“Production, purification and characterization of Extracellular Polymeric Substances from biofilm forming marine bacteria”

A dissertation thesis submitted to

Institute of Science Nirma University

for partial fulfilment of the requirements for

Degree of Master of Science (M. Sc.) in Biotechnology

(2020 – 2022)

BY

Pooja Saran (20MBT037)

Under the guidance of

Dr. D.R. Chaudhary, Principal Scientist

CSMCRI

CSIR – Central Salt and Marine Chemical Research Institute

Bhavnagar – 364002, Gujarat

NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
NAAC ACCREDITED ‘A’ GRADE

Institute of Science, Nirma University

Ahmedabad – 382481, Gujarat

April 2022

केन्द्रीय नमक व समुद्री रसायन अनुसंधान संस्थान
गिजुभाई बधेका मार्ग, भावनगर- ३६४ ००२
CENTRAL SALT & MARINE CHEMICALS RESEARCH INSTITUTE
Gijubhai Badheka Marg, Bhavnagar 364 002, Gujarat, India
CSIR-CSMCRI

PO/HR/2022
Dated: 22.04.2022

Dr. Doongar R. Chaudhary
Principal Scientist
Division of Plant Omics

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Pooja Saran** a student of M.Sc. Biotechnology at **Nirma University, Ahmedabad, Gujarat** has worked under my supervision for her M. Sc. dissertation thesis entitled **"Production, Purification and Characterisation of Extracellular Polymeric Substances (EPS) from Biofilm Forming Marine Bacteria"** at the Plant Omics Division of CSIR-Central Salt & Marine Chemicals Research Institute (CSIR-CSMCRI), Bhavnagar as partial fulfilment of M. Sc. Degree in Biotechnology. The thesis embodies original work done by her. Her work was found satisfactory and I wish her very good luck for her future endeavors.

(D.R. Chaudhary)

☎ : (O) 0278-2569496 Fax : 0278-2567562 E-mail: drchaudhary@csmcri.res.in

DECLARATION

I hereby declare that, to the best of my knowledge and belief, the thesis entitled **"Production, Purification and Characterisation of Extracellular Polymeric Substances (EPS) from Biofilm Forming Marine Bacteria"** is being submitted to the Institute of Science, Nirma University, Ahmedabad, for the partial fulfilment of the degree of Master of Science in Biotechnology (2020-2022). The work was carried out under Dr. D.R Chaudhary's (Principal Scientist, Plant Omics Division) supervision of CSIR-CSMCRI, Bhavnagar. I assure that this data is for my practical experience and skill development; will not be published, presented, or used for any other purpose in the future. I ensure that all the data presented in this report will be treated with the utmost confidentiality.

Date: 22.04.22

Place: Bhavnagar

Pooja Saran

(Student's signature)

ACKNOWLEDGEMENT

I would like to express my gratitude to the CSIR - Central Salt and Marine Chemical Research Institute (CSIR-CSMCRI) for giving me this opportunity to complete my dissertation. This is an exceptional opportunity for me that I will cherish throughout my life.

I would like to sincerely thank **Dr. D.R. Chaudhary** (Principal Scientist at CSIR-CSMCRI, Bhavnagar) for allowing me to work under his guidance.

I would like to thank **Dr. Kannan Srinivasan** (Director, CSIR-CSMCRI) and **Dr. P.K. Agarwal** (Head, Division of Plant Omics) for permitting me to carry out my dissertation at CSIR-CSMCRI, Bhavnagar.

It is my privilege to express my heartiest regards and gratitude to **Dr. M.S. Rathore**, **Dr. Avinash Mishra** and **Dr. Moutusi Manna** for their kind support and guidance throughout my work.

I am grateful to **Mr. Madhav Kumar** (Ph.D. fellow) for his scholarly guidance, supervision, consistent encouragement, and invaluable support in completing my dissertation work.

My sincere thanks to the Institute of Science, Nirma University, **Prof. Sarat Dalai** (HOD, Institute of Science) and **Dr. Vijay Kothari** for giving me the opportunity of undergoing five months dissertation at CSIR-CSMCRI, Bhavnagar, Gujarat.

I would like to thank all fellow members of the Molecular Biology and Biotechnology laboratory for their constant support throughout my dissertation work. I also want to express my thanks to my beloved parents and family members for their love, support and blessings.

Pooja Saran

INDEX

List of Figures

List of Tables

List of Abbreviations

EPS	Extracellular Polymeric Substances
ZMA	Zobell marine agar
ZMB	Zobell marine broth
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
BHIB	Brain Heart Infusion Broth
CR	Congo Red
TCA	Trichloroacetic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
EtBr	Ethidium bromide
TBE	Tris/Borate/EDTA buffer
SEM	Scanning electron microscopy
EDX	Energy dispersive X-ray spectroscopy
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
mL	Millilitre
µg	Microgram
L	Litre
Nm	Nanometre
OD	Optical Density
Rpm	revolutions per minute
µL	Microlitre
Mg	Milligram
kDa	Kilodaltons
kV	Kilovolt
mm	Millimetre

ABSTRACT

The marine environment is home to different bacteria that can be exploited to produce useful compounds such as extracellular polymeric substances. EPS is currently being used as a promising biopolymer in a wide range of biotechnological applications due to its unique biological features. EPS secreted by bacterial strains BM14 and RM26 were isolated, purified, and characterised in this study. The bacterial strains BM14 and RM26 produced 1126.0 mg L^{-1} and 1187.2 mg L^{-1} of EPS, respectively. The total sugar content of BM14 and RM26 extracted EPS was found to be 50.14 µg mg^{-1} and 122.75 µg mg^{-1} respectively. Antimicrobial susceptibility assay for bacteria determined the sensitivity of particular antibiotics to bacterium and both the strains showed sensitivity to all the antibiotics tested. SEM analysis of EPS produced by BM14 and RM26 was observed to be amorphous in nature. Both EPSs exhibited strong antibacterial activity against seven pathogenic bacteria: Pseudomonas *putida, Serratia liquifaciens, Escherichia coli, Serratia marcense, Klebsiella pneumoniae* and *Pseudomonas auregenosa* in vitro. Elemental analysis by EDX confirms the presence of carbon content to be higher in EPS produced by BM14 and RM26. The bacteria selected for the current study, BM14 and RM26 were already identified by 16S rRNA gene sequencing as *Staphylococcus warneri* and *Bacillus megaterium* respectively. The heterogenous features of EPS that were discovered in this study could be used in various biotechnological applications.

1. Introduction

The marine hydrosphere constitutes ~70% of its total surface area and covers most of the earth's biosphere. The ocean comprises the world's largest ecosystem, with a surface area of 365 million km^2 and a maximum depth of almost 11,000 meters. The ocean constitutes an extensive range of plants and wildlife and has a prominent supply of natural resources (Singh et al., 2016). The marine environment is a fluctuating habitat with higher or lower temperatures and pH levels creating challenges for the survival of marine microorganisms. Nutrient-limiting conditions in seawater affect the growth and survival rate of microorganisms (Smith et al., 2018). Temperature, pH, carbon source, salinity, and nutrition availability are all external factors that influence the chemical framework of bacterial biopolymers (Caruso et al., 2018). Microbes are found in two phases in the marine environment: planktonic (free-living) and biofilm (attached to a surface and each other and embedded in a self-producing matrix called extracellular polymeric substances (EPS)) (Smith et al., 2018). To maintain its growth in the harsh conditions of the marine ecosystem, the microorganisms tend to attach to the surfaces. Bacteria adhere to the surface by forming a biofilm in extreme circumstances (Goel et al., 2021; Singh et al., 2016). Microorganisms prefer biofilm formation because it is a vital and robust structure with a wide range of advantages like adhesion capabilities, nutritional sources, cell-cell communication (quorum sensing), resistance to drugs, environmental stresses, etc. (dos Santos et al., 2018). Bacteria are exceedingly diverse in the marine environment and to live in such harsh conditions, most marine bacterial cells are surrounded by EPS (Singh et al., 2016). The term "exopolysaccharide" was coined by Sutherland (1972) to define high-molecular-weight carbohydrate polymers produced by marine bacteria, and it is now frequently used to describe extracellular polymeric substances (Ignatova-Ivanova, 2017; Singh et al., 2016).

1.1 Biofilm

In nature, biofilms are made up of various microorganisms, including bacteria, archaea, yeasts, moulds, algae, and protozoans (Ann Punnen et al., 2018). They're found in natural resources like the sea, rivers, and submerged rocks, as well as medical (plaque and medical implants) and engineered systems including water pipelines, sewage, and

offshore oil and gas (Kavita et al., 2014). Biofilms are described as a complex aggregation of single or heterogeneous microbial communities embedded in a self-produced EPS matrix on biotic and abiotic surfaces (Mgomi et al., 2021; Packiavathy et al., 2021). Bacteria living in biofilms can withstand host immunological responses and are particularly resistant to antibiotics (Packiavathy et al., 2021) because the EPS acts as a barrier, providing anchorage and support while prohibiting therapeutic drugs from penetrating (Feng et al., 2021). The continuous flow of nutrients and waste outside the biofilm is a basic requirement for biofilm (El-Tarabily et al., 2021)Microorganisms generally occupy only 10% of the dry matter in biofilms, with the EPS structure occupying the remaining 90% (Kavita et al., 2013; Satish et al., 2017; Srinivasan et al., 2017) which also facilitates the development of three-dimensional (3D) structure, adherence to surfaces, and biofilm cohesion (Wingender et al., 1999). Within the biofilm, EPS is distributed in a non-homogeneous fashion between cells. Microbial cells are enclosed in an EPS matrix that interacts with one another. The matrix's stability is maintained by non-covalent interaction between EPS, which includes weak physicochemical forces (di Martino, 2018). This matrix helps organisms adapt to their surroundings and protects them from stresses like desiccation, biocides, antibiotics, heavy metals, and UV radiation (Kavita et al., 2013). Most bacterial pathogens use the quorum sensing (QS) mechanism to control biofilm development and the synthesis of additional virulent components to build pathogenicity in the host. This QS mechanism is also termed as the cell-to-cell communication system because bacteria communicate at both the inter-and intra-species levels using small diffusible signal molecules called autoinducers (AIs) (Giaouris et al., 2015; Packiavathy et al., 2021). The first stage of biofilm growth is reversible sorption, which occurs because of intermolecular interactions and hydrophobicity, and the second stage is the production of polymeric substances, which enables the cells to adhere to a surface and proliferate (Caruso et al., 2018; Gillett et al., 2016). Microhabitats and oxygen-free environments are formed as a result of biofilm formation, and they serve as hotspots for microbial-assisted organic transformation and element cycling. Bacterial aggregation also helps in the sequestration of trace metals, nutrients and other essential elements, making them more available to germs (Caruso et al., 2018). Forming a biofilm matrix aids structural organization and protects the microbial community. It is also crucial for metal adsorption and immobilization (Jiao et al., 2010). Biofilm development plays an essential role in chronic or recurrent infections (Ascenzioni et al., 2021). As a result,

research has focused on establishing alternative strategies for preventing the formation of biofilms in order to remove pathogenic bacteria (Kanmani et al., 2011). According to one study, EPS from *Streptococcus phocae* PI80 can prevent pathogenic bacteria from forming biofilms (Kanmani et al., 2011). The discovery of biofilm-producing marine microbes may lead to greater use of environment-friendly EPS molecules in industry, reducing dependence on biohazardous, non-degradable synthetic polymers. (Kavita et al., 2011).

1.2 Extracellular Polymeric Substances (EPS)

Microorganisms like bacteria, cyanobacteria, fungus, marine microalgae, and some marine microbes secrete a slimy layer known as EPS (Costa et al., 2018). It is mostly made up of polysaccharides, structural proteins, DNA, enzymes, lipids, and other compounds like humic acids (Costa et al., 2018; Xiao & Zheng, 2016The EPS matrix is made up of 50 to 90% of total organic matter and is produced by microorganisms that release secretion after consuming various biochemicals (Siddharth et al., 2021). Proteins and polysaccharides are the most important EPS components, accounting for 1% to 60% and 40% to 95% of total EPS components, respectively. (Izadi et al., 2021). The molecular weight of EPS ranges from 1 kDa to 2000 kDa (Dong et al., 2020; Flemming & Wingender, 2010; Nakano et al., 2018). Bacteria produce EPS in two forms: a capsule enclosing the bacterial cell surface known as a capsular polysaccharide (Ai et al., 2016; Insulkar et al., 2018), and an extracellular polysaccharide which is another type of slime polysaccharide that is loosely linked to the cell's outer surface and discharged into the surrounding (Caruso et al., 2018). Capsular polysaccharides (CPS) are strongly linked to cells, organised in a polymeric structure, closely packed, and adherent to a cell wall via linkage between the carboxyl groups of exopolysaccharides and the hydroxyl groups of lipopolysaccharides, or via covalent bonding via phospholipids and glycoproteins (Caruso, Rizzo, Mangano, Poli, Donato, et al., 2018). CPS is produced by bacteria during their log phase of bacterial growth (Minimol et al., 2019; Morais et al., 2018), and is involved in pathogenesis (Caruso, Rizzo, Mangano, Poli, Donato, et al., 2018). Bacteria produce EPS when they're in the stationary phase of their growth cycle (Gongi et al., 2020, 2022; Minimol et al., 2019). Bacteria that live in the harsh environment produce EPS as a protective shield around the cell (Insulkar et al., 2018). During the starvation period, they also act

as carbon and energy reserves (Kumar Singha, 2011). The EPS layer within the bacterial cell maintains the osmotic environment, and bacteria colonise to increase their chances of survival (Insulkar et al., 2018). EPSs are classified as homopolysaccharides or heteropolysaccharides entirely depending on their monomer composition; homopolysaccharides are monomers, whereas heteropolysaccharides are repeating units of different monomers, such as l-fructose, d-glucose, d-galactose, d-glucuronic acid, l-rhamnose, l-guluronic acid, and d-mannuronic acid (Sharma et al., 2021). In heteropolysaccharides, the repeating unit of different monomers is branched or unbranched, regular, and linked together by glycosidic linkages (Boukhelata et al., 2019). EPS isolated from marine bacteria are predominantly heteropolysaccharides (Poli et al., 2010; Sran et al., 2019) which are formed of 3 or 4 distinct monosaccharide units like pentose, hexose, amino sugar, uronic acid, and so on (Joulak et al., 2019). In addition to monomeric units, functional groups like sulfate, phosphate, pyruvate, acetate, succinic acid, and others may be added to the polymer chain (Escárcega-González et al., 2018). The wide range of physicochemical properties of EPS is due to its structural composition. These structural differences are critical for gaining exopolysaccharide applications (Sran et al., 2019).

Nowadays, EPS is quickly becoming a novel and significant source of polymeric compounds for industry. There is a growing interest in studying biofilm-forming bacteria and the EPS they produce. More biochemical characterization of EPS is required to determine its potential industrial application. In a marine environment, tidal action, high wave, and irregular exposure to air may cause the selection of bacteria with water-holding, adhesive, and protective polysaccharide coating. The EPS is extensively researched due to its numerous environmental and industrial applications such as it is used as thickeners and gelling agents in the food industry, in the pharmaceutical industry for developing bacterial vaccines, and in wastewater treatment (Abid et al., 2021; de Carvalho & Fernandes, 2010; Mishra et al., 2011).

2. Review of Literature

2.1 Biofilm

2.1.1 Biofilm formation

Biofilm formation starts with planktonic bacteria attaching themselves to surfaces (solid substrates). Bacteria use adhesins (such as fimbriae and lipopolysaccharide) to completely adhere to surfaces, a process known as irreversible attachment. Bacteria begin to grow and produce EPS, which helps in matrix formation and attachment. Later bacterial cells begin to produce and release signalling molecules to identify each other and develop a biofilm. The biofilms continue to mature and develop into the complex 3-D structure. After the microbial cells have aggregated and colonised themselves to form a biofilm, they use Quorum sensing to communicate with one another. Finally, the biofilm can release some of its colonies into the environment, allowing them to colonise new surfaces. Therefore, the bacterial cells escape from biofilms and return to their planktonic life form (Fig 1) (Muhammad et al., 2020; Shukla et al., 2017; Vasudevan, 2014).

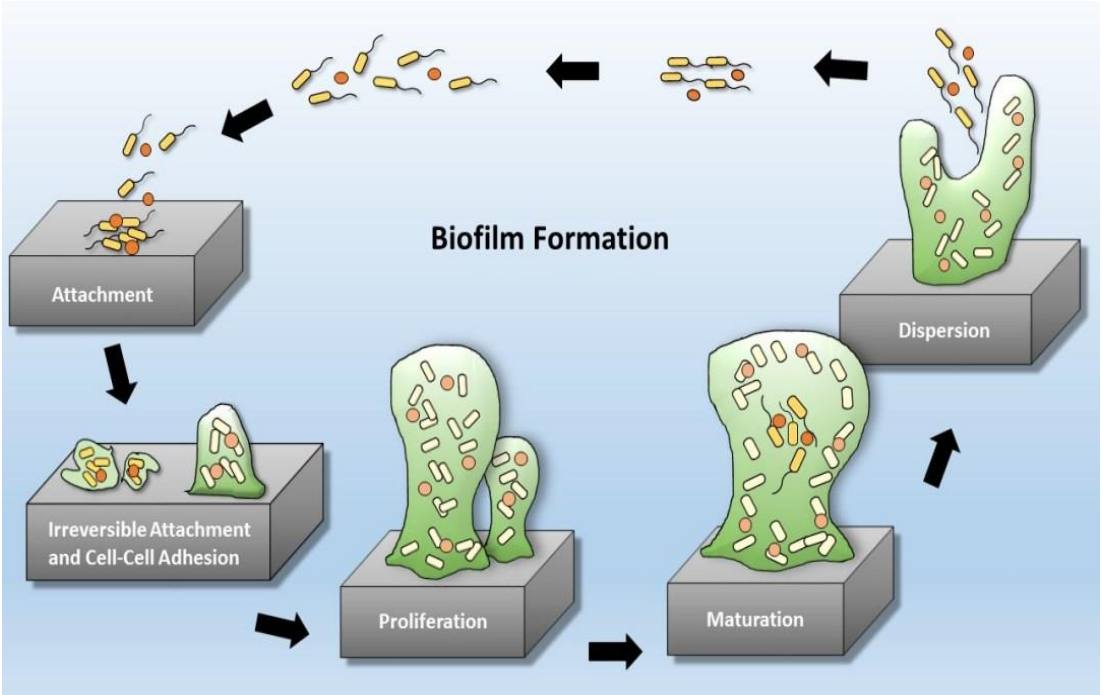

Fig 1: Biofilm Formation (Bakar et al., 2018)

2.1.2 Biotechnological Applications of Biofilm

According to Vu et al., (2009) EPS from biofilm is utilised in a wide range of biotechnology biomedical, and industrial uses, such as food engineering, pharmaceutical, and surgical applications, and bioremediation.

a) Biofilm is utilized in the remediation process due to its capability to sustain in its most hazardous environments and has more effective defense mechanisms because of specific genes (Shukla et al., 2017). Biofilm formed by bacteria can effectively eliminate dyes and metals through biosorption and bioaccumulation because the higher biomass density can lower the toxicity of certain metals and dyes using their enzyme activities (Sharma et al., 2021). Heavy metals can be effectively removed by bacteria such as *Escherichia coli, Bacillus subtilis*, and *Pseudomonas putida* CZ1 cultured from diverse metal-contaminated areas (Mohapatra et al., 2019).

b) Bacteria forming biofilm might be used to stimulate plant growth and also as a biocontrol agent in sustainable agriculture. It can be used to improve plant health and growth by increasing the production of growth hormones such as indole acetic acid (IAA) (Kour et al., 2019).

c) Recently biofilms have been used in agriculture and food industry because of their specific features like food fermentation, probiotics potential, inactivation of undesirable microbial growth, wastewater treatment, etc. (Turhan et al., 2019).

d) Biofilm-forming bacteria produce EPS, a biotechnologically important renewable source with a vast structural variety, as well as rheological, physical, and other distinguishing features (Vu et al., 2009).

   Understanding biofilm phenotype demands investigation and characterisation of a biofilm matrix. The EPS composition of the biofilm, interactions among EPS, and interactions among both EPS and microbial cells are all investigated as part of the characterization (di Martino, 2018).

2.2 Extracellular Polymeric Substance

Table 1: Bacterial EPS and the amount of EPS recovered from various bacteria

S.NO	Bacteria	Isolated from	Amount of EPS (mg L^{-1})	References
1.	*Vibrio parahaemolyticus*	Marine bacteria (Diu, India)	58.98	(Kavita et al., 2011)
2.	*Colwellia* sp. GW185	Sponge associated	120	(Caruso, Rizzo, Mangano, Poli, Donato, et al., 2018)
	Shewanella sp. CAL606	Antarctic bacteria	130	
	Winogradskyella sp. CAL396		52	
	Winogradskyella sp. CAL384		34	
3.	*Paenibacillus tarimensis* REG 0201M	Algerian Sahara (Rhizospheric soil of Triticum *durum*)	1310	(Boukhelata et al., 2019)
4.	*Marinobacter* sp. W1-16	Antarctic surface seawater	87	(Caruso et al., 2019)
5.	*Bacillus licheniformis*	Bacteria from Seaweed *G. dura*	576	(Singh et al., 2011)
6.	*Vibrio campbellii*	From Arabian Sea at Diu, India	400	(Kavita et al., 2013)
	Vibrio fortis		134	
7.	*Oceanobacillus iheyensis*	The coastal region of Sikka, India	400	(Kavita et al., 2014)
8.	*Pseudomonas* sp. PFAB4	Hot spring field, (in coal mine region)	2630	(Banerjee et al., 2018a)

Table 2: Types of EPS, their source, and functional properties

S.NO	EPS	Sources	Applications	References
1.	Xanthan	*Xanthomonas campestris*	Used as a cryoprotectant, stabilizing agent, thickening agent in the food industry High viscosity, pH, and temperature stability; Metal sorption; water retention; soil stability and strengthening; petroleum and pharmaceutical sectors; Also, it is used in cosmetics, agriculture, textile, and other industries.	(Julianti et al., 2017; Şengör, 2019; Sriprablom et al., 2019)
2.	Gellan	*Pseudomonas elodea, Sphingomonas spp*	In the food industry, it's used as a thickening agent, gelling agent, and emulsifier., a wide range of pH stability, hydrocolloids; Pharmaceutical industries; Soil strengthening	(Saha & Bhattacharya, 2010; Şengör, 2019; Yildiz & Karatas, 2018)
3.	Levan	*Zymomonas mobils, Alcaligenes viscosus, Paenibacillus polymyxa,*	Used as prebiotic in the food industry, anti-inflammatory & anti-tumor activities, film-forming capacity, low viscosity, high solubility; Soil aggregation;	(Esawy et al., 2013; Şengör, 2019; Vijayendra & Shamala, 2014)

		Bacillus subtilis	pharmaceutical industries	
4.	Curdlan	*Alcaligenes faecais*	To improve the viscoelasticity and stability of the food, it is used as a food additive. It's also utilised as a gelling agent and a matrix for immobilization.	(Shukla et al., 2019; Yildiz & Karatas, 2018)
5.	Dextran	Lactic acid bacteria (e.g., *Leuconostoc mesenteroides, Streptococcus mutans*, *Acetobacter capsulatus*)	Good stability, non-ionic; Water retention; Used in food, pharmaceutical industries It is used as a moisturiser and thickener in cosmetics. It is added to bread items and confectionery to enhance softness or moisture retention, prevent crystallization, and increase rheology viscosity, volume and texture, in the food sector.	(Şengör, 2019; Senpuku et al., 2018; A. Shukla et al., 2019; Yildiz & Karatas, 2018)
6.	Alginate	*Azotobacter* spp. and *Pseudomonas* spp.	Gelling capacity, anionic exchange, Hydrocolloids, Film-forming capacity; In pharmaceutical and medicines, In	(Cervino et al., 2019; A. Shukla et al., 2019; Thu et al., 2012)

			agriculture, Food hydrocolloids	
7.	Hyaluronan	*Pasteurella multocida* and *Streptococcus* spp	High hydrophilicity, biological activity, biocompatible and anionic charge; it's commonly utilised in food and cosmetics.	(Mandawe et al., 2018; Schulte et al., 2018; A. Shukla et al., 2019; Yildiz & Karatas, 2018)
8.	Pullulan	*Cytaria spp.*, *Aureobasidium pullulans*, *Rhodototula bacarum*, *Teloschistes flavicans*, *Cryphonectria parasitica*	Water solubility, water absorption capacity, and the ability to create strong, flexible films and fibres are desirable traits; Used as an edible covering to limit bacteria development and improve shelf life; important role in food, biomedical, and pharmaceutical fields;	(Widyaningrum & Meindrawan, 2020; Yildiz & Karatas, 2018)

2.2.1 Functions of Extracellular Polymeric Substances

a) Microorganisms that form biofilms and are surrounded by EPS play a key role in antimicrobial resistance (Everett & Rumbaugh, 2015; Fulaz et al., 2019). Biofilm matrices are negatively charged and adhere to positively charged molecules to guard the innermost cells against contact (Everett & Rumbaugh, 2014; Singh et al., 2021). Few investigations have indicated that *Staphylococcus* species produce slime that is an effective antagonist of pefloxacin, vancomycin, and teicoplanin, serving as a barrier to the compounds or affecting their activity in the cell membrane. (Costa et al., 2018).

b) Some of the primary functions of EPS involve bacterial cell aggregation, adherence to surfaces, floc production, cell-cell recognition, structural feature of biofilms, and the development of a protective shield for cells (More et al., 2014a; Solmaz et al., 2018; Tian, 2008). EPS are responsible for cohesion in microbes and biofilm attachment to surfaces, which controls spatial structure and allows the microorganism to interact, and act as adhesives (Donlan, 2002; Karygianni et al., 2020). Biofilms and flocs depend on these functions for their establishment (Costa et al., 2018). *Sphingomonas paucimobilis*, for example, has surface-active characteristics that promote and increase adhesion by forming polymeric substances (Azeredo & Oliveira, 2000). The amount of EPS in a cell can also have an impact on cell adhesion (Tsuneda et al., 2003).

c) EPS matrices have properties such as biodegradability, adsorption, and hydrophobicity or hydrophilicity due to their unique composition (Sheng et al., 2010; Solmaz et al., 2018).

d) EPS is also necessary for mass transfer between biofilms, adsorption of different metals and organic or inorganic chemicals by biofilms, and, most importantly, structural support for biofilms resistant to shear (Solmaz et al., 2018). EPS is generally anionic, which can help in the uptake of nutrients and vital minerals.

Anionic characteristics also promote the chelation of metals and ions, preventing them from entering cells (Choudhuri et al., 2020).

e) In the marine environment, EPS contributes in the elemental intake and breakdown of organic compounds, making them accessible for microbial development and other nearby microbial communities (Minimol et al., 2019).

f) Microorganisms that reside in low temperatures and high salinity rely on the EPS production for cryoprotection (Costa et al., 2018; Mancuso Nichols et al., 2005; Wang et al., 2019). The microorganism samples from the Arctic Sea Ocean revealed high EPS concentrations. The existence of EPS allows thermophilic bacteria to survive at extremely high temperatures. Bacillus sp. strain B3-72, for example, produces polymers that are resistant to degradation at high temperatures. (Costa et al., 2018).

2.2.2 Applications of Extracellular Polymeric Substances

EPS has numerous environmental and industrial uses due to its wide range of advantages and unique features such as biodegradability, biocompatibility and non-toxicity (Boukhelata et al., 2019; More et al., 2014). Different microorganisms produce EPS with different compositions, sizes, structures, numbers, and types of functional groups attached; thereby, it has many physiological functions (Aullybux et al., 2019).

a) EPS is extensively used as thickeners, viscosifying, gelling, stabilising, and emulsifying agents in the food industry (Abid et al., 2021; Insulkar et al., 2018; Korcz & Varga, 2021). *Lactobacillus* species, for example, produce EPS, which is used for gelling, thickening, and emulsifying properties in food industry (Insulkar et al., 2018).

b) EPS is mainly composed of polysaccharides with different structural properties. Some of which might have a unique feature that can be utilized in wastewater treatment for dewatering and sludge settling applications (Mishra et al., 2011; Sun et al., 2015). For example, the *Alteromonas* sp. strain produces EPS which

has a high affinity towards divalent ionic species and therefore can help in wastewater treatment. The *Zunongwangia profunda* strain's metal-binding ability for Cu (II) and Cd (II) is often used in treating wastewater (Sun et al., 2015).

c) It is exploited as a hydrophilic matrix in the pharmaceutical industry to ensure that the drugs are administered in a controlled manner for the production of bacterial vaccines and to boost nonspecific immunity (de Carvalho & Fernandes, 2010; Kavita et al., 2011). Some investigations have mentioned that *Lactobacillus* spp. EPS has been used to develop probiotics (Aullybux et al., 2019).

d) Some recent studies have reported the bacterial EPS to have antioxidative and antibacterial properties (Aullybux et al., 2019). The salt pan isolate *Halolactibacillus miurensis* shows high antioxidant properties (Insulkar et al., 2018).

e) Xanthan and Gellan, two well-known commercially available bacterial EPS, are Generally Regarded as Safe (GRAS) (Ali et al., 2020). Xanthan from the *Xanthomonas campestrisis* is widely used in food, the petroleum sector, medicines, cosmetics, personal care goods, and the agriculture field (Ali et al., 2020; Rana & Upadhyay, 2020). Gellan from *Sphingomonas* is used in pharmaceuticals, food, and research (agar substitute and gel electrophoresis) (Ali et al., 2020).

f) According to Satpute et al. (2010), EPS produced by marine microorganisms is a powerful biosurfactant and bioemulsifier with a wide range of structural and functional properties that might be used in a variety of industrial applications. EPS is a better alternative to the chemically synthetic compound, which are hazardous to the environment.

g) EPS also functions as an anti-biofilm agent as reported from the interaction between *Pseudomonas aeruginosa* and *Staphylococcus epidermis*. The exopolysaccharide produced by *P. aeruginosa* caused the dispersal of *S. epidermis* biofilm without killing the bacteria (Qin et al., 2009). Two species of

Lactobacillus were also reported for the production of anti-biofilm exopolysaccharides (Kim et al., 2009)

2.3 Future Prospects

Microorganism-produced EPS is used in treating wastewater, soil erosion prevention, soil remediation and removing heavy metals (Siddharth et al., 2021). It has thickening, and gelling, coagulation, stabilization, and water retention capabilities (Tiwari et al., 2020). Nevertheless, further research is required on the chemical nature, components, functional properties, and ideal conditions for the development and recovery of EPS (Siddharth et al., 2021). EPS production is proportional to the proliferation of microorganisms, which have ecological and physiological roles. There is a scarcity of information on genomics and proteomics, which could make EPS synthesis more economically feasible (Tiwari et al., 2020). Another unexplored area is the marine environment which possesses a diverse range of EPS with several advantages over other polysaccharides due to continuous tidal action, high waves, and irregular exposure to air, which selects bacteria with strong water-holding, adhesive, and protective polysaccharide coating. Marine bacterial EPSs' wide structural diversity and physicochemical features appropriate for industrial applications are poorly understood (Singh et al., 2016). As a result, the current study aims to understand better marine bacterial EPS, which is increasingly growing as a valuable source of polymeric substances for industrial applications.

Objectives of the study:

1. Screening of bacteria for their EPS production
2. Isolation, purification and characterization of EPS from selected marine bacteria
3. Applications of extracellular polymeric substances

3. Materials and Methods

3.1 Bacterial isolates and culture condition

Bacteria for the current experiment were previously isolated from surfaces such as plastic bottles and rubber from Shikka. The axenic bacterial cultures were preserved in 25% glycerol at -80 °C. A total of 38 bacteria were revived from a glycerol stock. Bacteria (100 µL from glycerol stock) were inoculated in Zobell marine broth (ZMB) media and cultured overnight at 30°C and 120 rpm. From the ZMB tubes, the bacteria were further streaked on Zobell marine agar (ZMA) media. The isolate was further sub cultured on ZMA media to obtain a pure culture of bacteria. The composition of ZMB is given in the appendix.

3.2 Screening for EPS producing isolates

The screening for EPS producing bacteria was done on brain heart infusion broth (BHIB) media supplemented with 1.5% agar, and 5% sucrose. The composition of BHIB is given in the appendix. The media was autoclaved at 121 °C and 15 psi. After autoclaving, when the media temperature reached ~60 °C, filter sterilized (0.22 µm syringe filter) 0.8 % congo red (CR) dye was added. A single bacterial colony from the ZMA plate was picked with the help of an inoculation loop and streak plated on ZMA prepared for screening. The plates were kept for incubation at 30 °C and observed at every 24 h up to 96 h. The bacterial colony which showed a black color colony with a slimy and mucoid appearance was selected as EPS positive isolate (Kırmusaoğlu, 2019).

3.3 Antimicrobial Susceptibility Testing

The ability of the isolates to withstand various antibiotic impregnated discs was evaluated using the disc diffusion method (Caruso et al., 2018). Muller-Hinton broth (MHB) and Muller-Hinton agar (MHA) media was prepared and autoclaved. The composition of MHB is given in the appendix. All the positive bacterial isolates were cultured in MHB medium and incubated overnight at 30 °C and 120 rpm. Selected positive isolates grown in MHB media were spread plated on MHA using a sterile cotton swab. Antibiotics discs (Himedia) with different concentrations were placed on

MHA media (Table 3). The plates were incubated for 24 h at 30° C and the zone of inhibition was determined using an antibiotic zone scale (Himedia).

Table 3: Antibiotics disc with respective concentrations used in the study

S.NO	Antibiotics	Concentration (mcg/disc)
1.	Ampicillin A	25
2.	Penicillin G	10*
3.	Vancomycin	10
4.	Chloramphenicol	25
5.	Kanamycin	30
6.	Erythromycin	10
7.	Clindamycin	2
8.	Azithromycin	15
9.	Gatifloxacin	5
10.	Imipenem	10
11.	Co –Trimoxazole	25

*unit/disc

3.4 Extraction and Purification of EPS

Bacterial isolates (EPS positive) were inoculated in a ZMB medium and incubated at 30 °C and 120 rpm for 3 days. Bacterial cultures were centrifuged for 10 min at 10,000 rpm at 4 °C to remove bacterial biomass. The supernatant was filtered twice and exopolysaccharides were obtained by adding two volumes of cold isopropanol to the supernatant and incubating at 4 °C overnight. (Kavita et al., 2011). The mixture was centrifuged again for 10 min at 10,000 rpm at 4 °C. The supernatant was discarded and crude EPS pellet was dissolved in a minimum amount of distilled water. The proteins were precipitated by adding an equal amount of 20% (w/v) trichloroacetic acid (TCA) and incubated overnight at 4 °C. The solution was centrifuged at 10,000 rpm for 10 min at 4 °C and the protein pellet was discarded. The supernatant was further purified by dialysis against distilled water with a 12 kDa pore size dialysis membrane (Sigma Aldrich) for 24 h at 4 °C (Aullybux et al., 2019). Before dialysis, the dialysis membrane was activated by washing with hot water followed by acidifying with a 0.2% (v/v)

solution of H_2SO_4. The membrane was again washed with normal water to remove traces of acid. After 24 hours, the dialyzed EPS sample was placed in a lyophilizer bottle and solidified with the help of liquid nitrogen. Purified EPS were lyophilized at –70 °C for 10–12 h with lyophilizer VirTis freezemobile 25EL (Kavita et al., 2011). The weight of EPS was recorded after complete lyophilisation.

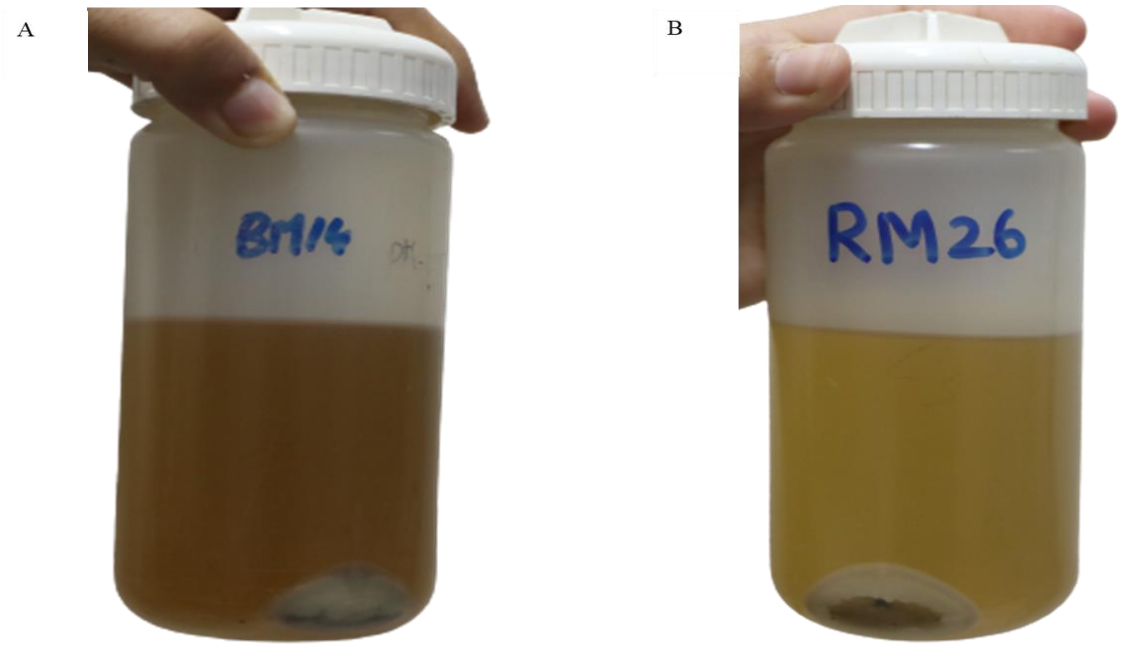

Fig 2: EPS extraction (liquid media contains the dissolved EPS while cell pellet contains bacterial biomass) of selected isolate BM14 and RM26

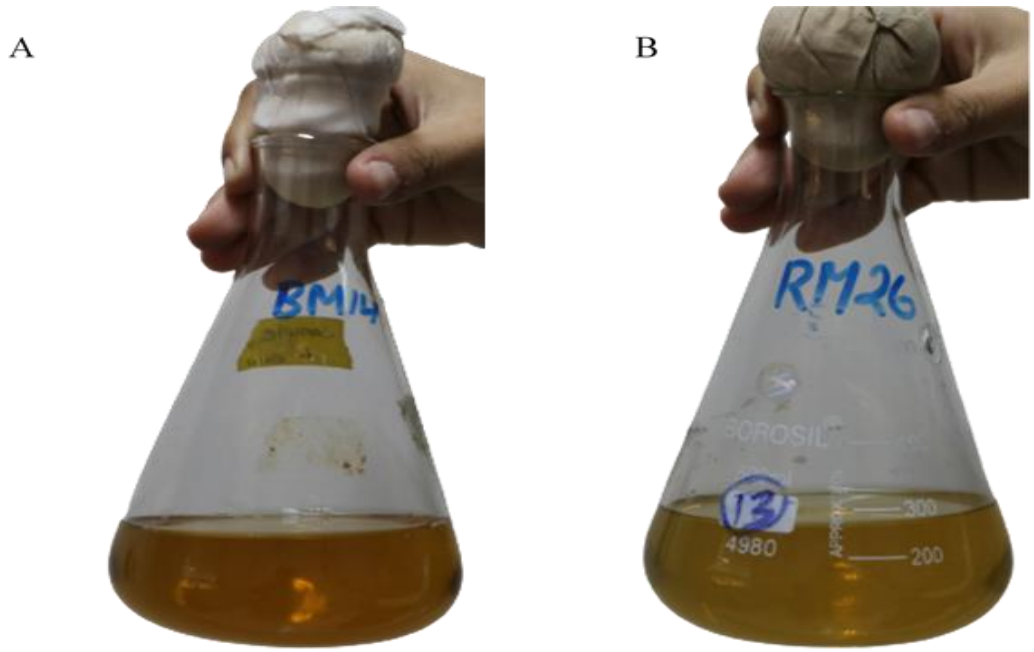

Fig 3: ZMB media containing dissolved EPS

Fig 4: VirTis freezemobile 25EL Lyophilizer

Fig 5: EPS sample solidified with the help of liquid nitrogen

3.5 Total Sugar Estimation

The total sugar (carbohydrate) content of extracted EPS was determined using the phenol sulphuric acid method taking glucose as a standard (Dubois et al., 1951). 200 µL EPS solution (1 mg mL^{-1}) in an eppendorf tube was treated with 200 µL of 5 % phenol and 1 mL concentrated H_2SO_4 (in triplicates). The standard curve was prepared using different concentrations of glucose (0 – 100 µg mL^{-1}). 200 µL from each tube were transferred into 96 well plate (quartz). The absorbance was measured at 490 nm using a spectrophotometer (EPOCH UV-VIS Spectrophotometer). The carbohydrate concentrations were determined using the standard curve (Sran et al., 2019).

3.6 Antibacterial Activity of EPS

The EPS with antibacterial properties was identified according to Balouiri et al. (2016). Muller-Hinton broth (MHB) and Muller-Hinton agar (MHA) media were prepared and autoclaved. All the pathogenic test strains were inoculated in MHB medium and incubated overnight at 30 °C at 120 rpm. The pathogenic strains used were - *Pseudomonas putida, Serratia liquefaciens, Escherichia coli, Proteus mirabilis, Shigella flexneri, Serratia marcense, Klebsiella pneumoniae, Staphylococcus epidermis, Pseudomonas auregenosa, and Enterococcus fecalis.* The pathogenic strains grown in MHB media were spread plated on MHA using a sterile cotton swab. Freeze-dried EPS was dissolved in filter-sterilized distilled water to a final concentration of 1 mg mL^{-1}. Filter paper discs were autoclaved, impregnated with 10

µl of the EPS, and placed on MHA seeded with test strains. A zone of inhibition was observed after 24 hours of incubation at 30°C (Aullybux et al., 2019).

3.7 Antioxidant Activity of EPS

3.7.1 Scavenging activity of DPPH radicals

The scavenging effect of the EPS on DPPH-free radicals was determined using Xie et al. (2019) method with minor modifications. DPPH solution was prepared by mixing 12 mg DPPH in 50 mL methanol, and OD was adjusted to 0.98 with methanol at 517 nm. Freshly prepared 100 µL DPPH was mixed with 100 µL of diluted EPS (0, 1, 2, 4 and 6 mg mL^{-1}) in distilled water in a 96-well plate and incubated in the dark for 30 min. Distilled water with DPPH was used for the blank control, and 100 µL of diluted ascorbic acid (0, 1, 2, 6, 4, 8, and 10 mg/mL) with DPPH was used as a positive control. The plate was read at 517 nm using a 96-well microplate reader (EPOCH UV-VIS Spectrophotometer). DPPH scavenging activity was calculated by the following formula (Xu et al., 2021):

$$\text{Scavenging activity (\%)} = (1-As/Ab) \text{ X } 100\%$$

As: Absorbance of sample
Ab: Absorbance of blank

3.7.2 Scavenging activity of ABTS radicals

ABTS radical scavenging activity was measured using the method described by Ma et al. (2018) with minor modifications. The ABTS was prepared by reaction of 90.05 mg ABTS in 25 mL distilled water with 16.53 mg Potassium persulfate in 25 mL distilled water. The resulting solution was then stored in the dark at room temperature for 16 hours. The ABTS solution was then diluted with distilled water to the absorbance of 0.70 nm at 734 nm before use. 100 µL ABTS solution was mixed with 100 µL of diluted EPS (0, 1, 2, 4 and 6 mg mL^{-1}) in distilled water in a 96-well plate and incubated for 5 minutes in the dark at room temperature. The absorbance of the mixture solution was determined at 734 nm (EPOCH UV-VIS Spectrophotometer), and ascorbic acid (0, 1,

2, 4, and 6 mg mL^{-1}) was used as a positive control. ABTS scavenging activity was determined using the following formula (Bomfim et al., 2020) :

Scavenging activity (%) = (1-As/Ab) X 100%

As: Absorbance of sample

Ab: Absorbance of blank

3.8 Characterization of EPS

3.8.1 Scanning Electron Microscopy (SEM) and Energy dispersive X-ray spectroscopy (EDX)

Scanning electron microscopy was used to examine the surface morphology of isolated EPS. The EPS sample was mounted on the stub and placed in a desiccator to prevent water retention by the EPS. The JSM – 7100 F Field Emission Scanning Electron Microscope was used to examine the surface morphology of EPS at 1000 x magnification with an acceleration voltage of 15.0 kV. The weight and atomic percentage of different elements present in the EPS sample were determined using energy dispersive X-ray spectroscopy (EDX) with the same instrument.

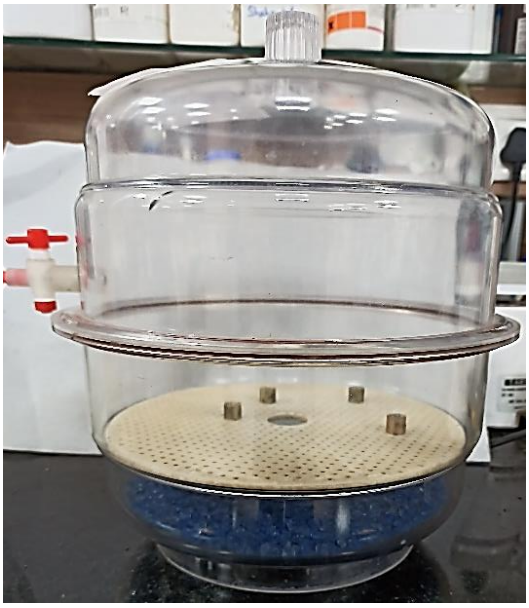

Fig 6: SEM and EDX sample preparation

3.8.2 Elemental Analysis

EPS sample (5 mg) was weighed and submitted to CSIR-CSMCRI central instrumentation facility to determine the total carbon, hydrogen, nitrogen, and sulfur contents using a CHNS analyser (Vario Micro Cube).

3.9 Biofilm formation assay

The biofilm-forming activity of EPS was determined using a slightly modified method described by O'Toole (2011). Briefly, all EPS-positive isolates and a positive biofilm culture of *Pseudomonas aeruginosa* were grown in ZMB media overnight. The overnight grown culture was diluted (1:100) in a fresh medium for biofilm assay. 100 µL from the dilution was added per well in a 96 well plate (in 4 replicates). The microtiter plate was incubated at 30 ℃ for 24 hours. Following incubation, the plate was turned over to remove out cells and submerged in a small tub of water to remove loosely attached bacterial cells. The unattached cells or media components were removed by shaking off water, and this step was repeated twice. 125 µL of a 0.1 % solution of crystal violet in water was added to each well of the microtiter plate. The plate was incubated at room temperature for 10-15 min and rinsed 3-4 times again with water. The excess cells and dyes were removed by shaking off water and the plate was blotted vigorously on a blotting sheet. The microtiter plate was dried overnight at 55 °C in an oven. 125 µL of 30 % acetic acid in water was added to a dried microtiter plate and incubated at room temperature for 10 to 15 min. The solubilized CV was transferred to a new microtiter plate, and absorbance was determined at 550 nm using 30 % acetic acid in water as blank (EPOCH UV-VIS Spectrophotometer).

3.10 DNA isolation and polymerase chain reaction (PCR)

3.10.1 DNA isolation

The Fast DNA Spin Kit was used to extract DNA from the bacterial sample, following the manufacturer's protocol. The overnight grown bacterial culture was transferred to an Eppendorf tube and centrifuged at 10,000 rpm for 5 min at room temperature. The pellet was resuspended in water and 1 mL of cell lysis buffer (CLS-TC) was added. The

homogenization was executed in the FastPrep instrument for 40 seconds at a speed setting of 6.0 followed by 5 to 10 min centrifugation at 14,000 rpm to pellet debris. The supernatant (700-800 µL) was transferred to a 2.0 mL microcentrifuge tube and mixed with an equal volume of the binding matrix. Inverted to mix. Incubated with gentle agitation for 5 minutes at room temperature on a rotator. Then half of the suspension was transferred to a SPIN Filter and centrifuged for 1 min at 14,000 rpm for. The catch tube was emptied and the remaining suspension was added to the SPIN Filter and centrifuged as before. The catch tube was emptied once a and 500 µL of prepared SEWS-M was added (ethanol must be added to concentrated SEWS-M). The pellet was gently resuspended using the force of the liquid from the pipet tip and centrifuged at 14,000 rpm for 1 minute. The contents of the catch tube were discarded and replaced. Centrifuged a second time at 14,000 rpm for 2 minutes and the catch tube was replaced with a new, clean tube. DNA was eluted by gently resuspending the binding matrix above the SPIN filter in 50 µL of DES (nuclease free water, supplemented with kit). The tube was closed and incubated at 55 ˚C in a heat block or water bath for 5 min. The eluted DNA was centrifuged at 14,000 rpm for 1 minute to bring it into the clean catch tube. The SPIN filter was discarded. Stored at -20 °C for extended periods or 4 °C until use.

3.10.2 DNA visualization on Agarose Gel Electrophoresis

The bands of DNA were observed on 1.2 % (w/v) agarose gel. Agarose gel was prepared by 1.2 g agarose added to 100 mL 0.5X TBE buffer. The agarose was microwaved for 1-3 min until completely dissolved and the solution was then cooled. 0.4 µL of Ethidium bromide (EtBr) was added to it. Agarose was poured into a gel tray with the well comb in place. When the gel solidified, it was placed into the electrophoresis unit filled with TBE buffer. The DNA sample was loaded into wells of the gel and was run for 1-1.5 hours. The gel was carefully removed from the electrophoresis unit and placed in the Gel Doc system to observe bands of bacterial DNA bands.

3.10.3 DNA Quantification by Nano-Drop

The concentration of DNA was measured using Nanodrop spectrophotometric ND-1000. Before measuring the sample concentration, 1 µL of DES was placed to set

'blank'. Then 1 µL of DNA sample was placed onto the pedestal and absorbance of the DNA sample was measured at 260 and 280 nm. The purity of DNA was measured by calculating the ratio of A260/A280.

3.10.4 PCR amplification

PCR amplification for RM26 isolate was performed by preparing the master mix (Table 4). The master mix was transferred to PCR tubes and template DNA was added. The reaction mixture was gently mixed by tapping PCR tubes and spun to settle tube content. The thermocycler for PCR reaction was programmed as described in Table 5. The amplified PCR product was analyzed using gel electrophoresis.

Table 4 - PCR reaction mixture

S. No	Components	Volume (µL)
1.	Milli –Q	37.50
2.	Buffer (10X)	5.00
3.	dNTPs	5.00
4.	Forward primer	0.50
5.	Reverse primer	0.50
6.	Taq polymerase	0.50
7.	Template DNA	1.00
	Total Volume	50 µL

Table 5 - PCR thermal cycle program:

Step	Parameters	Temperature (°C)	Time (min)	
1.	Initial Denaturation	94	8.0	
2.	Denaturation	94	1.0	34 cycles
3.	Annealing	58	1.0	
4.	Extension	72	1.3	
5.	Final extension	72	7.0	
6.	Infinite hold	4	∞	

3.10.5 PCR purification using Qiagen gel elution kit

The bands of DNA were visualized using a UV transilluminator and with a clean, sterile razor blade, the desired DNA fragment from the gel was sliced and transferred to the Eppendorf tube. Buffer QG (three volumes) was added to 1 volume of gel. The gel slice was incubated for 10 min at 50°C. The gel was dissolved completely by vortexing every 2-3 min during incubation. The color of the mixture changed to yellow. One gel volume of isopropanol was added and mixed to the sample. In 2 mL collection tube QIAquick spin column was placed. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 minute. The flow-through was removed and the QIAquick column was put back into the same tube. After centrifugation for 1 min, 0.5 mL of Buffer QG was added to the QIAquick column and flow-through was again discarded. QIAquick column was placed back into the same tube. 0.75 mL of Buffer PE was added to the QIAquick column for washing and the column was left undisturbed for 2-5 min. After 1 min of centrifugation, the flow-through was discarded. The QIAquick column was put back into the same tube. The column was centrifuged for 1 min at 13,000 rpm in a 2 mL collection tube. A clean 1.5 mL microcentrifuge tube was used to place the QIAquick column. The DNA was eluted by adding 50 µL of Buffer EB into the center of the QIAquick membrane and centrifuging the column for 1 min. 30 µL of elution buffer was added to the center of the QIAquick membrane to increase DNA concentration. This column was left undisturbed for 1 min and then centrifuged for 1 min.

4. Results and Discussion

4.1 Screening and identification of EPS producing isolates

A total of 38 bacterial isolates were checked for their EPS-producing activity (Fig 7). Based on colony characteristics only 2 bacteria were found to be EPS positive isolates and selected for further study (Fig 8). The EPS positive isolates were named BM14 and RM26. The bacteria selected for the current study were previously identified by Sanger's sequencing method targeting the 16S rRNA gene sequence. BM14 was identified as *Staphylococcus warneri* while RM26 was identified as *Bacillus megaterium*. *Staphylococcus warneri* belongs to the genus *Staphylococcus,* which is made up of gram-positive coccoid bacteria appearing in clusters (Sizar and Unakal.; 2022). *Bacillus megaterium* is a gram-positive rod-shaped bacterium that belongs to the genus *Bacillus* (Thubiani et al., 2018).

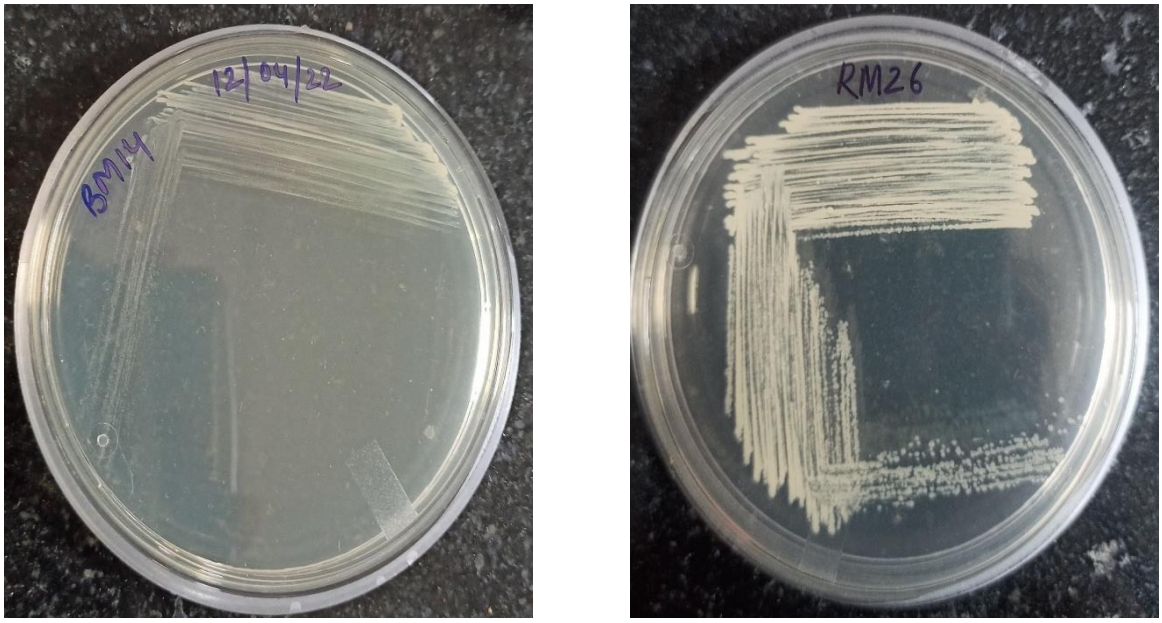

Fig 7: BM14 and RM26 bacteria grown on ZMA media

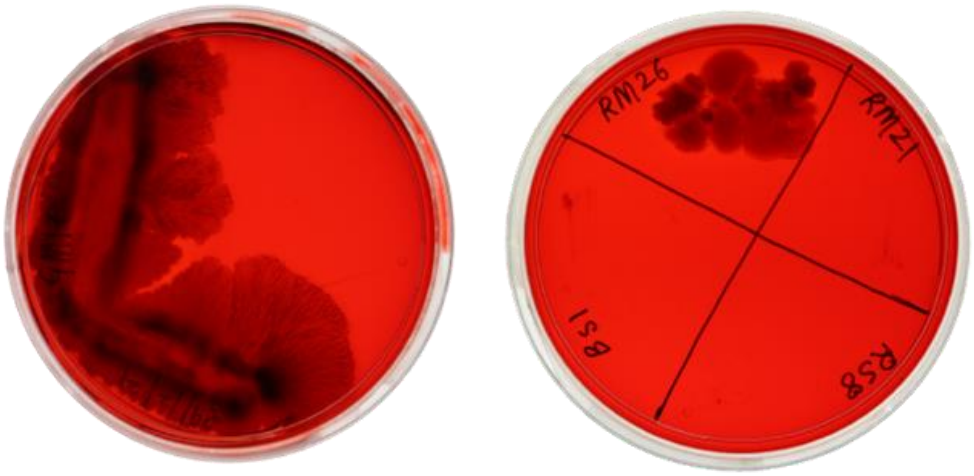

Fig 8: Screening for EPS production

4.2 Antimicrobial susceptibility assay

Antimicrobial susceptibility tests (antibiotic sensitivity) for bacteria are generally used to determine the sensitivity of particular antibiotics to the bacterium. The BM14 and RM26 strains were tested for antibiotic susceptibility using the disc diffusion method on MHA medium with various antibiotics. Both the strains showed sensitivity to all the antibiotics tested (Fig). Imipenem was observed to be the most effective against BM14 and RM26. Imipenem showed maximum zone of inhibition i.e., >40 mm and 39 mm in BM14 and RM26 respectively. BM14 also showed susceptibility to erythromycin (29 mm) and chloramphenicol (28 mm) whereas RM26 was susceptible to gatifloxacin (30 mm) followed by Erythromycin (28 mm) and co-trimoxazole (28 mm). The minimum zone of inhibition in BM14 was found to be 10 mm for Penicillin G followed by ampicillin A (13 mm) and vancomycin (14 mm). RM26 showed minimum sensitivity for clindamycin (6 mm) followed by penicillin G (10 mm) and vancomycin (15 mm). Previous research showed the sensitivity of different antibiotics such as penicillin and erythromycin on different Staphylococcus species (Pinna et al., 1999). Another study found that ampicillin had the highest overall resistance among *Staphylococcus* species, followed by co-trimoxazole and chloramphenicol (Kitara et al., 2011). Previously it was found that the bacteria *Bacillus anthracis* showed resistance against co-trimoxazole while the same bacteria were found susceptible to vancomycin, clindamycin, and imipenem (Cavallo et al., 2002). *Bacillus cereus* also showed resistance against

ampicillin and penicillin earlier, while they were found susceptible to chloramphenicol, imipenem, and erythromycin (Fiedler et al., 2019).

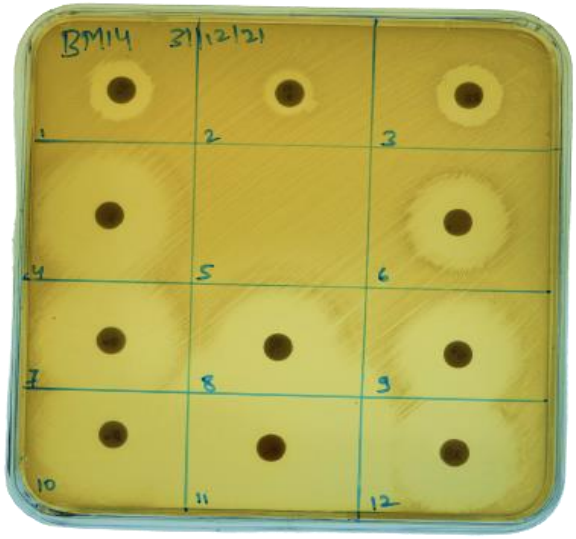

Fig 9: Antibiotics susceptibility assay for isolate BM14

(1- Ampicillin A, 2- Penicillin G, 3- Vancomycin, 4- Chloramphenicol, 5- Blank, 6- Erythromycin, 7- Clindamycin, 8- Azithromycin, 9- Gatifloxacin, 10- Imipenem, 11- Co –Trimoxazole)

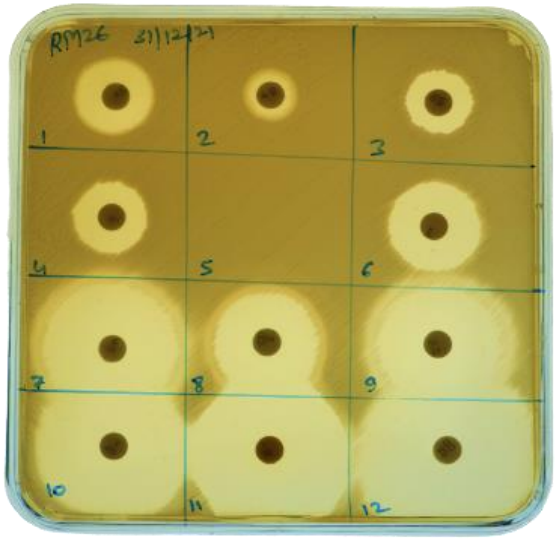

Fig 10: Antibiotics susceptibility assay for isolate RM26

(1- Ampicillin A, 2- Penicillin G, 3- Vancomycin, 4- Chloramphenicol, 5- Blank, 6- Erythromycin, 7- Clindamycin, 8- Azithromycin, 9- Gatifloxacin, 10- Imipenem, 11- Co –Trimoxzole)

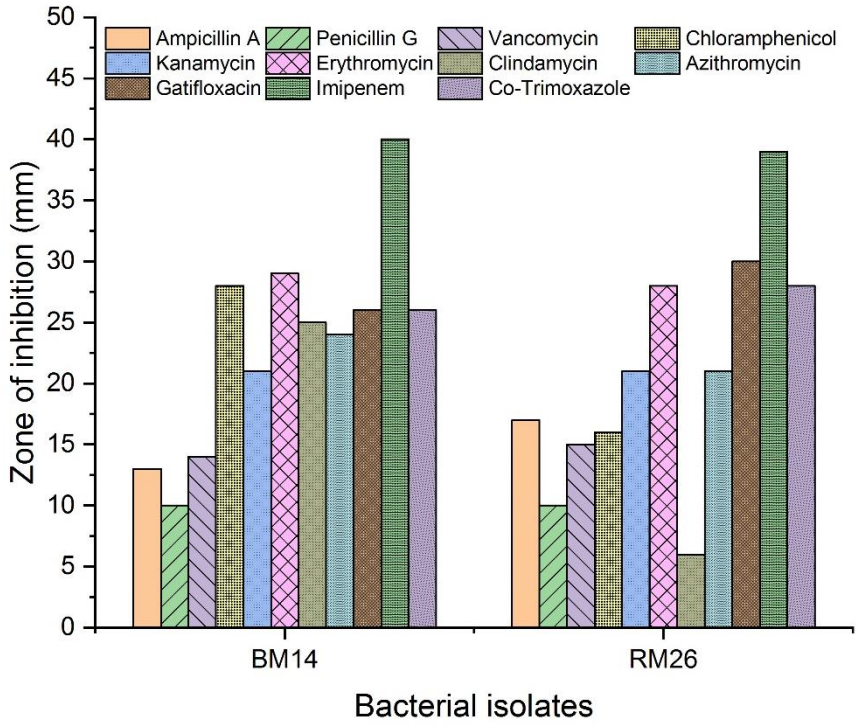

Fig 11: A graph showing antibiotic susceptibility pattern for BM14 and RM26

4.3 Extraction of extracellular polymeric substances

The dry weight of EPS produced by BM14 and RM26 was 1126 mg $L^{-1}$and 1187 mg L^{-1} respectively (Table 6). The amount of EPS obtained by BM14 and RM26 was significantly higher than that of previously reported bacteria such as *L. plantarum* JLAU103 (75 mg L^{-1}) (Min et al., 2019), *B. pseudomycoides* (316.46 mg L^{-1}) (Solmaz et al., 2018) and *Bacillus licheniformis* PASS26 (67 mg L^{-1}) (Singh et al., 2011). Previous research on *Bacillus subtilis* showed the effects of various synthetic nutrient sources on EPS production and it was found that among the synthetic carbon sources tested, EPS production was highest with 2 % sucrose (2660 mg L^{-1}) (Biol et al., 2013). Changes in culture conditions may stimulate EPS production rather than bacterial growth (Sánchez et al., 2006). Bacteria produce a large amount of EPS to protect themselves from adverse environmental conditions (increased osmotic pressure of culture media, water stress) by establishing a physicochemical barrier. (Boukhelata et al., 2019).

Fig 12: Extracted EPS from BM14

Fig 13: EPS extracted from RM26

Table 6: Final weight of purified EPS

S. No	Bacterial isolate	Weight of EPS (mg L^{-1})
1.	BM14	1126.0
2.	RM26	1187.2

4.4 Total Sugar Estimation

The phenol-sulfuric acid method is a colorimetric method for determining the total carbohydrate content of a sample. (Masuko et al., 2005). The chemical composition of microbial EPS is significant because it influences both its functional properties and its industrial potential. (Joulak et al., 2020). The total carbohydrate content of the extracted EPS from BM14 and RM26 was estimated to be 50.14 µg mg^{-1} and 122.75 µg mg^{-1} (Table 7). According to previous research, the total carbohydrate content of B. licheniformis was found to be 343.14 mg L^{-1} (Singh et al., 2011). Five *Staphylococcus* spp ATCC strains were studied for carbohydrate concentrations, and they showed significantly higher carbohydrate concentrations in EPS, ranging from 56 µg mL-1 to 372 µg mL-1 in EPS (Cruz et al., 2020).

Table 7: Total Sugar Estimation

S. No	Bacterial isolate	Sugar concentration (µg mg^{-1} of EPS)
1.	BM14	50.14687
2.	RM26	122.7522

4.5 Antibacterial Activity of EPS

The antibacterial activity of extracted EPS was tested against a list of pathogenic bacteria. The zone of inhibition around the discs showed the sensitivity of bacteria against tested EPS. EPS from BM14 and RM26 showed sensitivity against seven pathogenic bacteria while three bacteria (*Shigella flexneri*, *Staphylococcus epidermis* and *Enterococcus fecalis*) were found resistant (Table). For the EPS extracted from

BM14 two strains showed maximum zone of inhibition while EPS from RM26 showed maximum zone of inhibition for three pathogenic strains. *Proteus mirabilis* was more sensitive to EPS produced by RM26 than EPS produced by BM14. EPS from microorganisms have shown strong antimicrobial activity against a variety of pathogens and according to these findings, the antimicrobial activity increased with increasing EPS concentrations. In a study, the indicator organisms *Staphylococcus epidermidis* and *Escherichia coli* were inhibited by *B. subtilis* GAS101 (G. Sharma et al., 2018). *Lactobacillus* EPSs were found to have potent antibacterial activity against *E. coli* and *Salmonella typhimurium* in vitro (Rajoka et al., 2018). Dwivedi et al (2018) also studied the antibacterial activity of EPS for Gram positive *Bacillus* spp. and Gram-negative *Pseudomonas* spp. using test microorganisms - *E. coli, B. cereus, B. subtilis,* and *V. cholerae*. It was observed that pathogenic strains showed sensitivity to *Bacillus* spp. and *Pseudomonas* spp. EPS but the sensitivity varied for each pathogenic strain.

Table 8: Antibacterial activity of EPS

Pathogenic test strains	BM14	RM26
Pseudomonas putida	++	++
Serratia liquifaciens	+	+
Escherichia coli	+	+
Proteus mirabilis	+	++
Shigella flexneri	-	-
Serratia marcense	++	++
Klebsiella pneumoniae	+	+
Staphylococcus epidermis	-	-
Pseudomonas auregenosa	+	+
Enterococcus fecalis	-	-

4.6 Antioxidant Activity of EPS

DPPH radical scavenging activity of purified EPS from BM14 increased as their concentration increased from 0 to 6 mg mL^{-1}. BM14 showed 28.7 % scavenging activity at 6 mg mL^{-1}. The DPPH scavenging activity of EPS isolated from RM26 was maximum at 2 mg mL^{-1} concentration i.e. 27.2 %. At a concentration of 6 mg mL^{-1}, the scavenging activity decreased to 19.5 %. The scavenging capacity of ABTS radicals increased with the increase in EPS concentration from 0 to 10 mg mL^{-1}. EPS of BM14 could scavenge up to 31.3 % ABTS radicals while EPS extracted from RM26 showed 26.7 % scavenging activity at 10 mg mL^{-1} concentration. Previous research on EPS produced by *Lactobacillus plantarum* KX041 estimated DPPH elimination activity as 37.48 % (6 mg mL^{-1}) (Xu et al., 2019). Min et al. (2019) found that EPS produced by *Lactobacillus plantarum* showed an ABTS radical scavenging rate of 65.5 % at a concentration of 10 mg/mL.

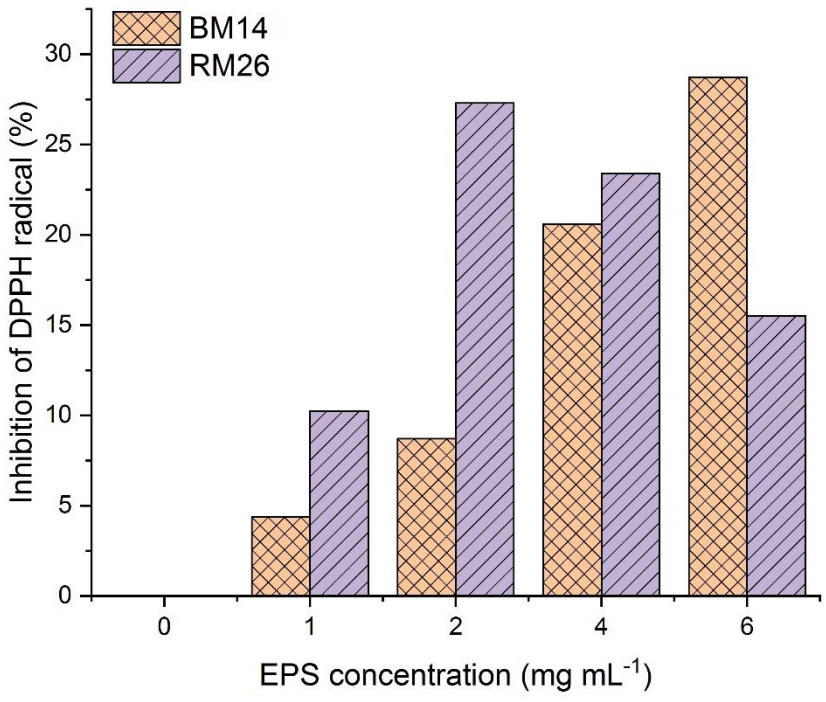

Fig 14: DPPH radical-scavenging activity of EPS from BM14 and RM26

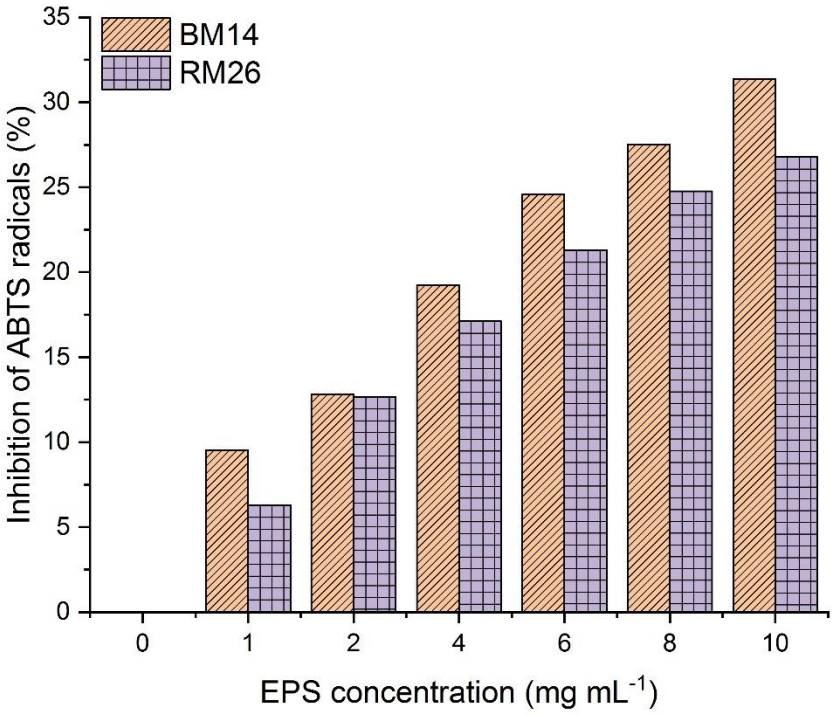

Fig 15: ABTS radical-scavenging activity of EPS from BM14 and RM26

4.7 Scanning Electron Microscopy (SEM)

SEM analysis showed that EPS produced by BM14 is loosely bound and smooth in shape. The SEM image of RM26 showed that the EPS was irregular in shape and was tightly bound. Both the EPS was found to be amorphous in nature**.** Kavita et al. (2013) also observed that the EPS from marine bacteria *Vibrio fortis* was irregular in shape and amorphous in nature.

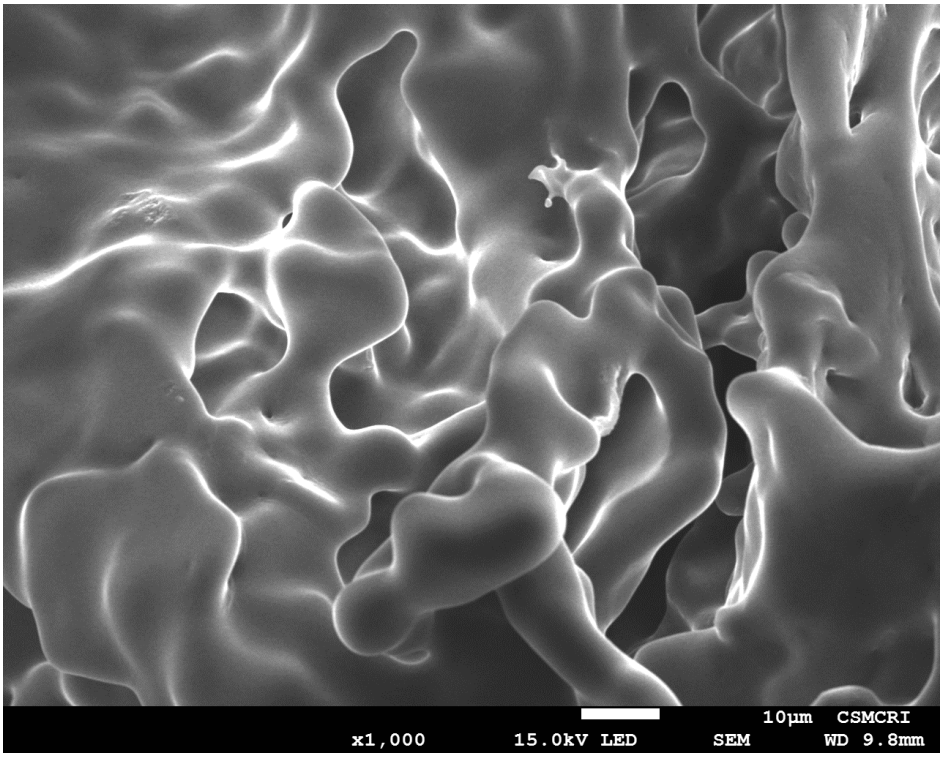

Fig 16: SEM image of BM14

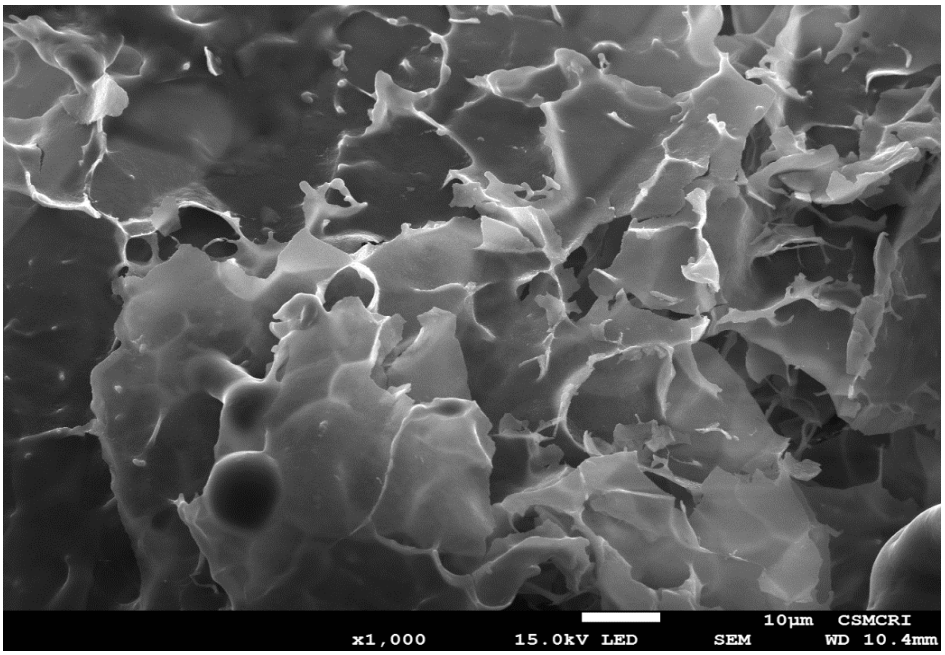

Fig 17: SEM image of RM26

4.8 Energy dispersive X-ray spectroscopy (EDX)

EDX was used to determine the elemental composition of EPSs which revealed the presence of weight and atomic percentage of different elements (Table 9). C, N, O, Na, S, and Cl were some of the common elements found in EPS produced by BM14 and RM26. Carbon (32.42 % w/w) was the most abundant element in EPS produced by BM14, followed by oxygen (13.09 % w/w) and chlorine (8.33 % w/w). In the EPS produced by RM26, carbon was found to be the most abundant component (36.41 % w/w), followed by oxygen (32.18 % w/w) and Chlorine (20.31 % w/w). Some traces of Mg and P were also found in RM26, while the same was not detected in BM14. Kavita et al. (2013) also found the C, N, O, Na, Mg, and P in the purified EPS sample of *Oceanobacillus iheyensis* in different proportions.

Table 9: Elemental EDX microanalysis of EPS

Elements	EPS (BM14)		EPS (RM26)	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
C	32.42	34.23	36.41	47.97
N	5.11	4.63	8.54	9.64
O	13.09	10.38	32.18	31.83
Na	0.26	0.14	0.87	0.60
S	0.26	0.10	0.59	0.29
Cl	8.33	2.98	20.31	9.07
Mg	ND	ND	0.30	0.20
P	ND	ND	0.80	0.41

4.9 Elemental Analysis

The EPS produced by BM14 showed the abundance of carbon (27.11%) followed by hydrogen (3.743 %) and nitrogen (3.08 %) using the CHNS analyser. The EPS produced by RM26 also had carbon as the most dominant element (14.91%) followed by hydrogen (1.467 %) and nitrogen (2.32 %). Sulfur was found in both the EPS samples but in trace amounts (0.52% in both). A previous study in *Bacillus anthracis* reported the presence of a higher percentage of carbon, hydrogen, and nitrogen (Banerjee et al., 2018).

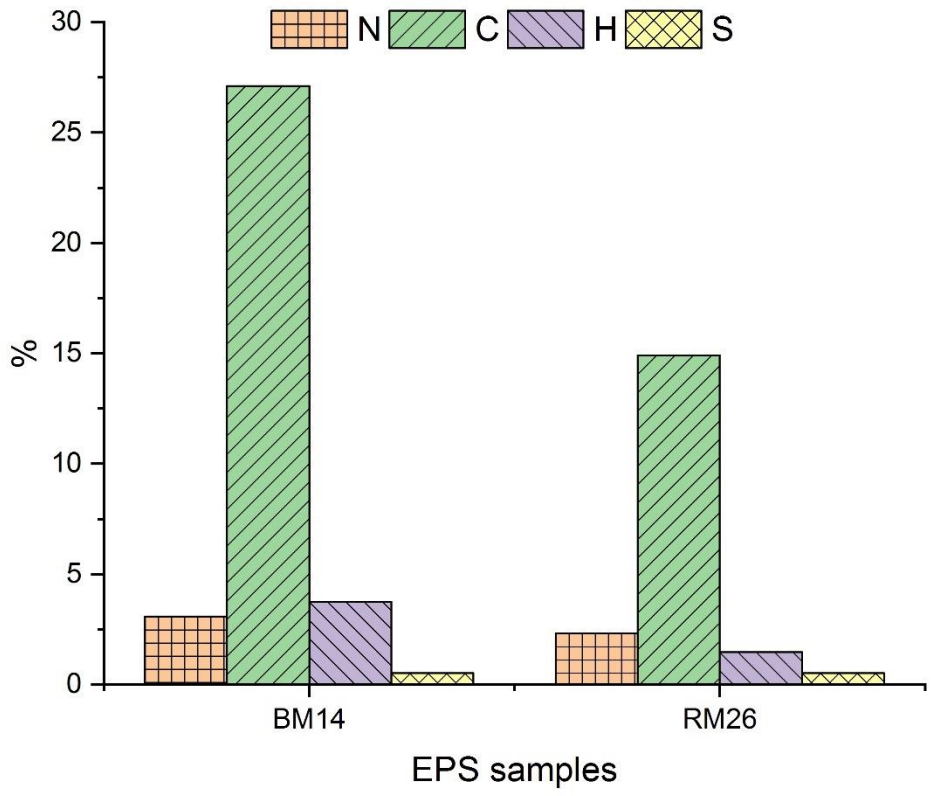

Fig 18: Elemental analysis of purified EPS

4.10 Biofilm Formation Assay

The bacterial strains BM14 and RM26 were checked for their biofilm forming activity. Figure 19 shows that BM14 was a strong biofilm former, while RM26 was a

comparatively weak biofilm former. The biofilm-forming activity was checked with reference to *Pseudomonas aeruginosa* as a positive control. Biofilm forming bacteria secrete EPS which helps bacteria to adhere to the surfaces (Limoli et al., 2015). *Pseudomonas aeruginosa* is a known biofilm-forming bacteria and has been extensively used in several types of research (Thi et al., 2020). Previous reports show that *Bacillus*, as well as *Staphylococcus*, are known to form biofilm in the marine environment (Arnaouteli et al., 2021; Otto, 2008).

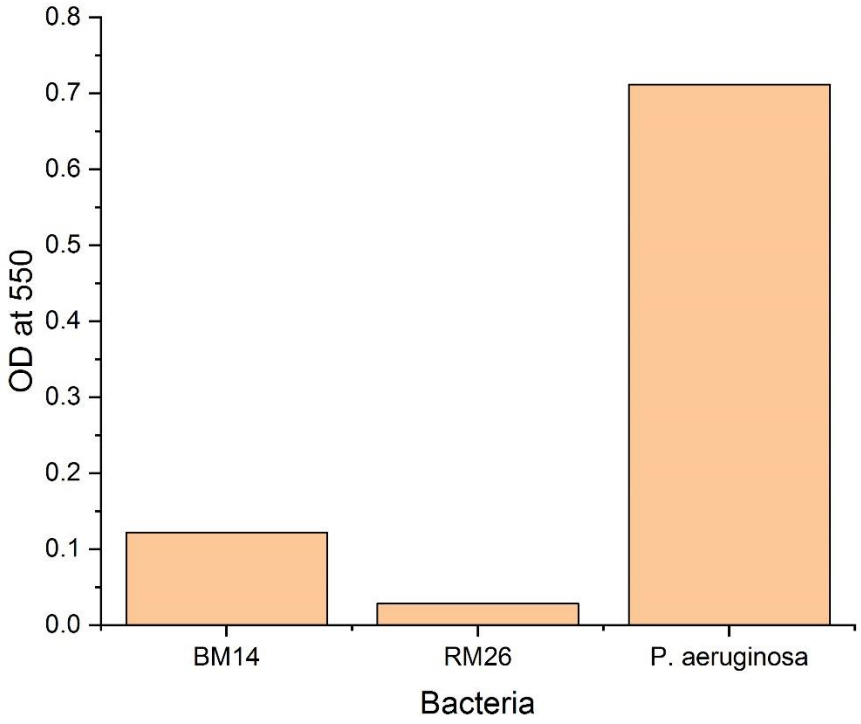

Fig 19: Biofilm forming activity of BM14 and RM26

Conclusion

The marine environment is an ideal environment for biofilm forming bacteria. Biofilm is primarily composed of EPS produced by bacteria. The difference in EPS composition and structure is important for promising biotechnological applications. EPS produced by microorganisms are natural polymers and considered to be eco-friendly due to their non-toxicity compared to synthetic polymers. Though EPS is found abundantly in biofilm forming bacteria there are few reports on EPS from marine bacteria. In this study, *Staphylococcus warneri* and *Bacillus megaterium* produced 1126.0 mg L^{-1} and 1187.2 mg L^{-1} of EPS respectively. SEM analysis of EPS produced by BM14 and RM26 was observed to be amorphous in nature. Elemental analysis revealed carbon as a major constituent of EPS produced by both bacteria. Some common elements such as O, N, Na and Mg were also determined. Furthermore, the in vitro antibacterial activity of these two EPS suggests that they may have promising potential against pathogenic bacteria. Though further studies are required to determine its different biotechnological and industrial uses.

References

Abid, Yousra, et al. "Rheological and emulsifying properties of an exopolysaccharide produced by potential probiotic Leuconostoc citreum-BMS strain." *Carbohydrate Polymers* 256 (2021): 117523.

Ali, Pervaiz, et al. "A glacier bacterium produces high yield of cryoprotective exopolysaccharide." *Frontiers in microbiology* 10 (2020): 3096.

Arnaouteli, Sofia, et al. "*Bacillus subtilis* biofilm formation and social interactions." *Nature Reviews Microbiology* 19.9 (2021): 600-614.

Ascenzioni, Fiorentina, et al. "Microbial biofilms in chronic and recurrent infections." *Frontiers in Microbiology* (2021): 3760.

Aullybux, Aadil Ahmad, et al. "Phylogenetics and Antibacterial Properties of Exopolysaccharides from Marine Bacteria Isolated from Mauritius Seawater." *Annals of Microbiology* 69.9 (2019): 957–972.

Azeredo, Joana, and Rosário Oliveira. "The role of exopolymers in the attachment of *Sphingomonas paucimobilis*." *Biofouling* 16.1 (2000): 59-67.

Bakar, Muhamad Abu et al. "Chronic Tonsillitis and Biofilms: A Brief Overview of Treatment Modalities." *Journal of Inflammation Research* 11 (2018): 329–337.

Balouiri, Mounyr, et al. "Methods for in Vitro Evaluating Antimicrobial Activity: A Review." *Journal of Pharmaceutical Analysis* 6.2 (2016): 71–79.

Banerjee, Aparna, et al. "Structural and functional properties of exopolysaccharide excreted by a novel *Bacillus anthracis* (Strain PFAB2) of hot spring origin." *Indian journal of microbiology* 58.1 (2018): 39-50.

Bomfim, Vanessa Barbosa, et al. "Partial characterization and antioxidant activity of exopolysaccharides produced by *Lactobacillus plantarum* CNPC003." *LWT - Food Science and Technology* (2020): 109349.

Boukhelata, Nadia, et al. "Characterization of an Extracellular Polysaccharide Produced by a Saharan Bacterium *Paenibacillus Tarimensis* REG 0201M." *Annals of Microbiology* 69.2 (2019): 93–106.

Caruso, Consolazione, et al. "Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria." *Applied and environmental microbiology* 84.4 (2018): e01624-17.

Caruso, Consolazione, et al. “Isolation, Characterization and Optimization of EPSs Produced by a Cold- Adapted Marinobacter Isolate from Antarctic Seawater.” *Antarctic Science* 31.2 (2019): 69–79.

Cavallo, Jean-Didier, et al. "Antibiotic susceptibilities of 96 isolates of *Bacillus anthracis* isolated in France between 1994 and 2000." *Antimicrobial agents and chemotherapy* 46.7 (2002): 2307-2309.

Cervino, Gabriele, et al. "Alginate materials and dental impression technique: A current state of the art and application to dental practice." *Marine drugs* 17.1 (2018): 18.

Choudhuri, Indranil et al. “Structural Characterization of an Exopolysaccharide Isolated from *Enterococcus Faecalis*, and Study on Its Antioxidant Activity, and Cytotoxicity Against HeLa Cells.” *Current Microbiology* 77.10 (2020): 3125–3135.

Costa, Ohana YA, et al. "Microbial extracellular polymeric substances: ecological function and impact on soil aggregation." *Frontiers in microbiology* 9 (2018): 1636.

Cruz, Cristina D., et al. “*A brief exploration of EPS composition in biofilms of Staphylococcus spp ATCC reference strains*.” Copernicus Meetings (2020) 9-129.

De Carvalho, Carla CCR, and Pedro Fernandes. "Production of metabolites as bacterial responses to the marine environment." *Marine drugs* 8.3 (2010): 705-727.

Di Martino, Patrick. "Extracellular polymeric substances, a key element in understanding biofilm phenotype." *AIMS microbiology* 4.2 (2018): 274.

Dong, Bin et al. “Roles of Molecular Weight-Fractionated Extracellular Polymeric Substance in Transformation of Au (III) to Au Nanoparticles in Aqueous Environments.” *Science of the Total Environment* 728 (2020): 138889.

Donlan, Rodney M. "Biofilms: microbial life on surfaces." *Emerging infectious diseases* 8.9 (2002): 881.

Santos, André Luis Souza dos, et al. "What are the advantages of living in a community? A microbial biofilm perspective!." *Memórias do Instituto Oswaldo Cruz* 113 (2018).

Dubois, M., et al. "A colorimetric method for the determination of sugars." *Nature* 168.4265 (1951): 167-167.

Dwivedi and Mitesh. "Exopolysaccharide (EPS) producing isolates from sugarcane field soil and antibacterial activity of extracted EPSs." *Acta Scientific Microbiology* 1 (2018): 06-13.

El-Tarabily, Khaled A., et al. "Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance." *Saudi Journal of Biological Sciences* 28.9 (2021): 5145-5156.

Esawy, Mona A. et al. "Levansucrase Optimization Using Solid State Fermentation and Levan Biological Activities Studies." *Carbohydrate Polymers* 96.1 (2013): 332–341.

Escárcega-González, Carlos Enrique et al. "Bacterial Exopolysaccharides as Reducing and/or Stabilizing Agents during Synthesis of Metal Nanoparticles with Biomedical Applications." *International Journal of Polymer Science* (2018)

Everett, Jake A., and Kendra P. Rumbaugh. "Biofilms, Quorum Sensing and Crosstalk in Medically Important Microbes." *Molecular Medical Microbiology: Second Edition* 1–3 (2015): 235–247.

Feng, Xi, et al. "Antibiotic Modulation of Capsular Exopolysaccharide in *Pelagicoccus enzymogenes sp*. nov. Isolated From Marine Sediment." *Frontiers in Marine Science* 8 (2021): 297.

Fiedler, Gregor et al. "Antibiotics Resistance and Toxin Profiles of *Bacillus* Cereus-Group Isolates from Fresh Vegetables from German Retail Markets." *BMC Microbiology* 19.1 (2019): 1–13.

Flemming, Hans Curt, and Jost Wingender. "The Biofilm Matrix." *Nature Reviews Microbiology* (2010): 623–633.

Fulaz, Stephanie, et al. "Nanoparticle–biofilm interactions: the role of the EPS matrix." *Trends in Microbiology* 27.11 (2019): 915-926.

Giaouris, Efstathios, et al. "Intra-and inter-species interactions within biofilms of important foodborne bacterial pathogens." *Frontiers in microbiology* 6 (2015): 841.

Gillett, A., David G. Waugh, and Jonathan Lawrence. "Laser surface modification of polymeric surfaces for microbiological applications." *Laser Surface Modification of Biomaterials*. Woodhead Publishing, 2016. 197-220.

Goel, Nikky, et al. "Antimicrobial resistance in biofilms: Exploring marine actinobacteria as a potential source of antibiotics and biofilm inhibitors." *Biotechnology Reports* 30 (2021): e00613.

Gongi, Wejdene, et al. "Extracellular Polymeric Substances Produced by the Thermophilic Cyanobacterium *Gloeocapsa gelatinosa*: Characterization and Assessment of Their Antioxidant and Metal-Chelating Activities." *Marine Drugs* 20.4 (2022): 227.

Gongi, Wejdene, et al. "Extracellular polymeric substances with high radical scavenging ability produced in outdoor cultivation of the thermotolerant chlorophyte *Graesiella sp*." *Journal of Applied Phycology* 33.1 (2021): 357-369.

Ignatova-Ivanova, Tsveteslava. "Exopolysaccharides from Bacteria with Novel Application." *Immunotherapy-Myths, Reality, Ideas, Future*. IntechOpen, 2017.

Insulkar, Prajakta, et al. "Purification and Structural-Functional Characterization of an Exopolysaccharide from *Bacillus Licheniformis* PASS26 with in-Vitro Antitumor and Wound Healing Activities." *International Journal of Biological Macromolecules* 120 (2018): 1441–1450.

Izadi, Parin, et al. "Holistic insights into extracellular polymeric substance (EPS) in anammosx bacterial matrix and the potential sustainable biopolymer recovery: A review." *Chemosphere* 274 (2021): 129703.

Jiao, Yongqin et al. "Characterization of Extracellular Polymeric Substances from Acidophilic Microbial Biofilms." *Applied and Environmental Microbiology* 76.9 (2010): 2916–2922.

Joulak, Ichrak, et al. "Evaluation of the production of exopolysaccharides by newly isolated Halomonas strains from Tunisian hypersaline environments." *International journal of biological macromolecules* 138 (2019): 658-666.

Joulak, Ichrak, et al. "Hetero-exopolysaccharide from the extremely halophilic Halomonas smyrnensis K2: production, characterization and functional properties in vitro." *3 Biotech* 10.9 (2020): 1-12.

Julianti, Elisa et al. “Functional and Rheological Properties of Composite Flour from Sweet Potato, Maize, Soybean and Xanthan Gum.” *Journal of the Saudi Society of Agricultural Sciences* 16.2 (2017): 171–177.

Kanmani, Paulraj et al. “Production and Purification of a Novel Exopolysaccharide from Lactic Acid Bacterium *Streptococcus Phocae* PI80 and Its Functional Characteristics Activity in Vitro.” *Bioresource Technology* 102.7 (2011): 4827–4833.

Karygianni, L. et al. “Biofilm Matrixome: Extracellular Components in Structured Microbial Communities.” *Trends in Microbiology* 28.8 (2020): 668–681.

Kavita, Kumari et al. “Characterisation and Anti-Biofilm Activity of Extracellular Polymeric Substances from Oceanobacillus Iheyensis.” *Carbohydrate Polymers* 101.1 (2014): 29–35.

Kavita, Kumari, et al. "Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*." *Biofouling* 27.3 (2011): 309-317.

Kavita, Kumari, et al. “Extracellular Polymeric Substances from Two Biofilm Forming *Vibrio* Species: Characterization and Applications.” *Carbohydrate Polymers* 94.2 (2013): 882–888.

Kırmusaoğlu, Sahra. *"*The methods for detection of biofilm and screening antibiofilm activity of agents*." Antimicrobials, antibiotic resistance, antibiofilm strategies and activity methods (2019): 1-17.*

Korcz, Evelin, and László Varga. “Exopolysaccharides from Lactic Acid Bacteria: Techno-Functional Application in the Food Industry.” *Trends in Food Science & Technology* 110 (2021): 375–384.

Kour, Divjot et al. “Microbial Biofilms: Functional Annotation and Potential Applications in Agriculture and Allied Sectors.” *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Biofilms Current Research and Future Trends in Microbial Biofilms* (2019): 283–301.

Limoli, Dominique H., et al. "Bacterial extracellular polysaccharides in biofilm formation and function." *Microbiology spectrum* 3.3 (2015): 3-3.

Ma, Wenjin et al. “Characterization, Antioxidativity, and Anti-Carcinoma Activity of Exopolysaccharide Extract from *Rhodotorula Mucilaginosa* CICC 33013.” *Carbohydrate Polymers* 181 (2018): 768–777.

Mancuso Nichols, C. A., et al. “Bacterial Exopolysaccharides from Extreme Marine Environments with Special Consideration of the Southern Ocean, Sea Ice, and Deep-Sea Hydrothermal Vents: A Review.” *Marine Biotechnology* 7.4 (2005): 253–271.

Mandawe, John et al. “Directed Evolution of Hyaluronic Acid Synthase from *Pasteurella Multocida* towards High-Molecular-Weight Hyaluronic Acid.” *ChemBioChem* 19.13 (2018): 1414–1423.

Masuko, Tatsuya et al. “Carbohydrate Analysis by a Phenol-Sulfuric Acid Method in Microplate Format.” *Analytical biochemistry* 339.1 (2005): 69–72.

Meliani, Amina, and Ahmed Bensoltane. "Biofilm-mediated heavy metals bioremediation in PGPR *Pseudomonas*." *J Bioremediat Biodegrad* 7.2 (2016).

Mgomi, Fedrick C. et al. “Bacteriophages: A Weapon against Mixed-Species Biofilms in the Food Processing Environment.” *Journal of Applied Microbiology* (2021)

Min, Wei Hong et al. “Characterization and Antioxidant Activity of an Acidic Exopolysaccharide from *Lactobacillus Plantarum* JLAU103.” *Journal of Bioscience and Bioengineering* 127.6 (2019): 758–766.

Minimol, V. A. et al. “Isolation and Characterization of Exopolysaccharide Producing Bacillus Cereus from Brown Seaweed- *Sargassum Wightii*.” *International Journal of Current Microbiology and Applied Sciences* 8.09 (2019): 1302–1311.

Mishra, Avinash, et al. “Characterization of Extracellular Polymeric Substances Produced by Micro-Algae *Dunaliella Salina*.” *Carbohydrate Polymers* 83.2 (2011): 852–857.

Mohapatra, Ranjan Kumar et al. “Potential Application of Bacterial Biofilm for Bioremediation of Toxic Heavy Metals and Dye-Contaminated Environments.” *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Biofilms Current Research and Future Trends in Microbial Biofilms* (2019): 267–281.

Morais, Victor, et al. “Purification of Capsular Polysaccharides of *Streptococcus Pneumoniae*: Traditional and New Methods.” *Frontiers in Bioengineering and Biotechnology* 6 (2018): 145.

More, Tanaji. et al. “Extracellular Polymeric Substances of Bacteria and Their Potential Environmental Applications.” *Journal of Environmental Management* 144 (2014): 1–25.

Muhammad, Musa Hassan et al. “Beyond Risk: Bacterial Biofilms and Their Regulating Approaches.” *Frontiers in Microbiology* 11 (2020): 928.

Nakano, Yuriko, et al. "The competing effects of microbially derived polymeric and low molecular-weight substances on the dispersibility of CeO2 nanoparticles." *Scientific reports* 8.1 (2018): 1-11.

O'Toole, George A. "Microtiter dish biofilm formation assay." *JoVE (Journal of Visualized Experiments)* 47 (2011): e2437.

Otto, Michael. "*Staphylococcal* biofilms." Bacterial biofilms (2008): 207-228.

Packiavathy, Issac Abraham Sybiya Vasantha et al. “AHL-Lactonase Producing *Psychrobacter sp*. From Palk Bay Sediment Mitigates Quorum Sensing-Mediated Virulence Production in Gram Negative Bacterial Pathogens.” Frontiers in Microbiology 12 (2021): 748

Poli, Annarita, et al. “Bacterial Exopolysaccharides from Extreme Marine Habitats: Production, Characterization and Biological Activities.” *Mar. Drugs* 8 (2010): 1779–1802.

Punnen, Stessy Ann, et al. "Evaluation of Antioxidant Property Of Exopolysaccharides Obtained From Biofilm Producing Bacteria." *European Journal of Pharmaceutical and Medical Research* (2018): 572-577.

Rajoka, Muhammad Shahid Riaz et al. “Characterization, the Antioxidant and Antimicrobial Activity of Exopolysaccharide Isolated from Poultry Origin *Lactobacilli*.” *Probiotics and Antimicrobial Proteins* 11.4 (2018): 1132–1142.

Rana, Sonali, and Lata Sheo Bachan, Upadhyay. “Microbial Exopolysaccharides: Synthesis Pathways, Types and Their Commercial Applications.” *International Journal of Biological Macromolecules* 157 (2020): 577–583.

Razack, Sirajunnisa Abdul et al. "Medium optimization for the production of exopolysaccharide by *Bacillus subtilis* using synthetic sources and agro wastes." *Turkish Journal of Biology* 37.3 (2013): 280-288.

Saha, Dipjyoti, and Suvendu Bhattacharya. “Hydrocolloids as Thickening and Gelling Agents in Food: A Critical Review.” *Journal of Food Science and Technology* 47.6 (2010): 587–597.

Sánchez, Jorge Ignacio et al. “Culture Conditions Determine the Balance between Two Different Exopolysaccharides Produced by *Lactobacillus Pentosus* LPS26.” *Applied and Environmental Microbiology* 72.12 (2006): 7495.

Satish, Lakkakula et al. “Rapid Biosynthesized AgNPs from *Gelidiella Acerosa* Aqueous Extract Mitigates Quorum Sensing Mediated Biofilm Formation of *Vibrio* Species—an in Vitro and in Vivo Approach.” *Environmental Science and Pollution Research* 24.35 (2017): 27254–27268.

Şengör, S. Sevinç. “Review of Current Applications of Microbial Biopolymers in Soil and Future Perspectives.” *ACS Symposium Series* 1323 (2019): 275–299.

Senpuku, Hidenobu et al. “SMU.940 Regulates Dextran-Dependent Aggregation and Biofilm Formation in *Streptococcus* Mutans.” *Molecular Oral Microbiology* 33.1 (2018): 47–58.

Sharma, Garima et al. “Antibacterial Activity, Cytotoxicity, and the Mechanism of Action of Bacteriocin from *Bacillus Subtilis* GAS101.” *Medical Principles and Practice* 27.2 (2018): 186–192.

Sharma, Hemlata, Jyoti Pal, and Deepesh Kumar Neelam. “Bacterial Extracellular Polymers: A Review.” *Journal of Pure and Applied Microbiology* 15.3 (2021): 1072–1082.

Sheng, Guo Ping, et al. “Extracellular Polymeric Substances (EPS) of Microbial Aggregates in Biological Wastewater Treatment Systems: A Review.” *Biotechnology Advances* 28.6 (2010): 882–894.

Shukla, Arpit, et al. "Depicting the exemplary knowledge of microbial exopolysaccharides in a nutshell." *European Polymer Journal* 119 (2019): 298-310.

Shukla, Sudhir K., et al. "19-bacterial biofilms and genetic regulation for metal detoxification." *Handbook of metal-microbe interactions and bioremediation* 317 (2017).

Siddharth, T., et al. "Environmental applications of microbial extracellular polymeric substance (EPS): a review." *Journal of Environmental Management* 287 (2021): 112307.

Singh, Ravindra Pal, et al. “Isolation and Characterization of Exopolysaccharides from Seaweed Associated Bacteria *Bacillus Licheniformis*.” *Carbohydrate Polymers* 84.3 (2011): 1019–1026.

Singh, Vijay Kumar, et al. "Marine bacterial extracellular polymeric substances: characteristics and applications." *Marine Glycobiology*. CRC Press (2016): 389-398.

Singha, Tapan Kumar. "Microbial extracellular polymeric substances: production, isolation and applications." *IOSR J Pharm* 2.2 (2012): 271-281.

Smith, Heidi J., et al. "Impact of hydrologic boundaries on microbial planktonic and biofilm communities in shallow terrestrial subsurface environments." *FEMS microbiology ecology* 94.12 (2018): 191.

Solmaz, Kubra Betul et al. “Characterization and Production of Extracellular Polysaccharides (EPS) by *Bacillus pseudomycoides* U10.” *Environments 2018, Vol. 5, Page 63* 5.6 (2018): 63.

Sran, Kulwinder Singh et al. “Production, Characterization and Bio-Emulsifying Activity of a Novel Thermostable Exopolysaccharide Produced by a Marine Strain of *Rhodobacter Johrii* CDR-SL 7Cii.” *International Journal of Biological Macromolecules* 127 (2019): 240–249.

Srinivasan, Ramanathan, et al. “In Vitro Antibiofilm Efficacy of Piper Betle against Quorum Sensing Mediated Biofilm Formation of Luminescent *Vibrio Harveyi*.” *Microbial Pathogenesis* 110 (2017): 232–239.

Sriprablom, Jiratthitikan et al. “Influence of PH and Ionic Strength on the Physical and Rheological Properties and Stability of Whey Protein Stabilized o/w Emulsions Containing Xanthan Gum.” *Journal of Food Engineering* 242 (2019): 141–152.

Sun, Mei-Ling, et al. "Characterization and biotechnological potential analysis of a new exopolysaccharide from the Arctic marine bacterium *Polaribacter* sp. SM1127." *Scientific reports* 5.1 (2015): 1-12.

Thi, Minh et al. "*Pseudomonas aeruginosa* biofilms." *International Journal of Molecular Sciences* 21.22 (2020): 8671.

Thu, Hnin et al. “Alginate Based Bilayer Hydrocolloid Films as Potential Slow-Release Modern Wound Dressing.” *International Journal of Pharmaceutics* 434.1–2 (2012): 375–383.

Tian, Yu. “Behaviour of Bacterial Extracellular Polymeric Substances from Activated Sludge: A Review.” *International Journal of Environment and Pollution* 32.1 (2008): 78–89.

Tiwari, Onkar Nath, et al. "Application of microbial extracellular carbohydrate polymeric substances in food and allied industries." *3 Biotech* 10.5 (2020): 1-17.

Tsuneda, Satoshi et al. “Extracellular Polymeric Substances Responsible for Bacterial Adhesion onto Solid Surface.” *FEMS Microbiology Letters* 223.2 (2003): 287–292.

Turhan, Emel Ünal et al. “Beneficial Biofilm Applications in Food and Agricultural Industry.” *Health and Safety Aspects of Food Processing Technologies* (2019): 445–469.

Vasudevan, Ranganathan. "Biofilms: microbial cities of scientific significance." *J Microbiol Exp* 1.3 (2014): 00014.

Vijayendra, S. V. N., and T. R. Shamala. "Film forming microbial biopolymers for commercial applications—A review." *Critical reviews in biotechnology* 34.4 (2014): 338-357.

Vu, Barbara et al. “Bacterial Extracellular Polysaccharides Involved in Biofilm Formation.” *Molecules* 14.7 (2009): 2535–2554.

Wang, Jia, et al. “Extremophilic Exopolysaccharides: A Review and New Perspectives on Engineering Strategies and Applications.” *Carbohydrate Polymers* 205 (2019): 8–26.

Widyaningrum, Dwiyantari and Bayu, Meindrawan. "The application of microbial extracellular polymeric substances in food industry." *IOP Conference Series: Earth and Environmental Science*. Vol. 426. No. 1. IOP Publishing, 2020.

Wingender, Jost, et al. “What Are Bacterial Extracellular Polymeric Substances?” *Microbial Extracellular Polymeric Substances*. Springer Berlin Heidelberg, (1999): 1–19.

Xiao, Rui, et al. “Overview of Microalgal Extracellular Polymeric Substances (EPS) and Their Applications.” *Biotechnology Advances* 34.7 (2016): 1225–1244.

Xie, Jianhua et al. “Physico-Chemical Properties, Antioxidant Activities and Angiotensin-I Converting Enzyme Inhibitory of Protein Hydrolysates from Mung Bean (*Vigna Radiate*).” *Food Chemistry* 270 (2019): 243–250.

Xu, Xiaoqing, et al. "Antioxidant and immunomodulatory properties of partially purified exopolysaccharide from *Lactobacillus casei* isolated from Chinese Northeast Sauerkraut." *Immunological Investigations* (2021): 1-18.

Yildiz, Hilal, et al. “Microbial Exopolysaccharides: Resources and Bioactive Properties.” *Process Biochemistry* 72 (2018): 41–46.

APPENDIX

ZMB (Zobell Marine Broth)

Ingredients	**gm L^{-1}**
Peptic Digest of Animal Tissue	5.0
Yeast Extract	1.0
Ferric Citrate	0.10
Sodium Chloride	19.45
Magnesium Chloride	8.80
Sodium Sulphate	3.24
Calcium Chloride	1.80
Potassium Chloride	0.55
Sodium Bicarbonate	0.16
Potassium Bromide	0.08
Strontium Chloride	0.034
Boric Acid	0.022
Sodium Silicate	0.004
Sodium Fluorate	0.0024
Ammonium Nitrate	0.0016
Disodium Phosphate	0.008
Final pH (at 25°C)	7.6 ± 0.2

BHIB (Brain Heart Infusion Broth)

Ingredients	gm L^{-1}
Peptic digest of animal tissue	10.000
Calf brain, infusion (solids)	12.500
Beef heart, infusion (solids)	5.000
Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Final pH (at 25°C)	7.4 ± 0.2

MHB (Muller Hinton Broth)

Ingredients	gm L^{-1}
HiVeg infusion	2.00
HiVeg acid hydrolysate	17.50
Starch	1.50
Final pH (at 25°C)	7.3 ± 0.1

Pooja Saran - Final Thesis.docx

ORIGINALITY REPORT

15%

SIMILARITY INDEX

PRIMARY SOURCES

1	www.frontiersin.org Internet	122 words – 1%
2	www.tandfonline.com Internet	120 words – 1%
3	usir.salford.ac.uk Internet	105 words – 1%
4	link.springer.com Internet	99 words – 1%
5	mzuir.inflibnet.ac.in:8080 Internet	98 words – 1%
6	hdl.handle.net Internet	85 words – 1%
7	Hilal Yildiz, Neva Karatas. "Microbial exopolysaccharides: Resources and bioactive properties", Process Biochemistry, 2018 Crossref	75 words – 1%
8	www.biomarklabs.com Internet	74 words – 1%
9	espace.inrs.ca Internet	62 words – 1%

10	Wei-Hong Min, Xiao-Bin Fang, Tong Wu, Li Fang, Chun-Lei Liu, Ji Wang. "Characterization and antioxidant activity of an acidic exopolysaccharide from Lactobacillus plantarum JLAU103", Journal of Bioscience and Bioengineering, 2019 Crossref	55 words – 1%
11	Syeda Hajira Banu, Mukunda Chethan Kumar. "caP4: A 2.97 KDa Cationic Antibacterial Peptide from Curcuma pseudomontana L.", International Journal of Peptide Research and Therapeutics, 2019 Crossref	53 words – 1%
12	aem.asm.org Internet	51 words – 1%
13	www.esp.org Internet	50 words – 1%
14	discovery.ucl.ac.uk Internet	46 words – < 1%
15	jgeb.springeropen.com Internet	46 words – < 1%
16	ir.unilag.edu.ng Internet	44 words – < 1%
17	Junlong Wang, Ting Yang, Jia Tian, Tao Zeng, Xiaofang Wang, Jian Yao, Ji Zhang, Ziqiang Lei. "Synthesis and characterization of phosphorylated galactomannan: The effect of DS on solution conformation and antioxidant activities", Carbohydrate Polymers, 2014 Crossref	39 words – < 1%
18	"Encyclopedia of Marine Biotechnology", Wiley, 2020	37 words – < 1%