.9**). The MPS8 of *Sargassum* sp., MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp. could scavenge free radicals and inhibit the α-glucosidase enzyme.

The dietary fiber content and polysaccharide content analysis of MPS showed that these polysaccharides can be used as a supplement in functional food applications. The infrared spectroscopy results of three potential MPS, MPS8 of *Sargassum* sp., MPS9 of *Stoechospermum* sp. and MPS10 of *Sphacelaria* sp. showed the presence of C-O-S specific bending vibration at 821 cm^{-1}, representing sulfation and vibration at 1610-1620 cm^{-1}, represents the presence of uronic acid (Hung et al., 2021). Apart from this, the MPS8 and MPS9 polysaccharide showed the presence of fucose-specific bending vibration within 1135-1150 cm^{-1}, representing the presence of fucoidan residue. The monosaccharide composition analysis using gas chromatography showed fucose as a major monosaccharide in MPS8 of *Sargassum* sp. and MPS9 of *Stoechospermum* sp., which could be due to fucoidan, a polymer of fucose in brown algae. However, MPS10 of *Sphacelaria* sp. showed glucose as the main monosaccharide sugar, which could be due to the cell wall polysaccharide laminarin, a polymer of glucose. These sulfated fucose and glucose containing MPS are shown to contribute largely for bioactivity.

Apart from the MPS which showed good antioxidant and antiglycemic activities. The macroalgal polysaccharides porphyran (MPS5) and ulvan (MPS15) showed good dietary fiber contents but low antioxidant and antiglycemic activity. These unique polysaccharides can be hydrolyzed into their oligosaccharides using microbial enzymes for their better applications which is discussed in chapter 3.

Chapter 3

Isolation and characterization of macroalgae-associated bacteria for their polysaccharide hydrolyzing enzymes

3.1 Introduction

Macroalgae are primitive, aquatic plants with low lignocellulose content; hence a simple extraction method can be used to isolate their bioactive compounds (Cheong et al., 2018). Macroalgal biomass comprises about 50 % carbohydrates mainly comprising of complex macroalgal polysaccharides (MPS) including agar, carrageenan, porphyran, alginate, laminarin, fucoidan and ulvan in its cell wall. These MPS have a complex structure with sulfation and diverse sugar composition are a challenge for their commercial applications (Tanna and Mishra, 2019). MPS are composed of monomers such as pentoses (xylose and arabinose), hexoses (galactose, glucose and mannose) and rare sugars (rhamnose, uronic acids), which are unique to macroalgae. In the previous chapter, I have shown that these MPS are specific macroalgal genera, however, their complex nature and high molecular weight limit their applications (Cheong et al., 2018). Studies have shown that these polysaccharides have wider applications when they are broken down into low molecular weight oligosaccharides. It can either be hydrolysed through chemical or enzymatic processes. In chemical hydrolysis, polysaccharides are treated with concentrated acid and heated at high temperature for several hours to get the final product (Lee and Lee, 2016). This chemical treatment leads to the production of monomers and undesirable toxic by-products such as furfural, hydroxymethyl furfural and high amounts of reducing sugars, which reduces the purity of oligosaccharides (Wang et al., 2019). On the contrary, during enzymatic hydrolysis, the polysaccharides are broken down by specific enzymes that cleave or hydrolyze the specific glycosidic bonds to produce stereo-specific oligosaccharides (Xu et al., 2018a, b). The oligosaccharides produced through enzymatic hydrolysis are also known to have superior nutraceutical and healthcare applications than parent polysaccharide (Cheong et al., 2018).

Enzymatic hydrolysis of these MPS requires specific enzymes for its breakdown into oligosaccharides. These enzymes are grouped in the Carbohydrate-Active enZYmes (CAZy) database into Glycoside hydrolase (GH) and Polysaccharide lyase (PL) based on their cleavage mechanism. GH is the group of enzymes involved in the breakdown of polysaccharides through cleavage of β-1, 4 glycosidic linkages. However, PL hydrolyses the polysaccharides through a β-elimination mechanism. Studies have shown that microbes associated with live and decaying macroalgae, especially the macroalgae-associated bacteria (MAB) are the major source of these GH enzymes (Oh et al., 2010; Gupta et al., 2013; Lin et al., 2017; Chen et al., 2018; He et al., 2018; Zhu et al., 2018). The microorganisms associated with benthic organisms such as marine molluscans and marine sediments are also a major source of these algal polysaccharide hydrolysing enzymes as well as antimicrobial compounds (Huang et al., 2013; Karthick and Mohanraju, 2018, 2020).

Macroalgal polysaccharide hydrolyzing enzymes are very specific and a large majority of these enzymes belong to the GH family which includes agarase, carrageenase, porphyranase, laminarinase and fucoidanase. However, alginate lyase and ulvan lyase belongs to the polysaccharide lyase family (Belik et al., 2020; Li et al., 2020). The microbial agarase, carrageenase and alginate lyase are well-documented for their applications and various biological activities (Hong et al., 2017; Cheong et al., 2018; Lee et al., 2019). In contrast, certain porphyranase and ulvan lyase have been characterized to hydrolyze porphyran and ulvan into their oligosaccharides (Zhang et al., 2019b, 2020; Hung et al., 2021). A detailed review of the current understanding of these unique macroalgal polysaccharide hydrolysing enzymes from microorganisms is given below.

3.1.1 Microbial enzymes to hydrolyse polysaccharides from red algae

Sulfated polysaccharides such as agar, carrageenan and porphyran are unique and specific to red macroalgae (Cian et al., 2015). These polysaccharides can be enzymatically hydrolyzed through substrate-specific enzymes such as agarase, carrageenase and porphyranase (**Table 3.1**).

Agarase

Agarases are the enzymes that hydrolyses agar, composed of repeating residues of 3-O-linked β-D-galactose (G) and 4-O-linked 3, 6-anhydro-α-L-galactose (AG) (Fu and Kim 2010). Agarases grouped into the glycoside hydrolases (GH) family GH16, GH50, GH86, GH118, and GH96 in the carbohydrate-active enzymes (CAZy) database based on their amino acid sequences. Agarase can be divided into two types based on how they cleave; the first, α-agarase (EC 3.2.1.158), cleaves α-1, 3 linkages, and the second, β-agarase (EC 3.2.1.81), acts on β-1, 4 linkages. So far, only four α-agarases from the GH96 family are characterized, which include marine bacteria, *Alteromonas* sp. (Potin et al., 1993), *Thalassomonas* sp. (Ohta et al., 2005), *Thalassomonas* sp. (Zhang et al., 2018) and *Catenovulum sediminis* (Lee et al., 2019) belonging to phylum Proteobacteria. However, many β-agarases are reported from different microbes in the marine environment belonging to phylum Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria.

The β-agarases group into four GH families; GH16, GH18, GH50 and GH86 most of which belong to GH16 and GH50 families and members of the former shows conglomerate lichenase and carrageenase activity (Kim et al., 2010). The majority of bacteria able to produce β-agarase is reported from bacterial isolates belonging to phylum Proteobacteria such as *Microbulbifer* sp., *Vibrio* sp., *Alteromonas* sp., *Pseudoalteromonas* sp., *Acinetobacter* sp., *Agarivorans* sp., *Pseudomonas* sp., *Catenovulum* sp., *Gayadomonas* sp. and *Thalassospira profundimonas*. The β-agarase producing bacteria are also reported from phylum Bacteroidetes including *Zobellia*

galactanivorans, *Flammeovirga* sp. and *Aquimarina agarilytica*. The bacteria *Streptomyces coelicolor* of Actinobacteria phylum is also reported to produce β-agarase. The α-agarase and β-agarase are shown to have different biochemical properties, amino acid sequences, molecular mass and catalytic properties. Their substrate specificity to cleave the linkages of the agar proves to be an inevitable tool for the production of agaro-oligosaccharide with various degrees of polymerization, including agarotriose (AO3), agarotetrose (AO4), neoagarobiose (NAO2), neoagarotetraose (NAO4), neoagarohexaose (NAO6) and neoagarooctaose (NAO8) (**Table 3.1**).

Carrageenase

Carrageenases are the GH enzymes that can hydrolyze glycoside linkage in carrageenan, a sulfated galactan polysaccharide specific to carrageenophytic red macroalgae. Carrageenases are grouped into κ-, ι- and λ-carrageenase based on their cleavage pattern to break down the κ-, ι- and λ-carrageenan (Sun et al., 2015). κ-carrageenase (EC 3.2.1.83) is the GH involved in the breakdown of glycosidic linkage between sulfated 3-linked β-D-galactose and 4-linked α-D-galactose of κ-carrageenan (Yao et al., 2013; Zhang et al., 2019b), they predominantly belong to GH16 and GH82 families and a few are yet to be characterized. The majority of the bacterial genera producing κ-carrageenase belongs to phylum Proteobacteria, which includes *Pseudomonas, Pseudoalteromonas, Vibrio* and *Thalassospira* sp.; Bacteroidetes phylum includes *Cytophaga, Tamlana, Zobellia, Cellulophaga* and *Pedobacter* are also reported to produce κ-carrageenase. The κ-carrageenase producing bacterial species identified so far from marine habitat belonging to Actinobacteria phylum is *Cellulosimicrobium* species. ι-carrageenases (EC 3.2.1.157) hydrolyzes the β-1, 4 glycosidic linkages within 3-linked-4-O-sulfated-β-L-galactose and 4-linked-2-O-sulfated-α-D-3, 6 anhydrogalactose of ι-carrageenan. ι-carrageenases belong to GH16 and GH82

families (Barbeyron et al., 2000). ι-carrageenases are reported from bacterial genera *Alteromonas* and *Microbubifer* belonging to phylum Proteobacteria and *Cellulophaga*, *Flavobacterium* and *Wenyingzhuangia* belonging to phylum Bacteroidetes. λ-carrageenase (EC 3.2.1.162) is the least studied enzyme that cleaves β 1-4 linkage in the galactose and anhydrogalactose, which structurally consists of two sulfated residues per disaccharide unit (Ohta and Hatada, 2006). Only a few bacterial species have been reported to produce λ-carrageenase whose GH families are not yet incorporated in the CAZy database and those are from phylum Proteobacteria include *Pseudoalteromonas* and *Pseudomonas* (Ohta and Hatada, 2006; Guibet et al., 2007). A more detailed study is required to understand the mechanism of ι- and λ-carrageenase activity to explore its potential to utilize algal biomass efficiently (**Table 3.1**).

Porphyranase

Red macroalgae such as *Pyropia* and *Porphyra* have a long evolutionary history as a source of nutritional supplements. Porphyran is a sulfated polysaccharide of these species and is mainly composed of 6-O sulfated galactose and 6-O-methyl galactose. Porphyranase is a GH enzyme involved in the cleavage of β-1, 4 glycosidic linkages between methylated porphyran and categorized as β-porphyranase (EC 3.2.1.178) (Zhang et al., 2019b). It belongs to the GH16 and GH86 families (Hehemann et al., 2012) and are transcribed by PorA and PorB gene cluster (Hehemann et al., 2010). Most of the reported porphyranase belongs to the Bacteriodetes phylum and only a few are reported from Proteobacteria and Actinobacteria (**Table 3.1**).

Table 3.1 Microbial enzymes identified for hydrolysis of red macroalgal polysaccharides

Enzymes	Microbial source	Habitat	Reference
α-agarase	*Alteromonas agarolyticus*	Seawater	Potin et al., 1993
EC 3.2.1.158	*Thalassomonas* sp. LD5,	Marine sediment	Zhang et al., 2018;
	Catenovulum sediminis WS1-A		Lee et al., 2019
β-agarase	*Zobellia galactanivorans*	Marine algae	Jam et al., 2005;
EC 3.2.1.81	*Pseudoalteromonas* sp. AG4,		Oh et al., 2010;
	Pseudomonas sp.,		Gupta et al., 2013;
	Aquimarina agarilytica ZC1		Lin et al., 2017
	Agarivorans sp. HZ105	Marine molluscans	Lin et al., 2012
	Flammeovirga sp. OC-4,	Seawater	Chen et al., 2016;
	Thalassospira profundimonas		Zeng et al., 2016
	Vibrio sp. strain CN41	Marine sediment	Liao et al., 2011
	Bacillus sp. MK03,	Terrestrial soil	Suzuki et al., 2003;
	Alteromonas sp. SY37-12,		Wang et al., 2006;
	Acinetobacter sp. AG LSL-1		Lakshmikanth et al.,
	Paenibacillus sp. SSG-1		2009; Song et al.,
			2014
κ-carrageenase	*Cytophaga* sp.,	Marine algae	Potin et al., 1991;
EC 3.2.1.83	*Tamlana* sp. HC4,		Sun et al., 2010;
	Pseudoalteromonas porphyrae,		Liu et al., 2011;
	Cellulosimicrobium cellulans,		Youssef et al., 2012;
	Vibrio sp. NJ-2,		Zhu and Ning 2016;
	Thalassospira sp. Fjfst-32,		Guo et al., 2018;
	Zobellia sp. ZL-4		Zhang et al., 2019a
	Pseudomonas carrageenovora,	Seawater	McLean and
	Pedobacter hainanensis NJ-02		Williamson, 1979;
			Zhu et al., 2018
	Bacillus sp. HT19	Terrestrial soil	Li et al., 2019

ι-carrageenase EC 3.2.1.157	*Alteromonas fortis*, *Cellulophaga* sp. QY3	Marine algae	Michel et al., 2001; Ma et al., 2013
	Microbulbifer thermotolerans, *Flavobacterium* sp. YS-80-122	Marine sediment	Hatada et al., 2011; Li et al., 2017
λ-carrageenase EC 3.2.1.162	*Pseudomonas carrageenovora*	Marine algae	Guibet et al., 2007
	Pseudoalteromonas CL9	Marine sediment	Ohta and Hatada, 2006
	Bacillus sp.	Terrestrial soil	Li et al., 2014
β-porphyranase EC 3.2.1.178	*Zobellia galactanivorans*, *Pseudoalteromonas atlantica*	Marine algae	Hehemann et al., 2010; Przybylski et al., 2015
	Wenyingzhuangia fucanilytica	Seawater	Zhang et al., 2019b

Microbial β-porphyranase activity is exhibited in marine bacteria such as *Pseudoalteromonas atlantica*, *Zobellia galactanivorans*, *Arthrobacter* S-22 and *Wenyingzhuangia fucanilytica.* The human gut bacterium *Bacteroides plebeius*, has reported to have porphyranase activity, as it has entered the human gastrointestinal tract due to the consumption of macroalgae (Osumi et al., 2002; Hehemann et al., 2012; Przybylski et al., 2015; Zhang et al., 2019b). The β-porphyranase isolated from *Zobellia galactanivorans* and protein extract of the marine bacterium *Pseudoalteromonas atlantica* can hydrolyze porphyran into methylated disaccharides and tetrasaccharides (Correc et al., 2011; Przybylski et al., 2015). The porphyranase from genes Por16A_Wf and Por16A_Wf produced disaccharides of sulfated galactose and complete hydrolysis of porphyran, respectively (Zhang et al., 2019b). Few porphyranase genes have been expressed in *E. coli*, and the enzymes were characterized, but its bioactive potential is yet to be explored (Hehemann et al., 2010, 2012; Zhang et al., 2019b).

3.1.2 Microbial enzymes to hydrolyse polysaccharides from brown algae

Brown macroalgae is the second largest group of macroalgae, mainly composed of polysaccharides alginate, fucoidan and laminarin (Deniaud et al., 2017). Alginates are linear unbranched polymers containing mannuronic acid and glucuronic acid residues arranged in a non-regular block-wise pattern along the chain (Yamasaki et al., 2005). Fucoidan is the α-L-fucose containing sulfated polysaccharide and laminarin is made up of β-1-3 glucan that occasionally has β-1-6 branches (Chen et al., 2019). The enzymatic hydrolysis of brown MPS requires PL enzymes such as alginate lyase to break down alginate. The GH enzymes, fucoidanase and laminarinase require to cleave the glycosidic bonds in fucoidan and laminarin, respectively (**Table 3.2**).

Alginate lyase

The cell wall and intracellular matrix of brown macroalgae are composed mainly of hydrocolloid alginate. It is a linear hetero-polymer of uronic acid mainly composed of glucuronate and mannuronate residues. It can be either in homo-polymeric (GG/MM) blocks or hetero-polymeric (G/M) blocks (Li et al., 2011a). Alginate lyase is a PL enzyme that has been found in numerous microbes and is one of the well-researched carbohydrate modifying enzymes (Fu and Kim, 2010). Alginate lyase has been isolated from various bacteria associated with macroalgae, molluscans and a wide range of marine habitats such as marine sediment, saltmarshes etc. (Jagtap and Manohar, 2021). About 50,000 amino acid sequences of PL, also known as alginate lyase, have been reported in studies; of these, a vast majority of roughly 47,000 sequences are identical to the microbial genome. However, a tiny subset of 260 alginate lyases are characterized; 181 from poly M lyase, 70 from poly G lyase and 9 from oligo-alginate lyase. From these well-characterized alginate

lyases, the functional characteristics of only 27 alginate lyase are confirmed as per the CAZy database (Belik et al., 2020).

Alginate is hydrolyzed by alginate lyase based on the β-elimination reaction, which cleaves the glycosidic bond between two β-D-mannuronate or α-L-glucoronate (Fu and Kim, 2010). Alginate lyases are categorized into poly G lyase, poly M lyase and poly MG lyase based on their substrate specificities (**Table 3.2**). Endolytic alginate lyase enzymes cleave the internal glycosidic bonds between the alginate. In contrast, exolytic enzymes cleave the terminal glycosidic linkage and contribute to the complete hydrolysis of alginate. Alginate lyases belong to 7 PL families PL5, PL6, PL7, PL14, PL15, PL17 and PL18. Most bacterial endolytic alginate lyase is assigned to PL5 and PL7 and exolytic belongs to PL15 and PL17 families. The alginate lyase isolated from marine molluscans and viruses belongs to the PL14 family. Bifunctional alginate lyase or poly MG lyase is placed in the PL18 family, while many are yet to be characterized and are dispersed in the PL6 family (Zhu and Yin, 2015).

Fucoidanase

Fucoidan is a natural fucose containing sulfated polysaccharide present in the cell wall of brown macroalgae, which is composed of alternating fucose residues (Deniaud et al., 2017; Zhao et al., 2018). Fucoidanase (EC 3.2.1.44) is GH enzyme, capable of hydrolyzing α-L-fucoside linkage without removing sulfate groups (Kusaykin et al., 2016; Cao et al., 2018; Shen et al., 2020). Classifying these enzymes is a difficult task because of the complex nature of fucoidan, which is often sulfated polysaccharides. Fucoidanase mostly belongs to GH107 families, but recently it is also reported from family GH168, which exhibits endolytic cleavage at α-1, 3 glycosidic linkages within sulfated and non-sulfated fucose (Cao et al., 2018; Shen et al., 2020).

Table 3.2 Microbial enzymes identified for hydrolysis of brown macroalgal polysaccharides

Enzymes	**Microbial source**	**Habitat**	**Reference**
Alginate lyase EC 4.2.2.3 / EC 4.2.2.11	*Pseudoalteromonas* sp. *Pseudomonas alginovora*, *Formosa algae* *Flavobacterium* sp. S20, *Sphingobacterium* sp. *Microbulbifer* sp., *Vibrio furnissii*, *Sphingomonas* sp. ZH0, *Bacillus* sp. Alg07, *Cellulophaga algicola*, *Aspergillus oryzae* *Gilvimarinus agarilyticus*	Marine algae	Li et al., 2011b; Lundqvist et al., 2012; Huang et al., 2013; Falkeborg et al., 2014; Yang et al., 2018; Zhu et al., 2018b; He et al., 2018; Chen et al., 2018; Fischer and Wefers, 2019; Huang et al., 2019; Belik et al., 2020; Singh et al., 2011
	Pseudomonas sp. E03, *Flammeovirga* sp.	Seawater	Zhu et al., 2015; 2019
	Flavobacterium multivorum	Terrestrial soil	Boucelkha et al., 2017
	Bacteroides cellulosilyticus	Human gut	Stender et al., 2019
	Saccharophagus degradans	Saltmarsh cordgrass	Kim et al., 2012
Fucoidanase EC 3.2.1.44	*Fucobacter marina*, *Mariniflexile fucanivorans*, *Formosa algae*	Marine algae	Sakai et al., 2003; Cao et al., 2018
	Pseudoalteromonas citrea	Seawater	Bakunina et al., 2002
	Fusarium sp. LD8, *Dendryphiella arenaria*	Marine sediment	Shvetsova et al., 2014; Wu et al., 2011
	Sphingomonas paucimobilis	Terrestrial soil	Kim et al., 2015

Glucanase/ Laminarinase EC 3.2.1.6/ EC 3.2.1.39	*Formosa algae* KMM 3553, *Vibrio breoganii* 1C10	Marine algae	Becker et al., 2017; Badur et al., 2020
	Bacillus clausii NM-1, *Pseudocardium sachalinensis*	Marine molluscans	Miyanishi et al., 2003; Kusaykin et al., 2017
	Trichoderma harzianum	Terrestrial plant	Ribeiro et al., 2019
	Botryosphaeria sp., *Phanerochaete chrysosporium*	Terrestrial soil	Giese et al., 2006; Kawai et al., 2006
	Saccharophagus degradans	Saltmarsh cordgrass	Wang et al., 2017

Fucoidanase is reported from bacterial phylum Bacteroidetes represented by *Fucobacter marina*, *Formosa algae*, *Mariniflexile fucanivorans*, *Flavobacterium* sp., *Wenyingzhuangia fucanilytica* and *Formosa haliotis*, followed by Proteobacteria (*Vibrio* sp., *Pseudoalteromonas citrea*, *Sphingomonas paucimobilis* and *Psychromonas* sp.) and Actinobacteria, *Streptomyces* sp. (**Table 3.2**). Fucoidanase is also reported in marine fungi *Luteolibacter algae* H18, *Fusarium* sp. LD8 and *Dendryphiella arenaria* (Wu et al., 2011; Nagao et al., 2018). Very scarce information is available on fucoidanase characteristics, type of cleavage, substrate specificity and degree of substrate sulfation (Kusaykin et al., 2016). The nucleotide sequence of the genes which encode fucoidanase from the GH107 family and their deduced amino acid sequence has been reported only for fucoidanase from *Mariniflexile fucanivorans* and *Formosa Haliotis* (Silchenko et al., 2017; Vuillemin et al., 2020). Recently a fucoidanase belonging to the GH168 family was identified from *Wenyingzhuangia fucanilytica* (Shen et al., 2020). Only two structures of fucoidanase from *Psychromonas* sp. and *Mariniflexile fucanivorans* have been reported in the CAZy database (Vickers et al., 2018; Cao et al., 2018).

Laminarinase

Laminarin is the storage polysaccharide of brown macroalgae, which is composed of glucose residues, with β-1, 3 and β-1, 6 linkages and it is a relatively underutilized polysaccharide (Becker et al., 2017). Laminarinase is the glycoside hydrolase that catalyzes its hydrolysis either through endolytic (EC 3.2.1.6 and EC 3.2.1.39) or exolytic (EC 3.2.1.58) mode of action. Endolytic laminarinase belonging to EC 3.2.1.39 hydrolyze β-1, 3 glycosidic bonds between adjoining glucose residues (Badur et al., 2020). According to the CAZy database, laminarinase is categorized within the GH16 (Liberato et al., 2021), GH17 (Becker et al., 2017), GH30 (Wang et al., 2017), GH55 (Kawai et al., 2006), GH81 and GH157 (Bianchetti et al., 2015) families. The laminarinase has been reported from Proteobacteria such as *Saccharophagus degradans* and *Vibrio breoganii*, from *Zobellia galactanivorans, Formosa algae, Formosa agariphila* and *Flavobacterium* sp. belonging to Bacteriodetes (Labourel et al., 2015; Kusaykin et al., 2016; Becker et al., 2017; Qin et al., 2017) and Firmicutes such as *Bacillus clausii, Bacillus circulans* and *Bacillus halodurans* (Miyanishi et al., 2003; Kim et al., 2006) and Actinobacteria; *Streptomyces* sp. (Bianchetti et al., 2015). In addition, laminarinase has been widely studied from fungal phyla Ascomycota, including *Botryosphaeria rhodina* and *Sclerotinia sclerotiorum* and Basidiomycota, *Phanerochaete chrysosporium* (**Table 3.2**). Recently the laminarinase that belongs to the GH16 family is reported to have dual β-1, 3 or β-1, 4 hydrolase activity, which helps in the complete saccharification of laminarin into fermentable sugars (Liberato et al., 2021). However, very few studies have been carried out to explore the potential of laminarinase to hydrolyse laminarin into bioactive product.

3.1.3 Microbial enzymes to hydrolyse polysaccharides from green algae

Green macroalgae have the highest carbohydrate content, but it is seldom explored division for their bioactivity. The sulfated polysaccharide ulvan is the principal carbohydrate constituent of this group of macroalgae. Ulvan is a polydisperse hetero-polysaccharide, primarily consisting of glucuronic-xylo-rhamnans, glucuronic-xylo-rhamno-galactans or xylo-arabinogalactans (Lahaye and Robic, 2007). These complex polysaccharides can be hydrolysed using ulvan lyase, which is the least explored PL that depolymerizes ulvan by cleaving the β-(1→ 4)-glycosidic bond through the β-elimination mechanism. It has also been reported that the complex ulvan is broken down by enzymes having β-glucuronyl hydrolase, rhamnosidase, xylosidase, and sulfatase activity (Foran et al., 2017). The majority of microbial ulvan lyase is divided into the PL24, PL25, PL28, PL37, and PL40 groups (Li et al., 2020). Ulvan lyase is reported from the major genera *Alteromonas*, *Pseudoalteromonas* and *Glaciecola* belong to the phylum Proteobacteria and *Persicivirga ulvanivorans*, *Formosa agariphila* and *Nonlabens ulvanivorans* from phylum Bacteroidetes (**Table 3.3**).

Table 3.3 Microbial enzymes identified for hydrolysis of green macroalgal polysaccharides

Enzymes	**Microbial source**	**Habitat**	**Reference**
Ulvan lyase EC 4.2.2.-	*Persicivirga ulvanivorans, Formosa agariphila*	Marine algae	Collen et al., 2011; Reisky et al., 2018
	Nonlabens ulvanivorans, Alteromonas sp.	Marine molluscans	Ulaganathan et al., 2018; Gao et al., 2019
	Pseudoalteromonas sp.	Marine sediment	Qin et al., 2018

Ulvan lyase of *Alteromonas* sp. isolated from the gut of the *Gammarus insensibilis*, an amphipod, produces ulvan oligosaccharides of <10 kDa molecular weight (Coste et al., 2015). Similarly, *Alteromonas* sp. isolated from feces of tiny marine organisms contains one long and one short ulvan lyase. The long ulvan lyase degrades the ulvan into ulvan oligosaccharides with 2, 4 or 6 degrees of polymerization (He et al., 2017).

Overview of these microbial enzymes observed that some enzymes such as agarase, carrageenase and alginate lyase are structurally well characterized and genes required for recombinant production are also well studied. However, some algal carbohydrase enzymes such as porphyranase, laminarinase, fucoidanase and ulvan lyase are not yet well characterized. There is a continuous need to isolate and characterize bacteria for their specific high activity enzymes. Hence in this chapter, I have focused on the characterization of bacteria associated with various macroalgae for their enzymatic potential to hydrolyse major algal polysaccharides such as porphyran, alginate and ulvan. This study will help to understand the diversity of potential macroalgae-associated bacteria and their microbial enzymes that exist in the natural environment capable of hydrolyzing algal polysaccharides.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Analytical grade sodium alginate (Sigma Aldrich, USA), ulvan extracted from *Ulva lactuca* as described by (Lahaye et al., 1999) and porphyran extracted from *Porphyra* sp. as illustrated by (Zhang et al., 2019b) were used for all experimental analysis. Zobell marine broth procured from Himedia laboratories, India of the microbiological grade was used for isolation of MABs and other chemicals used were of analytical grade.

3.2.2 Isolation of macroalgae-associated bacteria

Macroalgal samples were collected from major macroalgal beds along the Indian coast to isolate macroalgae-associated bacteria. The sampling stations included Okha and Porbandar along the Gujarat coast, Malvan and Kunkeshwar along the Maharashtra coast, Vagator, Dona Paula, Palolem from the Goa coast and Mandapam along the Tamil Nadu coast (**Figure 1.2**). The isolation of epiphytic, macroalgae-associated bacteria was carried out by the shake tube method (Pawar et al., 2018). For this, macroalgal samples were rinsed with sterile seawater to remove the attached debris, sand particles and seashells. Approximately 1 gm of the washed macroalgal sample (fresh weight) was re-suspended in 9 ml of sterile seawater and consequently vortexed for 10 minutes to release all the epiphytic bacteria into suspension and allowed to settle down. Then, the clear supernatant was collected, serially diluted (10^{-3}-10^{-4}) and 100 µl of diluted sample was plated on Zobell Marine agar microbiological plates under sterile conditions and incubated at 28 °C for 24 - 48 hours to obtain well-isolated colonies. After incubation, morphologically distinct colonies were picked, streak purified and maintained on Zobell Marine agar plates for further analysis.

3.2.3 Qualitative analysis of MABs to hydrolyze macroalgal polysaccharides

The purified MABs were grown on algal polysaccharide substrate plates to check their potential to hydrolyse porphyran, alginate and ulvan. In brief, the pure bacterial cultures were inoculated in a sterile Zobell Marine Broth (20 ml) and incubated in a shaker incubator for 24 h at 28°C. The culture suspension (10 µl) with an absorbance of 0.8 to 0.9 at 600 nm was spot inoculated on specific substrate media plates supplemented with 0.5% of porphyran or alginate or ulvan in the basal salt solution, composed of 5% sodium chloride, 0.5% ammonium sulphate, 0.3% dipotassium hydrogen phosphate, 0.1% magnesium sulphate, 0.01% ferrous sulphate and 1.5% agar. The spot

inoculated plates were incubated at 28°C for 48 h; after incubation, the plates were stained with substrate-specific staining solution; Lugol's iodine (0.5%) for porphyran, grams iodine (0.5%) for alginate and cetylpyridinium chloride (0.05%) for ulvan (Sawant et al., 2015). The zone of clearance around the bacterial colony was measured within 10 minutes of staining (**Appendix 3.1**). The bacterial isolates were tested for their ability to catalyse the polysaccharide breakdown activity and grouped based on the zone of clearance. Those isolates with the highest zone of clearance (20-35 mm) were categorised as "potential isolates" with very good activity and with decreasing zone of clearance, the isolates were grouped as good (11-20 mm), minimum (1-10 mm) and no activity group. The potential MABs with good efficiency in breaking down representative algal polysaccharides were used for further analysis and identified using molecular techniques.

3.2.4 Phylogenetic characterization of potential polysaccharide hydrolyzing MABs

The potential MABs (19 isolates) with the highest polysaccharide hydrolyzing activity were identified based on the 16S rRNA gene sequence. Each purified potential bacterial isolate was grown in 10 ml Zobell Marine Broth at 28°C for 24 h. The bacterial cell pellets of the individually grown isolates with an absorbance of approximately 0.8 at 600 nm were collected through centrifugation (20000 g for 5 min) and used for genomic DNA extraction (**Appendix 3.2a**). The 16S rRNA gene fragment of the MABs was amplified (**Appendix 3.2b**) using 27F and 1492R universal bacterial primers (Liao et al., 2011). The amplified fragment was purified using a PCR clean-up kit (Promega, USA) and sequenced bidirectionally on Genetic Analyzer 3130xl (ABI) based on the Big Dye v 3.1 chain terminator chemistry. 16S rRNA gene sequence obtained for individual isolates was compared with the NCBI database using the nucleotide BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial 16S rRNA gene sequence of the 19

potential MABs is deposited in the NCBI GenBank database. Phylogenetic analysis of bacterial isolates was carried out on the MEGA X platform based on the sequence homology of the 16S rRNA gene to gene sequence from the NCBI database using the Maximum likelihood algorithm (Tamura et al., 2011) and the robustness was assessed with 1000 bootstrap replicates.

3.2.5 Quantitative estimation of enzyme activity in the potential MAB

The nineteen potential isolates were evaluated for their quantitative enzyme activity. For this, individual isolates were inoculated in basal salt solution amended with 0.5% polysaccharide substrates (porphyran, alginate or ulvan) and grown at 28°C for 48 h. The enzyme activity of the potential isolates was assessed by measuring the release of reducing sugar following the dinitrosalicylic acid method (Miller, 1959) and galactose (20-100 μM) was used as a standard. Enzyme activity was expressed in 'U' as the amount of enzyme required to liberate 1 μM of reducing sugar min^{-1} mg^{-1} of protein (Gao et al., 2019). The extracellular protein content in the broth was determined following the Bradford assay and varying concentrations of BSA (50-250 μg ml^{-1}) were used as a standard. The isolates NIOA181, NIOA284, NIOA379, NIOA323 and NIOA354, which showed very high enzyme activity, are deposited in the National Centre of Industrial Microorganisms, Culture collection at CSIR-National Chemical Laboratory, Pune, India; of this *Bacillus* sp. NIOA181 (NCIM 5804) and *Bacillus sp.* NIOA284 (NCIM 5816), which showed very high ulvan lyase and porphyranase activity. These enzymes were studied in detail and their ability to hydrolyse ulvan and porphyran into their respective oligosaccharides was characterized, which is explained in Chapter 4.

3.3 Results

3.3.1 Isolation and screening of bacterial isolates

MABs were isolated from the most commonly grown macroalgae along the Gujarat, Maharashtra, Goa and Tamil Nadu coast. MABs associated with red macroalgae such as *Gracilaria*, *Halymenia*, *Porphyra* and *Hypnea*; brown algae such as *Padina*, *Spatoglossum*, *Sargassum*, *Stoechospermum* and *Sphacelaria* and *Chaetomorpha*, *Caulerpa* and *Ulva* of green macroalgae were isolated. Approximately 421 pure bacterial strains were isolated and about 152, 151 and 118 bacterial isolates were obtained from red, brown and green macroalgae, respectively (**Appendix 3.3**). These bacterial isolates were qualitatively analysed to check their potential to hydrolyse algal polysaccharides using representative algal polysaccharides porphyran, alginate and ulvan. The zone of clearance of all the isolates on substrate plates was measured to determine their efficiency. Of the 421 bacterial isolates tested, around 85 – 95% of the isolate did not show any clearance zone on polysaccharide substrate plates, which signifies that a large majority of the isolates are incapable of breaking down the complex polysaccharides. Of the 421 bacterial isolates tested 5 to 15% of the bacterial strains showed enzyme activity and only 2 - 5% of the bacterial isolates exhibited very good activity with a zone of clearance in the range of 20 – 35 mm around the colony (**Figure 3.1**). Among the isolates tested, porphyranase activity was not seen in about 86% of bacterial isolates (360 isolates) and about 5% showed very good (19 isolates) activity. Similarly, 83% of the isolates showed no activity (351 isolates) for alginate lyase and only 4% showed very good (17 isolates) activity. The maximum number (96%) of isolates tested did not show ulvan lyase activity due to the heteropolysaccharide nature of ulvan and of the 4% of bacterial isolates which showed activity, only 2% (7 isolates) showed very good ulvan lyase activity (**Figure 3.1**).

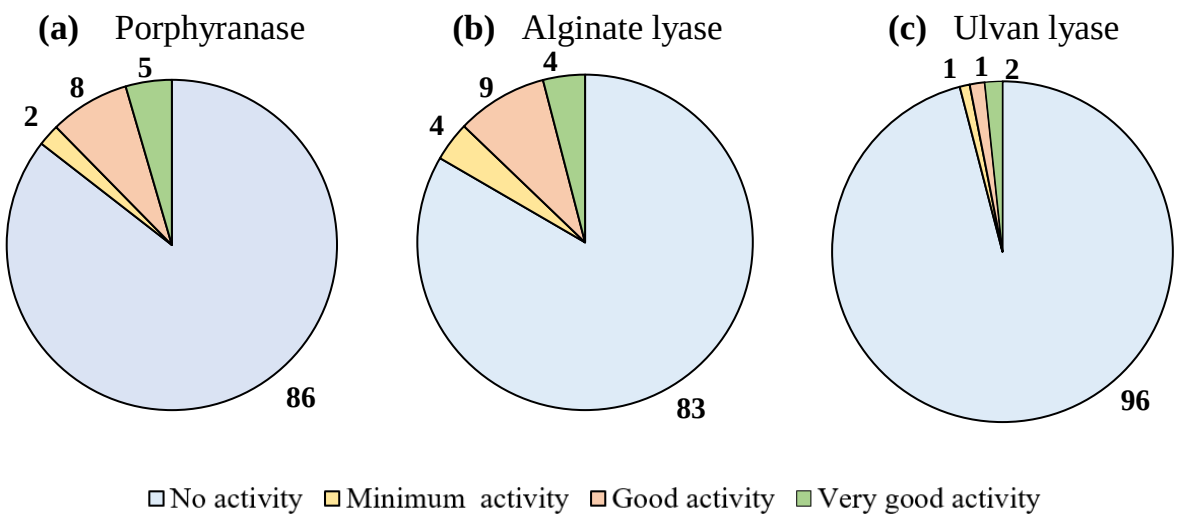


Figure 3.1 Percentage frequency of macroalgae-associated bacterial isolates with no activity, minimum, good and very good for hydrolysis of algal polysaccharides **(a)** porphyranase, **(b)** alginate lyase and **(c)** ulvan lyase based on qualitative plate assay.

The nineteen isolates, which showed very good activity with a maximum clearance zone (> 20 mm) were characterised as potential bacterial isolates to hydrolyse porphyran, alginate and ulvan substrates (**Appendix 3.4**). Maximum porphyranase activity with a zone of clearance of 32 mm was observed in bacterial isolate NIOA284 and isolates NIOA112, NIOA174, NIOA284, NIOA319, NIOA321, NIOA327 and NIOA398, which were associated with red macroalgae showed good porphyranase activity. Similarly, bacteria isolated from brown macroalgae NIOA29, NIOA118, NIOA169, NIOA170, NIOA323, NIOA328, NIOA354 and NIOA388 exhibited good alginate lyase activity. Ulvan lyase activity was observed in five isolates, NIOA181, NIOA302, NIOA325, NIOA329 and NIOA379, associated with green macroalgae, which are shown to produce ulvan lyase for hydrolysis of ulvan and maximum ulvan lyase activity was recorded in strain NIOA181 with a zone of clearance of 25 mm (**Appendix 3.4**).

3.3.2 Phylogenetic characterization of potential polysaccharide hydrolyzing bacteria

Phylogenetic analysis of the MABs was carried out based on the 16S rRNA gene sequences of the 19 potential and each of their closest bacterial taxa were identified using BLASTn sequence similarity (**Appendix 3.5, Figure 3.2**). The potential isolates were predominately grouped within the phylum Firmicutes and its diversity was represented by the genus *Bacillus* spp. (NIOA29, NIOA34, NIOA181, NIOA183, NIO284, NIOA321, NIOA323, NIOA329, and NIOA388), *Staphylococcus* spp. (NIOA118, NIOA174), *Exobacterium* spp. (NIOA302, NIOA96) and *Oceanobacillus* sp. (NIOA379). It was followed by representatives from Phylum Proteobacteria, which included *Acinetobacter* sp. (NIOA328), *Vibrio* sp. (NIOA169), *Pseudomonas* sp. (NIOA319), *Marinobacter* sp. (NIOA327) and genus *Micrococcus* sp. (NIOA354) belonging to Actinobacteria phylum (**Figure 3.2**). Maximum polysaccharide hydrolyzing bacteria (9 isolates) belonged to the phylum Firmicutes of the genus *Bacillus*.

3.3.3 Quantitative analysis of potential MAB to hydrolyze macroalgal polysaccharides

Based on qualitative analysis, a total of nineteen bacterial strains which showed very good activity were further characterised for quantitative estimation of specific enzyme activity. These bacterial isolates utilized the algal polysaccharides porphyran, alginate and ulvan as a carbon source by producing the specific enzyme to hydrolyse their glycosidic bonds. The enzyme activity in the potential MABs were estimated to quantify the specific enzymes porphyranase, alginate lyase and ulvan lyase produced by the individual isolates. The porphyranase activity recorded in the MABs was in the range of 61.9 - 208.6 U mg^{-1} (**Table 3.4**). Of the 19 isolates, 8 potential MABs (NIOA181, NIOA284, NIOA323, NIOA327, NIOA328, NIOA354, NIOA379 and NIOA388) showed >130 U of porphyranase activity and the highest activity was shown by red macroalgae-

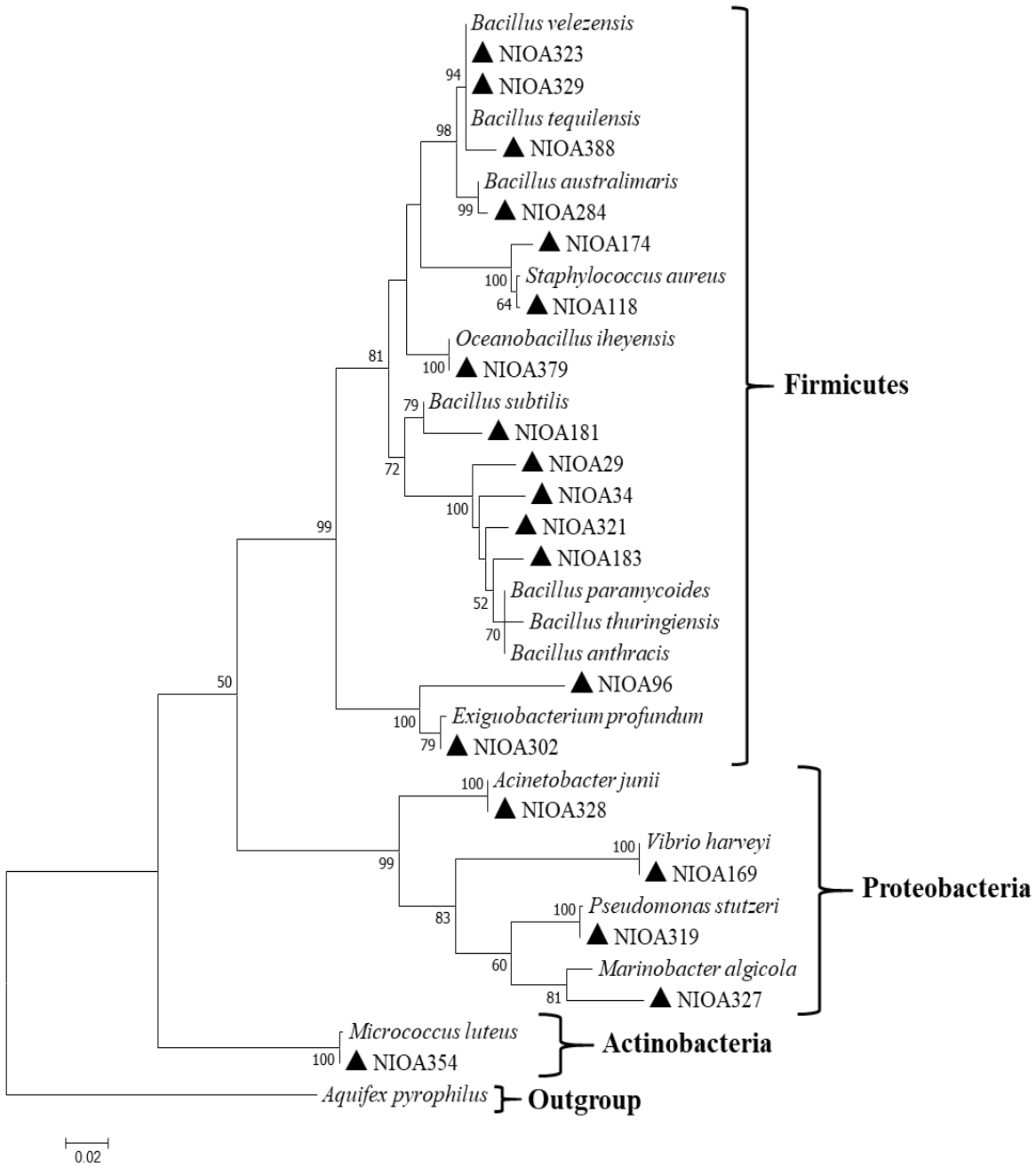


Figure 3.2 Phylogenetic tree showing the position of macroalgae-associated bacteria with potential to hydrolyse algal polysaccharides based on the maximum-likelihood analysis of the 16S rRNA gene sequences. Values at nodes indicate bootstrap support from 1000 replicates and scale represents the number of substitution per amino acid.

Table 3.4 Total porphyranase, alginate lyase and ulvan lyase activity (U mg^{-1} protein) of potential macroalgal associated bacteria

Isolates	Porphyranase	Alginate lyase	Ulvan lyase
NIOA29	61.9 ± 0.4	102.0 ± 5.4	62.7 ± 1.9
NIOA34	80.7 ± 4.8	110.7 ± 11.2	84.7 ± 1.1
NIOA96	67.6 ± 3.5	104.7 ± 9.7	37.7 ± 4.2
NIOA118	101.6 ± 11.0	105.0 ± 12.5	75.5 ± 2.7
NIOA169	77.6 ± 4.6	101.7 ± 11.3	75.4 ± 5.3
NIOA174	121.1 ± 1.0	100.8 ± 9.9	70.8 ± 1.2
NIOA181	174.7 ± 0.0	121.0 ± 1.6	**211.5 ± 0.3**
NIOA183	128.1 ± 4.3	102.7 ± 12.5	81.2 ± 0.6
NIOA284	**208.6 ± 1.7**	117.0 ± 3.5	140.1 ± 2.5
NIOA302	89.9 ± 4.3	115.6 ± 5.3	115.1 ± 3.3
NIOA319	81.7 ± 2.9	116.6 ± 12.5	69.3 ± 0.3
NIOA321	87.6 ± 13.4	117.9 ± 10.9	69.3 ± 1.1
NIOA323	190.1 ± 4.5	124.9 ± 3.6	164.3 ± 2.3
NIOA327	178.3 ± 0.7	138.6 ± 3.4	163.1 ± 3.7
NIOA328	170.2 ± 3.2	**140.2 ± 2.5**	144.4 ± 1.3
NIOA329	100.9 ± 0.3	123.2 ± 2.6	77.9 ± 4.1
NIOA354	182.9 ± 8.5	132.6 ± 2.63	116.7 ± 1.1
NIOA379	148.5 ± 3.8	122.1 ± 1.7	146.6 ± 3.8
NIOA388	132.6 ± 4.9	124.6 ± 8.6	78.8 ± 2.9

associated bacterial strain NIOA284 with 208.6 U mg^{-1} of enzyme activity. Similarly, bacterial isolates showed alginate lyase activity between 100.8 to 140.2 U mg^{-1} and *Acinetobacter* sp., NIOA328, showed the highest activity (**Table 3.4**). Due to the complex structural composition of ulvan, the ulvan lyase enzyme is rarely studied; the bacterial isolates in this study showed ulvan lyase activity in the range of approximately 40 - 200 U mg^{-1} (**Table 3.4**). Among these isolates, the most potential ulvan lyase and porphyranase producing bacterium was NIOA181 and NIOA284 which were identified as *Bacillus subtilis* and *Bacillus australimaris*, respectively and selected for characterization of their extracellular enzymes, which will be discussed in chapter 4.

Discussion

Macroalgae are known to harbor diverse microbial communities, contributing to their growth and defense. Many of these associated microbiomes are known to be the source of various extracellular enzymes, including the carbohydrase enzymes, which are in huge industrial demand, as they can catalyse the enzymatic breakdown of complex macroalgal polysaccharides into bioactive oligosaccharides (Selvarajan et al., 2019). These low molecular weight oligosaccharides are reported for various bioactivities such as antioxidant, immunomodulation, antiglycemic, anti-inflammatory, antitumor, prebiotic, antiobesity and antimicrobial (Jagtap and Manohar, 2021). However, the major bottleneck for the production of these bioactive sugars is the identification of potential microorganisms capable of efficiently hydrolyzing macroalgal polysaccharides into oligosaccharides.

In this chapter, the MABs were isolated and characterised for their ability to utilize the model polysaccharides representing all three macroalgal groups. The results show that only about

10 – 15% of bacteria could hydrolyse the complex polysaccharides such as porphyran, alginate and ulvan (Jagtap et al., 2022a). The bacteria that showed the potential to hydrolyse the unique and complex macroalgal polysaccharides are very scarce and known to contribute to the remineralization of macroalgae after the completion of a growth cycle (Florez et al., 2017; Naik et al., 2019). Studies in the water column of the seaweed ecosystem show the dominance of Firmicutes and Proteobacteria (Kizhakkekalam and Chakraborty, 2019; Parvathi et al., 2020). Our study showed the diversity of potential MABs belonging to phylum Firmicutes (68%), followed by Proteobacteria (26%) and Actinobacteria (5%). Molecular tools based on Illumina sequencing and analysis were carried out to understand the diversity of microbiomes associated with macroalgae and described the presence of bacterial taxa belonging to Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria and Verrucomicrobia (Selvarajan et al., 2019; Comba González et al., 2021). The application of metagenomics tools for understanding functional diversity can provide more insights into the ecological and biotechnological potential of macroalgae-associated microbial communities (Lin et al., 2018).

Macroalgal polysaccharide hydrolysing activity has been reported from a large number of bacterial and a few fungal isolates, mostly from marine habitats (Jagtap et al., 2021). In our study, MABs, which had the highest porphyranase, alginate lyase and ulvan lyase activity, was reported by taxa belonging to Firmicutes and Proteobacteria. Among these isolates, NIOA181 and NIOA284 which showed the highest ulvan lyase and porphyranase activity are identified as *Bacillus* sp. (Jagtap et al., 2022a). Various studies have reported the polysaccharide hydrolyzing activity of *Bacillus* sp., which showed their ability to hydrolyse agar, carrageenan and alginate, but there are no reports on ulvan and porphyran hydrolysis activity (Suzuki et al., 2003; Li et al., 2015, 2019; Chen et al., 2018). Very few studies have reported microbial ulvan lyase and

porphyranase activity to enzymatically hydrolyse ulvan and porphyran, respectively (**Table 3.1** and **Table 3.3**). Previous studies have been carried out to isolate ulvan hydrolyzing bacteria, including *Alteromonas* sp., *Formosa agariphila*, *Pseudoalteromonas* sp., *Pseudomonas vesicularis* and *Aeromonas salmonicida* (Coste et al., 2015; Konasani et al., 2018; Reisky et al., 2018; Qin et al., 2018, 2020; Gao et al., 2019; Hung et al., 2021). However, this is the first study, to the best of our knowledge, reporting ulvan lyase and porphyranase activity from a *Bacillus* sp., associated with green and red macroalgae respectively. Green macroalgae of ulvaceae are mainly composed of sulfated heteropolysaccharide ulvan, accounting for 8-29% of the dry weight of *Ulva* biomass (de Borba Gurpilhares et al., 2016; Balar et al., 2019). The poor solubility of ulvan due to its high molecular and polydisperse nature, with its complex composition of rare sugars such as rhamnose, uronic acid and xylose, limits its applications in various food, biofuel and pharmaceutical industries (Kidgell et al., 2019). Similar to ulvan due to the methylation and sulfation on galactose residue porphyran also requires specific enzymes for its enzymatic hydrolysis. Hence, the identification of novel ulvan lyase and porphyranase producing microbes with high specificity can be used for efficient utilization of macroalgal polysaccharides, which are not utilized to their full potential. This study concludes that the symbiotic mechanism exists in nature, with different bacteria contributing to the array of enzymes required to break down algal polysaccharides that exist as cell wall components of various macroalgae. These enzymes are in global demand to hydrolyze algal polysaccharides from natural algal biomass into bioactive oligosaccharides.

These potential MABs NIOA181and NIOA284 will be used in chapter 4 to characterize their extracellular ulvan lyase and porphyranase for enzymatic hydrolysis of ulvan and porphyran to produce respective macroalgal oligosaccharides.

Chapter 4

Enzymatic production and characterization of bioactive algal oligosaccharides

4.1 Introduction

Oligosaccharides are low molecular weight, hydrolyzed polysaccharides composed of 2-20 units of simple monomers. They are readily soluble in water, which makes them biocompatible and provides a wide range of bioactivity. Though they have been commercialized since the 1980s as low-calorie bulking agents. It has gained much interest in the food industry after research findings across the globe showed that non-digestible oligosaccharides with prebiotic status can be included as functional foods and were accredited with GRAS "Generally Recognized As Safe" status by the United States Food and Drug Administration (FDA, 2016). Marine macroalgae have very high carbohydrate content due to the presence of complex macroalgal polysaccharides (MPS) in their cell wall. Despite numerous bioactivities reports, the high molecular weight of MPS limits their applications in the food and pharmaceutical industries (de Borba Gurpilhares et al., 2016). Enzymatic hydrolysis is a green approach to hydrolyse these polysaccharides using specific enzymes that cleaves or hydrolyzes the glycosidic bonds to produce stereo-specific oligosaccharides (Xu et al. 2018a, b). These low molecular weight and biocompatible oligosaccharides have far-reaching applications in healthcare industries than the parent polysaccharides (Charoensiddhi et al., 2017; Cheong et al. 2018; de Borba Gurpilhares et al., 2019). Hence, polysaccharide hydrolysis using microbial enzymes is the best alternative approach to produce bioactive oligosaccharides for nutraceutical applications. In recent years, these enzymes have been in much demand due to the market potential of macroalgal oligosaccharides in functional foods and health drink formulations. Since then, the global requirement for these microbial polysaccharide degraders has been increasing for the production of functional oligosaccharides.

Microbial enzymes involved in the hydrolysis or cleavage of the complex and unique algal polysaccharides are grouped as glycoside hydrolases (GH) and polysaccharide lyase (PL). The GH is the group of enzymes that hydrolyze the glycosidic bond in the polysaccharides. A large majority of the oligosaccharides producing enzymes agarase, carrageenase, porphyranase, fucoidanase and laminarinase belong to the GH group and alginate lyase and ulvan lyase belong to PL group. These enzymes exhibit very high specific activity on the polysaccharide substrates and are known to have wide applications in structural analysis, controlling rheological properties of polysaccharides, protoplast formation and mainly in the production of bioactive oligosaccharides (Moreno et al., 2017). The current understanding of the enzymatic production of oligosaccharides from polysaccharides using these specific GH and PL enzymes is summarized below.

4.1.1 Enzymatic hydrolysis of red macroalgal polysaccharides into oligosaccharides

Agaro-oligosaccharides

Agarases are the group of GH enzymes that catalyze the hydrolysis of agar to produce oligosaccharides, which are mainly composed of basic alternating units of 3-O-linked-β-D-galactose (G) and 4-O-linked 3, 6-anhydro-α-L galactose (AG). The α-agarase hydrolyses the α-1, 3 linkages of agar and produces agarooligosaccharides with anhydro-galactose at the reducing end and this specific group of the enzyme is not very prevalent (Potin et al., 1993; Zhang et al., 2018; Lee et al., 2019). The β-agarase which hydrolyzes the β-1, 4 linkages and produced neoagarooligosaccharides with β-D-galactose at the reducing end are well studied and widely documented (Fu and Kim, 2010). The recombinant enzyme from cloning and expression of a β-agarase gene in *Bacillus subtilis* showed the production of neoagarooligosaccharides with 30-fold

greater activity than the original strain (Ohta et al., 2004). Recently Xu et al. (2018a, b) have reported the production of neoagarooctaose and neoagarodecaose from enzymatic hydrolysis of agar extracted from *Gelidium amansii* using recombinant β-agarase (**Table 4.1**). The neoagarooligosaccharides obtained using the recombinant β-agarase showed the production of specific products tetra, hexa or octa oligosaccharides with antioxidant, anti-inflammatory and prebiotic activity (Chen et al., 2006; Hu et al., 2006; Wang et al., 2017; Xu et al., 2018a).

Carrageenan oligosaccharide

Carrageenan is a linear sulfated polysaccharide mostly composed of repeating disaccharide units of sulfated galactose (G) and anhydro-α-L galactose (AG). They are categorized as κ-, ι- and λ-carrageenan based on the number of sulfate groups and hydrolyze internal β-1, 4 linkages by their specific enzymes, including κ-carrageenase (EC 3.2.1.83), ι-carrageenase (EC 3.2.1.157) and λ-carrageenases (EC 3.2.1.162) (Li et al., 2014; Kalitnik et al., 2016; Yermak et al., 2017). Studies on recombinant κ-carrageenase from *Zobellia* sp. expressed in methylated yeast *Pichia pastoris* and *Brevibacillus choshinensis* showed the production of κ-carrageenan tetra- and hexa-oligosaccharides (Yu et al., 2017; Xu et al., 2019). The recombinant λ-carrageenase from *Pseudolateromonas carrageenovora* was used in the production of tetra- and hexa-λ-carrageenan oligosaccharides (Shen et al., 2017). These enzymatically produced carrageenan oligosaccharides showed potential antioxidant, immunoregulatory and antitumor activity than parent carrageenan.

Porphyran oligosaccharide

Porphyranase (EC 3.2.1.178) is a GH enzyme that catalyzes the hydrolysis of porphyran, which is mainly composed of alternating 3-linked methylated β-D-galactose (Me-G) and sulfated 3, 6-

anhydro-α-L-galactose (SAG) at β-1, 4 glycosidic linkages to produce porphyran oligosaccharides with methylated G at the reducing end (Correc et al., 2011; Przybylski et al., 2015). Correc et al. (2011) used β-porphyranase isolated from *Zobellia galactanivorans* for the production of hexa porphyran oligosaccharides with SAG (1→3) MeG subunits. The β-methyl porphyranase isolated from *Pseudoalteromonas atlantica* hydrolyze the porphyran into porphyran oligomer of methylated disaccharide and dimethylated tetra-saccharides (Przybylski et al., 2015). Recently, the porphyran oligosaccharides produced using recombinant β-porphyranase cloned from *Wenyingzhuangia funcanilytica* at a wide pH range (pH 3.5-11) and showed antitumor and antiobesity activity (Osumi et al., 2002; Zhang et al., 2019b). Few porphyranase genes have been expressed in E. *coli* and the enzymes were characterized, but its bioactive potential was not explored (Hehemann et al., 2010, 2012; Zhang et al., 2019b).

4.1.2 Enzymatic hydrolysis of brown macroalgal polysaccharides into oligosaccharides

Alginate Oligosaccharides

Alginate lyase is the group of PL that cleave the β 1→4 linkage of α-L-glucuronic acid (GlcA) and its C5 epimer β-D-mannuronic acid (MnA) to produce alginate oligosaccharides. They are categorized into mannuronate lyase (EC 4.2.2.3) and glucoronate lyase (EC 4.2.2.11) based on their specificity to MnA- and GlcA-rich substrate, respectively (Zhu et al., 2015; Zhu et al., 2018b). Alginate lyase with bifunctional activity, which cleaves both MnA- and GlcA-rich substrates has been reported to hydrolyze alginate efficiently. Endolytic alginate lyase hydrolysis leads to the production of di-, tri-, tetra-, penta- and hexa-oligosaccharide as the main product, while exolytic alginate lyase further degrades oligosaccharides into monomers. The recombinant polyglucuronate

specific alginate lyase was shown to produce di-, tri-, tetra- and penta alginate oligosaccharides (Yang et al., 2018). Enzymatically produced alginate oligosaccharides exhibited antioxidant, immunoregulatory, anti-inflammatory and hypoglycemic properties (Zhou et al., 2015; Han et al., 2019; Tran et al., 2019; Xing et al., 2020).

Fucoidan oligosaccharides

Fucoidanase (EC 3.2.1.44) is the GH enzyme capable to catalyze the hydrolysis of fucoidan without the removal of substitute groups, which comprises of (1→4) and (1→3) linked α-L-fucose units and produced low molecular weight fucoidan oligosaccharides (Wang et al., 2007; Silchenko et al., 2013, 2018). So far, only one study has reported the ability of recombinant fucoidanase from *Formosa algae* to produce low molecular weight bioactive fucoidan oligosaccharide with immunoregulatory activity (Silchenko et al., 2013, 2018).

Laminarin oligosaccharides

Laminarinase or glucanase is the group of GH enzymes that hydrolyse laminarin composed of β-(1→3) and (1→6) linked glucose to produce laminarin oligosaccharides (Kadam et al., 2015; Lee and Lee, 2016). Laminarinase are categorized as exo-glucanase and endo-glucanase. The exo-β-(1→3)-glucanase hydrolyze laminarin at the terminal and formed monomer units and the endo-β-(1→3)-glucanase hydrolyze the β-1→3 linkage randomly within the chain of laminarin into β-(1→6) linked laminarin oligosaccharide. The side chain in laminarin limits the activity of endo glucanase, owing to steric hindrance and limits complete hydrolysis. Badur et al. (2020) have investigated four laminarinase isolated from *Vibrio breoganii* that can produce both low and high molecular weight laminarin oligosaccharide, with the degree of polymerization 3-4 and >8,

respectively. The endo-β-(1→3)-glucanase isolated from *Bacillus circulans* and *Bacillus clausii* NM1 (Kim et al., 2006) could hydrolyze laminarin into β-1→3 glucan oligomer with anti-inflammatory activity (**Table 4.1**).

Table 4.1 Bioactivities of enzymatically produced macroalgal oligosaccharides.

Algal oligosaccharides	**Bioactivity**	**References**
Agaro-oligosaccharides	Antioxidant, hepatoprotective, antiglycemic, prebiotic and anti-inflammatory activity	Wang et al. 2004; Chen et al. 2006; Hu et al. 2006; Wang et al. 2017; Xu et al. 2018a
Carrageenan oligosaccharides	Antioxidant, prebiotic, anti-inflammatory and immunoregulatory	Xu et al. 2012; Yao et al. 2014; Han et al. 2019
Porphyran oligosaccharides	Antiobesity activity	Osumi et al. 2002
Alginate oligosaccharides	Antioxidant, antiglycemic, prebiotic, anti-inflammatory, immunoregulatory and antiobesity activity	Hao et al. 2011; Falkeborg et al. 2014; Xu et al. 2014; Zhou et al. 2015; Han et al. 2019; Tran et al. 2019; Zhang et al. 2020
Fucoidan oligosaccharides	Antioxidant, immunoregulatory and antitumor activity	Wang et al. 2007; Silchenko et al. 2013; Silchenko et al. 2018
Laminarin oligosaccharides	Antitumor and immunoregulatory activity	Pang et al. 2005; Kim et al. 2006
Ulvan oligosaccharides	Antioxidant and angiotensin converting enzyme inhibition	Hung et al., 2021

4.1.3 Enzymatic hydrolysis of green macroalgal polysaccharides into oligosaccharides

Ulvan lyase (EC 4.2.2.) is the least explored PL enzyme that hydrolyses β-1, 4 glycosidic linkages between sulfated α-L-rhamnose (Rha3S) linked to either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) into ulvan oligosaccharides that have 4-deoxy-L-threo-hex-4-enopyranosiduronic acid (EPU) at the non-reducing end (Lahaye and Robic, 2007; Ulaganathan et al., 2018). Ulvan lyase isolated from *Alteromonas* species produced low molecular weight oligosaccharides, mainly composed of unsaturated rhamnose 3-sulfate and uronic acid. Similarly, ulvan lyase isolated from *Pseudoalteromonas* sp. showed potential to hydrolyze ulvan into ulvan oligomer, including disaccharide (EPU-Rha3S) and tetra-saccharides (EPU-Rha3S-Xyl-Rha3S) (Qin et al., 2018). Gao et al. (2019) studied the cloning, expression and characterization of recombinant ulvan lyase from *Alteromonas* sp. A321 and showed production of even numbered ulvan oligosaccharide. As of now, only antioxidant and angiotensin-converting-enzyme inhibition activities of enzymatically produced ulvan oligosaccharides were reported (**Table 4.1**).

Most of the oligosaccharides obtained from agar, carrageenan and alginate are shown to have very good bioactivity which has wide therapeutic applications. However, so far there only a few studies reported on the bioactivity of oligosaccharides obtained from enzymatic hydrolysis of porphyran and ulvan (Jagtap and Manohar, 2021). Hence, in this chapter, the underexplored polysaccharides such as porphyran and ulvan are enzymatically hydrolyzed using marine microbial enzymes ulvan lyase from NIOA181 and porphyranase from NIOA284. Their respective oligosaccharides were characterized using Mass spectrometry and NMR spectroscopy, which provide an accurate molecular mass and conformation of the compound. The antioxidant and prebiotic activity of these enzymatically produced oligosaccharides was compared with parent polysaccharides.

4.2 Materials and methods

4.2.1 Chemicals and reagents

The chemicals 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, fructooligosaccharides from chicory were purchased from Sigma Aldrich, USA. All other chemicals eused in this study were of analytical grade. The probiotic cultures *Lactobacillus acidophilus* (NCIM 2285/ATCC4963) and *Lactobacillus bulgaricus* (NCIM 2056/ATCC8001) and an enteric culture (E) of *Escherichia coli* (ATCC8739) were procured from National Collection of Industrial Microorganisms (NCIM), a national facility at CSIR-NCL, Pune, India.

4.2.2 Isolation of extracellular ulvan lyase from *Bacillus* sp. NIOA181 and porphyranase from *Bacillus* sp. NIOA284

The potential MABs, *Bacillus subtilis* NIOA181 and *Bacillus australimaris* NIOA284 identified through a qualitative and quantitative analysis were selected for the production of extracellular ulvan lyase and porphyranase enzymes (**Table 3.4**). For this, the isolates NIOA181 and NIOA284 strains were inoculated in 100 ml 0.5% ulvan and 0.5% porphyran substrate respectively in a basal salt solution comprising of 2 g K_2HPO_4, 30 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $FeSO_4 \cdot 7H_2O$ at pH 7 in 1L of media prepared in seawater and allowed to grow at 28 °C for 24 h. These bacteria were aerobically cultured in the 500 ml shake flasks containing 250 ml liquid selection medium at 28 °C and 150 rpm for 24 h. The enzyme activity was measured at 3, 6, 9 and 12 h intervals by determining the amount of reducing sugar released based on the dinitrosalicylic acid method. After incubation, the inoculum was centrifuged at 8000 rpm for 20 minutes to remove the bacterial cells from the supernatant. The cell-free supernatant was precipitated with saturated ammonium sulfate

to collect the crude enzymes, followed by dialysis against 50 mM Tris-HCl buffer (pH 8.0) for ulvan lyase and 50 mM phosphate buffer (pH 7) for porphyranase. After dialysis, the enzymes were loaded on the DEAE sepharose column and eluted using a linear gradient of NaCl (0.2 - 1.0 M) in respective buffers and active fractions were collected, pooled together and concentrated on a vacuum concentrator. The enzymes were further purified on gel permeation chromatography column Superdex 75 and eluted with deionized water. Fractions that showed enzyme activity were combined and lyophilized into ulvan lyase (ULB1) and porphyranase (PB1) powder.

4.2.3 Biochemical characterization of ulvan lyase ULB1 and porphyranase PB1

Extracellular ulvan lyase ULB1 from NIOA181 and β-porphyranase PB1 from NIOA284 were characterized for optimization of their potential to hydrolyse ulvan and porphyran, respectively. The influence of different parameters such as the pH, temperature, varying NaCl concentrations, metal ions and chelating agents on the relative activity of the ULB1 and PB1 was tested. The effect of different temperatures ranging from 20 - 60 °C by incubating the enzymes with 0.5% substrates for 30 minutes. Similarly, the effect of pH on ULB1 and PB1 activity was determined at optimum temperature in appropriate buffers (50 mM) for each of the pH ranges, such as acetate buffer (pH 3 - 6), phosphate buffer (pH 6 - 7), Tris-HCl buffer (pH 8 - 9) and carbonate-bicarbonate buffer (pH 10 - 11). The effect of different concentrations of NaCl on ULB1 and PB1 activity was evaluated at pH 7 and 35 °C for 1 h. The impact of different metal ions such as Li^+ (LiCl), K^+ (KCl), Mg^{2+} ($MgCl_2$), Ca^{2+} ($CaCl_2$), Ba^{2+} (BaCl2), Ni^{2+} ($NiSO_4$), Co^{2+} ($CoCl_2$), Cu^{2+} ($CuCl_2$) and Fe^{3+} ($FeCl_3$) at 10 mM concentration on ULB1 and PB1 activity was determined to understand their role as cofactors. The influence of chelating agents and detergents such as ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), urea, β-mercaptoethanol, sodium dodecyl sulfate

(SDS), Triton X-100, Tween 20, Tween 80 and cetyl trimethyl ammonium bromide (CTAB) at 0.1% concentration on ULB1 and PB1 activity was also determined.

4.2.4 Enzymatic hydrolysis of ulvan and porphyran using ULB1 and PB1

To estimate the enzymatic production of oligosaccharides, crude ulvan lyase ULB1 (10 µg/ml) was mixed with the 0.5% ulvan (10 ml) prepared in 50 mmol l^{-1} Tris-HCl buffer at pH 8.0 and supplemented with 1 mM $MgCl_2$ and incubated at 40°C for 24 h. Similarly, a 10 ml porphyran solution (0.5% w/v) in 100 mM phosphate buffer with 400 mM NaCl and 1 mM $CoCl_2$ was incubated with purified porphyranase PBI (10 µg/ml) for 24 h at 35 °C. The enzymatic hydrolysis of ulvan into ulvan oligosaccharides (UOS) and porphyran into porphyran oligosaccharides (POS) was determined using thin-layer chromatography (TLC) and the samples were taken at 0, 3, 6, 9 and 12 h time intervals and spotted on aluminum silica gel 60 mm plates. The liquid phase was composed of n-butanol: acetic acid: water (2:1:1) and the plates were conceded to run up to 5 cm from the sample location. For visualization of oligosaccharides, the plates were stained with 10% (v/v) H_2SO_4 in ethanol and heated at 100 °C for 10 minutes. The oligosaccharides were purified on Sephadex® G-100 medium (Sigma-Aldrich, USA) and eluted with milli-Q water and the fractions with reducing sugar were combined and freeze-dried for further analysis.

4.2.5 Structural characterization of ulvan and porphyran oligosaccharides

4.2.5.1 FTIR spectroscopy

The enzymatically produced UOS and POS using ULB1 and PB1, respectively were analysed based on Fourier Transform Infrared Spectroscopy (Shimadzu-8201PC spectrometer, Japan) and evaluated for their functional groups (Hung et al., 2021). For this, UOS and POS were individually

mixed with dry potassium bromide and the FTIR spectrum was recorded using 50 scans in the spectral range of 4000 – 400 cm^{-1}.

4.2.5.2 13C NMR analysis

The enzymatically produced and purified UOS and POS were treated with D_2O and freeze-dried for structural analysis. This process was repeated twice to exchange the deuterium and the ^{13}C NMR spectrum was recorded using BRUKER AVANCE III HD NMR 500 MHz in Liquid State at a concentration of 20 mg ml^{-1} in D_2O and acquisition parameters were set as a number of scan, 4000; D1, 2.0 sec and Pulse programme, zgpg30.

4.2.5.3 High Resolution Mass Spectroscopy Analysis

The enzymatically produced and purified UOS and POS were analysed using High-Resolution Mass Spectrometry (HRMS) on Bruker Impact HD Q-TOF spectrometer (Bruker Daltonics, Billerica, MA, USA) to determine the number of sugar moieties. Oligosaccharides at 0.1 mg ml^{-1} concentration prepared in acetonitrile/water (1:1 v/v) and injected (1 µL) through UHPLC (Dionex Ultimate 3000, Make-Thermo Scientific) to Electrospray Ionization (ESI)-Q-TOF system. The flow rate was set to 120 µL min^{-1} of mobile phase (90% acetonitrile in 0.1% formic acid prepared in water v/v) and the mass spectrum was recorded between 50 - 1500 m/z in positive ion polarity. The other MS parameters, such as capillary, endplate offset and charging voltage, were set as 4000 V, 500 V and 2000 V, respectively. The nebulizer was set at 1.7 bar, the dry heater at 200 °C and the dry gas at 7.0 L min^{-1}. The spectra visualization and baseline correction were analysed using Bruker compass Data Analysis software 4.2. The MS spectra were used to determine the molecular weight of oligosaccharides based on the m/z ratio and published literature.

4.2.6 Antioxidant and prebiotic potential of enzymatically produced oligosaccharides

The DPPH and ABTS radical scavenging activity of the ulvan and porphyran and its enzymatically hydrolysed products were evaluated at various concentrations (0.5 – 2 mg ml^{-1}) as reported in the method section 2.2.6.1 and 2.2.6.2, respectively. Similarly, the in vitro prebiotic activity of the ulvan and porphyran and its enzymatically hydrolysed products UOS and POS at 1% concentration were analyzed as reported in the method section 2.3.5.

4.2.7 Statistical analysis

All experimental studies were carried out in triplicates (n=3) and mean values are expressed along with standard deviations. Statistical significance was determined using one-way ANOVA and student t-test and the significance level was set as $P \leq 0.05$.

4.3 Results

4.3.1 Biochemical characterization of ulvan lyase ULB1

The characterization of ulvan lyase ULB1 activity was determined using the dinitrosalicylic acid method. The optimum pH for the ULB1 activity was determined by estimating the relative activity over a range of pH from pH 3.0 to pH 11. The ULB1 showed maximum activity at pH 8.0 (**Figure 4.1a**). Furthermore, the effect of temperature on ULB1 activity was estimated and it exhibited maximum activity at 40 °C and maintained more than 70% activity at 20 °C, suggesting that ULB1 is a cold-adapted enzyme (**Figure 4.1b**). Similarly, the effect of NaCl concentration on ULB1 activity was estimated at various concentrations (0.001 M - 1 M) where it showed a decrease in activity due to the addition of NaCl, suggesting that salt denatures the enzyme (**Figure 4.1c**).

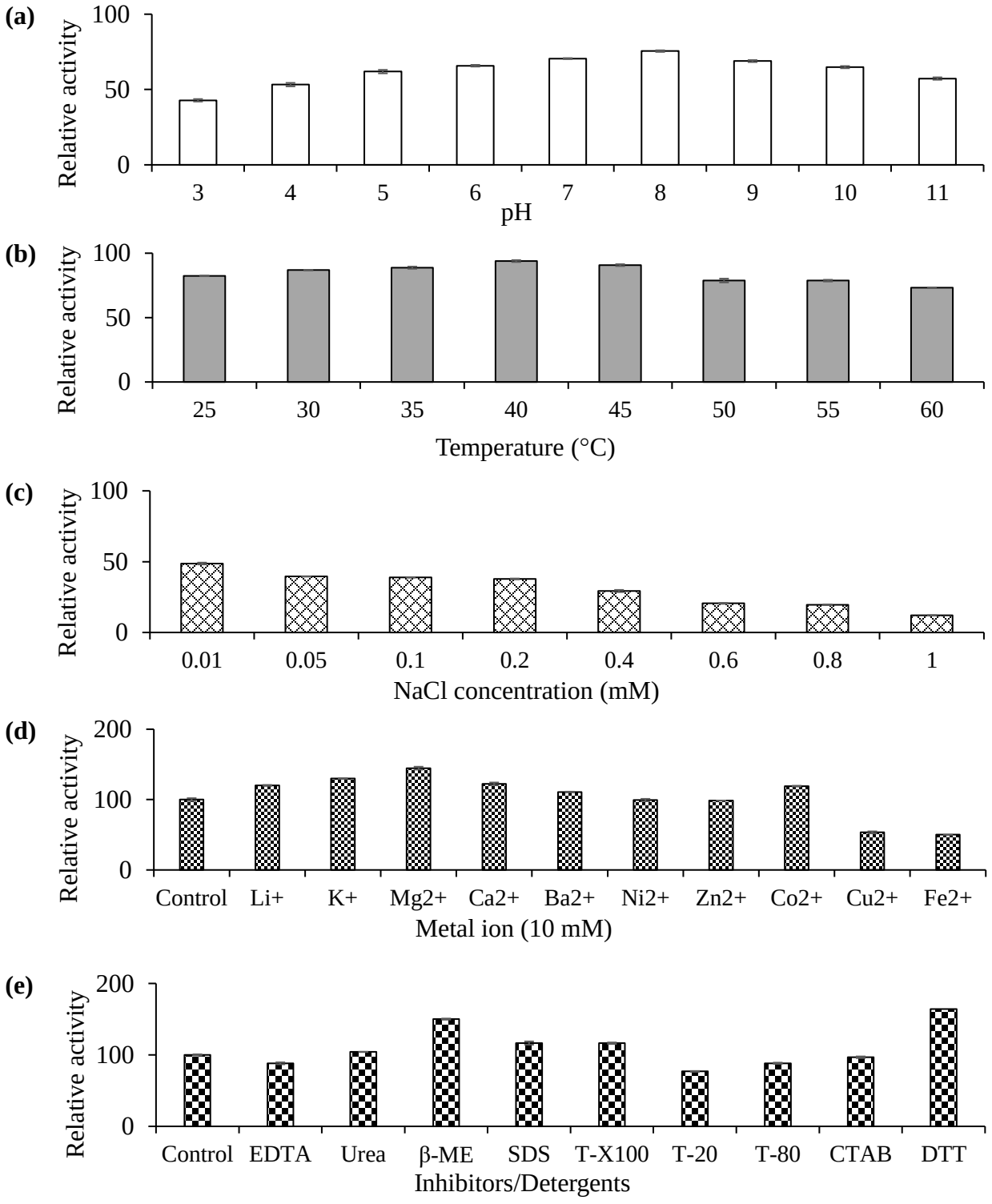


Figure 4.1 Influence of different parameters **(a)** pH **(b)** temperature **(c)** NaCl concentration **(d)** metal ions **(e)** chelating agents on the relative activity (%) of ulvan lyase ULB1.

The effect of metal ions on ULB1 activity is also estimated in the presence of various metal ions (Li^{2+}, K^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+}) and it showed that the ULB1 activity was greatly increased by the addition of 10 mM $MgCl_2$ (144%), KCl (129%), $CaCl_2$ (122%) and $CoCl_2$ (119%) (**Figure 4.1d**). The effect of chelating agent and detergent on ULB1 activity was determined by EDTA, DTT, Urea, β-mercaptoethanol, SDS, triton X-100, Tween 20, Tween 80, and CTAB and it showed a great increment in ULB1 activity in the presence of 0.1 % DDT (164%) and β-mercaptoethanol (150%), thus suggesting that preventing disulfide bond formation in the active site of the ulvan lyase can induce the enzyme activity (**Figure 4.1e**). However, EDTA inhibits the ULB1 activity, which suggests that the enzyme requires a divalent cation as a cofactor for its activity.

4.3.2 Biochemical characterization of porphyranase PB1

The sequential purification of porphyranase through ammonium sulfate precipitation increases the specific activity to 368 U mg^{-1}. Further, the consecutive DEAE anion exchange and gel permeation chromatography increased the specific activity of porphyranase to 562 and 1983 U mg^{-1}, respectively (**Appendix 4.1, 4.2**). The porphyranase specific activity and purification fold increased with each purification step. The purity of the porphyranase enzyme after each of these steps was confirmed through SDS-PAGE and the molecular mass of the three step purified protein was confirmed to be between 50 to 55 kDa (**Appendix 4.3**). The purification fold attained for the purified porphyranase PB1 was 10.5 fold. The effect of various parameters such as pH (3 to 11), temperature (20 to 60 ºC) and NaCl concentrations (0.001 to 1 M) on the PB1 activity was determined. The PB1 showed >50% activity retention at all tested temperatures and about 70% of relative activity was recorded at 25 to 45 °C.

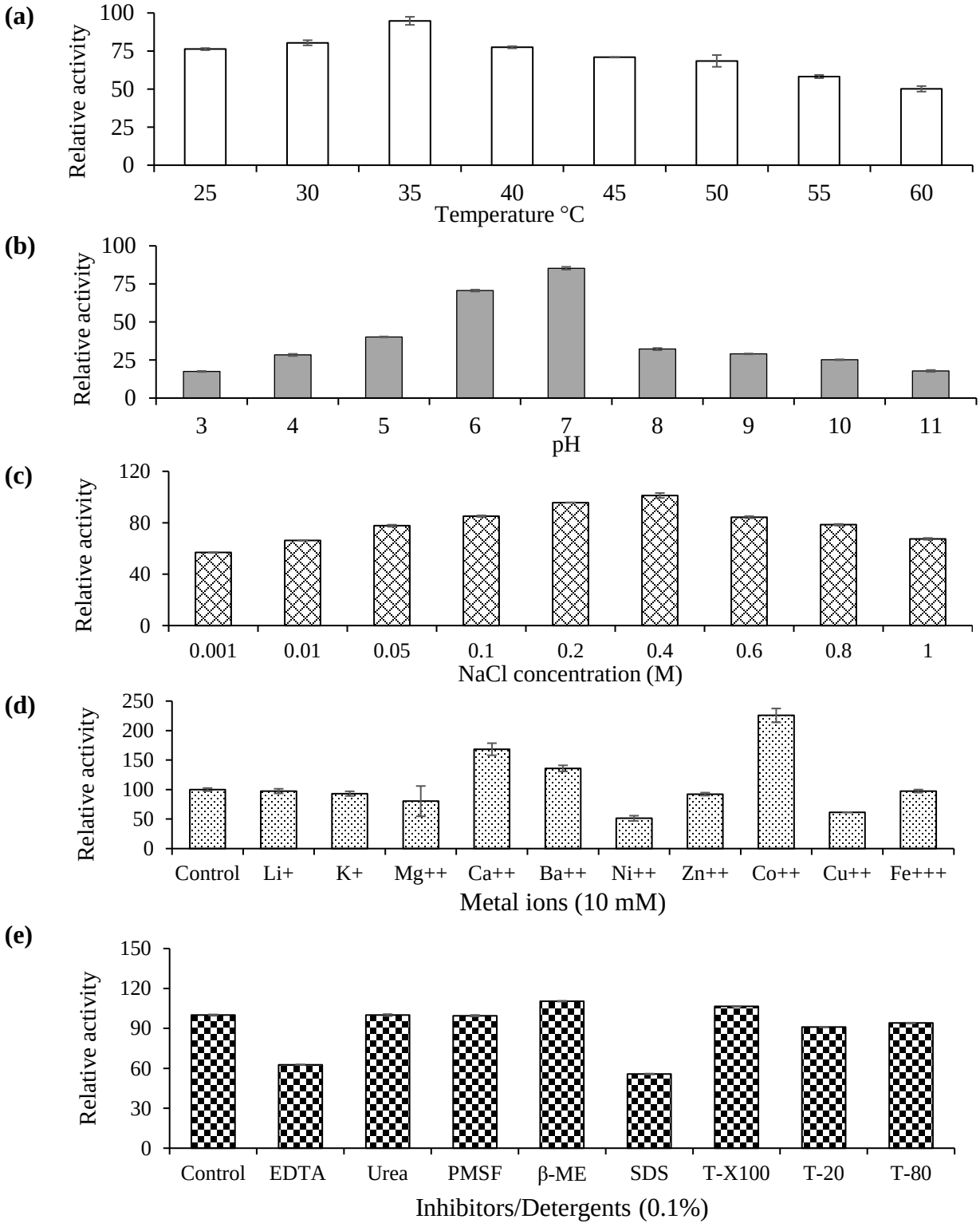


Figure 4.2 Influence of different parameters **(a)** pH **(b)** temperature **(c)** NaCl concentration **(d)** metal ions **(e)** chelating agents on the relative activity (%) of porphyranase PB1.

The highest PB1 activity was recorded at 35 °C with > 94% of relative activity (**Figure 4.2a)**. The PB1 activity was also measured in various buffers representing the pH range from 3 - 11 at 35 °C and it retained >70% of its activity at pH 6 with decreasing activity towards both acidic pH (3 - 5) and alkaline pH (8 - 11) ranges. The highest PB1 activity was recorded in a 50 mM phosphate buffer at pH 7.0 (**Figure 4.2b**). The effect of NaCl concentrations on PB1 activity was determined and the results show that >55% of PB1 activity was retained at all tested NaCl concentrations (0.001 M - 1 M). The highest PB1 activity of nearly 100% was recorded at 0.4 M NaCl concentration (**Figure 4.2c**). The effect of different metal ions (10 mM) and chelating agents or detergents (0.1%) on the PB1 activity was also estimated at optimum parameters. Metal ions such as Li^{+}, K^{+} and Fe^{3+} did not show any impact on PB1 activity. However, Ni^{2+} and Cu^{2+} declined the PB1 activity up to 51% and 61%. The PB1 activity was increased in the presence of Co^{2+}, Ca^{2+} and Ba^{2+} and recorded the highest (225%) PB1 activity in the presence of Co^{2+} metal ion (**Figure 4.2d**). The effect of inhibitors (0.1%) such as EDTA, urea, PMSF and β-mercaptoethanol on PB1 activity was investigated. The urea and PMSF did not show any impact on PB1 activity. In the presence of EDTA, the activity was reduced by up to 60%. However, β-mercaptoethanol showed activation of the PB1 with 110% activity retention (**Figure 4.2e**).

4.3.3 Enzymatic production of ulvan oligosaccharides using ULB1

The enzymatic hydrolysis of ulvan using ulvan lyase ULB1 showed the production of ulvan oligosaccharides with two different oligomers appearing below the rhamnose sugar, which represents that the UOS is composed of two different molecular weight oligosaccharides (**Appendix 4.4a**). The thin layer chromatography only provides the details on the size of the oligomers with the respect to the parent polysaccharides based on the Rf value. The degree of

polymerization, structural and molecular composition of the oligomers was determined based on NMR spectroscopy and HRMS spectrometry.

4.3.4 Structural characterization of ulvan oligosaccharides

4.3.4.1 IR characterization of UOS

The structural characterization of the UOS was carried out based on FTIR spectroscopy to study their functional groups (**Figure 4.3**). The spectrum shows characteristic peaks at 3280 cm^{-1} in UOS, attributed to the primary -OH group's stretching vibrations. Similarly, the absorption band at 2930 cm^{-1} can be associated with the -CH stretching vibration. The absorption band at 1417 cm^{-1} and 1658 cm^{-1} in the UOS represent symmetric and asymmetric carboxylate ($-COO^-$) group stretching.

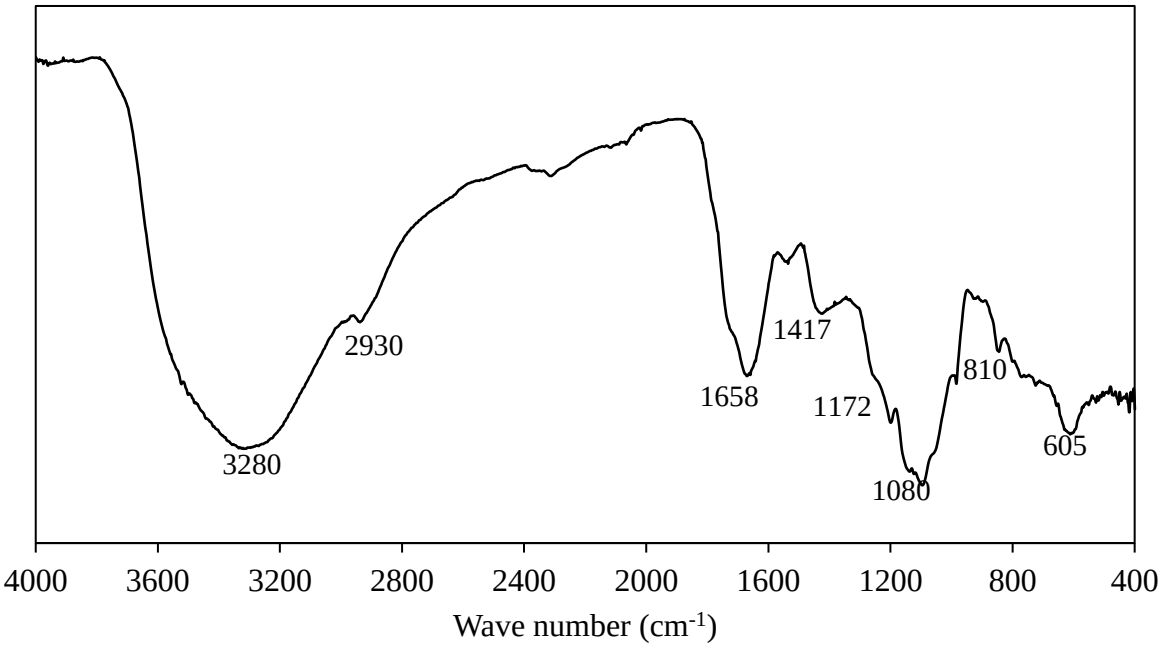


Figure 4.3 Functional group characterization of enzymatically produced ulvan oligosaccharide from ulvan based on FTIR spectrum.

The peaks at 1172 and 1080 cm^{-1} are assigned to C-OH and C-O-C stretching in the pyranose ring of the rhamnose sugar, which is the major monomer of ulvan. The characteristic of uronic acid residues was also observed in the band at 810 cm^{-1}. All these results infer that the enzymatically produced UOS retains its sugar residues and functional groups, similar to the ulvan IR spectrum described in green macroalgae (Lahaye et al., 1999).

4.3.4.2 NMR characterization of UOS

NMR spectrum based on ^{13}C was carried out for the enzymatically produced UOS to confirm the identity and configuration of the specific sugar moiety rhamnose, xylose and uronic acid (**Figure 4.4**).

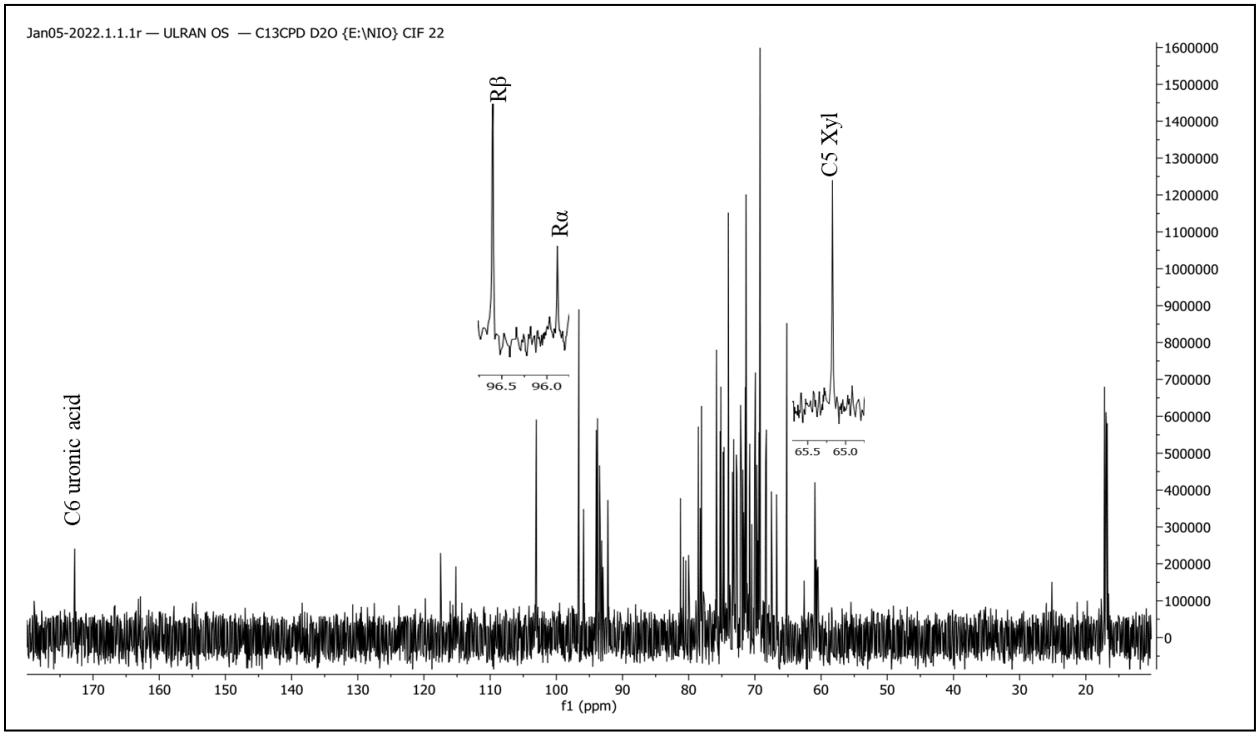


Figure 4.4 Chemical composition of enzymatically produced ulvan oligosaccharide from ulvan based on NMR spectrum.

The NMR spectrum confirmed the resonance at 96.7 and 95.9 ppm, which is attributed to the presence of α- and β-anomeric carbons in the reducing end of the rhamnose-3-sulfate residue, respectively. Methyl residue signals at 16.7, 16.8 and 16.9 ppm were observed, indicating the presence of L-rhamnose residue in UOS. The peak at 172.78 ppm represents C6 of 4-deoxy-L-threo-hex-4-enopyranosiduronic acid residue. The peak at 103 ppm implies the existence of C1 of β-D-xylose and the multiple peaks between 70-85 ppm represent C2-C5 of rhamnose, iduronic acid and glucuronic acid. These results of ^{13}C NMR analysis confirm the identity of the ulvan-specific sugars in UOS.

4.3.4.3 HRMS characterization of UOS

Enzymatically produced UOS was analysed by HRMS to determine the degree of polymerization, as the oligosaccharides are known to exist in 2 to 20 sugar units (**Figure 4.5**). From the HRMS spectrum studied, the highest significant peak at 269 m/z is attributed to Rha3S. The peaks at 295 m/z and 423 m/z are assigned to disaccharides of Rha-Xyl and ΔRha3S. The prominent peak at 599 m/z and 665 m/z represents the presence of trisaccharides composed of ΔRha3S-Xyl and Δ Rha3S-Xyl2S, respectively. The peaks at 699 m/z and 733 m/z are assigned to tetrasaccharides constituted by ΔRha3S-Xyl-Rha and ΔRha3S-ΔXyl. The HRMS spectrum of the enzymatically produced UOS using ulvan lyase shows that di, tri and tetrasaccharides were produced from ulvan heteropolysaccharide.

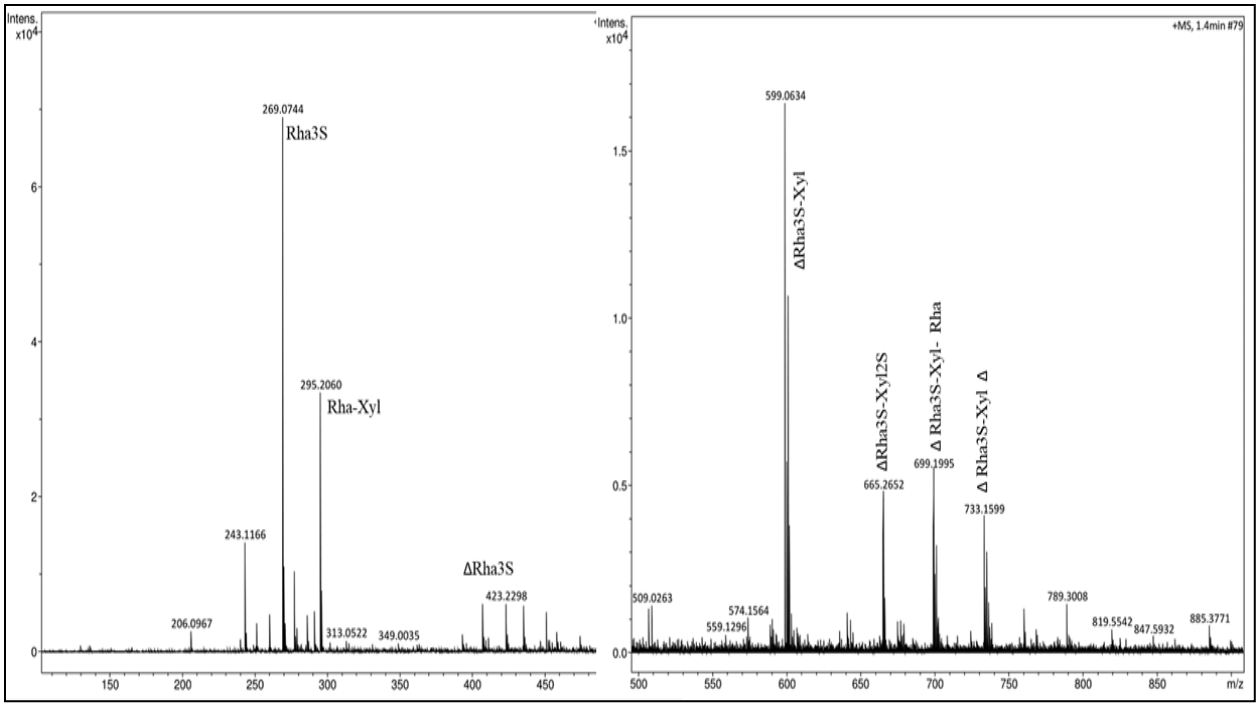


Figure 4.5 Molecular weight characterization of enzymatically produced ulvan oligosaccharide from ulvan based on HRMS spectrum.

4.3.5 Prebiotic activity of enzymatically produced UOS

The prebiotic potential of UOS was compared with ulvan and commercial prebiotic FOS based on the growth profiling of probiotic bacteria, *L. acidophilus*, *L. bulgaricus* and enteric *E. coli* and their prebiotic activity score (**Figure 4.6a**). The results show that the growth of probiotic bacteria, *L. acidophilus* and *L. bulgaricus*, was higher in the presence of 1% UOS (1.04 and 1.14) than ulvan (0.63 and 0.74) and equivalent to the commercial FOS (1.09 and 1.33) from chicory, respectively. For potential prebiotic activity, the bioactive compounds were tested for their ability to suppress the growth of enteric pathogenic strains of *E. coli.* The UOS showed prebiotic activity equivalent to FOS and was much higher than ulvan (**Figure 4.6a**). The prebiotic score was also calculated based on the CFU numbers obtained from the growth of probiotic and enteric bacteria in the

presence of ulvan, UOS and FOS. The results show that the score of UOS was 7.59 ± 0.16 for *L. bulgaricus* and 7.43 ± 0.16 for *L. acidophilus*, which was higher than ulvan and commercial prebiotic FOS (**Figure 4.6b**).

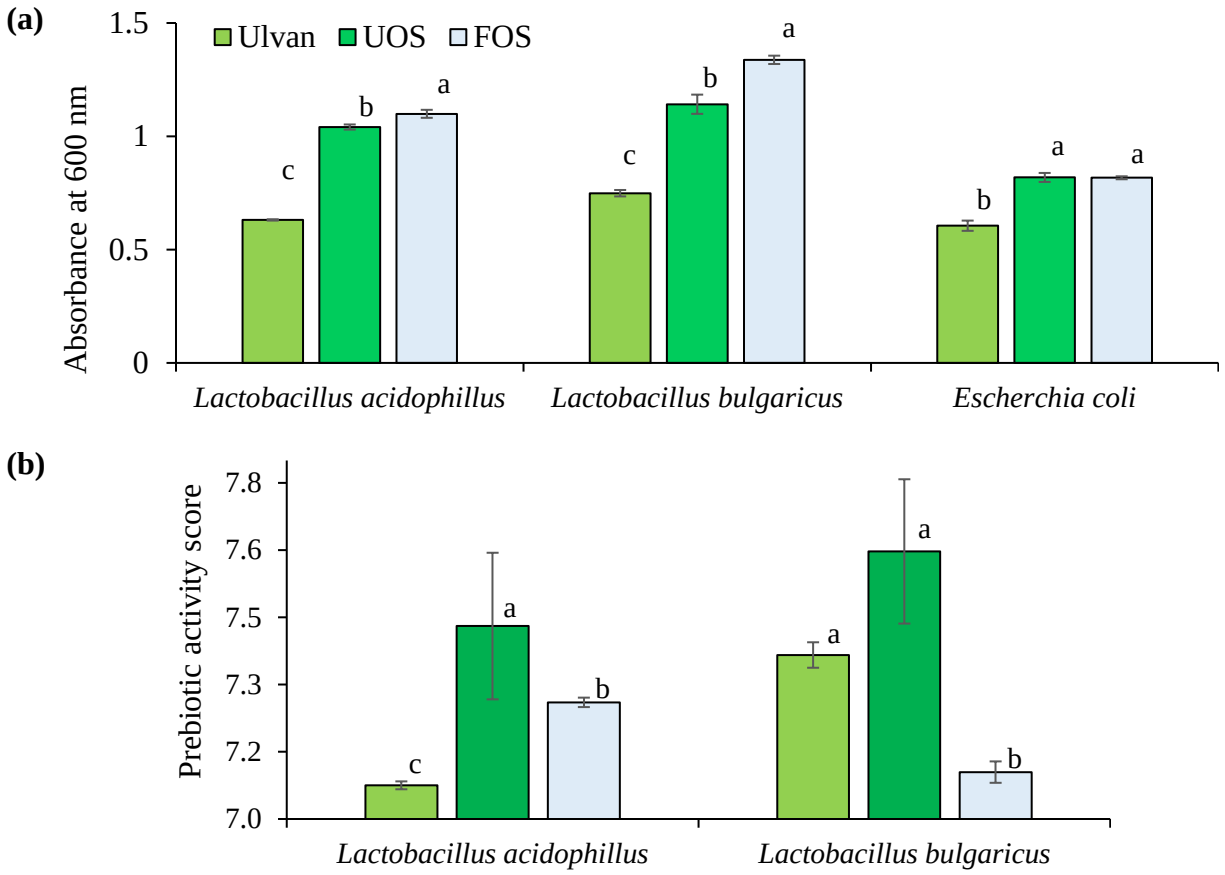


Figure 4.6 Prebiotic potential of ulvan oligosaccharides based on the (**a)** growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and (**b)** prebiotic activity score of probiotic bacteria in the presence of ulvan, ulvan oligosaccharides and fructooligosaccharides; a, b, c represents mean values within a bar with unlike superscript letters shows the significant difference ($p \leq 0.05$).

4.3.6 Enzymatic production of porphyran oligosaccharides using PB1

The purified porphyranase PB1 was used to hydrolyse porphyran into POS at the optimum temperature of 35 ºC, pH 7 and 0.4 NaCl concentration with 10 mM $CoCl_2$. The production of POS was monitored on a TLC plate with porphyran substrate and porphyranase. The results showed that porphyran was hydrolyzed into two different oligomers with different degrees of polymerization (**Appendix 4.4b**). Further, POS was purified and characterized using NMR spectroscopy and HRMS spectrometry to determine its structural composition and molecular mass.

4.3.7 Structural characterization of enzymatically produced POS

4.3.7.1 NMR characterization of POS

The ^{13}C NMR spectrum of the enzymatically produced POS was elucidated and the peaks were obtained from 15 to 105 ppm, with a peak at 20 ppm for methylation of galactose. As porphyran is composed of MeG and SAG, the signal at 100.9 ppm and 101.9 ppm attributes to the presence of AG and G, respectively. The resonance at 92.2 ppm and 96.2 ppm attributes to α- and β- anomeric carbon in the reducing end of the galactose, respectively (**Figure 4.7**). These results show that the enzymatically produced POS are mainly composed of α- and β- anomeric forms of galactose, indicating the production of oligosaccharides consisted of galactose with varying degrees of polymerization. The additional peaks at 98.0 ppm and 58.0 ppm confirm the presence of AG and methylation at C6 of galactose. The peaks at 74.7 ppm and 75.0 ppm were assigned for C4 and C5 of SAG. The peaks at 60.9 ppm and 61.2 ppm represent the methylation at C3 and C6 of galactose. The multiple signals between 68.5 ppm to 70.9 ppm represent the presence of numerous -C-OH bonds. The signal at 90.0 ppm was not detected, which is the characteristic of

hydrolysis of α (1-3) linkage in the porphyran chain, which confirms the cleavage site of the enzyme is β (1-4) and the enzyme acted on it is β-porphyranase.

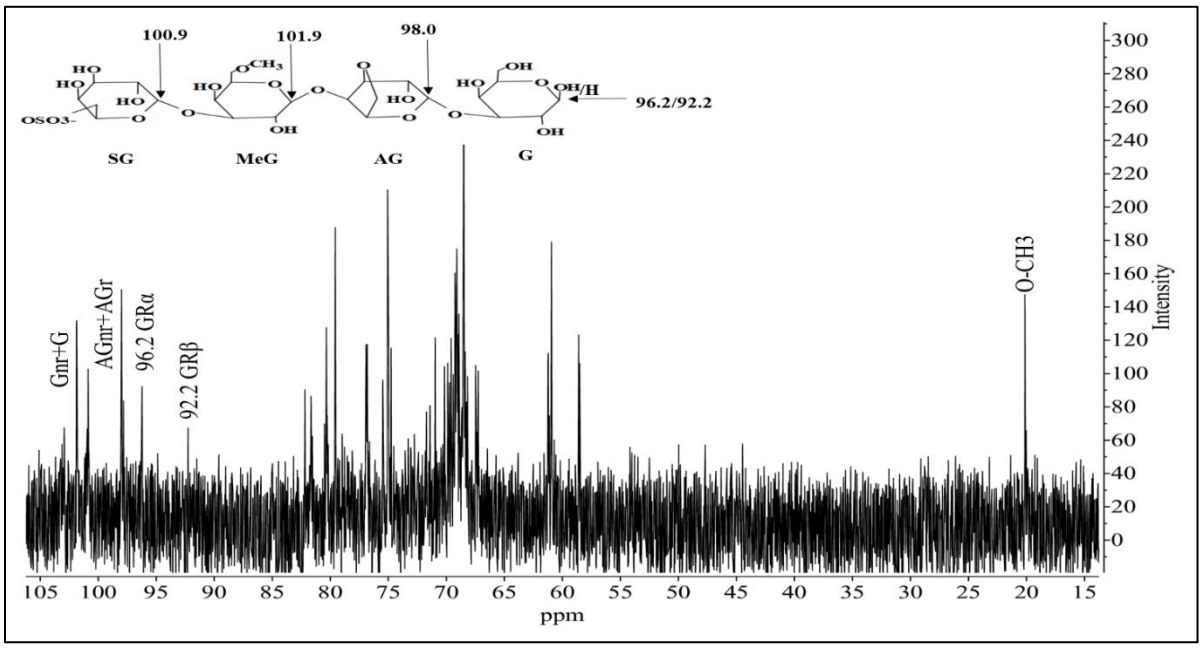


Figure 4.7 Structural characterization of enzymatically produced POS using NMR spectroscopy; in the spectrum of G, AG, SG, SAG and MeG represents galactose, anhydrogalactose, sulfated galactose, sulfated anhydrogalactose and methylated galactose, respectively. r and nr denote residues at the reducing and non-reducing end, respectively.

4.3.7.2 HRMS *characterization of POS*

The results of HRMS spectrometric analysis of enzymatically produced POS showed multiple peaks at various m/z ratios for different degrees of polymerization (**Figure 4.8)**. The highest peak at 475 m/z represents disaccharides made up of β (1-4) linked MeG and SAG, which are the major backbone of the porphyran structure (Przybylski et al., 2015; Zhang et al., 2019b). The peak at 665 m/z was attributed to the disaccharide; neoagarotetrose composed of the alternating residue of G

and AG (Zhang et al., 2018). The peak at 273, 365, 606, 665 and 701 m/z implies that the oligosaccharides also consist of monosaccharide sulfated galactose, a disaccharide of MeG-AG, trisaccharides of SG-MeG-AG, SG-MeG-SG and tetrasaccharides of MeG-AG-G-SAG, respectively. The HRMS spectrum represents the enzymatic breakdown of porphyran with the production of di-, tri- and tetrasaccharides composed of the backbone sugars G and AG.

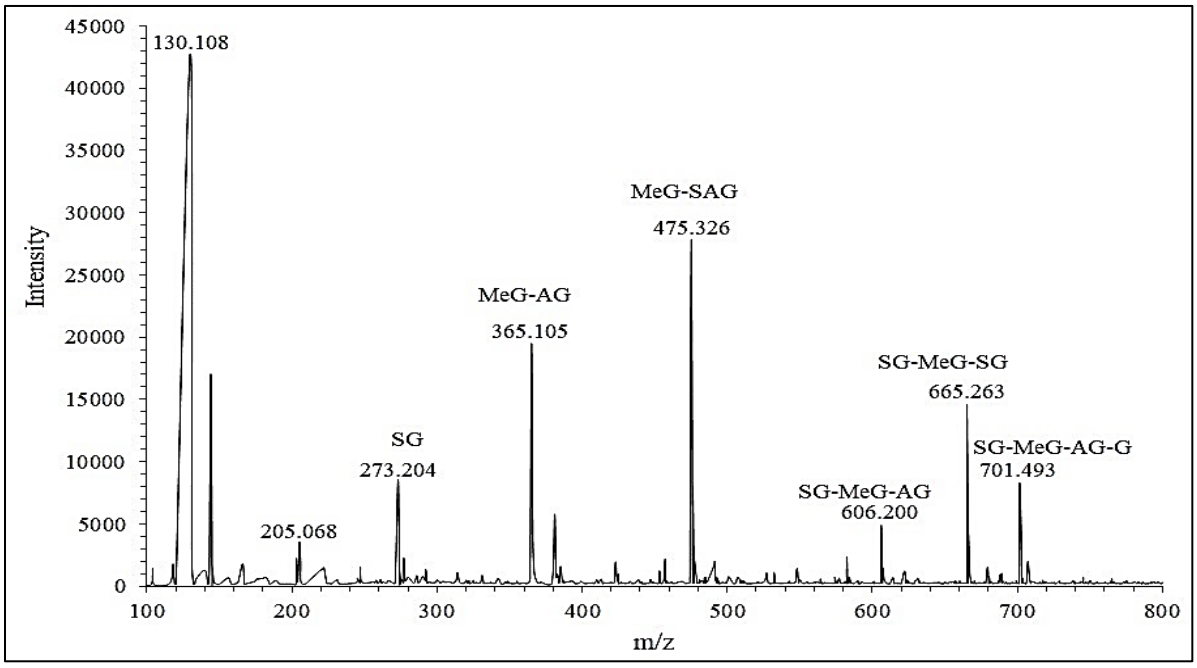


Figure 4.8 HRMS spectrum of enzymatically produced POS representing di-, tri- and tetrasaccharides. The alphabet G, AG, SG, SAG and MeG represents galactose, anhydrogalactose, sulfated galactose, sulfated anhydrogalactose and methylated galactose, respectively.

4.3.8 Antioxidant activity of enzymatically produced POS

The DPPH and ABTS radical scavenging assays were used to assess the antioxidant potential of POS in comparison with its polymer porphyran and commercial prebiotic FOS. The results show that POS showed good DPPH radical scavenging activity with inhibition of about 20% DPPH radicals than parent polysaccharide porphyran with <4% inhibition of DPPH radicals and FOS did

not show any radical scavenging activity at tested concentrations up to 10 mg ml^{-1} **(Figure 4.9a)**. Similarly, the ABTS radical scavenging activity results showed that the POS could scavenge ABTS radicals efficiently with >90% radical inhibition at 10 mg ml^{-1} concentration which was higher than the porphyran with <40% radical scavenging activity. POS showed concentration-dependent ABTS radical scavenging activity with a lower IC_{50} value of 4.72 mg ml^{-1} than porphyran (**Figure 4.9b**).

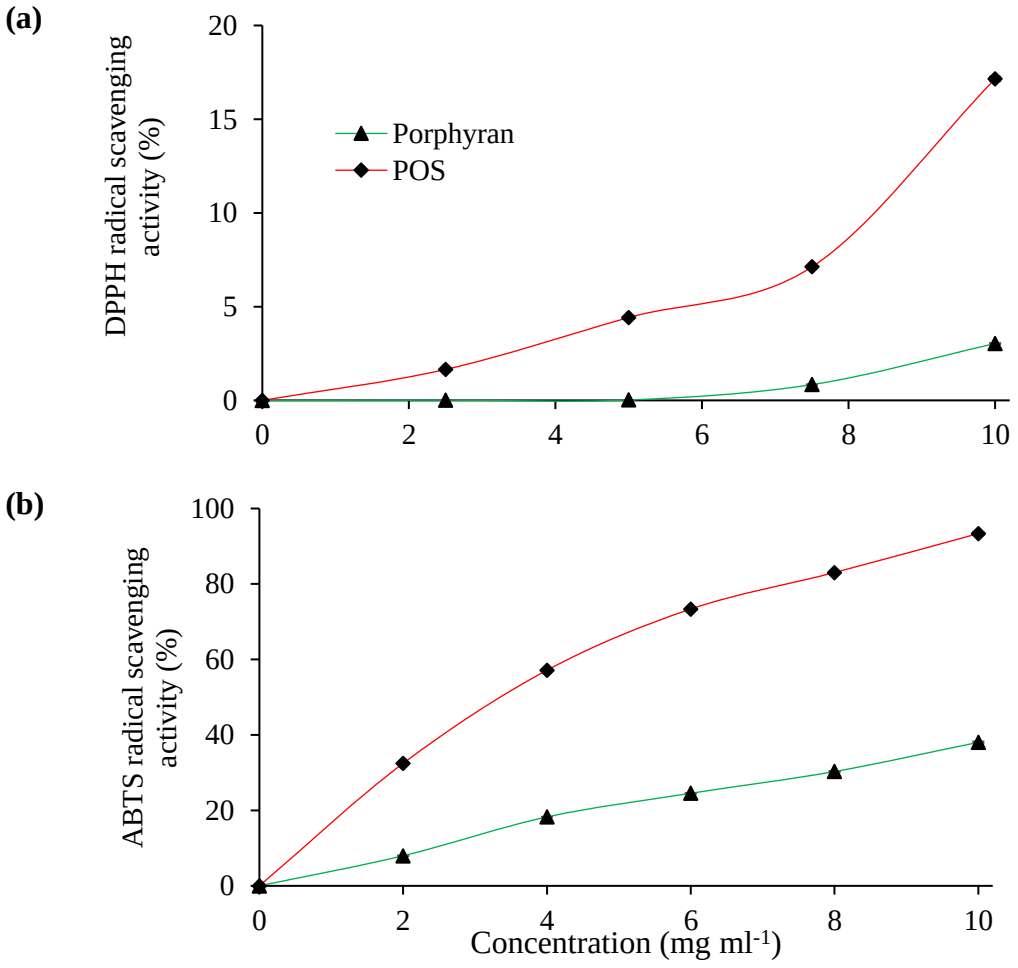


Figure 4.9 Antioxidant activity of porphyran and porphyran oligosaccharides (POS) **(a)** DPPH radical scavenging activity **(b)** ABTS radical scavenging activity**.**

4.3.9 Prebiotic activity of enzymatically produced POS

The prebiotic activity of enzymatically produced POS was measured based on the growth profiling of probiotic bacteria, *L. acidophilus*, *L. bulgaricus* and enteric *E. coli*, along with their prebiotic activity score and it was compared with porphyran and FOS.

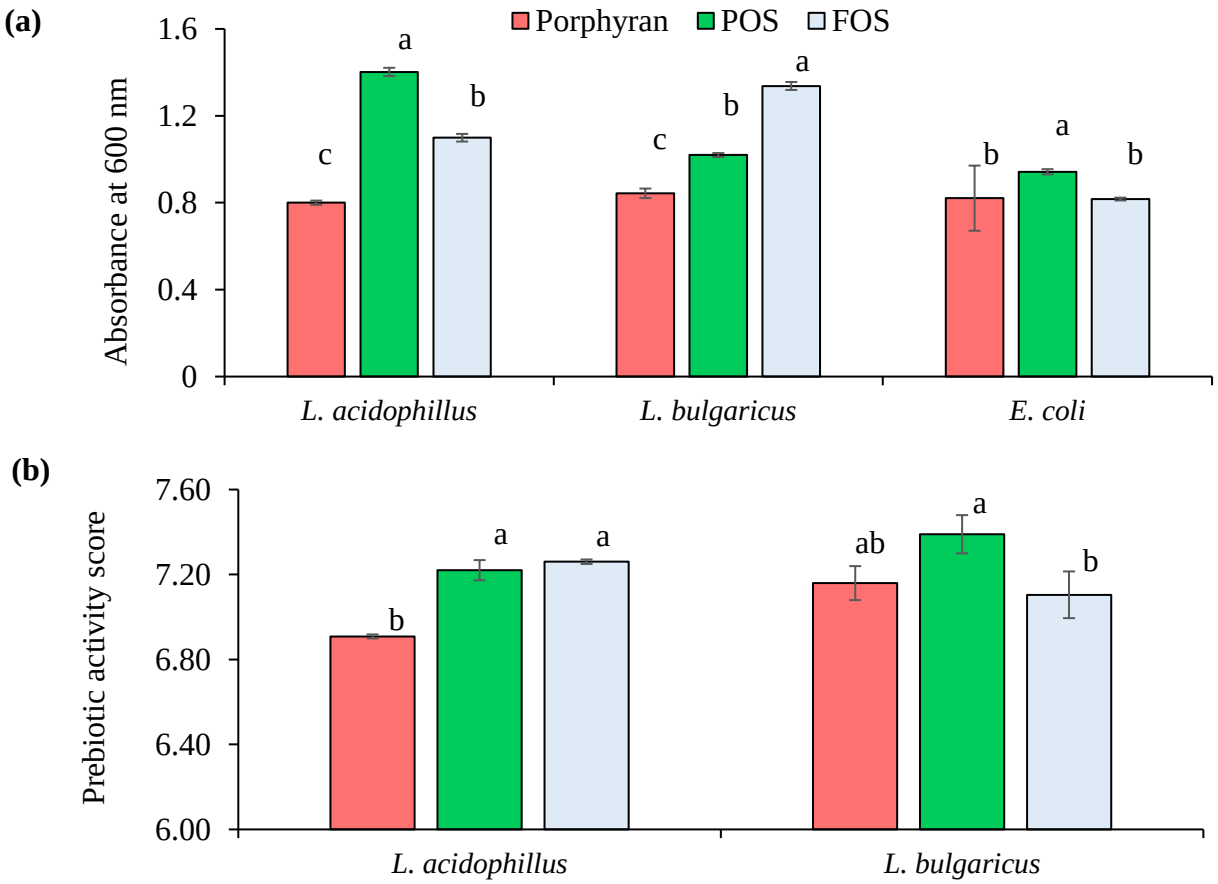


Figure 4.10 Prebiotic potential of porphyran oligosaccharides (POS) based on the **(a)** growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and **(b)** prebiotic activity score of probiotic bacteria in the presence of porphyran, porphyran oligosaccharides and fructooligosaccharides; a, b, c represents mean values within a bar with unlike superscript letters shows the significant difference ($p \leq 0.05$).

The results show that the growth of probiotic bacteria, *L. acidophilus* and *L. bulgaricus*, in the presence of porphyran, POS and FOS were significantly different (**Figure 4.10a**). The growth of *L. acidophilus* was high in POS (Abs 1.40) than in porphyran (Abs 0.8) and FOS (Abs 1.09). The POS was also able to promote the growth of *L. bulgaricus* higher than porphyran and equivalent to FOS. The prebiotic activity score of POS was calculated based on the CFU numbers obtained from probiotic and enteric bacteria growth and compared with porphyran and FOS. The results show that POS significantly promotes the growth of *L. bulgaricus* and *L. acidophilus* with a prebiotic activity score of 7.39 and 7.22, respectively, which was higher than porphyran (6.91 and 7.16) and equivalent to commercial prebiotic FOS (7.26 and 7.10). The difference in the growth of bacteria and prebiotic score in the presence of POS, porphyran and FOS was also supported by statistical analysis (**Figure 4.10b**).

Discussion

Microbial enzymes play an essential role in food and beverage processing, the pulp and paper industry, and bio-conversion applications in industries on commercial scales (Adrio and Demain, 2014). The sources of microbial enzymes include microbial communities belonging to bacteria, fungi and yeasts, which are globally used to produce economically viable enzymes for commercial applications (Zhang and Kim, 2010; Sarasan et al., 2020). Marine microbial enzymes are gaining enormous attention as they are the major source of enzymes that can hydrolyze algal biomass, including the complex algal polysaccharides like porphyran, alginate and ulvan in their cell wall (Hehemann et al., 2010). Despite numerous reports on their biomedical properties, their hydrocolloid and complex nature limits their applications. Enzymatically produced macroalgal oligosaccharides, which are hydrolyzed forms of complex polysaccharides, are gaining importance

due to their low molecular weight, biocompatibility, bioactivities, safety and solubility in water, making them a lucrative alternative. These oligosaccharides are reported for various bioactivities such as antioxidant, immunomodulation, antiglycemic, anti-inflammatory, antitumor, prebiotic, antiobesity and antimicrobial (Jagtap and Manohar, 2021). However, the major bottleneck for the production of these bioactive sugars is the identification of enzymes capable of efficiently hydrolyzing macroalgal polysaccharides into oligosaccharides.

In this chapter, the underexplored marine microbial enzymes ulvan lyase ULB1 and porphyranase PB1 to hydrolyse ulvan and porphyran have been studied. Various studies have reported the polysaccharide hydrolyzing activity of *Bacillus* sp., which showed their ability to hydrolyse agar, carrageenan and alginate, but there are no reports on ulvan hydrolyzing activity (Suzuki et al., 2003; Li et al., 2015, 2019; Chen et al., 2018). Very few studies have reported microbial porphyranase and ulvan lyase activity and enzymatic production of UOS and POS for various bioactivities. Only one recent study has reported antioxidant and angiotensin I converting enzyme inhibition activity of UOS produced using non-specific enzymes such as amylase, cellulase and xylanase (Hung et al., 2021). This is the first study, to the best of our knowledge, reporting the characterization of ulvan lyase ULB1 from a *Bacillus subtilis* associated with green macroalgae to hydrolyse ulvan into UOS at optimum conditions (Jagtap et al., 2022a).

The biochemical characterization of extracellular ulvan lyase ULB1 showed a pH optimum of 8.0 similar to the pH of seawater (Parvathi et al., 2020; Qin et al., 2020). The low range of NaCl concentration (10 - 200 mM) showed retention of 40% ulvan lyase activity. This lower concentration is due to the maximum growth of *Ulva* sp. in coastal areas with the influx of freshwater. Ulvan lyase showed the highest activity at an optimum temperature 40 °C with stability (>85%) between 25 - 35 °C temperature, other ulvan lyase were also reported to have optimum

pH and temperature of approximately 8.0 and 40 °C, respectively (Coste et al., 2015; Qin et al., 2018; Qin et al., 2020). PL24 and PL25 family ulvan lyases are reported to bind the divalent cations such as Ca^{2+} and Zn^{2+} to stabilize the enzyme (Reisky et al., 2018; Ulaganathan et al., 2018). In our study, apart from Ca^{2+} and Zn^{2+} other Li^{2+}, Mg^{2+} and Ba^{2+} also increased the activity (20%) of ulvan lyase, similar to those previously reported by Qin et al., 2020. The reducing agents β-mercaptoethanol and dithiothreitol (DTT) enhanced the activity (50%) of ulvan lyase. This may be because the DTT reacts with sugar to produce a glycosyl thiol and could shift the equilibrium of the reaction towards hydrolysis, making the ulvan lyase more effective.

Structural characterization of the UOS produced using ulvan lyase, ULB1 from MAB isolate, NIOA181 was carried out to confirm its structural composition based on FTIR, HRMS and NMR. The FTIR characterization of enzymatically produced UOS shows functional group-specific to ulvan such as -OH stretching vibration, -CH stretching vibration, -COOH asymmetric and symmetric stretching vibrations, -S=O stretching vibration band, C-O vibration associated with a -C-O-SO_3 and bending vibration of -C-O-S, were detected in UOS at wavenumber 3280 cm^{-1}, 2930 cm^{-1}, 1658 and 1417 cm^{-1}, 1172 cm^{-1}, 1080 cm^{-1} and 810 cm^{-1}, respectively. These peaks were similar to the earlier reports on UOS produced through enzymatic hydrolysis (Hung et al., 2021), which suggests that the enzymatic hydrolysis of ulvan did not remove the functional group-specific for parent polysaccharides. ^{13}C NMR and HRMS spectrometric analysis of UOS indicate that the end products of enzymatic hydrolysis of ulvan using ulvan lyase ULB1 from NIOA181 were disaccharides of ΔRha3S, trisaccharides of ΔRha3S-Xyl, ΔRha3S-Xyl-2S and tetrasaccharides of ΔRha3S-Xyl-Rha, ΔRha3S-XylΔ with 4-deoxy-L-threo-hex-4-enopyranosiduronic acid at non-reducing end, which was similar to UOS produced using broad-spectrum ulvan lyase, from *Formosa agariphila* KMM 3901T (Lahaye et al., 1997; Konasani et

al., 2018). The similarity in unsaturated (4-deoxy-L-threo-hex-4-enopyranosiduronic acid) end products and intermediate products without uronic acid composition confirms that the ulvan lyase ULB1 isolated from NIOA181 cleaves the 1, 4 linkages between Rha3S and GlcA or IdoA through endolytic cleavage (**Table 4.2**).

Table 4.2 Studies on microbial ulvan lyase and bioactivity of enzymatic hydrolysis products

Source or enzyme	**Degree of polymerization**	**Bioactivity**	**Reference**
Persicivirga ulvanivorans	DP2 - DP4	-	Collen et al. 2011
Alteromonas sp.	NA	-	Coste et al. 2015
Alteromonas sp.	DP2, DP4, DP6	-	He et al. 2017
Alteromonas sp. LOR	DP2, DP4	-	Foran et al. 2017
Pseudoalteromonas sp. PLSV	Di-, Tetra-	-	Qin et al. 2018
Formosa agariphila KMM 3901T	Di-, Tetra-	-	Reisky et al. 2018; Konasani et al. 2018
Alteromonas sp. A321	Di-, Tetra-	-	Gao et al. 2019
Alteromonas sp.	Di-, Tetra-	-	Qin et al. 2020
Glaciecola KUL10	DP2 - DP8	-	Mondal and Ohnishi 2020
Pseudomonas vesicularis MA103 and *Aeromonas salmonicida* MAEF108	NA	Antioxidant and ACE inhibition activity	Hung et al. 2021
Bacillus sp. NIOA181	Di, Tri and Tetra	Prebiotic activity	**This study**

Similar to ulvan lyase the porphyranase PB1 from isolate *Bacillus austalimaris* NIOA284 was characterized and the results show that the optimum conditions for maximum PB1 activity were at a temperature of 35 °C and pH 7. High salt concentrations in the marine water, 35.5 ppt corresponding to 1 M NaCl, decreased the enzyme affinity. However, during monsoon, the salinity is significantly lower in coastal waters with an influx of freshwater, in which *Porphyra* is reported to grow luxuriantly; this may be the reason that PB1 showed good activity at 0.4 M NaCl concentration (Pereira et al., 2006). Previous reports have shown that β-porphyranase activity was enhanced the in the presence of Ca^{2+}, Mg^{2+} and Cu^{2+}. However, our data demonstrate that the porphyranase is highly activated in the presence of Co^{2+} and it could act as a cofactor for the enzyme, indicating that the nature of the enzyme could be of metalloenzyme with the requirement of divalent cations. Porphyranase activity was significantly inhibited by EDTA; this may be due to the depletion or chelation of the cofactor required for its activity. The decrease in porphyranase activity in SDS indicates the presence of positively charged amino acids in the active site of the enzyme. Except for EDTA and SDS, the porphyranase exhibited good resistance towards inhibitors and detergents tested, which expands their applications in various industrial processes.

The enzymatic hydrolysis of porphyran led to the production of two different oligomers based on TLC analysis. These oligomers were further characterized using NMR spectroscopy and mass spectrometry. Very few studies have been carried out to explore the potential of porphyranase to hydrolyse porphyran into bioactive oligosaccharides. The NMR spectroscopy revealed that porphyranase predominately produces sulfated galactose and hybrid sulfated galactose/AG oligosaccharides. These results suggest that the enzymatic hydrolytic products were oligosaccharides of porphyran and their conformation indicates that the enzyme acted was β-

porphyranase. Previous studies on porphyranase-mediated hydrolysis products using porphyranase from *Wenyingzhuangia* sp. were different from that of PB1 (Zhang et al., 2019b; 2020).

HRMS analysis of enzymatically produced POS showed a significant peak at 475 m/z, indicating the presence of disaccharides composed of MeG and SAG, representing that the β-porphyranase cleave the β-1→4 glycosidic bond in porphyran (Correc et al., 2011; Przybylski et al., 2015). The additional peak at 365 m/z was due to the β-1→4 glycosidic cleavage between the G and AG, producing MeG and AG disaccharides. Similarly, the peak at 665 m/z represents trisaccharides units of SAG, MeG and SAG, formed due to the β-1→4 glycoside bond cleavage. The peak at 701 m/z implies that the POS is also composed of tetrasaccharides of MeG, AG, G, and SAG. This structural characterization analysis of the POS showed that the oligosaccharides are composed of di-, tri- and tetrasaccharides and the enzyme acted as β-porphyranase (**Table 4.3**).

Table 4.3 Studies on microbial porphyranase and bioactivities of enzymatic hydrolysis products

Source or enzyme	Degree of polymerization	Bioactivity	Reference
Pseudoalteromonas atlantica	PO2, PO4	NA	Correc et al. 2011; Przybylski et al. 2015
Zobellia galactanivorans$^{\varepsilon}$	PO4, PO8	NA	Hehemann et al. 2010
Bacteroides plebeius$^{\varepsilon}$	PO4, PO6	NA	Hehemann et al. 2012
Wenyingzhuangia fucanilytica$^{\varepsilon}$	PO2, PO4, PO6	NA	Zhang et al. 2019, 2020
Arthrobacter sp. S-22	PO2, PO4	Antiobesity	Osumi et al. 2002
Bacillus sp. NIOA284	PO2, PO4	Antioxidant, Prebiotic	**This study**

The bioactive potential of these enzymatically produced UOS and POS was also studied for their prebiotic activity, which was evaluated based on their capacity to increase the growth of probiotic bacteria. The growth of probiotic strains *L. acidophilus* and *L. bulgaricus* in the presence of UOS and POS, which were equivalent to FOS and higher than parent polysaccharides. The prebiotic activity tested based on their ability to suppress the growth of enteric bacteria showed that the activity of UOS and POS were equivalent to commercial prebiotic FOS and the prebiotic score UOS and POS was recorded to be higher than parent polysaccharides and FOS. These oligosaccharides with prebiotic activities are known to produce small chain fatty acids, favoring the growth of probiotics and suppressing oxidative stress caused by enteric pathogens (Hamdy et al., 2018; Guarino et al., 2020). This provides protection against colon infections and inflammation, enhances mineral absorption and lipogenesis and prevents any other gastrointestinal disorders, including colon cancer (Davani-Davari et al., 2019; Han et al., 2019). Antioxidant activity is routinely used for the evaluation of the bioactive potential of molecules to scavenge free radicals and reduce oxidative stress. This study shows the antioxidant potential of enzymatically produced POS and it showed higher radical scavenging activity than parent polysaccharide porphyran and FOS. The enzymatically produced POS showed DPPH and ABTS radical activity, similar to previous studies on enzymatically produced alginate and agar oligosaccharides (Zhu et al., 2016; Hong et al., 2017). Previous studies have shown that gut probiotic bacteria and their metabolites can regulate oxidative stress and protect the cells from oxidative damage (Lin et al., 2022). Hence, oligosaccharides with prebiotic and antioxidant activity are in great demand in the healthcare sector as they can proliferate the probiotic bacteria to boost the gastrointestinal tract and protect the cells from oxidative stress by inhibiting free radicals.

In this study, the macroalgal extracts and their polysaccharides were evaluated for their antioxidant, antiglycemic and prebiotic activities. The macroalgae-associated bacteria were isolated for their extracellular enzymes to hydrolyse macroalgal polysaccharides. Two potential bacteria which showed the highest ulvan lyase and porphyranase activity were identified and used for the enzymatic production of bioactive oligosaccharides. Hence, exploring the potential of macroalgal polysaccharides, macroalgae-associated bacteria for their extracellular enzymes for enzymatic production of oligosaccharides can increase the commercial value of the algal biomass. Based on these applications, the macroalgae collected from the wild or from aquaculture practices can pave way for its sustainable utilization and its bio-prospection which is an underexplored marine bioresource.

Chapter 5
Summary

5.1 Summary

The current generation has been greatly impacted due to lifestyle changes such as the consumption of carbohydrate-rich diets and decreased physical activity. This results in the accumulation of high glucose in the blood for a prolonged period causing hyperglycemia. Increased glucose levels in the blood lead to its autoxidation, oxidative phosphorylation and formation of chronic glycation products, all of this result in the production of excess reactive oxygen species. In an individual with a healthy physiological condition, reactive oxygen species are effectively eliminated by antioxidant enzymes produced by the defense system of the body. However, excessive reactive oxygen species production overwhelms the natural detoxification system and generates oxidative stress. The activation of cellular pathways by oxidative stress causes the onset of various chronic diseases, including diabetes, due to pancreatic β-cell dysfunction, lack of insulin production and malfunction of insulin receptors. Diabetes is one of the major chronic ailment and after its onset, the first line of treatment is to inhibit α-amylase and α-glucosidase enzyme activity. These are the primary enzymes involved in the release and absorption of glucose. Inhibiting these enzymes can significantly lower blood glucose levels. As a result, it is the most common therapeutic approach to delay glucose release and absorption. Oxidative stress also imbalances the probiotic and enteric gut microbiota, resulting in gastrointestinal disorders. Hence, preventing oxidative stress with antioxidant rich macroalgal biomolecules combined with antiglycemic and prebiotic activity could be an effective strategy to prevent dietary and metabolic disorders (**Figure 5.1**). In recent years, there has been a growing interest in natural biomolecules for their potential preventive role in the management of chronic diseases, especially those associated with oxidative stress.

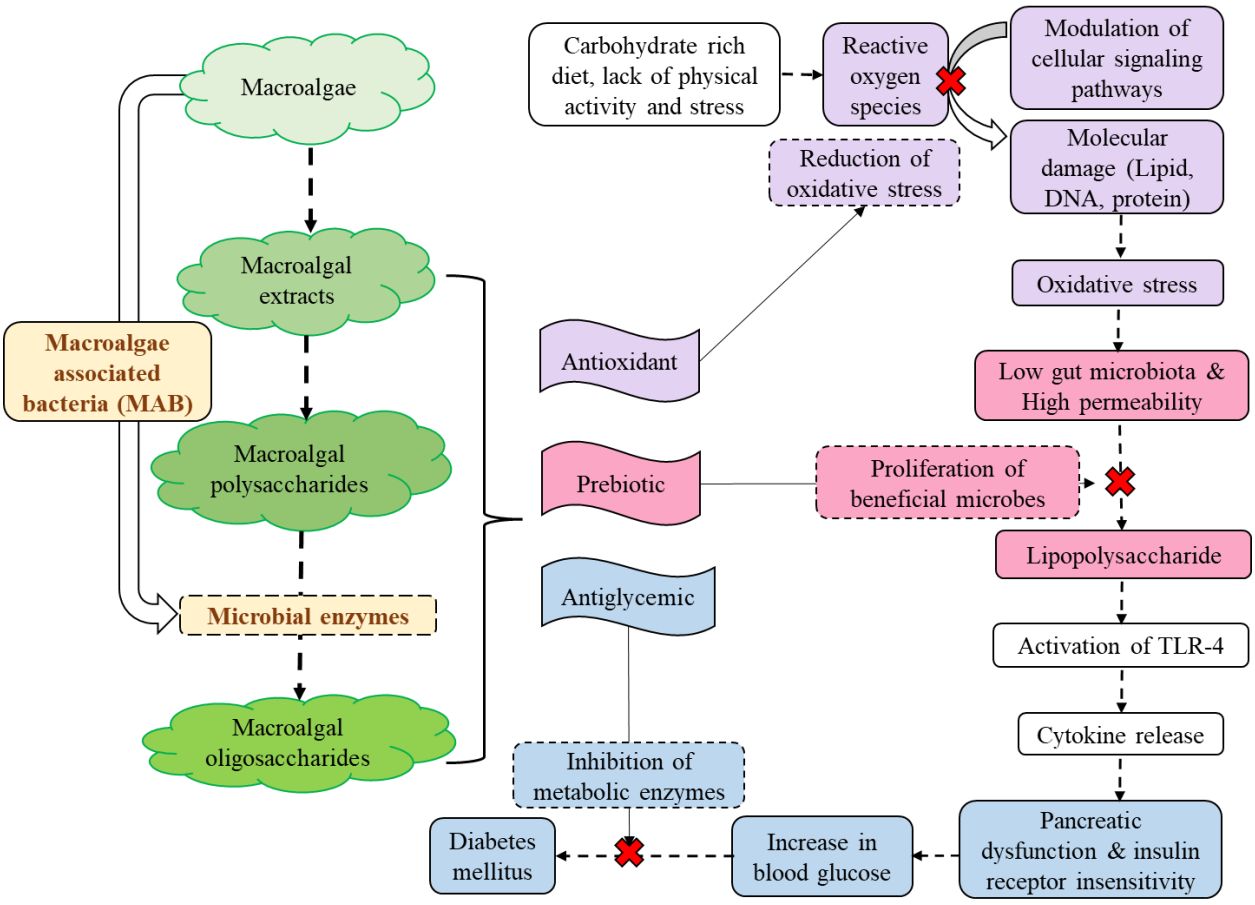


Figure 5.1 Summary of the potential of macroalgal extracts, polysaccharides, macroalgae-associated bacteria producing bioactive oligosaccharides to prevent the onset of chronic diseases.

Macroalgae are a sustainable source of various bioactive molecules, including proteins, pigments, small-chain fatty acids, metabolites and unique polysaccharides. Enzymatic hydrolysis of these unique polysaccharides into biocompatible and soluble, low molecular weight oligosaccharides has far-reaching applications. Microbial enzymes play a major role to produce specific oligosaccharides and microbes especially those associated with the marine macroalgae and benthic organisms grazing on macroalgae, are known to produce the extracellular enzymes that can break down the complex macroalgal polysaccharides into bioactive oligosaccharides. This highlights the significance of macroalgae-associated microbes as a source of enzymes capable of hydrolysing

complex polysaccharides into simple oligosaccharides with high bioactivity. In this research work, an attempt was made to study the importance of unexplored microbial enzymes in the production of biomolecules from macroalgae for healthcare applications. Field survey and experimental analysis to characterize the composition and bioactivity of unique macroalgal biomolecules carried out and the salient findings and its implications are listed below:

Salient findings and their implications:

- The fifteen macroalgae were collected from the Indian coast and processed for the preparation of aqueous extract. Among the macroalgal extracts studied, MEX14 and MEX15 of *Ulva* sp. showed the highest polysaccharide content. The brown macroalgal extracts MEX8 of *Sargassum* sp., MEX9 of *Stoechospermum* sp. and MEX10 of *Sphacelaria* sp. also showed good total phenolic content with highest radical scavenging and total antioxidant activity (Jagtap et al., 2021).
- The inhibition of α-glucosidase enzyme using antidiabetic compounds is the first line of treatment for diabetes management. Acarbose and voglibose are the major antidiabetic compounds that are commercially used. However, they have limited potency and lead to side effects like flatulence, bloating, abdominal pain or discomfort and nausea. Hence, there is a demand for natural α-glucosidase inhibitors and our results show that the MEX10 of brown algae *Sphacelaria* sp. has higher α-glucosidase inhibition activity than acarbose even at a very low concentration.
- The polysaccharides from macroalgae have high dietary fibre content, this property prevents them from being readily digested by the pancreatic enzymes and hence they reach the gastrointestinal tract intact and promote the growth of probiotic bacteria. The antioxidant potential of the MPS8 of *Sargassum* sp., MPS9 of *Stoechospermum* and MPS10 of *Sphacelaria*

sp. showed that they were able to scavenge DPPH, ABTS and H_2O_2 radicals. These potential MPS also showed α-glucosidase inhibition activity at tested concentrations. The prebiotic activity results showed that the MPS10, MPS14 and MPS15 aided in the proliferation of probiotic bacteria and suppressed the growth of enteric bacteria. These potential polysaccharides with no toxic effect on normal cell lines have potential to be used in functional food applications.

- Approximately 421 MABs were studied for their ability to use model polysaccharides representing all three macroalgal groups and qualitatively analysed for their enzyme activity. Only about 5 - 15% of the 421 MABs tested were found to have the ability to produce specific enzymes to hydrolyze porphyran, alginate, and ulvan (Jagtap et al., 2022b).
- Among the isolates tested, about 5% (19 isolates) of the MABs had very good porphyranase activity and only 4% (17 isolates) had very good alginate lyase activity. Due to the heteropolysaccharide nature of ulvan, the majority of isolates tested (96%) did not show ulvan lyase activity and only 2% (7 isolates) showed very good ulvan lyase activity.
- Phylogenetic analysis of the potential polysaccharide hydrolysing MABs using the Maximum likelihood algorithm revealed that these potential MABs could be classified as Firmicutes (68%), Proteobacteria (26%), and Actinobacteria (5%). Isolates belonging to *Bacillus* genera exhibited the maximum ability to hydrolyse the macroalgal polysaccharides tested.
- The partial 16S rRNA gene sequence of the 19 potential MABs, which showed production of porphyranase, alginate lyase and ulvan lyase are deposited in the NCBI GenBank database. From this, NIOA181 isolated from *Ulva* sp. showed the highest ulvan lyase activity, it was identified as *Bacillus subtilis*. The isolate NIOA284 isolated from the surface of *Porphyra* sp. showed the highest porphyranase activity, it was identified as *Bacillus australimaris*. These

potential isolates are kept in “Safe Deposit” at the National Collection of Industrial Microorganisms (NCIM), a national facility at CSIR-NCL, Pune, India.

- Enzymatic hydrolysis of macroalgal polysaccharides requires the use of specific enzymes capable of cleaving the glycosidic bond for the production of oligosaccharides.
- The novel microbial enzymes, ulvan lyase ULB1 from NIOA181 and porphyranase PB1 from NIOA284 isolated from this study were characterised, to optimise their ability to hydrolyse ulvan and porphyran. The effect of temperature, pH, NaCl concentration, metal ions, and inhibitors on the activity of ulvan lyase ULB1 and porphyranase PB1 was also studied.
- Ulvan lyase, ULB1 showed maximum activity at an optimum pH of 8.0 and temperature of 40 °C in the presence of Mg^{2+} and β-mercaptoethanol. The porphyranase, PB1 showed the highest activity at optimum pH 7.0, temperature 35 °C, at 0.4 M NaCl concentration and Co^{2+} ion was shown to act as a cofactor (Jagtap et al., 2022b).
- Ulvan was hydrolysed using ulvan lyase ULB1 isolated from *Bacillus subtilis* NIOA181 and their hydrolysis pattern showed the production of ulvan oligosaccharides (UOS) with two oligomers with different degrees of polymerization.
- Similarly, porphyran was extracted from *Porphyra* sp. and subject to enzymatic hydrolysis using porphyranase PB1 isolated and purified from *Bacillus australimaris* NIOA284 at optimum reaction conditions to produce porphyran oligosaccharides (POS).
- These enzymatically produced UOS and POS were further characterized using NMR spectroscopy and HRMS spectrometry to elucidate their chemical composition and structure. UOS showed the production of di, tri and tetrasaccharides with conserved functional groups of the parent ulvan. Structural and chemical analysis of POS showed that the enzyme acted as β-porphyranase and produced di, tri and tetrasaccharides.

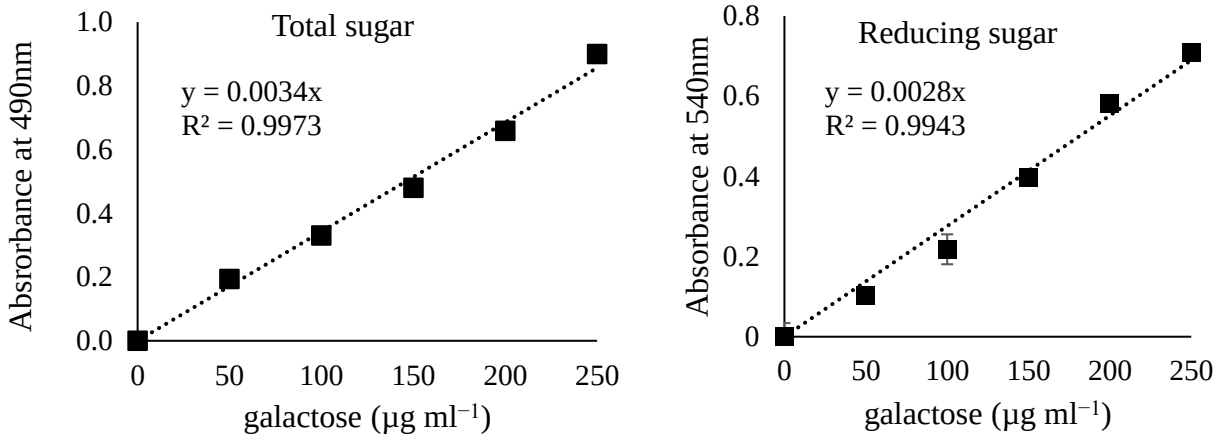
- The enzymatically produced and characterised UOS and POS exhibited radical scavenging activity and prebiotic activity equivalent or higher than the polysaccharides and commercially available fructooligosaccharides of terrestrial origin. These oligosaccharides with antioxidant and prebiotic activity have the potential commercial application in the healthcare sector.

5.2 Conclusion and future prospectus

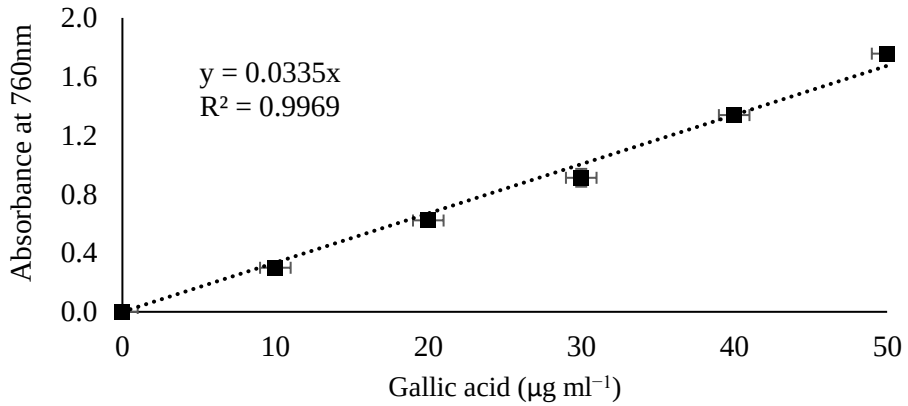
The global requirement for functional foods is increasing progressively and macroalgae are the major bioresource that can be utilized for multiple applications. Macroalgae representing all the major groups were studied and their aqueous extracts exhibited diverse bioactivities that can be used as an additive in various formulations to delay and prevent the onset of chronic diseases such as diabetes. The macroalgal polysaccharides with their high dietary fiber content with antioxidant, antiglycemic and prebiotic potential can be used as an active pharmaceutical ingredient. However, enzymatic hydrolysis of these polysaccharides into oligosaccharides can greatly improve the bioutilisation efficiency of algal products. Hence there is a great demand for the identification of efficient microbial enzymes for the production of stereospecific, bioactive oligosaccharides. The significant contribution of this study is the identification of novel MABs producing specific enzymes for the production of UOS and POS with bioactivity. These algal oligosaccharides with good bioactivity can be efficiently used as it provides protection against colon infections and inflammation, enhances mineral absorption and lipogenesis and prevents any other gastrointestinal disorders, including colon cancer. To maximize the production of oligosaccharides from macroalgae using these enzymes, the best-recommended approach is to transform the genes from the potential MABs into *Escherichia coli*. It is a major challenge to transform the complete gene complex for the production of these specific enzymes using recombinant technology. This could

be attempted as a continuation of this study and it can pave way for wider industrial applications of these enzymes. It can increase the commercial value of the macroalgal biomass and its bio-prospection which is an underexplored Blue Economy bioresource.

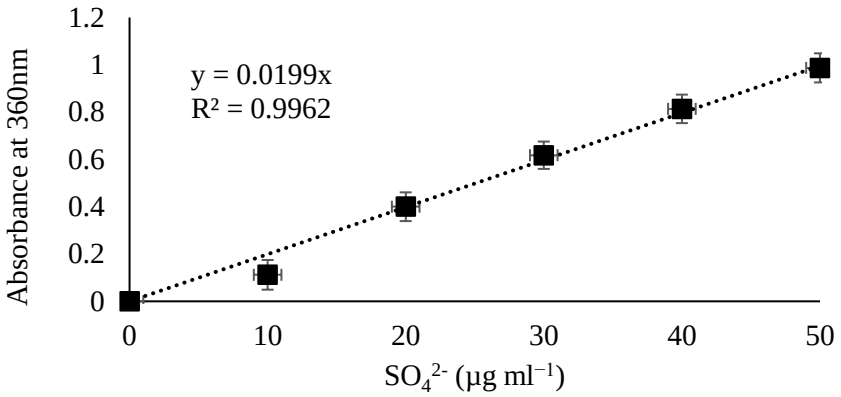
Appendix



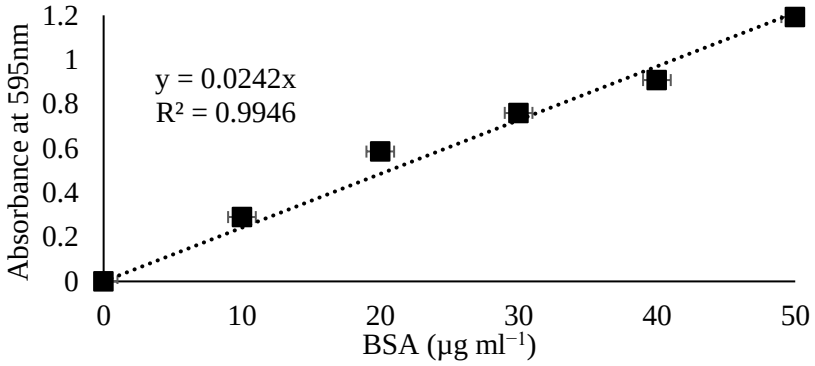
Appendix 2.1 Standard curve of galactose for determination of total and reducing sugar in macroalgal extracts and polysaccharides by Phenol-sulfuric acid and 3, 5 dinitrosalicylic method, respectively.



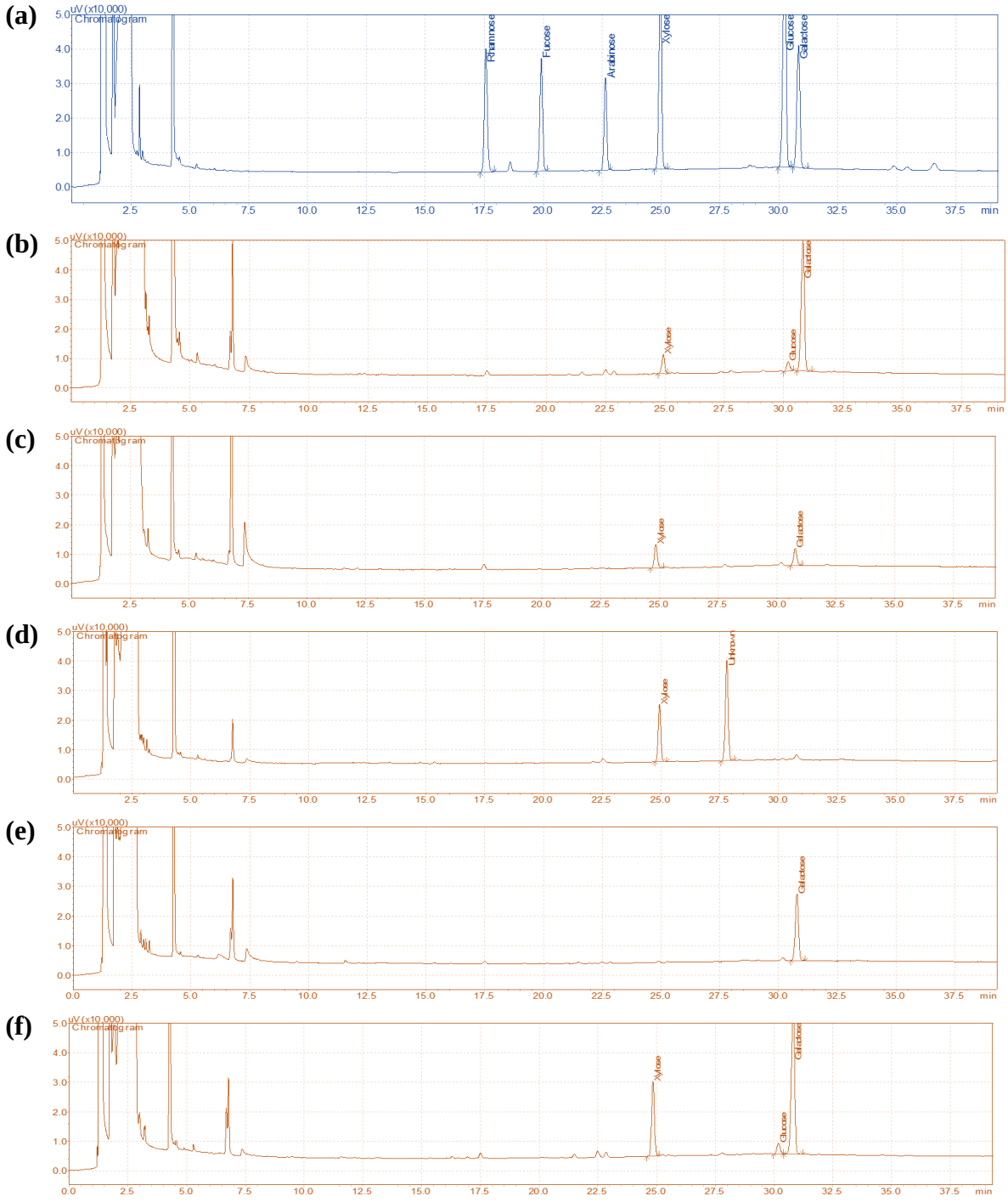
Appendix 2.2 Standard curve of gallic acid for determination of total phenolic content in seaweed polysaccharides by Folin-Ciocalteu method.



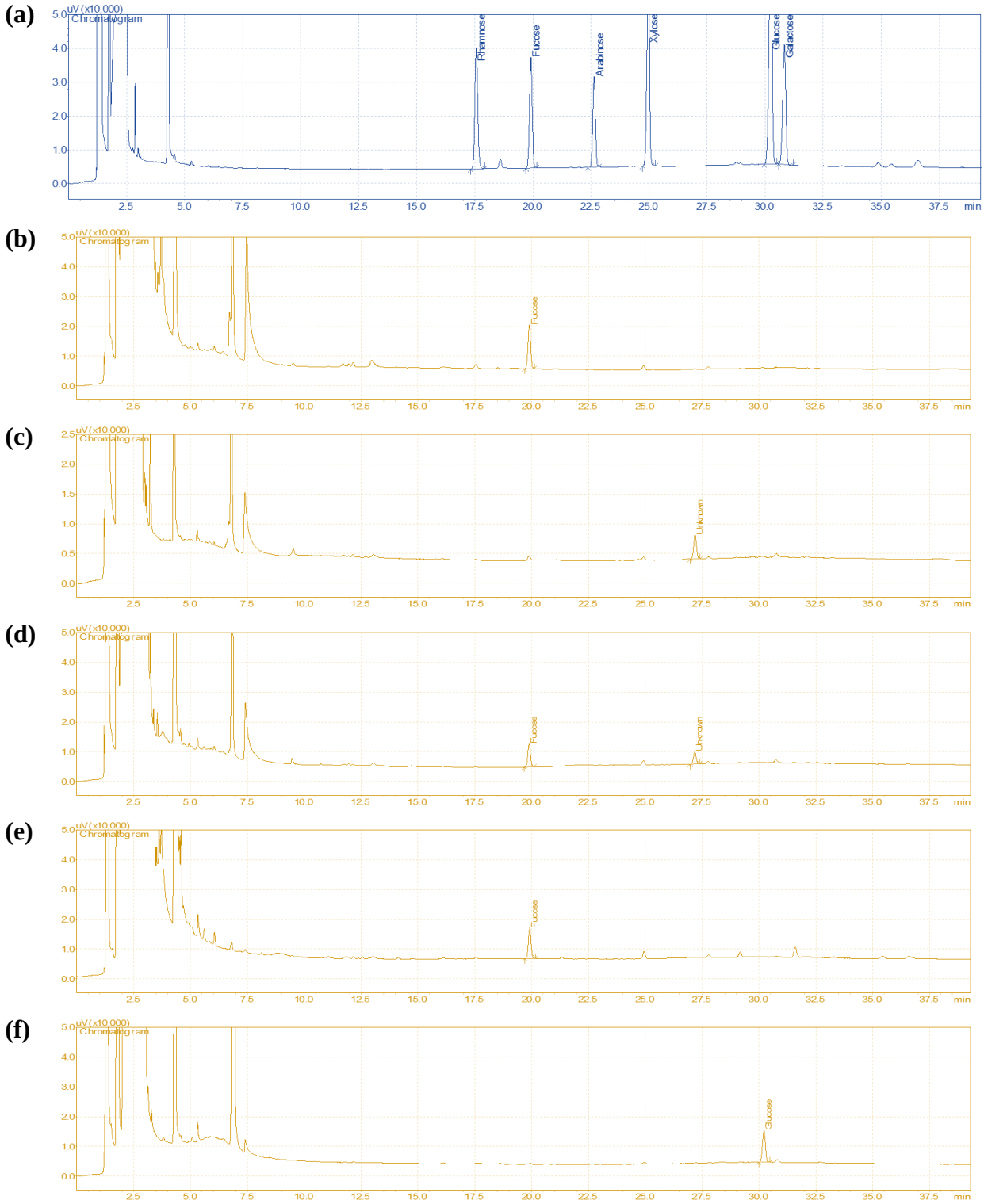
Appendix 2.3 Standard curve of potassium sulfate to determine sulfate content in macroalgal polysaccharides by $BaCl_2$-gelatin turbidity method.



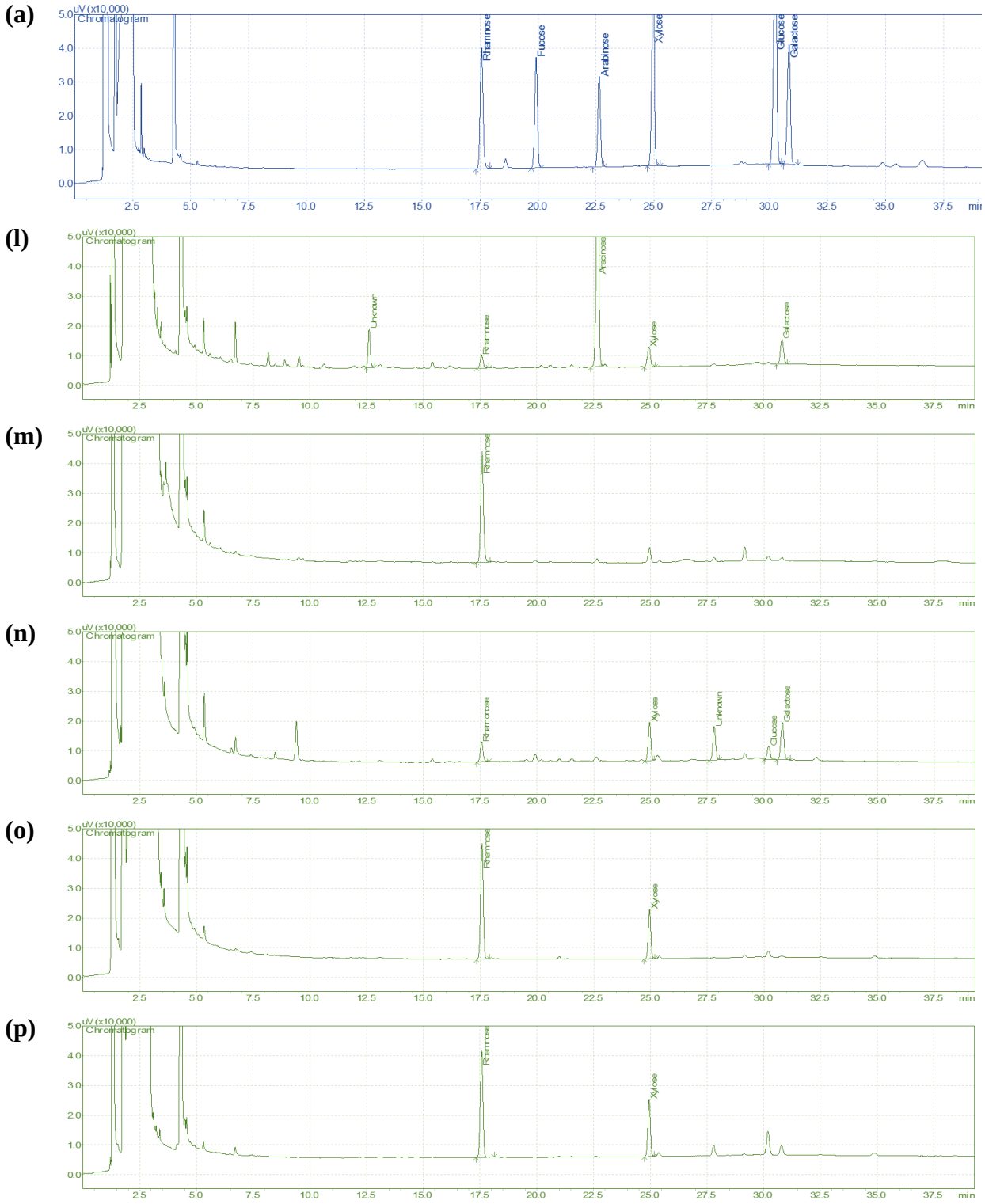
Appendix 2.4 Standard curve of Bovine serum albumin for determination of protein content in seaweed polysaccharides by Bradford method.



Appendix 2.5 Gas chromatogram of standard sugars **(a)** and MPS1 **(b)** MPS2 **(c)** MPS3 **(d)** MPS4 **(e)** and MPS5 **(f)** of red macroalgae.



Appendix 2.6 Gas chromatogram of standard sugars **(a)** and MPS6 **(b)** MPS7 **(c)** MPS8 **(d)** MPS9 **(e)** and MPS10 **(f)** of brown macroalgae.



Appendix 2.7 Gas chromatogram of standard sugars **(a)** and MPS11 **(b)** MPS12 **(c)** MPS13 **(d)** MPS14 **(e)** and MPS15 **(f)** of green macroalgae.

Appendix 2.8 Pearson correlation between polysaccharide content (PC), total phenolic content (TPC) with the antioxidant and antiglycemic activity.

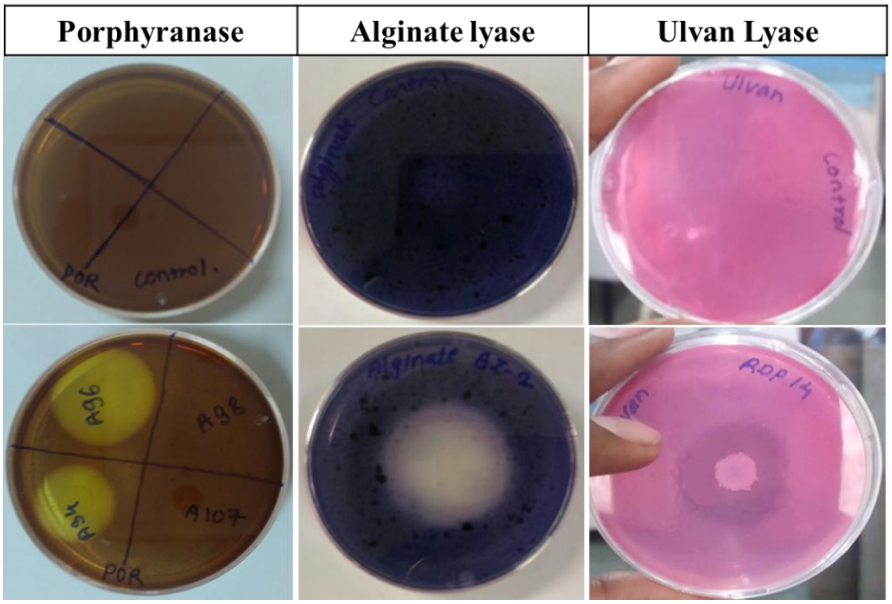
	PC	TPC	DPPH	ABTS	H_2O_2	TAC
TPC	-0.220					
DPPH	0.226	0.668[b]				
ABTS	-0.106	0.829[c]	0.656[a]			
H_2O_2	0.225	0.728[b]	0.755[b]	0.686[b]		
TAC	-0.043	0.774[c]	0.743[b]	0.575[a]	0.822[c]	
αGI	0.004	0.797[c]	0.784[c]	0.652[a]	0.850[c]	0.960[c]

[a]moderately significant at $P < 0.05$; [b]significant at $P < 0.01$; [c]highly significant at $P < 0.001$, PC; polysaccharide content, TPC; total phenolic content, TAC; total antioxidant capacity, αGI; α-glucosidase inhibition

Appendix 2.9 Pearson correlation coefficient between the biochemical composition of macroalgal polysaccharides and bioactivity.

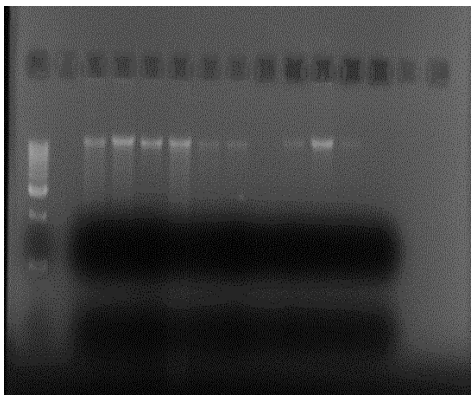
	PC	TDF	SC	NS	DPPH	ABTS	H_2O_2	TAC
TDF	-0.308[*]							
SC	-0.338[*]	0.690[**]						
NS	0.498[**]	-0.246	-0.384[**]					
DPPH	-0.239	-0.026	-0.243	-.045				
ABTS	-0.476[**]	-0.119	-0.118	-.113	.423[**]			
H_2O_2	0.184	0.107	-0.202	.003	.484[**]	-.097		
TAC	-0.165	-0.146	-0.301[*]	.098	.880[**]	.620[**]	.488[**]	
αGI	-0.101	-0.024	-0.202	.002	.327[*]	.137	.803[**]	.434[**]

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). PC; polysaccharide content, TDF; total dietary fibers, SC; sulfate content, NS; neutral sugar, TAC; total antioxidant capacity, αGI; α-glucosidase inhibition

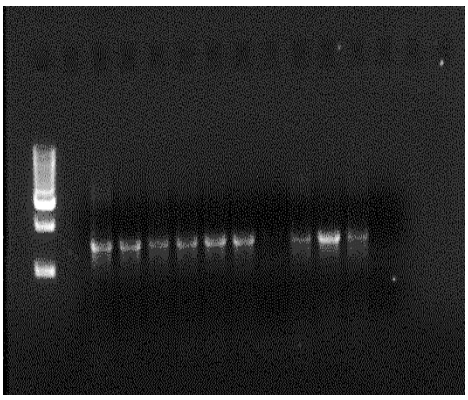


Appendix 3.1 Representative plates of the qualitative assay to determine the porphyranase, alginate lyase and ulvan lyase activity in macroalgae-associated bacteria.

(a)



(b)



Appendix 3.2 Agarose gel electrophoresis of **(a)** genomic DNA from potential MABs and **(b)** PCR amplified product.

Appendix 3.3 Counts of MABs isolated from red, brown and green macroalgae collected from major macroalgal beds of India

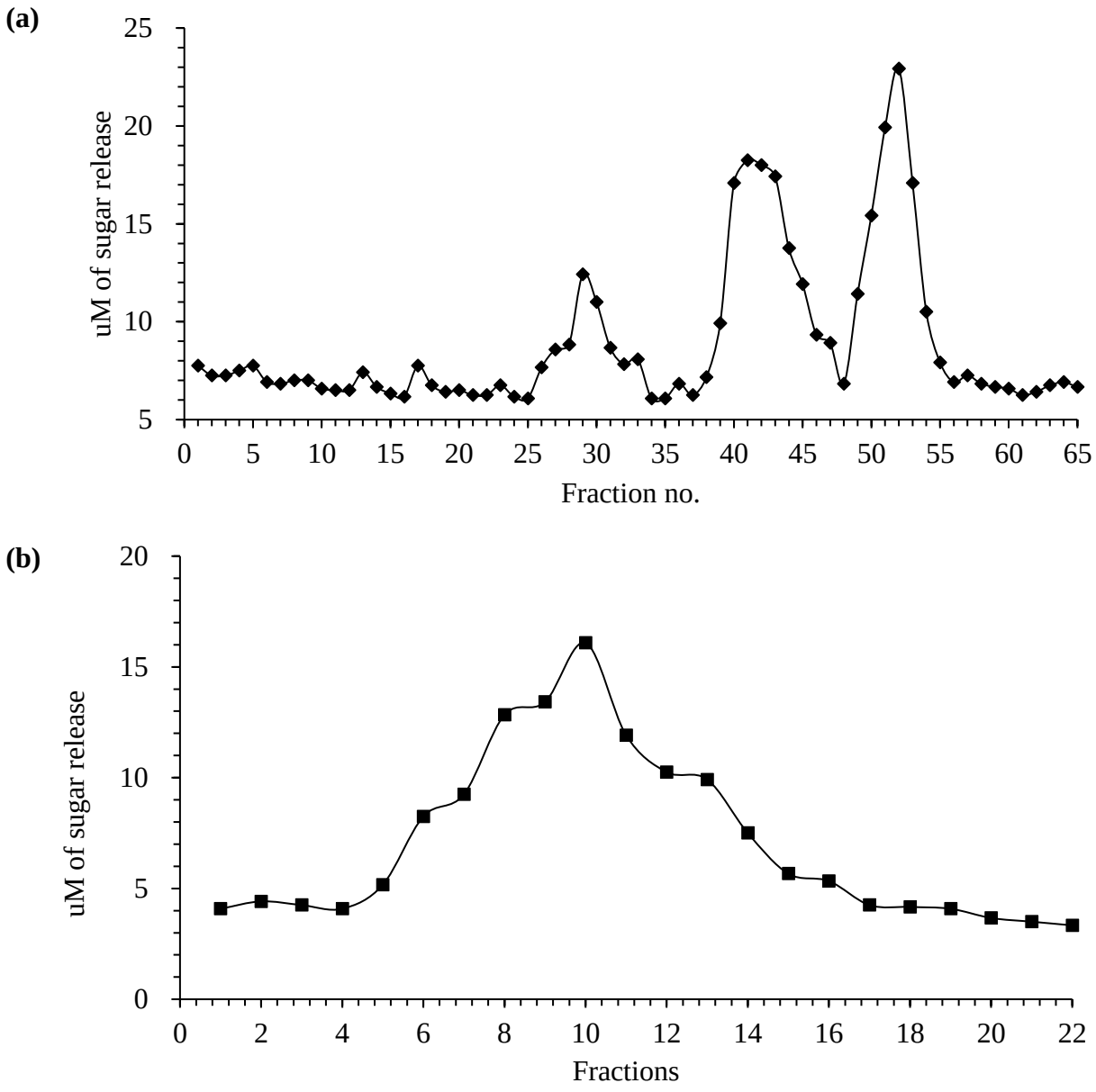
Sampling	Gujarat		Maharashtra		Goa			Tamil Nadu	Total
locations	Okha	Porbandar	Kunkeshwar	Malvan	Vagator	Dona Paula	Palolem	Mandapam	
Red macroalgae	**12**	**6**	**7**	**7**	**67**	**40**	**3**	**10**	**152**
Brown macroalgae	**14**	**4**	**9**	**11**	**32**	**55**	**14**	**12**	**151**
Green macroalgae	**10**	**14**	**7**	**6**	**42**	**33**	**4**	**2**	**118**
Total number of bacteria isolated									**421**

Appendix 3.4 Qualitative analysis of potential MAB on substrate plate assay (Zone of clearance)

Strains	Zone of clearance (mm)		
	Porphyranase	**Alginate lyase**	**Ulvan lyase**
NIOA29	22.0	21.0	0
NIOA34	25.0	18.0	0
NIOA96	26.0	20.0	0
NIOA118	26.0	20.0	0
NIOA169	24.0	22.0	0
NIOA174	26.0	15.0	0
NIOA181	20.0	16.0	25.0
NIOA183	21.0	20.0	21.0
NIOA284	32.0	25.0	0
NIOA302	22.0	24.0	21.0
NIOA319	21.0	20.0	0
NIOA321	20.0	20.0	0
NIOA323	24.0	26.0	20.0
NIOA327	21.0	20.0	23.0
NIOA328	23.0	26.0	23.0
NIOA329	20.0	21.0	0
NIOA354	21.0	21.0	14.0
NIOA379	21.0	20.0	21.0
NIOA388	20.0	23.0	0

Appendix 3.5 Molecular identification of MABs with potential to hydrolyze algal polysaccharides based on 16S rRNA gene sequence

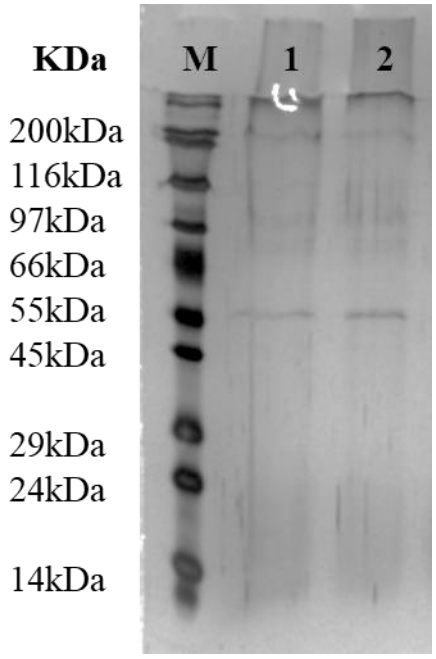
Isolate code	GenBank Accession no.	Nearest identified taxa (Accession number) in NCBI GenBank database	% identity
NIOA29	MZ295244	*Bacillus anthracis* (KC790242)	94
NIOA34	MZ295245	*Bacillus salmalaya* (KM051837)	96
NIOA96	MZ295247	*Exiguobacetrium profundum* (KM215140)	92
NIOA118	MZ295248	*Staphylococcus aureus* (MW175708)	99
NIOA169	MZ295249	*Vibrio harveyi* (MG719991)	99
NIOA174	MZ295250	*Staphylococcus sciuri* (MT550814)	99
NIOA181	MW487389	*Bacillus subtilis* (MT110998)	96
NIOA183	MZ295251	*Bacillus cereus* (DQ289055)	99
NIOA284	ON287193	*Bacillus australimaris* (MN077148)	99
NIOA302	MZ295252	*Exiguobacetrium profundum* (KM215140)	99
NIOA319	MZ295253	*Pseudomonas stutzeri* (MT356167)	99
NIOA321	MZ295254	*Bacterium strain* (MH151240)	95
NIOA323	MW487391	*Bacillus tequilensis* (MG651220)	99
NIOA327	MZ295255	*Marinobacter* sp. (KY628816)	99
NIOA328	MW487393	*Acinetobacter junii* (MT613873)	100
NIOA329	MZ295256	*Bacillus subtilis* (KX453895)	100
NIOA354	MW487394	*Micrococcus luteus* (MT533935)	100
NIOA379	MW487395	*Oceanobacillus iheyensis* (MN880490)	99
NIOA388	MZ295257	*Bacillus subtilis* (KX453895)	99



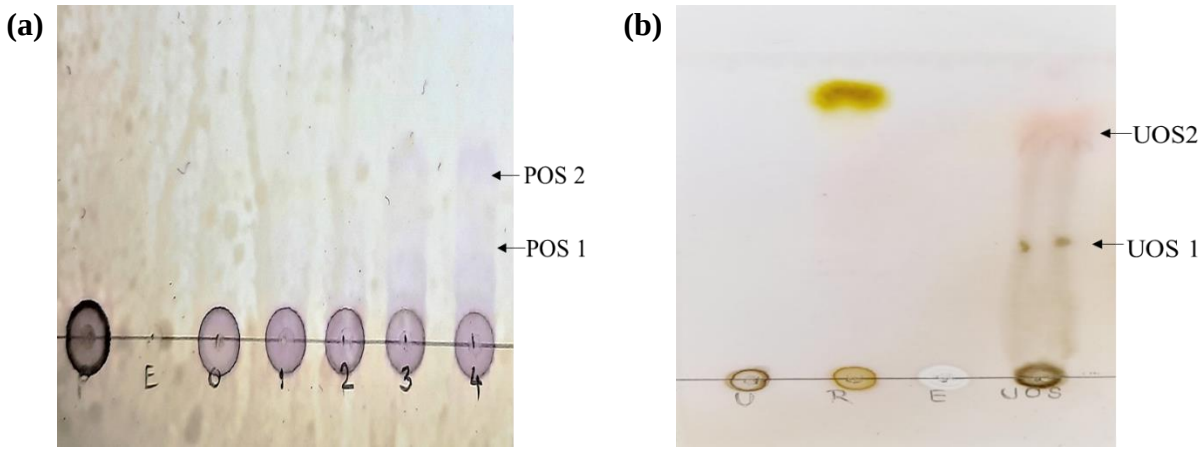
Appendix 4.1 Purification of porphyranase through **(a)** ion exchange chromatography using DEAE sephadex column and elution with NaCl gradient (0, 0.2, 0.4, 0.6, 0.8 and 1 M) and **(b)** Size exclusive chromatography using superdex 75 column eluted with sodium phosphate buffer (pH 7.0, 50 mM).

Appendix 4.2 Purification of porphyranase PB1 from *Bacillus* sp. NIOA284.

Purification Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	% Yield
Culture supernatant	250	49.7	9335.0	188	1.0	100
$(NH_4)_2SO_4$ precipitation	10	5.0	1851.1	368	1.95	19.8
Ion exchange	5	1.3	719.7	562	2.98	7.7
Gel permeation	1	0.1	198.3	1983	10.54	2.1



Appendix 4.3 Analysis of Porphyranase by SDS-PAGE. Lane M: protein marker; Lane 1 Enzyme after ion exchange chromatography; Lane 2: Enzyme after gel permeation chromatography.



Appendix 4.4 Thin layer chromatography analysis enzymatic production of **(a)** ulvan oligosaccharides (U; ulvan substrate, R; rhamnose sugar, E; ulvan lyase, UOS; ulvan oligosaccharides) and **(b)** porphyran oligosaccharides (P; porphyran substrate, E; porphyranase, 0=0hr; 1=3hr; 2=6hr; 3=9hr ;4=12hr time interval).

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Ocean Society of India (OSI) Kochi (Regd.) and Centre for Marine Living Resources and Ecology (CMLRE), Kochi

CERTIFICATE

This is to certify that Mr/~~Ms~~/~~Dr~~ ASHOK SHIVAJI JAGTAP, NIO

~~Participated~~ / Presented a paper (~~Invited~~ / Oral / ~~Poster~~) during the 6th Biennial Conference of Ocean Society of India (OSICON-19): Indian Ocean Processes and Resources- A Key to Blue Economy held at Centre for Marine Living Resources and Ecology (CMLRE), Kochi, during December 12 -14, 2019.

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General Convener
OSICON-19

Dr M Sudhakar
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Rituma Pal		
Convener	**President, PCRF**	**HOD, Dept. of Botany, C.U.**






सीएसआईआर एकीकृत कौशल विकास पहल
CSIR Integrated Skill Development Initiative
समुद्री शैवाल कृषि एवं प्रसंस्करण प्रौद्योगिकी (सी-सीपीटी)
Seaweed Cultivation and Processing Technology (SEA-CPT)

सीएसआईआर-केंद्रीय नमक व समुद्री रसायन अनुसंधान संस्थान
CSIR-Central Salt and Marine Chemicals Research Institute
फ़रवरी / February 24 – 26, 2021

सहभागिता प्रमाणपत्र / Certificate of Participation

प्रमाणित किया जाता है कि

जगताप अशोक शिवाजी

ने २४-२६ फ़रवरी, २०२१ के दौरान सीएसआईआर-सीएसएमसीआरआई, भावनगर, गुजरात में आयोजित
"सीएसआईआर एकीकृत कौशल विकास पहल और जिज्ञासा कार्यक्रम"
योजना के तहत प्रशिक्षण कार्यक्रम में भाग लिया और समुद्री शैवाल संवर्धन और प्रसंस्करण प्रोद्योगिकी (सी-सीपीटी) मे प्रमाण पत्र पाठ्यक्रम को सफलतापूर्वक पूरा किया

Jagtap Ashok Shivaji

has participated in the training programme under the scheme
"CSIR Integrated Skill Development Initiative and Jigyasa Programme"
and has successfully completed the course and entitles for the certificate
in Seaweed Cultivation and Processing Technology (SEA-CPT) during 24-26 February, 2021 held at CSIR-CSMCRI, Bhavnagar, Gujarat

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(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)

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वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद् (सीएसआईआर) द्वारा

"सीएसआईआर-एकीकृत कौशल पहल कार्यक्रम" के अंतर्गत

सीएसआईआर-केंद्रीय नमक व समुद्री रसायन अनुसंधान संस्थान, भावनगर द्वारा

सीएसआईआर-समुद्री शैवाल अनुसंधान स्टेशन, मंडपम कैंप, तमिलनाडु में

दिनांक ..... से ..... तक आयोजित

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प्रशिक्षण कार्यक्रम में भाग लिया और सफलतापूर्वक पूर्ण किया.

CERTIFICATE

This is to certify that Jagtap Ashok shivaji daughter/son of Mr Shivaji chintamani Jagtap has participated and successfully completed training program on "Seaweed Cultivation and Processing Technology", from 08-03-2021 to 10-03-2021 organized by CSIR-CSMCRI, Bhavnagar at CSMCRI - Marine Algal Research Station, Mandapam Camp, Tamil Nadu under the scheme "CSIR-Integrated Skill Initiative Program" a national program on skill development initiated by the Council of Scientific & Industrial Research (CSIR).

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Marine Biotechnology (2021) 23:159–176
https://doi.org/10.1007/s10126-021-10027-6

REVIEW

Overview on Microbial Enzymatic Production of Algal Oligosaccharides for Nutraceutical Applications

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Received: 17 November 2020 / Accepted: 15 February 2021 / Published online: 24 March 2021

Abstract

Global requirement for algal foods is increasing, as they are progressively consumed for its nutrition and health. Macroalgae is a proven source of metabolites, proteins, pigments, bioactive compounds, and algal polysaccharides. The unique polysaccharides such as agar, carrageenan, porphyran, alginate, fucoidan, laminarin, and ulvan are known for its wide range of bioactivities and extensively used for applications from tissue engineering to drug delivery. However, there are few limitations due to its high molecular size, low compatibility, and hydrocolloid nature. Hence, the enzymatically produced algal oligosaccharides have drawn tremendous attention due to its green synthesis, solubility, and lower molecular size. They are reported to have bioactivities including antioxidant, antiglycemic, immunostimulatory, anti-inflammatory, and prebiotic activities, which can be used in the healthcare and nutraceutical industry for the manufacture of functional foods and dietary supplements. However, identification of potential microorganisms, producing polysaccharide hydrolyzing enzymes, remains a major bottle neck for efficient utilization of bioactive algal oligosaccharides. This review summarizes the recent developments in the identification and characterization of microbial enzymes for the production of bioactive algal oligosaccharides. This can improve our understanding of bioactive algal oligosaccharides and pave way for efficient utilization of macroalgae to prevent various chronic diseases.

Keywords Macroalgae · Microbial enzymes · Algal oligosaccharides · Nutraceutical applications

Introduction

Macroalgae, also known as seaweeds, are primitive, multicellular, aquatic plants. They usually occur along the coasts in the intertidal, sublittoral-to-littoral zones and grow on rocks or other hard substrata where photosynthetic light is available. They are a sustainable source of various bioactive molecules, which has diverse health benefits and wide applications as pharmaceutical and nutraceutical compounds (Holdt and Kraan 2011; Tanna and Mishra 2018, 2019). Their unique polysaccharide, pigment, and polyphenolic content are largely known to be responsible for its bioactivity. Macroalgae is well accepted as a marine vegetable and consumed regularly in many South East Asian countries. Studies show that regular consumption of macroalgae can reduce the occurrence of chronic diseases including cancer, obesity, and diabetes and it also reduces the mortality rate (Tanna and Mishra 2018; Nunes et al. 2020). However, the bioactive potential of macroalgae are not fully explored for its nutraceutical applications though there is a vast diversity, of about 7500 of red, 2000 of brown, and 1800 of green macroalgal species across the globe (Keith et al. 2014). Macroalgae does not require arable land, freshwater, or fertilizers for its cultivation. They play a significant ecological role through carbon fixation and can sequester higher levels of carbon dioxide per unit area than terrestrial plants and microalgae (Mongin et al. 2016). The ability of these ecosystems to reduce the carbon foot print is widely recognized as an important process for mitigating the effects of climate change. Large-scale cultivation of macroalgae can also contribute to the welfare of the community and associated industries by providing jobs and employment. This can improve the economic status of the coastal communities, fishermen, and rural population by developing alternate employment opportunities. However, the benefits of

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this important, renewable, and sustainable bioresource for generation of "Blue Economy" are not efficiently utilized (Buschmann et al. 2017).

Algal Polysaccharides and Its Limitations for Nutraceutical Applications

Macroalgae is classified into three major groups: red algae (Rhodophyta), brown algae (Phaeophyta), and green algae (Chlorophyta) based on their pigment and polysaccharide composition (Keith et al. 2014). Red algae are phylogenetically the oldest division and have the largest number of species. Cell wall of red algae is mainly composed of sulfated galactan such as agar, carrageenan, and porphyran (Surget et al. 2017). Brown algae, which is the second largest macroalgal group, is rich in phytochemicals, polysaccharides, soluble fibre, and iodine and is known to have enormous bioactive potential (Baghel et al. 2020). They are mainly composed of three unique polysaccharides: alginate, fucoidan, and laminarin. Among the macroalgae, green algae are the least explored division of macroalgae for their bioactive potential and in food application (Surget et al. 2017). The green algae of genera *Ulva* and *Enteromorpha* are mainly composed of sulfated polysaccharide ulvan. Ulvan is a polydisperse hetero-polysaccharide, with rare sugars such as iduronic, glucuronic, and sulfated rhamnose. The high molecular weight of ulvan, which is approximately 189 to 8200 kDa, limits their applications in food and pharmaceutical industries. Though, it is reported to have anti-influenza, hepatotoxic, antioxidant, and anti-proliferative property (Lahaye and Robic 2007; Kidgell et al. 2019).

Algal polysaccharides from various macroalgae have enormous potential to be used as dietary fibres, hydrocolloids, anticancer, anticoagulant products, drug delivery, and tissue engineering applications (Singh et al. 2011; Tanna and Mishra 2019). However, their utility in nutraceutical and functional food industries is restricted due to their high molecular weight, low biocompatibility, and water solubility properties (Lovegrove et al. 2017; Gopinath et al. 2018). Hydrolysis of these polysaccharides lead to the production of low molecular weight, biocompatible, and soluble oligosaccharides, which has far reaching applications in health care industries than the parent polysaccharide (Charoensiddhi et al. 2017; Cheong et al. 2018).

Complex polysaccharides of macroalgae can be hydrolyzed into algal oligosaccharides (AOS) either by chemical or through enzymatic processes. In chemical hydrolysis, polysaccharides are treated with concentrated acid and heated at high temperature for several hours to get the final product (Lee and Lee 2016). This leads to the production of monomers and undesirable toxic by-products such as furfural, hydroxymethyl furfural, and high amounts of reducing sugars like glucose, xylose, arabinose, and galactose along with the oligosaccharides, which reduces the purity of oligosaccharides. These undesirable by-products limit the application of AOS in functional food and nutraceutical applications (Wang et al. 2019). On the contrary, during enzymatic hydrolysis, the polysaccharides are broken down by specific enzymes that cleave or hydrolyze the glycosidic bonds to produce stereo specific oligosaccharides (Xu et al. 2018a, b). The AOS produced through enzymatic hydrolysis are also shown to possess superior nutraceutical and health care applications than parent polysaccharide (Cheong et al. 2018). Hence, polysaccharide hydrolysis using microbial enzymes is the best alternative approach to produce bioactive oligosaccharides for nutraceutical applications. However, identification of novel microbial enzymes is the bottle neck for efficient bioutilization of the unique complex algal polysaccharides.

Microbes especially those associated with the marine macroalgae and benthic organisms grazing on seaweeds are known to produce extracellular enzymes to break down the complex algal polysaccharides into oligosaccharides. An understanding of the microbial enzymes capable of converting algal polysaccharides to AOS through enzymatic hydrolysis is vital for efficient bio-prospection of macroalgae which is an underexplored marine bioresource.

Microbial Enzymes for the Production of Algal Oligosaccharides

Research on the marine microorganisms, its physiology, and adaptations has contributed for the growth of the biotechnology sector. Microbes are universally distributed, and they are recognized as the most potential reservoir of diverse enzymes. The use of microbial enzymes in food fermentation is an age-old process, and fermentation are still applied in many food preparations. The robust nature of microbial enzymes such as stability and tolerance to varied range of pH and temperature, multi-functionality, economic feasibility, consistency, easy product modification, and optimization makes them potential candidate for food industries than plant and animal-based enzymes (Zhang and Kim 2010; Moreno et al. 2017). Microbial enzymes such as amylase, protease, lipases, and xylanase from bacteria and fungi are widely used in various food applications to improve taste and texture of food (Raveendran et al. 2018).

Microbial enzymes involved in the hydrolysis or cleavage of the complex and unique algal polysaccharides can be grouped as glycoside hydrolases and polysaccharide lyases. The glycoside hydrolase (GH) are the group of enzymes which hydrolyze the glycosidic bond between the algal sugars in the polysaccharides. A large majority of the AOS producing enzymes agarase, carrageenase, porphyranase, fucoidanase, and laminarinase

belong to this group. Polysaccharide lyase (PL) is the second group of enzymes which break down the polysaccharides as they cleave the (1 →4) glycosidic bond between anionic sugars through β-elimination mechanism as seen in alginate lyase and ulvan lyase. These enzymes exhibit very high specific activity on the polysaccharide substrates and are known to have wide application in structural analysis, controlling rheological properties of polysaccharides, protoplast formation, and mainly in the production of bioactive AOS (Moreno et al. 2017). In recent years, these enzymes are in much demand due to the market potential for algal oligosaccharides in functional foods, as they were accorded GRAS "Generally Recognized As Safe" status by US Food and Drug Administration. Since then, the global requirement for these microbial polysaccharide degraders are increasing for the production of functional oligosaccharides and this is actively driving the research in this area; some examples of hydrolyzing enzymes are given in Table 1.

Studies have shown that microbes associated with live macroalgae (Oh et al. 2010; Gupta et al. 2013; Lin et al. 2017), decaying forms (Li et al. 2011b; Zhu et al. 2018a; He et al. 2018; Chen et al. 2018), and seaweed sludge (Huang et al. 2013) are the major sources of algal polysaccharide degrading enzymes (Table 1). Reports also show that microbes associated with marine molluscans are also capable to producing these enzymes (Miyanishi et al. 2003; Lin et al. 2012; Kusaykin et al. 2017; Ulaganathan et al. 2018; Gao et al. 2019). Both bacteria and fungi isolated from seawater (Potin et al. 1993; Zhu et al. 2015, 2018a, 2019; Zeng et al. 2016) and sediment (Wu et al. 2011; Liao et al. 2011; Li et al. 2017; Qin et al. 2018; Zhang et al. 2018; Lee et al. 2019) both from coastal (Zhang et al. 2019b) and deep sea environment (McLean and Williamson 1979; Hatada et al. 2011; Chen et al. 2016) are shown to produce these enzymes to breakdown algal polysaccharides. Few reports show that microbes associated with terrestrial plants (Ribeiro et al. 2019), soil (Suzuki et al. 2003; Giese et al. 2006; Kawai et al. 2006; Wang et al. 2006a, b; Lakshmikanth et al. 2009; Song et al. 2014; Kim et al. 2015; Boucelkha et al. 2017), and human gut (Stender et al. 2019) are also capable to produce these enzymes. Carrageenase followed by agarase and alginate lyase are the well-studied enzymes for the production of AOS. Porphyranase and ulvan lyase are the least explored enzymes, and they are so far reported to be produced only by bacteria associated with marine algae and from marine sediment and seawater (Hehemann et al. 2010; Przybylski et al. 2015; Zhang et al. 2019a, b).

Enzymatic Production of Algal Oligosaccharides

AOS are low molecular weight carbohydrates composed of 2–20 units of simple monomer residues. They are readily soluble in water, which makes it biocompatible and provides with a wide range of bioactivity. Though they have been commercialized since the 1980s as low-calorie bulking agents, it has gained much interest in food industry after research findings across the globe showed that non-digestible oligosaccharides can be included as functional food and were credited with the prebiotic status (Moreno et al. 2017; Gurpilhares et al. 2019). Among the non-digestible carbohydrates, functional oligosaccharides are gaining out-standing popularity owing to their physiological benefits to the consumers (Table 2). Recently, they have garnered much interest in food and pharmaceutical sector owing to overwhelming consumer preference for healthier food (Zhu et al. 2020). The complex nature of the algal polysaccharides and the different moieties present require specific enzymes for the production of each AOS. The complex nature of the various algal polysaccharides and the different enzymes identified and characterized for the production of each AOS are summarized below.

Agaro-oligosaccharides

Agar is a linear polysaccharide mainly composed of basic alternating units of 3-O-linked-β-D-galactose (G) and 4-O-linked 3, 6-anhydro-α-L galactose (AG) (Fig. 1). Agarases are the group of enzymes which belong to the GH family that catalyzes the hydrolysis of agar. They are classified into α-agarase and β-agarase based on their cleavage mechanism (Gupta et al. 2013; Lin et al. 2017). α-agarases (EC 3.2.1.158) belong to the GH96 family which hydrolyzes the α-1, 3 linkage of agar and produces agaro-oligosaccharides with anhydro-galactose at the reducing end (Potin et al. 1993; Zhang et al. 2018; Lee et al. 2019). β-agarase (EC 3.2.1.81) hydrolyzes the β-1, 4 linkages, and the neoagaro-oligosaccharides with β-D-galactose at the reducing end are produced. β-agarase are mainly classified into four GH families in Carbohydrate-Active Enzyme (CAZy) database which includes GH16, GH50, GH86, and GH118 based on their amino acid sequence (Lin et al. 2017).

Agarase activity has been reported in a wide range of bacteria isolated from seawater, associated with marine sediments, marine algae, marine molluscs, and from freshwater and soil (Fu and Kim 2010). Only a few α-agarases obtained from marine bacteria *Alteromonas agarilyticus*, *Thalasomonas* sp., and *Catenovulum sediminis* (Potin et al. 1993; Zhang et al. 2018; Lee et al. 2019) are known. However, β-agarase activity has been reported from various bacteria including *Zobellia* sp., *Bacillus* sp., *Alteromonas* sp., *Acinetobacter* sp., *Pseudoalteromonas* sp., *Vibrio* sp., *Agarivorans* sp., *Pseudomonas* sp., *Paenibacillus* sp., *Flammeovirga* sp., *Thalassospira* sp., and *Aquimarina* sp. which produces either extracellular or intracellular

Table 1 Microbial enzymes identified for the production of algal oligosaccharides

Enzymes	Microbial source	Habitat	Reference
α-agarase EC 3.2.1.158	*Alteromonas agarolyticus*	Seawater	Potin et al. (1993)
	Thalassomonas sp. LD5, *Catenovulum sediminis* WS1-A	Marine sediment	Zhang et al. (2018); Lee et al. (2019)
β-agarase EC 3.2.1.81	*Zobellia galactanivorans* *Pseudoalteromonas* sp. AG4, *Pseudomonas* sp., *Aquimarina agarilytica* ZC1	Marine algae	Jam et al. (2005); Oh et al. (2010); Gupta et al. (2013); Lin et al. (2017)
	Agarivorans sp. HZ105	Marine molluscans	Lin et al. (2012)
	Flammeovirga sp. OC-4, *Thalassospira profundimonas*	Seawater	Chen et al. (2016); Zeng et al. (2016)
	Vibrio sp. strain CN41	Marine sediment	Liao et al. (2011)
	Bacillus sp. MK03, *Alteromonas* sp. SY37-12, *Acinetobacter* sp. AG LSL-1, *Paenibacillus* sp. SSG-1	Terrestrial soil	Suzuki et al. (2003); Wang et al. (2006a, b); Lakshmikanth et al. (2009); Song et al. (2014)
κ-carrageenase EC 3.2.1.83	*Cytophaga* sp., *Tamlana* sp. HC4, *Pseudoalteromonas porphyrae*, *Cellulosimicrobium cellulans*, *Vibrio* sp. NJ-2, *Thalassospira* sp. Fjfst-32, *Zobellia* sp. ZL-4	Marine algae	Potin et al. (1991); Sun et al. (2010); Liu et al. (2011); Youssef et al. (2012); Zhu and Ning (2016); Guo et al. (2018); Zhang et al. (2019a, b)
	Pseudomonas carrageenovora, *Pedobacter hainanensis* NJ-02	Seawater	McLean and Williamson (1979); Zhu et al. (2018a)
	Bacillus sp. HT19	Terrestrial soil	Li et al. (2019)
ι-carrageenase EC 3.2.1.157	*Alteromonas fortis*, *Cellulophaga* sp. QY3	Marine algae	Michel et al. (2001); Ma et al. (2013)
	Microbulbifer thermotolerans, *Flavobacterium* sp. YS-80–122	Marine sediment	Hatada et al. (2011); Li et al. (2017)
λ-carrageenase EC 3.2.1.162	*Pseudomonas carrageenovora*	Marine algae	Guibet et al. (2007)
	Pseudoalteromonas CL9	Marine sediment	Ohta and Hatada (2006)
	Bacillus sp.	Terrestrial soil	Li et al. (2014)
β-porphyranase EC 3.2.1.178	*Zobellia galactanivorans*, *Pseudoalteromonas atlantica*	Marine algae	Hehemann et al. (2010); Przybylski et al. (2015)
	Wenyingzhuangia fucanilytica	Seawater	Zhang et al. (2019a)
Alginate lyase EC 4.2.2.3 / EC 4.2.2.11	*Pseudoalteromonas* sp. *Pseudomonas alginovora,* *Formosa algae* *Flavobacterium* sp. S20, *Sphingobacterium* sp. *Microbulbifer* sp., *Vibrio furnissii,* *Sphingomonas* sp. ZH0, *Bacillus* sp. Alg07, *Cellulophaga algicola,* *Aspergillus oryzae* *Gilvimarinus agarilyticus*	Marine algae	Li et al. (2011b); Lundqvist et al. (2012); Huang et al. (2013); Falkeborg et al. (2014); Yang et al. (2018); Zhu et al. (2018b); He et al. (2018); Chen et al. (2018); Fischer and Wefers (2019); Huang et al. (2019); Belik et al. (2020); Singh et al. (2011)
	Pseudomonas sp. E03, *Flammeovirga* sp.	Seawater	Zhu et al. (2015); Zhu et al. (2019)
	Flavobacterium multivorum	Terrestrial soil	Boucelkha et al. (2017)
	Bacteroides cellulosilyticus	Human gut	Stender et al. (2019)
	Saccharophagus degradans	Saltmarsh cordgrass	Kim et al. (2012)
Fucoidanase EC 3.2.1.44	*Fucobacter marina*, *Mariniflexile fucanivorans*, *Formosa algae*	Marine algae	(Sakai et al. 2003); (Cao et al. 2018); Silchenko et al. (2013)
	Pseudoalteromonas citrea	Seawater	Bakunina et al. (2002)
	Fusarium sp. LD8, *Dendryphiella arenaria*	Marine sediment	Shvetsova et al. (2014); Wu et al. (2011)
	Sphingomonas paucimobilis	Terrestrial soil	Kim et al. (2015)

Table 1 (continued)

Enzymes	Microbial source	Habitat	Reference
Glucanase/Laminarinase EC 3.2.1.6/ EC 3.2.1.39	*Formosa algae* KMM 3553, *Vibrio breoganii* 1C10	Marine algae	Becker et al. (2017); Badur et al. (2020)
	Bacillus clausii NM-1, *Pseudocardium sachalinensis*	Marine molluscans	Miyanishi et al. (2003); Kusaykin et al. (2017)
	Trichoderma harzianum	Terrestrial plant	Ribeiro et al. (2019)
	Botryosphaeria sp., *Phanerochaete chrysosporium*	Terrestrial soil	Giese et al. (2006); Kawai et al. (2006)
	Saccharophagus degradans	Saltmarsh cordgrass	Wang et al. (2017a, b)
Ulvan lyase EC 4.2.2.-	*Persicivirga ulvanivorans*, *Formosa agariphila*	Marine algae	Collen et al. (2011); Reisky et al. (2018)
	Nonlabens ulvanivorans, *Alteromonas* sp.	Marine molluscans	Ulaganathan et al. (2018); Gao et al. (2019)
	Pseudoalteromonas sp.	Marine sediment	Qin et al. (2018)

β-agarase (Table 1). The recombinant enzyme from cloning and expression of β-agarase gene in *Bacillus subtilis* showed production of neoagaro-oligosaccharides with 30 fold greater activity than the original strain (Ohta et al. 2004). Recently Xu et al. (2018a, b) have reported the production of neoagaro-octaose and neoagaro-decaose from enzymatic hydrolysis of agar extracted from *Gelidium amansii* using recombinant β-agarase. The neoagaro-oligosaccharides obtained using the recombinant β-agarase showed production of specific products tetra, hexa, or octa oligosaccharides with antioxidant, anti-inflammatory, and prebiotic activity (Wang et al. 2004; Chen et al. 2006; Hu et al. 2006; Wang et al. 2017a, b; Xu et al. 2018a).

Carrageenan Oligosaccharide

Carrageenan is a linear sulfated polysaccharide mainly composed of repeating disaccharide units of 3-O-linked-β-D-galactose (G) and 4-O-linked 3, 6-anhydro-α-L galactose (AG). They are categorized as κ-, ι-, and λ-carrageenan based on the number of sulfate groups present per disaccharide unit (Yermak et al. 2017). The κ-carrageenan is shown to have only one sulfate group, ι- carrageenan has two sulphate groups, and λ-carrageenan has three sulfate groups per disaccharide (Fig. 2). Each of the carrageenan are hydrolyzed by their specific enzymes including κ-carrageenase (EC 3.2.1.83), ι-carrageenase (EC 3.2.1.157), and λ-carrageenases (EC 3.2.1.162) which subsequently leads to the production of κ-, ι-, and λ-carrageenan oligosaccharides, respectively (Kalitnik et al. 2016). They are all endohydrolases and belong to the GH family, which cleave the internal β-1, 4 linkage in κ-, ι-, and λ-carrageenan (Li et al. 2014; Shen et al. 2017). Studies have shown that κ-carrageenase belongs to the family of GH16 and ι-carrageenase belongs to GH82 family and λ-carrageenases is classified within GH150 family in the CAZy database (Guibet et al. 2007; Gobet et al. 2018).

The κ-carrageenase is the well-studied enzyme class (Table 1) and reported from gram negative bacterial genera *Pseudomonas*, *Cytophaga*, *Tamlana*,

Table 2 Bioactive potential of enzymatically produced algal oligosaccharides

Algal oligosaccharides	Bioactivity reported	References
Agaro-oligosaccharides	Antioxidant, hepatoprotective, antiglycemic, prebiotic and anti-inflammatory activity	Wang et al. (2004); Chen et al. (2006); Hu et al. (2006); Wang et al. (2017a, b); Xu et al. (2018a)
Carrageenan oligosaccharides	Antioxidant, prebiotic, anti-inflammatory and immunoregulatory	Yuan et al. (2005); Yuan et al. (2011); Xu et al. (2012); Yao et al. (2014); Han et al. (2019)
Porphyran oligosaccharides	Antioxidant, antitumor and antiobesity activity	Osumi et al. (2002); Zhao et al. (2006); Gong et al. (2018)
Alginate oligosaccharides	Antioxidant, antiglycemic, prebiotic, anti-inflammatory, immunoregulatory, and antiobesity activity	Wang et al. (2006a, b); Hao et al. (2011); Falkeborg et al. (2014); Xu et al. (2014); Zhou et al. (2015); Han et al. (2019); Tran et al. (2019); Zhang et al. (2020)
Fucoidan oligosaccharides	Antioxidant immunoregulatory and antitumor activity	Wang et al. (2007); Silchenko et al. (2013); Silchenko et al. (2018)
Laminarin oligosaccharides	Antitumor and immunoregulatory activity	Pang et al. (2005); Kim et al. (2006)
Ulvan oligosaccharides	No study	

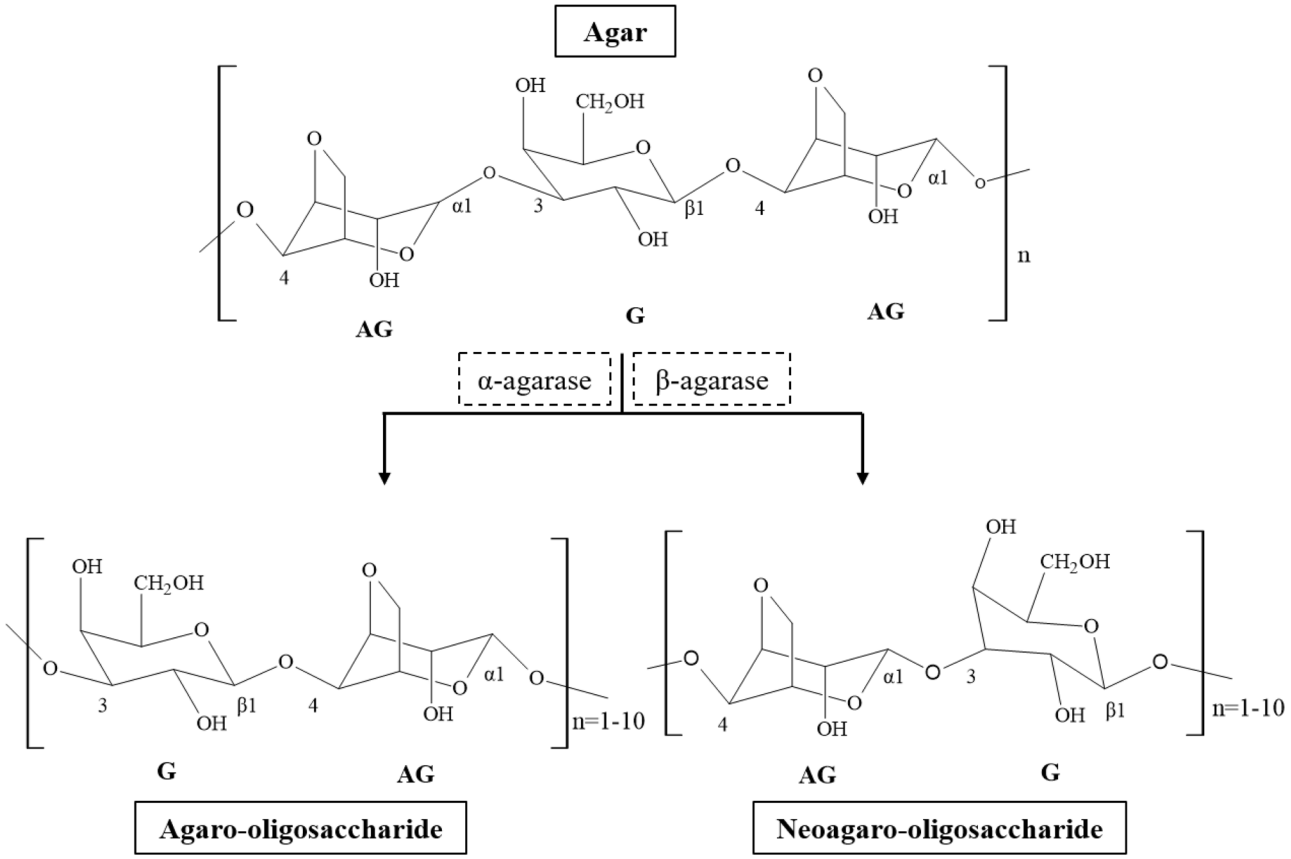

Fig. 1 Structure of agar and its enzymatic hydrolysis to produce agaro-oligosaccharide and neoagaro-oligosaccharide, where G is galactose and AG is 3, 6 anhydro-L-galactose

Pseudoalteromonas, *Vibrio*, *Pedobacter*, *Thalassospira*, and *Zobellia* (Potin et al. 1991; Sun et al. 2010; Liu et al. 2011; Zhu and Ning 2016; Guo et al. 2018; Zhu et al. 2018a; Zhang et al. 2019a, b). κ-carrageenase production was also reported in gram-positive bacteria *Cellulosomicrobium cellulans* and *Bacillus* sp. (Youssef et al. 2012; Ghanbarzadeh et al. 2018). The ι-carrageenase production are reported in bacteria *Alteromonas fortis*, *Microbulbifer thermotolerans*, *Cellulophaga* sp., *Flavobacterium* sp. (Michel et al. 2001; Hatada et al. 2011; Ma et al. 2013; Li et al. 2017), and λ-carrageenases from *Pseudolateromonas*, *Pseudomonas*, and *Bacillus* sp. (Ohta and Hatada 2006; Guibet et al. 2007; Li et al. 2014; Gobet et al. 2018). Studies on recombinant κ-carrageenase from *Zobellia* sp. expressed in methylated yeast *Pichia pastoris* showed production of κ-carrageenan tetra- and hexa-oligosaccharides (Yu et al. 2017). Xu et al (2019) reported production of recombinant ι-carrageenase in *Brevibacillus choshinensis* to form ι-carrageenan oligomer. The recombinant λ-carrageenase from *Pseudolateromonas carrageenovora* used in the production of tetra- and hexa-λ-carrageenan oligosaccharides (Shen et al. 2017). These enzymatically produced oligosaccharides showed potential antioxidant, immunoregulatory, and antitumor activity than parent carrageenan (Table 2).

Porphyran Oligosaccharide

Porphyran is a sulfated polysaccharide mainly composed of a linear backbone of alternating 3-linked methylated β-D-galactose (G) and sulfated 3, 6-anhydro-α-L-galactose (AG) units (Correc et al. 2011; Przybylski et al. 2015). β-Porphyranase (EC 3.2.1.178) are the group of GH enzymes studied so far belonging to the GH16 and GH86 family which catalyzes the hydrolysis of porphyran at β-1, 4 glycosidic linkage (Gong et al. 2018). β-porphyranase specifically recognizes and cleaves β-1, 4 linkage between methylated G and sulfated AG to produce porphyran oligosaccharides with methylated G at the reducing end (Fig. 3).

Microbial β-porphyranase enzymes are reported so far only from marine habitats (Table 1), in bacteria associated with macroalgae and coastal waters such as *Zobellia galactanivorans*, *Pseudoalteromonas atlantica*, and *Wenyingzhuangia funcanilytica*

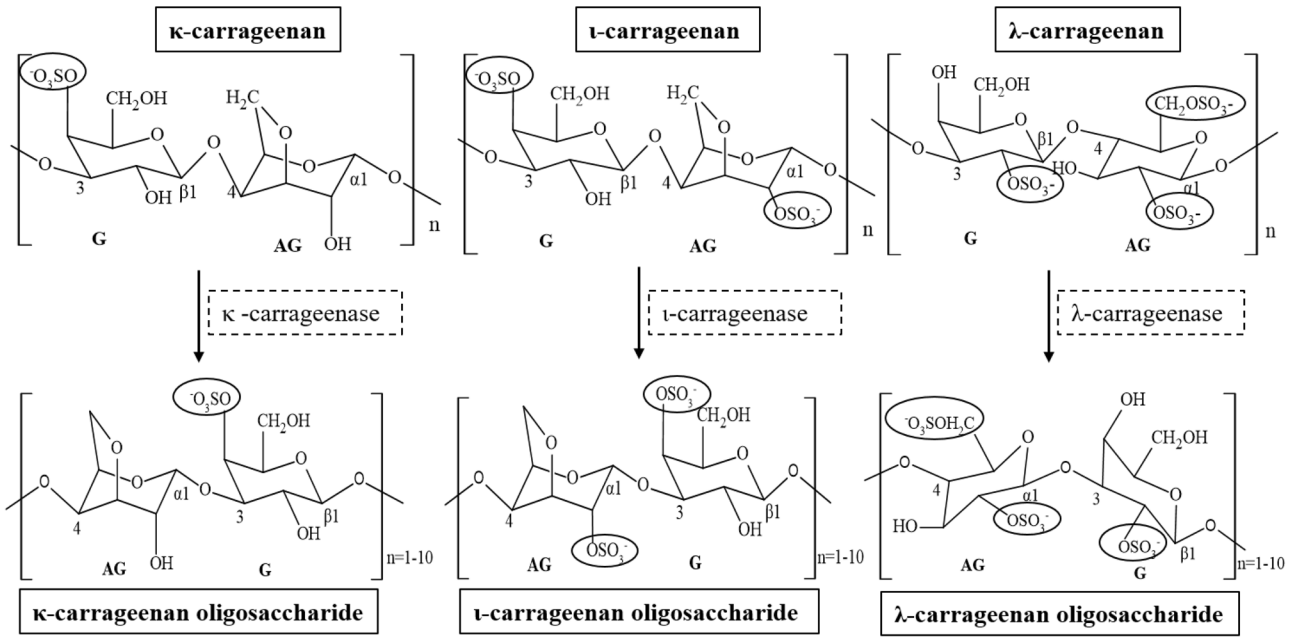

Fig. 2 Structure of carrageenan and its enzymatic hydrolysis to produce κ-, ι-, or λ-carrageenan oligosaccharides, where G is galactose and AG is 3, 6 anhydro-L-galactose

(Hehemann et al. 2010; Przybylski et al. 2015; Zhang et al. 2019a). Correc et al. (2011) used β-porphyranase isolated from *Zobellia galactanivorans* for the production of hexa-porphyran oligosaccharide with 6-sulphated AG (1 → 3) 6-methylated G subunits. The β-methyl porphyranase isolated from *Pseudoalteromonas atlantica* hydrolyze the porphyran into porphyran oligomer of methylated disaccharide and dimethylated tetra-saccharides (Przybylski et al. 2015). Recently, GH16 β-porphyranase was cloned from *Wenyingzhuangia funcanilytica* and expressed in *Escherichia coli*. This endo acting hydrolase gradually converts porphyran into low molecular weight porphyran oligosaccharides at wide pH range (pH 3.5–11), and its antitumor and antiobesity activity

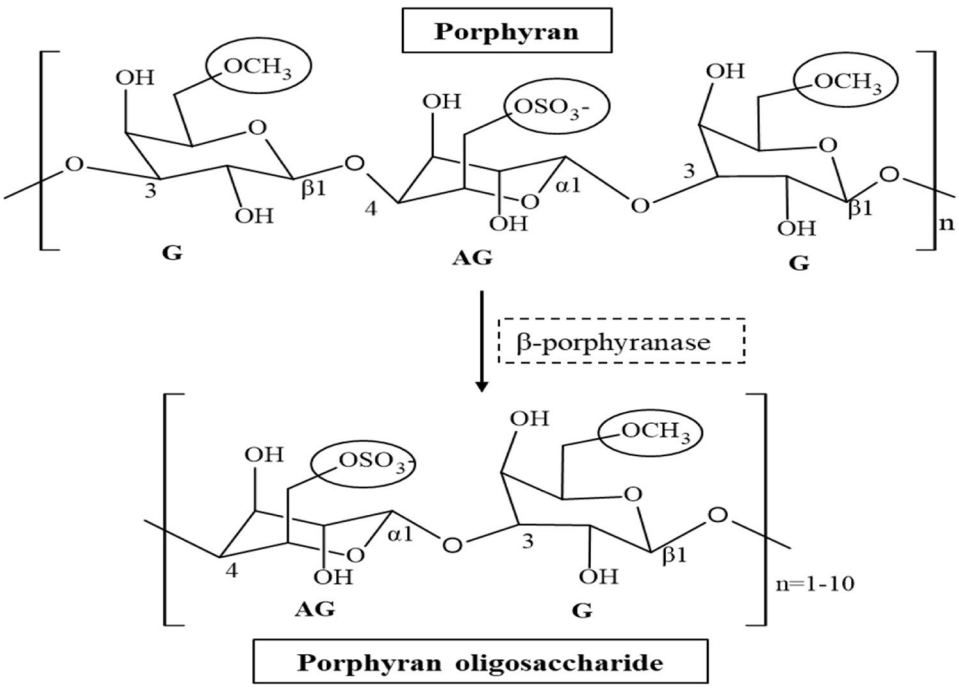

Fig. 3 Structure of porphyran and its enzymatic hydrolysis to produce porphyran oligosaccharide, where G is galactose and AG is 3, 6 anhydro-L-galactose

was confirmed through cell line studies (Osumi et al. 2002; Zhang et al. 2019a).

Alginate Oligosaccharides

Alginate is a complex polymer of α-L-glucuronic acid (GlcA) and its C5 epimer β-D-mannuronic acid (MnA). Alginate lyase are the group of polysaccharide lyases involved in the cleavage of alginate at β 1 → 4 linkage to produce alginate oligosaccharides (Fig. 4). It largely belongs to polysaccharide lyase family PL5, PL6, PL7, PL14, PL15, PL17, or PL18. They are categorized into mannuronate lyase (EC 4.2.2.3) and glucoronate lyase (EC 4.2.2.11) based on their specificity to MnA- and GlcA-rich substrate respectively (Zhu et al. 2015, 2018b). Few alginate lyase from PL17 and PL18 family exhibits bifunctional activity and can cleave both MnA- and GlcA-rich substrate, and they are reported to hydrolyze alginate efficiently. Based on the mode of action, alginate lyase are grouped into endolytic and exolytic alginate lyase. Endolytic alginate lyase hydrolysis leads to the production of di-, tri-, tetra-, penta-, and hexa-oligosaccharide as main product, while exolytic alginate lyase further degrade oligosaccharide into monomers.

Alginate lyase activity is reported from wide range of microorganisms isolated from seaweeds, seaweed factory sludge, seawater, terrestrial soil, and human gut (Table 1). Various major bacterial genera produces alginate lyases which includes *Pseudoalteromonas*, *Pseudomonas*, *Flavobacterium*, *Vibrio*, and *Bacillus* (Li et al. 2011a, b; Lundqvist et al. 2012; Huang et al. 2013; Zhu et al. 2018b). Alginate lyase was also reported from fungi *Aspergillus oryzae* isolated from marine macroalgae and also from the saltmarsh cordgrass associated marine bacterium *Saccharophagus degradans* (Kim et al. 2012) (Table 1). Polymannuronate specific lyase activity was reported in *Pseudomonas* sp. (Zhu et al. 2015) and *Bacteroides cellulosilyticus* (Stender et al. 2019) microorganism isolated from degrading seaweed such as *Flavobacterium* sp. (Huang et al. 2013) and *Microbulbifer* sp. Q7 (Yang et al. 2018) and *Cellulophaga algicola* (Fischer and Wefers 2019) showed polyglucuronate specific activity. Few microbial enzyme from

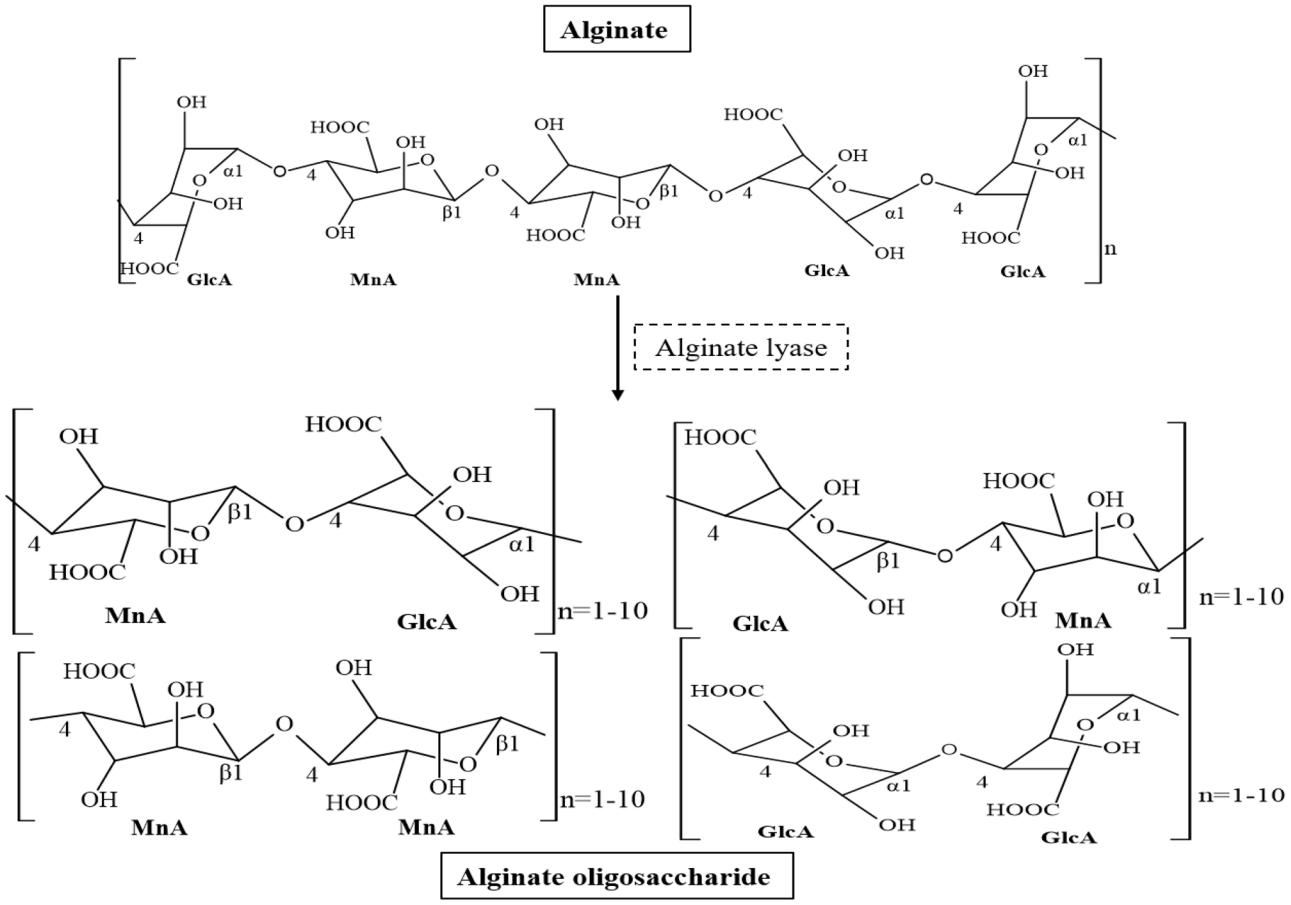

Fig. 4 Structure of alginate and its enzymatic hydrolysis to produce alginate oligosaccharide, where GlcA is glucuronic acid and MnA is mannuronic acid

Pseudoalteromonas sp. (Li et al. 2011b), *Vibrio furnissi* (Zhu et al. 2018b), *Gilvimarinus agarilyticus* (Huang et al. 2019), and *Flammeovirga* (Zhu et al. 2019) showed bifunctional activity. The gene from *Microbulbifer* sp. was expressed in *E. coli* to produce recombinant polyglucuronate specific alginate lyase, and the enzyme was tested for production of alginate oligosaccharide with degree of polymerization 2–5 (Yang et al. 2018). Enzymatically produced alginate oligosaccharides exhibited antioxidant, immunoregulatory, anti-inflammatory, and hypoglycemic properties (Zhou et al. 2015; Han et al. 2019; Tran et al. 2019; Xing et al. 2020).

Fucoidan Oligosaccharides

Fucoidan is a highly sulfated polysaccharide and is composed of α-L-fucose units linked by (1→4) and (1→3) glycosidic bond with sulfation at C2, C3, or C4 which varies in each algal species (Yuan and Macquarrie 2015). Fucoidanase (EC 3.2.1.44) are the enzymes of GH107 family that catalyze the hydrolysis of α-L-fucoside linkage without removal of substitute groups and produce low molecular weight fucoidan oligosaccharides (Fig. 5) (Wang et al. 2007; Silchenko et al. 2013, 2018).

The isolation, purification, and characterization of fucoidanase have been reported from bacteria *Pseudoalteromonas citrea* (Bakunina et al. 2002), *Fucobacter marina* (Sakai et al. 2003), *Formosa algae* (Silchenko et al. 2013), *Sphingomonas paucimobilis* (Kim et al. 2015), *Mariniflexile fucanivorans* (Cao et al. 2018), and also from marine fungi *Dendryphiella arenaria* (Wu et al. 2011) and *Fusarium* sp. LD8 (Shvetsova et al. 2014). So far, only one study reported recombinant fucoidanase from *Formosa algae* to produce low molecular weight bioactive fucoidan oligosaccharide with immunoregulatory activity (Silchenko et al. 2013, 2018).

Laminarin Oligosaccharides

Laminarin is a water soluble storage glucan found in brown macroalgae, and it consists of glucose as monomeric units inter-connected through β-(1→3) and some β-(1→6) links (Kadam et al. 2015; Lee and Lee 2016).

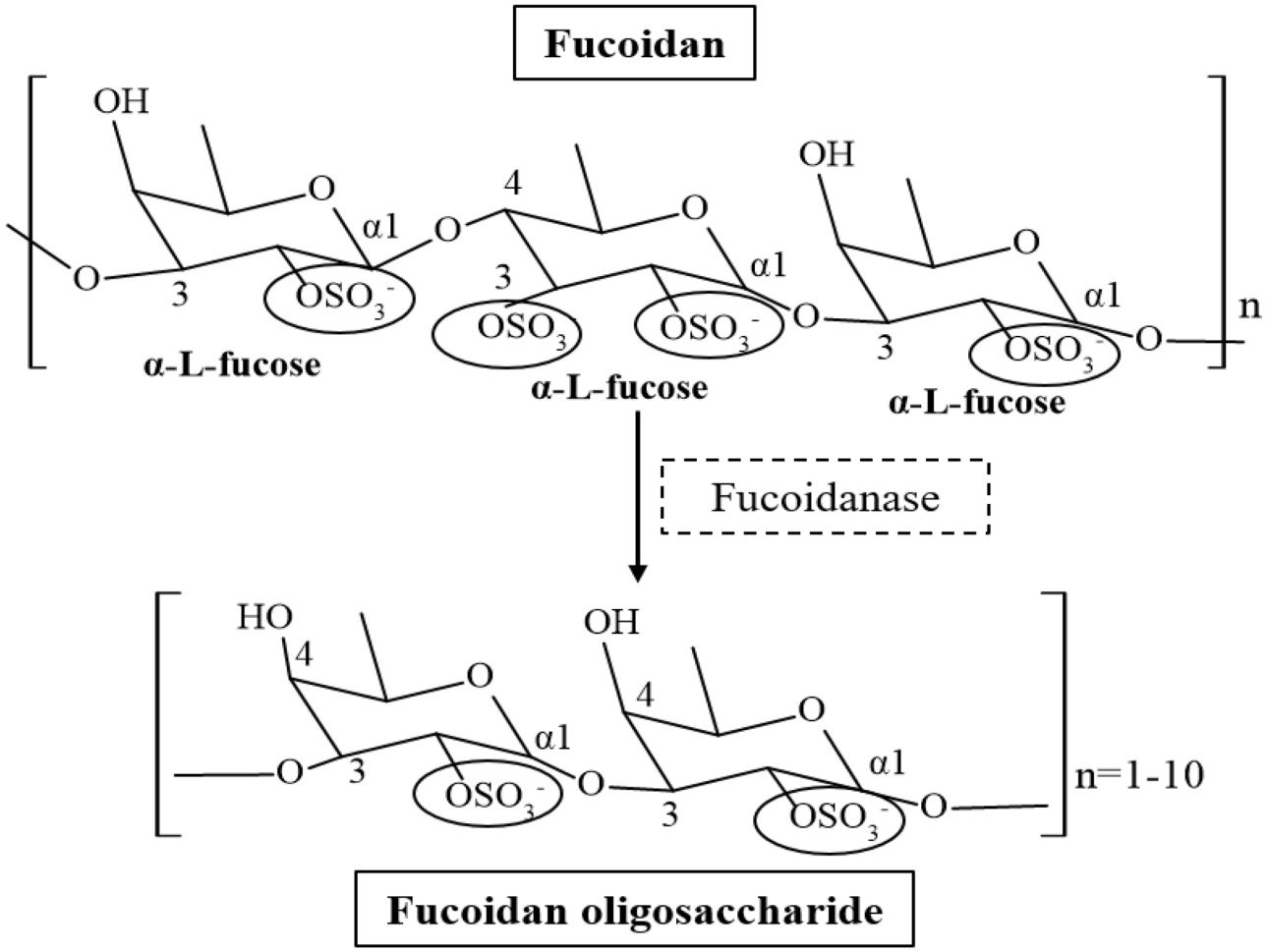

Fig. 5 Structure of fucoidan and its enzymatic hydrolysis to produce fucoidan oligosaccharide

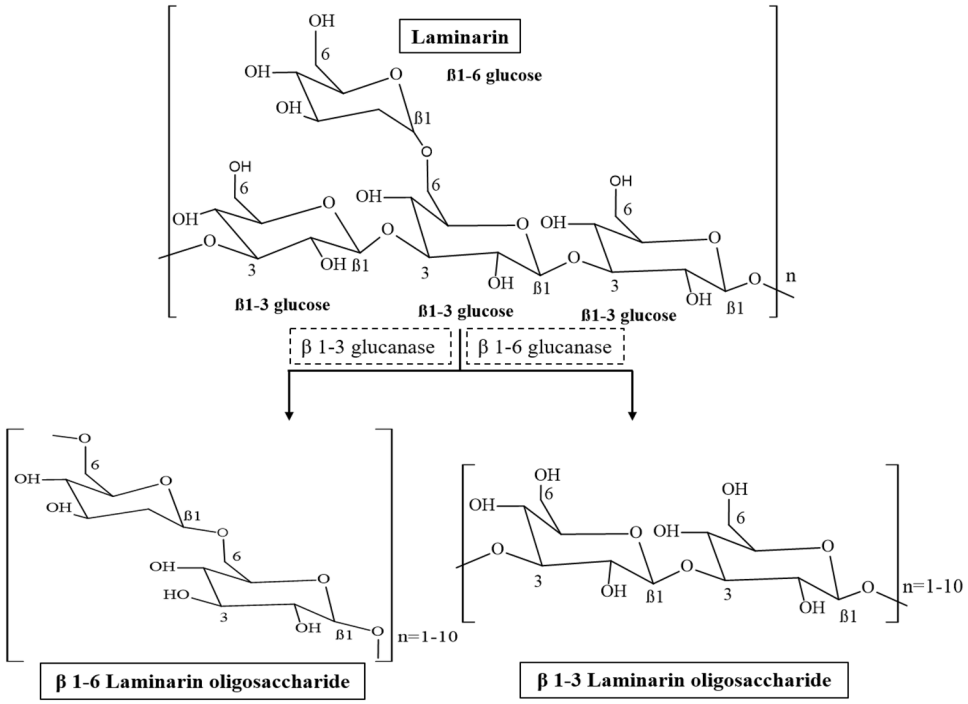

Fig. 6 Structure of laminarin and its enzymatic hydrolysis to produce β1-3 or β1-6 laminarin oligosaccharide

Laminarinase or glucanase are the group of GH enzymes involved in the hydrolysis of laminarin to produce laminarin oligosaccharides (Fig. 6) (Pang et al. 2005; Kim et al. 2006). They are classified as exo-β-(1 → 3)-glucanase (EC 3.2.1.58) and endo-β-(1 → 3)-glucanase (EC 3.2.1.6 and EC 3.2.1.39). The exo-β-(1 → 3)-glucanase hydrolyze laminarin at terminal and formed monomer units and the endo-β-(1 → 3)-glucanase hydrolyze the β-1 → 3 linkage randomly within the chain of laminarin into β-(1 → 6) linked laminarin oligosaccharide. The side chain in laminarin limits the activity of endo glucanase, owing to steric hindrance and limits complete hydrolysis. Endo-β-(1 → 3)-glucanase belongs to different GH families including GH16, GH17, GH55, GH64, and GH81. Though β-(1 → 3)-glucanase is widely studied, few studies have also reported β-(1 → 6) glucanase enzyme production from marine bacterium *Formosa algae* and *Saccharophagus degradans* which belongs to the GH 30 and GH 5 family respectively (Becker et al. 2017; Badur et al. 2020).

The glucanase producing microorganisms were isolated from both marine and terrestrial niches associated with macroalgae, molluscs, and terrestrial plants (Table 1). The β-(1 → 3) glucanase producing bacterium includes *Bacillus clausii* NM1, *Formosa algae*, and *Vibrio breoganii* 1C10. Some fungi such as *Botryosphaeria* sp., *Phanerochaete chrysosporium*, *Pseudocardium sachalinensis*, and *Trichoderma harzianum* are reported to produce laminarinase. Wang et al. (2017a, b) first time reported both β-(1 → 3) and β-(1 → 6)-glucanase activity from marine bacterium *Saccharophagus degradans* isolated from decaying saltmarsh cordgrass belongs to GH5 family. Badur et al. (2020) has investigated four laminarinase belonging to GH16 and GH17 family, isolated from *Vibrio breoganii* that can produce both low and high molecular weight laminarin oligosaccharide, with degree of polymerization 3–4 and > 8, respectively. The endo-β-(1 → 3)-glucanase isolated from *Bacillus circulans* and *Bacillus clausii* NM1 could hydrolyze laminarin into β-1 → 3 glucan oligomer with anti-inflammatory activity (Kim et al. 2006).

Ulvan Oligosaccharides

Ulvan is a sulphated heteropolysaccharide mainly extracted from green algae and structurally constituted by alternating units of D-glucuronic acid (GlcA) or L-iduronic acid (IdoA), linked to sulfated α-L-rhamnose (Rha3S) (Lahaye and Robic 2007; Gurpilhares et al. 2019). Ulvan lyase (EC 4.2.2.) are the least explored enzymes involved in the hydrolysis of ulvan, which belong to the polysaccharide lyase family PL24 or PL 25 or PL28 as described in the CAZy database (Ulaganathan et al. 2018).

Ulvan lyase activity have been reported from microorganisms isolated from green algal surface, marine molluscs grazing on alga and sediment including *Persicivirga ulvanivorans*, *Pseudoalteromonas* sp., *Formosa agriphila*, *Nonlabens ulvanivorans*, and *Alteromonas* sp. (Qin et al. 2018; Gao et al. 2019; Reisky et al. 2018). Ulvan lyase cleave β-1, 4 glycosidic bond between Rha3S linked to either GlcA or IdoA by β-elimination mechanism with the release of ulvan oligosaccharides that have 4-deoxy-L-threo-hex-4-enopyranosiduronic acid (EPU) at the non-reducing end (Fig. 7) (Reisky et al. 2018).

Ulvan lyase isolated from *Alteromonas* species produced low molecular weight oligosaccharides, mainly composed of unsaturated rhamnose 3-sulfate and uronic acid. Similarly, ulvan lyase isolated from *Pseudoalteromonas* sp. showed potential to hydrolyze ulvan into ulvan oligomer including disaccharide (EPU-Rha3S) and tetra-saccharides (EPU-Rha3S-Xyl-Rha3S) (Qin et al. 2018). Gao et al (2019) studied the cloning, expression, and characterization of recombinant ulvan lyase from *Alteromonas* sp. A321 and showed production of even numbered ulvan oligosaccharide. As of now, there are no studies on the bioactivity of enzymatically produced ulvan oligosaccharides.

Bioactivity of Enzymatically Produced Algal Oligosaccharides

In recent years, there has been a growing interest in natural biomolecules for their potential, preventive role in managing chronic diseases, especially those associated with oxidative stress. Hence, many researchers are driven to explore algal products as its nutritional importance is brought to light. Bioactive oligosaccharides, especially AOS, are investigated in depth as a source of nutraceutical or functional food as they have multiple bioactivities. Agaro, carrageenan, and alginate oligosaccharides are among the well-studied AOS for their bioactivity such as antioxidant, antiglycemic, anti-inflammatory, prebiotic along with hepatoprotective, and antitumor activity (Table 2). Recent studies are exploring the enzymatic production and bioactivity of other AOS such as porphyran, fucoidan, laminarin, and ulvan oligosaccharides (Table 1).

Antioxidant Activity

Biomolecules that scavenge reactive oxygen species (ROS) prevent a cascade of physiological stress as it is the key signalling molecule which elicit the metabolic processes that leads to the progression of numerous chronic diseases (Fig. 8). Almost all the AOS studied have reported radical scavenging activity based on 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azino-bis (3-etilbenzotiazolin)-6-sulfonic acid (ABTS), hydroxyl, or superoxide radical assays (Chen et al. 2006; Xu et al. 2018a, b)

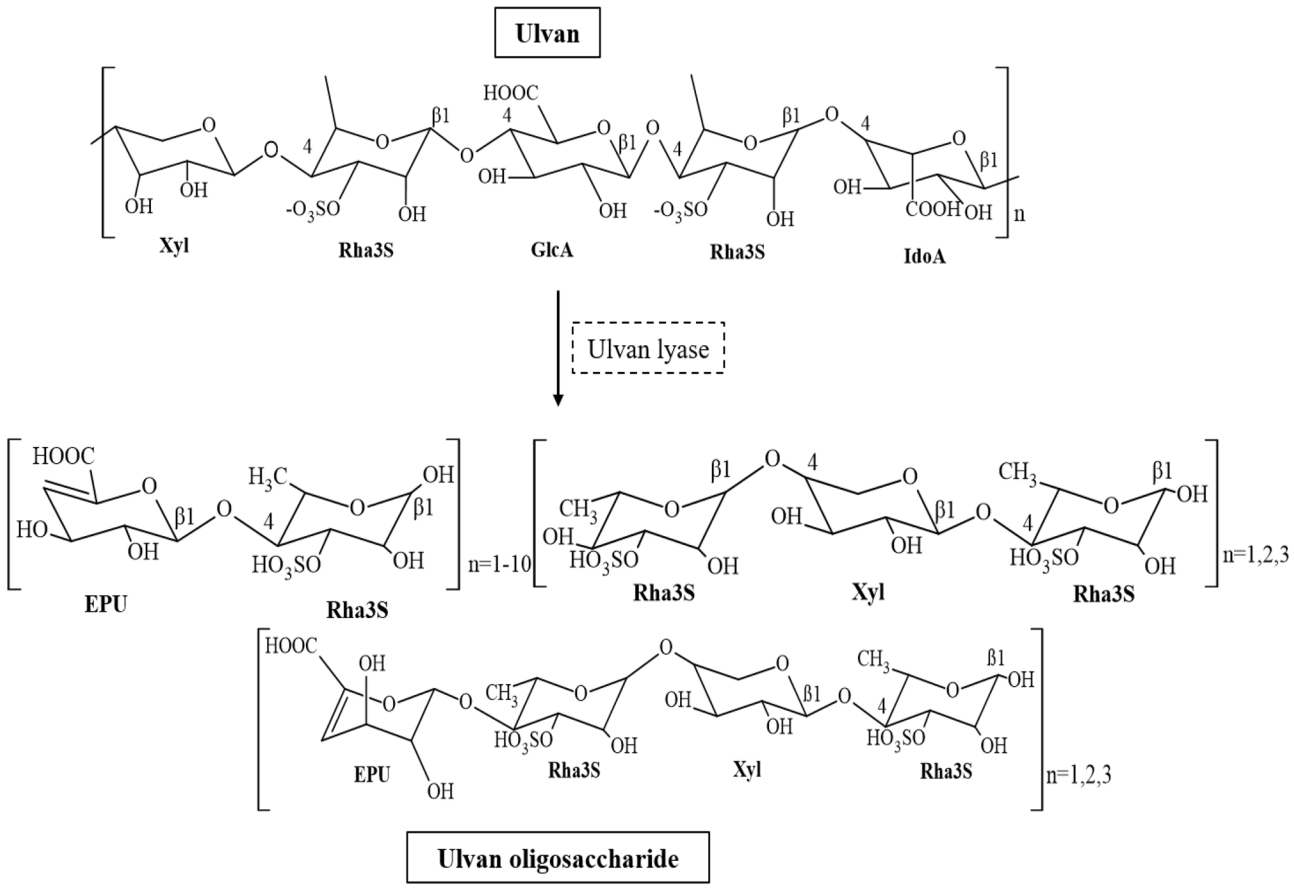

Fig. 7 Structure of ulvan and its enzymatic hydrolysis to produce ulvan oligosaccharide (Xyl = xylose, Rha3S = α-L-rhamnose 3-sulphate, GlcA = glucuronic acid, IdoA = iduronic acid, EPU = 4-deoxy-L-threo-hex-4-enopyranosiduronic acid)

(Table 2). Studies have shown that AOS of carrageenan (Yuan et al. 2005), porphyran (Zhao et al. 2006), and agar (Wang et al. 2004) reported higher antioxidant activity than parent polysaccharides. The enzymatically hydrolyzed AOS of agar (Xu et al. 2018a, b) and carrageenan (Sun et al. 2015) showed higher inhibition of free radicals, compared with acid and hydrogen peroxide hydrolyzed polysaccharide. Zhang et al. (2020) have been reported the antioxidant potential of enzymatically produced alginate oligosaccharide, shows good inhibitory activity of up to > 88%, > 81%, and > 61% towards hydroxyl, ABTS, and DPPH radicals. The alginate oligosaccharide was shown to inhibit (89%) lipid oxidation along with hydroxyl, ABTS, and superoxide free radical inhibition activity.

Antiglycemic Activity

Oligosaccharides are able to inhibit the enzymes involved in the metabolism of carbohydrates including α-amylase, α-glucosidase, and dipeptidyl peptidase-4. These are the target enzymes which can be inhibited for treatment or prevention of type 2 diabetes (T2D). So far, only AOS of agar and alginate are reported to have antiglycemic activity mostly accompanied along with antioxidant activity (Hao et al. 2011; Han et al. 2019). In vitro antiglycemic studies on neoagaro-oligosaccharides showed significant inhibition of α-glucosidase and relatively lower inhibition of α-amylase enzymes (Hong et al. 2017). In vivo mice model studies of neoagaro-oligosaccharides showed stimulation of growth in mice suffering from T2D. Administration of different doses of neoagaro-oligosaccharides in T2D mice model showed lowering of blood glucose and improvement in glucose tolerance level. It also able to alleviate hepatocyte swelling and recovered pancreatic β-cells involved in insulin secretion (Lin et al. 2019). Alginate oligosaccharides showed antiglycemic activity as it can stimulate the expression of genes for GLUT4 and insulin receptor. Additionally, it also

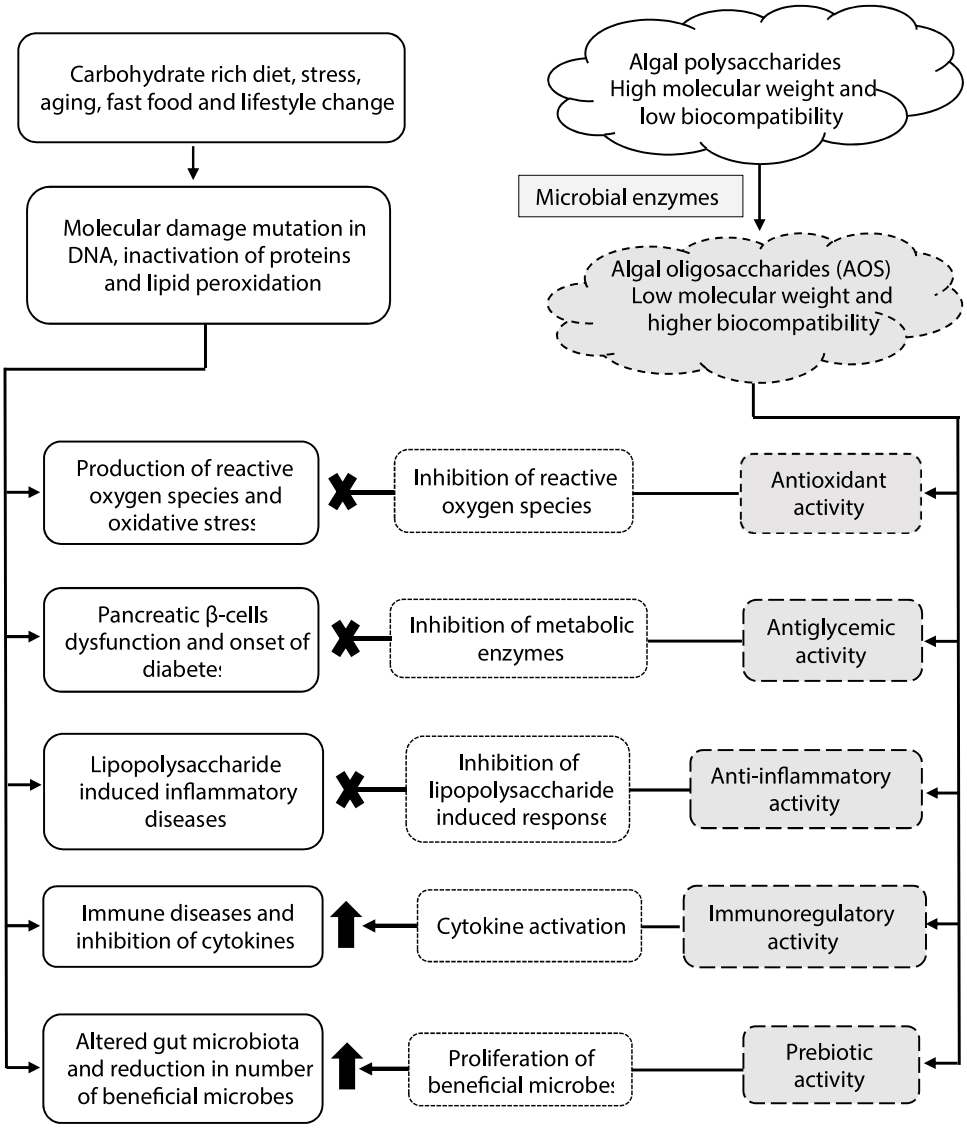

Fig. 8 Schematic representation of bioactive potential of algal oligosaccharides in nutraceutical applications

can enhance phosphorylation of proteins involved in insulin signalling pathways to prevent T2D (Hao et al. 2011). Antiglycemic activity is also reported from crude algal extracts of brown and red algae, and studies show that antioxidant along with antiglycemic activity can aid to control diabetes more efficiently (Holdt and Kraan 2011).

Anti-inflammatory Activity

The oxidative stress leads to the generation of lipopolysaccharide-induced inflammation through elevation of nitric oxide and prostaglandin level. The agaro, neoagaro, and alginate oligosaccharides suppress the elevated level of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines including tumour necrosis factor-α, interleukin-1β, interleukin 6 in lipopolysaccharide-induced murine macrophage, and human monocytes (Enoki et al. 2010; Zhou et al. 2015). Xu et al (2012) reported significant correlation between κ-carrageenan oligosaccharide concentration and inhibition of viability and content of nitric oxide, tumour necrosis factor-α, and interleukin 10 released by lipopolysaccharide-activated microglial cells. Agaro-oligosaccharide administration in colitis induced mice showed the upregulation in expression of heme oxygenase-1 in colonic mucosa. Irrespective to the length of oligosaccharides, the 3, 6-anhydro-α-L-galactose at the reducing end, showed similar induction of heme oxygenase expression (Enoki et al. 2010; Higashimura et al. 2013). In vivo studies on neoagaro-oligosaccharides showed significant inhibition of lipopolysaccharide induced inflammatory response in mouse macrophage cells. The inhibition may be due to the reduction of lipopolysaccharide, induces nitric oxide synthase and interleukin-1β expression through downregulation of both pathways including mitogen activated protein kinase and nuclear factor-κB signalling pathways (Wang et al. 2017a, b). This suggests that the degree of polymerization and the number of sulfate group of oligosaccharides play an important role on their anti-inflammatory activity (Guo et al. 2018).

Immunoregulatory Activity

Prolonged oxidative stress leads to inflammation which leads to excessive production of cytokines and macrophages which can supress the immune response of the individual. The effect of laminarin oligosaccharides on the expression of genes involved in the regulation of immune function showed upregulation of genes for interleukin 7, 15, and interleukin 2 receptor, T-cell receptor beta, which is linked with immunity improvement (Kim et al. 2006). Studies found that the low molecular weight AOS, especially the carrageenan oligosaccharides (1.7 kDa) showed increased secretion of tumour necrosis factor α, involved in immune modulation. Treatment of murine macrophage cell line with carrageenan oligomer results in the upregulation of expression of toll-like receptor 4, cluster of differentiation 14, and myeloid differentiation 2 (Yuan et al. 2011). Sulfated fucoidan oligosaccharides produced after enzymatic hydrolysis of fucoidan showed induction in the expression level of antigen activation involved in innate and adaptive immunity (Silchenko et al. 2013; Yao et al. 2014). The unsaturated alginate oligosaccharide induced nitric oxide production, nitric oxide synthase, and stimulated tumour necrosis factor-α production. It also induces macrophage activation to release nitric oxide and tumour necrosis factor-α involved in nuclear factor-κB and mitogen-activated protein kinase signalling pathways (Xu et al. 2014). The carrageenan oligosaccharide showed higher induction of interleukin 10 and the cytokine level in both human cell lines and mice blood cells both human cell lines and mice blood cells which improves its immune function (Kalitnik et al. 2016).

Prebiotic Activity

Beneficial gut microbes like *Lactobacillus* sp. and *Bifidobacterium* sp. play an important role as they inhibit the growth of pathogenic bacteria including *Salmonella* sp. or *Escherichia coli*, *Shigella* and *Peptoniphilus* (Han et al. 2019). Any change in the composition of the gut microflora due to oxidative or physiological stress can destabilise the metabolic activity of the individual. Some of the AOS are categorized as prebiotic compounds as they are not digested by gastrointestinal enzymes and helps in the proliferation of beneficial gut microbes. The neoagaro-oligosaccharides found to have high inhibition to enzymes of upper gastrointestinal tract, so it can reach to the colon and serve as substrate for intestinal microflora (Hong et al. 2017). The administration of neoagaro (Hu et al. 2006) and alginate oligosaccharides (Wang et al. 2006a, b) is shown to significantly proliferate the number of *Bifidobacterium* and *Lactobacillus* anaerobically with no side effect during in vivo and in vitro studies. These AOS were able to stimulate better growth of beneficial bacteria in comparison with fructooligosaccharides (FOS) or galactooligosaccharides (Hu et al. 2006). In addition to this, reports show that these AOS are safe for consumption as they do not cause any adverse effects like belching and abdominal bloating and improves the general physiological well-being of the individual (Wang et al. 2006a, b).

Most of the AOS from red and brown algal polysaccharides shows very good bioactivity which has wide therapeutic applications. However, so far, there are no studies on the bioactivity of green algal AOS, which is the least explored group of AOS (Table 2). The antioxidant,

antiglycemic, anti-inflammatory, immunoregulatory, and prebiotic properties are essential criteria for an efficient nutraceutical or functional food (Fig. 8) which is in high demand globally.

Multiple Bioactive Potentials of Enzymatically Produced Algal Oligosaccharides for Nutraceutical Application

The present generation is greatly affected due to the changes in lifestyle and frequent consumption of carbohydrate rich diet and decreased physical exercise. This leads to accumulation of high glucose in blood for a prolonged period of time causing hyperglycaemia. Increased glucose accumulation in the blood leads to its autoxidation, oxidative phosphorylation and formation of advanced glycation end products which lead to generation of ROS (Fig. 8). In an individual with a healthy physiological condition, ROS are effectively eliminated by antioxidant enzymes produced by the defense system of the body. However, excessive ROS production overwhelms the natural detoxification system and leads to oxidative stress. The induction of cellular pathways by oxidative stress leading to the onset of various chronic diseases including diabetes due to pancreatic β-cell dysfunction, lack of insulin production, and malfunction of insulin receptors. It is one of the major chronic ailments, and after its onset, the first-line of treatment is through the inhibition of α-amylase and α-glucosidase enzyme activity. These are the primary enzymes which are involved in the metabolism of dietary carbohydrates into glucose. Inhibition of these enzymes can significantly decrease the blood glucose level. Therefore, it is the most common therapeutic approach to delay glucose release and absorption (Lin et al. 2019). However, preventing the oxidative stress using AOS with antioxidant property in combination with antiglycemic activity could be a much effective strategy for diabetes management. Hence, there is a huge demand for natural sources of bioactive compounds with ROS scavenging and antiglycemic activity.

Oxidative stress also causes immune diseases, inflammatory disorders, and alteration in gut microbiota (Chen et al. 2006). AOS are known to prevent all of these chronic disorders as they play a significant role to inhibit the lipopolysaccharide induced response and activate cytokine to prevent inflammatory and immune diseases respectively. It also can proliferate the beneficial gut microbiota and inhibit the growth of pathogenic bacteria (Fig. 8). Hence, algal oligosaccharides have gained a lot of attention in food industry as it can greatly aid in preventing and managing chronic diseases and to improve the general health of the individuals. An understanding of the various enzymes and AOS produced through enzymatic hydrolysis from diverse algal polysaccharides is of utmost importance. This can facilitate its potential to be widely explored for functional food applications which is the need of the hour as there are increasing reports of chronic diseases among individuals of age groups starting from toddlers to aging adults globally.

Conclusions

Algal sugars and bioactive products of macroalgae have been studied and well known for its nutraceutical and pharmaceutical properties. However, there are a few limitations for efficient utilisation of macroalgal biomass globally. Lack of efficient processing methods and low yield limits the complete utilisation of algal based products. Identification of efficient microbial enzymes for production of bioactive algal oligosaccharides can greatly improve the bio utilisation efficiency of algal products. This can increase the commercial value of the algal biomass collected from the wild or from aquaculture practices and pave way for sustainable utilization of macroalgae and its bio-prospection which is an underexplored marine bioresource.

Acknowledgements This is NIO contribution number 6685.

Funding Ashok S Jagtap received Junior Research Fellowship, Grant number 31/026/(0300)/2017-EMR-I, from Council of Scientific and Industrial Research, India.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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ISSN 1063-0740, Russian Journal of Marine Biology, 2021, Vol. 47, No. 6, pp. 489–497. © Pleiades Publishing, Ltd., 2021.

ORIGINAL PAPERS

Antioxidant and Antiglycemic Properties of Macroalgae, an Underutilized Blue Economy Bioresource in India[1]

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Received May 14, 2021; revised July 14, 2021; accepted September 17, 2021

Abstract—Macroalgae are marine bioresources with a myriad of bioactive potential to treat various chronic diseases such as cancer, obesity, diabetes and coronary heart diseases. Though there is a vast diversity of macroalgal species along the coastal states of India, they are not fully explored for their nutraceutical benefits. In this study, the antioxidant and antiglycemic properties along with its polysaccharide and total phenolic content of fifteen selected macroalgae from the Indian coast was evaluated. The antioxidant activity was assessed based on the radical scavenging and reducing power efficiency and the antiglycemic activity was determined based on its α-glucosidase inhibition capacity. The results show that *Pterocladia capillacea* (Rhodophyta) and *Stoechospermum marginatum* and *Sphacelaria rigidula* (Phaeophyta) exhibited good antioxidant and antiglycemic activity, equivalent to model compounds. Statistical analysis shows a highly significant correlation between both these activities and the total phenolic content. Characterizing the bioactivity can benefit the current generation as they are greatly affected by various chronic diseases due to oxidative stress. Exploring the macroalgae can provide potential natural alternatives, which are of great demand in the healthcare industry and pave way for its sustainable utilization for creating the Blue Economy.

Keywords: Antiglycemic activity, antioxidants, bioresource, marine macroalgae, oxidative stress

DOI: 10.1134/S1063074021060067

INTRODUCTION

Macroalgae or seaweeds are a sustainable source of various bioactive molecules with diverse health benefits and have wide applications as pharmaceutical and nutraceutical compounds [9, 23]. Their polysaccharide, pigment, protein and phenolic contents are largely known to be responsible for their bioactivity [26]. These biochemical components of macroalgae are unique and are not present in any of the other aquatic or terrestrial plants [19]. They are well accepted as a marine vegetable and consumed regularly only in South East Asian countries and some parts of Europe, America and Australia; but seldom explored in India [2, 7]. Studies show that regular consumption of macroalgae can reduce the occurrence of chronic diseases including cancer, obesity, and diabetes and also reduces the mortality rate, as they have higher activity of antioxidant enzymes [23, 28]. However, the bioactive potential of macroalgae are not fully explored for its nutraceutical benefits, though there is a vast diversity, of about 5500 macroalgal species across the globe [16]. Along the 7500 km long coastline of India, approximately 900 species and subspecies of seaweeds are recorded. Major seaweed diversity are reported from Tamil Nadu (302), followed by Gujarat (202), Maharashtra (152), Lakshadweep (89), Andhra Pradesh (79) and Goa (75) coast [25]. But in India, major emphasis is made only on red algae, *Gracilaria* sp. and *Kappaphycus* sp., which are commercially utilized for its nutritional value.

In recent years, there has been a growing interest in natural antioxidants as the present generation is greatly affected by changes in lifestyle and frequent consumption of carbohydrate rich diet, decreased physical exercise and aging, leading to oxidative stress. Prolonged exposure to oxidative stress can lead to the onset of many chronic diseases. In an individual with healthy physiological condition, reactive oxygen species (ROS) are effectively eliminated by antioxidant enzymes produced by the defence system of the body. However, an excessive production of ROS overwhelms the natural body defence system, which is not capable to detoxify the large production of free radicals. This triggers various cellular pathways and leads

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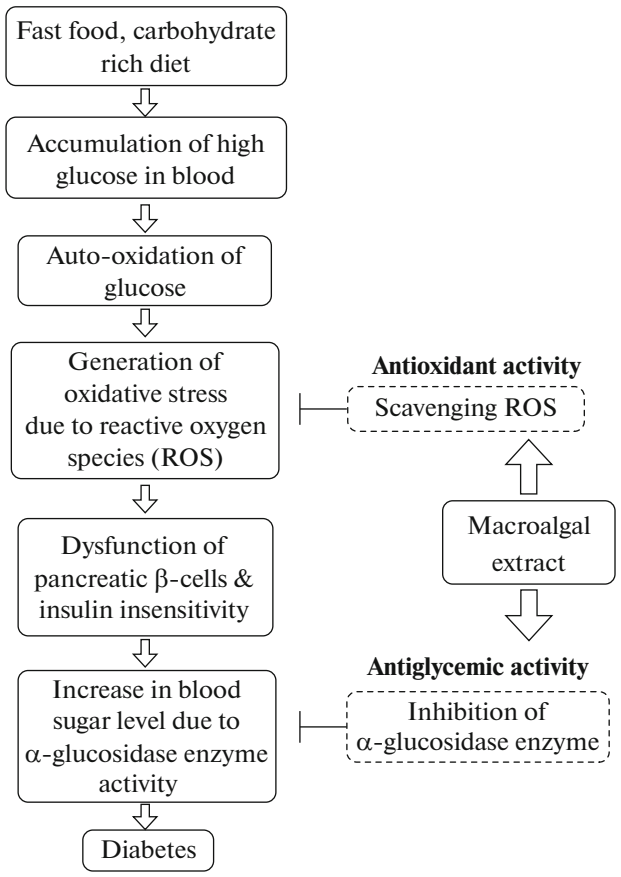

Fig. 1. Schematic representation of effective diabetes management by macroalgal biomolecules with antioxidant and antiglycemic activities.

to several physiological damage such as pancreatic β-cells dysfunction leading to the onset of diabetes, lipopolysaccharide induced inflammatory diseases, immune diseases and inhibition of cytokines [27].

According to the recent report of the International Diabetes Federation, India is the second country in the world with the highest maximum number of people affected by diabetes (77 million), only preceded by China (116.4 million). Diabetes mellitus is one of the major chronic disorders that affects a large majority of today's population across all age groups including infants. Estimates say that there are nearly 463 million people who are currently affected worldwide by diabetes and the counts could increase up to 700 million by 2045. It is characterized by persistent high blood glucose level, which sets in due to improper carbohydrate metabolism caused by inadequate pancreatic insulin production, insulin action or both [3, 8].

Diabetes is a chronic ailment and after its onset, the first-line of treatment is through the inhibition of α-amylase and α-glucosidase enzyme activity [20]. These are the primary enzymes which are involved in the metabolism of dietary carbohydrates into glucose. Inhibition of these enzymes can significantly decrease the blood glucose level and therefore is the most common therapeutic approach to delay glucose release and absorption. Inhibiting the α-glucosidase activity is widely used as it is the key enzyme catalyzing the final step of carbohydrate metabolism, involved in the release and elevation of blood glucose level [8, 13]. However, preventing ROS stress using algal biomolecules with antioxidant properties in combination with antiglycemic activity could be a much effective strategy for diabetes management (Fig. 1). Therefore, it is very important to identify natural sources of bioactive compounds that exhibit ROS scavenging and antiglycemic activity.

In this study, a few less studied macroalgae were evaluated for their *in vitro* antioxidant and antiglycemic activity which can be used effectively for diabetes management (Table 1). Macroalgae *Pterocladia capillacea*, *Gracilaria corticata*, *Halymenia venusta, Hypnea musciformis, Porphyra umbilicalis* (Rhodophyta), *Padina tetrastromatica, Spatoglossum asperum, Sargassum cinereum, Stoechospermum marginatum, Sphacelaria rigidula* (Phaeophyta) and *Chaetomorpha antennina, Caulerpa peltata, Caulerpa racemosa, Ulva fasciata, Ulva lactuca* (Chlorophyta) were selected on the basis of their biomass availability in post-monsoon season along the west coast of India [25]. Reports on the bioactivity of *Kappaphycus alvarezii, Gracilaria opuntia* and *Enteromorpha* species are well documented [6, 15], but there is a lack of knowledge about the bioactivity of many other abundantly available seaweeds. Understanding of the bioactive potential of macroalgae especially for treatment of chronic diseases and for improving the general physiological wellbeing of the individual can promote the widespread consumption of macroalgae in India. There is the need of the hour for efficient utilization and bioprospection of macroalgae, which are an underexplored marine bioresource.

MATERIALS AND METHODS

Sampling of Macroalgae and Preparation of Macroalgal Extract

Macroalgal samples were collected from the western coast of India at selected rocky shore locations, along Gujarat coast at Okha and Porbandar; at Malvan from Maharashtra coast and from Goa coast at Vagator, Anjuna, Dona Paula and Palolem (Fig. 2), during low tide in the post-monsoon season between September to December of 2017 and 2018. The water temperature in the sampling sites was approx. 28 ± 2°C, the salinity was 36 ± 1 psu . Fifteen different macroalgal samples were selected for this study (Table 1), they were manually collected and thoroughly rinsed to remove the attached sand particles, epiphytes and seashells. The macroalgal samples belonging to all the three algal phyla Rhodophyta, Phaeophyta and Chlorophyta were identified morphologically based on tax-

Table 1. List of macroalgae evaluated in this study

Extract Id	Macroalgae identification	Phylum	Sampling location
MEX1	*Pterocladia capillacea* (S.G.Gmelin) Santelices & Hommersand	Rhodophyta	Dona Paula, Goa
MEX2	*Gracilaria corticata* (J.Agardh) J. Agardh	Rhodophyta	Vagator, Goa
MEX3	*Halymenia venusta* Børgesen	Rhodophyta	Okha, Gujarat
MEX4	*Hypnea musciformis* (Wulfen) J.V. Lamouroux	Rhodophyta	Palolem, Goa
MEX5	*Porphyra umbilicalis* Kützing	Rhodophyta	Vagator, Goa
MEX6	*Padina tetrastromatica* Hauck	Phaeophyta	Anjuna, Goa
MEX7	*Spatoglossum asperum* J. Agardh	Phaeophyta	Palolem, Goa
MEX8	*Sargassum cinereum* J. Agardh	Phaeophyta	Dona Paula, Goa
MEX9	*Stoechospermum marginatum* (C. Agardh) Kützing	Phaeophyta	Palolem, Goa
MEX10	*Sphacelaria rigidula* Kützing	Phaeophyta	Dona Paula, Goa
MEX11	*Chaetomorpha antennina* (Bory) Kützing	Chlorophyta	Anjuna, Goa
MEX12	*Caulerpa peltata* J.V. Lamouroux	Chlorophyta	Malvan, Maharashtra
MEX13	*Caulerpa racemosa* (Forsskål) J. Agardh	Chlorophyta	Okha, Gujarat
MEX14	*Ulva fasciata* Delile	Chlorophyta	Porbandar, Gujarat
MEX15	*Ulva lactuca* Linnaeus	Chlorophyta	Vagator, Goa

onomic keys [12]. The macroalgal specimens were individually shade dried at an ambient temperature of 28 ± 5°C for 2–3 days followed by further heat drying in a ventilated oven at 40 ± 2°C for 12 h and finely ground using an electrical mixer-grinder into a fine powder. Macroalgal extracts (MEX) were prepared by suspending approximately 10 gm of dry powdered samples of each algae in 400 mL of the distilled water and refluxed in the Soxhlet apparatus at 80°C for 12 h. The macroalgal extracts of 15 macroalgae samples belonging to all the three phyla were used for this study (Table 1). Each of the MEX was concentrated using a rotary evaporator (Roteva, India), lyophilized on a freeze-dryer (Labconco, USA) and used for further experiments. All fine biochemicals were purchased from Sigma Aldrich, USA; and the enzyme and substrates were obtained from Sisco Research Laboratories, India.

Estimation of Polysaccharide Content

Total sugars present in the MEX were estimated by phenol sulfuric acid method. Briefly, the MEX was mixed with 5% phenol, followed by the addition of 96% sulfuric acid and cooled for 10 min. The intensity of the colored complex was measured at 490 nm with a UV-Visible spectrophotometer (Shimadzu, Japan). Reducing sugar was also determined using dinitrosalicylic acid method. For this, the MEX was mixed with 3, 5-dinitrosalicylic acid reagent followed by incubation for 5 min in a boiling water bath to stop the reaction. Then the tubes were cooled and the absorbance of the colored complex formed was measured at 540 nm. The non-reducing sugar, which is the polysaccharide content (PC) of the macroalgae, was determined from the total and reducing sugar concentrations estimated for each MEX. Glucose was used as a standard, and the PC content of the MEX is expressed as μg mg^{-1} dry weight of the MEX.

Estimation of Total Phenolic Content

The total phenolic content (TPC) of the MEX was determined using the Folin–Ciocalteu method reported by Martins with slight modifications [17]. In brief, MEX were mixed with Folin–Ciocalteu reagent and allowed to stand for 5 min at 25°C to facilitate reaction. Then, Na_2CO_3 (7.5% in solution in water) was added to destroy the residual reagent and kept for 2 h incubation. At the end of the incubation period, the absorbance was measured at 760 nm, and the TPC content was estimated from the gallic acid calibration curve and expressed as mg GAE g^{-1} dry weight of the MEX.

Bioactivity Assay for Estimating the Antioxidant Potential

Antioxidant activity of MEX was tested at four different concentrations between 0.1–2 mg mL^{-1} based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide (H_2O_2) radical scavenging potential and reducing power (RP) efficiency. Ascorbic acid was used as a model compound to compare antioxidant capacity.

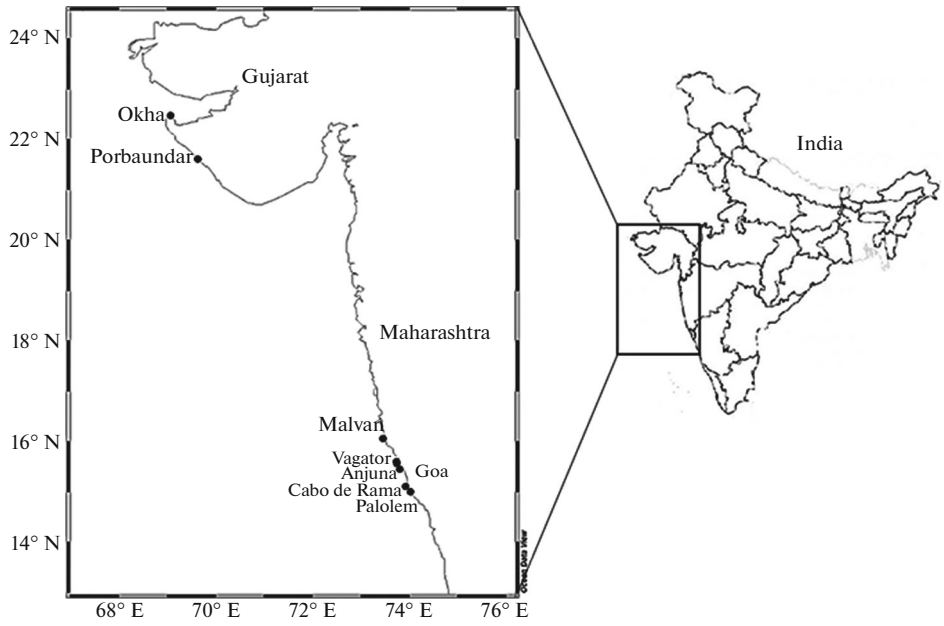

Fig. 2. Location of sampling sites along the North West coast of India.

The DPPH radical scavenging activity of the MEX was evaluated basing on the modified method of Kedare and Singh [10]. Briefly, 0.2 mM DPPH-methanol solution was mixed with various concentrations of MEX. The reaction mixture was incubated for 30 min at 25°C in dark and absorbance was measured at 517 nm to calculate percentage of DPPH radical scavenging activity. The ABTS radical scavenging potential of MEX was determined by an improved ABTS decolorization assay [4]. Oxidation of 7 mM ABTS and 2.45 mM potassium persulphate leads to generation of ABTS radicals. The ABTS radical solution was mixed with different concentrations of MEX, and the reaction mixture was incubated for 30 min at 25°C in dark. A decrease in absorbance was measured at 734 nm, and the percentage of ABTS radical scavenging activity was calculated. The potential of MEX to scavenge H_2O_2 radicals was determined by the method reported by Fernando [5]. The reaction mixture consisted of MEX, 9 mM $FeSO_4$, 9 mM salicylic acid, and 9 mM H_2O_2. The mixture was incubated at 37°C for 10 min, the absorbance was recorded at 510 nm, and the H_2O_2 percentage radical scavenging activity was calculated.

The radical scavenging/inhibition activity (%) was calculated using the below formula:

$$\text{Scavenging activity}\,(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100.$$

Where A_0 is the absorbance of control without a sample, A_1 is the absorbance in the presence of the sample.

RP of the fifteen MEXs was evaluated by the method reported by Chakraborty [1] with slight modifications. In brief, MEX was mixed with sodium phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After incubation, 10% trichloroacetic acid was added to the mixture and centrifuged at 8000 × g for 5 min followed by the addition of 0.1% ferric chloride to the supernatant. The absorbance of the colored complex was measured at 700 nm and the increment in absorbance indicates an increase in RP.

Bioactivity Assay for Estimating the Antiglycemic Potential

The antiglycemic activity of the fifteen MEXs was evaluated through α-glucosidase inhibition (αGI) assay carried out using the method described by Zaharudin [29]. For this, the enzyme α-glucosidase (0.1U/mL) was premixed with different concentrations (0.005–0.250 mg) of MEX and acarbose in 20 mM sodium phosphate buffer (pH 6.9). After 20 min incubation at 37°C, 3 mM p-nitrophenyl-α-D-glucopyranoside were added. The reaction was aborted by adding 0.1M Na_2CO_3 and the absorbance measured at 405 nm to estimate the inhibition of α-glucosidase activity and represented as percentage activity.

Statistical Analysis

All experimental studies were carried out in triplicates ($n = 3$), and mean values were expressed along with standard deviations. The IC_{50} value was calculated using GraphPad Prism 7 software. Statistical

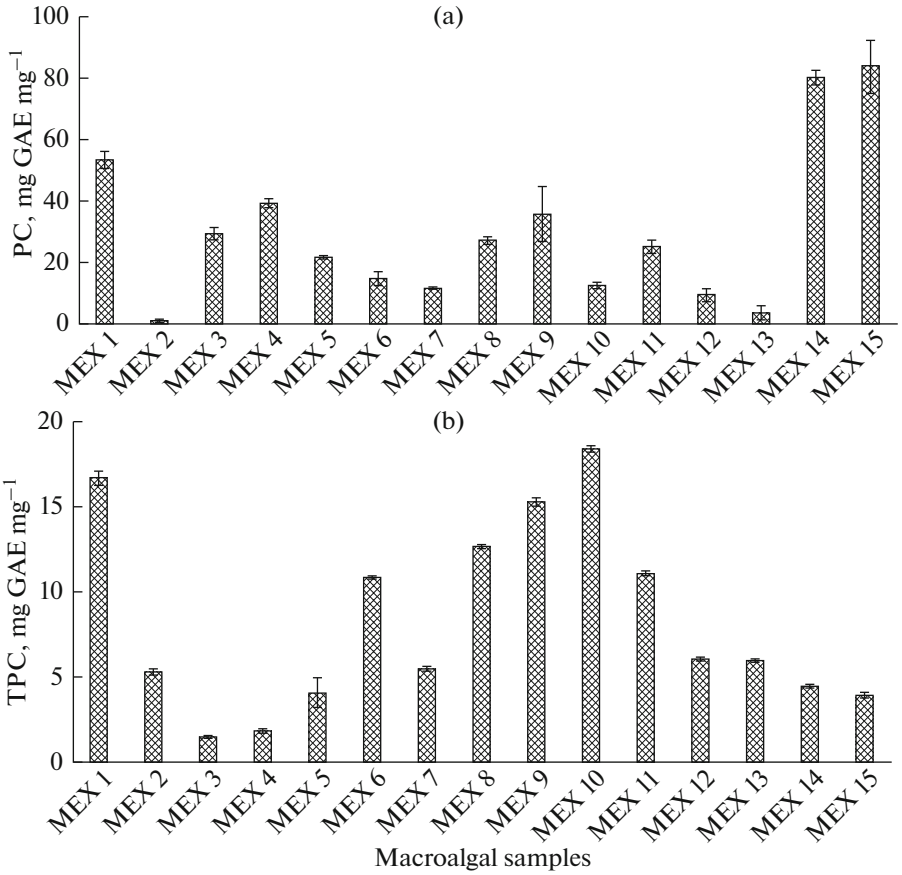

Fig. 3. (a) Polysaccharide content (PC) and (b) total phenolic content (TPC) of macroalgal extracts (MEX). The error bars denote standard deviations of means (means ± *SD*, $n = 3$).

analysis was carried out to evaluate the relationship between the variables, and the significance level was set as $P \leq 0.05$.

RESULTS

Polysaccharide Content

In this study, the PC of fifteen MEXs was determined (Fig. 3a); the MEX14 and MEX15 of the green algae *Ulva fasciata* and *Ulva lactuca* showed the highest PC of 83.68 and 80.2 mg g^{-1}, followed by MEX1 and MEX4 of the red algae *Pterocladia capillacea* and *Hypnea muciformis* with 53.43 and 39.12 mg g^{-1} of PC, respectively. The maximum PC content in the brown algae studied was recorded in MEX9 of *Stoechospermum marginatum* with 35.68 mg g^{-1}. In all other macroalgae, the PC was less than 30 mg g^{-1} (Fig. 3a).

Total Phenolic Content

Among the macroalgal specimens analyzed in this study, MEX10 of *Sphacelaria rigidula* showed the highest TPC of 18.40 mg g^{-1}GAE. It was followed by MEX1 and MEX9 of *Pterocladia capillacea* and *Stoechospermum marginatum* with 16.68 mg g^{-1}GAE and 15.30 mg g^{-1}GAE of TPC, respectively. The TPC in all other macroalgae tested was less than 13 mg g^{-1} GAE (Fig. 3b).

Antioxidant Activity

The DPPH, ABTS and H_2O_2 radical scavenging activity were determined at different concentrations in all the fifteen MEXs studied (Table 1). MEX9 and MEX1 of *Stoechospermum marginatum* and *Pterocladia capillacea* showed the highest DPPH radical scavenging activity of 98 and 93%, respectively, which was close to ascorbic acid, followed by MEX10 (*Sphacelaria rigidula*) with 68% scavenging activity at 2 mg mL^{-1} concentration. In ABTS assay, the MEX9 of the brown algae *S. marginatum* showed the highest ABTS radical scavenging activity of 65%, followed by *S. rigidula* MEX10, with 36% radical scavenging. MEX9 and MEX10 of the brown algae *S. marginatum* and *S. rigidula* also showed the highest H_2O_2 radical scavenging activity with 89 and 75%, respectively. RP assay is also used to determine the antioxidant potential, which is a rapid and robust screening method. It

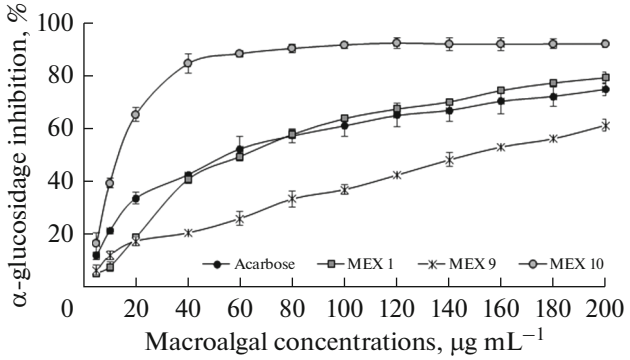

Fig. 4. Antiglycemic activity of macroalgal extracts of *Pterocladia capilacea* (MEX1), *Stoechospermum marginatum* (MEX9) and *Sphacelaria rigidula* (MEX10) at different concentrations. The error bars denote standard deviations of means (means ± *SD*, $n = 3$).

is based on the capacity of the antioxidant molecules to reduce ferric chloride to form a ferric-ferrous colored complex, which can be quantified as it has the maximum absorption at 700 nm. The concentration-dependent antioxidant capacity of the MEX was estimated as a function of RP (Supplementary Table S1). The MEX1 of the red macroalga *Pterocladia capillacea* showed the highest RP of 29.3 mg g^{-1}AAE followed by MEX10 and MEX9 of the brown macroalgae *Sphacelaria rigidula* and *Stoechospermum marginatum* with 28.6 mg g^{-1}AAE and 22.9 mg g^{-1}AAE, respectively.

The IC_{50} values for each of the radicals were calculated at four different concentrations between 0.1 to 2 mg mL^{-1} of the MEX along with their radical scavenging activity (Supplementary Table S1). The highest DPPH radical scavenging activity was shown by MEX of *Stoechospermum marginatum* with IC_{50} 0.22 mg mL^{-1}, followed by *Pterocladia capillacea* and *Sphacelaria rigidula* having an IC_{50} value of 0.5 and 0.9 mg mL^{-1}, respectively. The ABTS radical scavenging activity of MEXs of the brown macroalgae *S. marginatum* and *S.rigidula* showed an IC_{50} value of 1.33 and 3.9 mg mL^{-1}, respectively. In H_2O_2 radical scavenging assay, the IC_{50} value observed in the MEX of *S. marginatum* was the lowest at 0.38 mg mL^{-1} followed by *S. rigidula* (1.05 mg mL^{-1}) and *P. capillacea* (1.49 mg mL^{-1}).

Antiglycemic Activity

Antiglycemic activity based on αGI assay was estimated for all the fifteen MEXs at 0.5 mg mL^{-1} concentration. Among the macroalgae tested, only three MEXs—MEX1 of *Pterocladia capillacea*, MEX9 of *Stoechospermum marginatum*, and MEX10 of *Sphacelaria rigidula* showed positive results and were further tested for enzyme inhibition activity at different concentrations (0.005 to 0.2 mg mL^{-1}). The brown macroalga *S. rigidula* (MEX10) showed a very good αGI activity of 91.84%, followed by *P. capillacea* (MEX1) and *S. marginatum* (MEX9) with 66% and 56% inhibition, respectively (Fig. 4). The highest αGI activity with the lowest IC_{50} value was shown by MEX10 of the brown algae *Sphacelaria rigidula* at 0.013 mg mL^{-1}, which was lower than positive control acarbose with an IC_{50} value of 0.054 mg mL^{-1}. The MEX1 and MEX9 of macroalgae *Pterocladia capillacea* and *Stoechospermum marginatum* also showed good αGI activity with IC_{50} value 0.062 and 0.151 mg mL^{-1}, respectively. *S. rigidula* with a very low IC_{50} value (0.013 mg mL^{-1}) and high (91.84%) αGI proved to have the highest αGI activity, surpassing the model compound acarbose, which is widely used for diabetes control (Supplementary Table S2).

Correlation between the antioxidant and antiglycemic activities with PC and TPC was carried out to understand the contribution of these biomolecules to the bioactivity. A significant to highly significant correlation was observed between TPC and antioxidant activity based on all the four assays and also with antiglycemic activity. A highly significant correlation observed between RP and αGI shows that antioxidant and antiglycemic activities are significantly correlated, and TPC has a significant contribution to the antioxidant and antiglycemic activity of the MEXs studied (Table 2).

DISCUSSION

Macroalgae are known to have the most generous reserve of marine natural products in the ocean. They generally grow in rocky intertidal regions and are constantly exposed to solar radiation, temperature and tidal fluctuations creating oxidative stress [14]. Hence, they have developed a strong defense system and many novel biomolecules to adapt to harsh environmental conditions. Macroalgal biomolecules such as pigments, fatty acids, polyphenols, sterols, carotenoids, dietary fibers and polysaccharides are known to have a wide range of bioactivity as antioxidants, antidiabetic, immuno-regulatory, prebiotic, anticancer and antiviral agents [21, 23]. The polysaccharide content of the seaweeds is different from that of terrestrial plants as they are devoid of hemicellulose. Seaweeds are composed of unique polysaccharides such as carrageenan, agar, porphyran, alginate, fucoidan, laminarin and ulvan. The PC content estimated shows that *Ulva* spp. have the highest PC followed by *Pterocladia capillacea, Hypnea muciformis* and *Stoechospermum marginatum* with more than 35 mg g^{-1}. Among the macroalgae studied, *Sphacelaria rigidula, P. capillacea* and *S. marginatum* had the maximum TPC content. These biomolecules showed good correlation with the biological activity (Table 2).

DPPH, ABTS and H_2O_2 are simple, rapid and extensively used free radicals to evaluate the antioxi-

Table 2. Pearson correlation between the algal polysaccharide content (PC), total phenolic content (TPC) and the bioactivity assays for antioxidant and antiglycemic activities

	PC	TPC	DPPH	ABTS	H_2O_2	RP	αGI
PC	1						
TPC	−0.220	1					
DPPH	0.226	0.668[b]	1				
ABTS	−0.106	0.829[c]	0.656[a]	1			
H_2O_2	0.225	0.728[b]	0.755[b]	0.686[b]	1		
RP	−0.043	0.774[c]	0.743[b]	0.575[a]	0.822[c]	1	
αGI	0.004	0.797[c]	0.784[c]	0.652[a]	0.850[c]	0.960[c]	1

[a]Moderately significant at $P < 0.05$, [b]significant at $P < 0.01$, [c]highly significant at $P < 0.001$.

dant potential of biomolecules. During the radical scavenging assay, the efficiency of the antioxidant molecules to stabilize the free radicals by accepting a hydrogen ion to produce a non-radical compound was tested. Antioxidant activities are studied extensively and reported in various macroalgal biomolecules such as pigments, sugars and phenolic compounds. Macroalgal biomolecules such as pigments, TPC and PC are largely studied for their antioxidant activity [24]. Results of our study show that the brown macroalgae *Stoechospermum marginatum* (MEX9), and *Sphacelaria rigidula* (MEX10) and the red macroalga *Pterocladia capillacea* (MEX1) had the maximum radical scavenging and RP activity. Among these, *S. marginatum* shows the highest antioxidant activity with the lowest IC_{50} value (Supplementary Table S2). One of the major applications for the antioxidant biomolecules is to prevent chronic diseases, especially diabetes which is widely distributed [27].

Studies from across the globe have tried to understand the efficiency of various algal antioxidant biomolecules for the management of diabetes [29]. Since the reduction of ROS activity along with the antiglycemic activity can play a crucial role in controlling the sugar levels, the current emphasis of the research is to study and identify biomolecules, which have both antioxidant and antiglycemic activity. A few studies have specifically targeted the antiglycemic activity of the phenolic compounds and MEX [8, 20]. Antiglycemic activity based on the inhibition of α-glucosidase, the major gastroinstential enzyme, is considered as one of the approaches to the prevention and treatment of diabetes. The α-glucosidase can be inhibited by several oral α-glucosidase inhibitors such as acarbose and voglibose [22]. They have limited potency and common side effects like flatulence, bloating, abdominal pain or discomfort, abdominal fullness, and nausea [3]. Hence there is a demand for natural α-glucosidase inhibitors, and our results show that macroalgae have very good potential. Results from this study show that the IC_{50} values for αGI activity of the three best macroalgae (MEX1, MEX9 and MEX10) ranged from 0.013 to 0.151 mg mL^{-1}. The potential of these MEXs to inhibit α-glucosidase activity is higher than those reported earlier for *Halimeda macroloba* with an IC_{50} value of 6.38 mg mL^{-1} [2]; *Sargassum siliquosum* (0.57 mg mL^{-1}) and *Sargassum polycystum* (0.69 mg mL^{-1}) and *Undaria pinnatifida* with IC_{50} 0.080 mg mL^{-1} [18, 29]. The MEX10 of the brown alga *Sphacelaria rigidula* even showed a better αGI activity than that of the standard drug acarbose even at a low concentration from aqueous extraction (Fig. 4). Previous reports of antiglycemic activity from *Fucus vesiculosus* was reported to have a very high α-glucosidase inhibition activity at low IC_{50} value of 0.32 μg mL^{-1} through solvent (80% ethanol) extraction [13]. However, three macroalgae *Pterocladia capillacea*, *S. rigidula* and *Stoechospermum marginatum* are shown to have good antioxidant and antiglycemic activities (Supplementary Table S2) from the biomolecules obtained through aqueous extraction, which has far reaching applications as functional foods.

A significant correlation was observed between PC and TPC for antioxidant and antiglycemic activity (Table 2). Ganesan [6] reported the radical scavenging activity of macroalgae to be mostly related to their TPC. Lordan [13] have shown that TPC is contributing to both antioxidant and antiglycemic activities. Earlier reports on polysaccharides show that PC correlates with antiglycemic activity [11, 15]. Our results show that TPC has significantly contributed to the antioxidant and antiglycemic activities of the macroalgae *Pterocladia capillacea*, *Sphacelaria rigidula* and *Stoechospermum marginatum*. Such macroalgae with multiple bioactivities have wider applications and an edge over other macroalgae. They can be used effectively for diabetes management and as functional foods (Fig. 1). The present generation can be greatly benefited by the algal biomolecules as they are under constant oxidative stress and affected by many chronic diseases and allied physiological disorders [9].

CONCLUSIONS

This study shows that macroalgae with a combined antioxidant and antiglycemic activities have good potential for diabetes management. The aqueous macroalgal extracts show promising results and can be used for various nutraceutical formulations. It can play an important role in preventing the onset of diabetes and also as an alternative therapy for diabetes management. Seaweeds remains to be one of the most unutilized traditional marine bioresource. The huge nutritional potential of seaweeds is largely unexplored and identification of value-added applications in the nutraceutical and pharmaceutical industry can boost the seaweed aquaculture industry.

ACKNOWLEDGMENTS

The first author is thankful to Council of Scientific and Industrial Research, India, for the award of Junior Research Fellowship. Authors would also like to thank the Director of the CSIR-National Institute of Oceanography, Goa, for the laboratory facilities and encouragement to carry out this work. This is NIO contribution number 6764.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S1063074021060067 and are accessible for authorized users.

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Folia Microbiologica (2022) 67:175–191
https://doi.org/10.1007/s12223-021-00943-4

REVIEW

Marine microbial enzymes for the production of algal oligosaccharides and its bioactive potential for application as nutritional supplements

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Received: 29 September 2021 / Accepted: 20 December 2021 / Published online: 7 January 2022

Abstract

Marine macroalgae have a very high carbohydrate content due to complex algal polysaccharides (APS) like agar, alginate, and ulvan in their cell wall. Despite numerous reports on their biomedical properties, their hydrocolloid nature limits their applications. Algal oligosaccharides (AOS), which are hydrolyzed forms of complex APS, are gaining importance due to their low molecular weight, biocompatibility, bioactivities, safety, and solubility in water that makes it a lucrative alternative. The AOS produced through enzymatic hydrolysis using microbial enzymes have far-reaching applications because of its stereospecific nature. Identification and characterization of novel microorganisms producing APS hydrolyzing enzymes are the major bottlenecks for the efficient production of AOS. This review will discuss the marine microbial enzymes identified for AOS production and the bioactive potential of enzymatically produced AOS. This can improve our understanding of the biotechnological potential of microbial enzymes for the production of AOS and facilitate the sustainable utilization of algal biomass. Enzymatically produced AOS are shown to have bioactivities such as antioxidant, antiglycemic, prebiotic, immunomodulation, antiobesity or antihypercholesterolemia, anti-inflammatory, anticancer, and antimicrobial activity. The myriad of health benefits provided by the AOS is the need of the hour as there is an alarming increase in physiological disorders among a wide range of the global population.

Abbreviations

APS	Algal polysaccharides
AOS	Algal oligosaccharides
OS	Oligosaccharides
GH	Glycoside hydrolases
AO	Agaro oligosaccharide
NAO	Neo agaro oligosaccharide
NCO	Neo carrageenan oligosaccharide
PO	Porphyran oligosaccharide
ALO	Alginate oligosaccharide
FO	Fucoidan oligosaccharide
LO	Laminarin oligosaccharide
UO	Ulvan oligosaccharide
EC	Enzyme Commission
PL	Polysaccharide lyase
DP	Degree of polymerization

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Introduction

Macroalgae have a long history of being used as a food ingredient and for its therapeutic value. There is a wide range of literature, from ancient texts to recent studies, which appertain their broad applications throughout history to treat various illnesses and disorders (Cian et al. 2015). Their complex polysaccharides, pigments, and polyphenols are extensively characterized for their bioactivity (Baghel et al. 2014; Nunes et al. 2020). Macroalgae are primitive, aquatic plants with low lignocellulose content; hence, a simple extraction method is applicable to isolate their bioactive compounds (Cheong et al. 2018). They are considered as an important and sustainable source of bioactive molecules having various health benefits. Their biomass comprises about 50% of carbohydrates due to complex algal polysaccharides (APS) agar, carrageenan, alginate, porphyran, laminarin, fucoidan, and ulvan in its cell wall. These polysaccharides are a challenge for various commercial applications because of their complex structure, sulfation, unusual, and

diverse sugar composition (Tanna and Mishra 2019). Algal polysaccharides (APS) are composed of monomers such as pentoses (xylose and arabinose), hexoses (galactose, glucose and mannose), and rare sugars (rhamnose, uronic acids), which are not present in other plant biomass. These APS are unique to macroalgae, but their complex nature limits their applications. They can be depolymerized into bioactive oligosaccharides enzymatically, which have far-reaching applications in health care industries (Cheong et al. 2018).

Oligosaccharides (OS) are carbohydrate complexes of 2–20 monosaccharide units linked by O-glycosidic bonds. Based on the solubility and digestibility of OS, it can be classified into the digestible and nondigestible OS (Patel and Goyal 2011). Digestible OS is the sugar that can be further broken down by the gastrointestinal enzymes and absorbed in the intestinal region. However, nondigestible OS is resistant to digestion by these enzymes and facilitates the growth of probiotic bacteria; hence, they are primarily referred to as prebiotics (Mussatto and Mancilha 2007; Kothari et al. 2014). Oligomer produced through synthesis from simple sugars is called primary OS, such as fructooligosaccharides produced using fructose transferases enzyme from fruits, vegetables, and honey (Kothari et al. 2014). However, OS are largely produced by the hydrolysis of complex polysaccharides and are referred to as secondary OS such as xylooligosaccharides, which are produced from hardwood, softwood, bamboo shoots, wheat bran, and grasses either through enzyme treatment or chemical hydrolysis. OS of terrestrial origin includes glucooligosaccharides, maltooligosaccharides, mannanoligosaccharides, galactooligosaccharides, xylooligosaccharides, and lactooligosaccharides. Chitosan and algal oligosaccharides (AOS) are of marine origin obtained from benthic organisms such as shrimps and macroalgae, respectively (Patel and Goyal 2011). Macroalgae are a rich source of unique polysaccharides that are widely used as emulsifiers and stabilizers to produce confectionery products. Marine microbes have a highly promising potential to hydrolyze these polysaccharides into bioactive oligosaccharides. Enzymatic hydrolysis of APS using marine microbial enzymes is a greener approach to produce bioactive AOS. These enzymatically produced AOS have extensive usage for healthcare applications as nutritional supplements.

Enzymatic production of algal oligosaccharides

Microbial enzymes play an essential role in food and beverage processing, pulp and paper industry, and bio-conversion applications in industries on commercial scales (Adrio and Demain 2014). The sources of microbial enzymes include microbial communities belonging to bacteria, fungi and yeasts, which are globally used to produce economically viable enzymes for commercial applications (Zhang and Kim 2010). The ocean covers more than 70% area on earth, and it has countless microorganisms to produce various biomolecules, which can provide new insights and understanding of enzymes. Marine microbial enzymes are gaining enormous attention as they are the major source of enzymes that can hydrolyze algal biomass, including the complex polysaccharides (Hehemann et al. 2010). Marine microorganisms associated with macroalgae are the promising source to produce biocatalysts for converting complex APS to AOS. They are much likely to produce enzymes that break down these unique polysaccharides (Ghanbarzadeh et al. 2018; Jagtap and Manohar 2021). Studies have shown that the bioactive potential of enzymatically produced OS has better activity than OS made through acid hydrolysis (Xu et al. 2018). Hence, understanding the importance of microbial enzymes in AOS production is vital for the efficient utilization of algal biomass, which is an underutilized blue economy bioresource.

Microbial enzymes for the production of algal oligosaccharides from red algae

Red macroalgae are phylogenetically the oldest division and have the largest number of macroalgal species. Sulfated polysaccharides such as agar, carrageenan, and porphyran are unique and specific to red macroalgae (Cian et al. 2015). These polysaccharides can be enzymatically hydrolyzed into OS through substrate-specific enzymes such as agarase, carrageenase, and porphyranase (Table 1).

Agarase

Agarases are the enzymes that can hydrolyze agar, widely used in the commercial food industry, cosmeceutical, and medical field (Fu and Kim 2010). Agar is a hydrophilic polysaccharide of red macroalgae, composed of repeating residues of 3-O-linked β-D-galactose and 4-O-linked 3, 6-anhydro-α-L-galactose. Agarases grouped into the glycoside hydrolases (GH) family GH16, GH50, GH86, GH118, and GH96 in the carbohydrate-active enzymes (CAZy) database based on its amino acid sequences. Based on their cleavage pattern, agarase can be classified into α-agarase (EC 3.2.1.158) and β-agarase (EC 3.2.1.81), the former cleaves the α-1, 3, and the latter acts on the β-1, 4 linkages. It leads to the production of agaro oligosaccharide (AO) and neoagaro oligosaccharide (NAO) with various degrees of polymerization (Chen et al. 2016). So far, only four α-agarases from the GH96 family are characterized, which includes marine bacteria, *Alteromonas agarlyticus* (Potin et al. 1993), *Thalassomonas* sp. (Ohta et al. 2005), *Thalassomonas* sp. (Zhang et al. 2018), and *Catenovulum sediminis* (Lee et al. 2019) belonging to phylum Proteobacteria (Table 1). However, many β-agarases are reported from

Table 1 Sources of microbial enzymes, agarase, carrageenase, and porphyranase for the production of oligosaccharides from red algae

Source of enzyme	Degree of polymerization in oligosaccharides	Reference
α-agarase (EC 3.2.1.158)		
Proteobacteria		
Alteromonas agarilyticus GJ1B	AO3, AO4	Potin et al. (1993)
Thalassomonas sp. JAMBA33	AO6	Ohta et al. (2005)
Thalassomonas sp. LD5$^{\varepsilon}$	AO4	Zhang et al. (2018)
Catenovulum sediminis WS1A$^{\varepsilon}$	AO4	Lee et al. (2019)
β-agarase (EC 3.2.1.81)		
Proteobacteria		
Microbulbifer elongatus JAMBA7$^{\varepsilon}$	NAO6	Ohta et al. (2004)
Vibrio sp. PO-303$^{\varepsilon}$	NAO2, NAO6	Dong et al. (2006)
Alteromonas sp. SY37-12	NAO4, NAO6	Wang et al. (2006a)
Pseudoalteromonas sp. CY24$^{\varepsilon}$	NAO8, NAO10	Ma et al. (2007)
Acinetobacter sp. AG LSL-1	NAO2	Lakshmikanth et al. (2009)
Pseudoalteromonas sp. AG4$^{\varepsilon}$	NAO2, NAO4, NAO6^{x}	Oh et al. (2010)
Vibrio sp. strain CN41$^{\varepsilon}$	NAO4	Liao et al. (2011)
Agarivorans sp. HZ105$^{\varepsilon}$	NAO4	Lin et al. (2012)
Pseudomonas sp.	NAO2	Gupta et al. (2013)
Catenovulum agarivorans YM01T$^{\varepsilon}$	NAO4, NAO6	Cui et al. (2014)
Thalassospira profundimonas fst	NAO2, NAO4, NAO6	Zeng et al. (2016)
Gayadomonas joobiniege$^{\varepsilon}$	NAO2, NAO4, NAO6	Jung et al. (2017)
Microbulbifer sp. Q7$^{\varepsilon}$ *Microbulbifer* sp. Q7$^{\varepsilon}$	NAO4, NAO6^{p}	Su et al. (2017); Han et al. (2019)
Saccharophagus degradans 2–40	NAO2, NAO4, NAO6^{i}	Kim et al. (2017)
Bacteroidetes		
Zobellia galactanivorans$^{\varepsilon}$	NAO4, NAO6	Jam et al. (2005)
Flammeovirga sp. OC-4$^{\varepsilon}$	NAO4, NAO6	Chen et al. (2016)
Aquimarina agarilytica ZC$^{\varepsilon}$	NAO8	Lin et al. (2017)
Flammeovirga pacifica	NAO2^{x}	Gao et al. (2019a)
Actinobacteria		
Streptomyces coelicolor	NAO2, NAO4, NAO6^{x,m,g}	Hong et al. (2017)
Streptomyces lividans	NAO2^{b}	Yang et al. (2017)
κ-carrageenase (EC 3.2.1.83)		
Proteobacteria		
Pseudomonas carrageenovora	κ-NCO2, κ-NCO4, κ-NCO6	McLean and Williamson (1979)
Pseudoalteromonas sp. AJ5	κ-NCO8, κ-NCO10	Ma et al. (2010)
Pseudoalteromonas porphyrae LL1	κ-NCO4	Liu et al. (2011)
Pseudoalteromonas sp. QY203$^{\varepsilon}$	κ-NCO2, κ-NCO4	Xu et al. (2015)
Vibrio sp. NJ-2	NCO2, NCO4, NCO6, NCO8^{u}	Zhu and Ning (2016)
Thalassospira sp. Fjfst-32	κ-NCO2, κ-NCO4, κ-NCO6	Guo et al. (2018)
Bacteroidetes		
Cytophaga sp.	κ-NCO4	Potin et al. (1991)
Cytophaga sp. MCA-2	κ-NCO4, κ-NCO6^{c}	Haijin et al. (2003)
Tamlana sp. HC4	κ-NCO2	Sun et al. (2010)
Zobellia sp. ZM-2$^{\varepsilon}$ *Zobellia* sp. ZM-2$^{\varepsilon}$	κ-NCO4, κ-NCO6^{p}	Liu et al. (2013); Han et al. (2019)
Cellulophaga lytica N5-2	κ-NCO2, κ-NCO4	Yao et al. (2013)
Pedobacter hainanensis NJ-02$^{\varepsilon}$	κ-NCO4, κ-NCO6	Zhu et al. (2018b)
Zobellia sp. ZL-4$^{\varepsilon}$	κ-NCO4, κ-NCO6	Zhang et al. (2019b)
Actinobacteria		
Cellulosimicrobium cellulans	NCO2	Youssef et al. (2012)

Table 1 (continued)

Source of enzyme	Degree of polymerization in oligosaccharides	Reference
ι-carrageenase (EC 3.2.1.157)		
Proteobacteria		
Alteromonas fortis$^{\varepsilon}$		Michel et al. (2001)
Microbulbifer thermotolerans$^{\varepsilon}$	ι-NCO4^{m}	Hatada et al. (2011)
Bacteroidetes		
Cellulophaga sp. QY3$^{\varepsilon}$	ι-NCO2, ι-NCO4^{x}	Ma et al. (2013)
Flavobacterium sp. YS-80–122	ι-NCO4^{x}	Li et al. (2017a, b)
Wenyingzhuangia fucanilytica	ι-NCO4	Shen et al. (2017)
λ-carrageenase (EC 3.2.1.162)		
Proteobacteria		
Pseudoalteromonas CL19	λ- NCO4	Ohta and Hatada (2006)
Pseudomonas carrageenovora	λ- NCO4, λ-NCO6	Guibet et al. (2007)
β-porphyranase (EC 3.2.1.178)		
Proteobacteria		
Pseudoalteromonas atlantica	PO2, PO4	Przybylski et al. (2015); Correc et al. (2011)
Bacteriodetes		
Zobellia galactanivorans$^{\varepsilon}$	PO4, PO8	Hehemann et al. (2010)
Bacteroides plebeius$^{\varepsilon}$	PO4, PO6	Hehemann et al. (2012)
Wenyingzhuangia fucanilytica$^{\varepsilon}$	PO2, PO4, PO6	Zhang et al. (2019a)
Actinobacteria		
Arthrobacter sp. S-22	PO2, PO4^{b}	Osumi et al. (2002)

Bioactivity of oligosaccharides studied: ^{x}antioxidant, ^{g}antiglycemic ^{b}antiobesity, ^{i}anti-inflammation, ^{m}antimicrobial, ^{u}immunomodulation, ^{c}anti-cancer, and $^{\varepsilon}$source of recombinant enzyme for oligosaccharide production

different microbes in the marine environment belonging to phylum Firmicutes, Proteobacteria, Bacteriodates and Actinobacteria (Table 1).

The β-agarases group into four GH families: GH16, GH18, GH50, and GH86; most of which belong to GH16 and GH50 families and members of the former show conglomerate lichenase and carrageenase activity (Kim et al. 2010; Fu and Kim 2010). The majority of bacteria able to produce β-agarase activity is reported from bacterial isolates belonging to phylum Proteobacteria such as *Microbulbifer elongates*, *Vibrio* sp., *Alteromonas* sp., *Pseudoalteromonas* sp., *Acinetobacter* sp., *Agarivorans* sp., *Pseudomonas* sp., *Catenovulum agarivorans*, *Gayadomonas joobiniege*, and *Thalassospira profundimonas* (Table 1). The β-agarase producing bacteria are also reported from phylum Bacteroidetes including *Zobellia galactanivorans*, *Flammeovirga* sp., and *Aquimarina agarilytica*. *Streptomyces coelicolor* of Actinobacteria phylum are also reported to produce β-agarase (Table 1). α-agarase and β-agarase are shown to have different biochemical properties, amino acid sequences, molecular mass, and catalytic properties. Their substrate specificity to cleave the linkages of the agar proves to be an inevitable tool for the production of agaro-oligosaccharide (AO) with various degrees of polymerization, including agarotriose (AO3), agarotetrose (AO4), neoagarobiose (NAO2), neoagarotetraose (NAO4), neoagarohexaose (NAO6), and neoagarooctaose (NAO8) (Table 1). The AO produced by acid hydrolysis is non-specific; however, β-agarase is reported to produce specific NAO (Xu et al. 2018). These OS are also shown to have application in the plant biotechnology sector to produce plant growth promoters (Gonzalez et al. 2013). Enzymatically produced AO are proven to have eminent antioxidant, antiglycemic, and anti-inflammatory activities used for broad biological functional benefits for human health (Kim et al. 2017; Hong et al. 2017; Gao et al. 2019a). Few of the α-agarase and β-agarase have been characterized at a molecular level and expressed in *Escherichia coli* for the production AO, which has bioactive potential (Oh et al. 2010).

Carrageenase

Carrageenases are the GH enzymes that can hydrolyze glycoside linkage in carrageenan, a sulfated galactan polysaccharide specific to carrageenophytic red macroalgae. Carrageenases are grouped into κ-, ι-, and λ-carrageenase based on their cleavage pattern to break down the κ-, ι-, and λ-carrageenan (Sun et al. 2015). κ-carrageenase (EC

3.2.1.83) is the GH involved in the breakdown of glycosidic linkage between sulfated 3-linked β-D-galactose and 4-linked α-D-galactose of κ-carrageenan (Yao et al. 2013; Zhang et al. 2019b), they predominantly belong to GH16 and GH82 families, and a few are yet to be characterized. The majority of the bacterial genera producing κ-carrageenase belongs to phylum Proteobacteria, which includes *Pseudomonas*, *Pseudoalteromonas*, *Vibrio*, and *Thalassospira* sp.; bacterial genera *Cytophaga*, *Tamlana*, *Zobellia*, *Cellulophaga*, and *Pedobacter* belonging to Bacteriodetes phylum are also reported to produce κ-carrageenase. *Cellulosimicrobium* sp. are the κ-carrageenase producing bacterial species identified so far from marine habitat belonging to Actinobacteria phylum (Table 1). ι-carrageenases (EC 3.2.1.157) hydrolyzes the β-1,4-glycosidic linkages within 3-linked-4-O-sulfated-β-L-galactose and 4-linked-2-O-sulfated-α-D-3,6-anhydrogalactose of ι-carrageenan. ι-carrageenases belong to GH16 and GH82 families can produce carrageenan oligosaccharides (CO) of neo-carrabiose series (Barbeyron et al. 2000). ι-carrageenases are reported from bacterial genera *Alteromonas* and *Microbubifer* belonging to phylum Proteobacteria and *Cellulophaga*, *Flavobacterium*, and *Wenyingzhuangia* belonging to phylum Bacteroidetes. λ-carrageenase (EC 3.2.1.162) is the least studied enzyme that cleaves β-1–4 linkage in the backbone of λ-carrageenan which structurally consists of two sulfated residues per disaccharide unit (Ohta and Hatada 2006). Only a few bacterial species have been reported to produce λ-carrageenase whose GH families are not yet incorporated in the CAZy database (Table 1). Those λ-carrageenase producing bacterial genera from phylum Proteobacteria include *Pseudoalteromonas* and *Pseudomonas* (Ohta and Hatada 2006; Guibet et al. 2007). A more detailed study is required to understand the mechanism of ι- and λ-carrageenase activity to explore its potential to utilize algal biomass efficiently. Few carrageenases have been characterized at a molecular level and expressed in *E. coli* to produce CO with antimicrobial and antioxidant activities (Hatada et al. 2011; Ma et al. 2013).

Porphyranase

Red macroalgae such as *Pyropia* and *Porphyra* have a long evolutionary history as a source of nutritional supplements. Porphyran is a sulfated polysaccharide of these species and is mainly composed of sulfated galactose. Porphyranase is a GH enzyme involved in the cleavage of β-1, 4 glycosidic linkages between methylated porphyran and categorized as β-porphyranase (EC 3.2.1.178) (Zhang et al. 2019a). It belongs to the GH16 and GH86 families (Hehemann et al. 2012) and is transcribed by PorA and PorB gene cluster (Hehemann et al. 2010). Most of the reported porphyranase belongs to the Bacteriodetes phylum, and only a few are reported from Proteobacteria and Actinobacteria (Table 1). Microbial β-porphyranase activity is exhibited in marine bacteria such as *Pseudoalteromonas atlantica*, *Zobellia galactanivorans*, *Arthrobacter* S-22, and *Wenyingzhuangia fucanilytica.* The human gut bacterium *Bacteroides plebeius*, which has the enzyme activity, has entered the human gastrointestinal tract due to consumption of macroalgae (Osumi et al. 2002; Hehemann et al. 2012; Przybylski et al. 2015; Zhang et al. 2019a). β-porphyranase isolated from *Zobellia galactanivorans* and protein extract of the marine bacterium *Pseudoalteromonas atlantica* can hydrolyze porphyran into methylated disaccharides and tetrasaccharides (Correc et al. 2011; Przybylski et al. 2015). The porphyranase from gene Por16A_Wf and Por16A_Wf produced disaccharides of sulfated galactose and complete hydrolysis of porphyran, respectively (Zhang et al. 2019a). The porphyran oligosaccharide (PO) is produced using an enzyme from *Arthrobacter* species. It has antiobesity or antihypercholesterolomic activity as it reduces the serum cholesterol level in mice (Osumi et al. 2002). Few porphyranase genes have been expressed in *E. coli*, and the enzymes were characterized, but its bioactive potential is not explored (Hehemann et al. 2010, 2012; Zhang et al. 2019a).

Microbial enzymes for the production of algal oligosaccharides from brown algae

Brown macroalgae is the second largest group of macroalgae, mainly composed of polysaccharides alginate, fucoidan and laminarin (Deniaud et al. 2017). Alginates are linear unbranched polymers containing mannuronic acid and glucuronic acid residues arranged in a non-regular block-wise pattern along the chain (Yamasaki et al. 2005). Fucoidan is the α-L-fucose containing sulfated polysaccharide. Laminarin is made up of β-1–3 glucan that occasionally has β-1–6 branches (Chen et al. 2019). The enzymatic hydrolysis of brown APS requires polysaccharide lyase (PL) enzymes such as Alginate lyase to break down alginate. The glycoside hydrolase enzymes, fucoidanase and laminarinase, require to cleave the glycosidic bonds in fucoidan and laminarin, respectively (Table 2).

Alginate lyase

The cell wall and intracellular matrix of brown macroalgae are composed mainly of the hydrocolloid alginate. It is a linear hetero-polymer of uronic acid mainly composed of glucuronate and mannuronate residues. It can be either in homo-polymeric (GG/MM) blocks or hetero-polymeric (G/M) blocks (Li et al. 2011a). Because of alginate's metal ion chelating ability, it forms a highly viscous solution, so it has restricted applications. Hence, their hydrolyzed oligomers are in much demand, which can be used widely in the food and pharmaceutical industries as they are easily

Table 2 Sources of microbial enzymes, alginate lyase, fucoidanase, and laminarinase for the production of oligosaccharides from brown algae

Source of enzyme	Degree of polymerization in oligosaccharides	Reference
Alginate lyase: Poly M lyase (EC 4.2.2.3)		
Proteobacteria		
Pseudomonas alginovora X107[ε]	ALO3, ALO4	Lundqvist et al. (2012)
Pseudomonas sp. E03[ε]	ALO2–ALO5	Zhu et al. (2016a)
Nitratiruptor sp. SB155-2[ε]	ALO3, ALO4, ALO5	Inoue et al. (2016)
Serratia marcescens NJ-07	DP2–DP5	Zhu et al. (2018a)
Psychromonas sp. C-3	ALO2–ALO6	Xu et al. (2020)
Bacteriodetes		
Sphingobacterium sp. [ε]	ALO2, ALO3, ALO4	Yoon et al. (2000)
Formosa algae KMM 3553T[ε]	DP1–20[c]	Belik et al. (2020)
Firmicutes		
Bacillus sp. Alg 07	ALO2, ALO3, ALO4	Chen et al. (2018)
Actinobacteria		
Streptomyces ecolicolor A3(2)	ALO2–ALO6	Cheng et al. (2020)
Alginate lyase: Poly G lyase (EC 4.2.2.11)		
Proteobacteria		
Vibro sp. 510	ALO2, ALO3, ALO4	Zhang et al. (2004)
Shewanella sp. Kz7	ALO2, ALO3	Li et al. (2016)
Microbulbifer sp. Q7[ε]	DP2–DP5[p]	Yang et al. (2018);
Microbulbifer sp. Q7[ε]		Han et al. (2019)
Alteromonas portus HB161718T	ALO1, ALO3[x]	Huang et al. (2021)
Bacteriodetes		
Flavobacterium sp. S20[ε]	ALO2, ALO3, ALO5	Huang et al. (2013)
Flavobacterium multivorum	Oligoguluronate	Boucelkha et al. (2017)
Flammeovirga sp. MY04[ε]	DP2–DP7	Han et al. (2016)
Cellulophaga algicola DSM 14237[ε]	Oligomer	Fischer and Wefers (2019)
Alginate lyase: Poly MG lyase (EC 4.2.2.-)		
Proteobacteria		
Sphingomonas sp. A1[ε]	ALO2, ALO3	Hashimoto et al. (2005)
Pseudoalteromonas elyakovi[ε]	ALO4	Ma et al. (2008)
Pseudoalteromonas sp. SM0524	ALO2, ALO3, ALO4	Li et al. (2011a)
Pseudomonas sp. HJZ16	ALO2, ALO3	Li et al. (2011b)
Saccharophagus degradans[ε],	DP2–DP5	Kim et al. (2012)
Stenotrophomonas maltophilia KJ-2[ε]	ALO2, ALO3, ALO4	Lee et al. (2012)
Halomonas sp. QY114[ε]	ALO2, ALO3	Yang et al. (2016)
Microbulbifer sp. ALW1	ALO2, ALO3[x]	Zhu et al. (2016b)
Vibrio sp. NJU03[ε]	DP2–DP5	Zhu et al. (2018d)
Gilvimarinus sp. BP-2	DP2–DP6	Huang et al. (2019)
Pseudoalteromonas carrageenovora[ε]	ALO2, ALO3[x]	Zeng et al. (2019); Zhang et al. (2020)
Vibrio sp. W13[ε]	DP2–DP5	Hu et al. (2021)
Bacteriodetes		
Cellulophaga sp. NJ-1	ALO2, ALO3	Zhu et al. (2016a)
Flammeovirga sp. NJ-04[ε]	DP2-DP6	Zhu et al. (2019)
Actinobacteria		
Isoptericola halotolerans NJ-05[ε]	ALO2, ALO3	Zhu et al. (2018c)
Alginate lyase: yet to be characterized (EC 4.2.2.-)		
Proteobacteria		
Vibrio sp. QY102	ALO3, ALO4, ALO5, ALO6[p]	Wang et al. (2006b)
Agarivorans sp. L11[ε]	ALO2, ALO3, ALO4, ALO5[c]	Chen et al. (2017)

Table 2 (continued)

Source of enzyme	Degree of polymerization in oligosaccharides	Reference
Agarivorans gilvus *Streptomyces coelicolor*	ALO[c, x]	Jiang et al. (2021)
Bacteriodetes		
Flavobacterium sp. LXA	ALO6	An et al. (2008)
Firmicutes		
Bacillus subtilis KCTC11782	Molecular weight < 10 kDA[u]	Bang et al. (2015)
Fucoidanase (EC 3.2.1.44)		
Proteobacteria		
Vibrio sp.	FO2	Furukawa et al. (1992)
Pseudoalteromonas citrea		Bakunina et al. (2002)
Sphingomonas paucimobilis		Kim et al. (2015)
Psychromonas sp. SW5A		Vickers et al. (2018)
Bacteroidetes		
Fucobacter marina	FO3	Sakai et al. (2003)
Formosa algae	FO[u]	Silchenko et al. (2013)
Mariniflexile fucanivorans	DP 4–10	Cao et al. (2018)
Flavobacterium RC2-3	FO[x]	Chen et al. (2019)
Wenyingzhuangia fucanilytica[ε]		Shen et al. (2020)
Formosa haliotis		Vuillemin et al. (2020)
Actinobacteria		
Streptomyces sp.		Manivasagan and Oh (2015)
Verrucomicrobia		
Luteolibacter algae H18[ε]		Nagao et al. (2018)
Ascomycota		
Fusarium sp. LD8, *Dendryphiella arenaria*[ε]		Wu et al. (2011)
Laminarinase (EC 3.2.1.6/EC 3.2.1.39)		
Proteobacteria		
Saccharophagus degradans[ε]	LO2	Wang et al. (2017)
Vibrio breoganii 1C10[ε]	DP 3–4 and DP > 8	Badur et al. (2020)
Bacteroidetes		
Zobellia galactanivorans		Labourel et al. (2015)
Formosa algae KMM 3553[ε]	LO2, LO4	Kusaykin et al. (2016)
Formosa agariphila GH17A[ε]	LO2	Becker et al. (2017)
Flavobacterium sp. UMI-01		Qin et al. (2017)
Firmicutes		
Bacillus clausii NM-1	DP > 4[u]	Miyanishi et al. (2003)
Bacillus circulans	LO2, LO5[c]	Kim et al. (2006)
Bacillus halodurans		Pluvinage et al. (2017)
Actinobacteria		
Streptomyces sp.		Bianchetti et al. (2015)
Ascomycota		
Botryosphaeria rhodina	DP3-4	Giese et al. (2006)
Sclerotinia sclerotiorum		Ezzine et al. (2016)
Basidiomycete		
Phanerochaete chrysosporium[ε]		Kawai et al. (2006)

Bioactivity of oligosaccharides studied: [x]antioxidant, [p]prebiotic, [u]immunomodulation, [c]anticancer, and [ε]source of recombinant enzyme for oligosaccharide production

soluble and biocompatible (Yamasaki et al. 2005). Alginate lyase is a PL reported from many microorganisms capable of hydrolyzing alginate, and it is one of the well-studied carbohydrate modifying enzymes of algal biomass (Fu and Kim 2010). Alginate lyase has been isolated from various bacteria associated with macroalgae, molluscans and a wide range of marine habitats (Jagtap and Manohar 2021). Studies report about fifty thousand amino acid sequences of PL, which are identified as alginate lyase; among them, a large majority of forty-seven thousand sequences are similar to the microbial genome. However, a tiny subset of 260 alginate lyases are characterized: 181 from poly M lyase, 70 from poly G lyase, and 9 from oligo-alginate lyase. From these well-characterized alginate lyases, the functional characteristics of only 27 alginate lyases are confirmed as per the CAZy database (Belik et al. 2020).

Alginate is hydrolyzed by alginate lyase based on the β-elimination reaction, which cleaves the glycosidic bond between two β-D-mannuronate or α-L-glucoronate (Fu and Kim 2010). Alginate lyases are categorized into poly G lyase, poly M lyase, and poly MG lyase based on their substrate specificities, and they can act as endolytic or exolytic forms. Endolytic enzymes cleave the internal glycosidic bonds between the alginate and release disaccharides, trisaccharides, or tetrasaccharides, as the main products. In contrast, exolytic enzymes cleave the terminal glycosidic linkage releasing only monomers and contributing to the complete hydrolysis of alginate. Alginate lyases belong to 7 PL families PL5, PL6, PL7, PL14, PL15, PL17, and PL18. Most bacterial endolytic alginate lyase is assigned to PL5 and PL7, and exolytic belongs to PL15 and PL17 families. The alginate lyase isolated from marine molluscans and viruses belongs to the PL14 family. Bifunctional alginate lyase or poly MG lyase is placed in the PL18 family, while many are yet to be characterized and are dispersed in the PL6 family (Zhu and Yin 2015).

Alginate lyases are reported from bacterial Phylum Proteobacteria, Bacteriodetes, Firmicutes, and Actinobacteria (Table 2). Poly M and poly G specific lyases mostly belong to the phylum Proteobacteria and Bacteriodetes (Yoon et al. 2000; Zhang et al. 2004; Lundqvist et al. 2012; Huang et al. 2013; Li et al. 2016). Poly MG specific lyase, which can break down alginate efficiently, is reported from the bacteria belonging to Proteobacteria, Bacteriodetes, and Actinobacteria (Hashimoto et al. 2005; Zhu et al. 2016a, 2018a). These alginate lyases are predominantly used to produce alginate oligosaccharides (ALO) from the alginate (Wang et al. 2006b; An et al. 2008; Zhu et al. 2016a; Zeng et al. 2019; Belik et al. 2020; Zhang et al. 2020). Other methods such as acid hydrolysis and microwave treatment are also studied for ALO production, which has limited biomedical application (Xu et al. 2018). Similarly, alginate lyase from *Microbulbifer* sp., *Pseudoalteromonas carrageenovora*, *Formosa algae*, and *Alteromonas portus* could produce bioactive ALO with antioxidant and anticancer activity (Zhu et al. 2016b; Zeng et al. 2019; Zhang et al. 2020; Belik et al. 2020; Huang et al. 2021). Enzymatically produced ALO using alginate lyase from *Vibrio* sp. QY102 was studied for its prebiotic potential as it can proliferate the growth of probiotic cultures (Wang et al. 2006b). Alginate lyase gene isolated from *Agarivorans* sp. L11 and *Formosa algae* KMM 3553 T expressed in *E. coli* was shown to produce ALO with anticancer activities (Chen et al. 2017; Belik et al. 2020). Besides, ALO produced from uncharacterized alginate lyase was reported to have immunomodulatory activity (Bang et al. 2015).

Fucoidanase

Fucoidan is a natural fucose containing sulfated polysaccharide present in the cell wall of brown macroalgae, which is composed of alternating fucose residues (Deniaud et al. 2017; Zhao et al. 2018). Fucoidanase (EC 3.2.1.44) is the glycoside hydrolase of GH107 and GH168 families capable of hydrolyzing α-L-fucoside linkage without removing sulfate groups (Silchenko et al. 2013; Kusaykin et al. 2016; Cao et al. 2018; Shen et al. 2020). Classifying these enzymes is a difficult task because of the complex nature of fucoidan, which is often sulfated hetero-polysaccharides. Fucoidanase mostly belongs to GH107 families, but recently, it is also reported from family GH168, which exhibits endolytic cleavage at α-1, 3 glycosidic linkages within sulfated and non-sulfated fucose (Cao et al. 2018; Shen et al. 2020). Fucoidanase is reported from bacterial phylum Bacteroidetes represented by *Fucobacter marina*, *Formosa algae*, *Mariniflexile fucanivorans*, *Flavobacterium* sp., *Wenyingzhuangia fucanilytica*, and *Formosa haliotis*, followed by Proteobacteria (*Vibrio* sp., *Pseudoalteromonas citrea*, *Sphingomonas paucimobilis*, and *Psychromonas* sp.) and Actinobacteria, *Streptomyces* sp. (Table 2). Fucoidanase is also reported in marine fungi *Luteolibacter algae* H18, *Fusarium* sp. LD8 and *Dendryphiella arenaria* (Wu et al. 2011; Nagao et al. 2018). Very scarce information is available on fucoidanase characteristics, type of cleavage, substrate specificity, and degree of substrate sulfation (Kusaykin et al. 2016). The nucleotide sequence of the genes which encodes fucoidanase from the GH107 family and their deduced amino acid sequence has been reported only for fucoidanase from *Mariniflexile fucanivorans* and *Formosa Haliotis* (Silchenko et al. 2017; Vuillemin et al. 2020). Recently, a fucoidanase belonging to the GH168 family was identified from *Wenyingzhuangia fucanilytica* (Shen et al. 2020). Only two structures of fucoidanase from *Psychromonas* sp. and *Mariniflexile fucanivorans* have been reported in the CAZy database (Vickers et al. 2018; Cao et al. 2018). Enzymatically produced fucoidan oligosaccharides (FO) are poorly characterized; however, they are shown to have immunomodulation activity as they activate blood lymphocytes higher than native

fucoidan (Silchenko et al. 2013). Similarly, FO obtained using fucoidanase isolated from *Flavobacterium* RC2-3 showed antioxidant, tyrosinase inhibition, and anti-melanogenesis activity, which can be further used for cosmeceutical applications (Chen et al. 2019).

Laminarinase

The storage polysaccharide laminarin of brown macroalgae is composed of glucose residues, with β-1, 3 and β-1, 6 linkages, and it is a relatively underutilized polysaccharide (Becker et al. 2017). Laminarinase is the glycoside hydrolase that catalyzes its hydrolysis either through endolytic (EC 3.2.1.6 and EC 3.2.1.39) or exolytic (EC 3.2.1.58) mode of action (Badur et al. 2020). Endolytic laminarinase belonging to EC 3.2.1.39 hydrolyze β-1, 3 glycosidic bonds between adjoining glucose residues (Badur et al. 2020). According to the CAZy database, laminarinase are categorized within the GH16 (Liberato et al. 2021), GH17 (Becker et al. 2017), GH30 (Wang et al. 2017), GH55 (Kawai et al. 2006), GH81 (Pluvinage et al. 2017), and GH157 (Bianchetti et al. 2015) families. The laminarinase have been reported from Proteobacteria such as *Saccharophagus degradans* and *Vibrio breoganii* (Wang et al. 2017; Badur et al. 2020), from Bacteriodetes, *Zobellia galactanivorans*, *Formosa algae*, *Formosa agariphila* and *Flavobacterium* sp. (Labourel et al. 2015; Kusaykin et al. 2016; Becker et al. 2017; Qin et al. 2017), Firmicutes, *Bacillus clausii*, *Bacillus circulans*, and *Bacillus halodurans* (Miyanishi et al. 2003; Kim et al. 2006; Pluvinage et al. 2017), and Actinobacteria, *Streptomyces* sp. (Bianchetti et al. 2015). In addition, laminarinase has been widely studied from fungal phyla Ascomycota, including *Botryosphaeria rhodina* and *Sclerotinia sclerotiorum* and Basidiomycota, *Phanerochaete chrysosporium* (Table 2). Recently, the laminarinase that belongs to the GH16 family is reported to have dual β-1, 3 or β-1, 4 hydrolase activity, which help in the complete saccharification of laminarin into fermentable sugars (Liberato et al. 2021). The enzymatic hydrolysis of laminarin using laminarinase from *Saccharophagus degradans* showed the production of laminarin oligosaccharide (LO), gentibiose (Wang et al. 2017). The LO produced by the action of laminarinase from Firmicutes is shown to have immunomodulation activity as it was able to induce tumor necrosis factor α production from human monocytes and suppress the apoptotic death of thymocytes. Therefore, these LO could be helpful in developing immunomodulation and anticancer agent (Miyanishi et al. 2003; Kim et al. 2006).

Microbial enzymes for the production of algal oligosaccharides from green algae

Green macroalgae have the highest carbohydrate content, but it is seldom explored division for their bioactivity. The sulfated polysaccharide ulvan is the principal carbohydrate constituent of this group of macroalgae. Ulvan is a polydisperse hetero-polysaccharide, primarily consisting of glucuronic-Xylo-rhamnans, glucuronic-Xylo-rhamno-galactans, or Xylo-arabinogalactans (Lahaye and Robic 2007), which can be enzymatically hydrolyzed by ulvan lyase (Table 3).

Ulvan lyase.

Ulvan lyase is a PL that depolymerizes ulvan by cleaving the β-(1 → 4)-glycosidic bond through the β-elimination

Table 3 Sources of microbial enzyme, ulvan lyase for the production of oligosaccharides from green algae

Source of enzyme	Degree of polymerization in oligosaccharides	Reference
Ulvan lyase (EC 4.2.2.-)		
Proteobacteria		
Alteromonas sp. GIIUL2[ε]	Molecular weight < 10 kDa	Coste et al. (2015)
Alteromonas sp.[ε]	UO2, UO4, UO6	He et al. (2017)
Alteromonas sp. LOR[ε]	UO2, UO4	Foran et al. (2017)
Pseudoalteromonas sp. PLSV[ε]	UO2, UO4	Qin et al. (2018)
Alteromonas sp. A321[ε]	UO2, UO4	Gao et al. (2019b)
Alteromonas sp.[ε]	UO2, UO4	Qin et al. (2020)
Glaciecola KUL10[ε]	DP2—DP8	Mondal and Ohnishi (2020)
Pseudomonas vesicularis	UO[x]	Hung et al. (2021)
Aeromonas salmonicida		
Bacteroidetes		
Persicivirga ulvanivorans	UO2, UO4	Collen et al. (2011)
Formosa agariphila KMM 3901T[ε]	UO2, UO4	Reisky et al. (2018)
Formosa agariphila KMM 3901[ε]	UO2, UO4	Konasani et al. (2018)
Nonlabens ulvanivorans[ε]		Ulaganathan et al. (2018)

Bioactivity of oligosaccharides studied: [x]antioxidant and [ε]source of recombinant enzyme for oligosaccharide production

mechanism. Enzymes with β-glucuronyl hydrolase, rhamnosidase, xylosidase, and sulfatase activity are also reported to breakdown the complex ulvan (Foran et al. 2017). Most microbial ulvan lyases are categorized into five PL families PL24, PL25, PL28, PL37, and PL40 (Li et al. 2019). Ulvan lyase is reported from the major genera *Alteromonas*; *Pseudoalteromonas* and *Glaciecola* belong to the phylum Proteobacteria and *Persicivirga ulvanivorans*, *Formosa agariphila*, and *Nonlabens ulvanivorans* from phylum Bacteroidetes (Table 3). Ulvan lyase of *Alteromonas* sp. isolated from the gut of the *Gammarus insensibilis*, an amphipod, produces ulvan OS of < 10 kDa molecular weight (Coste et al. 2015). Similarly, *Alteromonas* sp. isolated from feces of tiny marine organisms contains one long and one short ulvan lyase. The long ulvan lyase degrades the ulvan into ulvan oligosaccharides (UO) with 2, 4, or 6 degrees of polymerization (He et al. 2017). The polysaccharide utilization loci from the genome of ulvan degrading bacterium *Alteromonas* sp. LOR is shown to play a significant role in the production of OS with the degree of polymerization 2 and 4 (Foran et al. 2017). The cloned ulvan lyase from *Pseudoalteromonas* sp. PLSV is used to produce UO composed of disaccharides and tetrasaccharides (Qin et al. 2018). Ulvan lyase gene, ALT3695 from *Alteromonas* sp. A321 is recently identified as a potential ulvan lyase to produce disaccharides and tetrasaccharides of ulvan (Gao et al. 2019b). From the genome of ulvan utilizing bacterium *Glaciecola* KUL10, two genes, namely KUL10_26540 and KUL10_26770, which has β-glucuronyl hydrolase activity to produce Rha3S and UO, are identified (Mondal and Ohnishi 2020). The endolytic ulvan lyase from *Formosa agariphila* can depolymerize ulvan into UO containing disaccharides and tetrasaccharides (Reisky et al. 2018; Konasani et al. 2018). Enzymatically produced UO has been studied only for its antioxidant activity (Hung et al. 2021).

Characterization of enzymatically produced algal oligosaccharides

Recently AOS have achieved significant consideration in various bioactivities based on their structure and properties. Furthermore, the chemical composition and stereospecific structural analysis of AOS is still a challenge, because of their complex structures (Cheong et al. 2018). Extensive research to recognize the structural composition of AOS is crucial to utilize them fastidiously. Thin-layer chromatography (TLC) is a basic, simple, fast, and economically feasible technique, most commonly used to analyze AOS obtained through the hydrolysis of polysaccharides (Coste et al. 2015; Zhu and Ning 2016; Qin et al. 2018; Zhu et al. 2018a). Characterization based on TLC gives the basic information about the approximate molecular weight and the number of sugar moieties of the AOS; however, it cannot differentiate the isomeric AOS. Hence, a mass spectroscopy–based approach is required, as it is a sensitive and powerful technique to elucidate the structural composition of AOS. It determines the accurate molecular weight, monosaccharide composition, glycosidic linkage, and chain length of AOS (Zeng et al. 2016; Gao et al. 2019a; Zhang et al. 2019b). For structural analysis of low molecular weight AOS, advanced techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are in use (Przybylski et al. 2015; Han et al. 2016; Lee et al. 2019). Mass spectroscopy does not discriminate between the stereoisomers of AOS; hence, nuclear magnetic resonance (NMR) spectroscopy is widely used to get information about the stereospecificity and the number and position of carbon and hydrogen atoms of the OS (Foran et al. 2017; Zhang et al. 2018; Belik et al. 2020). It can also provide information about the monomers, their structural confirmation and glycosidic bonds. The region in 1H NMR from 5.1 to 5.7 ppm is assigned for α-configuration, whereas 4.5–4.8 for β-configuration (Lahaye and Robic 2007). Hence, mass spectroscopy along with NMR spectroscopy is the best approach to explicate the structural characterization of AOS. These advanced spectroscopic techniques are used to characterize the structural composition of AOS, which will be of great importance and can pave the way to elucidate their bioactive potential using an in-silico modeling approach.

Bioactive potential of enzymatically produced algal oligosaccharides for application as dietary supplements

Dietary supplements are drug-like substances extracted from natural sources and are known to augment health. Nutritional supplements such as omega-3 fatty acids from marine sources and fructooligosaccharides from fruit extracts are commercially used. These supplements are in huge demand as the current generation is at high risk because of fast food consumption, decreased physical activity, and exposure to environmental stress. This leads to the generation of reactive oxygen species and its accumulation causes oxidative stress, which leads to a cascade of disorders, including diabetes, chronic inflammation, cancer, cardiovascular diseases, intestinal, and immune disorders. Healthy individuals effectively eliminate reactive oxygen species using the body's defense system. However, excessive reactive oxygen species causes an imbalance in the molecular pathways, which can lead to the onset of various chronic diseases (Salim et al. 2014). Enzymatically produced, bioactive, OS of algal polysaccharides can prevent the onset and provide treatment for various chronic diseases (Fig. 1).

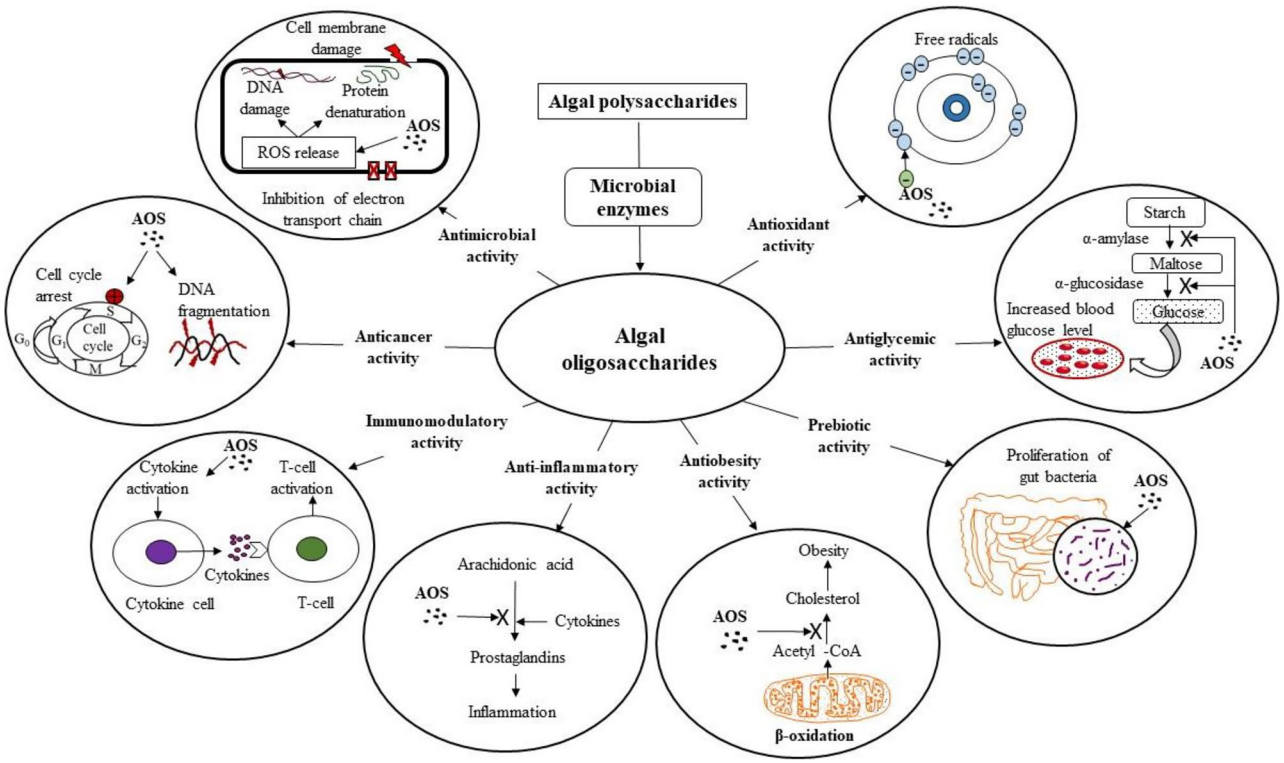

Fig. 1 Bioactive potential of enzymatically produced algal oligosaccharides for application as nutritional supplements: schematic representation

Antioxidant activity, which is the first line of defense, is the most vital biological activity for a bioresource to be used as a nutritional supplement. Enzymatically produced AOS such as AO (Oh et al. 2010; Hong et al. 2017; Gao et al. 2019a), ι-CO (Ma et al. 2013; Li et al. 2017a, b), and ALO (Zhu et al. 2016b; Zeng et al. 2019; Zhang et al. 2020; Huang et al. 2021) are shown to have efficient capacity to donate electrons and stabilize and (or) neutralize the free radicals (Fig. 1). Least studied FO and UO are also shown to counter the effect of oxidative damage (Chen et al. 2019; Hung et al. 2021). The AOS prepared using recombinant β-agarase and ι-carrageenase were also tested and found to show significant oxidative radical scavenging activity (Oh et al. 2010; Ma et al. 2013). Enzymatically produced NAO4 and NAO6 were shown to exert antioxidant activity and antiglycemic activity as they could inhibit major enzymes α-amylase and α-glucosidase (Hong et al. 2017). In diabetes, the first line of treatment is through the inhibition of carbohydrase enzymes α-amylase and α-glucosidase as they are involved in the metabolism of dietary carbohydrates into glucose. Inhibition of these enzymes can significantly decrease the blood glucose level and therefore is the most common therapeutic approach to delay glucose release and absorption (Fig. 1). AO is shown to have the potential to inhibit α-amylase as well as α-glucosidase to reduce the blood glucose level in in-vitro studies (Hong et al. 2017). Prebiotics are the non-digestible sugars that stimulate the growth of the probiotic bacteria *Lactobacillus* sp. and *Bifidobacterium* sp. in the colon and protect against gastrointestinal disorders. AOS such as AO, ALO, and CO are reported to proliferate the growth of these gut bacteria by increasing the release of small chain fatty acids (Wang et al. 2006b; Han et al. 2019). Combined antioxidant, antiglycemic and prebiotic activity is shown to play a major role in controlling diabetes (Fig. 1).

AOS has various applications which make it a lucrative choice such as pharmacological ingredient and dietary supplement. They are shown to control oxidative stress, elevated glucose levels in diabetic patients, reduce the serum lipid levels in hyperlipidemia, and prevent the onset of these chronic diseases (Cheong et al. 2018). The mitochondrial β-oxidation is the catabolic process in that fatty acid molecules are broken down to generate acetyl-CoA, which is the first molecule of the cholesterol biosynthetic pathway, catalyzed by HMG CoA synthase enzyme (Fig. 1). AO and PO are shown to have antiobesity activity as they can reduce cholesterol by inhibiting the HMG CoA synthase enzyme and promoting the expression of low-density lipoprotein receptors through in-vivo and in-vitro studies (Yang et al. 2017; Osumi et al. 2002). AO and CO are also shown to potentially decrease the binding of lipopolysaccharides to the cell surface and inhibition of lipopolysaccharide induced activation of nuclear factor kB and mitogen activated protein

kinase pathways through in vivo studies (Kim et al. 2017; Guo et al. 2018). Microbial infections and internal injuries spontaneously trigger macrophages; this leads to the production of free radicals and lipopolysaccharide induced inflammations due to elevation of nitric oxide and prostaglandin levels (Fig. 1). AOS regulates the phagocytosis ratio and phagocytosis index of macrophages and protects microglial cells induced by lipopolysaccharides. The AO (NAO4, NAO6) produced using both endo, and exo-type β-agarase showed anti-inflammatory activities as they can inhibit tyrosinase enzyme preventing DNA damage, inflammation, and other injuries (Kim et al. 2017). Immunomodulators are the molecules that act on the pathway that regulates the immune system by activating the T cells through cytokine production (Fig. 1). CO (Zhu and Ning 2016), ALO (Bang et al. 2015), FO (Silchenko et al. 2013), and LO (Miyanishi et al. 2003) are shown to activate cytokine cells to release cytokines to activate T cells and improve the immunity against foreign substances.

Cancer is a major ailment affecting a large population, which is caused due to the uncontrolled growth of cells, and many AOS are tested for their ability to delay cell proliferation. The uncontrolled growth of cells leads to the development of tumors which can be treated by AOS by regulating the DNA fragmentation, mitochondrial depolymerization and cell cycle arrest (Fig. 1). CO, ALO, and LO reduce the progression of tumor cells and are shown to have low to moderate toxicity on human melanoma cells (Haijin et al. 2003; Kim et al. 2006; Chen et al. 2017; Belik et al. 2020; Jiang et al. 2021). In addition to all these activities, AOS are also reported to have antimicrobial activity, an additional property that can improve its application. Antimicrobial molecules are known to control the growth of pathogen microbes which can cause many diseases. CO is shown to inhibit the electron transport chain, cell membrane damage, DNA damage, and protein denaturation in pathogenic bacteria (Hatada et al. 2011). AOS with all these activities has the potential to be used in the healthcare industry. Though the various bioactive potentials of AOS have been characterized, a systematic study using an in-silico approach based on the chemical structures of various oligosaccharides can provide insights into their unexplored bioactivity. These studies can also provide information on the mechanism of action and the interaction of AOS to elicit multiple health benefits.

Conclusion

Algal polysaccharides are unique and specific to macroalgae and have immense bioactive potential, but it is not utilized efficiently. Macroalgae are rich in sulfated polysaccharides, which can be enzymatically broken down into bioactive AOS. These oligosaccharides have various activities to control oxidative stress, decrease the blood glucose level in hyperglycemic conditions, modulate the immune system, proliferate the probiotic bacteria in the gut, and decrease the cholesterol level. Enzymes required for the production of these AOS are unique because specific enzymes are required to break down each of the APS. Marine microbes and those associated with seaweeds and benthic fauna are the major sources to isolate these enzymes. Some enzymes such as agarase, carrageenase, and alginate lyase, and their respective oligosaccharides are well studied. These enzymes are structurally characterized, and their bioactivity and genes required for recombinant production are also well studied. However, some algal carbohydrase enzymes such as porphyranase, laminarinase, fucoidanase, and ulvan lyase are not yet well characterized. Studying these enzymes for structural characterization and substrate specificity will help to utilize macroalgal biomass for health care applications efficiently.

Acknowledgements This is NIO contribution number 6853.

Author contribution Ashok S. Jagtap (ORCID 0000–0003-4089–4718) made substantial contributions to the design of the work and wrote the first draft of the manuscript. N. P. Vijay Sankar (ORCID 0000–0001-7882–4593) and Rehnuma I. Ghori (ORCID 0000–0003-1560–0001) performed the literature search, and Cathrine S. Manohar (ORCID 0000–0003-0178–7784) contributed to the study conception and critically revised the work. All the authors have read and approved the final manuscript.

Funding Ashok S. Jagtap received Senior Research Fellowship, grant number 31/026/(0300)/2017-EMR-I from Council of Scientific and Industrial Research, India. N. P. Vijay Sankar (82–44/2020(SA-III)) and Rehnuma I. Ghori (191620064838) also received research fellowship from University Grants Commission, India.

Declarations

Conflict of interest The authors declare no competing interests.

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Received: 5 June 2022 | Revised: 18 July 2022 | Accepted: 9 August 2022

DOI: 10.1111/jam.15775

Journal of Applied Microbiology

ORIGINAL ARTICLE

Prebiotic potential of enzymatically produced ulvan oligosaccharides using ulvan lyase of *Bacillus subtilis*, NIOA181, a macroalgae-associated bacteria

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Funding information
Council of Scientific and Industrial Research, India, Grant/Award Number: 31/026/(0300)/2017-EMR-I; University Grants Commission, India, Grant/Award Number: F.16-6(Dec.2016)/2017

Abstract

Aims: To characterize the polysaccharide hydrolyzing potential of macroalgae-associated bacteria (MABs) for the enzymatic production of oligosaccharides and determining their prebiotic potential.

Methods and Results: Approximately 400 MABs were qualitatively characterized for polysaccharide hydrolyzing activity. Only about 5%–15% of the isolates were found to have the potential for producing porphyranase, alginate lyase and ulvan lyase enzymes, which were quantified in specific substrate broths. One potential MAB, *Bacillus subtilis*, NIOA181, isolated from green macroalgae, showed the highest ulvan lyase activity. This enzyme was partially purified and used to hydrolyse ulvan into ulvan oligosaccharides. Structural characterization of ulvan oligosaccharides showed that they are predominantly composed of di-, tri- and tetrasaccharide units. Results showed that the enzymatically produced ulvan oligosaccharides exhibited prebiotic activity by promoting the growth of probiotic bacteria and suppressing the enteric pathogens, which were higher than the ulvan polysaccharide and equivalent to commercial fructooligosaccharides.

Conclusions: A potential MAB, NIOA181, producing ulvan lyase was isolated and used for the production of ulvan oligosaccharides with prebiotic activity.

Significance and Impact of the Study: Rarely studied ulvan oligosaccharides with prebiotic activity can be widely used as an active pharmaceutical ingredient in nutraceutical and other healthcare applications.

KEYWORDS

macroalgae, macroalgae-associated bacteria, prebiotic potential, ulvan lyase, ulvan oligosaccharide

INTRODUCTION

Macroalgae are a sustainable source of bioactive molecules, which have wide applications as functional foods in the nutraceutical and healthcare industry. Their global demand is progressively increasing and driving the expansion of its aquaculture industry. Cultivating macroalgae is also the need of the hour from the ecological perspective, as it facilitates CO_2 sequestration and is a widely accepted measure for reducing the carbon footprint (Duarte et al., 2017; Hurd et al., 2022). Macroalgae have the edge over other plants for their bioactive molecules: they are devoid of lignin and are composed of unique structural and storage polysaccharides, which are not commonly present in terrestrial plants (Jagtap et al., 2021; Li, Hu, Zhu, et al., 2020). They grow predominantly in the coastal, rocky shore areas

across all geographical locations, including the tropical, subtropical, temperate and polar regions (Hung et al., 2021; Selvarajan et al., 2019). The macroalgal polysaccharides have various bioactive potentials, but due to their high molecular weight and low solubility, they are primarily used only as hydrocolloids (Tanna & Mishra, 2019). Studies have shown that these algal polysaccharides have wider applications when broken down enzymatically into low molecular weight oligosaccharides. These bioactive sugars of macroalgal origin are in great demand in the healthcare sector as oligosaccharides are accredited with "GRAS" (generally recognized as safe) status by the United States Food and Drug Administration (FDA, 2016).

Enzymatic hydrolysis of complex, macroalgal polysaccharides requires specific enzymes for its breakdown into oligosaccharides. Macroalgae-associated bacteria (MABs) are the major source of these enzymes, such as agarase, carrageenase, porphyranase, alginate lyase, fucoidanase, laminarinase and ulvan lyase (Jagtap et al., 2022; Jagtap & Manohar, 2021). Enzymes catalysing algal polysaccharide breakdown are grouped into glycoside hydrolase (GH) and polysaccharide lyase (PL). The GH is the major group of enzymes that hydrolyse the breakdown of polysaccharides through the cleavage of β-(1 → 4)-glycosidic linkages and the PL enzymes acts through the β-elimination mechanism (Belik et al., 2020; Li, Hu, Wang, et al., 2020). Agarase, carragenase, porphyranase, fucoidanase and laminarinase are the GH enzymes that cleave β-(1 → 4)-glycosidic linkages in agar, carrageenan, porphyran, fucoidan and laminarin, respectively (Przybylski et al., 2015; Zhang et al., 2019). Alginate lyase and ulvan lyase belong to the PL group, which can hydrolyse alginate and ulvan polysaccharides through the β-elimination into respective oligosaccharides. Alginate lyase is one of the well-studied carbohydrate modifying enzyme, catalysing the hydrolysis of alginate into bioactive oligosaccharides mainly comprising of di-, tri- and tetrasaccharide units (Belik et al., 2020; Jagtap et al., 2022). However, porphyranase, fucoidanase, laminarinase and ulvan lyase are the least studied enzymes and there are very few reports on the bioactive potential of oligosaccharides produced using these enzymes (Qin et al., 2020; Reisky et al., 2018). Though chemical processes are used to hydrolyse the algal polysaccharides into low molecular weight oligosaccharides, it involves treating polysaccharides with concentrated acid and heating at high temperatures for several hours, releasing monomers and undesirable toxic by-products such as furfural and 5-hydroxymethylfurfural into a final product, which reduces the purity of oligosaccharides (Cheong et al., 2018; Lee & Lee, 2016). The oligosaccharides produced through chemical processes have limitations in functional food and nutraceutical applications as chemical treatment removes sulfation and methylation, which can reduce their bioactivity. Therefore, enzymatic hydrolysis of algal polysaccharides to produce stereo-specific bioactive oligosaccharides are gaining utmost importance because of the versatile nature of microbes producing these enzymes, which can be rapidly grown through batch or continuous cultivation technique in the laboratory with strong adaptability for commercial applications (Charoensiddhi et al., 2017; Gurpilhares et al., 2019).

Macroalgal diversity from India is well studied and documents approximately 5000 macroalgal species along the 7500 km coastal stretch. Extensive research on the diversity, aquaculture practices, production of value added products and extraction of bioactive molecules from different macroalgal groups are reported from the Indian sub-continent and its adjoining islands. Previous studies have reported the diversity and interactions of microbes in the macroalgal ecosystem from these regions (Naik et al., 2019; Singh & Reddy, 2014). They have depicted that macroalgae provide a suitable substratum for the settlement of microorganisms and release various organic substances that promote bacterial growth and biofilm formation (Selvarajan et al., 2019; Singh et al., 2011). However, the biotechnological potential of the microbiome associated with macroalgae for its enzymes to produce algal oligosaccharides is not fully explored.

This study focuses on the characterization of bacteria associated with various macroalgae for their enzymatic potential to hydrolyse major algal polysaccharides such as porphyran, alginate and ulvan. One of the potential MAB, *Bacillus subtilis*, NIOA181, isolated from green macroalgae, showed the highest ulvan lyase activity, which was used to hydrolyse ulvan into ulvan oligosaccharides (UOS). Structural characterization of the UOS based on Fourier transform infrared (FTIR), high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy was carried out. The prebiotic activity of the enzymatically produced UOS was also determined to identify their potential to increase the growth of probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus bulgaricus*) and suppress the growth of enteric *Escherichia coli* strains, which is known to elicit varied health benefits (Fernandez et al., 2011). This study will help to understand the diversity of bacteria associated with macroalgae and their enzymatic potential to hydrolyse algal polysaccharides into low molecular weight bioactive oligosaccharides.

MATERIALS AND METHODS

Chemicals and bacterial strains

Analytical grade sodium alginate (Sigma Aldrich), ulvan extracted from *Ulva lactuca* as described by Lahaye

et al. (1999) and porphyran extracted from *Porphyra* sp. as illustrated by Zhang et al. (2019) were used for all experimental analysis. Zobell Marine Broth was procured from Himedia laboratories, India and Man-Rogosa-Sharp broth from Sisco Research Laboratories Pvt. Ltd. India of the microbiological grade. Fructooligosaccharides (FOS) from chicory, used as a model prebiotic compound, was purchased from Sigma Aldrich and other chemicals used were of analytical grade. The potential probiotics *L. acidophilus* (NCIM 2285/ATCC 4963) and *L. bulgaricus* (NCIM 2056/ATCC8001) and an enteric culture of *Escherichia coli* strain DSM1576 (ATCC 8739) were procured from the National Collection of Industrial Microorganisms, CSIR-National Chemical Laboratory, Pune, India.

Isolation of MABs

Macroalgal samples were collected from major macroalgal beds along the Indian coast to isolate MABs. The sampling stations included Okha and Porbandar along the Gujarat coast; Malvan and Kunkeshwar along the Maharashtra coast; Vagator, Dona Paula and Palolem from the Goa coast; and Mandapam along the Tamil Nadu coast (Figure 1). Representative macroalgae belonging to all three major groups of red, brown and green macroalgae were collected (Jagtap et al., 2021) and associated epiphytic MABs were isolated following the shake tube method (Singh et al., 2011). For this study, macroalgal samples were rinsed with sterile seawater to remove the attached debris, sand particles and seashells. Approximately 1 g of the washed macroalgal sample (fresh weight) was re-suspended in 9 ml of sterile seawater to release all the epiphytic bacteria into suspension and serially diluted (10^{-3}–10^{-4}) samples were spread plated on Zobell Marine agar microbiological plates under sterile conditions and incubated at 28°C for 24–48 h to obtain well-isolated colonies. At the end of the incubation period, morphologically distinct colonies were picked, streak purified and maintained on Zobell Marine agar plates for further analysis with designated isolate numbers NIOA1 to NIOA421 in sequence.

Qualitative analysis of MABs for enzymatic hydrolysis of algal polysaccharides

The purified MABs were grown on algal polysaccharide substrate plates to check their potential to hydrolyse porphyran, alginate and ulvan. In brief, the pure bacterial cultures were inoculated in a sterile Zobell Marine Broth (20 ml) and incubated in a shaker incubator for 24 h at 28°C. The culture suspension (10 μl) with the absorbance of 0.8–0.9 at 600 nm was spot inoculated on specific substrate media plates supplemented with 0.5% of porphyran or alginate or ulvan in the basal salt solution, composed of 5% sodium chloride, 0.5% ammonium sulphate, 0.3% dipotassium hydrogen phosphate, 0.1% magnesium sulphate,

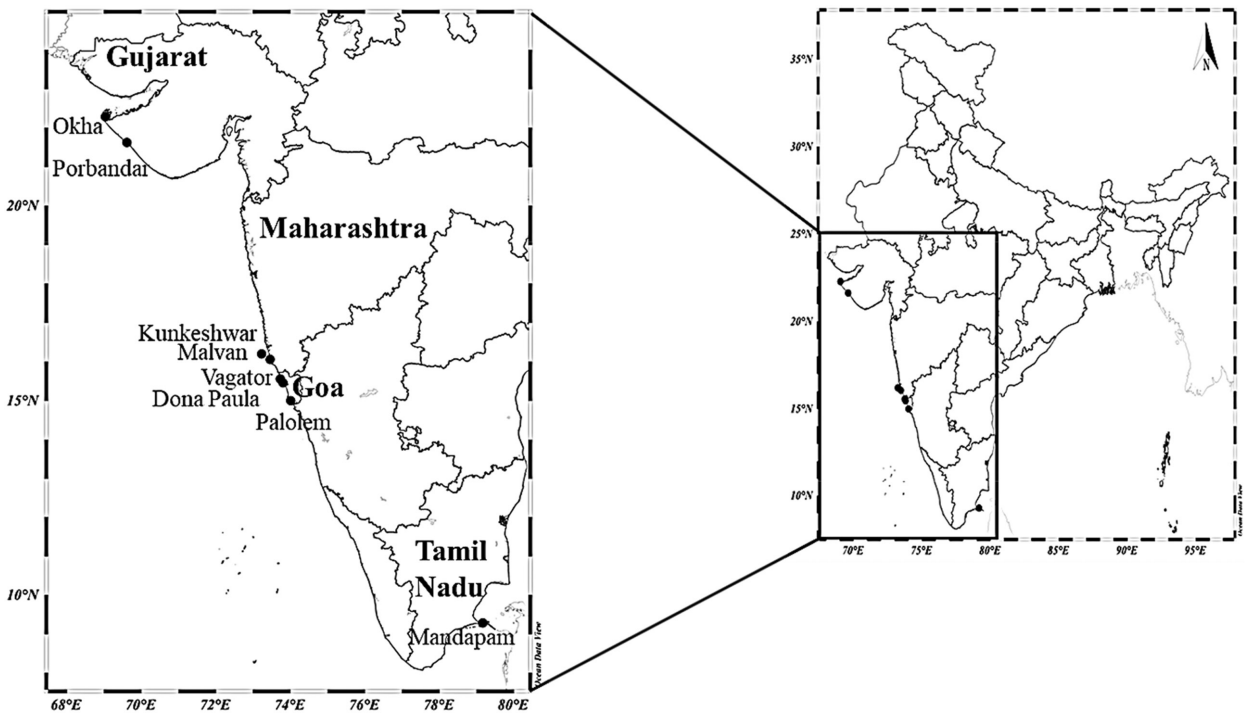

FIGURE 1 Sampling locations at the major macroalgal beds of the Indian coast along Gujarat, Maharashtra, Goa and Tamil Nadu coast

0.01% ferrous sulphate and 1.5% agar. The spot inoculated plates were incubated at 28°C for 48 h; after incubation, the plates were stained with substrate-specific staining solution; Lugol's iodine (0.5%) for porphyran, Gram's iodine (0.5%) for alginate and cetylpyridinium chloride (0.05%) for ulvan (Sawant et al., 2015). The zone of clearance around the bacterial colony was measured within 10 min of staining (Figure S1). The bacterial isolates were tested for their ability to catalyse the polysaccharide breakdown activity and grouped based on the zone of clearance. Those isolates with the highest zone of clearance (20–35 mm) were categorized as "potential isolates" with very good activity and with decreasing zone of clearance, the isolates were grouped as good (11–20 mm), minimum (1–10 mm) and no activity group. The potential MABs with good efficiency in breaking down representative algal polysaccharides were used for further analysis and identified using molecular techniques.

Phylogenetic characterization of potential polysaccharide hydrolyzing MABs

The potential MABs (19 isolates) with the highest polysaccharide hydrolyzing activity were identified based on the 16S rRNA gene sequence. Each purified potential bacterial isolate was grown in 10 ml Zobell Marine Broth at 28°C for 24 h. The bacterial cell pellets of the individually grown isolates with an absorbance of approximately 0.8 at 600 nm were collected through centrifugation (20,000 *g* for 5 min) and used for genomic DNA extraction. The 16S rRNA gene fragment of the MABs was amplified using 27F and 1492R universal bacterial primers (Liao et al., 2011). The amplified fragment was purified using a PCR clean-up kit (Promega) and sequenced bidirectionally on Genetic Analyzer 3130xl (ABI) based on the Big Dye v 3.1 chain terminator chemistry. 16S rRNA gene sequence obtained for individual isolates was compared with the NCBI database using the nucleotide BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial 16S rRNA gene sequence of the 19 potential MABs are deposited in the NCBI GenBank database. Phylogenetic analysis of bacterial isolates was carried out on the MEGA X platform based on the sequence homology of the 16S rRNA gene to gene sequence from the NCBI database using the maximum likelihood algorithm (Tamura et al., 2011) and the robustness was assessed with 1000 bootstrap replicates.

Quantitative estimation of enzyme activity in the potential MABs

The 19 potential isolates were evaluated for their quantitative enzyme activity. For this, individual isolates were inoculated in basal salt solution amended with 0.5% polysaccharide substrates (porphyran, alginate or ulvan) and grown at 28°C for 48 h. The enzyme activity of the potential isolates was assessed by measuring the release of reducing sugar following the dinitrosalicylic acid method (Miller, 1959) and galactose (20–100 μM) was used as a standard. Enzyme activity was expressed in *U* as the amount of enzyme required to liberate 1 μM of reducing sugar min^{-1} mg^{-1} of protein (Gao et al., 2019). The extracellular protein content in the broth was determined following the Bradford assay and varying concentrations of BSA (50–250 μg ml^{-1}) were used as a standard. The isolates NIOA181, NIOA284, NIOA379, NIOA323 and NIOA354, which showed very high enzyme activity, are deposited in the National Centre of Industrial Microorganisms, Culture collection at CSIR-National Chemical Laboratory, Pune, India. Of this, NIOA181 (NCIM 5804), *Bacillus* sp., which showed very high ulvan lyase activity, was studied in detail and its ability to hydrolyse ulvan was characterized.

Biochemical characterization of ulvan lyase (ULB1) from *Bacillus* sp. NIOA181

Ulvan lyase, ULB1 from *Bacillus* sp. NIOA181 was characterized for optimization of its potential to hydrolyse the algal polysaccharide ulvan. For this, *Bacillus* sp. NIOA181 was inoculated in 100 ml of basal salt solution, amended with 0.5% ulvan and allowed to grow at 28°C for 24 h. The cell-free supernatant containing the extracellular ulvan lyase was collected after centrifugation of the culture broth at 5000 ***g*** for 15–20 min. The supernatant was precipitated with saturated ammonium sulphate to collect the crude enzyme and the excess ammonium sulphate was removed by dialysis. The crude ulvan lyase was loaded on the DEAE sepharose column and eluted with a 0–1 M NaCl gradient and fractions were collected at regular intervals. Those fractions with enzyme activity were combined and dialysed against 50 mM tris–HCL buffer (pH 8.0) and freeze-dried into powder form. The influence of different parameters such as the pH, temperature, varying NaCl concentrations, metals ions and chelating agents on the relative activity of the lyophilized enzyme was tested. The effect of pH (3–11) on ulvan lyase activity was determined at 50 mM of different buffers such as acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), tris–HCl buffer (pH 8.0–9.0) and carbonate–bicarbonate buffer (pH 10.0–11.0) and temperatures ranging from 20–60°C by incubating the lyophilized enzyme with 0.5% substrate for 30 min. Similarly, the effect of NaCl concentrations (0.01–1 M), different metal ions (Li^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+}) at 10 mM concentration, 0.1% detergent or inhibitors (ethylene diamine tetraacetic acid [EDTA], dithiothreitol [DTT], urea,

β-mercaptoethanol, sodium dodecyl sulphate [SDS], triton X-100, Tween 20, Tween 80 and cetyl trimethyl ammonium bromide [CTAB]) on ulvan lyase activity was determined at optimum conditions along with appropriate controls with no addition of metal ions or detergents.

Enzymatic production of UOS using ulvan lyase ULB1 from *Bacillus* sp. NIOA181

Enzymatic hydrolysis of ulvan was carried out to produce UOS using partially purified ulvan lyase, ULB1 from *Bacillus* sp. NIOA181 at the optimum conditions obtained from the characterization studies (pH 8.0, temperature 40°C, 0.01 M NaCl, 10 mM Mg^{2+} and 0.1% of β-mercaptoethanol). For this, 1 ml (10 μg) of the enzyme was mixed with the 0.5% ulvan (100 ml) prepared in 50 mM Tris–HCl buffer at pH 8.0 and supplemented with 10 mM $MgCl_2$ and incubated at 40°C for 24 h and UOS production was determined using thin-layer chromatography. The UOS was purified on Sephadex® G-100 medium (Sigma-Aldrich) and fractions (2 ml) were eluted with 50 mM Tris–HCL buffer (pH 8.0). The fractions were analysed for reducing sugar content using the dinitrosalicylic acid method (Miller, 1959). The fractions with the highest reducing sugar content were combined and freeze-dried and the white, crystalline ulvan oligosaccharides obtained were used for structural characterization and bioactivity analysis.

Structural characterization of enzymatically produced UOS

FTIR spectroscopy

The enzymatically produced UOS was analysed based on Fourier transform infrared spectroscopy (Shimadzu-8201PC spectrometer) and evaluated for its functional groups (Hung et al., 2021). For this, lyophilized powder of UOS was individually mixed with dry potassium bromide and the FTIR spectrum was recorded using 50 scans in the spectral range of 4000–400 cm^{-1}.

HRMS spectrometry

HRMS analysis of UOS was performed using Bruker Impact HD Q-TOF spectrometer (Bruker Daltonics). For this, UOS (100 μg ml^{-1}) prepared in acetonitrile/water (1:1 v/v) was injected through UHPLC (Dionex Ultimate 3000; Make-Thermo Scientific) to electrospray ionization (ESI)-Q-TOF system with a flow rate of 120 μl min^{-1} and 90% acetonitrile in water containing 0.1% formic acid v/v was used as a mobile phase. The high-resolution mass spectrum was recorded for 10 min between 50–1500 m/z in positive ion polarity. The other MS parameters used were as follows: set capillary, 4000 V; set endplate offset, −500 V; set charging voltage, 2000 V; set nebulizer, 1.7 bar; set dry heater, 200°C; and set dry gas, 7.0 L min^{-1}. The spectra visualization and baseline correction were analysed using Bruker compass Data Analysis software 4.2 and the mass was confirmed by the m/z of the sample based on the available literature (Gao et al., 2019).

NMR spectroscopy

UOS at 20 mg ml^{-1} concentration in deuterated chloroform was used for ^{13}C NMR analysis on a BRUKER AVANCE III HD NMR 500 MHz a Liquid State 500 MHz NMR spectrometer. The acquisition parameters were set as the number of scans, 4000; D1, 2.0 s and Pulse program, zgpg30 for analysis.

Prebiotic activity of enzymatically produced UOS

The prebiotic activity of the enzymatically produced UOS was analysed based on their ability to promote the growth of two potential probiotics *L. acidophilus* and *L. bulgaricus* and suppress the growth of enteric culture, *E. coli*. The growth was determined by growing these isolates in a dextrose-free Man-Rogosa-Sharp medium supplemented with UOS (1%) as the sole carbon source by measuring the absorbance of the culture medium at 600 nm at 0 and 24 h. The prebiotic activity of UOS was compared with ulvan and commercial FOS from chicory, following the same methodology described above for UOS. To calculate the prebiotic score, 100 μl of starter and 24 h grown probiotic and enteric cultures were spread plated on basal Man-Rogosa-Sharp medium and incubated at 37°C for 24 h under anaerobic conditions in a RUSKINN CONCEPT 400M workstation. The growth of probiotic and enteric cultures was measured by enumerating the number of colony-forming units (CFUs).

The prebiotic activity score (PA) was calculated using the following equation:

$$PA = \left[\left(\log Px^{24} - \log Px^{0}\right) \div \left(\log Pg^{24} - \log Pg^{0}\right)\right] - \left[\left(\log Ex^{24} - \log Ex^{0}\right) \div \left(\log Eg^{24} - \log Eg^{0}\right)\right],$$

where Px^{24} and Px^{0} are the CFU measured of probiotic cultures from the 24 h grown and starter culture (at 0 h), respectively with 1% ulvan/UOS or FOS supplement; Pg^{24} and Pg^{0} are the CFU measured from the 24 h grown and starter culture (at 0 h) respectively with 1% glucose supplement in the broth. Similarly, Ex^{24} and Ex^{0} and Eg^{24} and Eg^{0} is the

CFU measure of enteric culture, *E.coli*. The CFU was enumerated by plating the broth on Man-Rogosa-Sharp plates (Hu et al., 2006).

Statistical analysis

All experimental studies were carried out in triplicates ($n = 3$) and mean values are expressed along with standard deviations. Statistical significance was determined using one-way ANOVA and Student's *t*-test and the significance level was set as $p \leq 0.05$.

RESULTS

Isolation and screening of bacterial isolates

MABs were isolated from the most commonly grown macroalgae along the Gujarat, Maharashtra, Goa and Tamil Nadu coast. MABs associated with red macroalgae such as *Gracilaria*, *Halymenia*, *Porphyra* and *Hypnea*; brown algae such as *Padina*, *Spatoglossum*, *Sargassum*, *Stoechospermum* and *Sphacelaria* and *Chaetomorpha*, *Caulerpa* and *Ulva* of green macroalgae were isolated. Approximately 421 pure bacterial strains were isolated and about 152, 151 and 118 bacterial isolates were obtained from red, brown and green macroalgae, respectively (Table S1). These bacterial isolates were qualitatively analysed to check their potential to hydrolyse algal polysaccharides using representative algal polysaccharides porphyran, alginate and ulvan. The zone of clearance of all the isolates on substrate plates was measured to determine their efficiency. Of the 421 bacterial isolates tested, around 85%–95% of the isolate did not show any clearance zone on polysaccharide substrate plates, which signifies that a large majority of the isolates are incapable of breaking down the complex polysaccharides. Of the 5%–15% of the bacterial strains that showed enzyme activity, only 2%–5% of the bacterial isolates exhibited very good activity with a zone of clearance in the range of 20–35 mm around the colony (Figure 2). Among the isolates tested, porphyranase activity was not seen in about 86% of bacterial isolates (360 isolates) and about 5% showed very good (19 isolates) activity. Similarly, 83% of the isolates showed no activity (351 isolates) for alginate lyase and only 4% showed very good (17 isolates) activity. The maximum number (96%) of isolates tested did not show ulvan lyase activity due to the heteropolysaccharide nature of ulvan and of the 4% of bacterial isolates that showed activity, only 2% (7 isolates) showed very good ulvan lyase activity (Figure 2). The 19 isolates, which showed very good activity with a maximum clearance zone (>20 mm) were characterized as potential bacterial isolates to hydrolyse porphyran, alginate and ulvan substrates (Table S2). Maximum porphyranase activity with a zone of clearance of 32 mm was observed in bacterial isolate NIOA284 and isolates NIOA112, NIOA174, NIOA284, NIOA319, NIOA321, NIOA327 and NIOA398, which were associated with red macroalgae showed good porphyranase activity. Similarly, bacteria isolated from brown macroalgae NIOA29, NIOA118, NIOA169, NIOA170, NIOA323, NIOA328, NIOA354 and NIOA388 exhibited good alginate lyase activity. Ulvan lyase activity was observed in five isolates, NIOA181, NIOA302, NIOA325, NIOA329 and NIOA379, associated with green macroalgae, which are shown to produce ulvan lyase for hydrolysis of ulvan and maximum ulvan lyase activity was recorded in strain NIOA181 with a zone of clearance of 25 mm (Table S2).

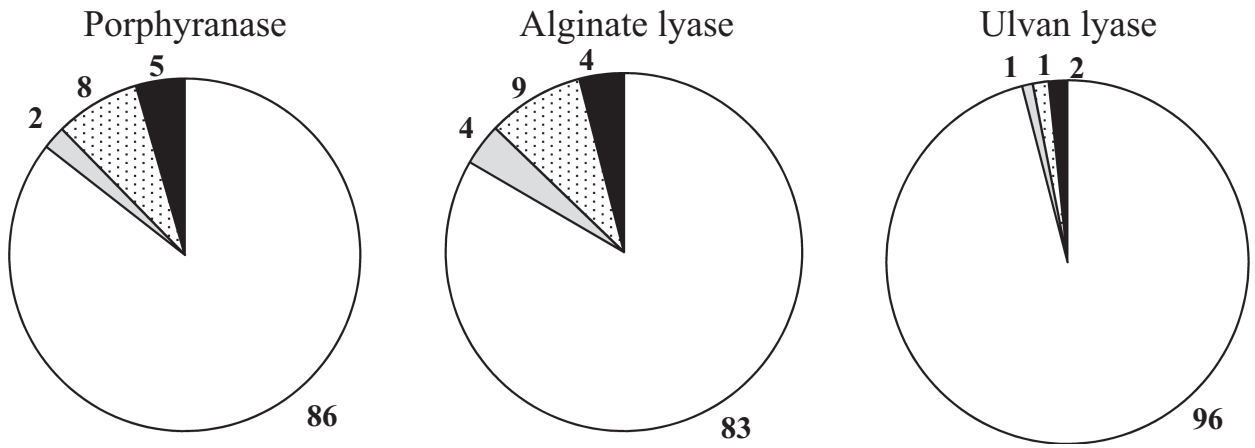

FIGURE 2 Percentage frequency of macroalgae associated bacterial isolates with no activity (white), minimum (grey), good (dotted) and very good (black) for hydrolysis of algal polysaccharides (a) porphyranase, (b) alginate lyase and (c) ulvan lyase based on qualitative plate assay

Phylogenetic characterization of potential polysaccharide hydrolyzing MABs

Phylogenetic analysis was carried out based on the 16S rRNA gene sequences of the 19 potential MABs and each of their closest bacterial taxa was identified using BLASTn sequence similarity (Table S3; Figure 3). The potential isolates were predominately grouped within Phylum Firmicutes and its diversity was represented by the genus *Bacillus* spp. (NIOA29, NIOA34, NIOA181, NIOA183, NIO284, NIOA321, NIOA323, NIOA329, NIOA388), *Staphylococcus* spp. (NIOA118, NIOA174), *Exobacterium* spp. (NIOA302, NIOA96) and *Oceanobacillus* sp. (NIOA379). It was followed by representatives from Phylum Proteobacteria, which included *Acinetobacter* sp. (NIOA328), *Vibrio* sp. (NIOA169), *Pseudomonas* sp. (NIOA319), *Marinobacter* sp. (NIOA327) and genus *Micrococcus* sp. (NIOA354) belonging to Actinobacteria phylum (Figure 3). Maximum polysaccharide hydrolyzing bacteria (nine isolates) belonged to the phylum Firmicutes of the genus *Bacillus*.

Quantitative estimation of enzyme activity in the potential MABs

Based on qualitative analysis, a total of 19 bacterial strains that showed very good activity were further characterized for quantitative estimation of specific enzyme activity. These bacterial isolates utilized the algal polysaccharides porphyran, alginate and ulvan as a carbon source by producing the specific enzyme to hydrolyse their glycosidic bonds. The enzyme activity in the potential MABs were estimated to quantify the specific enzymes porphyranase, alginate lyase and ulvan lyase produced by the individual isolates. The porphyranase activity recorded in the MABs was in the range of 61.9–208.6 U mg^{-1} (Table 1). Of the 19 isolates, 8 potential MABs (NIOA181, NIOA284, NIOA323, NIOA327, NIOA328, NIOA354, NIOA379 and NIOA388) showed >130 U of porphyranase activity and the highest activity was shown by red macroalgae-associated bacterial strain NIOA284 with 208.6 U mg^{-1} of enzyme activity. Similarly, bacterial isolates showed alginate lyase activity between 100.8 to 140.2 U mg^{-1} and *Acinetobacter* sp., NIOA328, showed the highest activity (Table 1). Due to

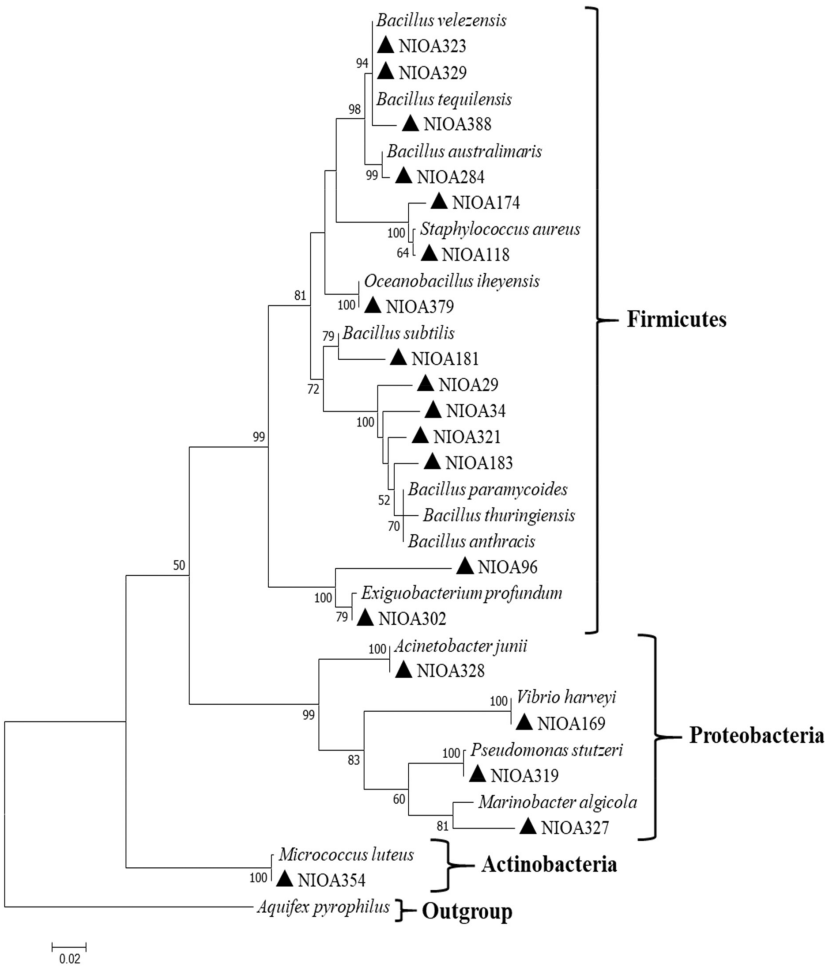

FIGURE 3 Phylogenetic tree showing the position of macroalgae associated bacteria with potential to hydrolyse algal polysaccharides based on the maximum-likelihood analysis of the 16S rRNA gene sequences. Values at nodes indicate bootstrap support from 1000 replicates and scale represents the number of substitution per amino acid.

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TABLE 1 Quantitative estimation of enzyme activity (U mg^{-1} protein) in potential macroalgae-associated bacteria

Isolates	Porphyranase	Alginate lyase	Ulvan lyase
NIOA29	61.9 ± 0.4	102.0 ± 5.4	62.7 ± 1.9
NIOA34	80.7 ± 4.8	110.7 ± 11.2	84.7 ± 1.1
NIOA96	67.6 ± 3.5	104.7 ± 9.7	37.7 ± 4.2
NIOA118	101.6 ± 11.0	105.0 ± 12.5	75.5 ± 2.7
NIOA169	77.6 ± 4.6	101.7 ± 11.3	75.4 ± 5.3
NIOA174	121.1 ± 1.0	100.8 ± 9.9	70.8 ± 1.2
NIOA181	174.7 ± 0.0	121.0 ± 1.6	211.5 ± 0.3
NIOA183	128.1 ± 4.3	102.7 ± 12.5	81.2 ± 0.6
NIOA284	208.6 ± 1.7	117.0 ± 3.5	140.1 ± 2.5
NIOA302	89.9 ± 4.3	115.6 ± 5.3	115.1 ± 3.3
NIOA319	81.7 ± 2.9	116.6 ± 12.5	69.3 ± 0.3
NIOA321	87.6 ± 13.4	117.9 ± 10.9	69.3 ± 1.1
NIOA323	190.1 ± 4.5	124.9 ± 3.6	164.3 ± 2.3
NIOA327	178.3 ± 0.7	138.6 ± 3.4	163.1 ± 3.7
NIOA328	170.2 ± 3.2	140.2 ± 2.5	144.4 ± 1.3
NIOA329	100.9 ± 0.3	123.2 ± 2.6	77.9 ± 4.1
NIOA354	182.9 ± 8.5	132.6 ± 2.63	116.7 ± 1.1
NIOA379	148.5 ± 3.8	122.1 ± 1.7	146.6 ± 3.8
NIOA388	132.6 ± 4.9	124.6 ± 8.6	78.8 ± 2.9

the complex structural composition of ulvan, the ulvan lyase enzyme is rarely studied; the bacterial isolates in this study showed ulvan lyase activity in the range of approximately 40–200 U mg^{-1} (Table 1). The isolate, NIOA181, a green macroalgal epiphytic bacteria, showed the highest (211.5 mg^{-1}) ulvan lyase activity, identified as *B. subtilis* and selected in this study for enzymatic production of UOS.

Biochemical characterization of ulvan lyase (ULB1) from *Bacillus* sp. NIOA181

The effect of different parameters such as pH (3–11), temperature (20–60°C), NaCl concentrations (0.01–1 M), different metal ions (10 mM) and chelating agents (0.1%) on the ulvan lyase activity was determined. The maximum ulvan lyase activity was estimated to be at a mildly alkaline pH of 8, with a decrease in activity at highly acidic (3–4) and alkaline pH (10–11) (Figure 4a). Optimum enzyme activity was in the temperature range from 30 to 45°C, with maximum activity at 40°C, reduction in activity was observed at low temperature (20°C) and at 50 and 60°C (Figure 4b). Maximum ulvan lyse activity was recorded at the lowest NaCl concentration tested at 0.01 M and a decrease in activity was seen from 0.05 to 1 M (Figure 4c). The effect of different metal ions Li^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+} tested showed that the ULB1 activity was the highest in the presence of Mg^{2+} (10 mM) which was about 144% in comparison with the control which had no metal ion supplementation. About 120% of activity was recorded in the presence of K^+, Ca^{2+} and Co^{2+} (Figure 4d). Metal ions such as Li^+, Ba^{2+}, Ni^{2+} and Zn^{2+} showed no impact on the ULB1 activity, whereas Cu^{2+} and Fe^{2+} had a negative effect. Of all the chelating agents tested for their influence on the ULB1 activity, β-mercaptoethanol and DTT had a positive impact, with the former showing maximum activity. Chelating agents urea, SDS and Triton X-100 had no effect and the activity was almost similar to the control with no addition of chelating agents. However, EDTA, Tween 20, Tween 80 and CTAB negatively influenced the ULB1 activity (Figure 4e). Of the different parameters tested, optimum ULB1 activity was recorded at pH 8.0, temperature 40°C, 0.01 M NaCl concentration and in the presence of metal ion Mg^{2+} and chelating agent β-mercaptoethanol, which was used for the enzymatic breakdown of ulvan into its UOS.

Structural characterization of enzymatically produced UOS

FTIR spectroscopy analysis

The structural characterization of the UOS was carried out based on FTIR spectroscopy to study their functional groups (Figure 5a). The spectrum shows characteristic peaks at 3280 cm^{-1} in UOS, which may be attributed to the primary -OH group stretching vibrations. Similarly, the absorption band at 2930 cm^{-1} can be associated with the -CH stretching vibration. The absorption band at 1417 and 1658 cm^{-1} in the UOS represent symmetric and asymmetric carboxylate ($-COO^-$) group stretching. The peaks at 1172 and 1080 cm^{-1} are attributed to C-OH and C-O-C stretching in the pyranose ring of the rhamnose sugar, which is the major monomer of ulvan. The characteristic of uronic acid residues was also observed in the band at 810 cm^{-1}. All these results infer that the enzymatically produced UOS retains its sugar residues and functional groups, similar to the ulvan IR spectrum described in green macroalgae (Lahaye et al., 1999).

HRMS spectrometry analysis

Enzymatically produced UOS was analysed by HRMS to determine the degree of polymerization, as the oligosaccharides are known to exist in dimer to 20 mers (Figure 5b). From the HRMS spectrum studied, the highest significant peak at 269 m/z is attributed to Rha3S. The peaks at 295 and 423 m/z are assigned to disaccharides of

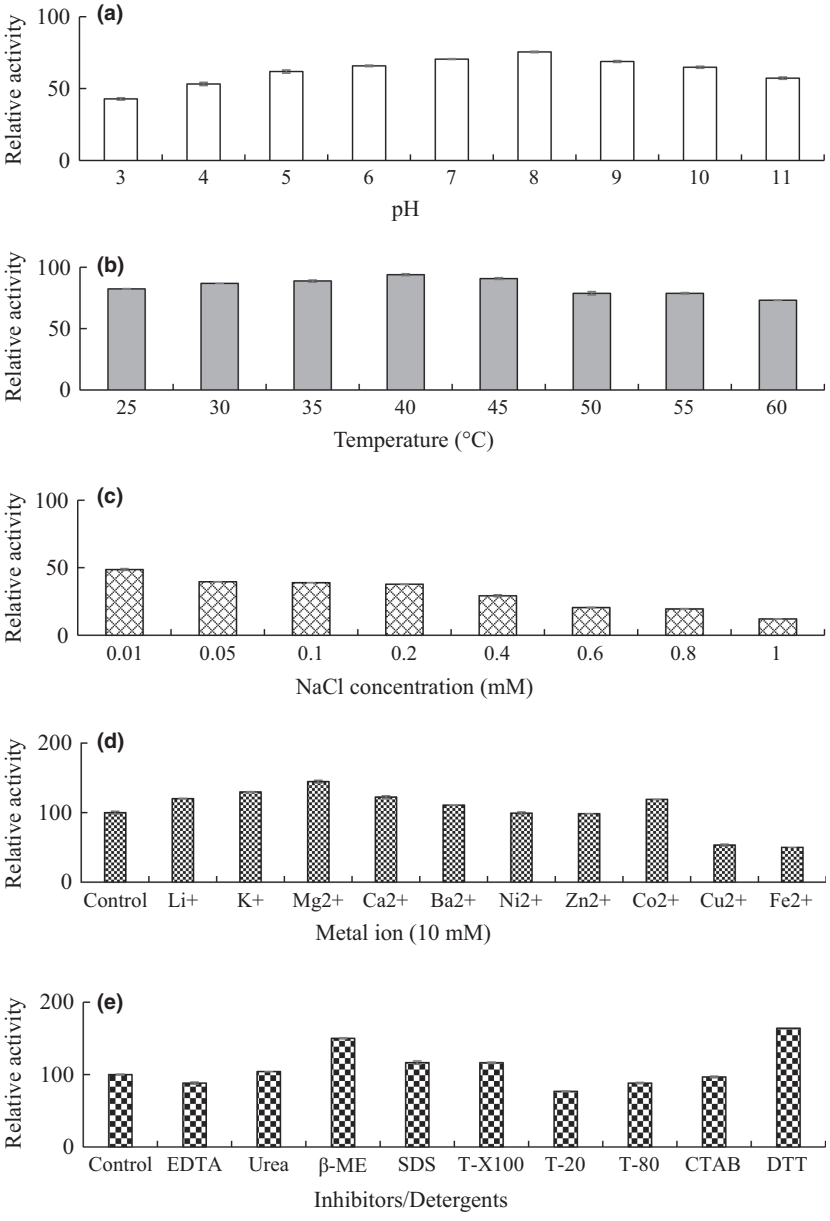

FIGURE 4 Influence of different parameters (a) pH (white bars) (b) temperature (grey bars) (c) NaCl concentration (dotted bars) (d) metal ions (grid bars) (e) chelating agents (black bars) on the relative activity (%) of ulvan lyase

Rha-Xyl and ΔRha3S. The prominent peaks at 599 and 665 m/z represent the presence of trisaccharides composed of ΔRha3S-Xyl and Δ Rha3S-Xyl2S, respectively. The peaks at 699 and 733 m/z are assigned to tetrasaccharides constituted by ΔRha3S-Xyl-Rha and ΔRha3S-ΔXyl. The HRMS spectrum of the enzymatically produced UOS using ulvan lyase shows that di-, tri- and tetrasaccharides were produced from ulvan heteropolysaccharide.

NMR spectrum analysis

NMR spectrum based on ^{13}C was carried out for the enzymatically produced UOS to confirm the identity and configuration of the specific sugar moieties rhamnose, xylose and uronic acid (Figure 5c). The NMR spectrum confirmed the resonance at 96.7 and 95.9 ppm, which is attributed to the presence of α- and β-anomeric carbons in the reducing end of the rhamnose 3 sulphate residue, respectively. Methyl residue signals at 16.7, 16.8 and 16.9 ppm were observed, indicating the presence of L-rhamnose residue in UOS. The peak at 172.78 ppm represents C6 of 4-deoxy-L-threo-hex-4-enopyranosiduronic acid residue. The peak at 103 ppm implies the existence of C1 of β-D-xylose and the multiple peaks between 70–85 ppm represent C2-C5 of rhamnose, iduronic acid and glucuronic acid. These results of ^{13}C NMR analysis confirm the identity of the ulvan-specific sugars in UOS.

Prebiotic potential of enzymatically produced UOS

The prebiotic potential of UOS was compared with ulvan and commercial prebiotic FOS based on the growth

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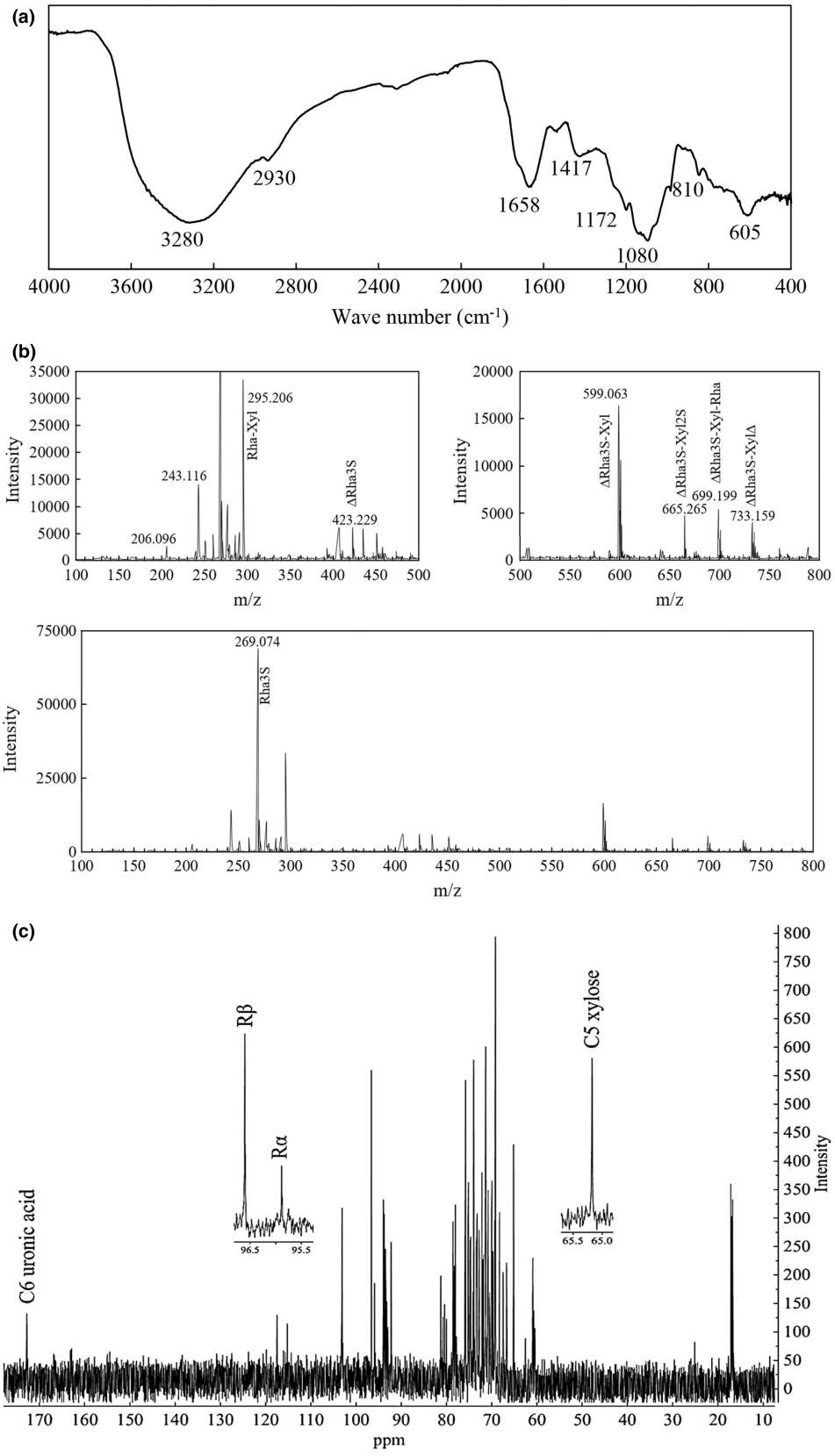

FIGURE 5 Structural characterization of enzymatically produced ulvan oligosaccharide from ulvan based on (a) FTIR (b) HRMS and (c) NMR spectrum

profiling of probiotic bacteria, *L. acidophilus*, *L. bulgaricus* and enteric *E. coli*, along with their prebiotic activity score. The results show that the growth of probiotic bacteria, *L. acidophilus* and *L. bulgaricus* in the presence of ulvan, UOS and FOS were significantly different (Figure 6a). The ability of UOS (0.81) and FOS (0.81) to suppress the growth of *E. coli* was significantly equal and higher than ulvan (0.6). Such bioactive compounds, which show the ability to suppress the growth of enteric pathogenic strains of *E. coli* prove to be much efficient in their prebiotic activity. The prebiotic score was also calculated based on the CFU numbers obtained from the growth of probiotic and enteric bacteria in the presence of ulvan, UOS and FOS. The results show that UOS significantly promoted the growth of *L. bulgaricus* and *L. acidophilus* with a prebiotic score of 7.59 and 7.43, respectively, which was higher than ulvan and commercial prebiotic FOS; this was also supported by statistical analysis (Figure 6b).

DISCUSSION

Macroalgae are known to harbour diverse microbial communities, contributing to their growth and defence. Many of these associated microbiomes are known to be the source of various extracellular enzymes, including the carbohydrase enzymes, which are in huge demand, as they can catalyse the enzymatic breakdown of complex macroalgal polysaccharides into bioactive oligosaccharides (Selvarajan et al., 2019). These low molecular weight oligosaccharides are reported for various bioactivities such as antioxidant, immunomodulation, antiglycemic, anti-inflammatory, antitumor, prebiotic, antiobesity and antimicrobial (Jagtap & Manohar, 2021). However, the major bottleneck for the production of these bioactive sugars is the identification of potential microorganisms capable of efficiently hydrolyzing macroalgal polysaccharides into oligosaccharides. Hence there is a continuous need to study the natural habitats for such microbes.

In this study, the MABs were isolated and characterized for their ability to utilize the model polysaccharides representing all three macroalgal groups. The results show that only about 10%–15% of bacteria could hydrolyse the complex polysaccharides such as porphyran, alginate and ulvan. The bacteria that showed the potential to hydrolyse the unique and complex macroalgal polysaccharides are very scarce and known to contribute to the remineralization of macroalgae after the completion of a growth cycle (Florez et al., 2017; Naik et al., 2019). Studies in the water column of the seaweed ecosystem show the dominance of Firmicutes and Proteobacteria (Kizhakkekalam & Chakraborty, 2019; Parvathi et al., 2020). Our study showed the diversity of potential MABs belonging to phylum Firmicutes (68%), followed by Proteobacteria (26%) and Actinobacteria (5%). Molecular tools based on Illumina sequencing and analysis were carried out to understand the diversity of microbiomes associated with macroalgae and described the presence of bacterial taxa belonging to

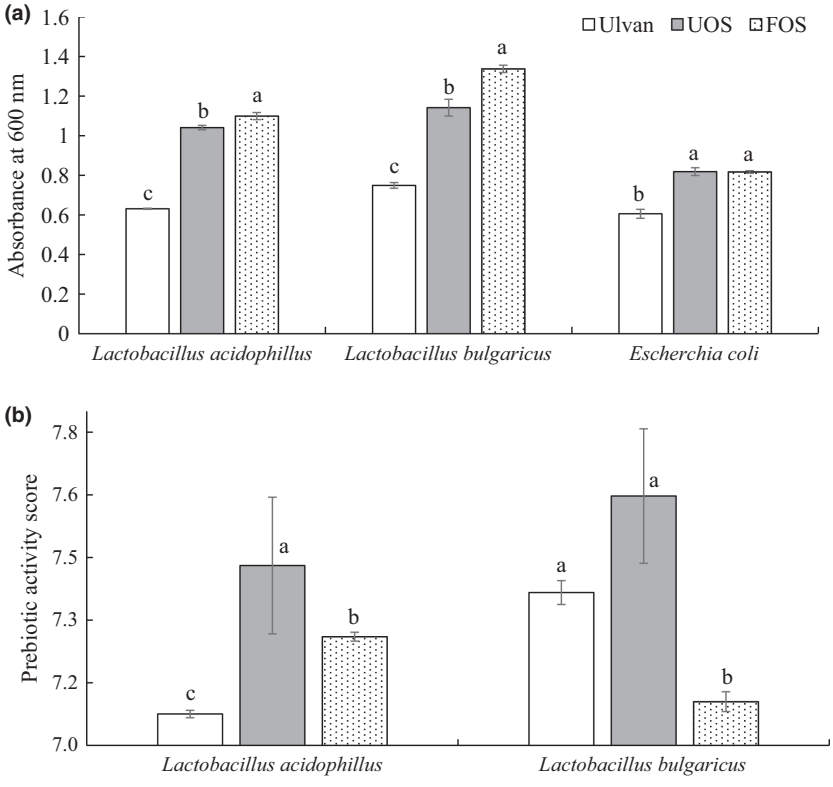

FIGURE 6 Prebiotic potential of ulvan oligosaccharides based on the (a) growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and (b) prebiotic activity score of probiotic bacteria in the presence of ulvan (white bars), ulvan oligosaccharides (grey bars) and fructooligosaccharides (dotted bars); a–c represents mean values within a bar with unlike superscript letters shows significant difference ($p \leq 0.05$)

Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria and Verrucomicrobia (Comba González et al., 2021; Selvarajan et al., 2019). The application of metagenomic tools for understanding functional diversity can provide more insights into the ecological and biotechnological potential of macroalgae-associated microbial communities (Lin et al., 2018).

Macroalgal polysaccharide hydrolysing activity has been reported from a large number of bacterial and a few fungal isolates, mostly from marine habitats (Jagtap et al., 2022). In our study, MABs, which had the highest porphyranase, alginate lyase and ulvan lyase activity, were reported by taxa belonging to Firmicutes and Proteobacteria. Among these isolates, NIOA181, *Bacillus* sp., and its enzyme ulvan lyase were studied in detail to produce UOS. Various studies have reported the polysaccharide hydrolyzing activity of *Bacillus* sp., which showed their ability to hydrolyse agar, carrageenan and alginate, but there are no reports on ulvan activity (Chen et al., 2018; Li et al., 2015, 2019; Suzuki et al., 2003). Very few studies have reported microbial ulvan lyase activity and enzymatic production of UOS (Table 2). Only one recent study has reported antioxidant and angiotensin I converting enzyme inhibition activity of UOS produced using non-specific enzymes such as amylase, cellulase and xylanase (Hung et al., 2021). This is the first study, to the best of our knowledge, reporting ulvan lyase activity from a *Bacillus* sp., associated with green macroalgae. Green macroalgae of ulvaceae are mainly composed of the sulphated heteropolysaccharide ulvan, accounting for 8%–29% of the dry weight of *Ulva* biomass (Gurpilhares et al., 2016). The poor solubility of ulvan due to its high molecular and polydisperse nature, with its complex composition of rare sugars such as rhamnose, uronic acid and xylose, limits its applications in various food, biofuel and pharmaceutical industries (Kidgell et al., 2019). The identification of ulvan lyase producing microbes can be used for efficient utilization of macroalgal polysaccharides, which are not utilized to their full potential.

Structural characterization of the UOS produced using ulvan lyase, ULB1 from MAB isolate, NIOA181 was carried out to confirm its structural composition based on FTIR, HRMS and NMR. The FTIR characterization of enzymatically produced UOS shows functional group-specific to ulvan such as -OH stretching vibration, -CH stretching vibration, -COOH asymmetric and symmetric stretching vibrations, -S=O stretching vibration band, C-O vibration associated with a -C-O-SO_3 and bending vibration of -C-O-S, were detected in UOS at wavenumber 3280 cm^{-1}, 2930 cm^{-1}, 1658 and 1417 cm^{-1}, 1172 cm^{-1}, 1080 cm^{-1} and 810 cm^{-1}, respectively. These peaks are similar to the earlier reports on UOS produced through enzymatic hydrolysis (Hung et al., 2021), which suggests that the enzymatic hydrolysis of ulvan could not remove the functional group-specific for parent polysaccharides. As ulvan is a polydisperse heteropolysaccharide constituted of repetitive disaccharide units of Rha3S-GlcA, Rha3S-IduA and Rha3S-Xyl (Lahaye et al., 1997). Enzymatic breakdown of ulvan can be achieved via hydrolysis or β-elimination mechanism using polysaccharide lyase (PL). Only a few PL families of ulvan lyase have been reported in the carbohydrate-active enzymes database (http://www.cazy.org), including PL24, PL25, PL28, PL37 and PL40 (Li, Hu, Wang, et al., 2020). PL24 and PL25 family ulvan lyase cleaves the glycosidic bond between GlcA-Rha3S and Rha3S-GlcA/IduA, respectively (Gao et al., 2019; Qin et al., 2018). Furthermore, PL28 family ulvan lyase cleaves the glycosidic bond next to both GlcA and IduA

TABLE 2 Studies on microbial ulvan lyase from marine sources and characterization of the enzymatically hydrolysed product

Source or enzyme	Degree of polymerization	Bioactivity	References
Persicivirga ulvanivorans	DP2–DP4	—	Collén et al. (2011)
Alteromonas sp.	NA	—	Coste et al. (2015)
Alteromonas sp.	DP2, DP4, DP6	—	He et al. (2017)
Alteromonas sp. LOR	DP2, DP4	—	Foran et al. (2017)
Pseudoalteromonas sp.	Di-, Tetra-	—	Qin et al. (2018)
Formosa agariphila	Di-, Tetra-	—	Reisky et al. (2018); Konasani et al. (2018)
Alteromonas sp. A321	Di-, Tetra-	—	Gao et al. (2019)
Alteromonas sp.	Di-, Tetra-	—	Qin et al. (2020)
Glaciecola KUL10	DP2–DP8	—	Mondal and Ohnishi (2020)
Pseudomonas vesicularis and *Aeromonas salmonicida*	NA	Antioxidant and ACE inhibition activity	Hung et al. (2021)
Bacillus sp. NIOA181	Di, Tri and Tetra	Prebiotic activity	This study

(Ulaganathan et al., 2018). The endolytic cleavage of ulvan through β-elimination using PL24 ulvan lyase from *Alteromonas* sp. (AsPL) forming disaccharides (ΔRha3S) and tetrasaccharides (ΔRha3S-Xyl-Rha) composed of unsaturated uronic acid at the non-reducing end (Lahaye et al., 1997; Qin et al., 2020). Similarly, ulvan hydrolysis using ulvan lyase of PL25 family from *Alteromonas* sp. A321 produced disaccharides of ΔGlcA-Rha3S and tetrasaccharides of ΔGlcA-Rha3S-IdoA-Rha3S (Gao et al., 2019). The ulvan depolymerization using ulvan lyase from *Formosa agariphila* KMM 3901T overexpressed in *Escherichia coli* results in the release of unsaturated disaccharides such as ΔRha3S and a tetrasaccharide of ΔRha3S-Xyl-Rha as the principal end products (Konasani et al., 2018). The enzymatically produced UOS was characterized to confirm its structural configuration. FTIR spectroscopy confirmed its functional groups and the ^{13}C NMR analysis was used to confirm the identity of the ulvan specific sugars in UOS. Further, mass spectrometry analysis was performed to estimate the sugar moiety based on the molecular weight. It indicated that the end products of enzymatic hydrolysis of ulvan using ulvan lyase ULB1 from NIOA181 were disaccharides of ΔRha3S, trisaccharides of ΔRha3S-Xyl, ΔRha3S-Xyl-2S and tetrasaccharides of ΔRha3S-Xyl-Rha, ΔRha3S-XylΔ with 4-deoxy-L-threo-hex-4-enopyranosi duronic acid at non-reducing end, which was similar to UOS produced using broad-spectrum ulvan lyase, from *F. agariphila* KMM 3901T (Konasani et al., 2018; Lahaye et al., 1997). The similarity in unsaturated (4-deoxy-L-th reo-hex-4-enopyranosiduronic acid) end products and intermediate products without uronic acid composition confirm that the ulvan lyase ULB1 isolated from NIOA181 cleaves the 1, 4 linkages between Rha3S and GlcA or IdoA through endolytic cleavage.

The bioactive potential of the UOS was also studied based on the prebiotic activity, which was evaluated based on their capacity to increase the growth of probiotic bacteria. The enzymatically produced UOS showed one to two-fold higher activity than ulvan to proliferate the probiotic strains *L. acidophilus* and *L. bulgaricus*, which was equivalent to FOS. The prebiotic activity tested based on their ability to suppress the growth of enteric bacteria showed that UOS was equivalent to commercial prebiotic FOS and its prebiotic score UOS was recorded to be higher than ulvan and FOS. These enzymes are in global demand to hydrolyse the complex polysaccharides to produce bioactive oligosaccharides. The ulvan lyase of MAB, NIOA181, has the ability for enzymatic depolymerization of ulvan for the production of UOS with the prebiotic activity that could potentially be used in the healthcare sector for the efficient proliferation of gut microbiota. These prebiotic compounds produce small chain fatty acids, favouring the growth of probiotics and suppressing oxidative stress caused by enteric pathogens (Guarino et al., 2020; Hamdy et al., 2018). This provides protection against colon infections and inflammation, enhances mineral absorption and lipogenesis and prevents any other gastro-intestinal disorders, including colon cancer (Davani-Davari et al., 2019; Han et al., 2019). Identification and characterization of potential bacteria and its enzyme ulvan lyase in this work can be used efficiently for enzymatic hydrolysis and bioconversion of ulvan containing green macroalgae biomass to produce oligosaccharides with prebiotic activity for various healthcare applications.

ACKNOWLEDGEMENTS

Ashok S. Jagtap received Junior and Senior Research Fellowship, grant number 31/026/(0300)/2017-EMR-I from the Council of Scientific and Industrial Research, India and Ashutosh S Parab (F.16-6 [Dec. 2016]/2017) acknowledges the research fellowship from University Grants Commission, India. This is NIO contribution number 6958.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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How to cite this article: Jagtap, A.S., Parab, A.S., Manohar, C.S. & Kadam, N.S. (2022) Prebiotic potential of enzymatically produced ulvan oligosaccharides using ulvan lyase of *Bacillus subtilis*, NIOA181, a macroalgae-associated bacteria. *Journal of Applied Microbiology*, 133, 3176–3190. Available from: https://doi.org/10.1111/jam.15775