Isolation, Characterization and Applications of Exopolysaccharides produced from marine microorganisms

By

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Abbreviations

Nm	Nano meter
h	Hour/s
°C	Degree centigrade
RT	Room temperature
g	Gravity
g, mg, µg	Gram, milligram, microgram
L,mL, µL	Liter, milliliter, microliter
M, mM, µM	Molar, millimolar, micromolar
N	Normal
Min	Minute
s	Second
HCl	Hydrocholric Acid
PS	Polysaccharides
DP	Degree of polymerization
HoPS	Homopolysaccharides
HePS	Heteropolysaccharides
IPS	Intracellular polysaccharide
SPS	Structural polysaccharide
LPS	Lipopolysaccharides
EPS	Extracellular polysaccharide
rEPS	Released exopolysaccharide
GTFs	Glycosyltransferases
sPS	Sulphated polysaccharides
scFOS	Short-chain fructooligosaccharide
sp	Spicies
SCFAs	Short-chain fatty acids
GH	Glycoside hydrolase
DMSO	Dimethyl sulphoxide

LAB	Lactic acid bacteria
MEGA	Molecular evolutionary genetics analysis
NCBI	National center for biotechnology information
CLSM	Confocal laser Scanning Microscopy
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
GPC	Gel Permeation Chromatography
AFM	Atomic force microscopy
PCR	Polymerase chain reaction
ANI	Average Nucleotide Identity
XRD	X-ray diffraction
DSC	Differential Scanning Calorimetric
TGA	Thermal Gravimetric Analysis
TLC	Thin-layer Chromatograohy
UHPLC	Ultra high-performance Liquid Chromatography
TFA	Trifluoroacetic Acid
HPLC	High-performance Liquid Chromatography
LC-HRMS	Liquid chromatography high-resolution mass spectrometry
FE-SEM	Field Emission Scanning Electron Microscopy
FT-IR	Fourier Transform Infrared
SEC	Size Exclusion Chromatography
DEPT	Distortion less Enhancement by Polarization Transfer
NOESY	Nuclear Overhauser Effect Spectroscopy
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
FFD	Full Factorial Design
CCD	Central Composite Design
MTP	Microtitre plate
DoE	Design of Experiments
OFAT	One Factor At a Time

OD	Optical density
CFU	Colonies Forming Unit
RSM	Response Surface Methodology
DO	Dissolved oxygen
ANOVA	Analysis of Variance
CDW	Cell Dry Weight
CFU	Colony forming unit
dpEPS	Deproteinized exopolysaccharides
cEPS	Crude exopolysaccharides
LD	Low dose
ID	Intermediate dose
HD	High dose
TC	Total cholesterol
LDLC	Low density lipoprotein cholesterol
HDLC	High density lipoprotein cholesterol
FOS	Fructooligosaccharides
GOS	Galactooligosaccharides
MW	Molecular weight
LMW	Low Molecular Weight
HMW	High Molecular Weight
kDa	Kilo Dalton
NCIM	National Collection of Industrial Microorganisms
IAEC	Institutional Animal Ethics Committee
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
OECD	Organization for Economic Co-operation and Development
fgDNA	Fecal genomic DNA
DNA	Deoxyribonucleic acid
rRNA	Ribosomal RNA
NFW	Nuclease free water
qPCR	Quantitative PCR

OUT	Operational taxonomic unit
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide

Contents

Chapter 1:

Levan exopolysaccharide: A leading biopolymer in food and pharmaceuticals

1. General introduction: Polysaccharide

Polysaccharides (PSs) are high molecular weight compounds, which consisting of different monosaccharide units connected by glyosidic linkage in multiple forms. These are often made up of hexose and/or pentose sugars in multiple proportions, patterns and degree of polymerization (DP). Polysaccharides can be acidic, neutral, or basic in nature, based on the monosaccharide combinations and are often found heterogeneously configured with different or modified monosaccharide units. They are the most abundant biopolymers on the Earth's surface, present everywhere with vast diversity in their chemistry pattern. Based on the structural organizations, it exhibits different properties, such as, solubility, as some are soluble in hot, cold, or ambient water (Varki et al., 2015; Guo et al., 2017) . PSs accounts for an important class of biological polymers and play a vital role in cell's survival, adhesion or signaling. Based on chemical nature, they are classified into two classes: firstly, homopolysaccharides (HoPS), made of repeating units of identical monosaccharides which can be further categorized into glucans, fructans, and polygalactans (Zhang et al., 2014). Secondly, heteropolysaccharides (HePS) consist of repeating units of different monosaccharides. These repeating units can be of disaccharide to heptasaccharides arranged in orderly manner (Mollakhalili Meybodi & Mohammadifar, 2015) . Natural biopolymers such as polysaccharides are widely used in many industries based on their suitable physicochemical, mechanical, and biological properties. Viscosifying and pseudo-plasticity properties are resistant to high temperature, salinity, and pH which qualifies polysaccharides to use in packaging and transportation (Lee et al., 2001). PSs have been extensively applied in drug delivery. Therefore, it is known as an "intelligent drug delivery system" (Patel et al., 2011). PSs can be extracted from bacteria, fungi, algae, plants, and animals (Zhao et al., 2020). Depending on the source of isolation, it plays various important functions, such as energy-storing, maintaining structural integrity and protecting against various cell physiological stresses. According to their morphological locations, polysaccharides are categorized into three groups: Intracellular-, structural-, and extracellular- polysaccharides (Wilkinson, 1958).

1.1 Intracellular polysaccharide (IPS)

Polysaccharides which provide energy to the cell are called intracellular, endopolysaccharide or cytosolic polysaccharides. IPSs are generally found in plants and animals, with some exceptions

in microbes. These functions as reserve energy in specialized cells in the form of starch or glycogen in plants and animals respectively. In bacterial system, they are located in the protoplast and perform a similar function. *Streptococcus mutants* have been known to accumulate IPS in the presence of excess sugar and utilize them during starvation conditions (Busuioc et al., 2009).

1.2 Structural polysaccharide (SPS)

SPSs provides structural integrity and acts as a cell boundary; thus, they are also called cell wall polysaccharide. Polysaccharides, including lipopolysaccharides (LPS), techoid acids and peptidoglycan are types of microbial cell walls, whereas cellulose, hemicellulose, and pectins belong to plant cell wall polysaccharides. These are the fundamental structure of the plants as well as microbial cell walls, which provides a support and protection to the cells.

1.3 Extracellular polysaccharide (EPS)

Polysaccharides are excreted outside the cell as slime or capsules called extracellular or exopolysaccharides. EPSs are majorly known from a microbial system primarily involved in a defense against various stresses, it is also involved in forming cell walls and maintaining structural integrity. EPSs helps in adaptation and nutrient uptake. EPSs also facilitates selective diffusion allowing useful material, cryoprotection etc. Dextran polysaccharide is the first industrially important EPS produced by *Leuconostoc mesenteroides* (Crescenzi, 1995). Looking at its biological applications, EPSs are widely explored in the industrial and pharmaceutical sectors.

2. Exopolysaccharide (EPS)

EPSs are extracellular, high molecular weight polymeric compounds produced by microorganisms in their surrounding environment. EPS is a general term for all forms of exopolysaccharide found outside the cell, either attached to the cell or free living. Depending on the relationship to the cell, these are termed slime, capsular or micro capsular polysaccharides (Duguid, 1951). The form of EPS covalently bound to the surface is called capsular EPS (cEPS), while loosely bound or secreted in the form of slime is called released EPS (rEPS). rEPS are excreted into their surrounding environment, free and easily extractable from the media. EPSs are made up of monosaccharides and/or their derivatives which can be simple or decorated with various groups (organic or inorganic). Sutherland in 1972, coined the term exopolysaccharide for the carbohydrate polymers

synthesized and secreted outside by marine microbes, especially bacteria (Sutherland, 1972). These are fundamental and most studied components of microbial biofilms besides lipids, nucleic acids and proteins (Dolfi et al., 2015). EPSs consist of identical or different repeating units of monosaccharides or their derivatives held by glycosidic linkages. Although exopolysaccharides are ubiquitous, their chemistry varies significantly from one EPS to another.

Comparatively, EPSs are very diverse than IPS and SPS. They exist in branched or linear patterns, which are associated with the activity of specific glycosyltransferases (GTFs, EC 2.4) (Sran et al., 2019).Two decades ago, RA, 1994 estimated that there are likely more than 10^{12} possible combinations that can be made alone from hexose sugars (RA, 1994). The complexity of EPSs increases by various structural organizations, such as different sugars, linkage and branching patterns and variation in sugar configuration and their sequences. In addition to saccharide moieties, EPSs are decorated with non-carbohydrate components. Generally, succinyl, acetyl and pyruvyl are linked with polysaccharides as organic compounds. At the same time, phosphate and sulfate are two main inorganic groups that take part in the decoration of the backbone structure of EPSs (Su et al., 2007). EPS applications can directly relate to their physicochemical properties, which decide the applicability of the candidate molecule. This property plays an essential role in designing a new drug candidate. Physicochemical are intrinsic molecular properties of compounds including molecular weight, morphology, structural arrangement, density, solubility, boiling point etc. Considering applications, solubility and molecular weight are two major properties owned by EPSs. Exopolysaccharides are categorized into low molecular weight (LMW, 8-50 kDa) and high molecular weight (HMW, >50 kDa) compounds. It has been stated that, the biological activities of EPSs are affected by their molecular weight (Calazans et al., 2000; Colliec-Jouault & Delbarre-Ladrat, 2014). Understanding the solubility of EPS is the most important factor for its applications. Stabilizing, emulsifying, film forming, gelling, thickening, water retention, adhesion, coagulating and drug delivery can only be achieved with an aqueous solvent system. The structural arrangement determines ionic interaction, solubility and rheology of compounds. Solubility and biological activity depend upon structural configuration, molecular weight, type and branching of monosaccharides linked with different glycosidic bonds, e.g. Pullulan (soluble) and cellulose (insoluble). Though both are homopolymers of glucose, one is water soluble and another is insoluble. Similarly, levan and inulins are homopolymers of fructose with different water solubility

indexes. The HMW bio macromolecule imparts optimal physical activity in therapeutic use (Petit et al., 2007), such as bio adhesive, emulsification, cryoprotection, oil recovery, heavy metal removal, antitumor, antioxidant etc. There are a few highly recognized and well-studied HMW EPS, e.g. Xanthan, dextran, curdlan, cellulose, gellan and bacterial alginate. The most common linkages found between monosaccharides are β-1, 3 or β-1, 4 or α-1, 6 or α-1, 2, which forms a rigid structure giving a stronger backbone. α-1, 6 or α-1, 2 provides flexibility to the structure, it can be suggested that wherever the support system is required, a structural polysaccharide will be applied. Apart from the basic chemical structure, EPSs are embellished with amine, uronic acid, sulphate, etc., which add benefits to applications. EPS decorated with various groups such as acetate, sulphate and phosphate, alkyl, sulphonyl, and carboxy methyl have shown noticeable applications in various fields.

The overall scopes of commercially available exopolysaccharides from the microbial origin are represented in Table 1, and the emerging exopolysaccharides from marine bacteria with their potential applications are summarized in Table 2. These microbial polymers show great interest in various industrial applications. Thus the microbial-produced EPS can be further explored for well-beings of the society.

Table. 1 Well characterized commercial exopolysaccharides from microbial origin

Sr. No.	EPS	Producer	Properties/ activity	Application	References
1	Alginate	*P.aeruginosa*, *A. vinlandii*	Gelling capacity Film-forming Emulsifying Thickening Stabilizing	Treatment of osteoarthritis, Disintegrating agent for tablets, Wound dressing and bandages, Dental impressions Antacid, Food hydrocolloid, Surgical dressings, Microspheres for drug delivery	Anderson et al., 1987; Mukherjee and Atala., 2005; Nwodo et al., 2012; Peyrom et al., 1974

2	Cellulose	*Acetobacter* sp., *Gluconacetobacter hansenii*	High tensile strength, High crystallinity Moldability Insolubility in most solvents	Artificial skin and blood vessels, Drug delivery Wound healing, Scaffolds for tissue engineering, Audio speaker diaphragms	Beliah et al., 2020; Jung et al., 2002
3	Dextran	*L. mesenteroides, L. dextranicum Weissella cibaria*	Antioxidant Stabilizing Newtonian fluid Behavior	Blood volume expander Column packing material	Ahmed et al., 2012; Du et al., 2018
4	Emulsan	*Acinetobacter* sp.	Emulsifying	Vaccine adjuvant Drug delivery	Dams et al 2008, Pines et al., 1988
5	Fucoidan	*Cladosiphon okamuranus*	Anti-oxidant Anti-coagulant Anti-tumor Anti-inflammatory	Free radical scavenging, Cancer treatment	Azuma et al., 2012
6	Gellan	*Pseudomonas elodea*	Gelling Stabilizing Drug carrier	Excipient in oral ophthalmic and nasal drug formulations Tablet disintegration Controlled drug release Wound healing and tissue engineering	Felt et al., 2001; Hagerstrom, 2003; Robinson et al., 1991
7	Hyaluronan	*Streptococcus equisimilis*	Anti-reflux Wound healing Drug carrier	Eye surgery (vitreous substitution/replacement), Wound dressing material, Osteoarthritis treatment (intraarticular injection), Dental impressions, Matrix for tablets, Arthritis treatment	Kogan et al; 2007, Necas et al., 2008

8	Levan	*B. licheniformis*, *L. reuteri*,	Prebiotic Hypocholesterolemia Anti-oxidant Low viscosity Anti-tumor activity Adhesivity, Anti inflammatory	Bio-thickener in food Film-forming capacity	Freitas et al., 2011
9	Rhamsan	*Alcaligenes*	Gelling	Food stabilization	Robinson et al., 1991; Tako, 1993
10	Xanthan	*X. campestris*	High viscosity Stabilizer Thickener Drug carrier	Suspension stabilizer in pharmaceutical creams Controlled drug release Personal care products	Morris and Harding., 2009
11	Pullulan	*Aureobasidium Pullulans*	Encapsulating Film forming	Tablet granulation and coating, binder, and oxygen impermeable Wound care products	Mocanu et al., 2011
12	Scleroglucan	*Sclerotium* sp	Antitumor Stabilizer	Stabilization of dressings, Ice creams	Survase et al., 2007

Table. 2 Exopolysaccharides from marine bacterial origin

Sr. No.	EPS	Producer	Properties/ activity	Application	Reference
1	Aeromonas Gum	*Aeromonas nichidenii*	Gelling Viscocifying Stabilizer Thickner	Food supplement	Xu et al., 2004; Zhang et al., 1999
2	Alternan	*L. mesenteroides*	Prebiotics	Dietary supplements Papermaking aids Extender in food	Kote, 2002; Leathers et a l., 1997
3	Clavan	*Clavibacter michiganensis*	Antitumor Moisturizing	cancer treatment Treatment for rheumatoid arthritis Cosmetics	Vanhooren and Vandamme, 1998, 2000
4	Curdlan	*Alcaligenes faecalis var. myxogenes*	Gelling Texturizing Stabilizing Newtonian fluid	Blood volume expander column packing	Zhang et al., 2018

			behavior Chromatographic media		
5	Cyclosophorans	*Agrobacterium*	Encapsulation drug carrier	Drug delivery	Hisamatrsu. ,1992; Vandamme, & Soetaert, 1995
6	Ethapolan	*Acinetobacter* sp.	Emulsifying	Oil recovery	Grinberg et al., 1995
7	Fucogel	*K. pneumoniae*	Moisturizing	Skin soother, moisturizer	Robert et al., 2003
8	FucoPol	*Enterobacter* sp.	Adhesive Emulsifying Stabilizing gelling thickening Flocculating Antioxidant	Photo protective sunscreen Cryoprotection Food supplement	Guerreiro et al., 2021; Torres et al., 2015
9	Galactan	*Weissella confusa*	Hydrocolloid Thickning Emulsifying Stabilizing Encapsulation	Food adjuncts	Kavitake et al., 2016
10	Gelrite	*Pseudomonas* sp.	Gelling	Thermal protection	Iyer et al., 2006
11	Glucuronan	*Gluconacetobacter hansenii etc.*	Gelling Thickening	Food supplement	Khan et al., 2007
12	Haloglycan	*Halomonas stenophila*	Flocculating Emulsifying	oil recovery	Amjres et al., 2015
13	Heparin	*Vibrio sp.*	Anticoagulant antithrombotic antitumor antiviral agent	Treatment and Prevention of blood cloting Cancer treatment	Guezzenec et al., 2002
14	Inulin	*Streptococcus mutans*	Prebiotics, Targeted drug delivery Immunomodulatory	substitute of fat in food products	Rosell & Birkhed., 1974

15	Mannan	*Brucella melitensis, Rhodotorula acheniorum, Edwardsiella tarda*	Antioxidant Pseudo plasticity	Food supplement	Arias et al., 2003
16	Peanan	*Paenibacillus polymyxa*	Rheology	NA	Rütering et al., 2018
17	Reuteran	*Lactobacillus reuteri*	Probiotic	Used in bakery food supplement	Meng et al., 2016
18	Simusan	*Arthrobacter*	Thickener Stabilizer Emulsying Strong flocculant	Food supplement	Soph'ya et l., 1995
19	Sphingan	*Sphingomonas pituitosa*	Stabilizer, Rheology	Stabilization of food product	Denner et al., 2001; Li et al., 2016
20	Succinoglycan	*Alcaligenes faecalis var. myxogenes*	High viscosity Acid stability drug carrier	Oil recovery tissue engineering drug delivery	Gao et al., 2021
21	Welan	*Alcaligenes*	Gelling Stabilizing Emulsifying Thickening	Food stabilizer Oil recovery	Robinson et al., 1991
22	Xylinun	*A. xylinum*	Viscoelasticity, Rheological Thickening, Gelling, Immunomodulatory, stabilizer, emulsifier	Stabilization Encapsulation Film formation Inhibition of release of water from foods,	Baines, & Seal., 2012, Saito et al., 2003
23	Zooglan	*Zoogloea ramigera*	Methanolysis ,Thickening, Stabilizing, Lubricating,	Metal chelator	Lee et al., 2006
24	Mucoidan	*Rhodococcus erythropolis*	Pristane degrading	toxic (organic) compound degradation	Urai et al., 2007
25	Mutan	*Streptococcus mutans,, Lactobacillus*	Antitumor anti-oxidant antimicrobial Immunomodulatory	Cancer treatment Activating immune cell	Koo et al., 2010

26	Cepacian	*Burkholderia cepacia*	Biofilm forming	NA	Nogueira et al., 2005; Sist et al., 2003
27	Spirulan	*Arthrospira platensis*	NA	NA	Trabelsi et al., 2009

2.1 Microbial exopolysaccharide

Exopolysaccharides from microorganisms has enormous qualities to stand in the industrial sector. Microbial EPSs shows extreme diversity in their chemical nature. Most of the EPS are composed uniformly or in a combination of glucose, galactose, mannose, fructose, rhamnose, etc. Microorganisms can produce two types of polysaccharides. For example, *Erwinia amylovora* produces levan and amylovoran. Microbial EPS have been shown to possess a multifaceted biological activities including, anticoagulant, antiviral, antitumor (Mahgoub et al., 2018), ant oxidative (Ye et al., 2012), immunomodulatory and hypocholesterolemia activities (Zhao et al., 2020). A few microbially produced polysaccharides have been commercially used in the industries, for example, alginate, xanthan gum, hyaluronic acids, cellulose, dextran, levan, and gellan gum were used due to their gelling, emulsifying, stabilizing, thickening and cryoprotection activities (Morris & Harding, 2009).

2.1.1 Algal EPS

Based on cellularity, algae are divided into macroalgae and microalgae. Microalgae are found in fresh and marine water bodies. Marine microalgae produce a unique form of extracellular polysaccharide, which can be observed as free form or adhered to the cells. Red and brown algae have been reported to produce EPS and are used in various fields. Marine microalgae generally produce sulphated polysaccharides (sPS) with different proportions of uronic acid. Thus the algal polysaccharides are acidic in nature (Marcati et al., 2014). Raposo et al. (2013) reviewed almost 120 different sulphated polysaccharides from marine microalgae with different proportions. Most of them are extracellular heteropolymers, which can be made up of 6 to 10 monosaccharides substituted with various organic derivatives (de Jesus Raposo et al., 2013). Some of them are

glycoproteins having 55% protein content (Trabelsi et al., 2009). The anionic nature of EPS makes them suitable for many applications, especially in pharmaceuticals, neutraceuticals, cosmeceuticals, food and environment. Due to their anionic nature, algal sPS used for environmental cleanup by chelating metal ions of effluents and wastewater (Freire-Nordi et al., 2005;Mona & Kaushik, 2015). Marine sPS are shown to have several applications such as antiviral, antibacterial (de Jesus Raposo et al., 2013;Yim et al., 2004), anti-thrombogenic (Mousavian et al., 2022), antiatherogenic, anticoagulant (Borowitzka, 2013), anti-inflammatory, antioxidant (Chen et al., 2010), hypocholesterolemic (Dvir et al., 2000), antitumor and immunomodulatory (Chen et al., 2012). Sulphated EPS have been shown to enhance the proliferation of T and B lymphocytes and can also improve food quality (Wang et al., 2019).

2.1.2 Fungal EPS

Fungal cells produces EPS in all phases of their growth cycle in the form of extracellular biofilm or capsules (Osińska-Jaroszuk et al., 2015). Based on the uronic acid content, fungal EPSs are classified into two types, acidic and neutral. EPS produced from marine fungi have been widely applied for their potential antioxidant activity (Sun et al., 2004; Chen et al., 2011; Guo et al., 2013) which can be used as a food supplement. Fungal polysaccharides have been shown to possess immunomodulatory (Sun et al., 2016), antitumor (Zhou et al., 2013) and metal-removal activities (Lotlikar et al., 2018). Schizophyllan, lantanas and krestin polysaccharides have been widely studied from *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor* respectively. These are clinically accepted as immunoceuticals in several oriental countries (Masuda et al., 2009; Tang et al., 2012).

2.1.3 Bacterial EPS

Bacterial EPS has many unique characteristics, such as biodegradability, biocompatibility, bio adhesive, etc. These are used in pharmaceutical, food, biomedical and chemical industries as flocculants, absorbents, drug delivery agents, etc. The most significant potential of bacterial EPSs is related to their use in high-demand market value, such as biomedicine, pharmaceuticals and cosmetics. Bacterial polysaccharides fulfil the criteria of purity, crystallinity and various functional groups, so bacterial EPSs gain preference at the industrial scale (Freitas et al., 2011). EPS-based

nanoparticles, such as silver, gold, etc., have been used as bactericidal, fungicidal drugs, manufacturing sensors and optoelectronics (Scala et al., 2019). Bacterial polysaccharides are requisite of many pharmaceutical and industrial applications based on their tremendous biological function and properties. These are demandable by industries and can be produced economically for high-added-value pharmaceuticals. The production can be achieved in controlled conditions in a short period of time and easily optimizes production parameters and downstream processing ((Delbarre-Ladrat et al., 2014).

2.1.3.1 Source of EPS-producing bacteria

The EPS-producing bacteria can be found in everywhere, such as soil, water (marine), and biological (milk, jaw, gut, and lungs) samples. The soil bacteria have contributed significantly to the soil with the help of EPSs. It assists plant-microbe interactions. Soil bacteria attach to plant roots with the help of EPSs. Rhizospheric bacteria have been known to associate with plant roots and help them. *Azospirillum, Rhizobium, Pseudomonas* and *Bacillus* are widely known for plant-microbe interaction. EPS plays a crucial role in enhancing soil fertility and aridity (Osemwegie et al., 2020), protecting against desiccation and maintaining sustainability in agriculture. In soils, EPS helps colonize rhizobial species, entraps nutrients and increases water-holding capacity to improve the plant's growth and associated microbes (Fujishige et al., 2006). EPS has been shown to produce from human-associated microbes of the gut, lungs, jaws and milk. Gut and milk resident microbes were shown to enhance colonization of healthy gut microbiota such as *Bifidobacterium, Bacteroides* and lactic acid bacteria (LABs) which are having immunomodulatory and anticancer activity. The marine source has been widely explored, yet it needs to be completed. Due to continuous changes in climatic conditions, marine sources have been witness to providing novel bioactive compounds.

2.1.3.2 Marine bacteria

Soil bacteria have yet to be explored much; they cannot provide novel EPSs. LAB from milk is a well-known producer of EPSs, for which exploration is saturated. Due to ethical restriction and cultural conditions, EPSs from the human body have yet to be explored to an extent. Marine microbial EPSs possess novel and unique physical properties. EPS from marine bacterial sources presents a broad range of applications in food industries. They have emerged as important biopolymers having potential applications in various industries such as paper, food, beverages,

textile, pharmaceutical, cosmetics, metal mining and oil recovery, etc (Becker et al., 1998; Finore et al., 2014). Marine habitat harbours unusual microorganisms with potential biotechnological applications (Querellou, 2003). These microbes are constantly encountered by various stress conditions, which may force them to adopt specific defensive mechanisms. However, marine bacterial EPSs are often exist as HMW polymers (Rigouin et al., 2012).

The marine biosphere is heterogeneous, covering an extensive range of ecosystems such as marine sediments and microbial mats, shallow and deep-sea hydrothermal vents, hypersaline environments, Antarctic sea ice, etc. Marine ecosystems present an immense microbial diversity and are still unexploited entirely, offering the opportunity to discover novel bio macromolecules (Roca et al., 2015) (Roca et al., 2016). therefore, it can be a promising source for the researcher to discover various novel molecules (Dufourcq et al., 2014; van Eldik & Hubbard, 1996). It hosts tremendous biodiversity and offers rich flora and fauna, representing a vast indispensable commercial-grade products (Jenkins & Hall, 1997; Satpute et al., 2010). Marine environments are highly complex habitats causing various stresses. Marine microorganisms have been reported to cope with extreme conditions, such as adverse environmental conditions Due to the structural diversity of marine EPS, it has gain considerable attention (Al-Nahas et al., 2011; Mancuso Nichols et al., 2004).

Marine organisms have developed unique metabolic processes to survive in extreme conditions, resulting in the ability to produce novel compounds compared to the microbes residing on the Earth's surface (Chi & Fang, 2005). Therefore, EPS from the marine environment exhibits great interest in medicine, such as drug discovery (Fenical, 1993; Hwang et al., 2008). Blunt et al. (2016) reported that > 25,000 novel bioactive compounds had been identified in the past five decades with approximately a 5% increment per year (Blunt et al., 2016). Increasing demand for natural biopolymers forces the exploration of marine microbial life, which can be the keystone to accomplishing the need of the time (Suresh Kumar et al., 2007).

2.2 Role of EPS

Exopolysaccharides play a fundamental role in many microorganisms for providing an environment for protection, nutrient entrapment, survival, cellular recognition, adherence and cell identity. Bacteria excrete polysaccharides into their surroundings, which forms an outer sheath and

act as an primary defense system against different biotic or abiotic stresses (Boyle & Reade, 1983). Biotic stress is exerted by pathogenic fungi, bacteria, viruses, viroids etc., while abiotic stresses, including desiccation, salinity, antibiotics, osmotic pressure, extreme pH and temperature or toxic compounds such as lead, mercury, cadmium, sulfur dioxide (Nichols et al., 2005). EPSs are also involved in cellular recognition by binding to lectins.

2.3 Biosynthesis of EPS

Bacterial EPSs are mostly synthesized intracellularly and then exported to the extracellular environment, except dextran, levan, alternan, reuteran and mutan PS (Ates, 2015; Vanhooren & Vandamme, 1998). Four different pathways have been reported for synthesis of EPSs, which include Wzx/Wzy pathway, ABC transporter pathway, synthase-dependent pathway and sucrase-dependent pathway. Generally, most of organisms utilize synthase-dependent pathways for homopolysaccharide synthesis. The so-called sucrase-dependent pathway is performed extracellularly. Biosynthesis of dextran, levan, alternan, reuteran and mutan is catabolized by outer membrane-bound enzymes, namely dextransucrase, levansucrase, alternansucrase, reuteransucrase, and mutansucrase, respectively (Boels et al., 2001; Vanhooren & Vandamme, 1998). These enzymes utilize sucrose as the sole carbon source. In intracellular biosynthesis of EPS, substrate is uptaken first by an organism and then catabolized, while in extracellular EPS biosynthesis, the substrate catabolizes outside by the cell-bound enzymes (Freitas et al., 2011). Heteropolysccharides are synthesized intracellularly and involves a series of enzymes for stepwise synthesis and export. Simple sugar cannot perform the synthesis of EPS. It needs to be charged by phosphorylation such as nucleotide monophosphate/diphosphate (NMP/NDP). The charged NMP/NDP-sugar gets transferred to lipid, undecaprenyl phosphate (C55-P) with the action of glycosyltransferases (GTFs), (Sutherland, 2001). Further assembly and transport carried out by other GTFs, the involvement of intermediate lipid pathway plays a key role. This pathway is reported in Gram-negative bacteria but it can also be observed in Gram-positive bacteria.

The Wzx/Wzy pathway and the ABC transporter pathways are most commonly utilized for the secretory (slime) EPSs and capsular polysaccharides. These pathways synthesize heteropolysaccharides, while synthase-dependent and sucrase-dependent pathways work to synthesize hompolysaccharides.

I. Wzx/Wzy pathway

Wzx/Wzy pathway operates in the cell cytoplasm, where several cytoplasmic membrane-bound enzymes are involved in EPS synthesis. After substrate uptake, it involves assembling repeating units by the action of high-specificity glycosyltransferases (GTFs). These transfers charged sugar nucleotide towards an undecaprenyl diphosphate linker molecule (C55-P, Und-P) step by step. The Und-P lipid linker remains anchored at the inner cell membrane. The oligomeric sequence of the final, mostly branched, the respected GTFs determines polymers. Other substituents, such as pyruvate, acetate and side chain are incorporated before final assembly. The stage of substitution has yet to be made clear (Schmid & Sieber, 2015). After the final assembly of repeating units, the Wzx protein, known as flippase, transports the assembly into the periplasmic space by the H^+ dependent antiport transport system. After that, polymerization takes place by the action of a polymerase called Wzy protein. Sometimes co-polymerase involves in the determination of chain length. Finally, the secretion of polymers functions with the help of OPX protein (outer membrane export protein), which has relatively low substrate specificity (Cuthbertson et al., 2009).

II. ABC transporter pathway

This pathway synthesizes capsular polysaccharides, where polymerization of linear polysaccharides is completed on poly - 2 - keto - 3 - deoxyoctulosonic acid linker (Kdo) linked Und-P linker at the cytosolic membrane by the action of highly specific GTFs and polymerase. Finally, Wza protein exports the polymer chain outside the membrane. Completing polysaccharides in the cytosol and adding a Kdo linker is the main difference between the ABC transport and Wzx/Wzy pathways.

III. Synthase-dependent pathway

In the synthase-dependent pathway, the complete polymerization and translocation of polymer strands occur in both the cell wall and cell membrane by the single synthase enzyme with the help of TPR (tetratricopeptide repeat). The TPR helps in the protection of polymers from degradation till their export through the outer membrane porin protein (β-barrel). The synthase-dependent pathway is involved in homopolysaccharide synthesis, e.g. bacterial cellulose and alginate, hyaluronan etc.

IV. Sucrase-dependent pathway

Extracellular glycosyltransferase enzymes, namely sucrase, synthesize some homopolysaccharides. This enzyme catalyzes disaccharide sugar, i.e. sucrose, and utilizes one of its monomers to synthesize homopolysaccharides. Depending on its activity as glucosyltransferase or fucosyltransferase, it produces glucan polysaccharide, i.e. dextran, reuteran, mutan, or alternan, by the action of dextransucrase, reuteransucrase, mutansucrase, or alternansucrase, respectively and fructan polysaccharides, i.e. levan or inulin by the action of levansucrase or inulosucrase, respectively. The pathways are schematically represented in Fig 1. According to Schmid et al. (2018) the underlying mechanism of microbial polysaccharides synthesis has been poorly understood.

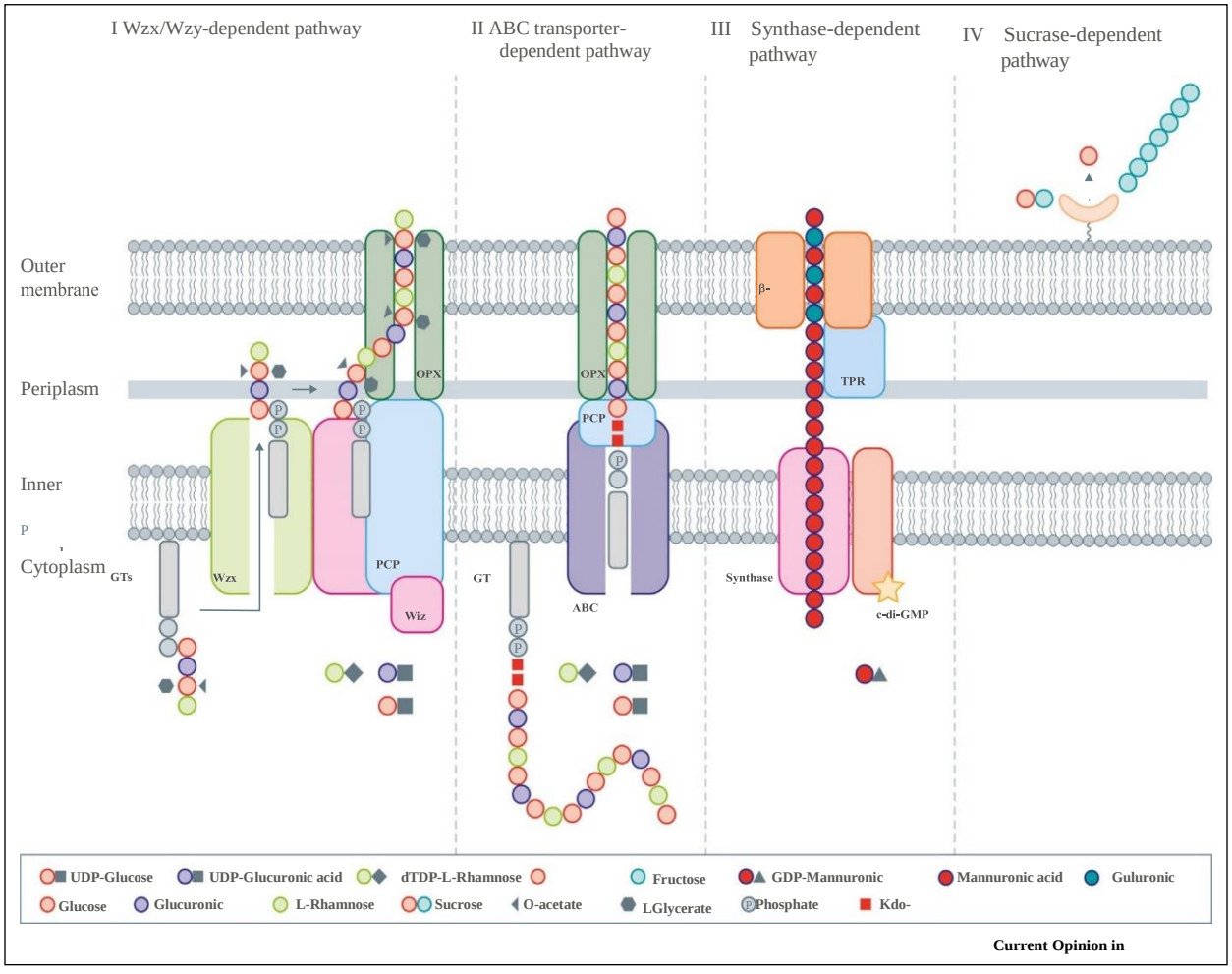

Fig. 1 Schematic representation of exopolysaccharide pathways found in microbes: (I) The Wzx/Wzy pathway. The steps involve assembling repeating units carried out by glycosyltransferases (GTFs), flipping Und-P linked oligomer repeating unit into periplasmic space

by flippase enzyme (Wzx) where it simultaneously polymerizes and transport to extracellular environment by Wzy and OPX, respectively. (II) In the ABC transporter pathway, the assembly of the whole polymer chain takes place on the Und-P linked Kdo linker in the cytoplasm by the action of different GTFs, followed by flipping out towards the periplasmic space. (III) The synthase-based pathway known to polymerize only a single type of monomer by the particular GTFs, and porin protein (β-barrel) finally transport the polymer. (IV) Sucrase-based biosynthesis takes place solely outside of the cell. The assembly of homopolysaccharides or oligosaccharides is carried out by extracellular membrane-bound sucrase enzyme (Adopted from Schmid et al., 2015).

3. Levan exopolysaccharide: A leading biopolymer

General introduction

The majority of homopolysaccharides are composed of glucose monosaccharides (Roca et al., 2015). Hence exploration of other sugar-based homopolysaccharides can be taken into account, which may contribute additional benefits compared to glucose-based homopolysaccharides. One such sugar is fructose, and fructose-based polysaccharides have gained attention for exploration.

3.1 Fructans

Fructans are homopolymers of fructofuranosyl residues joined by β-linkage, which are synthesized by the action of fructosylatransferase (FTase, EC 2.4..1.9). Fructans exist as a chain of polyfructose terminated with glucose residue. Based on the chain length, fructans have been classified into three distinct classes: short-chain fructooligosaccharide (scFOS), Oligofructose (fructooligosaccharide), polyfructose (inulin, levan and graminan). They are made up of either/or β (2,6) and β (2,1) linkages. They are further classified into five classes based on the glycosidic linkages between the initial trisaccharide: Inulin, having β (2,1) linkages with the initial trisaccharide 1-kestose; levan, having β (2,6) linkage, based on 6-kestose; graminan, having β (2,6) and β (2,1) linkages; the neo series based on neokestose of inulin with β (2,1) and levan with β (2,6) linkages, with an internal glucose moiety (Norio et al., 2007). Fructans produced by bacteria are mostly of levan type. Only a few bacteria synthesize inulins type (Nakapong et al., 2013). Fructans are resistant to gastric enzymes, which the action of endo-levanase and fructofuranosidase can only catabolize. Thus,

they are mostly applied as dietary fibers. Besides their function as dietary fibers, they possess many other properties, making them a potential candidate for improving human health.

3.2 Levan

Levans are homopolymers of fructose monosaccharides containing β- (2, 6) glycosidic bonds existing as linear or branched forms. These are the polyfructose among inulin and graminin. *Bacillus subtilis* have been widely explored for levan production. Levan can be classified as type-I and type-II. Type-I levans consist of β- (2, 6) linkage of only D-fructose units, whereas levan type II is a polymer of D-fructose with glucose at the terminus. Levan belongs to the nondigestible carbohydrate class of polysaccharides, which are unable to digest by digestive enzymes secreted by the pancreas and small intestinal mucosa. The colon microbiota is equipped with enzymes that have the potential to digest levans into short-chain fatty acids (SCFAs, Den Besten et al., 2013).

3.2.1 History of levan

In the early 19th century, Greig-Smith and Steel had found a microorganism producing viscous molecule from the secretion of *Eucalyptus stuarina*, which was found rotating levorotatory upon exposed to plan polarize light, which is a letter discovered as a fructose-based polysaccharide called levan (Greig-Smith & Steel, 1902; Kopeloff et al., 1920) . The enzymes that produce polysaccharide gum upon utilization of sucrose was coined as viscosaccharase. Letter on enzymes was nomenclated in accordance with the product they synthesize. Therefore levan-synthesizing enzyme called levansucrase, a dextran-synthesizing enzyme, is called dextransucrase. The fructan catabolic enzyme is distinct from anabolic enzymes. They belong to polyfructosidase. Levan hydrolytic enzymes are called levanases (Hestrin et al., 1943). Spies have reported a highly branched form of fructan called, Sinistrin from *Urginea maritima* (Spies et al., 1992;Praznik & Spies, 1993;). An investigation has found that sinistrin fructans have a structure similar to the fructan from garlic (Baumgartner et al., 2000).

3.2.2 Source of levan

Levan production has been reported from plants, bacteria, fungi and algae (Donot et al., 2012). In microbial systems, it has been suggested that levan forms a capsule to defend against biotic factors

(Paton, 1960). Plant levans (aka, Phlen) function as storage material, which also protects against stress tolerance (Pavis et al., 2001). Plants levans are commonly found in grasses such as *Dactilys glomerata* and *Phleum pretense, Triticum aestivum, Cocksfoot, Pachysandra terminalis and Allium sativum* (Norio et al., 2007). Plant levans are linear, while microbial levans are mostly branched with varied from low molecular weight to high molecular weight with a degree of polymerization (DP) up to 5×10^4 (Ortiz-Soto et al., 2019). Microbial levans shows variable degrees of branching up to 12-15% (Han & Clarke, 1990), Which is relatively high as compared to plant levans (having DP varying from 2-200 fructose residue) (Han & Clarke, 1990). Levan molecular weight differs from organism to organism. Bacterial levans are highly branched and comprises up to 30% of the total residues (Smelcerovic et al., 2008), which influence the overall properties of the molecule (Smelcerovic et al., 2008). The C-1 is a preferable position for branching. The branching is more often found after every 12 residues of fructose in the chain. Levan's molecular weight and degree of branching with an extension of fructose residues vary according to source and production parameters (Öner et al., 2016). Levan is strictly non-reducing and synthesized by several bacteria, including Bacillus, Aerobacter, Erwinia, etc. (Bekers et al., 2004). Most bacterial species contain a unique levansucrase gene usually found first in the operon.

3.3.3 Biosynthesis of levan

Levans is synthesized by an extracellular enzyme called levansucrase (EC 2.4.1.10), which can be either bound to the cell surface or released into the media (Arrieta et al., 2004). It performs a dual catalytic activity like transferase and hydrolase. Its β-fructosyl transferase activity catalyzes non-Leloir type of transfructosylation reaction. It belongs to the glycoside hydrolase (GH) family 68 (Szwengiel et al., 2016), this kind of fibrinolytic enzyme has been known to produce by Gram-positive and Gram-negative bacteria. More than 650 discrete sequences have been reported till date (González-Garcinuño et al., 2018). The fucosyltransferase of plants and fungi has been categorized into the glycoside hydrolase GH32 family.

The GH 68 family comprises a clan of GH-J, which comprise a five-bladed propeller structure with three identical catalytic (Asp, Glu, and Asp) residues retaining reaction mechanisms. After being translated into the cytosol, levansucrases adopt a final conformation in the periplasm and are secreted out of the cell at an acidic pH (5.0-6.0) (Arrieta et al., 2004). Levansucrase is secreted by

the SecA (Leloup et al., 1999). Levansucrase utilizes sucrose as the sole carbon source for the synthesis of levan. Generally, levansucrase initiates a reaction by sucrose, yet it can also utilize sucrose-containing units such as raffinose and stachylose (Iizuka et al., 2002; Kim et al., 2005). In some bacteria, levan metabolism is strictly regulated by two enzymes. The anabolic levansucrase and catabolic levanase (β-D-fructofuranosidase) encoded by SacB and SacC genes, respectively (Gay et al., 1983; Wanker et al., 1995). SacB is induced in sucrose (Marvasi et al., 2010) , while SacC is induced by low concentrations of fructose (Martin et al., 1989). The constitutive expression can be observed in the presence of glucose. It is active on levan, inulin and sucrose (Shida et al., 2002). In the bacterial system, levansucrase catalyzes three metabolic reactions, which lead to the synthesis of levan polysaccharides. It includes hydrolysis and polymerization of a substrate (sucrose), and hydrolysis of the product (levan).

Structurally levansucrase contains five fold β-propeller topology and four anti-parallel strands (Lombard et al., 2014). The active site of a levansucrase consists of two subsides, the substrate binding or hydrolytic side (aka -1 subside) and the transfer or polymerization side (aka -1 subside). The -1 subside has a high affinity towards fructose residues, while the +1 subsite occupies fructose and glucose residues. Initially, -1 subside occupies sucrose, and then hydrolyzed into glucose and fructose. Retaining fructose in the pocket, glucose is released in the medium (Jang et al., 2001). This process continues throughout the completion of the reaction. Then retained fructose attracted towards +1 subside and polymerizes to β- (2, 6)-linked oligofructans by fructosyltransferase reaction, which eventually leads to the formation of levan (Li, 2015). The reaction occurs in an enzyme's deep negative cavity via a double displacement or "ping-pong" mechanism (Kim et al., 2005). The levansucrase utilized energy released by the hydrolysis of glycosidic bonds (Perlot & Monsan, 1984). Sucrose can acts as a donor as well as an acceptor molecule. Depending on the circumstances, levansucrase can function generator or destroyer of levan. The C-2 position of sucrose, stachyose raffinose or oligofructans acts as an acceptor to couple with fructose of sucrose as a doner called priming reaction. Depending on the microbial sources, the reaction can happen in a processive or disproportionate (produce FOS) manner.

3.2.4 Structure of levan

Levans are naturally occurring polymers of D-fructofuranose with β - (2, 6) linkages between repeating fructofuranosyl units with terminal glucose (Tanaka et al., 1980) and branching at C-1 (Arvidson et al., 2006). Glycosidic bonds are formed by a reaction between the hemiacetal hydroxyl groups of one monomer unit with the hydroxyl group of another. The levan structure I depicted in Fig 2. The molecular shape of levan can be observed as spheroidal shape (Tanaka et al., 1980). Levans form densely packed spheres, presumably due to more intermolecular interactions (Jakob et al., 2013).

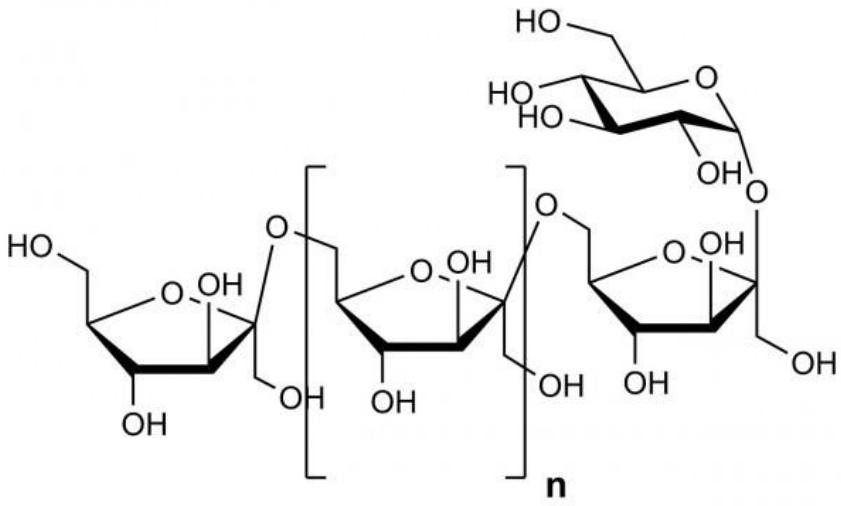

Fig. 2 Levan polysaccharide

3.2.5 Properties of levan

Levans exhibit unique physiochemical properties. Levans are strong adhesive, can retain water and nutrient (Mann & Wozniak, 2012), which helps microbes against desiccation and starvation. A viscous gel can be obtained by levan. Levans are amphiphilic polymers soluble in aqueous and fats (Öner et al., 2016).They are insoluble in organic solvents except for dimethyl sulphoxide (DMSO) (Manandhar et al., 2009). The aqueous solubility of levan can be increased in hot water (Gupta et al., 2011). Due to their amphiphilic nature, they can form a thin film which can be applied in various fields. They are stable in heat, acid and alkaline mediums, which distinguishes levans from other natural polysaccharides (Combie et al., 2004) . Further low intrinsic viscosity, compatibility with salts and detergents, water/chemical holding capacity, good rheological, pseudo plasticity (Bae et al., 2008) , and bioactive potential make levan a unique polysaccharide having

applications in many fields (Bekers et al., 2005). These all valuable properties make levan a versatile biopolymer.

3.2.6 Applications of levan

Levans has been applied in the food, pharmaceutical, cosmetic industries, and environment (Arvidson et al., 2006).

Health sector: Levans have been shown to have a potential health benefits. Levan extracted from *Bacillus subtilis* has shown antitumor and antioxidant activity which was further derivatized and found enhanced antitumor activity. Antitumor activity of levan has been shown against 8 different cell lines (Yoo et al., 2004). It is used in dental caries (Arvidson et al., 2006). It has been shown that, levan induces mitochondria-mediated cell apoptosis in cancer cells which were found to be more active against the HepG2 cell line (Abdel-Fattah et al., 2005). Levans can prevent colon cancer incidence by stimulating mineral absorption and activation of the immune system. Low molecular weight levans have been known for their cholesterol-lowering properties, are recognized for their bifidogenic effect, and are considered as prebiotic compounds (Adamberg et al., 2015). Studies have shown that, adding levan to the diet prevents atherosclerosis in rats by reducing oxidative stress (Belghith et al., 2012), obesity and hyperlipidemia (Kang et al., 2004). Levan blending in cosmeceutical products alleviates skin miniaturization and skin irritation. It has been shown to enhance levan activity by derivatization with sulphate, phosphate, and acetate. A derivatized form of levan has been shown to have an anti-HIVagent (Roberts & Garegg, 1998). The sustained drug release has been achieved by levan, which was applied to release curcumin (Bahadori et al., 2019), bovine serum albumin, vancomycin (Sezer et al., 2011) and O-acetyl-α-tocopherol (Nakapong et al., 2013), 5-fluorouracil (Tabernero et al., 2017).

Food sector: EPSs are receiving attention in dairy industries to substitute commercial stabilizers with a natural stabilizers to prevent syneresis and improve the texture of the fermented product (De Vuyst et al., 2001) . A traditional Japanese food dish called natto. It is a fermented product of whole soybeans reached in levan and has been consumed daily in the form of breakfast (Shih & Yu, 2005). Levan belongs to dietary fibers. Thus, low molecular weight levan is extensively used as a prebiotic supplement.

Environmental sector: The levan produced by *Paenibacillus polymyxa* CF43 expedites soil aggregation and water holding capacity enhancing mineral absorption and growth of wheat plants (Bezzate et al., 2000) .

Others: In other industries, levan has been applied as a plasma expander, sweetener, and gum. Levans are used as encapsulating agent, thickener, food stabilizer, emulsifier, and drug carrier. Levan-based nanostructured systems are prevalent, e.g. nanocapsules, nanogel, nanofibers, nanocomposites, nanofilm, and nanoparticles (De Siqueira et al., 2020). It has also been shown that, simple and acetylated levan crosslinked with epichlorohydrin can form a thin plastic film. The polysaccharides such as levan are often depolymerized by free radicals, chemical hydrolysis, or ultrasonic methods to enhance their activity (Petit et al., 2007).

4. Concluding remarks

Bacterial exopolysaccharides are leading-edge natural polymers with different physicochemical properties. Based on their physicochemical properties, they are applicable to various industries. The current market requires the finding of novel and unique exopolysaccharides from various habitats. Despite the many applications, commercialization is a major concern for EPS exploration. The extensive literature review reveals that most homopolysaccharides are composed of glucose monosaccharides. The finding of other homopolysaccharides will be a great alternative to explore. And another aspect is its commercialization. The high production of a product to production cost is the major obstacle. Therefore, optimization of culture conditions for high production allows for exploring the exopolysaccharides and applying them to various industries.

Significance of the research

To fulfil the increasing demand of natural biopolymers at various areas, novel microbial exopolysaccharides are majorly concern. Microbial exopolysaccharides have enormous application in food environment clean up, cosmeceutical, nutraceuticals and pharmaceuticals. Majority of homopolysaccharides are glucose based. A levan exopolysaccharide is only fructose homopolysaccharide having diverse range of molecular weight. The glucose polymers are saturated since several decades while the fructose homopolysaccharide are leading edge

biopolymer of today's era. Levan are currently extracted from plants and algal source which has high production cost, therefore to overcome these problem bacterial levan plays an alternative role. The production of bacterial levan and its downstream processing is simple and time saving as compare to plant or algae.

Organization of thesis

The thesis is organized into six different chapters. Describes. The *First chapter* provides the detailed comprehensive literature review emphasizing exopolysaccharide from various source. Several habitats were examined out of which the marine habitat has focused. The biosynthetic pathways, structure and many application of microbial EPS has emphasized. The *Second chapter* deals with the screening and identification of microorganisms from marine environment for exopolysaccharide (EPS). Marine sediment samples were collected from Chorao Island, Goa which coordinates the 15°25'N and 15°30'N between 73°45'E and 73°59'E. The *Third chapter* deals with the production, purification and structural characterization of produced EPS. The structure was characterized using various analytical techniques. The *Forth chapter* deals with the optimization of product yield using a cost-effective approach. Media components were amended to enhance product yield using Micro bioreactor (1.0 mL volume), BioLector pro, and Response Surface Methodology and validated at shake flask, 1.0L and 10L at lab scale fermenter. The *Fifth chapter* deals with the applications of levan polysaccharide to find out their prebiotic potential by *in-vitro* and *in-vivo* study using quantitative PCR and metagenomics approaches and for anticancer activity. And the *Sixth chapter* provides summery of thesis and future perspective of the levan polysaccharide.

Statement of problem

Exopolysaccharides (EPS) are bio macromolecules produced by microorganisms, which are reported mainly from bacteria, fungi, yeast, and algae. Bacteria are the major sources for the EPS production due to the ease of cultivation, manipulation, controlled production and downstream processing. The EPS production triggered by various stress conditions and marine ecosystems has been known for its various biotic and abiotic stresses such as high temperature, salinity, pressure, toxic elements and pathogens. The marine microbial EPS function to cope-up with such adverse

environmental conditions in the given habitat. More than hundred different types of EPSs have been reported in the literature. They are majorly isolated from marine environments with a wide applications due to their unique physicochemical properties. EPS's are widely applied in the food sector as well as pharmaceutical industries. Industrial demand has been increased for EPSs in large quantities to meet the needs. Despite the enormous applications of EPSs, only few of them have been commercialized because of their high production cost. However, searching for novel EPS and economical production requires more time to commercialize them at an industrial scale. EPS have been reported as an anticancer drug candidate but understanding its mechanism of anticancer is challenging. However researcher should look for cost effective production and reveal the mechanism of anticancer activity which are major concerns of EPS needs to be explored further.

Objectives

The bacterial EPSs are very important class of biopolymers beneficial to human kind. However, the searching of novel EPS producing bacteria with unique physicochemical properties is requisite to explore at higher scale. And the minimizing production cost is another aspect to commercialize and apply them at various fields.

Therefore, following are objectives of the current thesis:

I. Screening and identification of potent exopolysaccharide-producing bacteria from marine source
II. Production, purification and structural elucidation of exopolysaccharide
III. Optimization of fermentation process via Micro bioreactor (BioLector Pro)
IV. Potential applications of EPS in food and pharmaceuticals

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Chapter 2:

Screening and Identification of Exo-polysaccharide producing organism from marine habitat

Abstract

The study aimed to screen the Exopolysaccharide (EPS)-producing marine bacteria to search for novel or efficient EPSs from the diverse marine ecosystem. A total of 43 previously isolated marine strains were screened for EPS production; four strains, namely AC-11, SGD-03, V-74 and SGD-05, were found positive for EPS production. Preliminary studies have showed that, these bacteria are rod-shaped and Gram-positive in nature. Among the 4 potential strains, SGD-03 produced a high amount of EPS, i.e. 894 mg/L, which was taken further for detailed studies. Based on the polyphasic identification, the SGD-03 strain shared the highest 16S rRNA sequence similarity with the *Bacillus licheniformis* ATCC 14580 and named as *Bacillus* sp. SGD-03.

1. Introduction

Exopolysaccharides (EPS) are natural polymers and are defensive bio macromolecules produced by micro to macro, aquatic to terrestrial life forms for their survival (Kekez et al., 2015). Microorganisms produce EPS as a defense strategy to protect themselves against biotic and abiotic stresses (Boyle & Reade, 1983; Sanalibaba & Çakmak, 2016) EPSs can appear as covalently bound to the cell surface, called capsular PS or either loosely bound or secreted, called as “slime layer“, or “glycocalyx” (De Vuyst et al., 2001). Polysaccharides from microorganisms have gained attention considering their wide range of essential pharmacological and industrial properties, such as antiviral (Arena et al., 2006), anti-oxidative (Raza et al., 2011), immunomodulatory, antitumor (Ramamoorthy et al., 2018; Staniszewska et al., 2017), cholesterol-lowering (Korcz et al., 2018), wound healing (Sun et al., 2020), gelling and emulsifying activity (Iyer et al., 2006). In view of the increased demand for natural biopolymers for various industrial applications (Suresh Kumar et al., 2007) industries are looking for bacterial polysaccharides with unique characteristics-several bacterial species are known to be EPS producers, among them, lactic acid bacteria (LAB) are dominant ones. Certain *Bacillus* species have demonstrated higher EPS production yields when compared to the LAB (Lee et al., 1997). EPS-producing bacteria can be isolated from various sources such as soil, marine water, freshwater hydrothermal vent, shallow marine vent, and human gut. The marine ecosystem witnessed a diversified source of microbes, which could offer novel EPS-producing bacteria. The EPS are widely explored in the marine ecosystem, especially from hydrothermal vents. The microbial diversity of marine ecosystems is still largely unexplored,

allowing the discovery of novel bio macromolecules. Some islands are not yet explored or explored poorly; therefore, exploring such areas would be a promising source to discover novel molecules. EPSs are polyhydroxy alcohols which can retain very high amounts of water. It can retain 99 % water content. The hydrated EPS can be visualized by light or scanning electron microscopy with appropriate staining. Polysaccharides are charged molecules targeted to make ionic interactions with certain dyes to screen them from the microbial population. Anionic monosaccharides of EPS may be probable locations for cationic binding dyes, such as Ruthenium Red (Figueroa & Silverstein, 1989) and Alcian blue (Karlyshev & Wren., 2001). India ink can also stain the EPSs (Sutherland., 2001) and vice versa for cationic polysaccharides. The Ruthenium Red adsorption can also be measured to quantify the amount of EPS (Dabour et al., 2005). EPS is a coat of glycocalyx around the microbial cells. To screen the EPS-producing bacteria, this outer coat can be targeted. Surface-bound polysaccharides can be tested by Maneval staining followed by visualization under a light microscope. This property efficiently distinguishes between released and surface active polysaccharides (Corstvet et al., 1982). The EPS accumulation occurs in the presence of a high concentration of carbon source and limiting conditions of minerals (phosphorus, sulfur, nitrogen and magnesium), which are essential for the growth of an organism. EPS screening can be performed by liquid or solid culture media. The slime (mucoid) colonies of bacteria were observed on the solid nutrient plate. The liquid nutrient media supplemented with high sugar (5%) concentration has been studied to screen the EPS-producing isolate of *Lactobacillus* strain from milk (van Geel-Schutten et al., 1998). The dense nature of culture media is a characteristic of the presence of EPS. During fermentations, viscosity of liquid media increases due to the presence of EPS. Thus, viscometric analysis of culture media can be applied for EPS screening (Van den Berg et al., 1993, Sanni et al., 2002;). The rapid and easy screening method was developed, taking advantage of the viscosity of a sample. Microhaematocrit capillary-based measurement of efflux time of viscous solution can be measured. The sample's viscosity can directly be propositional to the molecule present in the sample; therefore, efflux time varies depending on the concentration of EPS. These are great alternatives for screening of EPS-producing bacteria and quantifying produced EPS. In brief, capillaries are immersed into fermented media and inverted afterwards after fermentation. Then they are allowed to efflux viscous media by gravity. At the end of

incubation, efflux time will be calculated, and the effluxed sample will be touched with a sterile inoculating loop to test the ropiness of colonies (Ricciardi et al., 1997).

Morphological-based screening of EPS-producing bacteria has also been studied. Some analytical techniques can be utilized to probe the location and distribution of EPS in media. Such as confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), ultrafiltration, gel permeation chromatography (GPC) and scanning electron microscopy (SEM) (Smitinont et al., 1999; Welman et al., 2003). The lectin conjugating dyes, such as Alexa Fluor 488 imaging, can also locate the EPS in media (Hassan et al., 2002; Goh et al., 2005). Lectin has a high affinity to galactopyranosyl residues in many *Lactobacilli* (Gruter et al., 1993). The CLSM, in conjunction with lectin SBA Alexa Fluor 488 conjugate can be an effective imaging technique to detect the location and distribution of EPS in a media. Atomic force microscopy by AFM (AFM-SMFS) can be a valuable tool to visualize the conformational analysis of polysaccharides on the live bacteria cell surface (Francius et al., 2008). The web-like structures or array of polysaccharides fiber can be viewed in SEM analysis (Whitfield & Roberts, 1999).

Molecular-level screening can be performed by targeting various genes involved in EPS production. Gene-specific primer pairs have been probed. Low et al., (1998) showed that, the molecular-based screening of EPS is in-efficient because even in the presence of a gene, the functional expression was not observed or sometimes a false positive result obtained which may be the case of non-specific amplification of the gene due to the low primer concentration in the reaction (Low et al., 1998). However, morphological analysis is unable to distinguish the homo-polysaccharides (HoPS) or hetero-polysaccharides (HePS); the PCR-based technique is certain to detect genes specific to HoPS (gtf and lev) or HePS (epsA, epsB, epsD/E, epsGTF) (Palomba et al., 2012). Despite the presence of EPS genes, some strains do not produce EPS. The gene expression was not found. Therefore molecular-level screening should always be accompanied by another screening method.

2. Materials and Methods

2.1 Reagents and kits

The majority of the chemical were procured from Hi-Media, Mumbai, Sigma-Aldric, New England Bio Labs

Sucrose

Yeast extract

Peptone

Agar Agar

Ethanol

Nuclease Free Water

HiPurA™ bacterial genomic DNA isolation kit

Exo-SAP (New England Bio Labs)

GelRed™ dye (Sigma-Aldrich)

Agarose

2.2 Screening and identification of EPS-producing bacteria

2.2.1 Sample collection and isolation of marine bacteria

Marine sediment samples were previously collected from the intertidal region of mangroves, Chorao Island, Goa which coordinates the 15°25'N and 15°30'N between 73°45'E and 73°59'E (Fig. 1) and processed by Syed's group (https://sites.google.com/site/drsyedgdastager/publications). Samples were successively diluted to meet low density, distinct and recognizable colonies by traditional serial dilution method.

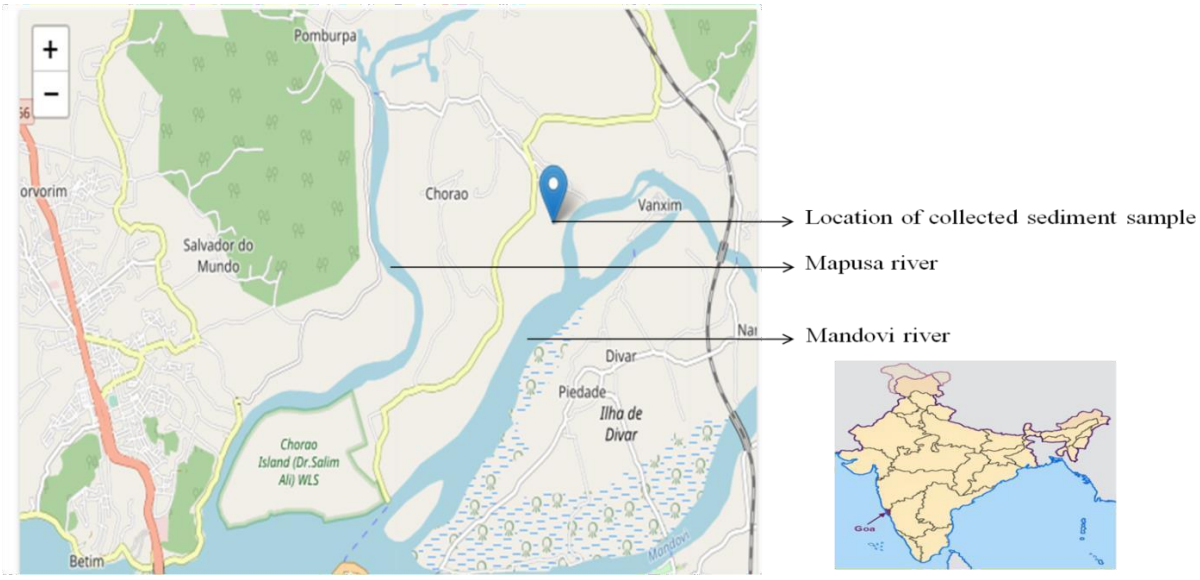

Fig.1 Map of the sampling site and Chorao Island, Goa, India.

2.2.2 Screening of EPS-producing bacteria

The smooth, mucoid colonies selection is the primary screening for isolating EPS-producing bacteria on solid culture media. Further, the production of EPS was confirmed by liquid submerged culture. The previously isolated bacterial strains were taken to screen EPS production (Table. 1). EPS production was confirmed by mucoid colony formation followed by precipitation of EPS using alcohol. A string test was also used for further confirmation of EPS. The screening was performed based on solid media. A sterile filter disc was kept on solid nutrient agar supplemented with different carbon sources with varying pH and incubated at different temperatures (Guimarães et al., 1999). Fresh cultures were grown on solid nutrient agar medium (Peptone, 1.0 %; Beef extract, 1.0 %; NaCl, 0.5 % and 1.5 % of agar) supplemented with different carbon sources (sucrose, maltose and lactose, 2.0 %) (Arrieta et al., 1996) and incubated separately at 28°, 30° and 37°C. After incubation, the mucoid colony formation was examined by observing the presence of slimy mucoid natures on or around the filter disc until 48 h with every 12 h of intervals. Further, loopful colonies were immersed in alcohol to observe the precipitation of EPS. When a loopful of culture is mixed with pre-chilled absolute ethanol (4°C), colonies precipitate out from the solution, confirming the EPS producer and solution turbid indicates negative for EPS (Paulo et al., 2012). Torching bacterial colonies with sterile inoculating loop or toothpick may form a sticky thread of

EPS called as string test. A string test was performed for further confirmation. Lifting a colony with a sterile inoculating loop to create a thin string of colonies >5 mm string formation can be considered positive for EPS producer (Hector et al., 2015).

Table. 1 List of marine isolates

Sr. No.	Isolates	Sr. No.	Isolates	Sr. No.	Isolates
1	AC-3 (*Lysinibacillus macrolides*)	16	V-88 (*Bacillus stratosphericus*)	31	NIO-1030 (*Bacillus stratosphericus*)
2	AC-7 (*Lysinibacillus pakistanensis*)	17	V-93 (*Bacillus aerophilus*)	32	NIO-1051 (*Pseudomonas stutzeri*)
3	AC-8 (*Lysinibacillus cresolivorans*)	18	D-9 (*Bacillus flexus*)	33	NIO-1057 (*Glutamicibacter mysorens*)
4	AC-9 (*Bacillus nealsonii*)	19	D-10 (*Bacillus cerues*)	34	NIO-1109 (*Exiguobacterium indicum*)
5	AC-11 (*Bacillus aryabhattai*)	20	D-12 (*Bacillus jeotgali*)	35	NIO-1123(*Bacillus aquimaris*)
6	AC-13 (*Bacillus velezensis*)	21	D-25 (*Bacillus cereus*)	36	NIO-1130 (*Bacillus aquimaris*)
7	AC-15 (*Lysinibacillus xylanilyticus*)	22	R-2 (*Bacillus circulans*)	37	19-a (*Paeni bacillus pabuli*)
8	V-17 (*Bacillus firmus*)	23	NIO-1002 (*Microbacterium zeae*)	38	M-37 (*Bacillus flexus*)
9	V-19 (*Bacillus badius*)	24	NIO-1008 (*Arthrobacter equi*)	39	SGD-03 (*Bacillus licheniformis*)

10	V-25 (*Bacillus badius*)	25	NIO-1009 (*Rhodococcus kroppenstedtii*)	40	SGD-05 (*Bacillus mojavensis*)
11	V-68 (*Bacillus flexus*)	26	NIO-1011(*Dietzia maris*)	41	SGD-7 (*Bacillus altitudinus*)
12	V-74 (*Cohnella ferri*)	27	NIO-1013 (*Bacillus aerius*)	42	SGD-11 (*Bacillus cereus*)
13	V-76 (*Bacillus flexus*)	28	NIO-1016 (*Domibacillus roboginosus*)	43	SGD-14 (*Bacillus endophyticus*)
14	V-82 (*Kytococcus sedentarius*)	29	NIO-1021 (*Kocuria marina*)		
15	V-85 (*Gordonia terrae*)	30	NIO-1023 (*Deinococcus ficus*)		

2.2.3. Selection of potent EPS producer

After the plate-based screening, a total carbohydrate quantification was performed to choose the potent EPS-producing bacteria. Fresh overnight grown cultures were inoculated into a 500 mL Erlenmeyer's flask containing 100 mL of sterile nutrient broth supplemented with 1.0 % sucrose and incubated at 28°C for 24h at 150 rpm in a rotatory shaking incubator. The fermentation reaction was stopped, and cultures were harvested by centrifugation at 10000 rpm for 20 min at 4°C. The supernatant was separated and precipitated by alcohol and kept overnight at 4°C. Further, the precipitated EPS was dissolved in deionized water and subjected to quantification using the Anthrone reaction described by Trevelyan & Harrison (Trevelyan & Harrison, 1952). In brief, 0.2 % of Anthrone was prepared in H_2SO_4 and cooled at RT with intermittent shaking to make a clear suspension. A serially diluted glucose sample was chosen as standard to generate a standard curve. The 5 mL of Anthrone solution was added to 1 mL of standard solutions and test samples in the test tube. The suspension was mixed and cooled on ice. It was boiled in a water bath for 15 minutes to develop the reaction's colour. Subsequently, colourimetric absorbance was taken at 620 nm

using deionized water as a reference (blank). The standard curve of the sample was generated to quantify the concentration of unknown samples.

2.2.4 Culture maintenance and preservation

After the screening, the fresh culture of EPS-positive isolates were grown and prepared in 25% glycerol suspensions or lyophilized using skimmed milk and stored at -80°C for long-term storage. The cultures were re-streaked on a nutrient agar plate or slant at 4°C, used during the experimentation.

2.2.5 Identification of EPS-positive bacteria

A multi-phase approach was used for the identification of an organism. Scanning electron microscopy (SEM) was performed to characterize the surface morphology of an organism, Biochemical characterization was carried out using VITEK-2 compact system (bioMerieux, India, Ltd.), and molecular identification was performed using 16S rRNA gene sequencing and whole genome analysis by calculating average nucleotide identity (ANI). For SEM analysis, the cell pellet was separated from the supernatant by centrifuging overnight grown culture at 10000 RPM for 2 min. The cells were washed thrice with 0.1 M phosphate buffer (pH 7.2). Subsequently, cells were suspended in 2.5% glutaraldehyde solution for 1 h at 4°C. After fixation, cells were washed thrice with the same buffer and dehydrated with graded ethanol (30%, 50%, 70% and 100%) with incubating for each dehydration step for 3 min. Then cells were spread on gelatin (0.1%) coated glass coverslip and incubated overnight at 50 °C for drying. Further cover slip was mounted on the sample holding stub, subjected to gold coating prior to analysis and observed under an FEI Quanta 200 3D dual beam scanning electron microscope with an accelerating voltage of 20 kV. Biochemical-based identification was carried out using VITEK-2 compact, an automated microbial identification system (Pincus, 2006). Inoculum suspension of 0.5 McFarland turbidity was prepared in saline solution. The VITEK BCL card were used for identification according to the manufacturer's standard protocol (bioMerieux). Molecular identification was carried out using 16S rRNA gene sequencing and whole genome sequencing. Genomic DNA was isolated using the HiPurA™ Bacterial Genomic DNA purification kit and amplified by polymerase chain reaction (PCR) using bacterial 16S rRNA universal primer 8F and 1492R (Galkiewicz & Kellogg, 2008).

PCR thermal cycling was carried out as: genomic DNA was denatured initially at 94°C for 7 min and 30s for subsequent denaturation cycles. Primer annealing and extension of new DNA strands were carried out at 59°C and 72°C for 30s and 90s, respectively. After 30 cycles, the final extension was set at 72°C for 7 min. The Exonuclease I-Shrimp Alkaline Phosphatase method (Exo-SAP) was used to clean up amplified products. It was further subjected to cycle sequencing using universal primers: 8F, 530F, 800R and 1492R in separate wells to achieve a full-length sequence. BigDye terminator V3.1 was used as a chain terminator. HiDi formamide was added after precipitation (purification) in each well of the sequencing plate. The plate was fixed in Genetic Analyzer 35xL Applied Biosystems (HITACHI High Technologies Carporation, Tokyo, Japan) to run the machine. Sequencing reads were assembled using ChromasPro software. The assembly (query) sequence was subjected to the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud Database for homology searching. Molecular phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Evolutionary analysis was performed using MEGA software version 6.0 (Tamura et al., 2013). Genome sequence relatedness to its type strains was performed. An average nucleotide identity (ANI) value was calculated using a web-based server (http://enve-omics.ce.gatech.edu/). The whole-genome sequencing and type strain sequences data were retrieved from Oxford Nanopore Technology-MinION and NCBI databases.

3. Results and Discussion

A total of 43 marine strains were screened for the EPS production. Out of 43, four strains were found positive for EPS production (Fig. 2). Microscopic observation has shown that cells were rod-shaped and Gram-positive when observed under the light microscopy (Fig. 3). To select the best strain among four, a total carbohydrate quantification was performed using Anthrone method. Out of four, strain SGD-03 was found to be the best producer of EPS, i.e, 894 mg/L (Fig. 4). Therefore it was further subjected for detailed study. It was found to produce slimy mucous colonies on the solid nutrient medium supplemented with 2% of sucrose (Fig. 5a). Colonies formed a thread-like appearance of EPS upon the addition of pre-chilled ethanol, which reconfirms the positive for EPS production (Fig. 5b). Upon lifting the colonies with a sterile loop, an approximately 7–8 mm string was observed, which further confirmed EPS (Fig. 5c)

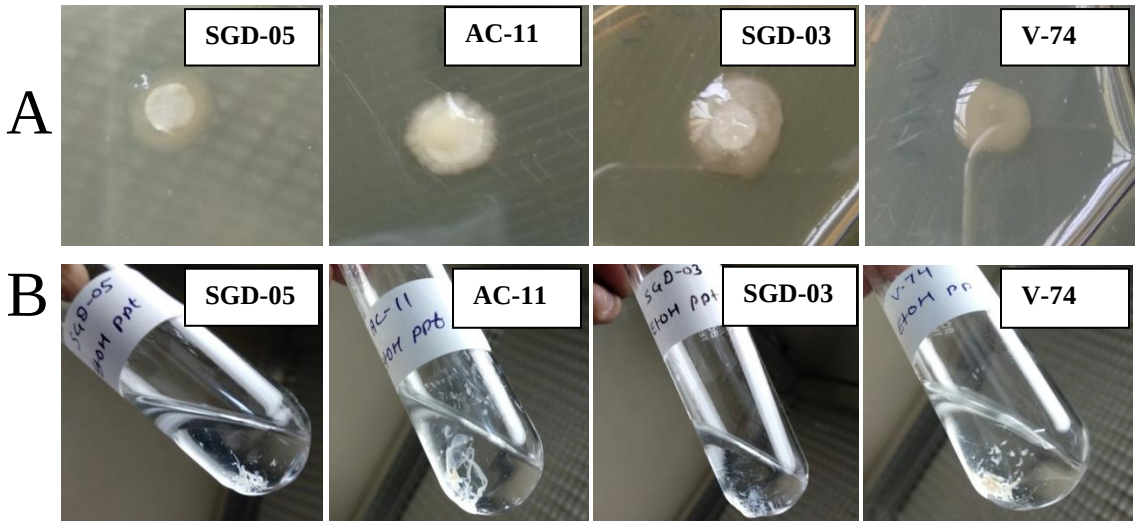

Fig. 2 Screening of exopolysaccharide-producing bacteria. A) Mucoid colony formation; B) Ethanol precipitation.

The surface morphology of EPS-positive strain was observed and confirmed using SEM analysis. The cells have rod-shaped morphology with 1.12 µM in diameter, indicating the characteristics of similarity of the genus *Bacillus* (Fig. 6) and biochemical characterization was performed by VITEK-2 compact system. The biochemical results suggested that the isolate showed a 98.0% probability with *Bacillus licheniformis* (Table. 2). Based on phylogenetic analysis using 16S rRNA gene sequence, strain SGD-03 has shared the highest sequence identity of 99.79% with *Bacillus licheniformis* ATCC 14580 (Fig. 7).

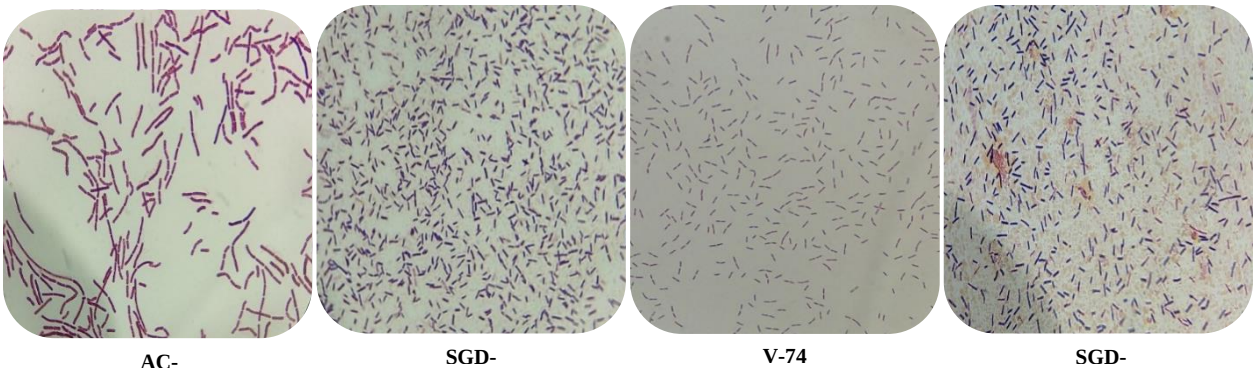

Fig. 3 Gram's Staining of EPS positive isolates

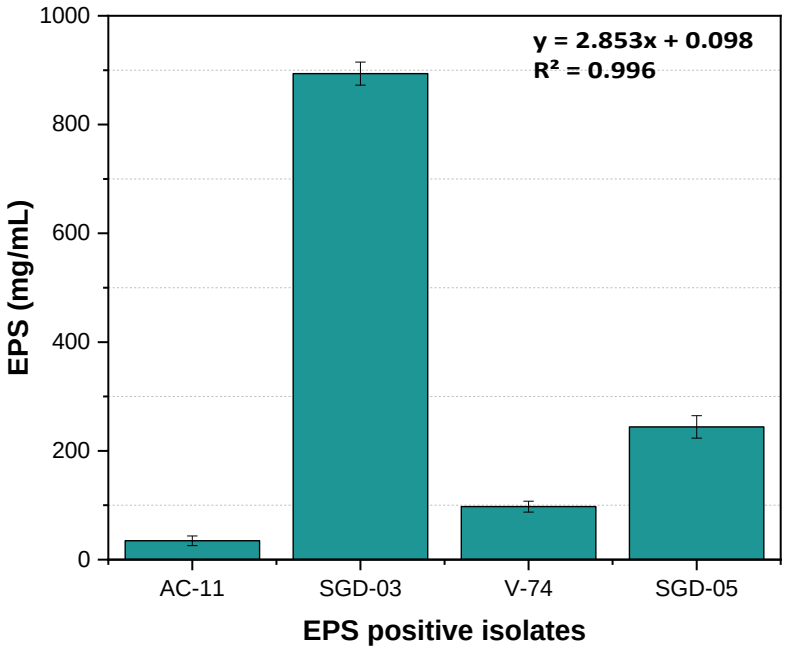

Fig. 4 EPS quantification produced from positive isolates

Genome-based ANI plot has demonstrated the similarity of SGD-03 with *Bacillus licheniformis* ATCC 14580 (Fig. 8a) and ANI matrix values of 99.0% have demonstrated the more than 95% ANI similarity with the type strains *Bacillus licheniformis* ATCC 14580 (Fig. 8b), which confirms the identification of the SGD-03 belongs to *Bacillus* genera.

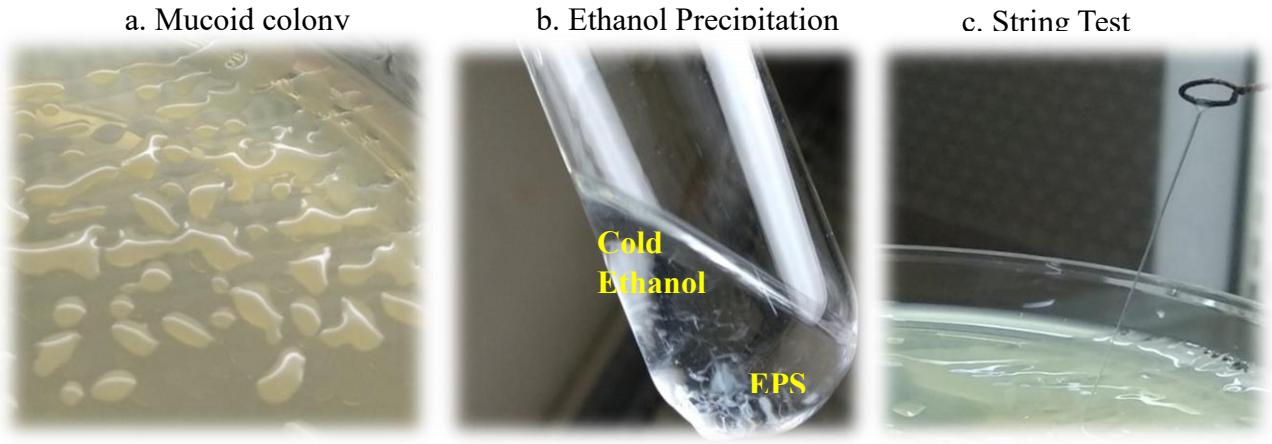

Fig. 5 Screening of EPS producing strain a) Mucoid colony; b) Ethanol precipitation; c) String test

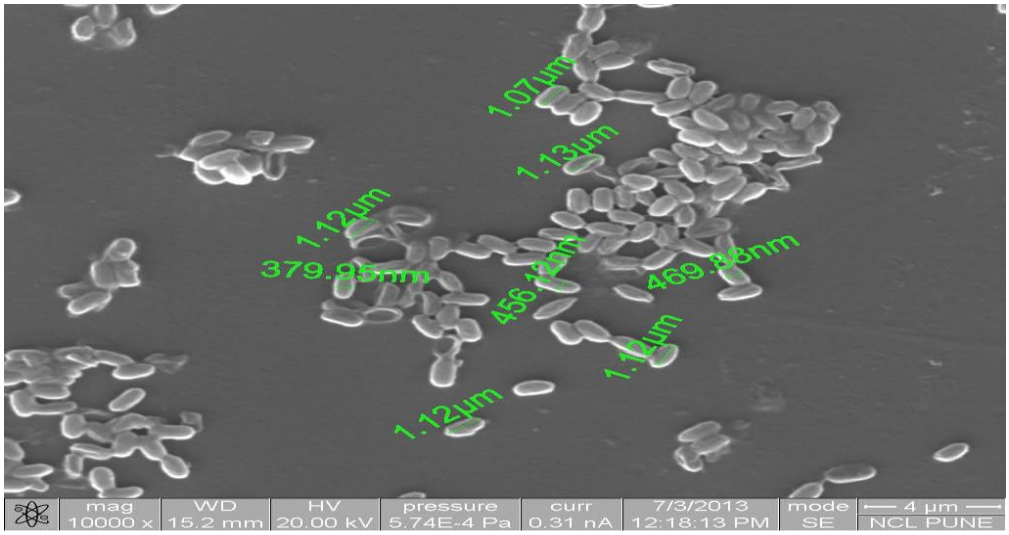

Fig. 6 Identification of *Bacillus* sp. SGD-03: a. Scanning electron microscopy; b. Phylogenetic analysis

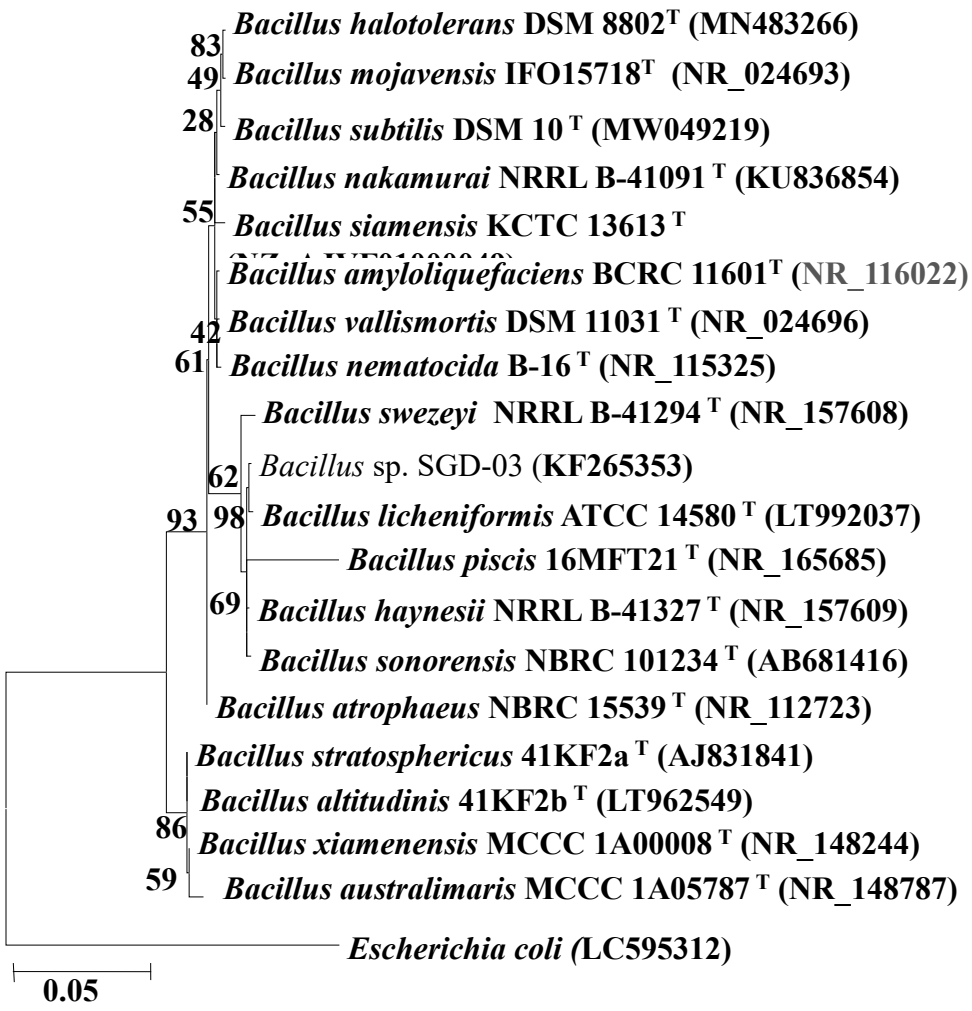

Fig. 7 Phylogenetic analysis of *Bacillus* sp. SGD-03

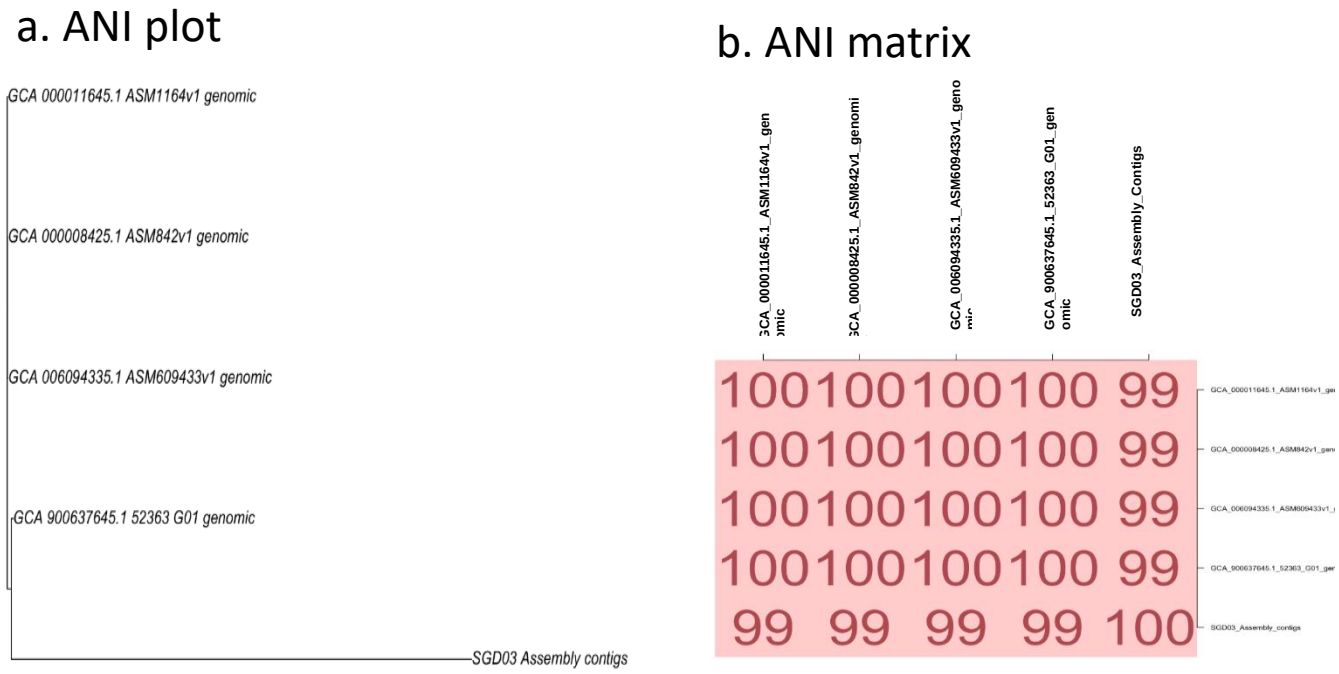

Fig. 8 a. ANI plot; b. ANI matrix

Table. 2 Biochemical/phenotypic identification of *Bacillus* sp. SGD-03

Identification Information	**Card:** BCL	**Lot Number:** 2390966403	**Expires:** Jul 19, 2022 12:00 IST
	Completed: Jan 6, 2022 06:50 IST	**Status:** Final	**Analysis Time:** 14.25 hours
Selected Organism	98% Probability **Bionumber:** 0332271715472671	**Bacillus licheniformis**	**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s) Bacillus licheniformis NAG(72),			

Biochemical Details																
1	BXYL	-	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	-	12	AlaA	-	13	TyrA	+	14	BNAG	-
15	APPA	-	18	CDEX	+	19	dGAL	-	21	GLYG	+	22	INO	+	24	MdG	+
25	ELLM	+	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	(-)	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	+	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	-	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

4. Conclusion

Headings to find EPS-producing bacteria exploring marine ecosystems, a total of 43 marine strains were subjected to screening of EPS-producing bacteria. Based on the initial screening, 4 out of 43 isolates could obtain positive for EPS production, namely AC-11, SGD-03, V-74, and SGD-05. Based on a preliminary analysis of EPS quantification, SGD-03 was found eminent for EPS production; therefore, it is taken for further detailed studies. SGD-03 can produce 894 mg/L of EPS. Based on the morphological, biochemical, and molecular (16S rRNA gene sequencing) identification, the strain belongs to rod-shaped Gram's positive bacteria, which shared the highest sequence identity of 99.79% with the type strains *Bacillus licheniformis* ATCC 14580, which confirms that SGD-03 belongs to *Bacillus* genera and named as *Bacillus* sp. SGD-03.

5. References

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Chapter 3:

Production, Purification and Structural elucidation of Exopolysaccharide

Abstract

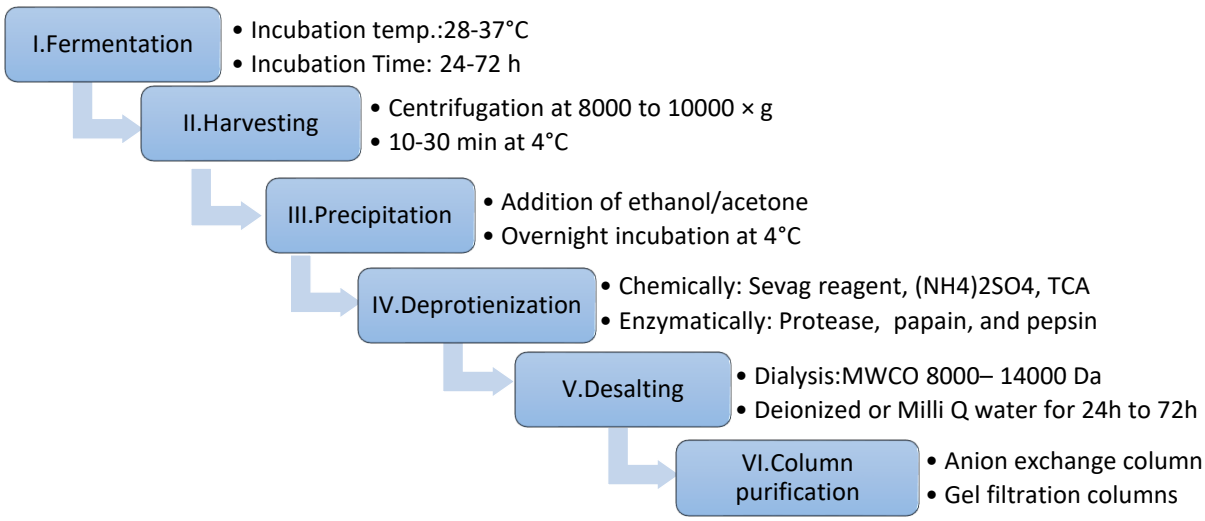
The EPS from *Bacillus* sp. SGD-03 was produced and purified sequentially. Produced EPS was characterized using various analytical techniques. Based on the morphological and thermogravimetric analysis, we found that EPS has a fibrillar network structure with 3.620 μm to 9.071 μm of diameter with a relatively smooth surface and thermostable up to 185°C. Analytic techniques such as SEC, TLC, HPLC, LC-HRMS, FTIR, and NMR analysis have revealed that, the produced EPS has a molecular weight of 1.0×10^4 Dalton and is composed of fructose monosaccharide units with hydroxyl, carbonyl and ether groups. NMR studies have confirmed that, the EPS has a linkage pattern of β-(2, 6) bonding, verifying its identity as a levan polysaccharide.

1. Introduction

Microbial exopolysaccharides (EPS) have gained much attention over decades due to their various physicochemical properties and making them as useful for various fields. EPS can be obtained by plants, fungi algae and bacteria, among them bacteria are the preferred for polysaccharides production. Due to continuously growing populations, industries are demanding expeditious and easily accessible sources of EPS producers. The production of bacterial EPS is very simple than algae and fungi. The bacterial polysaccharides are easily amenable and suitable for industrial applications according to the need. Bacterial polysaccharides are natural biopolymers which make them as safe and eco-friendly. Various bacterial genera have been known to produce EPS, including *Bacillus, Lactobacillus, Arthrobacter, Pseudomonas, Corynebacteria, Halomonas, Myroides, Acinetobacter,* etc. *Bacillus* species are widely explored because of its short generation time. Based on the environmental conditions, bacteria produces different types of EPSs irrespective of supplemented carbon source. The choice of carbon source can vary from bacteria to bacteria, whereas some bacteria utilize many carbon sources, for example, *Salmonella bongori* (Hector et al., 2015). A bacterium can produce two simultaneously, and two different polysaccharides, like *Bacillus subtilis* have been shown to produce levan and poly gamma glutamic acid in presence of sucrose and L-glutamic acid (Shih & Yu, 2005). It also produces 2 different molecular weights (85.23 kDa and 31.95 kDa) levans.

1.1 Production and purification of EPS

Different fermentations can be performed based on the microbial source and culture condition. A basal production level has been reported in bacteria; they can produce EPS throughout their growth cycle. In general, EPS production media is supplemented with a carbon source. After fermentation, several methods of extraction and purification of EPS have been reported. Following are the standard steps of EPS purifications:



1.2 Detection and quantification of EPS

Many colorimetric methods were used for carbohydrate detection and quantification, including Phenol-sulphuric acid, Anthrone-sulphuric acid, and UV-sulphuric acid. For the first time, Dreywood (1946) described the presence of carbohydrates based on the reaction color. Green color will develop when the carbohydrate is heated in the presence of Anthrone and acidic conditions. It was the first qualitative test for carbohydrate determination (Dreywood., 1946). Other color-developing reagents such as anthrone, phenol (Laurentin & Edwards., 2003), orcinol (Irwin & Leaver., 1956), or resorcinol (Monsigny et al., 1988) can also be used for carbohydrate detection. The phenol–sulfuric acid method is the most reliable, easiest, sensitive and simple one. This has been applied to quantify monosaccharides, oligosaccharides, glycoproteins and glycolipids.

Phenol–sulfuric acid is an efficient method in detecting as low as 10–80 nanomole of carbohydrate using 96 well plate using glucose as a standard.

1.3 Characterization of purified EPS

Biomolecules such as lipids, nucleic acids or proteins are well studied based on the molecular level by gene targeting, but the characterization of polysaccharides cannot be performed at the molecular level. Several analytical techniques used characterize EPSs. Based on structural arrangement PSs have gained different properties which can be used for various industries. The fingerprints of carbohydrates can be detected by GS-MS-based TMSA derivatives (Xia et al., 2018). The NMR (Qu et al., 2019) and High-resolution X-ray crystallography are widely used to determine the 3D structure of biomolecules (Dahech et al., 2013). Other new methods have also been reported for the determination of polysaccharides. The small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS) analysis will help to understand the confirmation of EPS in solution (Khan et al., 2017). Molecular weight can be determined by SEC HPLC using molecular markers of pullulans. MALDI TOF-TOF MS and gel permeation chromatography (GPC) can be used to determine the molecular weight of EPS. The monosaccharides can be detected by GC–MS analysis, TLC and HPLC (Singh et al., 2011). The X-ray diffraction (XRD) and differential scanning calorimetric (DSC) XRD observations reflect the amorphous nature of EPS. Thermal gravimetric analysis (TGA) studies displayed thermal stability over 30-350°C (Insulkar et al., 2018).

2. Material and Methodology

The majority of the chemical were procured from Hi-Media, Mumbai, and Sigma-Aldrich.

2.1 Reagents and chemicals

Peptone

Beef extract

Sodium chloride

Sucrose

Fructose

Ethanol (Merck)

Methanol (Qualigens)

Chloroform

n-Butanol

Acetonitrile

p-anisaldehyde

Trifluoroacetic acid

Sulphuric acid

Potassium bromide

Pullulan (Sigma-Aldrich)

Dextran

TLC plate (Silica 60, Merck)

2.2 Production and extraction of bacterial exopolysaccharide (EPS)

Based on organism's growth requirements, various media are used for the EPS production with the addition of a carbon source. Most often, sucrose is supplemented with the media. The production was carried out in an Erlenmeyer flask (500 mL) containing 200 mL of working volume. A fresh bacterial culture was inoculated in a flask containing nutrient broth comprising peptone, 1%; beef extract, 1%; Sodium chloride, 0.5%; pH 7.0 with supplementation of sucrose (2%). The culture was incubated at 28 °C at 150 rpm for 24 h. After incubation, bacterial cells were harvested by centrifuging the culture medium at 10000 rpm for 30 min at 4°C to obtain cell-free supernatant. The three-volume of pre-chilled ethanol (4°C) was added to the supernatant and left overnight at 4°C for precipitation. Further, the precipitate was recovered by centrifuging at 10000 rpm for 10 min at 4°C and washed with water and freeze-dried using 70 % ethanol.

2.3 Purification of bacterial exopolysaccharide (EPS)

The extracellular polymers, especially proteins, are the bottleneck for EPS purification. Chemical or enzymatic methods can remove the proteins. EPS was deproteinized using the Sevag reagent (chloroform: n-Butanol, 5:1). The volume ratio of a sample (5.0 mg/mL) to Sevag reagent 2:1 was used (Li et al., 2012). In brief, Sevag reagent was added to the sample and mixed for 20 min using rotospin with 50 rpm and centrifuged for phase separation. The upper aqueous phase was carefully

transferred to a fresh falcon tube and repeated for 3-4 times until a clear organic phase was obtained, which is free of the intermediate protein layer.

Further, deproteinized EPS was dialyzed against Milli Q water for 72 h and freeze-dried. Finally, size exclusion chromatography was performed using the SEC column and HPLC detection. A macroporous silica-based stationary phase was used in the SEC separation method, which works on the hydrodynamic volume of the analyst (Churms, 1996). UHPLC (Ultimate 3000, ThermoFisher) was equipped with a size exclusion column (Diol-200, YMC) to collect fractions of purified EPS. The concentration of 5.0 mg/mL; injection volume, 50.0 µL; flow rate, 1.0 mL/min; Detector, RI were applied. Purified EPS was lyophilized using a Labonco lyophilizer and stored for further chemical analysis.

2.4 Characterization of bacterial exopolysaccharide

The size, shape, and structure of biomolecules such as proteins are well studied due to genetically encoded by specific genes, whereas the characteristic size, shape, and structure of many polysaccharides remain unclear. The characterization of polysaccharides involves several analytical techniques for the complete structuriation of unknown molecules.

2.4.1 Monosaccharide composition Analysis

Monosaccharide analysis of EPS was performed by thin-layer chromatography (TLC), Ultra high-performance liquid chromatography (UHPLC), and liquid chromatography high-resolution mass spectrometry (LC HR-MS). An EPS of 1.0 mg/mL was hydrolyzed with intermediate shaking using 2 N trifluoroacetic acid (TFA) at 100°C for 1 h. Hydrolysates were neutralized by evaporating TFA using vacuum drying. Traces of TFA were removed by adding methanol, followed by water until it neutralized. The hydrolyzed sample was centrifuged at 13000 rpm to remove unhydrolyzed or charred material. The supernatant was subjected to carbohydrates analysis.

2.4.1.1 Thin layer chromatography

Fructose was used as a standard carbohydrate based on the previous TLC chromatogram performed with all available mono- and disaccharides. A standard carbohydrate (1.0 mg/mL) and hydrolyzed sample, 5.0 µL, was spotted on a TLC plate (Silica 60, Merck), air-dried, and placed in a TLC

chamber saturated with the mobile phase. Solvent system (mobile phase) acetonitrile: water in the ratio of 85:15 (v/v) were used to separate the carbohydrates (Robyt et al., 2000). The plate was removed, air-dried, and subjected to develop a chromatogram in presence of the staining solvent p-anisaldehyde: sulphuric acid: ethanol with a 1:1:20 ratio. Developed chromatogram was visualized by charring the plate in a hot air oven for 10 min at 110°C (Insulkar et al., 2018).

2.4.1.2 High-performance liquid chromatography (HPLC)

The hydrolyzed sample was filtered using a 0.22 µm syringe filter and subjected to Ultra high-performance liquid chromatography (UHPLC, Ultimate 3000, ThermoFisher) equipped with Phenomenex (Rezex RCM Monosaccharide) column set on 80°C. Milli Q water as a carrier with a flow rate of 0.6 mL/min, RI at 35°C conditions was used for detection. For comparison, an injection volume of 20 µL having a 1.0 mg/mL concentration was injected with standard carbohydrates.

2.4.1.3 Liquid chromatography high-resolution mass spectrometry

The LC-HRMS spectra were recorded with an HPLC system (Thermo Scientific Q-Exactive, Accela 1250 pump). The system was equipped with a hypersil GOLD column having a particle size of 3.0 µM. The 1.0 mg/mL sample was prepared in Milli Q blended with methanol and injected in a 1.0 µL volume. Methanol was used as a carrier with a flow rate of 0.5 mL.

2.4.2 Field Emission Scanning Electron Microscopy (FE-SEM) analysis

The surface morphology of purified EPS was observed and analyzed using FE-SEM. Dry polysaccharide samples (Test and standard) were mounted over carbon tape, and fixed on the sample holder stub. Before analysis, samples were subjected to gold coating using a sputter coater. The surface morphology was observed and photographed from 1000X to 4000X magnification with an accelerating voltage of 18 kV using the FEI Nova NanoSEM 4450 electron microscope.

2.4.3 Thermo gravimetric analysis (TGA)

After the morphological characterization, the thermal stability of EPS was determined by thermogravimetric analysis (TGA) using a Perkin Elmer-7 analyzer with the help of alumina pans under a nitrogen atmosphere (20 mL/min). The system was heated from 30 to 500°C with a heating rate of 5°C /min.

2.4.4 Fourier Transform Infrared (FT-IR) spectroscopy

Analysis of functional groups was carried out using FT-IR spectroscopy. KBr was activated in a hot air oven at 80°C for overnight. The sample was mixed in KBr in a ratio of 1:100 (mg) grounded to prepare the test KBr disc (13 mm). The disc was kept in a desiccator to resist moisture entrapment and subjected to record absorbance spectra. Spectra were recorded from the wavenumber 4000cm^{-1} to 400cm^{-1} using FT-IR spectrometer vertex70, Bruker. Background spectra were subtracted by recording spectra of the reference KBr disc.

2.4.5 Molecular weight determination

The exopolysaccharide average molecular weight was determined by the size exclusion chromatographic (SEC) column, combined with a UHPLC system with a RI detector. EPS sample 1.0 mg/mL was prepared in the mobile phase, and Milli Q water was filtered through a 0.45 µm syringe filter. At ambient temperature, an injection volume of 20 µL was passed through the column (Diol-200 YMC) with a 1.0 mL/min flow rate. Standard Pullulan with a peak molecular weight (Mp) ranging from ~350 to 700,000 Da (Sigma) was used for calibration. The calibration curve was used to determine the average molecular weight of EPS. Sample chromatogram was also compared with standard polysaccharides, Pullulan 10 kDa (P-10) and Dextran 10 kDa (D-10).

2.4.6 NMR spectroscopic analysis

NMR spectroscopy is a valuable qualitative and quantitative tool for biomolecules. The structural analysis of polysaccharides needs a type of content sugar and their absolute configurations. Two-dimensional NMR spectroscopy provided sequential information to predict the structure of unknown polysaccharides (Landersjö et al., 2002). The purified EPS was applied to 1D and 2D NMR analysis to elucidate the exact structure of a polysaccharide. A 20.0 mg powder of EPS was dissolved in 0.6 mL of D_2O and kept overnight at 4°C for proper disintegration. Spectra of 1D and 2D, including ^{1}H (500.13 MHz), ^{13}C (125.76 MHz), Distortion less Enhancement by Polarization Transfer (DEPT), Nuclear Overhauser Effect Spectroscopy (NOESY), Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence spectroscopy (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) were recorded with Bruker Avance spectrometer 500. As an internal standard, all chemical shifts (δ) are reported in parts per million downfield from D_2O (4.71

ppm). Spin multiplets are reported as d (doublet) and t (triplet). Coupling constant (J) is reported in hertz (Hz).

3. Results and Discussion

3.1 Monosaccharide composition analysis

Monomeric units of polysaccharides were analyzed using thin-layer chromatography, high-performance liquid chromatography and liquid chromatography high-resolution mass spectroscopy. TLC, HPLC, and LC HR-MS analysis have revealed that polysaccharide has solely made up of a single type of monosaccharide, D- Fructofuranose with a 180Da ($C_6H_{12}O_6$, 203.05 Na). Presence of D- Fructofuranose reveals that polysaccharide belongs to the homopolysaccharide type of polymer, so it can be confirmed that it is a fructan class of polysaccharide (Fig. 1a, 1b, and 1c).

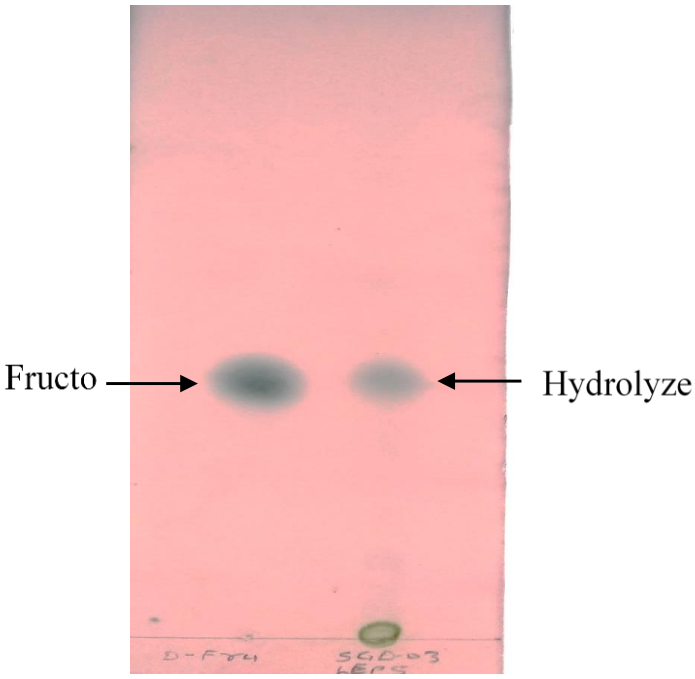


Fig. 1a. TLC chromatogram of hydrolyzed EPS produced from *Bacillus* sp. SGD-03

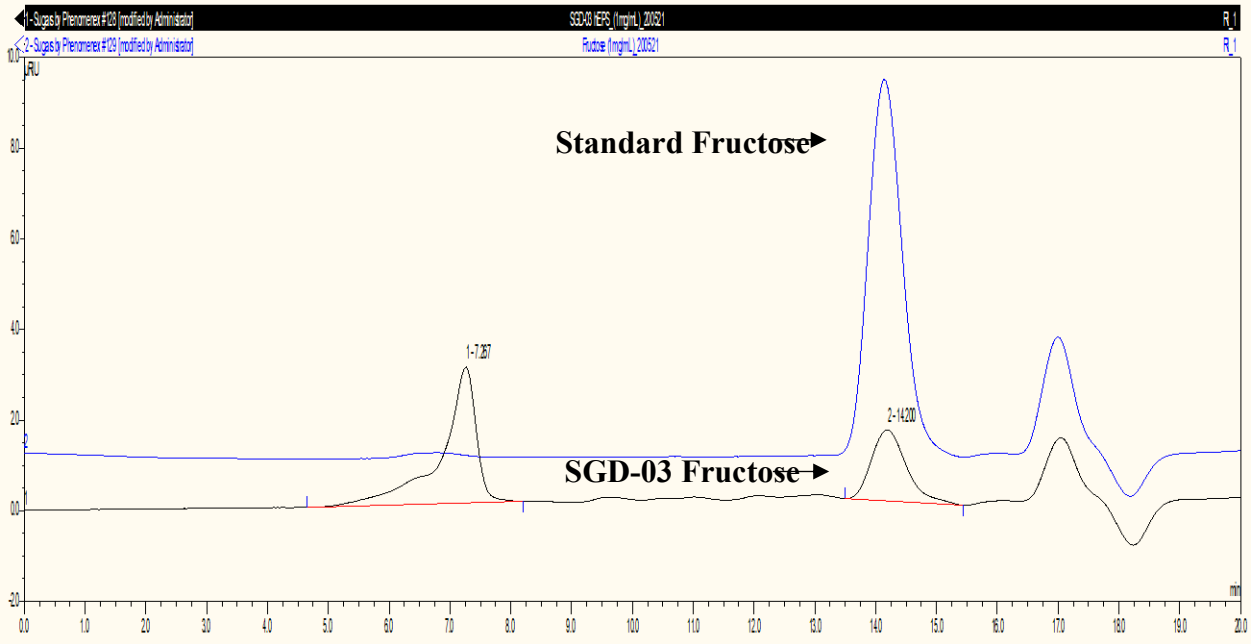


Fig. 1b. HPLC chromatogram of hydrolyzed EPS produced from *Bacillus* sp. SGD-03

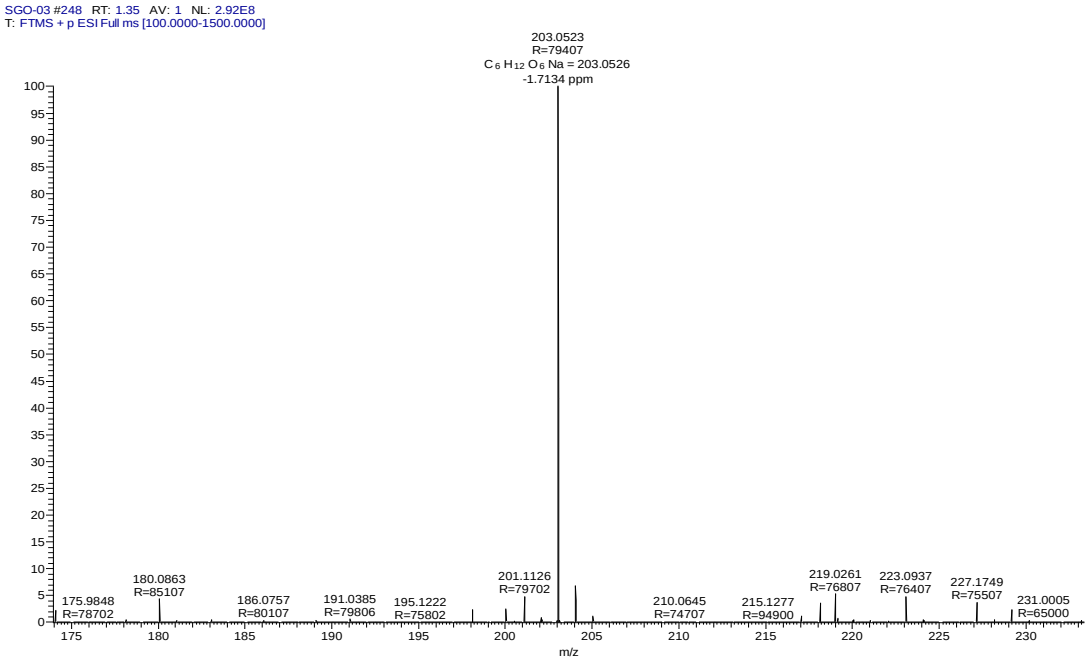


Fig. 1c. LC-HRMS chromatogram of hydrolyzed EPS produced from *Bacillus* sp. SGD-03

3.2 Field Emission Scanning Electron Microscopy (FE-SEM)

The scanning electron micrographs showed a significantly fibrous network structure with a relatively smooth surface of polysaccharides. The surface morphology of test EPS was compared with standard EPS (Levan) of *Erwinia herbicola*. A smooth surface was observed in the standard EPS (Fig. 2A) and test EPS (Fig. 2B) with various ranges of fiber diameters. The fiber diameters were in the range of 3.620 µm to 9.071 µm, forming different pore sizes of the structure. Test EPS showing comparable morphology and fibrillar diameter with the standard. Variations in diameter and surface structure could be due to various branching patterns and different degrees of polymerization of EPS from organism to organism (Fig. 2A and 2B).

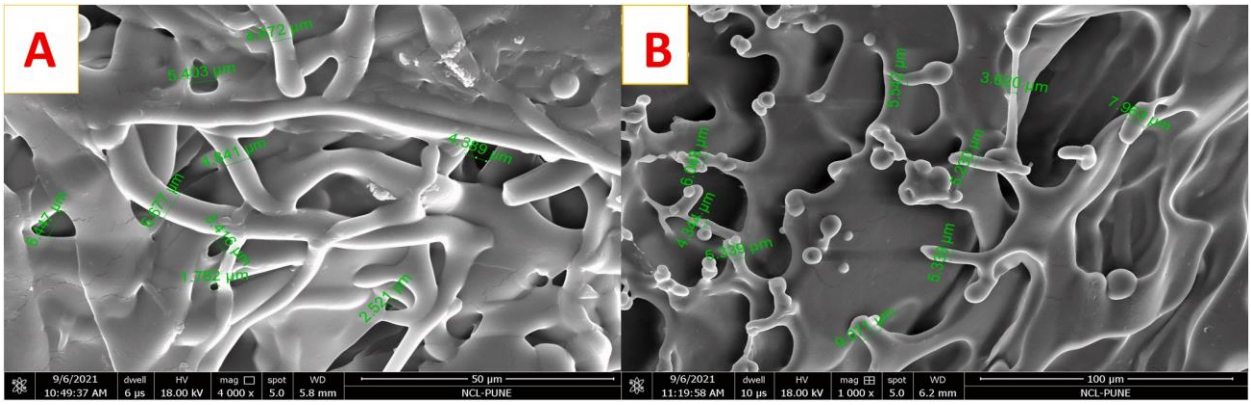


Fig. 2 Scanning Electron micrograph A. Standard EPS from *Erwinia herbicola*, B. Test EPS produced from *Bacillus* sp. SGD-03.

3.3 Thermo gravimetric analysis (TGA)

After morphological characterization, thermal stability was confirmed through thermo gravimetric analysis, showing that the purified EPS is thermostable up to 185°C with 10 % weight reduction at 188°C. The thermostability of the EPS makes it efficient for working in high temperatures (Fig. 3)

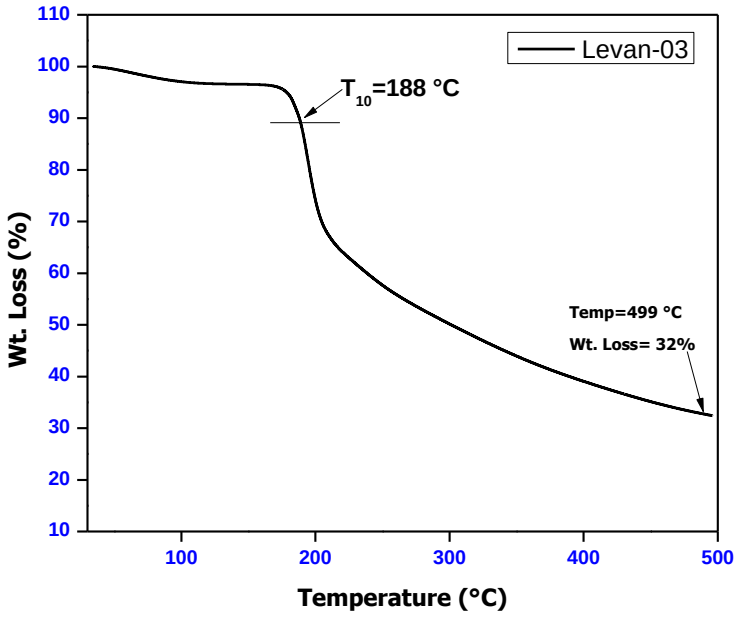


Fig. 3 TGA Analysis of EPS produced from *Bacillus* sp. SGD-03 EPS

3.4 FT-IR spectroscopy

Various functional groups in the produced EPS were analyzed and assigned using the Fourier transform infrared spectroscopy spectra. Characteristic absorption spectra of EPS were shown in the Figure. 4. The broad and strong intensity peak at 3371 cm^{-1} corresponds to Hydroxyl (O–H) stretching, characterizing the polyhydroxy nature of polysaccharides (Zhou et al., 2016). The weak absorption at 2938 cm^{-1} and 2886 cm^{-1} are corresponding peaks to C–H stretching, suggesting the presence of methylene groups vibrating asymmetric and symmetrically (Thakham et al., 2020). Further, an intense absorption at 1652 cm^{-1} is the characteristic carbonyl (C=O) stretching vibration peak. It could be due to the saccharides' Aldo/ketose nature (Vasanthakumari et al., 2015). Medium absorption peaks between 1420 cm^{-1} to 1191 cm^{-1} belong to the deformation vibration of the C–H and O–H groups (Ibrahim et al., 2006). A medium intense peak of C-O-H vibration appears at 1126 cm^{-1} (Thakham et al., 2020). An absorption peak at 1059 cm^{-1} suggests the presence of D-fructose. The strong absorption at 1013 cm^{-1}, C-O-C stretching, is the indicative

marker peak of the polysaccharides. The absorbance peak between 1000 and 800 cm^{-1} belonged to typical carbohydrates. The peaks at 928 and 811 cm^{-1} are corresponding peaks of the furanoid ring structure of the saccharide unit (Ni et al., 2018; Tanaka et al., 1980).

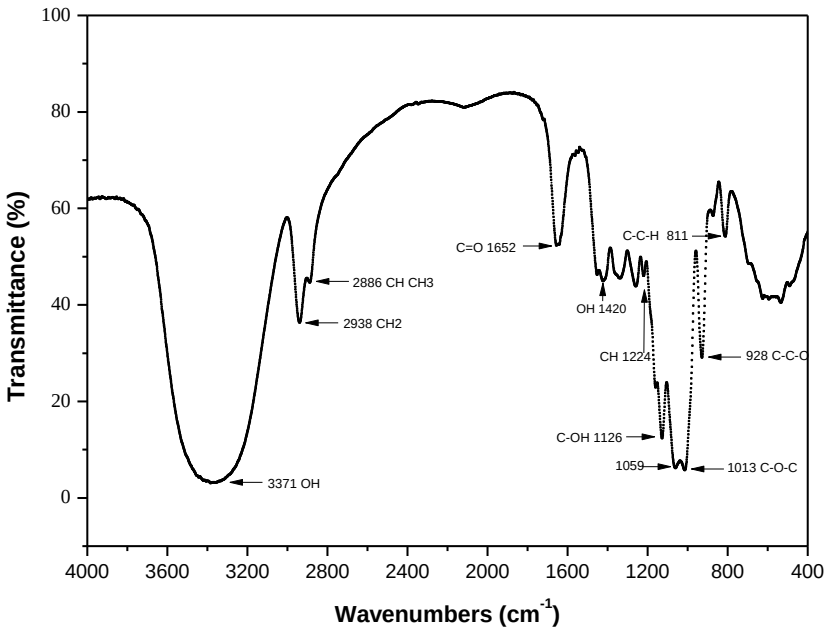


Fig. 4 FT-IR spectrum of EPS produced from the *Bacillus* sp. SGD-03

3.5 Molecular weight determination

The average molecular weight of purified EPS was determined using size exclusion chromatography. The calibration curve for molecular weight determination was made using several standards of Pullulan polysaccharide ranging from ~350–700 Mp. Based on calibration and comparison with standard Pullulan and Dextran, the size exclusion chromatogram has shown that the purified EPS comprises an average molecular weight of 1.0 × 104 Da. Supplementing the initial concentration of sucrose, 40%, and consumption of about a quarter of sucrose, Levan with less than 1.0×10^4 Da molecular weight was produced (Tanaka et al., 1980), so it can be assumed that upon utilization of 100% sucrose, the levan with molecular weight, 1.0×10^4 Da could be produced (Fig. 5).

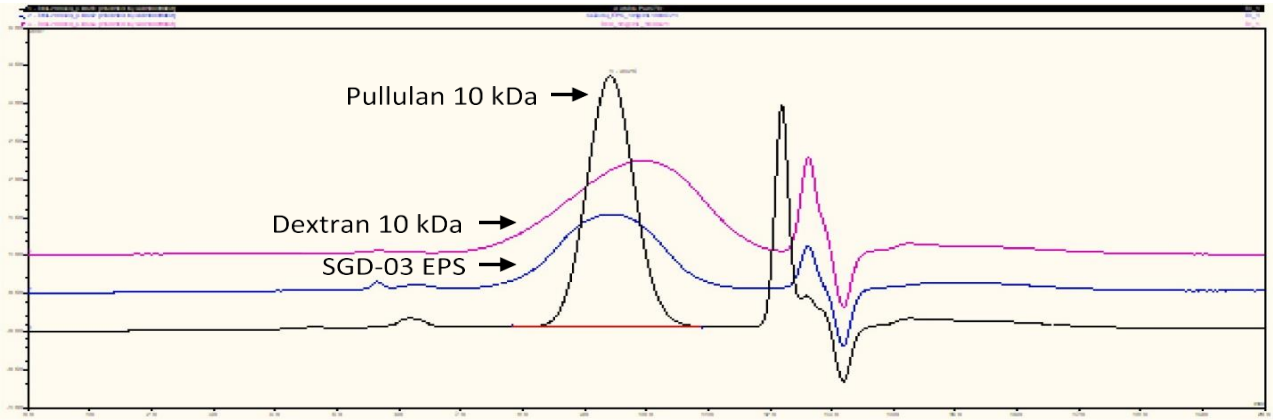


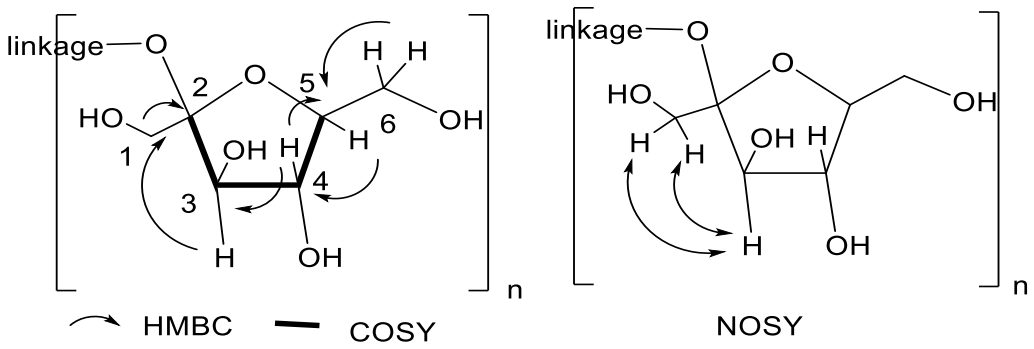
Fig. 5 Size exclusion chromatogram of EPS produced from *Bacillus* sp. SGD-03

3.6 NMR analysis

The spectra of 1D (^{1}H and ^{13}C) and 2D (NOESY, COSY, HSQC, and HMBC) NMR were analyzed to determine the glycosidic linkage pattern between the monosaccharide residues and elucidation of structure. The ^{1}H NMR seven signal at the range of δ 3.49 and δ 4.13 and 13C NMR shows six signals at a range of δ 59.9 to δ 80.29, shows characteristics peaks of sugar (Fig. 6B, 6C, 6D). Also, the ^{13}C NMR spectra signal at δ 104.2 shows the characteristic peaks of anomeric carbon present in the sugar (Fig. 6C and S6). The DEPT and HSQC spectra show two methylene peaks at δ 59.9 (δ 3.71 and 3.60) and δ 63.4 (δ 3.48 and δ 3.83), indicating the presence of the keto-sugars (Fig. 6G and 6I). In the COSY spectra, the signal at δ 3.48 and δ 3.83 shows a correlation of δ 3.89 protons. The proton at δ 3.89 correlation at δ 4.03 proton and further this δ 4.03 proton shoes correlation with δ 4.12 proton indicate that both are serially connected (Fig. 6A and Fig. 6E). After careful analysis of 1D and 2D NMR (Fig. 6A to 6H) and literature report, confirmed the fructose sugar (Taylan et al., 2019). The linkage of the fructose sugar was confirmed using NOESY NMR spectra. The proton on C-3 carbon δ 4.13 shows a NOESY correlation peak at proton present on C-1 carbon of δ 3.71 and δ3.61, indicating that both are in the same plane. Hence the sugar is linked by beta linkage (Fig. 6A and Fig. 6F). The NMR spectra confirm that the purified EPS is Levan, linked by β (2–6)-D-fructofuranosyl residue. The levan has named as

levan-03 (After the producing organism) The carbon chemical shifts were also compared with previously reported levan from other sources presented in Table 1 (Pei et al., 2020; Wahyuningrum & Hertadi, 2015).

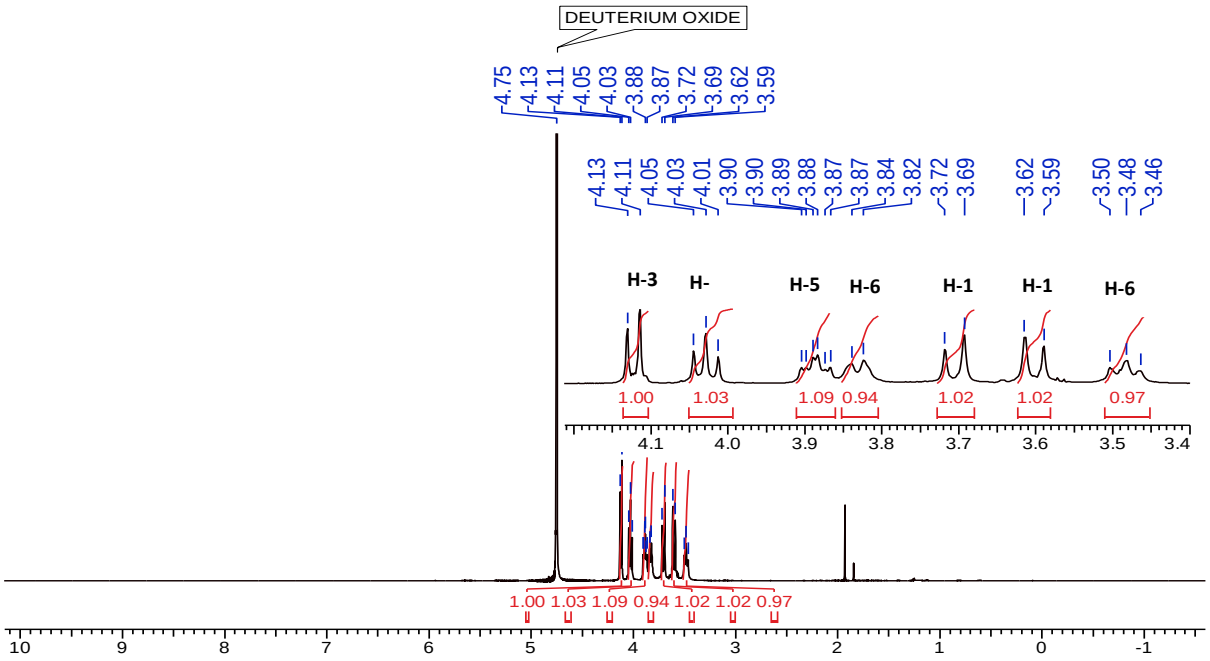
A.



B.

δC		δH
C-1	59.9	J = 13.0 Hz, 1 H), 3.60 1 H)
C-2	104.2	
C-3	76.3	4.12 (d, J = 8.4 Hz, 1 H)
C-4	75.2	4.03 (t, J = 8.0 Hz, 1 H)
C-5	80.3	3.89 (dt, J = 3.4, 7.8 Hz, 1 H)
C-6	63.4	3.48 (t, J = 9.9 Hz, 1 H) 3.83 (d, J = 7.6 Hz, 1 H)

C.


DEUTERIUM OXIDE
4.75
4.13
4.11
4.05
4.03
3.88
3.87
3.72
3.69
3.62
3.59
4.13
4.11
4.05
4.03
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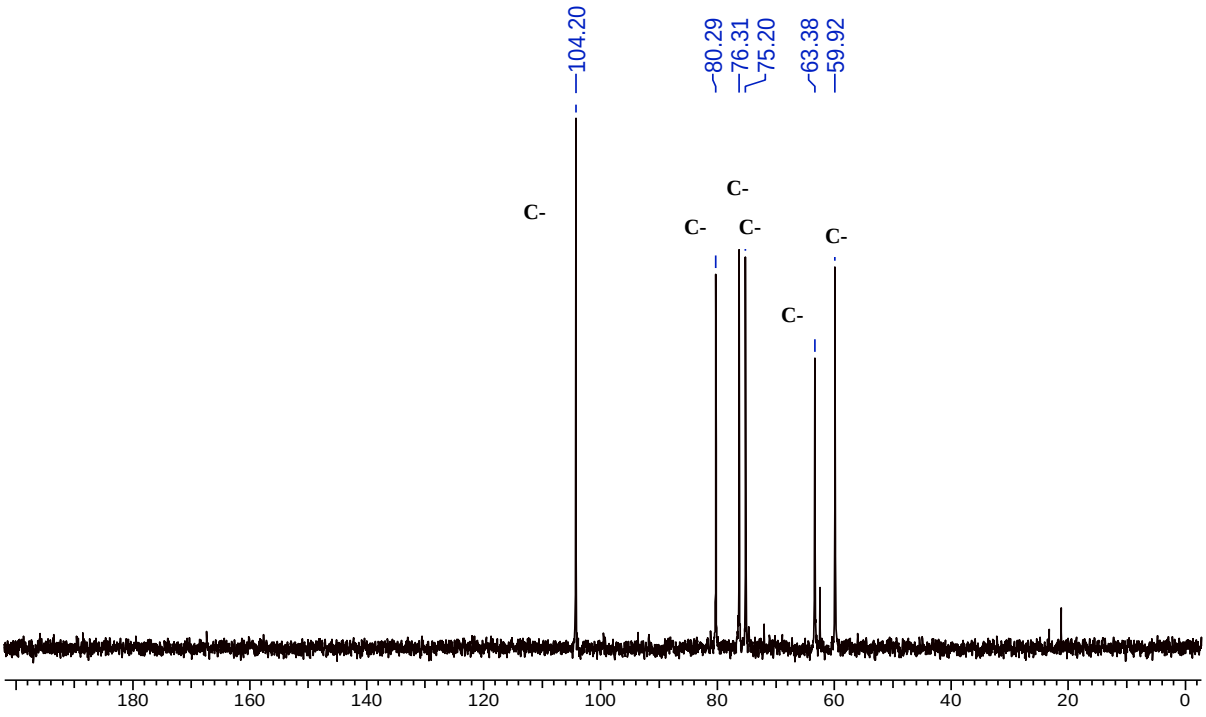
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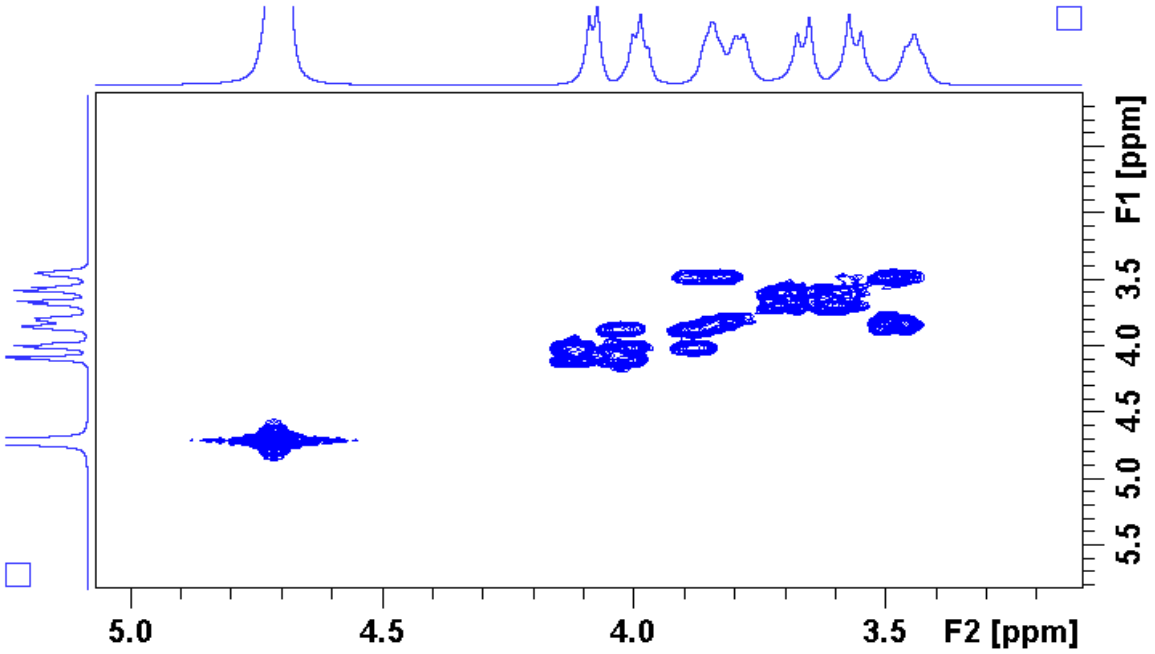
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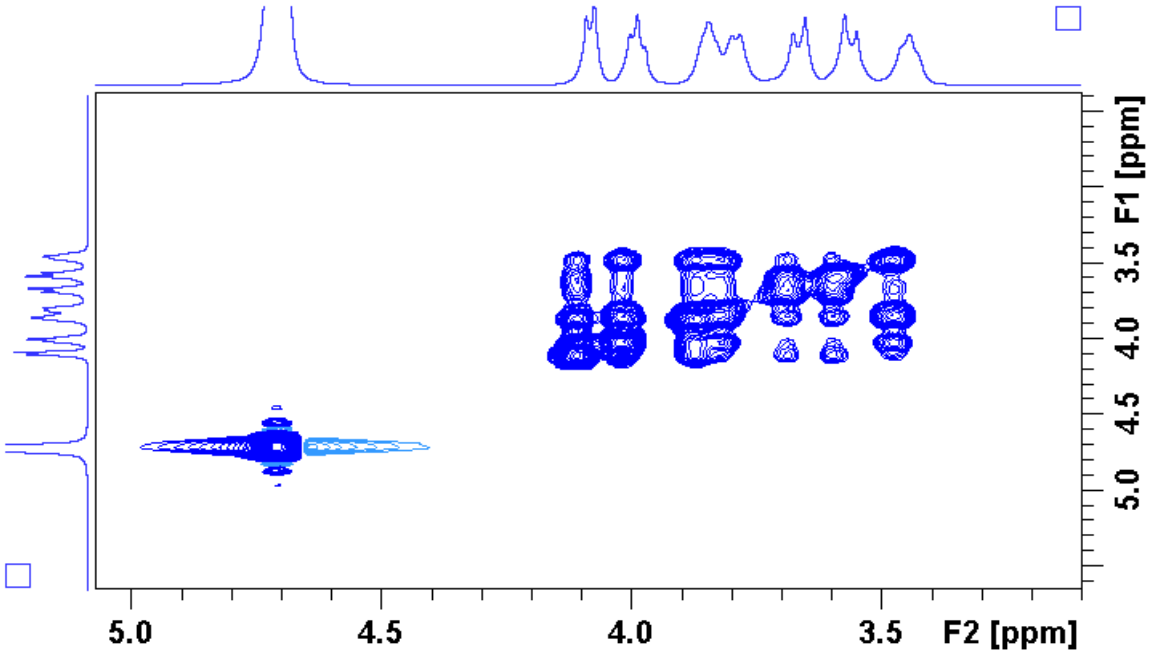
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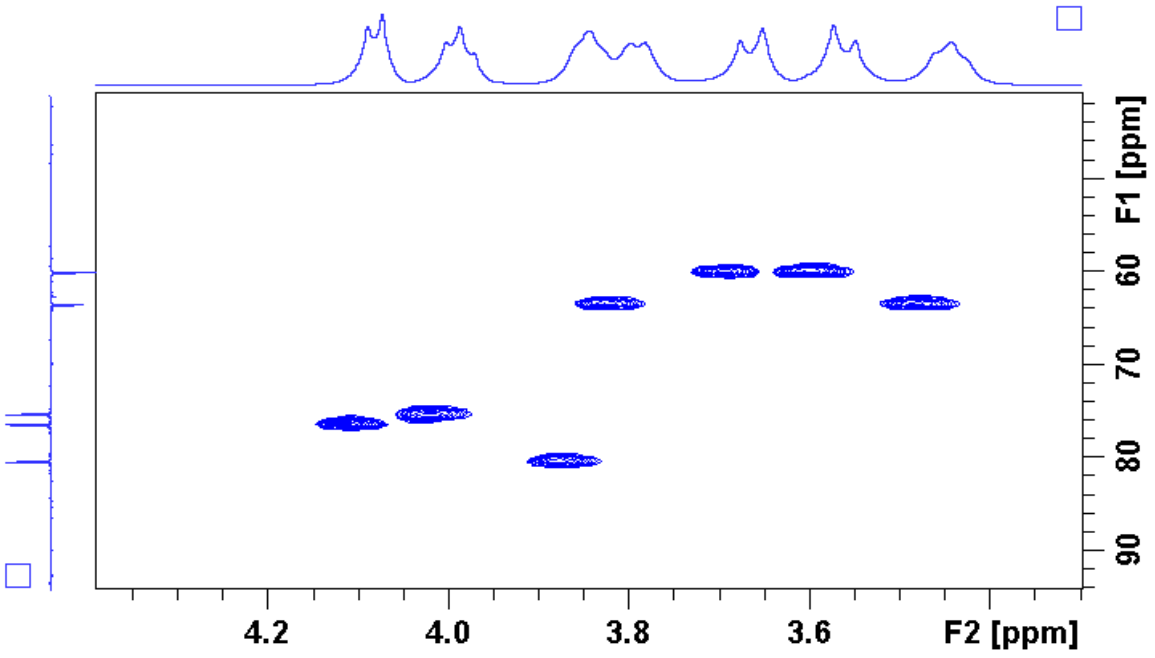
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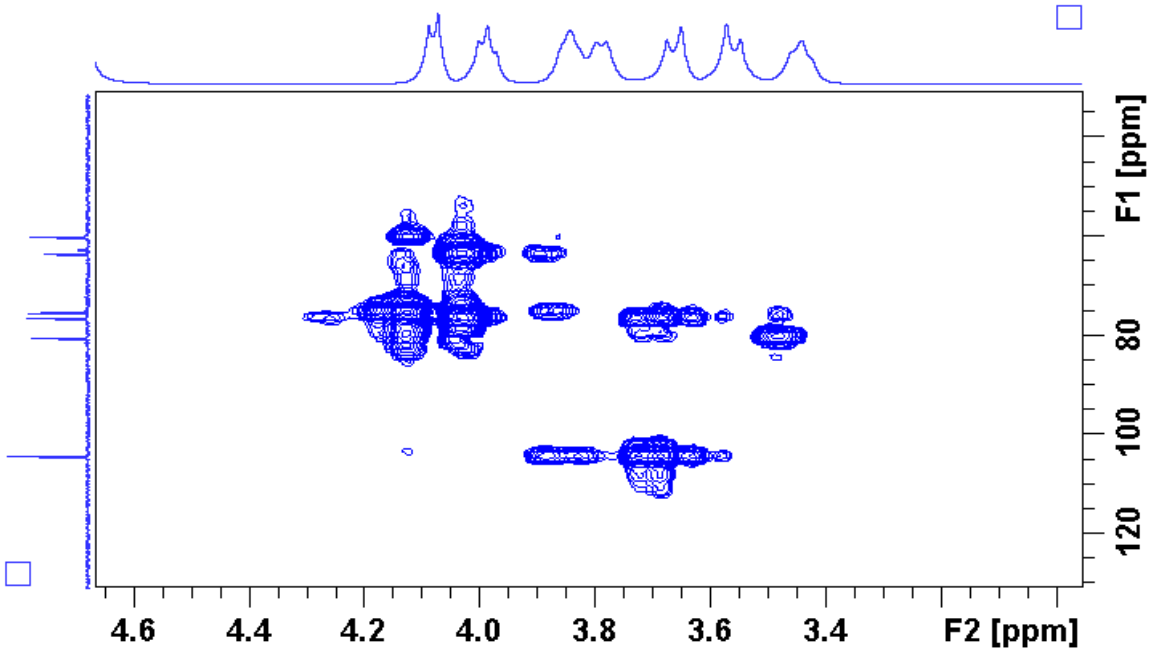
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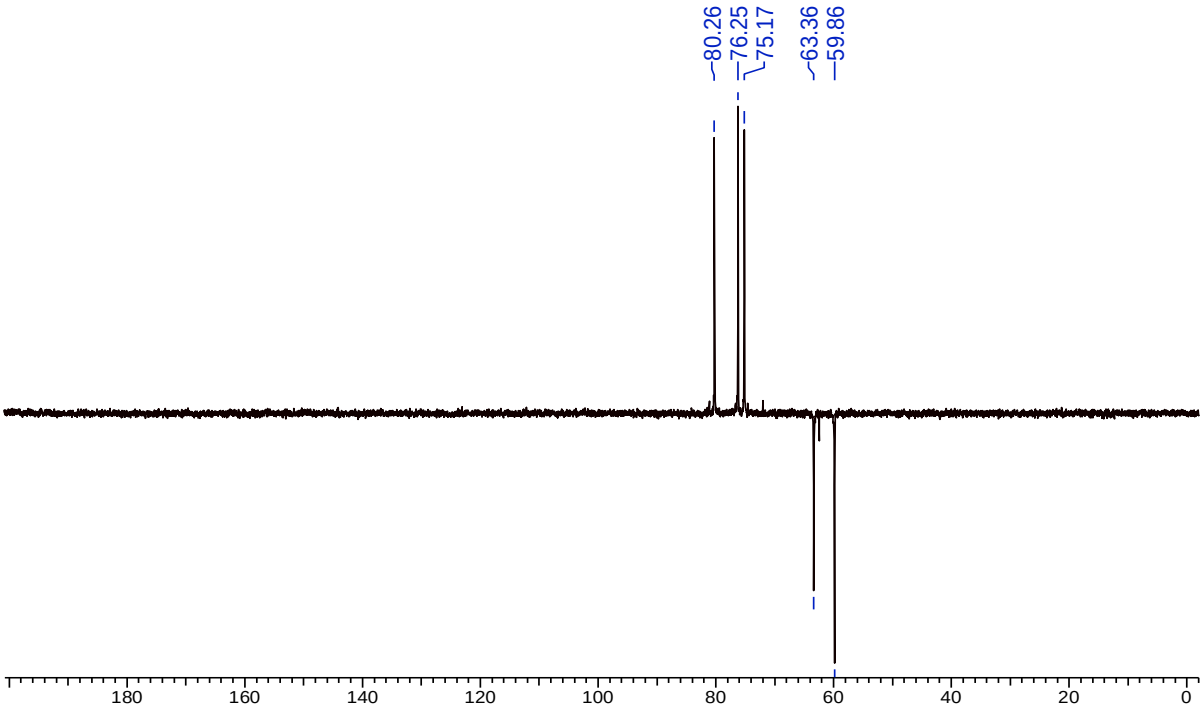

Fig. 6 NMR analysis of the EPS produced by Bacillus sp. SGD-03: A. Linkage; B. δ value of C & H; C. ^{1}H spectra; D. ^{13}C spectra; E. COSY; F. NOESY; G.HSQC; H.HMBC; I.DEPT.

Table.1 Comparison of Chemical shifts (ppm) for ^{13} C NMR with reported *Bacillus* sp. for Levan EPS

Carbon atom	*B. licheniformis* *	*B. megaterium* **	Bacillus sp SGD-03 (This study)
	δC (^{13}C-NMR)		
C1	59.85	59.90	59.92
C2	104.22	104.20	104.20
C3	76.24	76.30	76.31

C4	75.18	75.20	75.20
C5	80.31	76.38	80.29
C6	63.41	63.39	63.38

* Wahyuningrum et al., 2015; ** Pei et al., 2020

4. Conclusion

In concluson, we have successfully purified and characterized the EPS produced by a marine organisms *Bacillus* sp. SGD-03. Produced EPS was further purified sequentially, employing precipitation, dialysis, deproteinization, and column chromatography based on the size exclusion principle. Complete characterization of EPS was carried out using various analytical techniques. The morphological observation was that the EPS has a fibrous network structure with a relatively smooth surface of 3.620 μm to 9.071 μm fiber diameter. Thermogravimetric analysis revealed that the EPS can tolerate high temperatures and is stable up to 185°C which is a 10 % reduction after an increase in the temperature to 188°C. Size exclusion chromatography was used for molecular weight determination, revealed that; the produced EPS has a molecular weight of 1.0×10^4 Dalton. Interestingly, this unique-sized levan has not been reported in the literature before and will open the door for research requiring a specific 10 kDa levan. Further, analytical techniques such as TLC, HPLC, LC-HRMS, FTIR, and NMR analysis have revealed that the EPS has a molecular weight of and is composed of fructose monosaccharide units with hydroxyl, carbonyl, and ether groups. NMR confirmed the produced EPS has a β-(2, 6) linkage bonding, confirming its identity as a levan polysaccharide.

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Chapter 4:

Optimization of fermentation process via Micro bioreactor (BioLector Pro)

Abstract

This study is aimed to develop a cost-effective, robust high throughput process for levan production from a marine *Bacillus* species SGD-03 using a high throughput micro bioreactor system. The quantitative process parameters were optimized using response surface methodology (RSM) involving full factorial (FFD) followed by central composite design (CCD). The new medium was formulated by amending peptone, beef extract, sodium chloride and sucrose. Peptone 0.4%, beef extract 0.4%, and sucrose 4.0%, were found optimum process parameters for maximum EPS production, which is approximately valued at 1.12 USD/L of the media chemicals used for the production. The maximum yield of crude EPS obtained was 123.9 g/L, resulting in 20.7 g/L pure EPS, and the production has been validated with a shake flask, 1 L, and 10 L pilot scale fermentation.

1. Introduction

Several compounds have been produced by fermentation using microbial and animal cells. Production of bioactive compounds using fermentation technology is eco-friendly over chemical synthesis and is widely accepted approach. Microbial fermentation is the preferred source of product development due to the ease of alteration in the media and optimization process. Based on the high production rate and scale-up, product of microbial origin has gained attention in industry to scale up. Microbes are a simple yet efficient system for product development in a short time. Biomolecules such as enzymes, hormones, and other bioactive compounds of microbial sources have been optimized using fermentation technology. Process development is highly economical and efficient in fulfilling the market demands in terms of productivity and reproducibility. Several fermentation parameters have been studied to understand the scale-up processes, such as pH, temperature, dissolved oxygen, etc. Efficient optimization mainly depends on a thorough understanding of the process parameters. Defining process parameters to scale up are critical factors that need intense monitoring.

The production cost seems to be a bottleneck in EPS commercialization. Bacterial EPS possesses a unique property that allows commercialization. Plants and algal polysaccharides are currently being used in industries, which can be replaced by bacterial EPS. Bacterial EPSs confers more advantages in industrial applications because of ease of extraction and controllability of

production. Cell immobilization, solid-state fermentation and submerged fermentation can produce the levan in at larger scale. The packed-bed bioreactor has achieved continuous levan production by calcium-alginate immobilized *Zymomonas mobilis* cells (Silbir et al., 2014).

To accomplish the need in a shorter duration and commercialization of EPS, various strategies have been adopted, like media optimization, strain improvement and change in fermentation parameters (Lee et al., 1997; Song et al., 2013;Berekaa., 2014)). Different approaches were used for optimization. Several cost-effective media formulations by using cheap raw materials have been stated to enhance the production of levan, such as sugar beet molasses (Han & Watson, 1992; Gojgic-Cvijovic et al., 2019). sugarcane syrup and molasses, date syrup (de Oliveira et al., 2007; Moosavi-Nasab et al., 2010; Abou-Taleb et al., 2015; Moussa et al., 2017). The optimum levan production has been shown to be produced in high sucrose-containing media. Levan production has also been improved through the metabolic process by heterologous expression of levan-producing enzymes (sacB) (Trujillo et al., 2001;Gu et al., 2017), mutation and knocking out of gene of the sucrose phosphorylase gene scrP also shown to increased levan production (Teixeira et al., 2013; Asgher et al., 2020). Among these approaches, amending media components are an easy and rapid method for optimization of levan. Recently, fermentation technology focused on developing process parameters to scale up the production.

1.1 Fermentation process

Bioprocess technology works on two types of fermentation, i.e., submerged fermentation and solid-state fermentation. Fermentation is a metabolic process that converts the substrate into a product by the action of different catalysts according to physical circumstances. Several fermentation processes have been studied for scaling up the process. The process has been divided into three stages: upstream, fermentation, and downstream. The upstream process is a pre-fermentation stage that includes isolation, screening, strain improvement, media formulation, inoculum preparation, optimization, and inoculation in the production medium. Basal fermentation media is at least composed of carbon-nitrogen, micronutrients, vitamins, and water. The formulated media must have essential nutrients to support microbial growth and product formation. Fermentation requires two kinds of media; the inoculum media enriches the culture, and the production media contains carbon, nitrogen, minerals, and vitamins, enabling product formation.

The second stage is fermentation, which is nothing but the process operation of the fermenter, production, and monitoring of process parameters (pH, dissolved oxygen, biomass, air transfer rate, rpm, etc.) and the third, downstream process involves product harvesting, purification, and analysis of the product. The purification and analysis vary depending on the desired product. For instance, purification of secondary metabolite involves fermentation, cell harvesting, separation, concentration, and fractionation of metabolite by gravity-based column using different polarity-based solvents system. On the other hand, the purification of biomolecules such as proteins and polysaccharides involves precipitation separation of other contaminants by solvents using column chromatography and electrophoresis. The fermentation reaction is carried out in a closed vessel called a fermenter or bioreactor, ranging from one to thousands of liters. The design of the basic fermentor is depicted in Fig 1. The optimization is a challenging process that needs a high amount of media, glassware, shakers, etc.

The scale down experiments during bioprocessing are very important for optimization. For an established process, manufacturers use scale-down experiments to troubleshoot or de-bottleneck steps that are running sub-optimally. In this instance Micro bioreactors serve as a multitude of scale-down needs for bioprocesses. BioLector pro is an advanced, automated, robust, and high throughput technology efficient to overcome problems associated with the requirement of different media, glasswares, incubator shakers, etc. (BioLector depicted in Fig. 2). The micro-level fermentation is a prerequisite of time and offers many unique characteristics for optimization of the fermentation process. It is a well-developed product optimization instrument that assists fermentation's controlled operation and is used to cultivate cells to produce various metabolites. It provides an optimum condition for microbial growth and product formation. High levan production in a shake flask needs continuous attention till the completion of the process. This also requires human resources, a high amount of media, and separate utilities to optimize each process parameter and set of experiments. A micro bioreactor offers a high throughput optimization of process parameters in one go. It has in-built pre-calibrated optical sensors for online monitoring of pH, DO, biomass, and fluorescence of fermentation conditions. It utilizes different type’s 48-well MTP according to the applications. Some plates are featured with inbuilt control pH and feeding of pH solution to adjust the desired pH. The robotic arm can be attached to the primary instrument to sample in real-time automatically. These are ready-to-use plates.

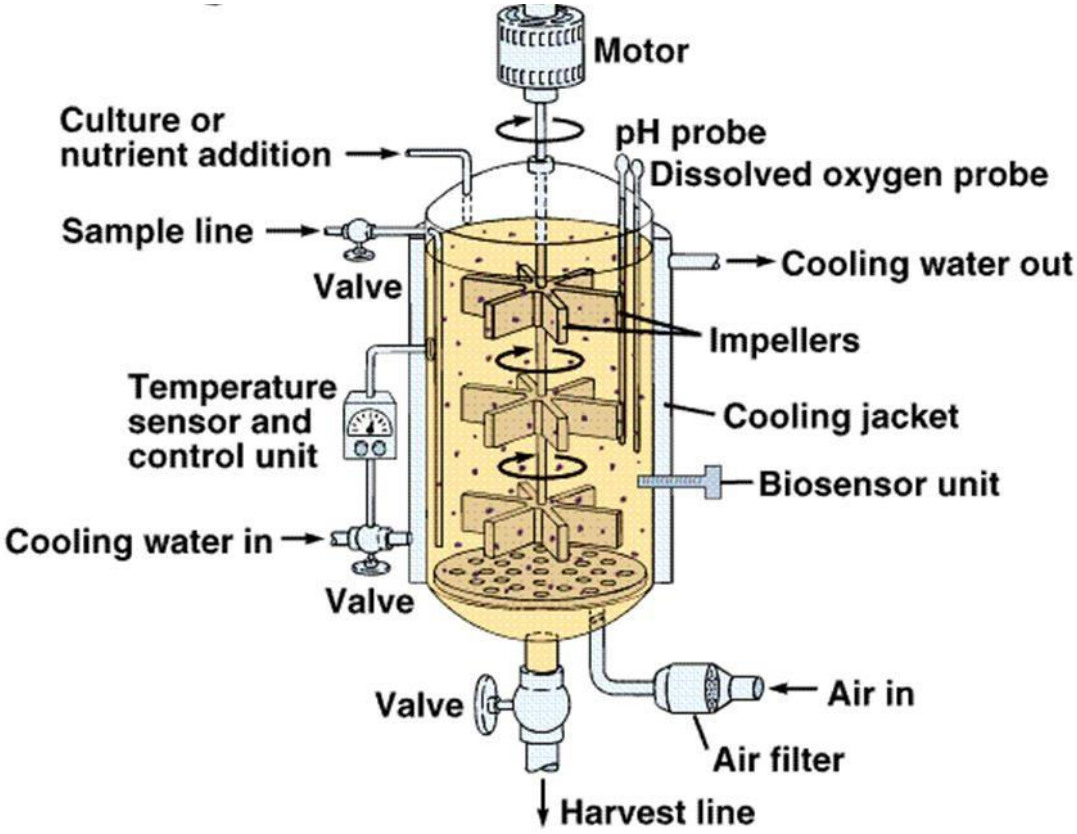

Fig.1 Design of fermentation (Prescott, 1999)

The advantages of Micro bioreactor (BioLector Pro) over the large-scale fermenter

BioLector Pro	Large scale fermenter
Multiple fermentation (up to 48) reactions can be performed in parallel.	Only one fermentation reaction can be performed at a time.
Real-time data visualization.	Endpoint data visualization.
A large number of data can be generated within a short period.	It takes a longer time to generate data.
The sample can be withdrawn automatically using a robotic arm.	The sample can be withdrawn manually using a sampling port.

It automatically maintains the pH of the solution through micro channels in each well of the plate.	pH needs to maintain manually using peristaltic pumps.
Measures and maintains required dissolved oxygen concentration automatically.	Dissolved oxygen concentration needs to monitor manually and supply.
Biomass concentration can be calculated automatically in ongoing reactions.	A sample has to collect to calculate biomass concentration in an ongoing reaction.
Filters can be added for metabolite analysis.	The large-scale fermenter has no option to add any filters.
A large number of data can be summarized automatically and plotted in graphs using the software.	Provide fewer data and need to plot the graphs manually.
A minimum of 1.0 mL of volume is sufficient to optimize the conditions.	A minimum of 1.0 L of volume is required to optimize the conditions.
It reduces capital expenditure costs.	It increases capital expenditure costs.
Process parameters can be developed according to the need of the process in a short duration.	The development of process parameters needs a longer time.

Applications of BioLector Pro

1. It is suitable for vaccine development and metabolite production.
2. It is used to screen and optimize the media, cell lines, microbial strain, and other parameters.
3. Oxygen transfer and feeding rate can be optimized.
4. It can be used for proteomics and genomics studies, etc.

Scale-up is the bottleneck in bioprocess development, which takes a lot of time and slows down the development of the primary screening media component. Shake flask-based screening can take several days to weeks, Whereas, Micro bioreactor assists in screening the process parameter in a

short duration. Running simultaneous 48 fermentation reactions can reduces space, human resources and cost.

Generally, media is composed of more than one component with varying concentrations. A statistical approach can be used to determine the interaction between media components and their concentration. The statistical experiment design is an efficient approach to optimize the media parameters compared to conventional methods. Most of the studies have been restricted to optimizing the medium composition by one factor at a time (OFAT), which is time-consuming and unable to understand the interaction between the variables (Sen & Swaminathan., 1997). Design of Experiments (DoE) can effectively understand the interactions among the various physiological parameters to optimize media conditions parallel. Response surface methodology (RSM) has been extensively used to optimize the responses altering the media composition (Kekez et al., 2015). Despite all the important benefits, low productivity is the bottleneck in commercializing EPS.

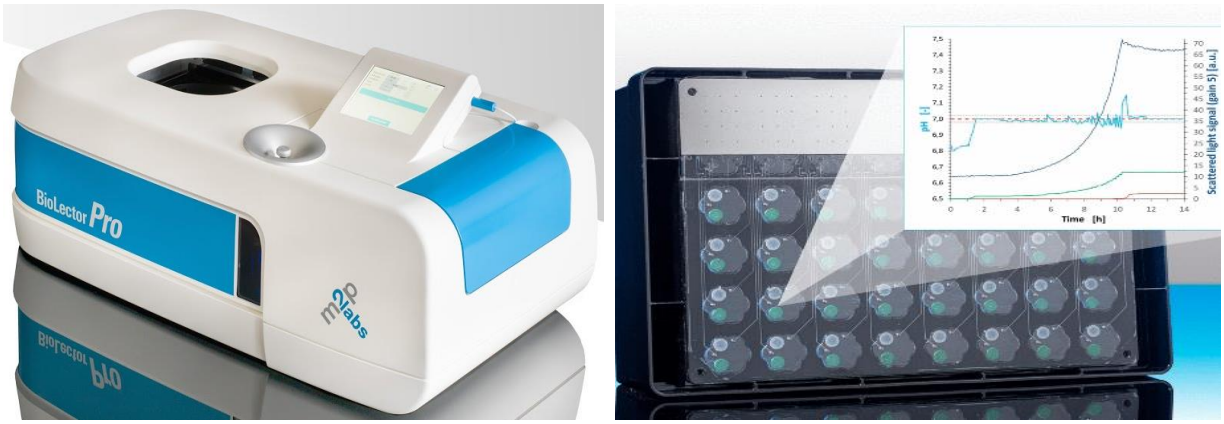

Fig. 2 BioLector Pro and the optode plate

In combination with response surface methodology, Micro bioreactor-based optimization will help to understand the possible interaction of various factors at different levels. It utilizes minimal media (1.0 mL) to optimize the production parameters. A conventional method of optimization relay on shake flask levels without control of pH, biomass, and dissolved oxygen, which are fundamental parameters for optimization. An automated Micro bioreactor facilitates online pH,

biomass, and dissolved oxygen monitoring. The Micro bioreactor's single optode plate efficiently runs 48 experiments simultaneously with various media combinations before going to large-scale fermentation. A time-saving and cost-effective approach for the optimization is requisite of the time.

This study describes the optimized production using a miniature version of the fermenter, a BioLector Pro, which uses a 1.0 mL working volume. It assists in online monitoring of pH, dissolved oxygen, and biomass, which are key parameters for optimizing the fermentation process. Optimization of fermentation parameters at a small scale helps minimization of media with multiple sets of experiments.

2. Materials and Methods

2.1 Reagents and kits

The majority of the chemicals were procured from Hi-Media, Mumbai, and Merck (Sigma-Aldrich)

Sugars (Sucrose, fructose, glucose, maltose, and lactose)

Peptone,

Beef extract,

Sodium chloride

Ethanol (Merck)

Phenol (Merck)

Sulphuric acid

2.2 Bacterial strain and culture conditions

Based on the initial screening and characterization, strain *Bacillus* sp. SGD-03 was taken for optimization studies. A nutrient broth (Peptone, 1.0%; Beef extract, 1.0%; NaCl, 0.5% with a pH of 7.0) was used to study the optimum temperature and inoculum titer. Temperature variables like 25°C, 28°C, 30°C and 37°C and inoculum OD of 0.1 to 0.5 were set to analyze the effect of temperature and inoculum size on organisms' growth profile. One percent (0.1 OD) of overnight

grown culture was inoculated in nutrient broth and incubated at various temperatures for 24h at 150 RPM under shaking conditions. At the same time, optical densities were adjusted from 0.1 to 0.5 to analyze the effect of inoculum titer on the growth of an organism for 48h. Cells, 0.1 OD (10^7 colonies forming unit, CFU/mL) were inoculated in a nutrient broth and incubated for 18h at 28°C temperature with 150 rpm to obtain fresh active starter culture for further experiments.

2.3. Salt and pH tolerance

Salt and pH tolerance were tested to check the effect of osmolarities and pH on the organism's growth profile. Nutrient broth, with different concentrations of saline solution 2.0–10.0% (w/v) and pH ranging from 5.0–10.0 (at an interval of 1.0 units), was tested. Fresh culture of *Bacillus* sp. SGD-03 was inoculated as 1.0% v/v in 96 microtiter well plates containing different sets of experiments and incubated for 24 h at 28 °C with shaking of 150 rpm. The salt and pH tolerance profiles were analyzed by absorbance at 600 nm using the Synergy H1 hybrid multi-mode microtiter plate reader.

2.4. Micro bioreactor-based optimization of EPS

2.4.1. Screening of the best carbons source for the EPS production

To examine the effect of carbon sources on EPS production, 2.0% of fructose, glucose, sucrose, maltose, and lactose were supplemented in nutrient broth. Setting 1.0 mL of working volume, an Inoculum of 1.0% (0.1 OD_{600}) was inoculated in an optode plate. The plate was fixed and operated at 28°C, 1400 rpm for 48 h in BioLector pro (m2p Labs). The optode plate measured the dissolved oxygen (DO), pH, and biomass. After confirming the pre-eminent carbon source, its concentration ranging from 2.0%, 4.0%, 6.0%, 8.0%, and 10.0% w/v were studied to evaluate the optimal concentration.

2.4.2. Screening of process optimization factor by full factorial design (FFD)

The full factorial experiments were designed to screen the desirable factors at various levels to optimize the response. Full factorial design of experiments (DoE) was modeled by Minitab19 statistical software involving three independent variables (Peptone, beef extract, and sucrose) at four levels (0% to 3.0%, low to high) with 5.0 g/L NaCl as a constant. A total number of 128

experiments in replicates were performed to analyze the effect of each factor and their interaction on EPS production.

2.4.3. Optimization of response by RSM

Based on the full factorial experiments, all the media components (peptone, beef extract, and sucrose) were further selected to enhance the response (All factors were chosen as they were found to have a positive effect on EPS production). Central composite design (CCD) was applied to study the impact of optimal points. The independent variables, peptone and beef extract with their low concentration of 0.1% to high 0.4%, were used. And sucrose with 1.0–4.0% concentration with 0.5% NaCl (w/v) as a constant was studied in a face-centered CDD. A total of 20 repeated experiments were performed, containing 8 factorial points, 6 axial points, and a sextuple at the center point (Dos Santos et al., 2016). Optimal media composition (obtained by CCD experiments) was further validated at fermentor scale for EPS production.

2.5. Production and quantification of EPS

Media optimization was performed using an optode embodied 48-well microtiter plate (Flower well plates, m2p-labs GmbH, Germany) in a BioLector Pro machine, which facilitates online monitoring of cell biomass, pH and dissolved oxygen (DO) saturation separately in each well without effusing (Dos Santos et al., 2016; Kensy et al., 2009). With working volume 1.0 mL, overnight grown culture (18 h), inoculated as 1.0% (0.1 OD_{600}) in each well containing various media combinations generated by experimental design. (Media components peptone, beef extract, and sucrose were prepared in stock concentrations, and pH was adjusted to 7.0). The plate was fixed and operated at a temperature of 28°C; rpm, 1400; humidity, 100% for 48h of incubation. Later, the plate was taken out from the Microbioreactor. Samples were centrifuged at 10000 rpm, 30 min at room temperature to obtain cell-free supernatant. The three-volume of pre-chilled ethanol (4°C) was added to the supernatant. Precipitated EPS (threaded appearance) was separated and washed with 70% ethanol. One milliliter of Milli Q water was added and kept overnight at 4°C for proper dissolution. The dissolved crude EPS was subjected to quantification. For the process validation, the optimized media composition of FFD and CCD for EPS production was subjected to a shake flask (500 mL) followed by 1–10 L fermentors. Erlenmeyer flask of 500 mL

containing 200 mL of working volume was set. The flasks were inoculated in optimal media composition and incubated at 28°C at 150rpm for 24h. Dissolved oxygen saturation, 10%, was maintained by cascading rpm from 250 to 500; pH 7.0; temperature, 28°C and 24 h of incubation were set for fermentor scale (1 L and 10 L).

2.6. Data analysis

Analysis of experimental designs (full factorial and face-centered composite), analysis of variance (ANOVA), framing of two-dimensional contour plot, and three-dimensional cubic model for response surface representing variable data points were performed using regression analysis of variables by Minitab statistical software version 19.0. The biomass concentrations were correlated and calculated by linear regression of light scattering (620nm) and optical density (600nm) using serial dilutions of a known concentration of cell dry weight (CDW) as standard. CFU was calculated from 12 to 48h of incubation in a shake flask (working volume of 100 mL) and BioLector (working volume 1.0 mL) using a plate spread technique to check an organism's cell viability. 100 µL of sequentially diluted culture was spread on a solid agar plate and incubated at 28°C for 24h. CFU/mL was noted after 12h and 24h incubation. pH and dissolved oxygen saturation were estimated by the BioLection version 3.19.4 data processing software (m2plabs GmbH, Germany). EPS quantification was determined by the phenol sulphuric acid method. A standard linear curve of pullulan was generated to quantify the concentration of EPS in each experimental setup (Rao & Pattabiraman., 1989).

3. Results and discussion

3.1 Effect of temperature and inoculum on the growth of *Bacillus* sp. SGD-03

The effect of temperature on the growth of an organism and its EPS production was analyzed. Depending on the temperature of the location from where the strain has been isolated (28–30°C), temperature variables of 25°C, 28°C, 30°C, and 37°C were used to analyze the optimum temperature for the growth. It was found that the organism can grow in all the tested temperatures, with an optimum at 28°C. We have also compared EPS production for the tested temperatures. It was observed that the organism had produced an optimum of 3.83 g/L of pure EPS at 28 °C with supplementing 2% of sucrose in media (Fig. 3a). An effect of various inocula on the organism's

growth was observed based on growth profile analysis of *Bacillus* sp. SGD-03 with different inocula. Inoculum OD ranging from 0.1 to 0.5 was set to obtain the inoculum titer value. The optimum growth of Bacillus sp. SGD-03 was found in the inoculum OD from 0.2 to 0.5 till 24h and started declining, as well as 0.1 inoculums OD, which has also shown comparable growth at 24h and continued till 32h. The desirable growth progress of *Bacillus* sp. SGD-03 was achieved with inoculum having 0.1 OD. An organism has shown a distinct, desirable growth pattern of its growth phases (Fig. 3b).

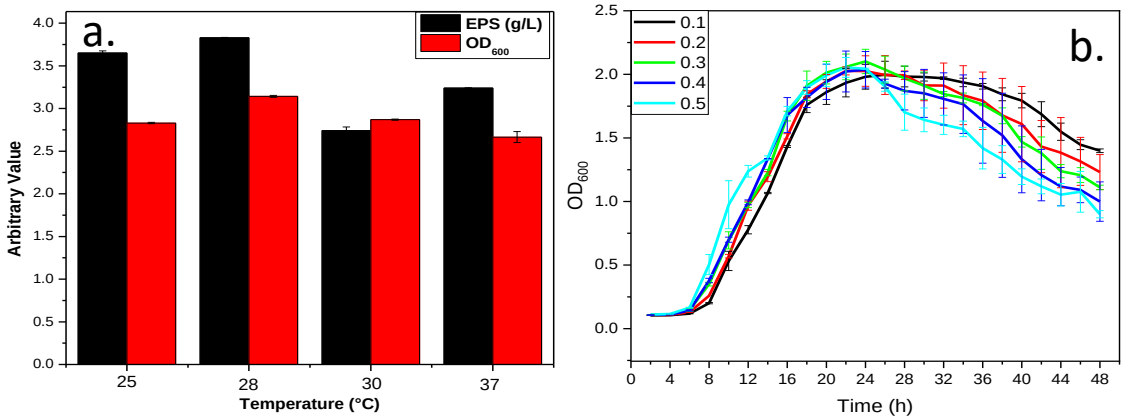

Fig. 3 Effect of temperature and inoculum OD on the growth profile of *Bacillus* sp. SGD-03: a. Temperature; b. Inoculum OD

3.2 Effect of salt and pH on the tolerance profile of the organism

The tolerance of salt and pH were analyzed based on the growth profile of an organism. An inverse relationship of growth to salt concentration was observed. As the concentration of salt increases, the growth of an organism decreases. It was observed that the organism showed optimum growth between 18 and 20 h in 2.0% salt and declined rapidly after 20 h onwards. The organism could not tolerate high salinity after 20h (Fig. 4a). On the observation of pH, an organism's growth was significantly affected at low pH 4.0 and high pH 10.0. The pH 6.0 and 7.0 is the optimum range for the development of an organism. A moderate growth profile was observed at pH 5.0, 8.0, and 9.0 (Fig. 4b). It can be concluded that the organism can tolerate a wide range of pH as well as salt.

However, the desirable growth phases were not observed. It may be an intervention of EPS in the absorbance.

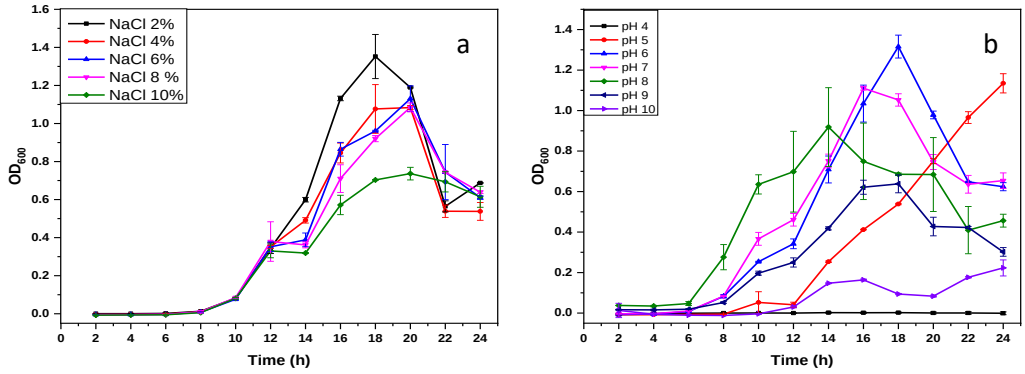

Fig. 4 Growth tolerance profile of *Bacillus* sp. SGD-03: a. Salt; b. pH

3.3. Optimization of process parameters for EPS production using BioLector Pro

3.3.1. Screening of carbon source for EPS production

The influence of various carbon sources on EPS production was investigated using a Micro bioreactor, i.e., BioLector Pro. Five carbon sources, including monosaccharides (fructose, glucose) and disaccharides (sucrose, maltose, and lactose), were studied to examine their effect on EPS production. Sucrose was found to be the best carbon source having a significant impact on EPS production. It showed almost a 10-fold increase compared to other carbon sources (Fig. 5a). Further, various concentrations of sucrose were studied. The 4.0% sucrose concentration was found to be an optimum concentration effect on EPS production. Though the observation seems to have a direct relationship between EPS production with increasing sucrose concentration, there is no significant increase in the EPS yield with higher concentrations of sucrose (Fig. 5b).

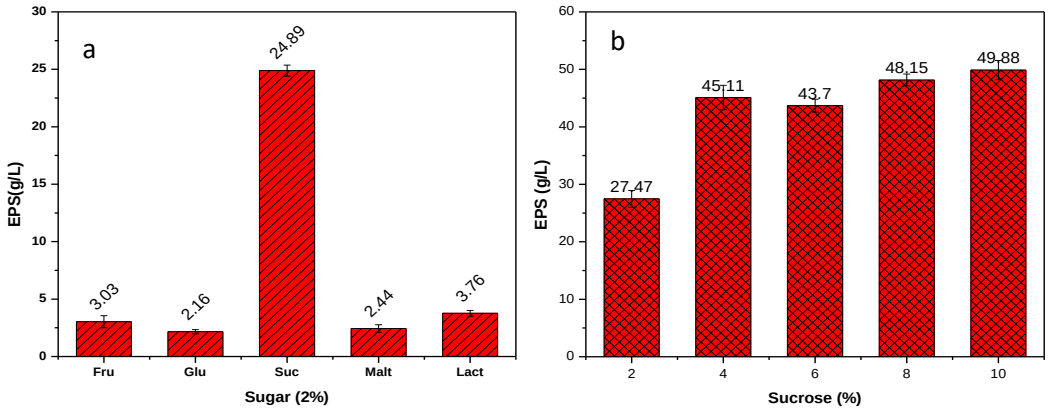

Fig. 5 Screening of carbon source: a. Effect of various carbon sources on EPS production; b. Effect of Sucrose concentrations on EPS production.

3.3.2. Screening of significant variables

To choose the best combination of nutrient media components, i. e. peptone, beef extract, and NaCl with an effect of supplemented carbon source (sucrose), were screened from low to high 0–3.0% concentration. Significant variables were obtained by the full factorial design regarding the influence on EPS production. Results were interpreted by linear regression analysis using the Minitab19 statistical software, which helps estimate each independent variable's effect. The optimum response, 67.0 g/L, was derived with the combination of peptone (2.0%), beef extract (3.0%), and sucrose (3.0%) with 0.5% NaCl as a constant (Table 1). The sucrose with a higher concentration (3%) has the main effect on EPS production (Fig. 6). Peptone and beef extract were also found to have a positive impact on enhancing EPS production. Statistical significance of the effect of each variable on EPS production was calculated and investigated by f-test, which has shown adequacy of the design with highlighting all the factors, i.e., factor (A), peptone; factor (B), beef extract, and factor (C), sucrose are having the statistically significant effect on EPS production. Based on this observation, it can be concluded that all the factors are essential in producing EPS. The p-value of <0.000 confirms that the overall model is very significant and productive.

Moreover, the value of R^2 0.584 for the model indicates that the 40% variation in the sample indicates the results of individual variables effect. The 2-way interaction of beef extract and sucrose was very significant. The nitrogen source (peptone) is also required for the growth of an organism to produce EPS. Therefore the model could not explain total variance. The probable cause could be the various interactions of variables, as mentioned in Table 2. The response of each run was calculated using the following regression equation:

$$Y = 2.13 + 2.83\ A + 2.27\ B + 8.31\ C - 0.84\ A*B - 0.15\ A*C + 1.29\ B*C + 0.236\ A*B*C$$

Where Y = Response, A = Peptone, B = Beef extract, and C = Sucrose. The correlation of the calculated response (EPS) to the biomass of each run has shown in Table 1. Microorganisms produce EPS in response to various stress conditions like nutrient deficiency, salt tolerance, heat tolerance, drought conditions, etc. (Sandhya & Ali., 2015). In defined media, EPS production is stimulated by nutrient limitations in the presence of excess carbohydrates (Duguid & Wilkinson., 1953). According to this, the concentration of selected optimum levels of input factors was modified by diluting 10 times to mimic the nutrient deficiency to induce the organisms EPS producing ability to maximize the yield of EPS. NaCl was kept constant (0.5%) to maintain the osmolarity balance. The concentration of sucrose was used undiluted as the carbon source acts as a substrate for the EPS production required for the growth of an organism. And it will work in place of the high salinity of seawater to maintain osmolarity.

Table. 1 **Experimentally designed runs with various levels of each variable and EPS -Biomass of each run with a mean value of replicates.**

	Variable																															
	A. Peptone (%)										B. Beef extract (%)										C. Sucrose (%)											
Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
A	**3**	**2**	**0**	**3**	**1**	**2**	**0**	**3**	**3**	**2**	**0**	**0**	**3**	**0**	**2**	**1**	**3**	**1**	**2**	**1**	**2**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**1**	**1**	**1**	**1**
B	**2**	**0**	**0**	**1**	**2**	**1**	**2**	**2**	**1**	**3**	**3**	**3**	**3**	**2**	**1**	**2**	**0**	**3**	**0**	**3**	**0**	**1**	**1**	**1**	**3**	**3**	**1**	**2**	**1**	**3**	**1**	**2**
C	**0**	**3**	**2**	**1**	**3**	**0**	**2**	**1**	**3**	**0**	**2**	**1**	**2**	**3**	**2**	**1**	**0**	**1**	**2**	**0**	**0**	**1**	**3**	**1**	**1**	**0**	**1**	**3**	**2**	**2**	**0**	**0**
EPS (g/L)	**13**	**43**	**3**	**18**	**20**	**7**	**26**	**42**	**47**	**10**	**30**	**30**	**28**	**46**	**30**	**16**	**5**	**21**	**30**	**6**	**3**	**26**	**25**	**22**	**24**	**5**	**19**	**32**	**37**	**44**	**5**	**6**
SD (g/L)	**2**	**3**	**2**	**1**	**1**	**1**	**6**	**10**	**13**	**2**	**4**	**5**	**9**	**4**	**7**	**2**	**1**	**3**	**5**	**2**	**1**	**6**	**2**	**4**	**0**	**0**	**11**	**3**	**12**	**26**	**3**	**2**
EPS (g/g CDW)	**5**	**7**	**5**	**4**	**14**	**4**	**15**	**13**	**7**	**5**	**21**	**22**	**7**	**22**	**6**	**6**	**2**	**8**	**5**	**4**	**1**	**19**	**6**	**6**	**7**	**5**	**7**	**9**	**9**	**18**	**3**	**4**
SD (g/g CDW)	**1**	**1**	**2**	**0**	**0**	**1**	**4**	**3**	**2**	**1**	**7**	**0**	**3**	**4**	**1**	**0**	**0**	**2**	**1**	**2**	**0**	**3**	**0**	**2**	**1**	**1**	**5**	**3**	**3**	**3**	**2**	**1**

Run	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
A	**3**	**3**	**2**	**3**	**1**	**0**	**0**	**2**	**0**	**0**	**2**	**0**	**2**	**1**	**2**	**1**	**3**	**0**	**2**	**3**	**1**	**1**	**3**	**0**	**3**	**3**	**3**	**0**	**1**	**2**	**2**	**0**
B	**2**	**0**	**2**	**1**	**0**	**2**	**0**	**2**	**2**	**1**	**3**	**0**	**2**	**0**	**0**	**0**	**1**	**1**	**3**	**3**	**2**	**0**	**3**	**0**	**2**	**0**	**0**	**1**	**3**	**1**	**3**	**3**
C	**3**	**1**	**1**	**2**	**1**	**1**	**3**	**2**	**0**	**3**	**1**	**0**	**0**	**0**	**1**	**3**	**0**	**2**	**2**	**3**	**2**	**2**	**0**	**1**	**2**	**3**	**2**	**0**	**3**	**3**	**3**	**3**
EPS (g/L)	**46**	**15**	**21**	**42**	**15**	**21**	**9**	**18**	**5**	**52**	**22**	**1**	**7**	**3**	**13**	**48**	**9**	**36**	**24**	**65**	**21**	**33**	**11**	**6**	**26**	**18**	**42**	**5**	**52**	**59**	**67**	**42**
SD (g/L)	**10**	**0**	**4**	**15**	**5**	**2**	**11**	**4**	**3**	**2**	**1**	**0**	**1**	**1**	**5**	**4**	**4**	**0**	**3**	**4**	**8**	**2**	**0**	**3**	**1**	**2**	**4**	**3**	**3**	**0**	**21**	**16**
EPS (g/g CDW)	**8**	**3**	**6**	**8**	**5**	**18**	**13**	**4**	**5**	**24**	**7**	**2**	**4**	**2**	**4**	**11**	**4**	**16**	**8**	**20**	**7**	**9**	**4**	**7**	**7**	**2**	**9**	**5**	**15**	**9**	**16**	**15**
SD (g/g CDW)	**1**	**0**	**1**	**2**	**2**	**1**	**4**	**1**	**2**	**12**	**1**	**0**	**1**	**1**	**1**	**1**	**2**	**0**	**3**	**3**	**3**	**0**	**1**	**4**	**2**	**1**	**1**	**3**	**2**	**0**	**1**	**8**

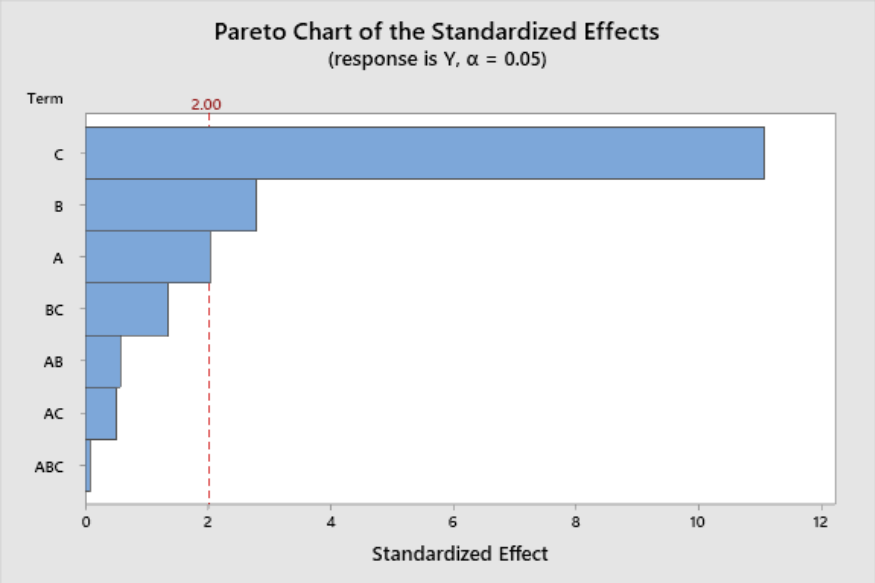

Fig. 6 Main effect of a variable on EPS production

Table. 2 Analysis of variance for anticipation of EPS-Biomass yields influencing by variables in screening design of experiment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	20965.0	2995.0	24.12	0.000
Non-Linear	3	20352.5	6784.2	54.63	0.000
A. Peptone	1	566.9	566.9	4.57	0.035
B. Beef extract	1	1936.8	1936.8	15.60	0.000
C. Sucrose	1	17848.8	17848.8	143.74	0.000
2-Way Interactions	3	598.6	199.5	1.61	0.191
AxB	1	47.3	47.3	0.38	0.538
AxC	1	8.6	8.6	0.07	0.793

BxC	1	542.6	542.6	4.37	0.039
3-Way Interactions	1	13.9	13.9	0.11	0.739
AxBxC	1	13.9	13.9	0.11	0.739
Error	120	14901.2	124.2		
Lack-of-Fit	56	12654.6	226.0	6.44	0.000
Pure Error	64	2246.6	35.1		
Total	127	35866.2			

3.3.3. Response surface methodology (RSM)

The selected optimum levels of a factor with some modifications were further optimized using face-centered composite design (FCCD). A total number of 20 different run with various combinations of peptone, beef extract, and sucrose in a set of factorials, axial and center points. The response of each run was calculated using the following regression equation: Y = 29.1 − 117 A + 79 B − 5.7 C + 161 A*A − 313 B*B + 0.78 C*C − 72 A*B + 22.6 A*C + 53.0 B*C where Y is the response variable; A, peptone; B, Beef extract and C is the sucrose The analysis of variance (ANOVA) results was analyzed to check the significance of the fit of the first-order polynomial equation of the FCCD experimental data. The model coefficient of determination R^2 value was calculated as 0.831, indicating that the model better explained the 83.11% variability in the response (Sharmila et al., 2013). The model's fisher F values of 5.47 and the p values of <0.007 indicate the model is statistically significant (Table 3). The CCD experimental runs observed and predicted responses are shown in Table 4. The optimum level of each variable and the effect of their interaction on response were studied by the 2D contour plots counter to any two independent variables withholding other variables at a constant of and 3D scattered plot. The interaction of beef extract and peptone showed that they are equally important to maximize EPS production (Fig. 7A). A nitrogen source factor A (peptone) has been demonstrated to act as a supporting role to maximize the response. In contrast, factor B (beef extract) has played a crucial role in the production of EPS. As a substrate, the factor C (sucrose) plays a fundamental building block for EPS production (Fig. 7A, B, C).

Table 3 Analysis of variance of and their influence on the response by CCD experimental design

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	8404.8	933.87	5.47	0.007
Non-Linear	3	6897.1	2299.02	13.46	0.001
A. Peptone	1	0.6	0.64	0.00	0.952
B. Beef extract	1	303.0	303.04	1.77	0.212
C. Sucrose	1	6593.4	6593.39	38.59	0.000
Square	3	139.9	46.62	0.27	0.844
AxA	1	36.1	36.10	0.21	0.656
BxB	1	136.3	136.34	0.80	0.393
CxC	1	8.5	8.46	0.05	0.828
2-Way Interaction	3	1367.9	455.95	2.67	0.105
AxB	1	21.3	21.28	0.12	0.731
AxC	1	207.6	207.59	1.21	0.296
BxC	1	1139.0	1138.98	6.67	0.027
Error	10	1708.6	170.86		
Lack-of-Fit	5	1263.7	252.75	2.84	0.138
Pure Error	5	444.9	88.98		
Total	19	10113.4			

It seems that all the factors work together to optimize EPS production. The factor sucrose with the high concentration, 4.0%, and 0.4% of the individual factor beef extract and peptone showed a maximizing effect on EPS production. (Fig. 7B, C, D). A nitrogen source factor A (peptone) has been shown to act as a supporting role to maximize the response, while factor B (beef extract) has shown a crucial role in EPS production. The factor C (sucrose), a substrate, plays the role of a fundamental building block for EPS production (Fig. 7A, B, C).

Table 4 CCD designs run with their expected and observed responses

Run Order	Peptone (%)	Beef Extract (%)	Sucrose (%)	Observed Value	Predicted Value
1	0.4	0.4	4	97.60	88.48
2	0.4	0.1	1	23.80	19.19
3	0.25	0.25	1	10.91	19.39
4	0.25	0.1	2.5	47.72	30.77
5	0.4	0.4	1	6.90	3.07
6	0.25	0.25	2.5	34.07	43.31
7	0.1	0.4	1	25.04	16.02
8	0.1	0.4	4	76.57	81.05
9	0.25	0.25	2.5	57.82	43.31
10	0.1	0.1	1	16.62	25.61
11	0.25	0.25	2.5	35.41	43.31
12	0.25	0.25	2.5	47.31	43.31
13	0.4	0.1	4	47.98	56.87
14	0.1	0.25	2.5	54.81	46.68
15	0.25	0.25	4	78.68	70.74
16	0.25	0.25	2.5	49.13	43.31
17	0.25	0.4	2.5	24.28	41.78
18	0.1	0.1	4	39.22	42.91
19	0.4	0.25	2.5	38.52	47.19
20	0.25	0.25	2.5	37.23	43.31

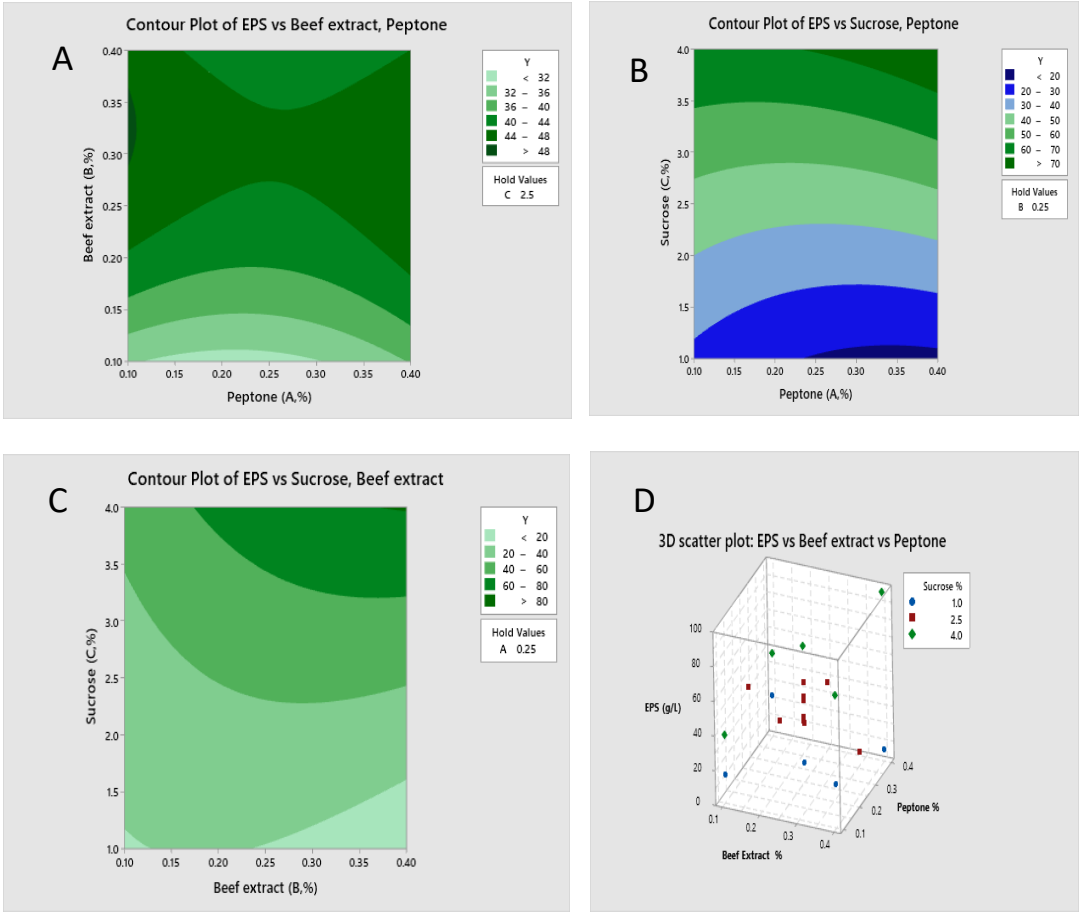

Fig. 7 Response of variables and 3D scatter plots on EPS production. a) peptone and beef extract Vs EPS, b) Sucrose, peptone Vs EPS, c) Sucrose, beef extract Vs EPS, and d) 3D scatter plot of central composite design data points.

It seems that all the factors works together to optimize EPS production. The factor sucrose with the high concentration, 4.0%, and 0.4% of the individual factor beef extract and peptone showed a maximizing effect on EPS production. (Fig. 7B, C, D).

3.4 Validation of the model at 1.0L and 10.0L fermenters level

To validate the continual of the model for predicting conditions and optimum response values, the model was validated with the selected optimal conditions of the full factorial and central composite

design in 500 mL of Erlenmeyer flask containing 200 mL of the optimal composition of media (Peptone, 0.4%, beef extract, 0.4%, and NaCl, 0.5% and 4% of sucrose) followed by 1 L and 10 L fermenters. Under the suggested conditions, the response value of EPS yield was 97.6 g/L. In shake flask studies, the results of FFD and CCD concerning BioLector have shown a variation of ±1.6 and ± 4.79 g/L, respectively (Fig. 8), which confirms the variation in the production and the reproducibility of the model process parameters. The comparative result of fermenters has also confirmed the significance of the model. The value of the Microbioreactor (BioLector Pro) to 1 L and 10 L fermenter levels showed a variation of ±9.9 g/L and ± 23.6 g/L of EPS. The variation of ±9.9 g/L and ± 23.6 g/L of EPS in the Microbioreactor to fermentor may cause the continuous supply and maintaining 10% oxygen saturation. In the validation at large scale 1 L and 10 L fermentation, optimum EPS 107.3 g/L and 123.9 g/L were observed, respectively. The optimum EPS synthesis was observed at 21h of fermentation and started decreasing (Shih et al., 2005). Once the carbon source depletes, organisms are expected to enter the stationary phase and utilize EPS as a carbon source for survival by activating its endolevanase activity. Therefore organism was observed growing continuously and could not attain the decline phase. The common condition in fermentation, as the organism metabolizes the nutrients; various organic acids are produced, indicating a drop in pH. In contrast, pH increases due to ammonia production initiated upon utilizing proteins (Fig. 9). A similar pattern of pH drop and rise was observed from 1.0 mL to 10.0L. However, the values of pH in 1.0 mL have differed. The difference in pH values cannot be explained well in 1.0 mL volume because the combinations of media components were made manually. It may be possible that upon adding stock components, pH showed variation. The added volume of the stock solution may lead to a minor variation in pH of 1.0 mL. And we have observed that the organism showed optimal growth in the range of pH 6–7 (Fig. 4b).

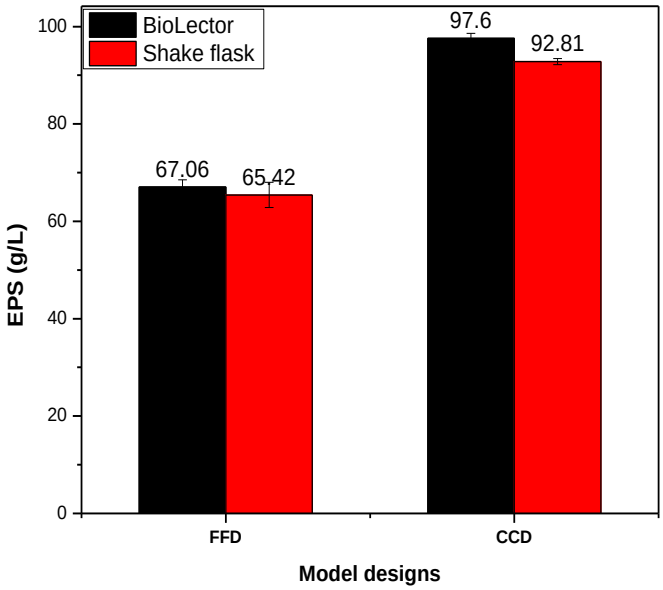

Fig.8 Microbiolector with shake flask

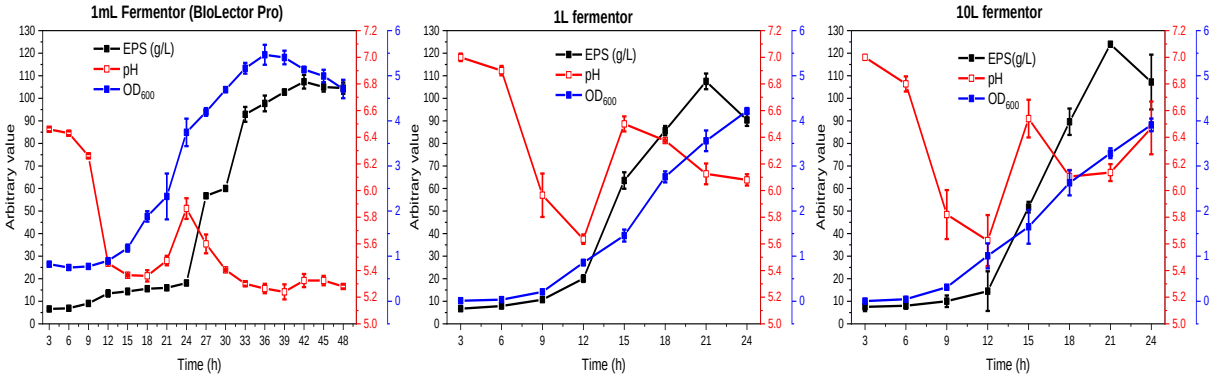

Fig. 9 Microbiolector comparative validation for EPS production. BioLector (1.0 mL) Vs Fermentor (1.0-10.0 L)

3.5 Biomass and EPS quantification

The biomass concentration was calculated by diluting the known concentration of dry weight of the cell as per the requirement. The value of optical density readings at 600 nm and light scattering

at 620 nm was plotted, and biomass concentration was calculated and correlated (Fig. 10); keeping a thumb rule, 1.0 unit of optical density corresponds to 1.0 g/L of biomass (Myers et al., 2013).

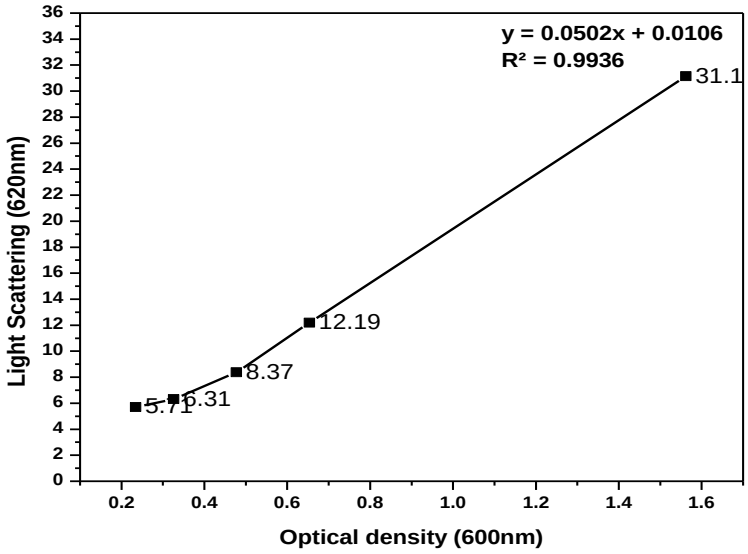

Fig. 10 Correlation of Optical density and light scattering

The viable cells were counted as CFU/mL and found that the viable cell count increased in the same pattern from 12 to 24h of cultured media in both the shake flask and BioLector (Microbioreactor). In the case of BioLector, the CFU drastically increased in 36 h, reached 1.4×10^9 CFU/mL, and declined further (Fig. 11). In the shake flask, the CFU declined after 24h. It can be inferred that the organism grows exponentially after 24h of incubation in Biolector Pro (Microbioreactor). All the EPS quantification was done by revaluated phenol sulphuric acid method by Rao and Pattabiraman (1989). The unknown concentration of EPS was estimated by a linear graph of standard pullulan polysaccharides (Fig. 12). EPS purification cannot be performed with 1.0 mL of fermented broth, as cleansing includes precipitation, deproteinization, and dialysis. The quantified EPS can be considered as partially purified or crude as it was a quantification of EPS (threaded appearance) after ethanol precipitation.

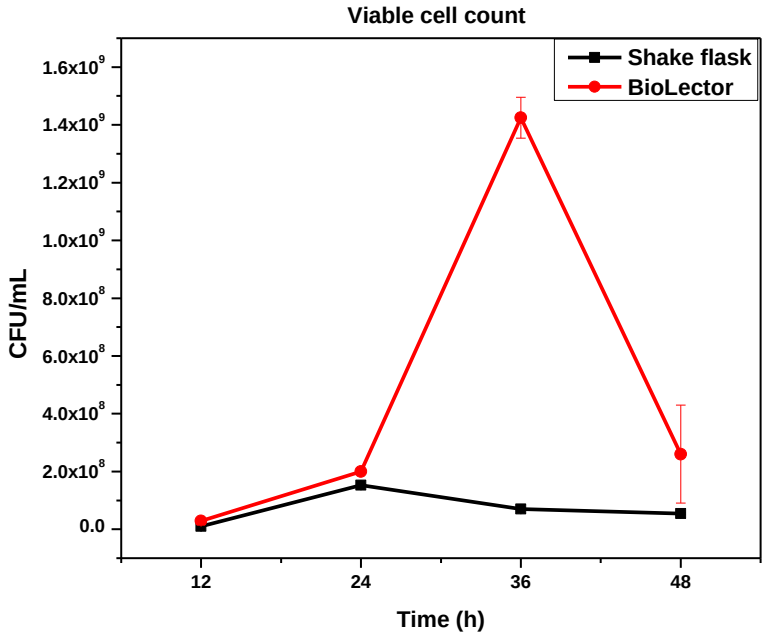

Fig. 11 Viable cell count (*Bacillus* sp. SGD-03)

Here we have shown a comparative profile of crude EPS (cEPS), deproteinized EPS (dpEPS), and purified EPS after dialysis and fraction collected by column chromatography. It was found that 20.74 g/L of pure EPS could be recovered from the crude EPS (Fig. 13).

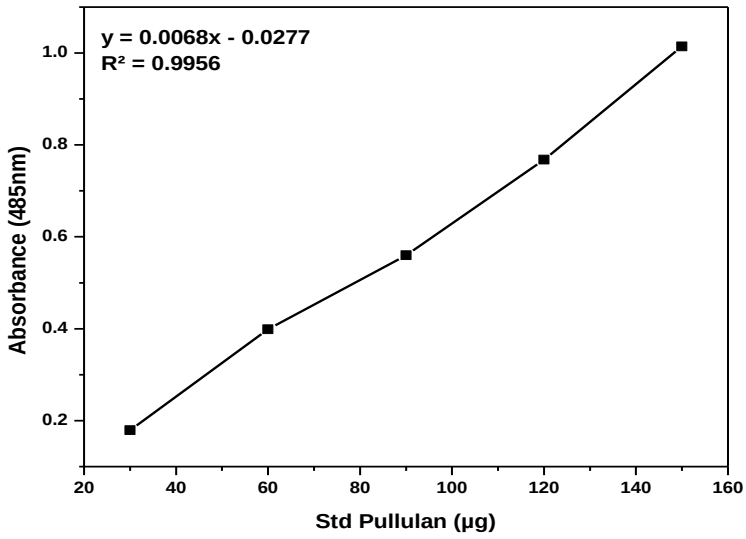

Fig. 12 EPS quantification using Standard graph for Pullulan

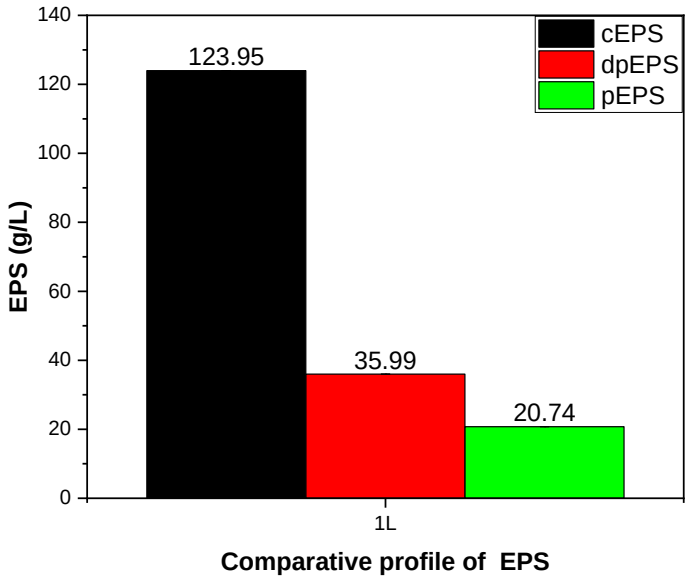

Fig. 13 Comparative profile of crude EPS (cEPS) Deprotienized (dpEPS) and Purified EPS (pEPS)

4. Conclusion

It is well known that a microorganism produces a vast range of compounds, from fuels to commodity chemicals and pharmaceuticals to fine chemicals (Zhang et al., 2016; Carbonell et al., 2018). However, despite developing microbial platforms to convert nearly any carbon source into the desired product, a modest number of these cases have seen the successful transition to industrial scale and marketed as products. Economic competitiveness with the established chemical or biosynthetic route is an important factor. Low titers and low yields in the laboratory setting also need to be overcome to proceed with the scale-up (Carbonell et al., 2018). The ability to perform the upstream processing in an automated system under sterile conditions allows for the preparing the cultivation media for small-scale cultivation directly on the robotic deck prior to cultivation (David et al., 2010; Kottmeier et al., 2010). Determination of quantitative process parameters for optimizing production strains and cultivation conditions is the key for bioprocess development for almost every new microbial product. In the EPS production processes in the MTP cultivation system BioLector Pro, the obtained growth and production behavior has been validated in 10,000 times magnitude from 1.0 mL to 10 L with similar production yield from 107 to 123 g/L of EPS,

respectively. The present study aimed to develop a cost-effective, robust process to produce EPS from a marine organism and optimize the EPS production by a high throughput Microbioreactor system. A potent EPS producer strain, *Bacillus* sp. SGD-03 was isolated from marine sediment and subjected further for optimization on the BioLector Pro system. Microbioreactor data was validated on a shake flask, followed by a 1.0 L and 10.0 L fermentor.

Production of EPS was enhanced sequentially, involving screening of the best carbon source and their different concentrations. Screening process parameters by full factorial design of experiment at various levels of factors were carried out, followed by the central composite design experiment of response surface methodology. The optimum media composition standardized with 1.0 mL of working volume in BioLector Pro was validated by ANOVA and reproduced by shake flask, 1.0L and 10.0L fermenter level, and found significant reproducibility from BioLector Pro to Fermenter, i. e., 1.0 mL: 1.0L: 10.0L fermentation. To support the hypothesis that to mimic the nutrient deficiency and osmolarity pattern, it was observed that the organism grows and produces EPS in optimal media constitutes peptone, 0.4%, beef extract, 0.4%, and sucrose, 4.0%. It was observed that the organism was grown well in 2.0–4.0% salt concentration from 18 to 20h and started declining afterward.

Similarly, EPS production was found to be maximum at 21h and decreased after. It can be concluded that the sucrose concentration (screening to optimization) 2.0–4.0% and diluted media was found suitable to mimic the pattern of osmolarity and nutrient deficiency enhancing EPS production by utilizing sucrose as a substrate. We have formulated minimal media for the complete conversion of substrate into product. At an optimized minimal production media, the marine strain produced 123.9 g/L of crude EPS, which resulted in 20.7 g/L of pure levan, and is approximately costing at 1.12 USD/L of the media chemicals used for the production. This cost will be drastically reduced when you scale-up the production and the possibility to explore for the industrial-scale

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Chapter 5:

Potential applications of levan in food and pharmaceuticals

5A. Prebiotic potential of levan

5B. Anticancer potential of levan

5A. Prebiotic Potential of Levan

Abstract

The role of fructan fiber in augmenting the healthy gut microbiota has been revealed in past decades. This study will help better understand the role of levan in the modulation of gut microbiota to improve human health. This investigated the effect of levan on probiotic fitness and its possible role in improving human health. The Albino Wistar rats were taken for the study and divided into three treatment groups and fed with different doses of levan (LD, 100mg/kg, ID, 250mg/kg, and HD, 500 mg/kg) concentration according to their body weight for 14 days. The Serum's TC, LDLC, HDLC, triglyceride, and glucose were estimated. The serum's biochemical profiling found that the triglycerides have been significantly increased in treatment groups. TC seems uninfluenced by the treatment and LDLC was found to decrease with LD and a slight increase with ID and HD. The glucose was also increased slightly, which can be attributed to the terminal glucose of levan. The probiotic abundance was quantified using qPCR and metagenomics analysis. The *Bifidobacterium*, *Prevotella*, *Bacteroides*, *Roseburia*, *Lactobacillus*, *Oscillibacter*, *Clostridium*, *Anaerovibrio*, and *Ruminicoccus* populations were significantly increased treated with levan polysaccharide. The significant increase in the probiotic group suggests levan's potential prebiotic characteristics.

1. Introduction

Levans has been reported for many applications in various streams. These are considerably more associated with human health. Due to the adherent property of levan, the human gut helps probiotic organisms for colonization. It has been stated that gut microbiota influences the function of intestinal epithelial cells, including gene expression, cell growth, and proliferation (Peterson et al., 2015). The human body comprises trillions of microbial cells, mainly part of the skin and intestine. These microbes are the savior of humans, maintaining gut homeostasis and strengthening the immune system. The gut microbes communicate with immune cells to train the host to respond to the infection (Rooks & Garrett, 2016). Probiotic microbes, such as *Bifidobacterium*, *Lactobacilli*, *Bacteroides*, and *Enterobacter*, are indispensable to the gut. Extensive use of antibiotics and unhealthy eating habits alter gut microbiota, which disturbs the relationship with the host organism. The perturbation in homeostasis of gut microbiota causes gastrointestinal diseases such

as gastroesophageal reflux, intestinal bowel disease, colorectal cancer and hemorrhoids, duodenal ulcer, etc. (DeGruttola et al., 2016; Wu & Lewis, 2013). Improvement of gut health has been an encouraging topic of human gut research. Several ways can improve gut health; among them, adopting a fiber-rich diet is easy and preferable to restore the gut microbiota imbalance. It is stated that a diet with enough fiber can prevent or minimize disease conditions. Regular intake of dietary fibers is a crucial food supplement that can maintain a healthy gut. A vegetarian diet can provide such fibers from fruits, fermented foods, whole grains, leafy vegetables, etc. Dietary fibers are non-digestible carbohydrates called prebiotics which are unable to digest by human digestive enzymes and thus cannot provide energy to the host for metabolic processes. Several non-digestible polysaccharides have been reported showing their prebiotic potential (De Vuyst & Degeest, 1999). Prebiotics resist gastric acidity, hydrolysis by mammalian enzymes, and intestinal absorption. They do not hydrolyze and are absorbed by the intestine and reach the colon. They are fermented into short-chain fatty acids (SCFAs) by the colonic microbes and assist the growth of healthy microflora (Den Besten et al., 2013). These fatty acids help prevent weight gain, diabetes, heart disease, and cancers.

Humans are fortunate to have certain gut bacteria called probiotics that efficiently metabolize such compounds producing valuable products (SCFAs). Xylo-oligosaccharides (XOS), fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose, lafinose, and inulins are common prebiotics. Among these, FOS is more common and encourages the growth of beneficial probiotic bacteria, which helps to control several gastrointestinal diseases, as low molecular weight fructans are known for their prebiotic potential. Levans is only a known diverse MW fructose-based polysaccharide composed of β 2-6 linkage. These are soluble in water, fats, and dimethyl sulfoxide (Manandhar et al., 2009), which supports their application in the environment, cosmetics, foods, and bio-medical sectors. The gelling property of levan may encourage colonization health, promoting colonic bacteria such as *Bifidobacteria*, *Bacteroides*, *Lactobacillus*, etc. Low molecular weight levans are now emerging as prebiotics that bacteria can easily obtain.

Recently, gut research has drawn attention to protecting the health of an individual caused due to unhealthy eating habits. Currently, the nutritional status of gut health is maintained by various medications with certain side effects. In search of natural substitutes for maintaining a healthy gut, fructans have paid much attention. Gut microbiota takes care of the physiology of human beings.

In reciprocation to the gut microbiota, an individual need to take care of their gut microbiota. A comprehensive understanding of gut microbiota is a primary need to protect them. To obtain a comprehensive profile of gut biodiversity, the whole metagenome sequencing approach is efficient in revealing microbial diversity to examine the probiotic microbes. To examine the indigenous population of the gut, metagenomics data are efficient in providing a comprehensive profile of gut microbes for a better understanding of microbial diversity. This study has characterized and optimized low molecular weight levan (10 kDa) from a marine Bacillus sp. which can be a promising prebiotic supplement. In this objective, we are heading towards knowing levan's influence on the abundance of probiotic community of gut microbiota using quantitative PCR targeting dominant probiotic groups. Understanding total population and metagenomics data will be an excellent tool for in-depth knowledge of total microbial biodiversity.

2. Material & Methods

2.1 *In-vitro* testing of levan

To test the prebiotic potential of levan, an initially *in-vitro* Study was carried out. Reference strains (*Bifidobacterium bifidum*, NCIM 5697; *Lactobacillus fermentum*, NCIM 2165) were procured from a culture collection NCIM resource center, Pune, India, and grown in MRS media (HiMedia) supplemented with different concentrations of levan (named after producing organism (0.1, 0.25, 0.5, and 0.75) and incubated at 37°C for 48h in anaerobic and microaerophilic conditions. After incubation, the optical density was measured at 600nm using 96 well plate reader.

2.2 Animals and drugs

Animals were obtained, and experiments were performed at the National Institute of Biosciences and MIT World Peace University, Pune, India, respectively. A total of 36 animals (both gender), including species and strain [Swiss mice, 25-35 g (6 in number) and Albino Wistar rats, 100-200 g (30 in number)] were procured and maintained under optimum growth conditions for adaptation of animals (15-20 days) with regularly feeding. The approval was taken from the institutional animal ethics committee (IAEC) prior to animal experimentations and submitted to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Animals were treated according to approved guidelines. (Animal experiment protocol no.: SOPMITWPU/IAEC/2021-22/M1/01).

2.3 Toxicity and efficacy testing

The compound was tested for its adverse effects on Swiss mice. The higher dose of 2000 mg/kg body weight was selected based on the OECD drug administration guidelines for oral toxicity. Using the gavage technique, the test compound (levan) was solubilized in deionized water and administered orally in a triplicate group. After dosing on the first day, the behavioral toxicity or mortality status was monitored regularly for the 14 days of the observation. After confirming the toxicity profile, Albino Wistar rats were treated with levan to assess their prebiotic potential. The study involved 30 animals (male and female, 1:1) divided into five groups; two were assigned for vehicle and standard control, and three for treatment groups. Based on acute toxicity, three doses of test compound were chosen; low dose (LD, 100 mg/kg); intermediate dose (ID, 250 mg/kg); and high dose (HD, 500 mg/kg) of body weight. Inulin (500 mg/kg) and sterile deionized water were used as standard and normal (vehicle) control, respectively. Freshly prepared levan solutions were administered regularly for 14 days by gavage technique.

2.4 Sample Collection

After the feeding period of 14 days, fresh fecal samples from experimental animals were collected in sterile micro centrifuges tubes. Samples were withdrawn by gently squeezing the rectal part of animals and stored at -80°C until processed further. Following 14 days of feeding, animals were anesthetized, and blood samples were collected in a heparinized tube from the retro-orbital sinus. After collection, whole blood was allowed to clot at RT for 30 min, serum was separated by centrifuging at 2000 × g for 15 min, and samples were stored at -80°C for biochemical estimation. Further, animals were euthanized and dissected to obtain liver, kidney, and intestine tissue and stored at 4°C for histopathological analysis.

2.5 Biochemical estimation

The serum's total cholesterol, LDL cholesterol (LDLC), HDL cholesterol (HDLC), triglyceride, and glucose were estimated calorimetrically using commercially available total cholesterol,

triglyceride, glucose (Erba Mannheim), direct LDL (Pathozyme Diagnostics) and direct HDL (UltiChem- Yucca) kits by endpoint method according to the manufacturer's instructions.

2.6 Histopathological analysis

The kidney, liver, and intestine were separated and washed thoroughly with a physiological saline solution. Tissues were fixed by immersing in a 10 % neutral formalin solution for 15 days. The residual fixative was removed, and tissues were washed with the aforementioned saline solution. Further, tissue samples were dehydrated with an ethanol gradient (30 %, 50 %, 70 %, and 100 %) and embedded using the molten paraffin blocks following the classical procedure. Further, tissues were sliced by the microtome to obtain thin sections for staining. Tissue sections were deparaffinized and rehydrated prior to staining. Tissue sections were stained by hematoxylin and eosin (H & E) staining protocol, observed under a light microscope, and results were noted.

2.7 DNA extraction

To examine the effect of levan on the probiotic population, two gut-dominating probiotic groups, namely *Bifidobacterium* and *Lactobacillus*, were chosen as the reference group. Previously procured reference strains were taken, and DNA was isolated for standard curve generation. The frozen fecal samples were thawed and used for DNA extraction. Extraction and purification of fecal genomic DNA (fgDNA) were performed using QIAamp® PowerFecal® Pro DNA Kit, Quigen. Briefly, the homogenized fecal samples were treated chemically and mechanically with lysis buffer CD1 and PowerBead. Suspensions were vortexed and centrifuged at maximum speed for 10 and 1 min, respectively. Supernatants were transferred to a sterile microfuge tube and vortexed for 5 s after adding solution, CD2. They were then centrifuged at 15000 ×g for 1 min. Solution CD3 was added to the supernatants, vortexed for 5 s, and loaded to the MB spin column. The suspension was centrifuged at 15000 ×g for 1 min, the flow-through was discarded, and the step was repeated for solution EA and solution C5. Finally, total DNA was eluted with solution C6 and stored at -20°C for further analysis. A similar protocol was followed for the extraction of DNA from reference strains. The DNA quality was confirmed by agarose gel electrophoresis and quantified using the Qubit quantification method applying high sensitivity assay.

2.8 qPCR-based quantification and analysis

Target gene, 16S rRNA, from reference cultures were amplified individually by polymerase chain reaction (PCR) using group-specific primers: *Bifidobacteria* primers, 5'CGCGTCYGGTGTGAAAG3' (F- Bifido) and 5'CCCCACATCCAGCATCCA3' (R- Bifido); *Lactobacilli* primers, 5'GAGGCAGCAGTAGGGAATCTTC3' (F-Lacto) and 5'GGCCAGTTACTACCTCTATCCTTCTTC3' (R-Lacto) (Delroisse et al., 2008). The reaction was carried out in a 25 µL reaction volume containing: 1 µL template (10-50 ng/µL), 2.5 µL of each primer (320 nM), nuclease-free water (NFW), and 12.5 µL 1X EmeraldAmp® GT PCR Master Mix, TaKaRa Bio India LTD. The amplicons were generated using the following parameters: initial denaturation and final extension of DNA were performed at 98°C for 5 min and 72°C for 10 min, respectively. Denaturation at 98°C, 5 s; annealing at 55°C, 10 s; and extension at 72°C, 30 s were applied for the amplification cycles (40 cycles). To purify the desired fragment, amplified products were electrophoresed on 2.0 % agarose gel and excised from the gel under aUV transilluminator. The desired DNA fragments were purified using the QIAquick® Gel Extraction Kit, Qiagen, and quantified using the Qubit method. Purified DNA was diluted serially at a logarithmic scale with 1×10^{-1} to 1×10^{-7} ng/µLgenerating a standard curve to quantify unknown DNA concentration. The qPCR amplification reaction was carried out in a 10 µL volume. Final concentration containing: template, 1 µL (1 ng/µL fgDNA) (); primers, 1 µL of each (320 nM) and Yellow sample buffer, PowerTrack™ SYBR™ Green Master Mix (Applied Biosystems), and sterile nuclease-free water (NFW) were added according to the manufacturer's instructions. The plate was spun for 30 s at 1000 rpm to settle minute droplets at the bottom. 40 amplification cycles were performed by following conditions; denaturation at 95°C for 10 min and 15 s was applied for initial and subsequent cycles. Annealing and extension were performed at 60°C for 1 min, and signals were captured at 80°C for 15 s. A melt curve was also generated to examine non-specific amplification. The analysis was carried out by the StepOnePlus Real-Time PCR system and the software Thermo Fisher (Applied Biosystems).

2.9 Metagenome sequencing and Data availability

Further, the purified individual fgDNA samples were subjected to whole metagenomic shotgun sequencing using a MinION sequencer. Briefly, library preparation of the extracted DNA was

performed by means of 1D Native barcoding genomic DNA (EXP-NBD103) and Ligation Sequencing Kit 1D (SQK-LSK108) protocol as suggested by the (Oxford Nanopore Technologies, Oxford, UK). *Pre-processing of the reads and Statistical analysis of the data.* Albacore (v2.2.7) was used to base-call the reads. Poretools, a flexible toolkit for analyzing the datasets produced by the MinION nanopore sequencer, was used for further Quality Check (QC) (Loman & Quinlan, 2014). Error correction of the reads was achieved with Canu to give good-quality long reads (Koren et al., 2017). Singleton was removed, and the OTU table was rarefied to even sampling depth based on the sample having the lowest sequencing reads. Then OTU distribution pattern was plotted in R, followed by applying the Shapiro-Wilk normality test was also calculated to confirm the normal distribution of data. Parametric tests were applied to calculate the beta diversity and alpha diversity. Alpha-diversity plot was constructed using Shannon and Simpson index, and a statistical test (t-Test/Anova) was also calculated based on the grouping of samples. Principal Component Analysis (PCoA) plot for beta diversity estimation was constructed using the Bray-Curtis dissimilarity matrix. Significance in the dissimilarity measure was evaluated using ANOISM and PERMANOVA (Adonis test p 0.05). Additionally, to estimate the compositional homogeneity between the groups Betadisper test (p 0.05) was applied to the datasets. To control the errors arising from multiple comparisons, Benjamini-Hochberg (BH.) FDR procedure and Bonferroni correction were used.

The metagenomic datasets used in this study are publicly available on Sequence Read Archive (SRA) portal under the bioproject PRJNA. The details of all the samples with their accession IDs have been collated in supporting information (SRA biosample accessions_ PRJNA).

3. Results and discussion

3.1 Effect of levan on animal physiology

After the administration of levan, animals were kept under observation for 14 days. The mortality and behavioral changes of animals were examined. Animals were found to be healthy at the higher dose of 2000 mg/kg and had no negative impact on their physiology. Levan was a safe drug candidate with no adverse effect on animal behavioral changes. The result indicates that the below 2000 mg/kg dose can be chosen for further study.

3.2 Effect of levan on serum biochemistry

On completion of treatment (14 days), the influence of levan was measured for the serum's total cholesterol, LDL cholesterol (LDLC), HDL cholesterol (HDLC), triglyceride, and glucose. The values (mg/dl) are expressed as Mean±SEM, n=6. Values of standard control and test compound treated groups are compared with normal control by ANOVA followed by Dunnett's test. GraphPad Prism 9.2 was used for the data analysis. The results of the biochemical estimation are summarized in Table 1. Comparing normal control and treated groups, the levan diet found the total cholesterol level uninfluenced. However, a slight rise in total cholesterol was observed in the standard control (Inulin), which is noteworthy for the levan as a test, so it can be concluded that the levan is more promising than inulin. It was stated that people who take enough prebiotics in their diet could be prevented cardiovascular disease. Triglyceride is blood fat and a very important component providing energy. It stores in body fat cells and is released when more energy is required (Bouhnik et al., 1994). A significant increase in triglyceride was observed in standard control and treated groups compared to normal control. The $p<0.0001$ have confirmed its statistical significance. Treatment of 100 mg/kg drug (levan) effectively reduced the LDLC. Surprisingly, LDLC increased slightly in the treatment group of 250 mg/kg and 500 mg/kg. The rise in LDLC was also observed in standard control (Inulin 500 mg/kg). Higher refined sugar intake, such as fructose or fructose-based polymers, affects the serum lipid profile and can increase LDLC levels (DiNicolantonio et al., 2016). A similar observation has been reported by Bahroudi et al., 2020. Upon comparing the LDLC of standard and treatment groups, it seems a high drug level can reverse the cholesterol level. It may be stated that a high intake of levan can increase LDLC; therefore, a low dose can be recommended. The HDL cholesterol (good cholesterol) was found to increase in the treatment of 100 mg/kg and 500 mg/kg of body weight, while HDLC level was uninfluenced in the treatment group of 250 mg/kg. The HDL was significantly increased in the treatment groups compared to the normal control, suggesting that levan positively impacts the HDLC profile. HDL cholesterol carries free cholesterol from peripheral tissues, which helps remove blockages of blood vessels. Levan's helps increase the HDL cholesterol in the bloodstream, which is a significant observation. A minimal amount of glucose rose in the treatment groups compared to the control groups. It may attribute to the presence of terminal glucose residue of levan.

Table 1 Estimations of serum biochemical

Groups	Glucose (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL-Cholesterol(mg/dl)	HDL-Cholesterol (mg/dl)
Group-I (Vehicle control)	85.17±2.05	63.25±2.40	60.14±2.55	73.76±7.19	7.52±0.59
Group-II (Standard control)	88.83±2.53	71.57±4.61	87.55±2.21 ***	80.92±5.93	8.2±0.75
Group-III (100 mg/kg of test compound)	96±2.14	62.41±2.20	77.37±1.04 ***	70.32±5.86	8.72±0.39
Group-IV (250 mg/kg of test compound)	92±3.41	63.62±4.16	87.64±1.92 ***	84.40±2.38	7.48±0.74
Group-V (500 mg/kg of test compound)	91±2.88	61.25±1.99	91.75±1.03 ***	76.71±7.52	9.96±0.39

***p<0.0001 when compared with normal control

3.3 Effect of levan on visceral organs

After the experimentations, visceral organs, including the liver, kidney, and intestine, were excised and stained with H&E stains for histopathological observations. It was observed that the levan has a negligible effect on the visceral organs. The results were compared with control groups. No abnormalities were detected in the mucosal epithelium of the intestine in both the sex. No/minimal abnormalities such as vascular congestion and degenerative changes were marked in renal tubules/glomerulus in the kidney of both sexes with high doses (250 mg/kg and 500mg/kg). In the case of the liver, no abnormalities were found with a lower dose (100 mg/kg), while minimal

degenerative changes were seen with intermediate (250 mg/kg) and higher (500 mg/kg doses in both sexes. The microscopic observation showed that overall pathological grades were minimal at focal points. Any foreign drug has some impact on the organs. No significant lesions on organs were observed upon treatment of levan; therefore, these results have proved levan is safe. The overall results are summarized in Fig. 1.

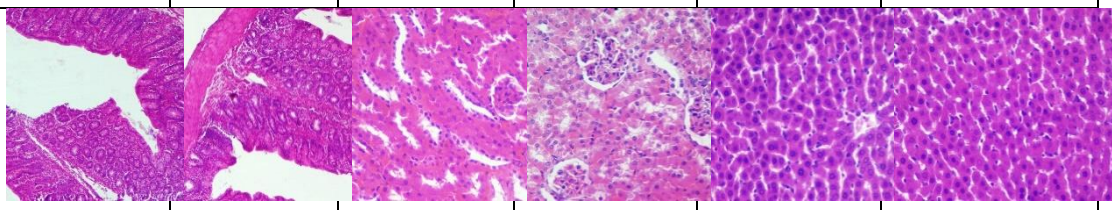
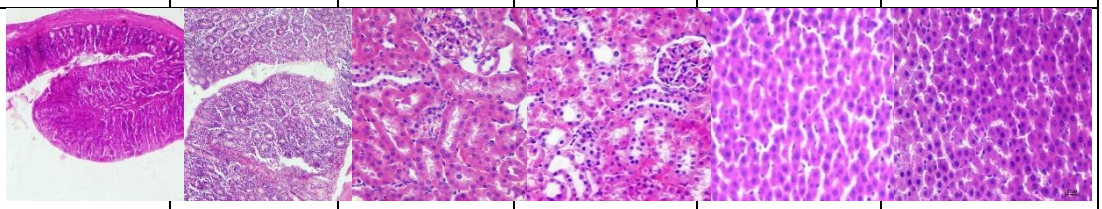
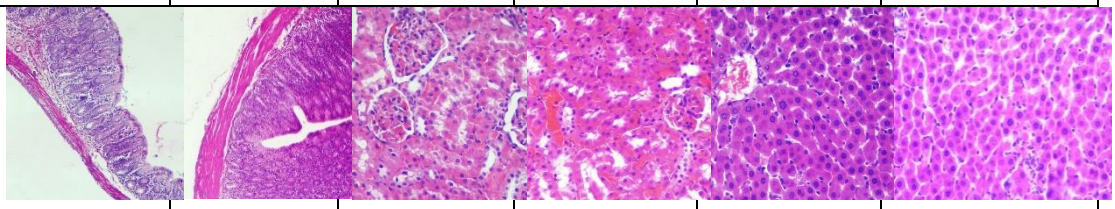
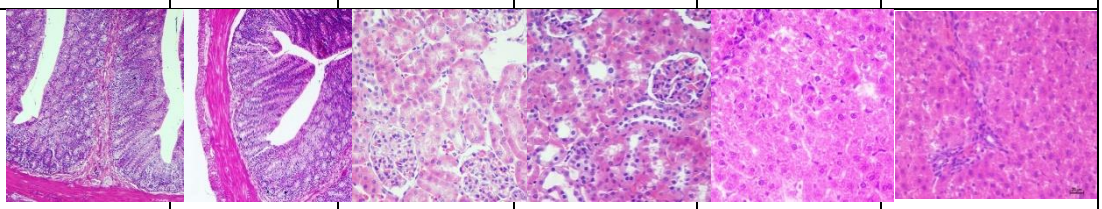
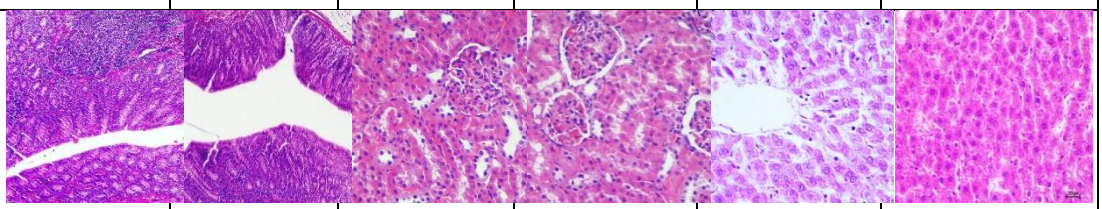
	Intestine (Female)	Intestine (Male)	Kidney (Female)	Kidney (Male)	Liver (Female)	Liver (Male)
Group-I (Vehicle control)						
Group-II (Standard control, 500 mg/kg)						
Group-III (100 mg/kg of test compound)						
Group-IV (250 mg/kg of test compound)						
Group-V (500 mg/kg of test compound)						

Fig. 1 Histopathological observations of liver, kidney, and intestine tissue

3. 4 Effect of levan on probiotic bacteria

Initially, the prebiotic potential of levan was confirmed by *In-vitro*, and it was found that levan is directly proportional to probiotic bacteria. As concentration increase, the growth of organism also increase. An *In-vitro* study has suggested that levan can be used as a prebiotic supplement for further animal studies (Fig. 2)

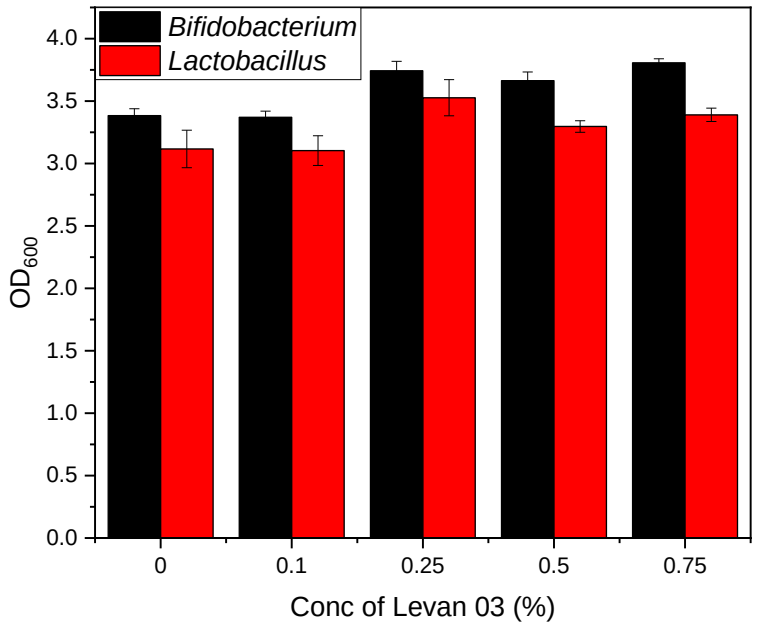


Fig. 2 Effect of levan on *Bifidobacterium* and *Lactobacillus* (*In-vitro*)

After confirming in-vitro studies, levan was fed to an animal to evaluate its prebiotic potential. Quantitative measurements of the probiotic bacterial population were assessed by qPCR utilizing the fecal DNA of experimental animal groups. The rats were fed a low dose (100 mg/kg), an Intermediate dose (250mg/kg), and a high (500 mg/kg) of levan. Water and inulin (500 mg/kg) were taken as control groups. Following treatment of 14 days, fecal samples were collected, and genomic DNA was extracted, purified, and subjected to qPCR. The standard curves with R^2 of

0.9998, 1, and 1 were generated for *Bifidobacterium* and *Lactobacilli*, respectively (Fig. 3A, 3B) to measure the unknown concentration of DNA copy number.

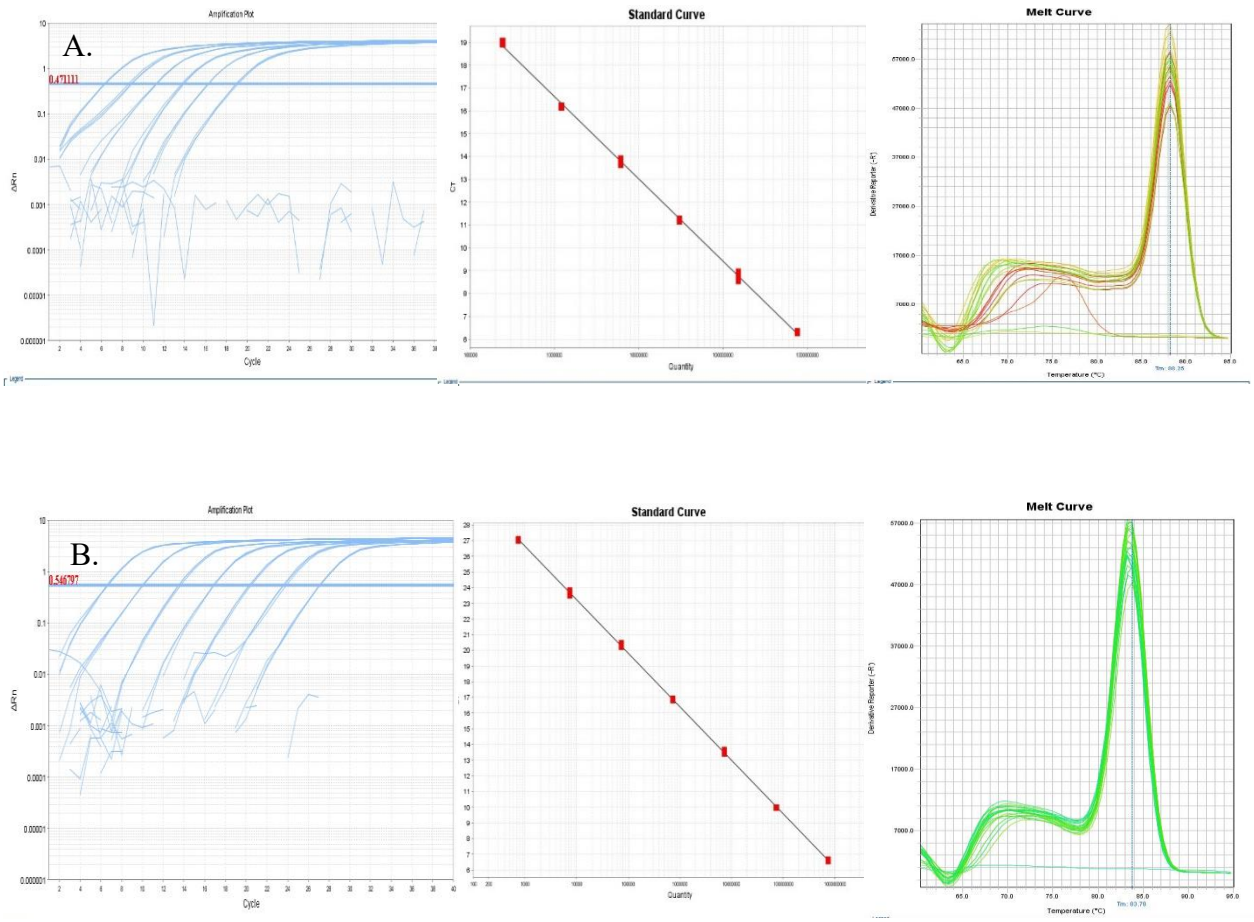


Fig. 3 qPCR curve (Amplification, standard, and melt curve): A. *Bifidobacterium;* B. *Lactobacilli*

The qPCR results revealed that the number of *Bifidobacterium* and *Lactobacilli* were significantly increased in the treatment groups compared to normal control at low and intermediate levels (copy number was reflected in logarithmic value). However, no significant gradual increase of bacterial populations was observed with an increasing levan dose. (Fig. 4A, 4B). *Bifidobacterium* is a dominant bacteria of gut microbes that begin to grow inside babies' intestines by digesting mothers' milk containing oligosaccharides (Turroni et al., 2012). It has also been stated that leaky gut syndrome can be cured by sealing the gaps between intestinal cells with the help of

Bifidobacterium and *lactobacilli*. It can be concluded that the low dose and intermediate dose of levan were enough probiotogenic potential.

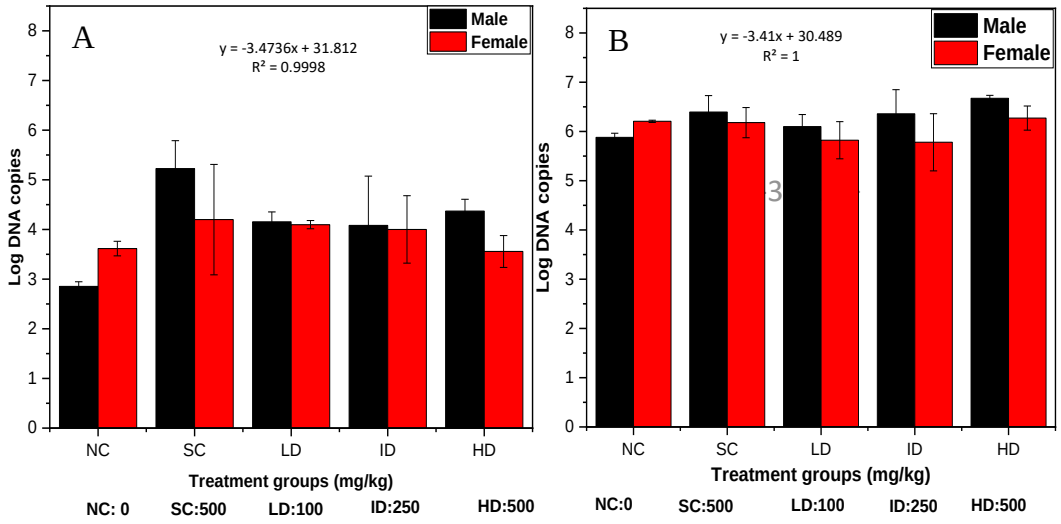


Fig. 4 Potential effect of levan on probiotic bacteria: A. *Bifidobacterium;* B. *Lactobacilli*

The *Bifidobacteria* and L*actobacillus* are well-known probiotics in the human gut. Further, other gut resident bacteria were accessed using a metagenomics study. The taxonomic classification of gut microbes was examined, and the top 20 phyla were analyzed. The Bacteroidetes and Firmicutes were found to be most abundant in all treatment groups.

3.5 Microbial Diversity indices

Alpha diversity was measured for the samples with control and treated groups. Compared to the other doses, the standard and low levan doses showed a higher microbial richness and evenness in terms of microbial diversity of males. In the case of females, a higher microbial richness and evenness were observed with an intermediate and high dose of levan together with the standard control (Fig. 5). Computation of the beta diversity indicated low dose has more microbial diversity as compared to the other doses and control along axis 1 (55.8%) (Fig. 6).

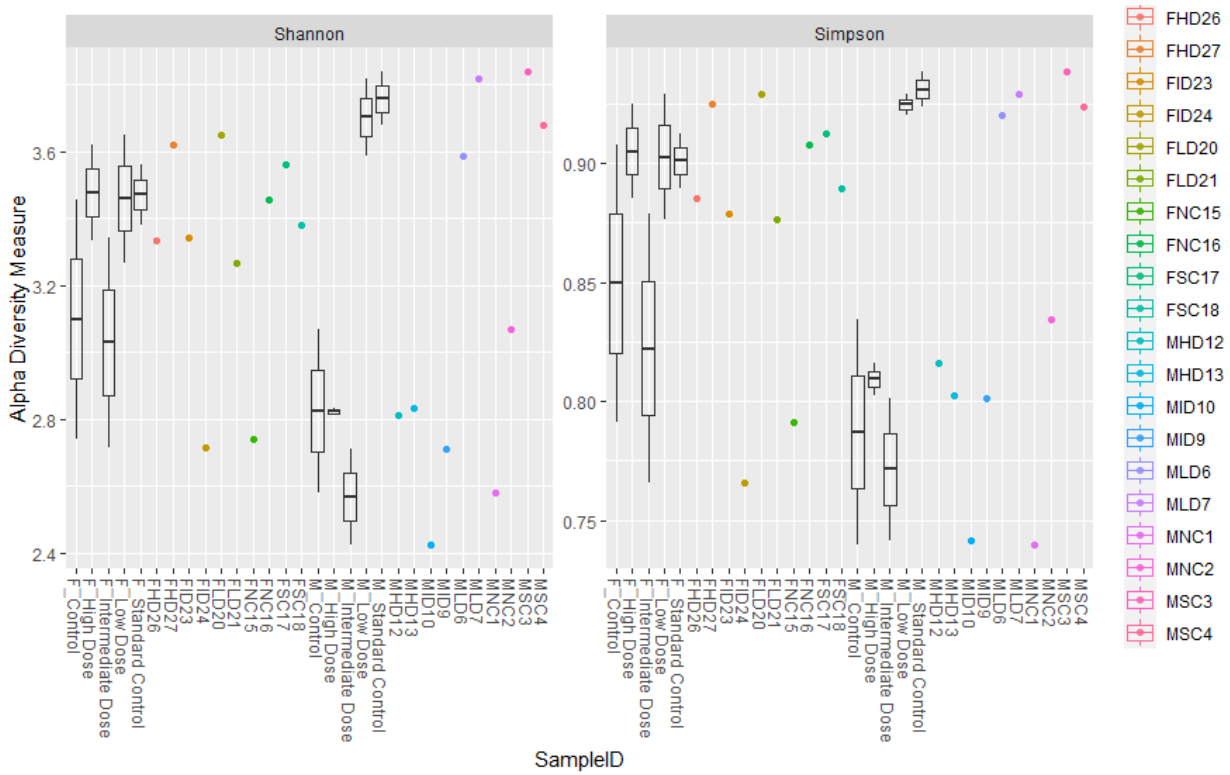


Fig. 5 Alpha diversity of gut microbiota

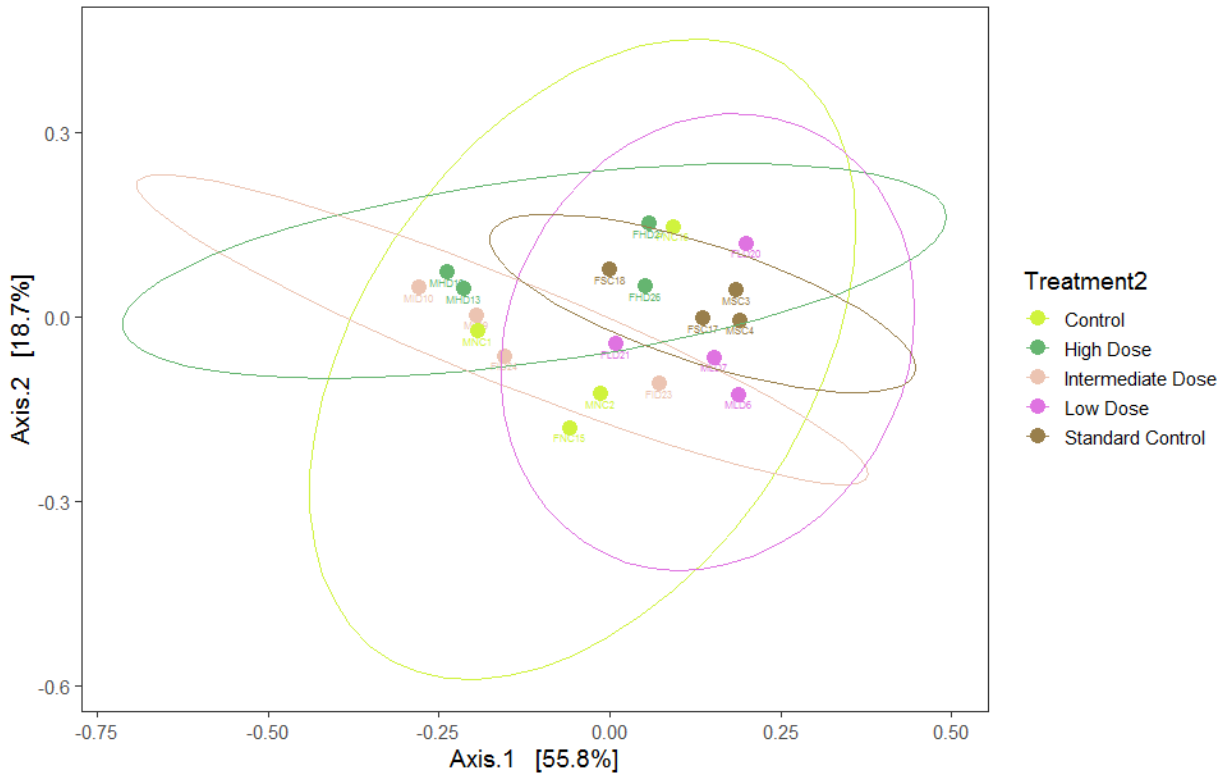


Fig. 6 Beta diversity of gut microbiota

3.6 Bacterial community composition

Metagenomic assessment of the bacterial community through nanopore sequencing revealed a predominance of phyla comprising of the probiotic bacteria Bacteroidetes (45-65%) and Firmicutes (25-30%) (Fig. 7). In recent years, there is considerable evidence of the importance of Firmicutes/Bacteroidetes (F/B) ratio in maintaining the intestinal homeostasis. A decreased F/B ratio is the marker for gut dysbiosis (Stojanov et al., 2020).

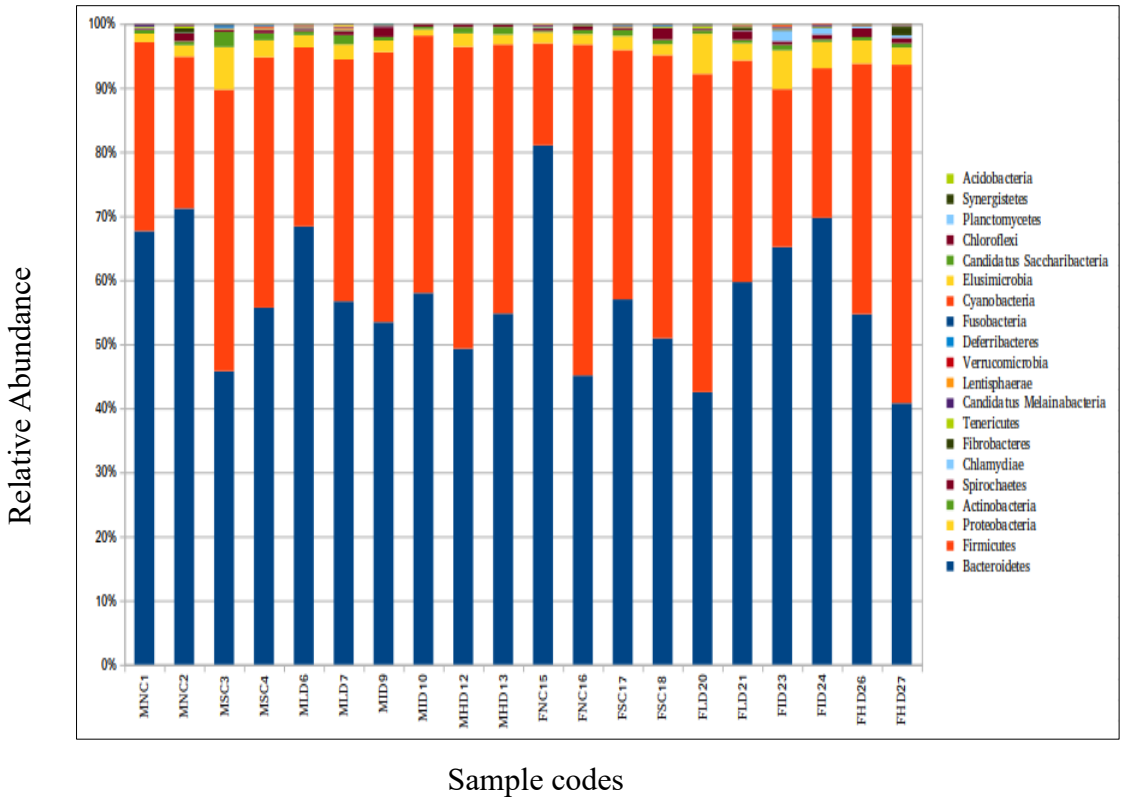


Fig. 7 Bacterial community composition of top 20 phyla

At the genus level, a predominance of *Prevotella* was observed in all the samples with a relative abundance of (42-78%) (Fig. 8a). However, compared to the control samples, a relative decrease in the abundance of *Prevotella* was observed for this group. However, this was not significant (Fig 8b). Additionally, this result corroborated with the qPCR data wherein the log DNA copies of control and test groups with respect to *Prevotella* showed no significant differences (Fig. 9). Besides *Prevotella*, the other dominant genus was *Bacteroides*. These two bacteria are reported to exceed their relative abundances beyond 40% in gut-microbiome studies (Gorvitovskaia et al., 2016). Several studies with microbiome sequencing of the gut microbial communities have reported a predominance of *Prevotella*, *Bacteroides*, *Acidminococcus*, *Roseburia*, *Faecalibacterium*, *Anaerostipes*, *Parabacteroides*, and *Clostridiales*. However, in this study, *Prevotella*, *Bacteroides*, *Anaerovibrio*, *Roseburia*, *Lactobacillus*, and *Clostridium* were found among the dominating taxa.

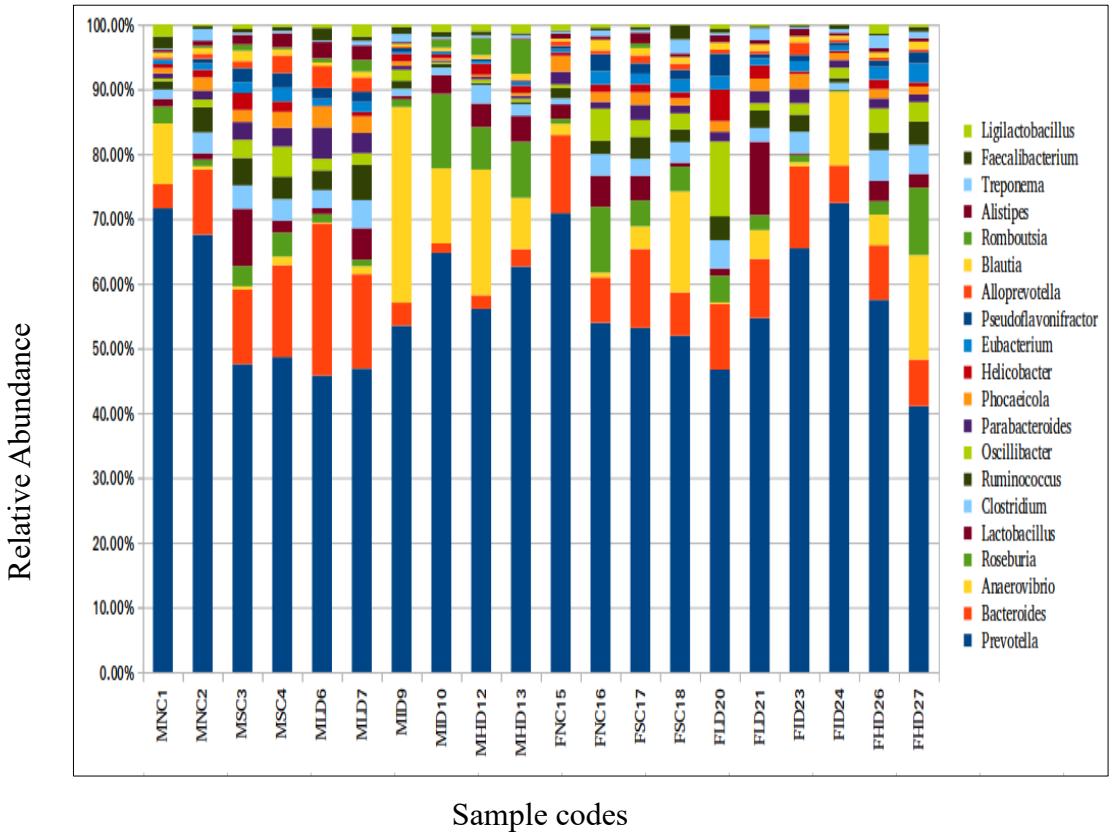


Fig. 8a Bacterial community composition of top 20 genus

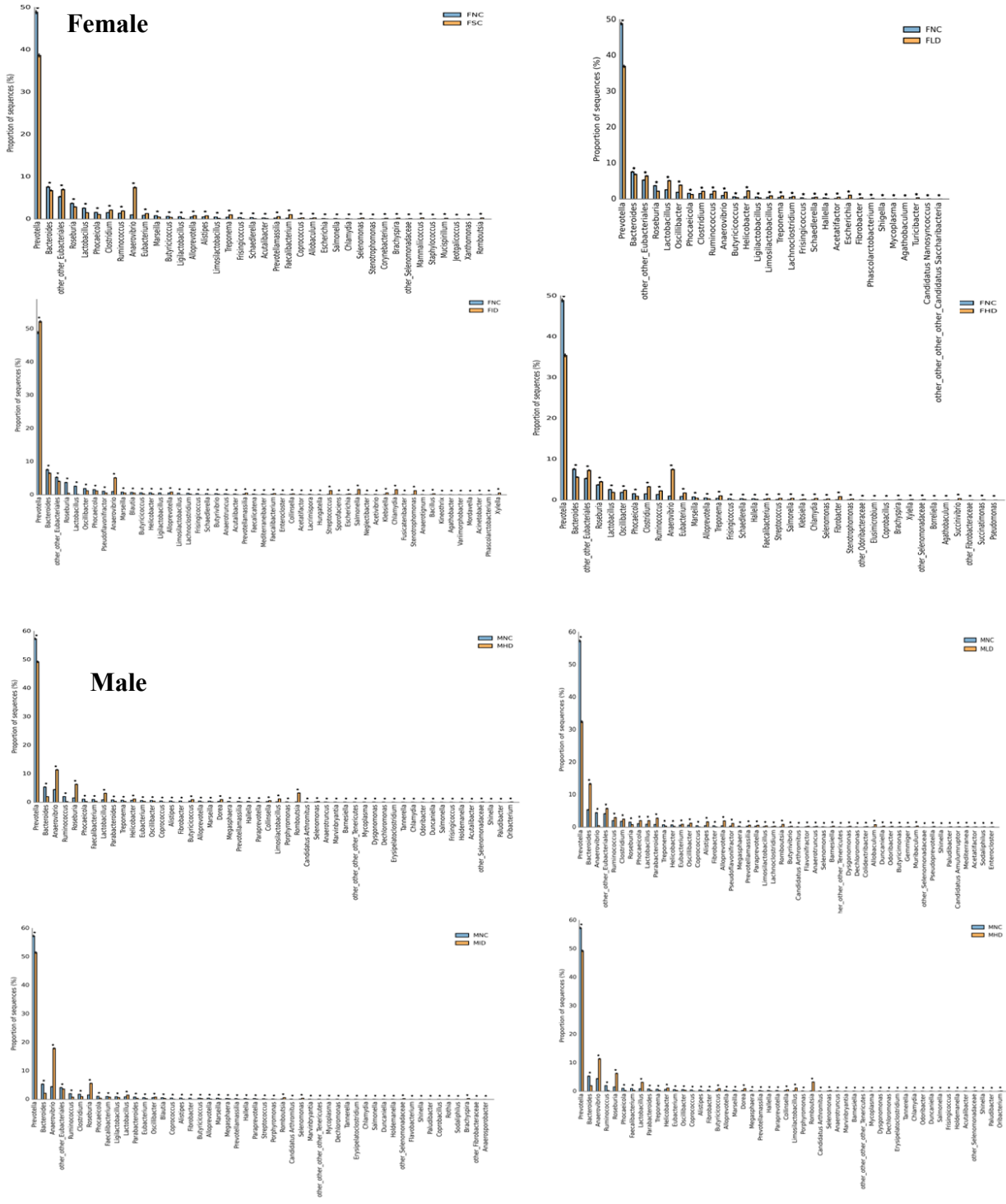


Fig. 8b Bacterial community composition of different taxa

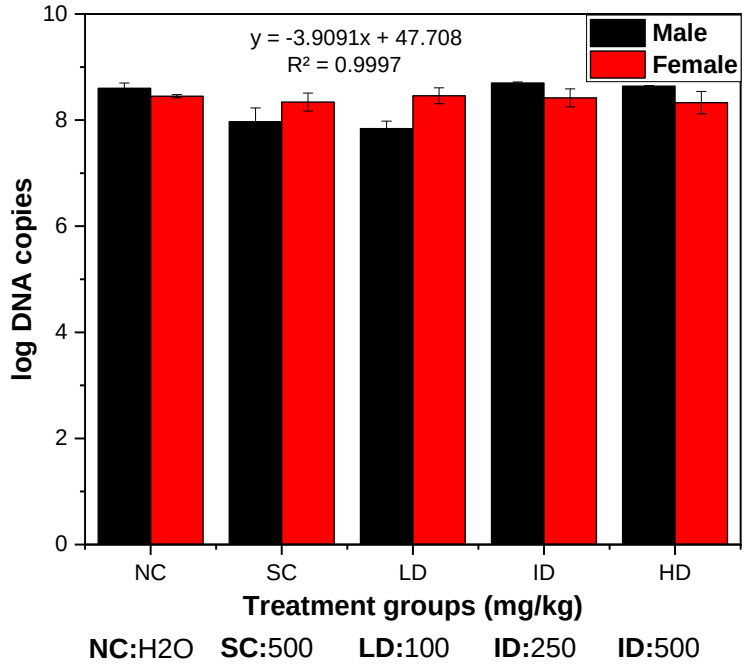


Fig. 9 Potential effect of levan on *Prevotella*

Further, analysis with respect to the proportion of sequences was carried out. Compared to normal control, a significant increase in the proportion percentage of *Prevotella*, *Bacteroides*, *Roseburia*, *Lactobacillus*, *Oscillibacter*, *Clostridium*, *Anaerovibrio*, and *Ruminicoccus* was observed. Anaerovibrio is a lipolytic bacteria that hydrolyzes triglycerides into glycerol and fatty acids. The addition of levan clearly promoted the growth of beneficial fecal microbiota. The 2-fold increase in the levels of microbiota belonging to the genus *Prevotella* is seen when the mice are exposed to intermediate and high doses of levan compared to low dosage. The genus *Anaerovibrio* showed similar results with a 15% increase in all dose intensities compared to control showing increased health benefits that also play an essential role in rumen lipid degradation (Strömpl et al., 1999). By producing extracellular lipase, *Anaerovibrio* decomposes lipids and glycerol into free VFAs.

On the contrary, the relative abundance of the genus *Bacteroides* is higher in the group with a low dosage of levan as compared to the control but reduces to less than 5% with increasing dose. Other genera belonging to *Lactobacillus* and *Roseburia* were also seen to be increased at intermediate and high doses rather than low dose. The intermediate dose of levan also promoted the growth of

Faecalibacterium can reduce the severity of inflammation and enhance intestinal barrier function by releasing metabolites (Lopez-Siles et al., 2017). The genus *Romboutsia* showed increased hits when subjected to a higher dosage of levan but not at intermediate or low doses. The genus *Romboutsia* covers a broad range of metabolic capabilities with respect to carbohydrate utilization, fermentation of single amino acids, anaerobic respiration, and metabolic end products (Gerritsen et al., 2014). The standard control showed increased hits as compared to normal control for most of the genera, such as *Bacteroides, Eubacteriales, Ruminicoccus, Clostridium, Lactobacillus, and Oscillibacter*. This showed that the presence of gut microbiota is almost changed when the host is exposed to probiotics such as levan, each genera having its' function towards the benefit of the host. All of these findings imply a positive correlation of the host towards metabolization of incorporated levan and its' effects in the enrichment of the gut microflora, giving it an edge in survival. An important link between host metabolic phenotype and nutritionally altered gut microbiota is that alterations in the full spectrum of metabolic pathways can be directed by probiotic supplementation. The modulation of the composition of gut microbiota stimulates the maturation of gut microbiota biological function and promotes the growth performance in animal models suggesting increased precisions in animal studies. Such clinical trials could corroborate the findings of enhanced and diversified treatment effects of multi-strain probiotics. In addition, the more heterogeneous responses to multi-strain probiotic treatment suggest that future precision microbiome modulation should consider the personalized response to probiotic formulas with caution.

4. Conclusion

Here we have studied *the in-vivo* prebiotic potential of LMW levan will undoubtedly help better understand the role of dietary fiber (levan) in the modulation of gut microbiota to improve human health. The main objective of this study was to assess the prebiotic potential of low molecular weight levan, determine a suitable dose and its effect on gut probiotome, visceral organs, and serum biochemistry, and determination of suitable dose of it. Our previous study characterized a low molecular weight levan, which was further applied for in vivo application to enhance the probiotic abundance. In this objective, an animal study was performed to analyze the effect of levan as a prebiotic supplement. Biochemical estimation of serum, histopathological observation, and probiotic populations was investigated. A biochemical estimation has shown that the test

compound significantly increased HDLC (good cholesterol) and triglyceride, the most critical parameters in regulating metabolism and reducing the risk of blockage of blood vessels. It also showed a drop in LDLC (bad cholesterol) at a low dose. Histopathological observations have confirmed that the drug is safe for animal use and can be used as a diet supplement. The quantitative measurement of the probiotic group was also analyzed by qPCR and metagenomics analysis. The result has shown that the candidate drug significantly increases many probiotic groups. The *Bifidobacterium*, *Prevotella*, *Bacteroides*, *Roseburia*, *Lactobacillus*, *Oscillibacter*, *Clostridium*, *Anaerovibrio*, and *Ruminicoccus* populations were increased treatment with levan polysaccharide. Irritable bowel syndrome is thought to be associated with lower Bifidobacterium levels (Taverniti & Guglielmetti, 2014). In non-breast-fed infants, prebiotic fiber formulations promote post-natal immune development, decrease respiratory infections and improve bowel function (Veereman, 2007). Therefore, supplementation of prebiotic compounds in the diet is necessary to maintain gut microbiota to improve healthy living.

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5B. Anticancer potential of levan

Abstract

Microbial exopolysaccharides are applied in many industries such as pharmaceutical, cosmetic, biomedical, etc. This study investigated the cytotoxic effect of low molecular weight levan on cancer cells. The MTT assay found that levan has significant activity on the treated cancerous cell line, i.e., MDA-MB231 and HeLa. Further, the cytotoxic effect of levan was insignificant on normal cells; thus, based on the preliminary study, it can be suggested that low molecular weight levan can be a promising drug for cancer treatment.

1. Introduction

Microorganisms from extreme habitats such as marine ecosystems are considered a rich source of novel bioactive compounds, which is important from a pharmaceutical perspective (De Philippis et al., 2001). EPSs of microbial origin have proven their ability in medical applications since mid of 20th century with dextran, the first clinical trial drug to expand blood plasma to control wounds shocks since 1953 (Nwodo et al., 2012). Microbial exopolysaccharides are applied in many industries such as pharmaceutical, cosmetic, biomedical, etc., as they possess important properties such as antioxidative, antiviral and immunomodulatory, antitumor and cholesterol-lowering, gelling and emulsifying, stabilizing, cryoprotection, etc. EPSs from marine bacteria are well known for their antitumor and immunomodulatory effect. Pullulan and alginate have been studied for their sustained drug delivery of numerous anticancer compounds (Matricardi et al., 2013). Several EPS are reported for their anticancer activities, such as fucoidan, levan scleroglucan, clavan, heparin, and mutan, but how they work is still unknown in bacteria; somehow, it is traced in fungus. Reports show that EPS activates mitochondria associated apoptosis path associated with loss of mitochondrial membrane potential, enhancement of mitochondrial cytochrome c release and intracellular ROS production, the elevation of p53 and Bax expression, down-regulation of Bcl-2, and the activation of caspase-9 and -3. Keeping this scenario in mind, it is necessary to discover the anticancer mechanism in bacteria. HMW EPSs seem more effective for antitumor activity than those LMW EPS (Peng et al., 2005).

As our strain has produced LMW weight levan, we considered investigating it for anticancer activity. For that, we initially performed an MTT assay to check its toxicity.

2. Materials and methods

2.1 *In-vitro* antitumor activity

Tumor cell lines were procured from National Center for Cell Science, Pune, India, which adopted a few days to test the antitumor activity. For breast cancer, MDA-MB231, and cervical cancer, HeLa cells for antitumor activity and kidney cell line (Vero) were used to test the cytotoxic effect of the compound. Paclitaxel drug was used as a positive control. Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10 % Fetal Bovine Serum, L glutamine, antibiotics, and other essential components according to the need of cells and incubated at 37°C in a humidified incubator at 5% CO_2 (v/v). Prior to culture, the sterility of the medium was confirmed by keeping them in an incubator for 48h. The range of levan from 2.5 to 200 µg/ml was applied as drug treatment. Each well of 96 well plates was added with 5×10^4 cells and growth media and incubated at 37°C for 48 h in a humidified incubator, applying 5% CO_2. The 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) assay was performed following the procedure that was explained by Hansen et al., 1989. The 3-(4, 5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide salt converts into insoluble formazon crystal upon reduction by mitochondrial dehydrogenases, which is an indicative marker for cell viability. The data is represented as a mean triplicate value of viable cells.

3. Result and Discussion

The result of the MTT assay indicated that no significant cell death was observed in a normal cell line (Vero) treated with levan concentration up to 200 µg/ml. The cell survival was observed at over 80%, which can be accepted in accordance with other cancer drugs (Fig.1). The cell proliferation inhibition of the cancer cell line was found significant. For MDA-MB231, a gradual decrease in cell viability was observed by increasing the concentration of levan up to 200 µg/ml. This seems further by increasing the concentration of the test drug (Fig. 2). Similar results were observed in the case of the HeLa cell (Fig. 3). Interestingly, the results showed that levan has a

positive impact on normal cell lines, whereas it inhibited the growth of cancer cells. The result indicates that a low molecular weight levan can be used for the treatment of different cancer.

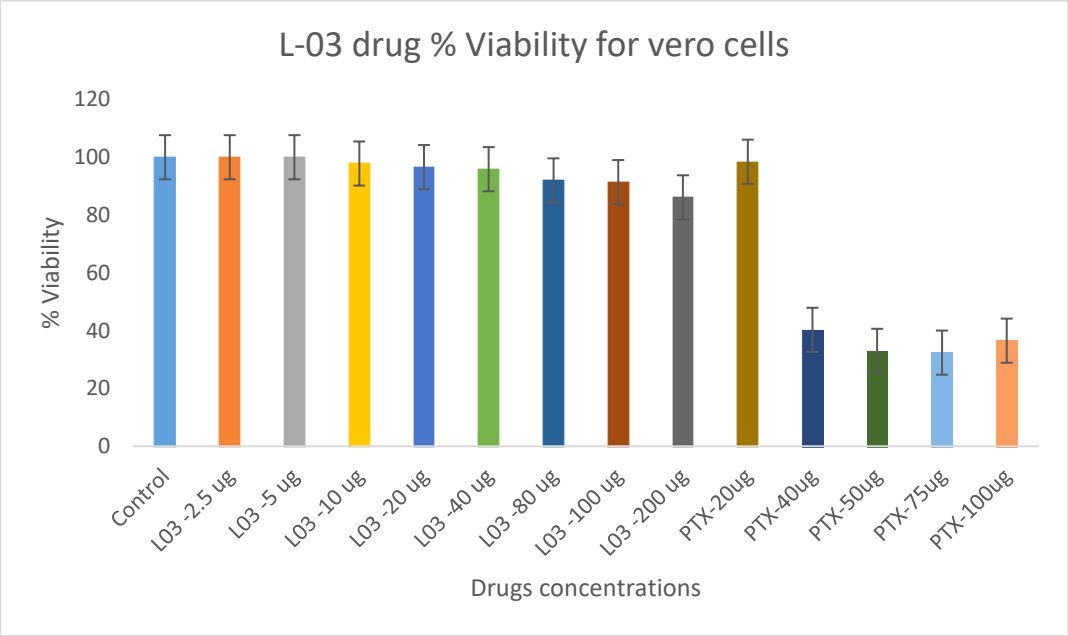

Fig.1 Effect of low molecular weight levan on the growth of Vero cell line

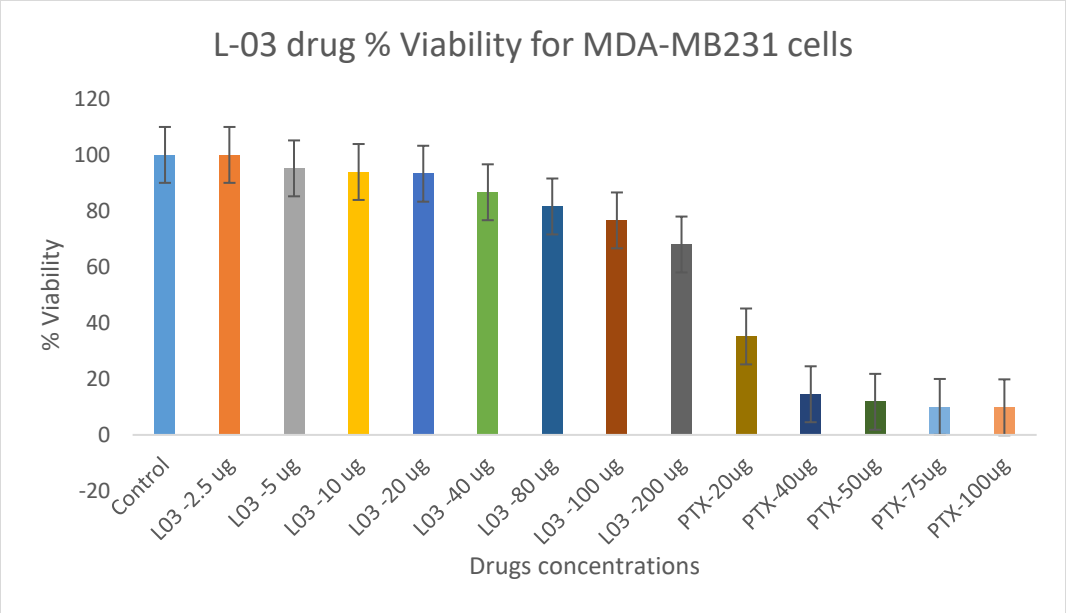

Fig.2 Effect of low molecular weight levan on the growth of MDA-MB231 cell line

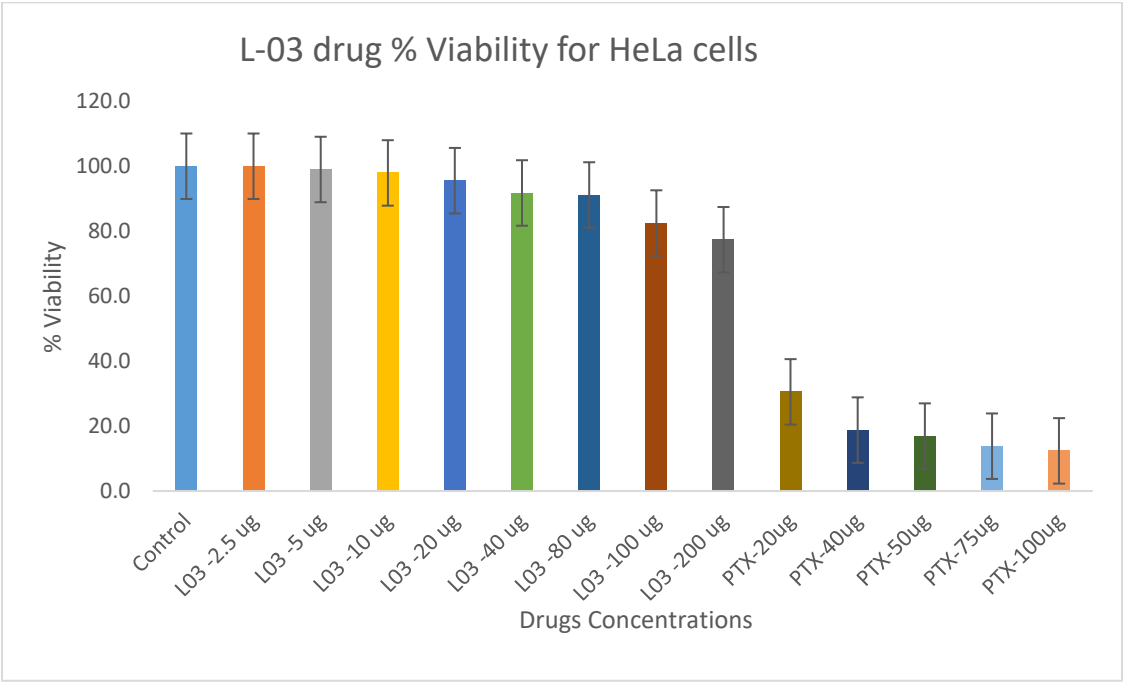

Fig.3 Effect of low molecular weight levan on the growth of HeLa cell line

4. Conclusion

The high molecular weight levans have been reported for their anticancer activity. It has also been reported that anticancer activity is depend on molecular weight and branching pattern. In this study, we have reported the anticancer activity by low molecular weight levan, which will help to explore the low molecular weight levan for cancer treatment. It is also noticeable that this low molecular weight levan is safe for normal cells while harmful for cancerous cells; therefore, it opens a new window to explore low molecular weight levan to find out its mechanism of action.

5. References

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Chapter 6:

Summary and Future Perspectives

Overall summary

Exopolysaccharides (EPS) are defensive biomacromolecules produced by micro to macro, aquatic to terrestrial life forms for their survival. Microbial exopolysaccharides have gained much attention over decades due to their significant physicochemical properties making their applicability to various fields. Plants and algal polysaccharides are currently being used in industries that are not feasible due to high production costs; therefore, industries are looking for bacterial polysaccharides considering their unique characteristics. These are natural biopolymers which makes them as safe and eco-friendly. Bacterial EPSs confer more advantages in industrial applications because of ease of extraction and controllability of production. Due to continuously growing populations, industries are demanding expeditious and easily accessible sources of EPS producers. The production cost seems to be a bottleneck in EPS commercialization; therefore, bacteria polysaccharides have gained attention considering their wide range of essential pharmacological and industrial applications. This study aimed to screen the exopolysaccharide (EPS) producing marine bacteria to search for novel or efficient EPSs from the diverse marine ecosystem and to develop a cost-effective, robust high throughput process for EPS production using a high throughput micro bioreactor system to apply them on various food and pharmaceutical industries. Given these, we have screened 43 previously isolated marine strains for EPS production and obtained four EPS-producing isolates. The AC-11, SGD-03, V-74, and SGD-05 were positive for EPS production. Among these, SGD-03 was found to have more potential to produce a high amount of EPS, i.e., 894 mg/L, which was taken further for detailed studies. Further, the strain was identified using a polyphasic approach, and it found that the SGD-03 strain shared the highest 16S rRNA sequence similarity with the *Bacillus licheniformis* ATCC 14580 and was named *Bacillus* sp. SGD-03.

SGD-03 was taken further for production, purification, and characterization of unknown EPS. After purification, a complete characterization of EPS was carried out using various analytical techniques. The analytical techniques such as TLC, HPLC, LC-HRMS, FTIR, and NMR analysis have revealed that the EPS is composed of fructose monosaccharide units with hydroxyl, carbonyl,

and ether groups. NMR confirmed the produced EPS has a β-(2, 6) linkage bonding, confirming its identity as a levan polysaccharide.

Further, the morphological observation revealed that the EPS has a fibrous network structure with a relatively smooth surface of 3.620 μm to 9.071 μm fiber diameter. Thermogravimetric analysis revealed that the EPS could tolerate high temperatures up to 185°C, and a 10 % reduction was observed after increasing the temperature to 188°C. The size exclusion chromatography determined the molecular weight and revealed that the produced EPS has a molecular weight of 1.0×10^4 Dalton. Interestingly, this unique-sized levan has not been reported in the literature before and will open the door for research requiring a specific 10 kDa levan.

Although *Bacillus* sp. SGD-03 produces a high amount of levan, but it is still not up to the benchmark production to commercialize it. Determination of quantitative process parameters for optimizing production strains and cultivation conditions is key for bioprocess development for almost every new microbial product. The quantitative process parameters were optimized by robust high throughput process micro-bioreactor (BioLector Pro) using response surface methodology (RSM) involving full factorial (FFD) followed by central composite design (CCD). The new medium was formulated by amending peptone, beef extract, sodium chloride, and sucrose. Peptone 0.4%, beef extract 0.4%, and sucrose 4.0% were found to be optimum process parameters for maximum EPS production, which is approximately valued at 1.12 USD/L of the media chemicals used. This cost will be drastically reduced when you scale up the production and the possibility of industrial-scale exploration. The maximum yield of crude EPS obtained was 123.9 g/L, resulting in 20.7 g/L pure levan, which is almost 6-7 fold increased compared to unoptimized condition. The production has also been validated with a shake flask, 1 L, and 10 L pilot scale fermentation.

After confirming the structure of unknown EPS (levan) having a low molecular weight (LMW, 10 kDa), it was applied as a potential prebiotic compound. The primarily *in-vitro* study targeted *Bifidobacterium* and *Lactobacillus* to test the prebiotic potential of LMW levan. The *in-vitro* study has found a positive effect on enhancing probiotic bacterial count. Successively, an *In-vivo* study was carried out by animal models (Albino mice and Albino Wistar rats). Animals were divided into five groups. Out of them, two were control groups; normal and positive treatment groups, and

three were treatment groups (low dose, 100mg/kg, intermediate dose, 250 mg/kg, and high dose, 500 mg/kg of body weight). After treatment, blood samples and visceral organs were extracted for serum biochemical analysis and histopathology, and DNA was extracted for qPCR analysis. The serum's biochemical estimation showed that the triglyceride significantly increased compared to the normal control. Surprisingly, glucose was also increased in serum, which can be attributed to terminal glucose in the levan polysaccharide. Estimating total cholesterol showed that the total cholesterol content significantly decreased compared to the standard control (inulin) and was not significant to the normal control. The histopathological observation showed a minimal lesion score in all treatment groups. The qPCR was used to quantify the abundance of *Bifidobacterium* and *Lactobacillus*, and the result indicated that low-dose and intermediate-dose treatment found significant increments in *Bifidobacterium* and *Lactobacillus*. The gut microbiota has immense diversity; therefore, procuring primer and amplifying a group-specific gene is much more complicated. Hence, to reveal the probiotome, the fecal DNA sample was subjected to metagenomics analysis to reveal all other probiotic profiles.

Future perspectives

The marine biosphere is a vast and diversified ecosystem to explore for isolation and screening of different bioactive compounds. The microbes from marine ecosystems constantly face challenges of adverse conditions that force them to change their metabolic processes and defense strategies. More than 50 different types of EPSs have been reported from marine sources, yet it is efficient to provide many more; therefore, extensive screening should be done for the screening of EPS-producing bacteria and other microbes. Characterization of EPS by various analytical methods provides the structure of EPSs. An analytical method like GPC determines the molecular weight of EPS, but the length of branching and bonding can not be measured, which is the fundamental need to apply EPSs in various fields. A finding of the branching pattern mechanism will certainly improve the application and synthesis of desirable EPS. In this study, scale-up for EPS production was performed using a regular-use nutrient medium by amending it. Several media are also reported for their high production. Besides known media, different cost-effective media such as sugar molasses, beet-root molasses, syrups, and sugarcane bagasse should have experimented. The levan polysaccharides show the unique property of solubility. It is soluble in water and oils. Using

this advantage, levan's derivatization with other cell impermeable compounds should be attempted. Another is the purification of other amphiphilic molecules, such as biosurfactants should be tried as levan is soluble in oil. Levan production can be performed by enzyme immobilization. In levan production, fructose is utilized to produce levan while glucose is left over in media; therefore, a tandem reaction can be developed for complete sucrose metabolism. Development of recombinant microorganisms for cost-effective production of levan on a large scale can be attempted.

Levan has many applications in various fields; here we applied them in food and medicine as prebiotic and anticancer drug molecules. This can be explored for several other applications based on their size, shape, molecular weight, and solubility.

ABSTRACT

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Faculty of Study: Biological Sciences **Year of Submission**: 2023

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Title of the thesis: Isolation, Characterization and Applications of Exopolysaccharides produced from marine microorganisms

Exopolysaccharides (EPS) are bio macromolecules produced by microorganisms. Composed of various monosaccharides especially glucose, galactose, mannose, fructose and rhamnose with different patterns. Majority of them are homopolymers or glucose. Bacteria are the major sources for the EPS production due to the ease of cultivation, manipulation, controlled production and downstream processing. EPS's has enormous applications in the food and pharmaceutical sector. Industrial demand has been increased for EPSs in large quantities to meet the needs. Despite the enormous applications of EPSs, only few of them have been commercialized because of their high production cost. However, searching for novel EPS and economical production is hot topic of research therefore researcher looking for cost effective production. Aiming to finding of potential bacteria for novel EPS, characterizations and optimization to fullfil the industrial demand for various application. These outcome are embodied in the current thesis, which are divided into four chapters. The extensive literature survey was carried out for the EPS from different environments and marine environment has be explored. The *first chapter* deals with the screening and identification of microorganisms from marine environment for exopolysaccharide production. *Second chapter* deals with the production, purification and structural characterization of produced EPS. *Third chapter* constructed for optimization of product yield using a cost-effective approach. Media components were amended to enhance product yield at Micro bioreactor using 1.0 mL working volume and validated at shake flask, 1.0L and 10L at lab scale fermenters and the *forth chapter* deals with the applications of levan polysaccharide to find out their prebiotic potential by *in-vitro* and *in-vivo* study using quantitative PCR and metagenomics approaches and for anticancer activity.

List of publications:

From thesis:

1. Wagh VS, Said MS, Bennale JS, Dastager SG. Isolation and structural characterization of exopolysaccharide from marine Bacillus sp. and its optimization by Microbioreactor. Carbohydrate Polymers. 2022 Jun 1;285:119241. DOI: https://doi.org/10.1016/j.carbpol.2022.119241

2. Vasudev Wagh, Mukul Tamb, Rachel Samson, Prajakta Murudkar, Vinay Rajput, Anil Pawar, Mahesh Dharne, Syed Dastager (2023) Investigating effect and dose of levan on gut microbiota, serum biochemistry and visceral organs in diet supplemented rats (Manuscript under preparation).

Patents:

1. "High throughput micro bioreactor process for efficient production of levan from marine Bacillus sp. SGD-03" Patent Application no. (Record No. 2022021621150 - 2021- INV-038 date of filing 14-09.2021)

Other than thesis:

3. Wagh VS, Ram H, Dastager SG. Priestia veravalensis sp. nov., isolated from coastal sample. Archives of Microbiology. 2021 Oct;203(8):4839-45.DOI: https://doi.org/10.1007/s00203-021-02418-z
4. Remesh, A. V., Raveendran, A., Bincy, K., Wagh, V. S., Dastager, S. G., & Babu, C. V. (2022). Insights on biorational potential of Ocimum gratissimum essential oil and its binary combination with monoterpene phenol for control of rice weevil (Sitophilus oryzae) and aflatoxigenic fungi. *Food Bioscience*, *50*, 102019. DOI: https://doi.org/10.1016/j.fbio.2022.102019

Conferences

Poster presented at international carbohydrate conference on " Emerging Frontiers on carbohydrate chemistry and glycobiology" at university of Lucknow, Lucknow on December 5-7, 2019.

Poster title: Purification and Characterization of an Exopolysaccharide from an unexplored marine environment.

Author: Vasudev S.Wagh and Syed G.Dastager

Abstract: Microbial polysaccharides are the polymers of monosaccharide units which can be simple or derivative sugar linked together with glycoside linkages with various degrees of polymerisation. Microbial polysaccharides can be categorised as exopolysaccharides , structural polysaccharides, and endopolysaccharides depending on their functions in microbial cells. Out of these, exopolysaccharides contribute immensely to the field of research and development due to their numerous applications in industrial as well as pharmaceutical sectors. Here we have isolated, screened and identified exopolysaccharide (EPS) producing bacteria from a marine environment at Chorao Island, Goa, India. EPS was purified and partially characterized using FE-SEM, GPC, FTIR and HPLC. Various degrees of polymerisation along with several branching patterns of EPS and a leaf clover like morphology was observed in Field Emission Scanning Electron Microscopy (FE-SEM). The molecular weight of EPS was found to be 41,244 Da. by Gel Permeation Chromatography (GPC). Functional groups were analysed with Fourier Transform Infrared Spectroscopy (FTIR) and the characteristic presence of OH, C=O, C-N and C-O-C was detected. High Performance Liquid Chromatography (HPLC) using ion exchange column revealed that the EPS was primarily composed of D-Glucosamine, D-Glucose, D-Mannose and D-Fructose with a ratio of 14:1:23:2.

Poster presented at international carbohydrate conference on "Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health" at CSIR-IIP, Dehradun on December 1-4, 2021.

Poster title: High-throughput Micro bioreactor based optimization of exopolysaccharide produced from a marine organism and its structural analysis

Authors: Vasudev S. Wagh, Madhukar S. Said, Jagadish S. Bennale, and Syed G. Dastager

Abstract:

In the present study, a cost-effective robust Micro bioreactor based optimization of exopolysaccharide and its structural characterization was analyzed. The FE-SEM analysis has shown the significant fibrillar structure of EPS. Size exclusion chromatography, TLC, HPLC, LC-HRMS, FTIR and NMR analysis revealed that EPS produced has a molecular weight of 1.0×10^4 Dalton and is composed of fructose monosaccharide with hydroxyl, carbonyl, and ether groups. NMR analysis has revealed that the backbone structure of EPS has a branching pattern of β-(2, 6) linkages which confirms that it shows similarity with Levan polysaccharide. We have demonstrated cost-effective media composition for EPS production by Micro bioreactor. The maximum yield of EPS obtained was 123.9 g/L by response surface methodology using robust Micro bioreactor, a BioLector Pro and production has been validated with shake flask, 1.0L and 10.0L pilot-scale fermentation.

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Isolation and structural characterization of exopolysaccharide from marine *Bacillus* sp. and its optimization by Microbioreactor

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ABSTRACT

In the present study, a cost-effective, robust Microbioreactor based production optimization of levan like exopolysaccharide from marine Bacillus sp. SGD-03 was analysed. FE-SEM analysis has showed the significant fibrillar structure of EPS. Size exclusion chromatography and other analytical data revealed that, produced EPS has a molecular weight of 1.0×10^4 Da and is composed of fructose monosaccharide with hydroxyl, carbonyl, and ether groups. The backbone structure of EPS has a branching pattern of β-(2,6) linkages which confirms the similarity with available levan like polymers. The cost-effective media composition for levan production was demonstrated. The maximum yield of crude levan obtained was 123.9 g/L by response surface methodology using robust BioLector Pro Microbioreactor, and same has been validated with shake flask, 1 L and 10 L pilot-scale fermentation.

1. Introduction

Exopolysaccharides (EPS) are natural polymers and are defensive biomacromolecules produced by micro to macro, aquatic to terrestrial life forms for the primary goal of survival (Kekez et al., 2015). In recent times, polysaccharides from microorganisms have captivated attention considering their wide range of essential pharmacological and industrial properties, such as antiviral (Arena et al., 2006), anti-oxidative (Raza, Makeen, Wang, Xu, & Qirong, 2011), immunomodulatory, antitumor (Ramamoorthy, Gnanakan, Lakshmana, Meivelu, & Jeganathan, 2018; Staniszewska, Szymanski, & Ignatowicz, 2017), cholesterol-lowering (Korcz, Kerényi, & Varga, 2018), wound healing (Sun et al., 2020), gelling and emulsifying activity (Iyer, Mody, & Jha, 2006). In view of the increased demand for natural biopolymers for various industrial applications (Suresh Kumar, Mody, & Jha, 2007). Several bacterial species known to be EPS producers. Among them lactic acid bacteria (LAB) are dominant. Certain *Bacillus* species have been demonstrated higher EPS production yields than the LAB (Lee et al., 1997). Another crucial aspect of the EPS is to determine the absolute configuration of the carbohydrate moieties and their derivatives. For an unknown structural exopolysaccharide, a combination of analytical methods such as Fourier Transform Infrared (FTIR) Spectroscopy, High-Resolution Mass Spectrometry (HRMS), and Nuclear Magnetic Resonance (NMR) spectroscopy serve as an excellent tool for determining its structure, these techniques demonstrate its importance for determining the absolute configurations of sugar residues of an EPS (Säwén, Zhang, Yang, & Widmalm, 2010). In order to accomplish the need and commercialisation of EPS, various strategies have been adopted like media optimisation, strain improvement, and fermentation parameters by many researchers (Berekaa, 2014; Lee et al., 1997; Song, Jeong, & Baik, 2013; Xu et al., 2019). Most of the studies have been restricted to optimise the medium composition by one-factor at a time (OFAT), which is time-consuming and unable to understand the interaction between the variables (Sen & Swaminathan, 1997). Design of Experiments (DoE) can effectively understand the interactions among the various physiological parameters to optimise media conditions. Response surface methodology (RSM) has been extensively used to optimise the responses altering the media composition (Kekez et al., 2015). Despite all the important benefits, low productivity is the bottleneck in commercialising EPS (Ale et al., 2020), microbioreactor based optimisation in combination with response surface methodology will help to understand the possible interaction of various factors at different levels. It utilises a minimal amount of media (1.0 mL) to optimise the production parameters. A conventional method of optimisation relay on shake flask levels without

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control of pH, biomass and dissolved oxygen which are fundamental parameters for optimisation. An automated microbioreactor facilitating online monitoring of pH, biomass and dissolved oxygen. The single optode plate of the microbioreactor is efficient to run 48 experiments at a time with various media combinations before going to large scale fermentation. Time saving and cost effective approach for the optimisation is requisite of the time. This study aimed to study the high-throughput optimisation of EPS production, its structural characterization from a marine *Bacillus* sp. SGD-03 and validation of 1.0 mL to 10.0 L fermentation by Microbioreactor.

2. Materials & methods

2.1. Screening and identification

Microbial cultures were isolated from marine sediment samples from Chorao Island, Goa, using the serial dilution method and EPS screening. EPS production was confirmed by mucoid colony formation followed by precipitation of EPS using alcohol. A string test was also utilised for further confirmation. Fresh cultures of marine isolates were applied on solid nutrient agar medium (Peptone, 1.0%; Beef extract, 1.0%; NaCl, 0.5% and 1.5% of agar) supplemented with different carbon sources (sucrose, maltose and lactose) and incubated separately at 28 °C, 30 °C and 37 °C. Mucoid colony formation was examined by observing the presence of slimy mucoid colonies on the plate until 48 h with every 12 h intervals.

Further, loopful colonies were immersed in alcohol to observe the precipitation of EPS. When a loopful of culture is mixed with pre-chilled absolute ethanol (4 °C), colonies precipitate out from the solution, confirming the EPS producer and solution turbid indicates negative for EPS (Paulo et al., 2012). A String test was performed for further confirmation. Lifting a colony with a sterile inoculating loop to form a thin string of colonies. >5 mm string formation was considered positive for EPS producer strains (Hector et al., 2015). A multiphase approach was used for the identification of an organism. Scanning electron microscopy (SEM) was performed to characterise surface morphology of an organism, Biochemical characterization was carried out using *VITEK*-2 compact system and molecular identification was performed using 16S rRNA gene sequencing and whole genome analysis by calculating average nucleotide identity (ANI).

For SEM analysis, cell pellet was separated from supernant by centrifuging overnight grown culture at 10000 RPM for 2 min. The cells were washed thrice with 0.1 M of phosphate buffer (pH 7.2). Subsequently cells were suspended in 2.5% glutaraldehyde solution for 1 h at 4 °C. After fixation, cells were washed thrice with same buffer and dehydrated with graded ethanol (30%, 50%, 70% and 100%) with incubating for each dehydration step for 3 min. Then cells were spread on gelatin (0.1%) coated glass cover slip and incubated overnight at 50 °C for drying. Then cover slip was mounted on sample holding stub and subjected to gold coating prior to analysis, critical-point dried, mounted on scanning electron microscope stubs, sputter-coated with gold and viewed on a FEI Qunta 200 3D dual beam scanning electron microscope with an accelerating voltage of 20 kV, Biochemical based identification was carried using *VITEK*-2 compact an automated microbial identification system (Pincus, 2006). Inoculum suspension of 0.5 McFarland turbidity was prepared in saline solution. By using *VITEK* BCL test card identification test was performed according to the manufacturer's standard protocol (bioMérieux).

Molecular identification was carried out using 16S rRNA gene and whole genome sequencing. Genomic DNA was isolated using the HiPurA™ Bacterial Genomic DNA purification kit and amplified by polymerase chain reaction (PCR) using bacterial 16S rRNA universal primer 8F and 1492R. PCR Thermal cycling was carried out using the following parameters: genomic DNA was denatured initially at 94 °C for 7 min and 30 s for subsequent denaturation cycles. Primer annealing and extension of new DNA strand were carried out at 59 °C and 72 °C for 30 s and 90 s, respectively. After 30 cycles, the final extension was set at 72 °C for 7 min. Exonuclease I-Shrimp Alkaline Phosphatase method (Exo-SAP) was utilised to clean up amplified products. It was further subjected to cycle sequencing using universal primers: 8F, 530F, 800R and 1492R in separate wells to achieve a full-length sequence. BigDye terminator V3.1 was used as a chain terminator. HiDi formamide was added after precipitation (purification) in each well of the sequencing plate. The plate was fixed in Genetic Analyser 35xL applied Biosystems (Sanger sequencer) to run the machine. Sequencing reads were assembled using ChromasPro software. Assembly (query) sequence was subjected to NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud Database for homology searching. Molecular phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Evolutionary analysis was performed using MEGA software version 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Genome sequence relatedness to its type strains was performed. An Average Nucleotide Identity (ANI) value was calculated using a web-based server (http://enve-omics.ce.gatech.edu/). The whole-genome sequencing and type strain sequences data were retrieved from Oxford Nanopore Technology-MinION and NCBI databases, respectively.

2.2. Bacterial strain and culture conditions

Based on the initial screening, best EPS producer strain *Bacillus* sp. SGD-03 was taken for further detailed study. A nutrient broth (Peptone, 1.0%; Beef extract, 1.0%; NaCl, 0.5% with a pH of 7.0) was used to study the optimum temperature and inoculum titer. Temperature variables like 25 °C, 28 °C, 30 °C and 37 °C and inoculum OD of 0.1 to 0.5 were set to analyse the effect of temperature and inoculum size on organisms growth profile. One percent (0.1 OD) of overnight grown culture was inoculated in nutrient broth and incubated at various temperature for 24 h at 150 RPM under shaking condition, while optical densities were adjusted from 0.1 to 0.5 to analyse the effect of inoculum titer on the growth of an organism for 48 h. Cells, 0.1 OD (10^7 colony forming unit, CFU/mL) were inoculated in a nutrient broth and incubated for 18 h at 28 °C temperature with 150 rpm to obtain fresh active starter culture for further experiments.

2.3. Salt and pH tolerance

Salt and pH tolerance were tested to check the effect of osmolarities and pH on the organisms growth profile. Nutrient broth, with different concentration of saline solution 2.0–10.0% (*w/v*) and pH ranging from 5.0–10.0 (at an interval of 1.0 units), was tested. Fresh culture of *Bacillus* sp. SGD-03 was inoculated as 1.0% *v*/v in 96 microtiters well plate containing different sets of experiments and incubated for 24 h at 28 °C with shaking of 150 rpm. The salt and pH tolerance profiles were analysed by absorbance at 600 nm using the Synergy H1 hybrid multi-mode microtiter plate reader.

2.4. Microbioreactor based optimisation of EPS

2.4.1. Screening of best carbons source for the EPS production

To examine the effect of carbon sources on the EPS production, 2.0% of fructose, glucose, sucrose, maltose, and lactose were supplemented in nutrient broth. Setting 1.0 mL of working volume, Inoculum of 1.0% (0.1 OD_{600}) were inoculated in optode plate. The plate was fixed and operated at 28 °C, 1400 rpm for 48 h in BioLector pro (m2p Labs). Dissolve oxygen (DO), pH, and biomass were measured by optode plate. After confirming the pre-eminent carbon source, its concentration ranging from 2.0%, 4.0%, 6.0%, 8.0% and 10.0% *w/v* were studied to evaluate the optimal concentration.

2.4.2. Screening of process optimisation factor by full factorial design (FFD)

The full factorial experiments were designed to screen the desirable factors at various levels to optimise the response. Full factorial design of experiments (DoE) was model by Minitab19 statistical software involving three independent variables (Peptone, beef extract and sucrose) at four levels (0% to 3.0%, low to high) with 5.0 g/L NaCl as a constant. A total no. of 128 experiments in replicates were performed to analyse the effect of each factor and their interaction on EPS production.

2.4.3. Optimisation of response by RSM

Based on the full factorial experiments, all the media components (peptone, beef extract and sucrose) were further selected to enhance the response (All factors was chosen as they were found a positive effect on EPS production). Central composite design (CCD) was applied to study the effect of optimal points. The independent variables peptone, beef extract with their low concentration of 0.1% to high 0.4% were used. And sucrose with 1.0–4.0% concentration with 0.5% NaCl (*w/v*) as a constant was studied in a face centred CDD. A total of 20 repeated experiments were performed, containing 8 factorial points, 6 axial points, and sextuple at the center point (Dos Santos et al., 2016). Optimal media composition (obtained by CCD experiments) was further validated at fermentor scale for EPS production.

2.5. Production and purification of EPS

Media optimisation was performed using optode embodied 48-well microtiter plate (Flower well plates, m2p-labs GmbH, Germany) in a BioLector Pro machine, which facilitates online monitoring of cell biomass, pH and dissolved oxygen (DO) saturation separately in each well without effusing (Dos Santos et al., 2016; Kensy, Zang, Faulhammer, Tan, & Büchs, 2009). With working volume 1.0 mL, overnight grown culture (18 h), inoculated as 1.0% (0.1 OD_{600}) in each well containing various media combinations generated by experimental design. (Media components peptone, beef extract and sucrose were prepared in stock concentrations, pH was adjusted to 7.0). The plate was fixed and operated at a temperature of 28 °C; rpm, 1400; humidity, 100% for 48 h of incubation. Later, the plate was taken out from the Microbioreactor. Samples were centrifuged at 10000 rpm, 30 min at room temperature to obtain cell-free supernatant. The three-volume of pre-chilled ethanol (4 °C) was added to the supernatant. Precipitated EPS (Threaded appearance) was separated and washed with 70% ethanol. One milliliter of Milli Q water was added and kept overnight at 4 °C for proper dissolution. For the process validation, the optimised media composition of FFD and CCD for EPS production were subjected to a shake flask (500 mL) followed by 1–10 L fermentors. Erlenmeyer flask of 500 mL containing 200 mL of working volume was set. The flasks were inoculated in optimal media composition and incubated at 28 °C at 150 rpm for 24 h. Dissolved oxygen saturation, 10%, was maintained by cascading rpm from 250 to 500; pH 7.0; temperature, 28 °C and 24 h of incubation were set for fermentor scale (1 L and 10 L). For purification, steps from harvesting to precipitation were repeated; further precipitated EPS was subjected to deproteinise by the Sevag reagent (chloroform: n-Butanol, 5:1). Most of the EPS are represented by proteins, and the protein amount significantly decreases after treatment with proteinases. The volume ratio of a sample (5.0 mg/mL) to sevag reagent 2:1 was used (Li, Zhao, Zhou, & Wu, 2012). Further, deproteinised EPS was dialysed against demineralised, followed by Milli Q water for 72 h. UHPLC (Ultimate 3000, ThermoFisher) was equipped with a size exclusion column (Diol-200, YMC) to collect fractions of purified EPS. Concentration of 5.0 mg/mL; injection volume, 50.0 μL; flow rate, 1.0 mL/min; Detector, RI were applied. Purified EPS was lyophilised and stored for further analysis.

2.6. Data analysis

Analysis of experimental designs (full factorial and face-centered composite), Analysis of variance (ANOVA), Framing of two-dimensional contour plot and three-dimensional cubic model for response surface representing variable data points was performed using regression analysis of variables by Minitab statistical software version 19.0. The biomass concentrations were correlated and calculated by linear regression of light $scattering_{(620nm)}$ and optical $density_{(600nm)}$ using serial dilutions of a known concentration of cell dry weight (DW) as standard. CFU were calculated from 12 to 48 h of incubation in shake flask (working volume of 100 mL) as well as BioLector (working volume 1.0 mL) using plate spread technique in order to check cell viability of an organism. 100 μL of sequentially diluted culture was spread on solid agar plate and kept for incubation at 28 °C for 24 h CFU/mL were noted after 12 h and 24 h of incubation, respectively. pH and dissolved oxygen saturation were estimated by the BioLection data processing software (m2plabs GmbH, Germany). EPS quantification was determined by the phenol sulphuric acid method. A standard linear curve of pullulan was generated to quantify the concentration of EPS of each experimental set-up (Rao & Pattabiraman, 1989).

2.7. Characterizations of exopolysaccharide

2.7.1. Field Emission Scanning Electron Microscopy (FE-SEM) analysis

The surface morphology of EPS was observed and analysed using FE-SEM. Dry polysaccharide samples (Test and standard) were mounted over carbon tape, fixed on the sample holder stub. Before analysis, samples were subjected to gold coating using a sputter coater. The surface morphology was observed and photographed from 1000× to 4000× magnification with an accelerating voltage of 18 kV using the FEI Nova NanoSEM 4450 electron microscope.

2.7.2. Monosaccharide composition analysis

Monosaccharide analysis of EPS was performed by thin-layer chromatography (TLC), Ultra high-performance liquid chromatography (UHPLC) and liquid chromatography high-resolution mass spectrometry (LC HR-MS). With intermediate shaking, an EPS of 1.0 mg/mL was hydrolysed using 2 N trifluoroacetic acid (TFA) at 100 °C for 1 h. Hydrolysates were neutralised by evaporating TFA using vacuum drying. Traces of TFA were removed by adding methanol followed by water until it neutralised. The hydrolysed sample was centrifuged at high-speed centrifugation to remove unhydrolysed or charred material. The supernatant was subjected to analyse the carbohydrates.

2.7.2.1. Thin layer chromatography. Fructose was used as a standard carbohydrate. A standard carbohydrate and hydrolysed sample, 5.0 μL, was spotted on a TLC plate (Silica 60, Merck), air-dried and placed in a TLC chamber saturated with the mobile phase. Solvent system (mobile phase) acetonitrile: water in the ratio of 85:15 (*v/v*) was used to separate the carbohydrates (Robyt, Wilson, Adlard, Poole, & Cooke, 2000). The plate was removed, air-dried and subjected to develop chromatogram in the staining solvent p-anisaldehyde: sulphuric acid: ethanol with 1:1:20 ratio. Then the chromatogram was visualised by charring the plate in a hot air oven for 10 min at 110 °C (Insulkar, Kerkar, & Lele, 2018).

2.7.2.2. High-performance liquid chromatography. The hydrolysed sample was filtered using a 0.22 μm syringe filter and subjected to Ultra high-performance liquid chromatography (UHPLC, Ultimate 3000, ThermoFisher) equipped with Phenomenex (Rezex RCM-Monosaccharide) column set on 80 °C. Milli Q water as a carrier with a flow rate of 0.6 mL/min, RI at 35 °C conditions was used for detection. For comparison, an injection volume of 20 μL having a 1.0 mg/mL concentration was injected with standard carbohydrates.

2.7.2.3. Liquid chromatography high-resolution mass spectrometry. The LC-HRMS spectra were recorded with a HPLC system (Thermo scientific Q-Exactive, Accela 1250 pump). The system was equipped with a hypersil GOLD column having a particle size of 3.0 μM. The 1.0 mg/mL sample was prepared in Milli blended with methanol and injected as 1.0 μL volume. Methanol with a flow rate of 0.5 mL was used as a carrier.

2.7.3. Fourier Transform Infrared (FTIR) spectroscopy

Analysis of functional groups was carried out using FTIR spectroscopy. KBr was activated in a hot air oven at 80 °C for overnight. The sample was mixed in KBr in a ratio of 1:100 (mg), ground to prepare the test KBr disc (13 mm). The disc was kept in a desiccator to resist moisture entrapment and subjected to record absorbance spectra. Spectra were recorded from the wavenumber 4000 cm^{-1} to 400 cm^{-1} using FT-IR spectrometer vertex70, Bruker. Background spectra were subtracted by recording spectra of reference KBr disc.

2.7.4. Molecular weight determination

The exopolysaccharides average molecular weight was determined by the size exclusion chromatographic (SEC) column, combined with a UHPLC system with a RI detector. EPS sample 1.0 mg/mL was prepared in the mobile phase, Milli Q water filtered through a 0.45 μm syringe filter. At ambient temperature, an injection volume of 20 μL was passed through the column (Diol-200 YMC) with a 1.0 mL/min flow rate. Standard Pullulan with a peak molecular weight (Mp) ranging from ~350 to 700,000 Da (Sigma) was used for calibration. The calibration curve was used to determine the average molecular weight of EPS. Sample chromatogram was also compared with standard polysaccharides, Pullulan 10 kDa (P-10) and Dextran 10 kDa (D-10).

2.7.5. NMR spectroscopic analysis

The purified EPS was applied to 1D and 2D NMR analysis to elucidate the exact structure of a polysaccharide. The 20.0 mg powder of EPS was dissolved in 0.6 mL of D_2O and kept overnight at 4 °C for proper disintegration. Spectra of 1D and 2D, including ^{1}H (500.13 MHz), ^{13}C (125.76 MHz), Distortionless Enhancement by Polarization Transfer (DEPT), Nuclear Overhauser Effect Spectroscopy (NOESY), Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence spectroscopy (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC), were recorded with Bruker Avance spectrometer 500. As an internal standard, all chemical shifts (δ) are reported in parts per million downfield from D_2O (4.71 ppm). Spin multiplets are reported as d (doublet) and t (triplet). Coupling constant (*J*) is reported in hertz (Hz).

3. Results and discussion

3.1. Screening and identification of EPS positive strains

A total of 43 marine strains were screened for EPS production. Based on screening, four strains were found positive for EPS production. Out of them, SGD-03 was found to be the pre-eminent EPS producer. It was further studies for its capability to produce EPS in all the media constituents and various incubation temperatures. It was found to produce slimy mucous colonies on the solid nutrient medium (Fig. S1a). Colonies formed a thread-like appearance of EPS upon addition of pre-chilled ethanol, which confirms positive strain for EPS production (Fig. S1b). Upon lifting the colonies with a sterile inoculating loop, an approximately 7–8 mm string was formed, which further confirmed EPS production (Fig. S1c). Microscopic observation has shown that SGD-03 belongs to the gram-positive, rod-shaped bacterium. The surface morphology of EPS positive strain was observed and confirmed using SEM analysis. The cells have rod shaped morphology with 1.12 μM in diameter which indicates the characteristics of genus *Bacillus* (Fig. S2a), and biochemical characterization was performed by substrate utilisation using by *VITEK*-2 compact system. The biochemical results indicated that, the isolate showed 98.0% probability with *Bacillus licheniformis* (Table S1). Based on phylogenetic analysis, the strain has shared the highest sequence identity of 99.79% with *Bacillus licheniformis* ATCC 14580 (Fig. S2b). Genome based ANI plot has demonstrated the similarity of SGD-03 with *Bacillus licheniformis* ATCC 14580 (Fig. S2c) and an ANI matrix values of 99.0% have demonstrated the more than 95% ANI similarity with the type strains *Bacillus licheniformis* ATCC 14580 (Fig. S2d), which confirms the identification of the SGD-03 belongs to *Bacillus* genera.

3.2. Effect of temperature and inoculum on the growth of Bacillus sp. SGD-03

The effect of temperature on the growth of an organism and its EPS production were analysed. Depending on the temperature of the location from where the strain has been isolated (28–30 °C), temperature variables of 25 °C, 28 °C, 30 °C, and 37 °C were used to analyse the optimum temperature for the growth. It was found that the organism can grow in all the tested temperatures, with an optimum at 28 °C. We have also compared EPS production concerning tested temperatures. It was observed that the organism has produced an optimum of 3.83 g/L of pure EPS at 28 °C with supplementing 2% of sucrose in media (Fig. S3a). An effect of various inocula on the organism's growth was observed based on growth profile analysis of *Bacillus* sp. SGD-03 with different inocula. Inoculum OD ranging from 0.1 to 0.5 was set to obtain the inoculum titer value. The optimum growth of *Bacillus* sp. SGD-03 was found in the inoculum OD from 0.2 to 0.5 till 24 h and started declining as well as 0.1 inoculums OD has also shown the comparable growth at 24 h and continued till 32 h. The desirable growth progress of *Bacillus* sp. SGD-03 was achieved with 0.1 inoculum OD. An organism has shown a distinct, desirable growth pattern of its growth phases (Fig. S3b).

3.3. Effect of salt and pH on tolerance profile of organism

The tolerance of salt and pH was analysed based on the growth profile of an organism. An inverse relationship of growth to salt concentration was observed. As the concentration of salt increases, the growth of an organism decreases. It was observed that the organism showed optimum growth between 18 and 20 h in 2.0% salt and declined rapidly after 20 h onwards. The organism could not tolerate high salinity after 20 h (Fig. 1a). On the observation of pH, it was found that the growth of an organism was significantly affected at low pH 4.0 and high pH 10.0. The pH 6.0 and 7.0 is the optimum range for the growth of an organism. A moderate growth profile was observed at pH 5.0, 8.0 and 9.0 (Fig. 1b). It can be concluded that the organism can tolerate a wide range of pH as well as salt. However, the desirable growth phases were not observed. It may be an intervention of EPS in the absorbance.

3.4. Optimisation of process parameters for EPS production using Biolector Pro

3.4.1. Screening of carbon source for EPS production

The influence of various carbon sources on EPS production was investigated using a microbioreactor, i.e. BioLector Pro. Five different carbon sources, including monosaccharide (fructose, glucose) and disaccharide (sucrose, maltose and lactose), were studied to examine their effect on EPS production. Sucrose was found to be the pre-eminent carbon source having a significant impact on EPS production. It showed almost a 10-fold increase as compared to other carbon sources (Fig. 2a). Further various concentrations of sucrose were studied. The 4.0% sucrose concentration was found to be an optimum concentration effect on EPS production. Though the observation seems direct relationship of EPS production with increasing sucrose concentration, there is no significant increase in the EPS yield with the higher concentrations of sucrose (Fig. 2b).

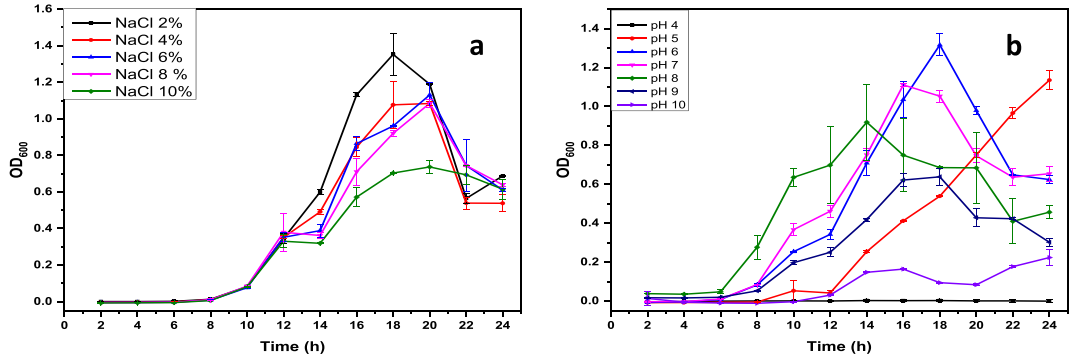

Fig. 1. Growth tolerance profile of *Bacillus* sp. SGD-03: a. Salt; b. pH.

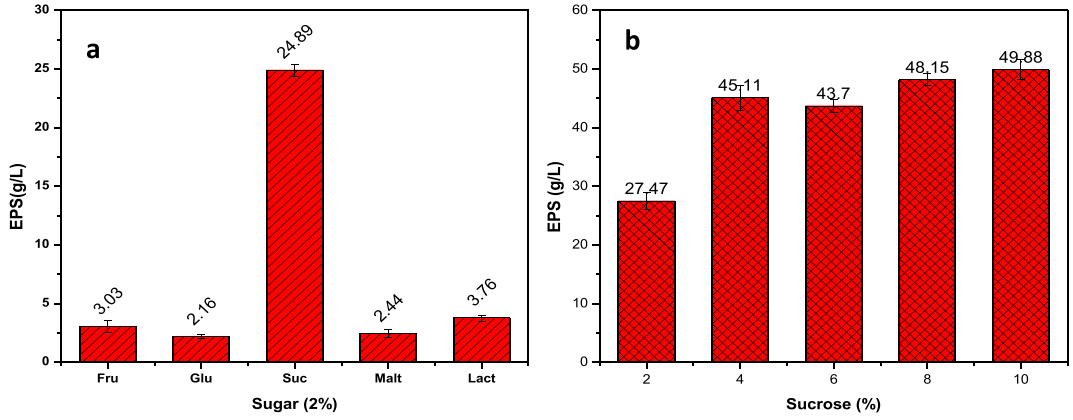

Fig. 2. Screening of carbon source: a. Effect of various carbon sources on EPS production; b. Effect of Sucrose concentrations on EPS production.

3.4.2. *Screening of significant variables*

To choose the efficient combination of nutrient media components, i.e. peptone, beef extract, and NaCl with an effect of supplemented carbon source (sucrose), were screened from low to high 0–3.0% concentration. Significant variables were obtained by the full factorial design with regards to influence on EPS production. Results were interpreted by linear regression analysis using the Minitab19 statistical software, which helps estimate each independent variable's effect. The optimum response, 67.0 g/L was derived with the combination of peptone (2.0%), beef extract (3.0%), and sucrose (3.0%) with 0.5% NaCl as a constant (Table S2). The sucrose with a higher concentration (3%) has the main effect on EPS production (Fig. S4). Peptone and beef extract were also found to have a positive impact on enhancing EPS production.

Statistical significance of the effect of each variable on EPS production was calculated and investigated by F-test, which has shown adequacy of the design with highlighting all the factors, i.e., factor (A), peptone; factor (B), beef extract, and factor (C), sucrose are having the statistically significant effect on EPS production. Based on this observation, it can be concluded that all the factors are essential in producing EPS. The *p*-value <0.000 confirms that the overall model is very significant and productive. Moreover, the value of R^2 0.584 for the model indicates that the 40% variation in the sample indicates the results of individual variables effect. The 2-way interaction of beef extract and sucrose was very significant. The nitrogen source (peptone) is also required for the growth of an organism to produce EPS. Therefore total variance could not be explained by the model. The probable cause could be the various interactions of variables as mentioned in Table 1. The response of each run was calculated using the following regression equation:

$$Y = 2.13 + 2.83\,A + 2.27\,B + 8.31\,C - 0.84\,A*B - 0.15\,A*C + 1.29\,B*C + 0.236\,A*B*C$$

Table 1
Analysis of variance for anticipation of EPS-Biomass yields influencing by variables in screening design of experiment.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	20,965.0	2995.0	24.12	0.000
Non-linear	3	20,352.5	6784.2	54.63	0.000
A. Peptone	1	566.9	566.9	4.57	0.035
B. Beef extract	1	1936.8	1936.8	15.60	0.000
C. Sucrose	1	17,848.8	17,848.8	143.74	0.000
2-Way interactions	3	598.6	199.5	1.61	0.191
AxB	1	47.3	47.3	0.38	0.538
AxC	1	8.6	8.6	0.07	0.793
BxC	1	542.6	542.6	4.37	0.039
3-Way interactions	1	13.9	13.9	0.11	0.739
AxBxC	1	13.9	13.9	0.11	0.739
Error	120	14,901.2	124.2		
Lack-of-Fit	56	12,654.6	226.0	6.44	0.000
Pure error	64	2246.6	35.1		
Total	127	35,866.2			

where, Y = Response, A = Peptone, B = Beef extract and C = Sucrose

The correlation of calculated response (EPS) to biomass of each run has shown in Table S2.

Microorganisms produce EPS in response to various stress conditions like nutrient deficiency, salt tolerance, heat tolerance, drought conditions etc. (Sandhya & Ali, 2015). In defined media, EPS production is stimulated by nutrient limitations in the presence of excess carbohydrate (Duguid & Wilkinson, 1953). According to this, the concentration of selected optimum levels of input factors was modified by diluting 10 times to mimic the nutrient deficiency to induce the organisms EPS producing ability to maximise the yield of EPS. To maintain the osmolarity balance, NaCl was kept constant (0.5%). The concentration of

sucrose was used undiluted as the carbon source acts as a substrate for the EPS production required for the growth of an organism. And it will work in place of the high salinity of seawater to maintain osmolarity.

3.4.3. Response surface methodology (RSM)

The selected optimum levels of a factor with some modifications were further optimised using face- centered composite design (FCCD). A total number of 20 different runs with various combinations of peptone, beef extract and sucrose in a set of factorials, axial and center points. The response of each run was calculated using the following regression equation:

$$Y = 29.1 - 117\ A + 79\ B - 5.7\ C + 161\ A*A - 313\ B*B + 0.78\ C*C - 72\ A*B + 22.6\ A*C + 53.0\ B*C$$

where Y is the response variable; A, peptone; B, Beef extract and C is the sucrose

The analysis of variance (ANOVA) results was analysed to check the significance of the fit of the first-order polynomial equation of the FCCD experimental data. The model coefficient of determination R^2 value was calculated as 0.831, indicating that the 83.11% variability in the response was better explained by the model (Sharmila, Nidhi, & Muthukumaran, 2013). The model's fisher F values of 5.47 and the *p* values <0.007 indicate the model is statistically significant (Table 2). The CCD experimental runs, observed as well as predicted response was shown in Table S3. The optimum level of each variable and the effect of their interaction on response were studied by the 2D contour plots counter to any two independent variables withholding other variables at a constant of and 3D scattered plot. The interaction of beef extract and peptone showed that they are equally important to maximise the EPS production (Fig. 3A). A nitrogen source factor A (peptone) have shown to acts as a supporting role to maximise the response, while factor B (beef extract) have shown crucial role for the EPS production. The factor C (sucrose), being a substrate plays a role of basic building block for the EPS production (Fig. 3A, B, C). It seems that all the factors works together to optimise the EPS production. The factor sucrose with the high concentration, 4.0%, and 0.4% of the individual factor beef extract and peptone showed a maximising effect on EPS production. (Fig. 3B, C, D).

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Table 2
Analysis of variance of and their influence on the response by CCD experimental design.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	8404.8	933.87	5.47	0.007
Non-linear	3	6897.1	2299.02	13.46	0.001
A. Peptone	1	0.6	0.64	0.00	0.952
B. Beef extract	1	303.0	303.04	1.77	0.212
C. Sucrose	1	6593.4	6593.39	38.59	0.000
Square	3	139.9	46.62	0.27	0.844
AxA	1	36.1	36.10	0.21	0.656
BxB	1	136.3	136.34	0.80	0.393
CxC	1	8.5	8.46	0.05	0.828
2-Way interaction	3	1367.9	455.95	2.67	0.105
AxB	1	21.3	21.28	0.12	0.731
AxC	1	207.6	207.59	1.21	0.296
BxC	1	1139.0	1138.98	6.67	0.027
Error	10	1708.6	170.86		
Lack-of-Fit	5	1263.7	252.75	2.84	0.138
Pure error	5	444.9	88.98		
Total	19	10,113.4			

3.5. Validation of the model at 1 L and 10 L fermenters level

To validate the continual of the model for predicting conditions and optimum response values, the model was validated with the selected optimal conditions of the full factorial and central composite design in 500 mL of Erlenmeyer flask containing 200 mL of the optimal composition of media (Peptone, 0.4%, beef extract, 0.4% and NaCl, 0.5% and 4% of sucrose) followed by 1 L and 10 L fermenters. Under the suggested conditions, the response value of EPS yield was 97.6 g/L. In shake flask studies, the results of FFD and CCD concerning BioLector have shown the variation of ±1.6 and ± 4.79 g/L, respectively (data not shown), which confirms the variation in the production and the reproducibility of the model process parameters. The comparative result of fermenters has also confirmed the significance of the model. The value of Microbioreactor (BioLector Pro) to 1 L and 10 L fermenter level showed the variation of ±9.9 g/L and ± 23.6 g/L of EPS. The variation of ±9.9 g/L and ± 23.6 g/L of EPS in Microbioreactor to fermentor may cause the continuous supply and maintaining 10% oxygen saturation. In the validation at large scale 1 L and 10 L fermentation, optimum EPS 107.3 g/L and 123.9 g/L were observed, respectively. The optimum EPS synthesis was observed at the 21 h of fermentation and started decreasing (Shih, Yu, Shieh, & Hsieh, 2005). It is expected that once the carbon source depletes, organisms enter the stationary phase and start utilising EPS as a carbon source for survival. Therefore organism was observed growing continuously and could not attain the decline phase. The common condition in fermentation, as the organism metabolises the nutrients, various organic acids are produced, indicating a drop in pH. In contrast, pH increases due to ammonia production initiate upon utilisation of proteins (Fig. 4). There is a similar pattern of pH drop and rise was observed from 1.0 mL to 10 L. However, the values of pH in 1.0 mL have differed. The difference in pH values cannot be explained well in 1.0 mL volume because the combinations of media components were made manually. It may possible that upon adding stock components, pH showed variation. The added volume of stock solution may lead to minor variation in pH of 1.0 mL. And we have observed that the organism showed optimal growth in the range of pH 6–7 (Fig. 1b).

3.6. Characterization of exopolysaccharide

3.6.1. Field Emission Scanning Electron Microscopy (FE-SEM)

The scanning electron micrographs showed a significantly fibrous network structure with a relatively smooth surface of polysaccharides. The surface morphology of test EPS was compared with standard EPS (Levan) of *Erwinia herbicola.* A smooth surface was observed in the standard EPS (Fig. 5A) as well as test EPS (Fig. 5B) with various ranges of fibre diameters. The fibre diameters were found in the range of 3.620 μm to 9.071 μm forming different pore sizes of the structure. Test EPS showing comparable morphology and fibrillar diameter with the standard. Variation in diameter and surface structure could be due to various branching patterns and different degrees of polymerisation of EPS from organism to organism (Fig. 5A and B).

3.6.2. Monosaccharide composition analysis

Monomeric units of polysaccharides were analysed using thin-layer chromatography, high-performance liquid chromatography and liquid chromatography high-resolution mass spectroscopy. TLC, HPLC and LC HR-MS analysis have revealed that polysaccharides have solely made up of a single type of monosaccharide, D- Fructofuranose with a 180 Da ($C_6H_{12}O_6$, 203.05 Na). Presence of D- Fructofuranose reveals that polysaccharide belongs to the homopolysaccharide type of polymer, so it can be confirmed that it is a fructan class of polysaccharide (Fig. S5a, S5b and S5c).

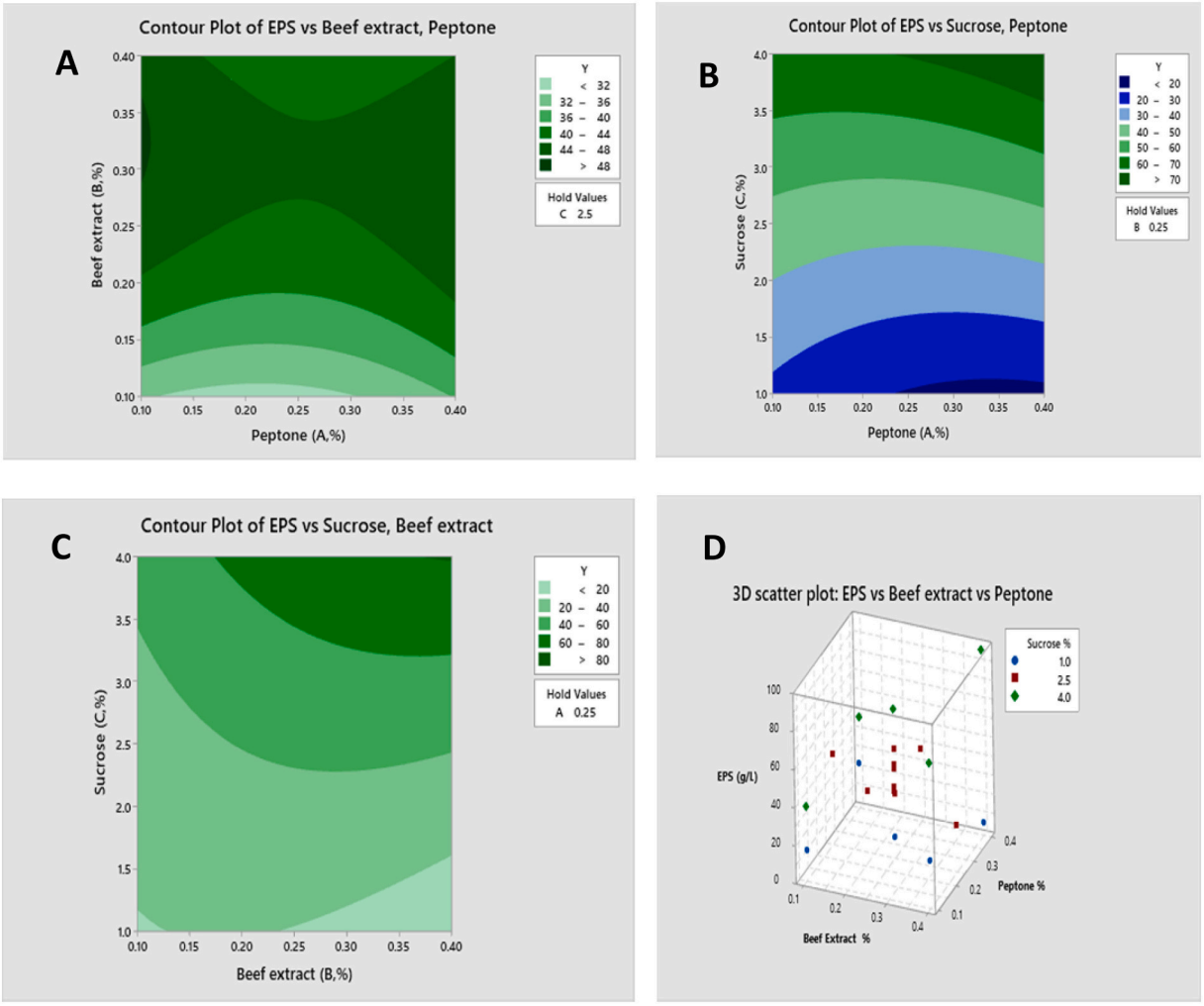


Fig. 3. Response of variables and 3D scatter plots on EPS production. A) peptone and beef extract Vs EPS, B) Sucrose, peptone Vs EPS C) Sucrose, beef extract Vs EPS and D) 3D scatter plot of central composite design data points.

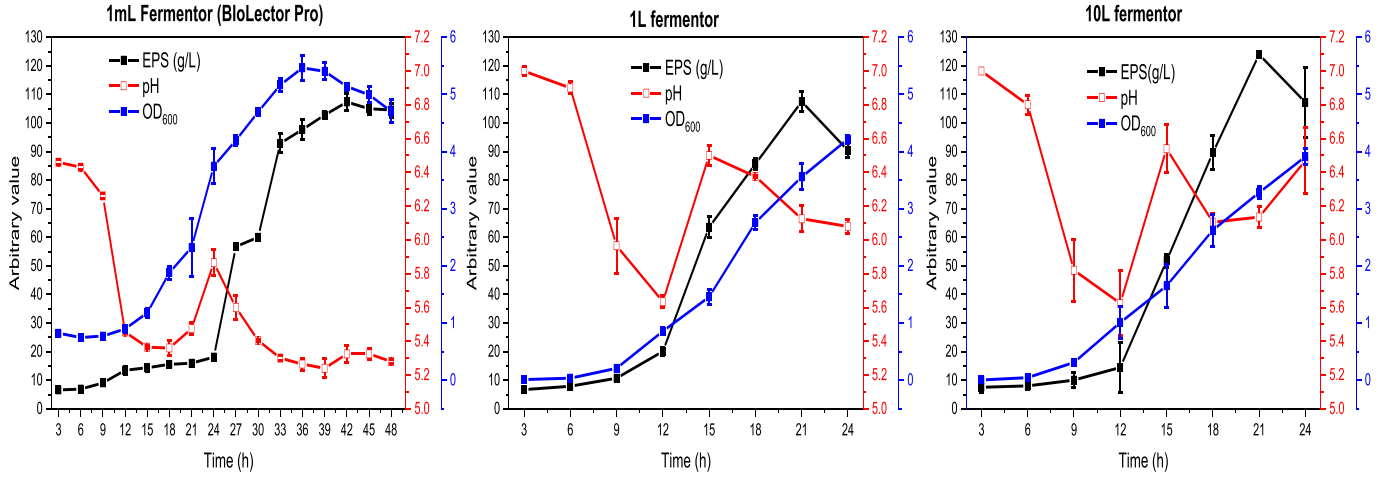


Fig. 4. Microbiolector comparative validation for EPS production. BioLector (1.0 mL) Vs Fermentor (1−10*L*).

3.6.3. FTIR spectroscopy

Various functional groups present in the polysaccharide were analysed and assigned using the Fourier transform infrared spectroscopy spectra. Characteristic absorption spectra of polysaccharides were shown in the results (Fig. 6). The broad and strong intensity peak at 3371 cm^{-1} corresponds to Hydroxyl (O—H) stretching, characterizing the polyhydroxy nature of polysaccharides (Zhou et al., 2016). The weak absorption at 2938 cm^{-1} and 2886 cm^{-1} are corresponding peaks to C—H stretching, suggesting the presence of methylene groups vibrating asymmetric and symmetrically (Thakham et al., 2020). Further, an

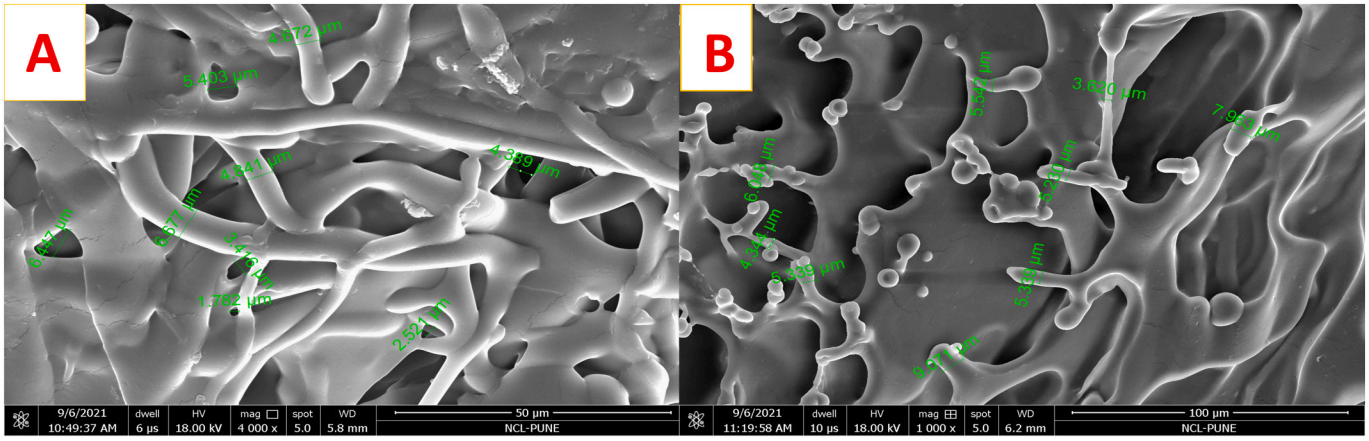


Fig. 5. Scanning Electron micrograph A. Standard EPS from *Erwinia herbicola*, B. Test EPS from *Bacillus* sp. SGD-03.

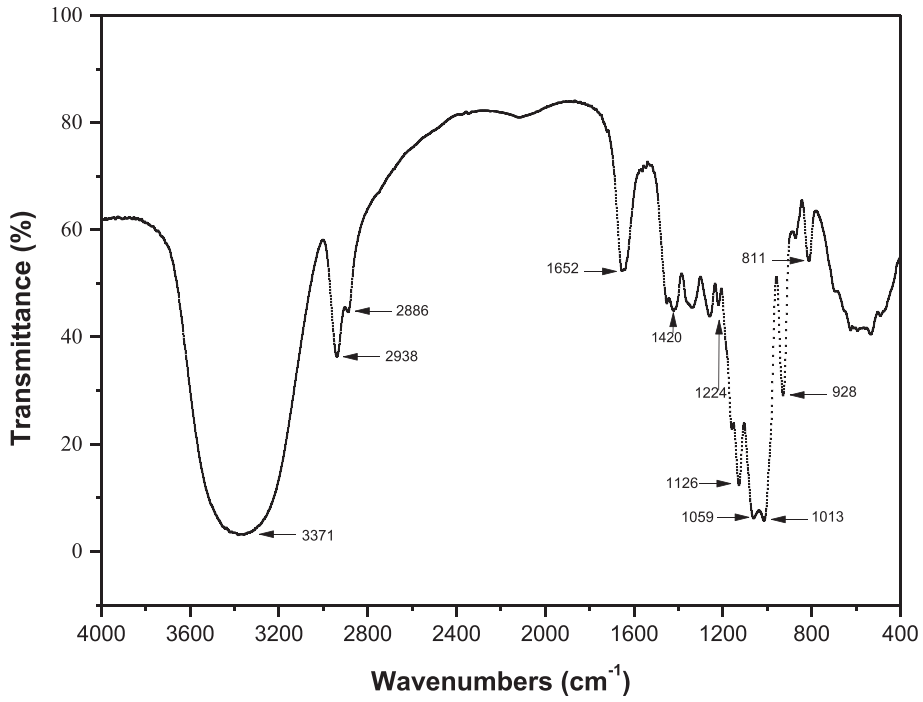


Fig. 6. Functional group determination of the EPS produced by Bacillus sp. SGD-03.

intense absorption at 1652 cm^{-1} is the characteristic carbonyl (C=O) stretching vibration peak. It could be due to the Aldo/ketose nature of saccharides (Vasanthakumari, Harikumar, Beena, Pandey, & Nampoothiri, 2015). Medium absorption peaks between 1420 cm^{-1} to 1191 cm^{-1} belong to deformation vibration of the C—H and O—H groups (Ibrahim, Alaam, El-Haes, Jalbout, & Leon, 2006). A medium intense peak of C-O-H vibration appears at 1126 cm^{-1} (Thakham et al., 2020). An absorption peak at 1059 cm^{-1} suggests the presence of D-fructose. The strong absorption at 1013 cm^{-1}, C-O-C stretching, is the indicative marker peak of the polysaccharides. The absorbance peak between 1000 and 800 cm^{-1} belonged to typical carbohydrates. The peaks at 928 and 811 cm^{-1} are corresponding peaks of the furanoid ring structure of the saccharide unit (Ni et al., 2018; Tanaka et al., 1980).

3.6.4. Molecular weight determination

The average molecular weight of purified EPS was determined using size exclusion chromatography. The calibration curve for molecular weight determination was made using several standards of Pullulan polysaccharide ranging from ~350–700 Mp. Based on calibration and comparing with standard Pullulan and Dextran, the size exclusion chromatogram has shown that the purified EPS comprises an average molecular weight of 1.0×10^4 Da. Supplementing the initial concentration of sucrose, 40%, and consumption of about a quarter of sucrose, Levan with less than 1.0×10^4 Da molecular weight was produced (Tanaka, Oi, & Yamamoto, 1980), so it can be assumed that upon utilisation of 100% sucrose, the Levan with molecular weight, 1.0×10^4 Da could be produced (Fig. 7).

3.6.5. NMR analysis

To determine the glycosidic linkage pattern between the monosaccharide residues and elucidation of structure, the spectra of 1D and 2D, ^1H and ^{13}C NMR were analysed. The ^1H NMR seven signal at the range of δ 3.49 and δ 4.13 and ^{13}C NMR shows six signals at a range of δ 59.9 to δ 80.29 shows characteristics peaks of sugar (Fig. 8B, C, S6, 8D and S8). Also, the ^{13}C NMR spectra signal at δ 104.2 shows the characteristic peaks of anomeric carbon present in the sugar (Fig. 8C and S6).

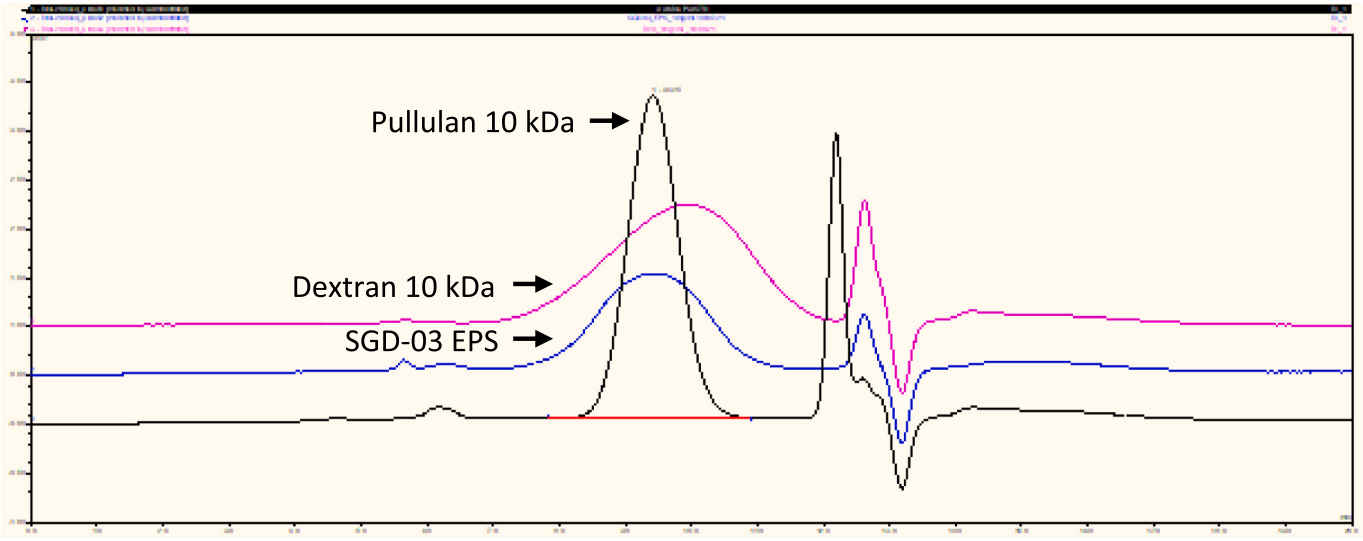


Fig. 7. Size exclusion chromatogram of the *Bacillus* sp. SGD-03 EPS.

The DEPT and HSQC spectra show two methylene peaks at δ 59.9 (δ 3.71 and 3.60) and δ 63.4 (δ 3.48 and δ 3.83), indicating the presences of the keto-sugars (Fig. S7 and 8G). In the COSY spectra, the signal at δ 3.48 and δ 3.83 shows a correlation of δ 3.89 protons. The proton at δ 3.89 correlation at δ 4.03 proton and further this δ 4.03 proton shoes correlation with δ 4.12 proton indicate that both are serially connected (Fig. 8A and Fig. 8E). After careful analysis of 1D and 2D NMR (Fig. 8A to 8H) and literature report, confirmed the fructose sugar (Taylan, Yilmaz, & Dertli, 2019). The linkage of the fructose sugar was confirmed using NOESY NMR spectra. The proton on C-3 carbon δ 4.13 shows NOESY correlation peak at proton present on C-1 carbon of δ 3.71 and δ3.61, indicating that both are in the same plane. Hence the sugar is linked by beta linkage (Fig. 8A and Fig. 8F). The NMR spectra confirm that the purified EPS is Levan, linked by β (2–6)-D-fructofuranosyl residue. The carbon chemical shifts were also compared with previously reported levan from other sources shown in Table S4 (Han & Clarke, 1990; Pei, Ma, Chen, & Liu, 2020; Wahyuningrum & Hertadi, 2015).

3.7. Biomass and EPS quantification

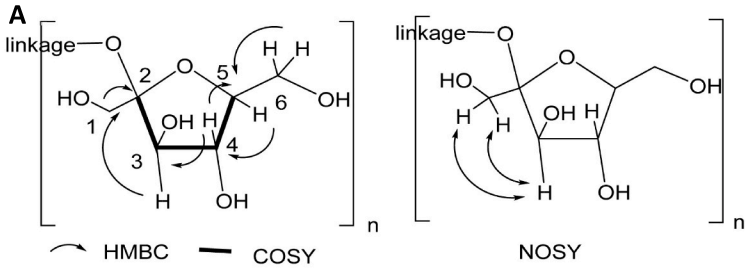
The biomass concentration was calculated by diluting the known concentration of dry weight of the cell as per the requirement. The value of readings of optical density at 600 nm and light scattering at 620 nm was plotted, and biomass concentration was calculated and correlated (Fig. S9), keeping a thumb rule, 1 unit of optical density corresponds to 1.0 g/L of biomass (Myers, Curtis, & Curtis, 2013). The viable cells were counted as CFU/mL and found that the viable cell count has increased in the same pattern from 12 to 24 h of cultured media in both shake flask and BioLector (Microbioreactor). In the case of BioLector, the CFU was drastically increased in 36 h and reached 1.4×10^9 CFU/mL and declined further (Fig. S10). While in shake flask the CFU was declined after 24 h. It can be inferred that the organism grows exponentially after 24 h of incubation in Biolector Pro (Microbioreactor). All the EPS quantification was done by revaluated phenol sulphuric acid method by Rao and Pattabiraman (1989). The unknown concentration of EPS was estimated by a linear graph of standard pullulan polysaccharides (Fig. S11). EPS purification cannot be performed with 1.0 mL of fermented broth, as cleansing includes precipitation, deproteinization, and dialysis. The quantified EPS can be considered as partially purified or crude as it was a quantification of EPS (threaded appearance) after ethanol precipitation. Here we have shown a comparative profile of crude EPS (cEPS), deprotienised EPS (dpEPS), and purified EPS after dialysis and fraction collected by column chromatography. It was found that 20.74 g/L of pure EPS could be recovered from the crude EPS (Fig. S12).

It is well known that microorganisms produces a vast range of compounds, from fuels to commodity chemicals and pharmaceuticals to fine chemicals (Carbonell et al., 2018; Harry, Lee, Taek, & Leonard, 2015; Zhang, Wang, Ang, & Zhao, 2016).

However, despite the development of microbial platforms to convert nearly any carbon source into the desired product, a modest number of these cases have seen the successful transition to industrial-scale and marketed as products. Economic competitiveness with the established chemical or biosynthetic route is an important factor. Low titers and low yields in the laboratory setting also need to be overcome to proceed with the scale-up (Arturo et al., 2018; Carbonell et al., 2018; Miguel, Barbara, Juan, Bernhard, & Adam, 2014). The ability to perform the upstream processing in an automated system under the sterile conditions allows for the preparing the cultivation media for the small-scale cultivation directly on the robotic deck prior to cultivation (David, Westphal, Bunk, Jahn, & Franco-Lara, 2010; Kottmeier, Müller, Huber, & Büchs, 2010).

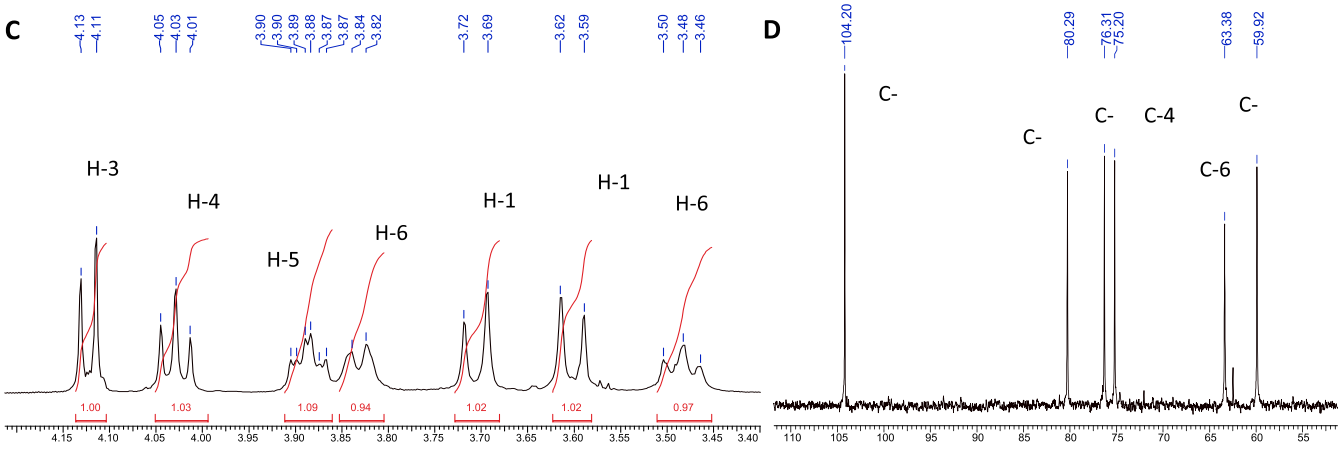
Considering the widespread use of levan in various industrial applications, the most common levan producer microorganisms are *Lactobacillus reuteri* (Van Geel-Schutten et al., 1999), *Streptococcus salivarius* SS2 (Simms, Boyko, & Edwards, 1990), *Zymomonas mobilis* (Chiang, Wang, Chen, & Chao, 2009; de Oliveira, da Silva, Buzato, & Celligoi, 2007), *Microbacterium laevaniformans* (Bae, Oh, Lee, Yoo, & Lee, 2008) and *Bacillus* sp. (Arvidson, Rinehart, & Gadala-Maria, 2006; Shih et al., 2005) and all reported from the terrestrial environment. Microbial levans are produced from sucrose-based substrates by various microorganisms and are polymers of fructose residues (fructans) synthesized by levansucrase enzymes (E.C. 2.4.1.10). These enzymes cleave their substrate sucrose and couple the fructose residue to a growing fructan chain. Based on molecular weights, levans are classified as low molecular weight (LMW) and high molecular weight (HMW). Molecular weight of 8 kDa to 50 kDa referred as LMW while product with a molecular weight $>$ 50 kDa referred as high molecular weight (HMW) levans (Ortiz-Soto, Porras-Domínguez, Seibel, & López-Munguía, 2019). In general, LMW levans are related to health applications in the food sector and function as cholesterol-lowering weight loss (Öner, Hernández, & Combie, 2016), and prebiotics which modulates intestinal microflora of humans (Kang, Chun, & Jang, 2005), while HMW levans possess antitumor, antioxidant property (Liu, Luo, Ye, & Zeng, 2012). A levan of 10 kDa (LMW) is a promising source of prebiotic, which can further generate FOS to enhance gut colonic microbiota by stimulating bifidobacteria.

A



B

	δC	δH
C-1	59.9	3.71 (d, J = 13.0 Hz, 1 H), 3.60 (d, J = 13.0 Hz, 1 H)
C-2	104.2	
C-3	76.3	4.12 (d, J = 8.4 Hz, 1 H)
C-4	75.2	4.03 (t, J = 8.0 Hz, 1 H)
C-5	80.3	3.89 (dt, J = 3.4, 7.8 Hz, 1 H)
C-6	63.4	3.48 (t, J = 9.9 Hz, 1 H) 3.83 (d, J = 7.6 Hz, 1 H)



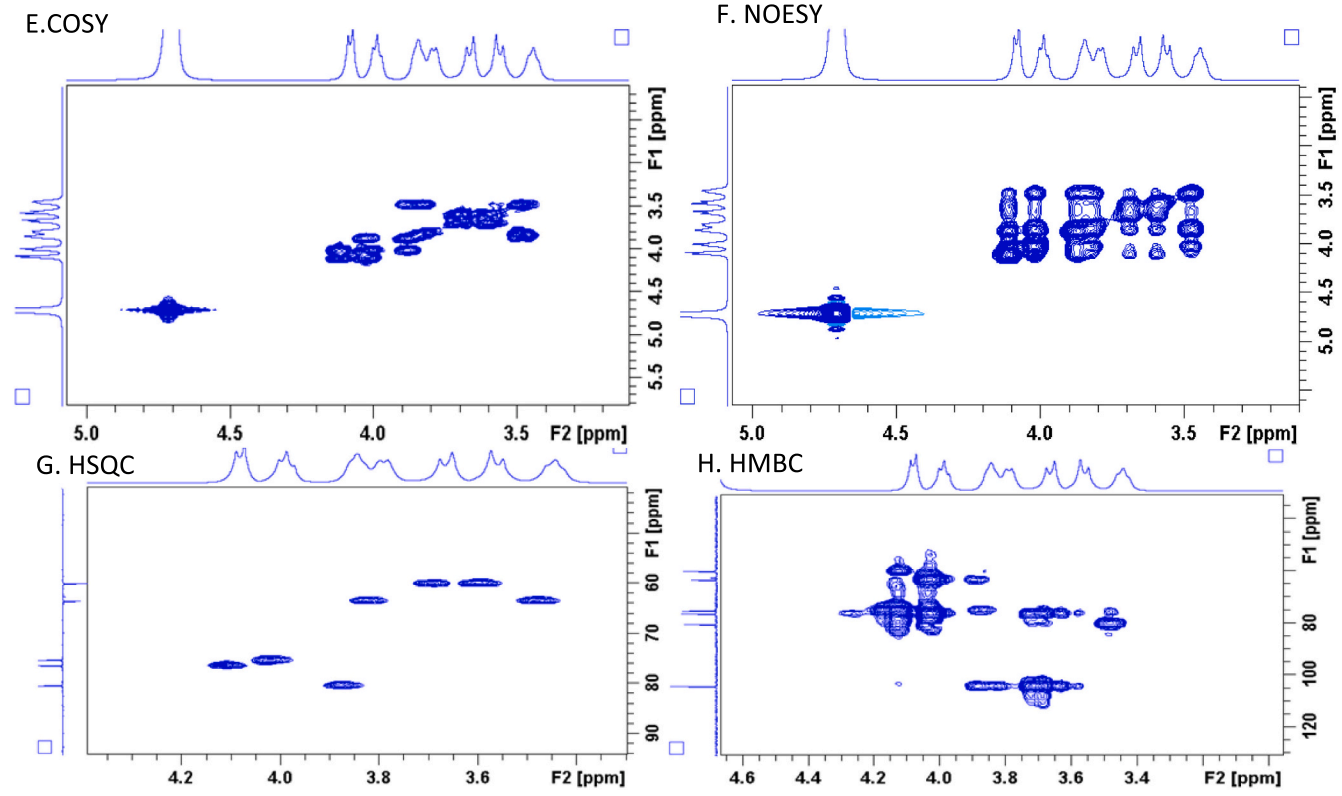


Fig. 8. NMR analysis of the EPS produced by Bacillus sp. SGD-03: **A.** Linkage; **B.** δ value of C & H; **C.** $_1$H spectra; **D.** $_{13}$C spectra; **E.** COSY; **F.** NOESY; **G.**HSQC; **H.**HMBC.

In the present work, marine microorganisms isolated from Chorao Island, Goa were screened for their EPS production abilities, and the new *Bacillus* strain SGD-03 was identified as the prominent producer of EPS. Characterization of EPS studies based on NMR analysis indicated that the nature of this EPS was a levan type with a repeating unit composed of β-(2,6)-D-fructofuranosyl residues. In addition, 123.9 g/L crude levan yields from the stationary phase of Microbioreactor condition using a defined media containing sucrose as sole carbon was achieved. Determination of quantitative process parameters for optimising production strains and cultivation conditions is key for bioprocess development for almost every new microbial product. The EPS production processes in the MTP cultivation system BioLector Pro, the obtained growth and production behaviour has been validated in 10,000 times magnitude from 1.0 mL to 10 L with similar production yield from 107 to 123 g/L of EPS respectively.

4. Conclusion

The present study aimed to develop a cost-effective, robust process to produce EPS from a marine organism and optimise the EPS production by a high throughput Microbioreactor system. A potent EPS producer strain *Bacillus* sp. SGD-03 was isolated from marine sediment and subjected further for optimization on the BioLector Pro system. Microbioreactor data was validated on shake flask, followed by 1 L and 10 L fermentor. Produced EPS was completely characterized by the FE-SEM, SEC, FTIR, TLC, HPLC, LC-HRMS, and NMR spectroscopy and has been identified as levan with a molecular weight of 1.0×10^4 Da. Production of EPS was enhanced sequentially, involving screening of best carbon source and their different concentrations. Screening process parameters by full factorial design of experiment at various levels of factors were carried out, followed by the central composite design experiment of response surface methodology. The optimum media composition standardized with 1.0 mL of working volume in BioLector Pro was validated by ANOVA and reproduced by shake flask, 1 L and 10 L fermenter level, and found significant reproducibility from BioLector Pro to Fermenter, i. e., 1.0 mL: 1 L: 10 L fermentation. To support the hypothesis that to mimic the nutrient deficiency and osmolarity pattern, it was observed that the organism grows and produces EPS in optimal media constitutes peptone, 0.4%, beef extract, 0.4%, and sucrose 4.0%. It was observed that the organism was grown well in 2.0–4.0% salt concentration from 18 to 20 h and started declining afterward. Similarly, production of EPS found maximum at 21 h and decreased thereafter. It can be concluded that the sucrose concentration (screening to optimisation) 2.0–4.0% and diluted media was found suitable to mimic the pattern of osmolarity and nutrient deficiency enhancing EPS production by utilising sucrose as a substrate. We have formulated minimal media for the complete conversion of substrate into product. At an optimised minimal production media, the marine strain produced 123.9 g/L of crude EPS which resulted in 20.7 g/L pure Levan, which is approximately valued at 1.12 USD/L of the media chemicals used for the production, this cost will be drastically reduced when you scale up the production and the possibility to explore for industrial-scale with different commercial applications.

CRediT authorship contribution statement

Vasudev S. Wagh and **Syed G Dastager** performed the literature search and wrote the manuscript. **Vasudev Wagh** and **Jagadish S. Bennale** performed the fermentation experiments and validation. **Vasudev S. Wagh** and **Madhukar S. Said** performed the experiments for structural elucidation. **Syed G. Dastager** conceived the idea of the work and supervised the project. All authors read and approved the final manuscript.

Declaration of competing interest

The author declares no conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119241.

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