Process Development, Molecular and Chemical Approaches to Improve the Production and Bioactivity of an Exopolysaccharide from a Probiotic *Lactobacillus plantarum* for Food Applications

by

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-Soumya M P

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg/L	Microgram per litre
a-EPS	Acetylated EPS
AGE	Agarose gel electrophoresis
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
CFU/mL	Colony Forming Units per Milliliter
cm	Centimetre
CMC	Carboxymethyl cellulose
Cm-EPS	Carboxymethylated EPS
Cmp	Chloramphenicol
Conc.	Concentration
CSH	Cassava Starch Hydrolysate
CTAB	Hexadecyl trimethyl ammonium bromide
Da	Dalton
DMEM	Dulbecco′s Modified Eagle′s Medium
DMSO	Dimethyl sulfoxide
DNS	3,5-Dinitrosalicylic acid
DPPH	1, 1-Diphenyl-2-picrylhydrazyl
DS	Degree of Substitution
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharides
Ery	Erythromycin
EtBr	Ethidium bromide
EtOH	Ethanol
F'/R'	Forward / Reverse Primers
FBS	Fetal Bovine Serum

Fig	Figure
FTIR	Fourier Transformed Infrared
g/L	Gram per liter
GIT	Gastrointestinal Tract
GPC	Gel Permeation Chromatography
GRAS	Generally Regarded as Safe
GTF	Glycosyltransferase enzyme
gtf	Glycosyltransferase gene
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
HePS	Heteropolysaccharides
HoPS	Homopolysaccharides
HPLC	High-Performance Liquid Chromatography
IBS	Irritable Bowel Syndrome
kb	Kilobase
kDa	Kilodalton
L	Litre
LAB	Lactic Acid Bacteria
LB medium	Luria Bertani medium
LD	Lethal Dose
MCS	Multiple Cloning Site
mg	Milligram
MHz	Mega Hertz
min	Minutes
mM	Millimolar
mMRS	Modified MRS
MRS	de Man, Rogosa and Sharpe
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl Tetrazolium bromide
Mw	Molecular weight

NaAl	Sodium alginate
NCBI	National Center for Biotechnology information
ng/µL	Nanogram per microliter
NICE	Nisin Controlled Expression System
nm	Nanometer
nmol $^{-1}$	Nanogram per mole
OD	Optical density
Pa-s	Pascal second
ppm	Parts per million
RID	Refractive Index Detection
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RSA	Radical Scavenging Activity
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
s-EPS	Sulphated EPS
SmF	Submerged Fermentation
SO_3.Py	Sulfur trioxide Pyridine complex
TA	Titratable acidity
TGA	Thermogravimetric analysis
TMHMM	Transmembrane prediction using hidden Markov models
UC	Ulcerative colitis
V	Voltage
v/v	Volume by volume
Vc	Vitamin C Equivalents
VCC	Viable Cell Count
vvm	Volume per volume per minute
w/v	Weight by volume
WHC	Water Holding Capacity

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Chapter 1

Chapter 1

Introduction and Review of Literature

1.1. Introduction

Exopolysaccharides (EPS) are high molecular weight polysaccharides produced by microbes and are secreted out of the cell to carry out various functions including cell adhesion, cell protection from external stress, and source of nutrients during shortage (De Vuyst and De Vin, 2007; Sengupta et al., 2018) Since bacteria can produce unique EPSs with specific properties, several researchers have identified and analyzed these EPSs in detail. Parameter optimization for fermentative production, exploration and exploitation of cheaper raw materials and use of a metabolically engineered strain are the three major possibilities for ameliorating the large-scale production that paves way for the commercial production and industrial attention of microbial polysaccharides (Welman and Maddox, 2003). One of the major challenges to bridle in this area is to increase the EPS production that must be remunerative and not idealistic.

Lactobacilli has acquired remarkable attention owing to their probiotic properties and as the EPS they produce are generally regarded as safe (GRAS) and have hardly any health risks. Exopolysaccharides secreted by LAB have been found to have unique physical and rheological properties that facilitate their most valuable application in the improvement of the rheology, texture, and mouthfeel of fermented dairy products (Welman and Maddox, 2003) that adds more value to these EPSes. Moreover, previous studies show that the EPS can withstand the conditions of the gastrointestinal tract enhancing and retaining the colonization ability of the probiotic bacteria. Exopolysaccharides are proven to have potential health benefits known especially for their varied biological properties like antioxidant, immunostimulatory, anti-diabetic, antitumor,

and cholesterol-adsorption to list a few. *In-situ* production of EPS, having potent biological activities, by Lactobacilli has drawn the attention of researchers to this field which foresees the possibilities of EPS as both food additives and fermented food ingredients.

Research pertaining to Lactobacilli is a flourishing area with lots of scopes and applications that benefit mankind. The indigenously isolated probiotic Lactobacilli serves as a potential source of prebiotic and postbiotic components where these cell factories are capable of producing lactic acid, acetic acid, sugar alcohols, antifungal molecules, oligopeptides, sugar polymers, and vitamins The industrial relevance of these compounds is pretty high considering their market demand and applications, of which exopolysaccharides of LAB are one such metabolite. Foreseeing the GRAS status of EPS produced by a probiotic lactobacillus, it has wide applications in food industries and the health sector. But the major limitation of LAB EPS is the yield and economic viability. The reported maximum production titer for an EPS from *Lactobacilli sp.* is limited to a range between 2.3-2.5g/L (Sørensen et al., 2022). To improve the yield and manage the cost of production, employing cheap and sustainable carbon sources can increase the feasibility of EPS bioprocess.

The EPS production level is directly correlated to the *gtf* gene expression level and could possibly be raised by over-expression (Boels et al., 2003) of certain genes. The glycosyltransferase genes and their gene products are directly involved in the EPS biosynthetic pathway of Lactobacilli as they play a pivotal role in the transfer of sugar moieties from the high-energy sugar nucleotides and thus help in the polymerization and formation of exopolysaccharides. This scenario can be exploited to make use of the least explored possibilities for a controlled homologous expression of these genes in Lactobacilli.

Modification of the innate EPS either chemically, or genetically, for enhanced biological activity is one of the alternate threshold areas to work on. Sulphonation, carboxymethylation, acetylation and phosphorylation are some of the reported chemical modifications that positively effects the physiochemical nature of the EPS. Specially, sulfonation is most widely used and confirmed to remarkably enhance the biological activities of polysaccharides, including anticoagulant and anti-inflammatory activities (Zhang et al., 2017). Modified EPS usually finds applications in pharmaceutical, and food industries. Introduction of suitable ionic groups with appropriate degree of substitution can significantly improve the bioactivity of polysaccharides, such as antioxidant, antitumor, antiviral and immunomodulating activities. However, chemical modifications of exopolysaccharide from Lactobacillus have been scarcely reported. Therefore, it is of great interest to investigate the methods to increase the EPS yield and subsequently enhance the bioactivity of exopolysaccharides from Lactobacillus *via* chemical modifications (Wang et al., 2015) and ultimately their application in food products.

Preliminary studies on the biological properties of *L. plantarum* BR2 revealed that it exhibits potent antioxidant properties which are impressive for its applications in food products. Considering all these parameters, the major objectives of this research work have been framed.

1.2. Objectives of the Study

The major objectives of the work addressed in this thesis are as follows:

- Characterization of exopolysaccharide-producing *Lactobacillus plantarum* BR2
- To explore alternate carbon sources for EPS production using indigenous *Lactobacillus plantarum* BR2
- To isolate glycosyltransferase genes (*gtf* genes) from the EPS-producing *Lactobacillus plantarum* BR2

- To clone and over-express the selected *gtf* genes and to study their role in EPS production in *Lactobacillus plantarum* BR2
- To chemically modify the *L plantarum* BR2 EPS for improving their biological properties
- Development of EPS-fortified probiotic functional yogurts with better organoleptic properties

The thesis is framed in eight different chapters where Chapter 1 gives a general introduction and highlights the significant and recent developments on probiotic LAB research focusing specially on exopolysaccharides production, purification and its characterization, functional role and relevance of glycosyltransferases in exopolysaccharide production by lactobacilli, physiochemical modifications of EPS for better biological properties and the diverse EPS applications. Chapter 2 provides the detailed methodologies of the general assays and techniques invariably used throughout the thesis. Chapter 3 describes the salient features of our indigenous isolate *L. plantarum* BR2 and its EPS. The exploration of an alternative carbon source for EPS production by *L. plantarum* BR2 is demonstrated in Chapter 4. Chapter 5 basically illustrates the over-expression of a glycosyltransferase gene (*gtf*) in *L. plantarum* BR2 thereby increasing the overall EPS production. This shows that in the EPS biosynthetic pathway, *gtf* plays a vital role in the transfer of sugar units from the high-energy sugar nucleotides and thus helps in the polymerization and formation of exopolysaccharides. Chapter 6 deals with the modification of native EPS by various chemical reactions. Preparation of EPS derivatives through acetylation, carboxymethylation and sulphonation of the *L. plantarum* BR2 EPS, its characterizations, and the subsequent evaluation of their biological properties have been reported in this chapter. Chapter 7 deals with the EPS application studies in the development of probiotic

functional yogurts employing skim milk using an indigenous LAB starter culture, *S. thermophilus* CUD3. Chapter 8 summarizes the major highlights of the research work, conclusions derived, and the future perspectives of the study followed by bibliography.

1.3. Review of Literature

The current section highlights the significant and recent developments in lactic acid bacterial research focusing especially on exopolysaccharides production, purification and its characterization, functional role, and relevance of glycosyltransferases in exopolysaccharide production by lactobacilli, physiochemical modifications of EPS for better biological properties and the diverse EPS application studies.

The global market of probiotic ingredients, supplements and food was worth $14.9 billion in 2007, $15.9 billion in 2008, and reached $58.2 billion in 2021, representing a compound annual growth rate of 8.7%. Extensive investigations of probiotics have been greatly enhanced by the research of new microbes for future probiotic bacteriotherapy applications (Soccol et al., 2010).

The emerging demand for the products having health benefits beyond nutrition has provided ample opportunity to explore relatively unexplored foods and beverages for isolation of lactic acid bacteria for their potential role in probiotic research. Due to the fact that these isolates were derived from fermented items and were deemed safe for consumption, traditional foods can be a source of lactic acid bacteria for probiotic use. Additionally, it is possible to suggest using these foods and drinks as probiotic delivery systems, which would stimulate demand for these traditional foods and, in turn, boost the rural economy (Angmo et al., 2016).

1.3.1. Probiotic Lactic Acid Bacteria

1.3.1.1. Common Probiotic Properties and Characteristics

A viable candidate for probiotics must be non-pathogenic, yield favourable *in vivo* effects, and endure the gastro-intestinal environment of hosts, including resistance to acidic pH, lysozyme and high bile salt concentrations. Such probiotics must also have strong intestinal epithelial adhesion and symbiotic colonisation with the native gut flora. One of the most prevalent forms of bacterial microorganisms that fulfil these qualities is lactic acid bacteria, which includes various species and strains of Lactobacillus and hence are being employed more frequently and increasingly used as probiotics which have the status of being generally regarded as safe (GRAS) (Gupta et al., 2023). Probiotics ought to be physiologically and genetically characterised, and they ought to be able to enter the gut in a viable state following product manufacturing, storage conditions, and gastric passage (Wegh et al., 2019).

Acid tolerance is crucial for the strains to endure stomach stressors for longer periods of time while being transported by high-acid meals like yoghurt without suffering a loss in population. When compared to pH 7, some isolates at pH 3 demonstrated an equivalent percentage of vitality, according to an in vitro investigation on low pH tolerance and according to some reports the resistance to low pH by *Lactobacillus* is due to the presence of F0F1-ATPase activity. In order to survive in the small intestine, they need to be able to tolerate bile salts, which are toxic to living cells. Most of the LAB strains are tolerant to bile salts up to 8% (Riaz Rajoka et al., 2017). The hypocholesterolemic effect that probiotics have on their hosts is another crucial characteristic, however it is not a requirement but rather one of the desired traits of the probiotic strain. It has been shown that Lactobacilli and *Bifidobacterium bifidum* exhibit a coprecipitation impact of cholesterol and deconjugated bile acids in relation to a reduction in cholesterol level

(Bhat and Bajaj, 2018; Wu et al., 2020). LAB promotes health through a number of different mechanisms. The property of adherence to intestinal epithelial cell is one of the mechanisms which involved different type of interaction. Several researchers have noted that aggregation capacity and hydrophobicity are connected to cell adherence qualities (Jang et al., 2019; Riaz Rajoka et al., 2017). Probiotic lactobacilli shows higher hydrophobicity towards hexadecane and other hydrocarbons (Angmo et al., 2016).

Exopolysaccharides, one of the fundamental components of the biofilm matrix, are produced by probiotics in response to stressful environmental conditions like an acid-alkaline GI situation, technological stresses, host immune response factors, and some environmental substances like ethanol and lysozyme, as well as oxidative conditions, making them more resistant. Their molecular make-up and placement on the cell surface, where it serves as a shield and traps toxins, are both the protective role of EPS (Pourjafar et al., 2022).

1.3.1.2. Health benefits of LAB

Consuming probiotics has been shown to help with a variety of clinical conditions, including cancer and female uro-genital infections as well as infantile diarrhoea, antibiotic-associated diarrhoea, relapsing *Clostridium difficile colitis*, *Helicobacter pylori* infections, inflammatory bowel disease, and relapsing colitis (De Vuyst et al., 2004). Probiotics may affect the gut microbiota by suppressing and inhibiting pathogens as well as limiting their adherence and establishment in the gut, according to some research. Immunomodulatory activities have been demonstrated for the probiotic members of the genera Lactobacillus, Bifidobacterium, and Streptococcus, with favourable effects on cell-mediated immunity and inflammation. A prospective use is the modulation of immunological development by probiotics. Particularly in infants where the most pronounced immune modulating effects have been documented. The

management and treatment of allergies, gut and respiratory infections, irritable bowel syndrome (IBS), ulcerative colitis (UC), and new-born colic have all shown encouraging benefits when probiotics are included in the diet of infants and young children. Probiotics, however, need more research and should be administered cautiously in children who are immunocompromised or have serious illnesses (Angmo et al., 2016; Wegh et al., 2019).

Over the past few decades, probiotic LAB have been the subject of the greatest research as firstly they are typically viewed as safe because they are beneficial for the gastrointestinal tract (GIT) (Naidu et al., 1999). Secondly, they contribute to fermentation and make up the bulk of the microflora in fermented products. Thus, isolation of novel probiotics from fermented milk and dairy products and their bioactive metabolite are very crucial for the production of novel value-added products (Soccol et al., 2010; Westerik et al., 2019). By producing lactic acid, acetic acid, H_2O_2, bacteriocin, diacetyl, and CO_2, they are recognised to be crucial in food preservation and to suppress spoilage bacteria or food-borne diseases. Probiotic supplements have therefore grown in favour as biotherapies for treating ailments and enhancing gut health (Wegh et al., 2019).

Fig.1.1 illustrates few of the lactic acid bacterial metabolites and its uses. Technical usability criteria, such as high sensory qualities, phage resistance, viability during technological treatment, stability during manufacturing and storage, in addition to safety and other functional attributes, should be taken into account when choosing a probiotic strain. Lactic acid probiotics contain a number of health-improving properties, including the ability to lower blood cholesterol, have antioxidant, antibacterial and anti-diabetic properties, and to inhibit the progression of hypertension where EPS also plays a significant role in contributing these health promoting benefits (Ali et al., 2023; Janahi et al., 2018).

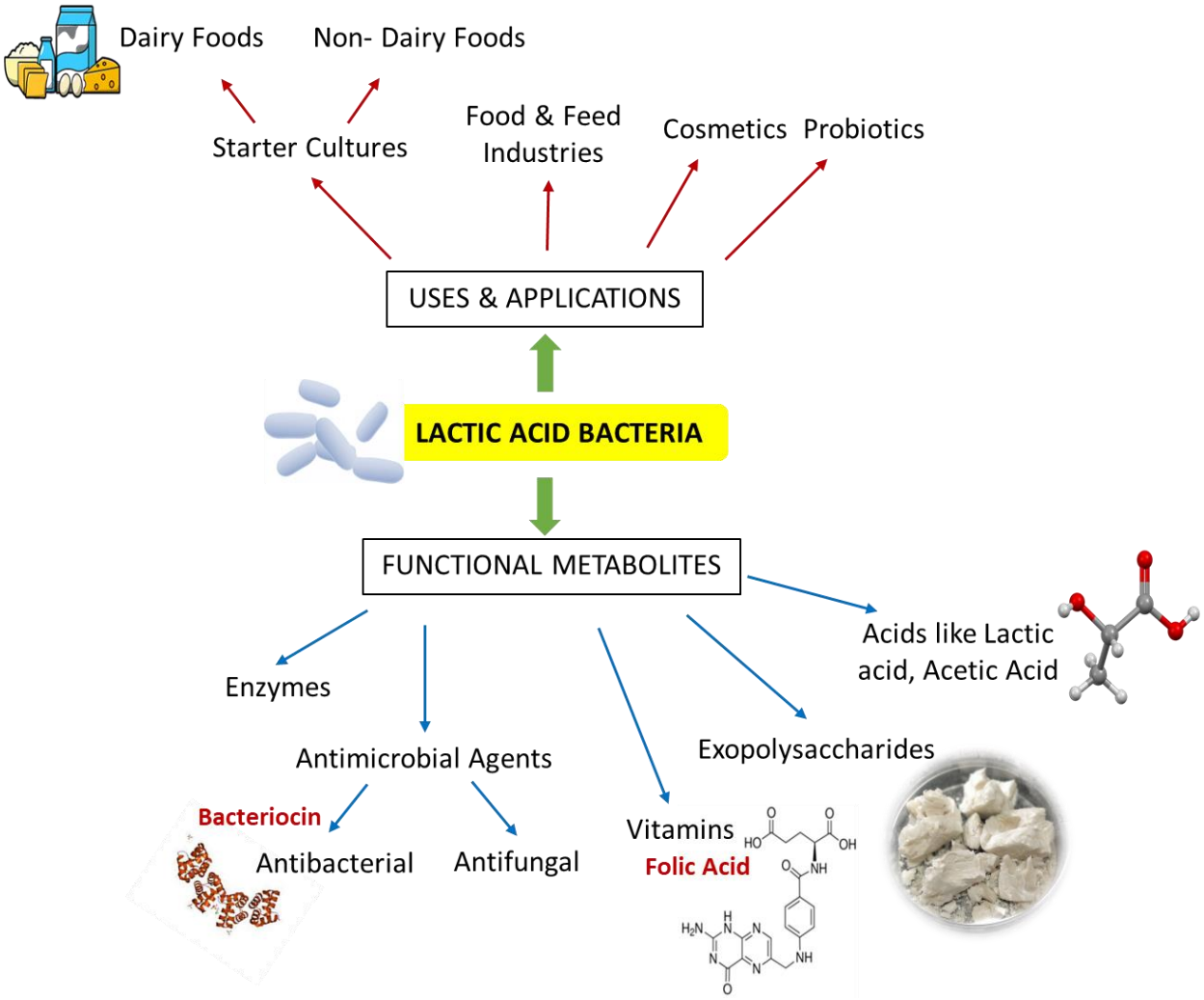


Fig.1.1. Functional metabolites of lactic acid bacteria and its use and applications

Food, pharmaceuticals, cosmetics, and the biomedical industry are just a few of the industries where bioactive EPS is used (Ayyash et al., 2020a). However, because probiotic lactobacilli found in food have not been extensively studied, EPS has mostly gone underexploited (Saif and Sakr, 2020). Probiotics producing EPS are beneficial to human health and are involved in biological activities like enhancing various immune responses *in vivo* and *in vitro* as they are known to have anti-tumorigenic and anti-immunolabelling activities (Divya et al., 2012; Vidhyalakshmi and Nachiyar, 2011).

1.3.2. Exopolysaccharides from Lactic Acid Bacteria

1.3.2.1. EPS Production from LAB

Lactic acid bacteria are the well-known mesophilic group of EPS producers. Among mesophilic bacteria genera, *Bacillus* spp., *Lactobacillus Bulgaricus, Lactobacillus helveticus, Lactobacillus brevi, Lactococcus lactis, Leuconostoc mesenteroides, and Streptococcus sp.* are good EPS producing lactic acid bacteria. The other potential EPS producers are *Pseudomonas* spp. *Acetobacter* spp. *Aureobasidium* spp. *Sinorhizobium* spp. *Escherichia* spp. *Acetobacter* spp. (Kumar Singha, 2012). Table 1.1 lists few of the EPS producing lactic acid bacteria.

EPS produced by LABs can be used as additives or produced naturally in food *in situ*. The process of *in-situ* EPS synthesis is less controllable than the *ex-situ* production and use of EPS because it heavily depends on fermentation circumstances, such as medium composition, pH value, temperature, incubation period, agitation, etc. (Abarquero et al., 2022; Korcz and Varga, 2021). Wider applications of EPS are made possible by its *ex-situ* production, which involves isolation and purification of EPS and then using it as a food additive. In this form, the polymer can be added to the food in greater quantities than could be produced *in situ*. The amount of EPS incorporated as a bioingredient should be determined based on the desired functional and physical characteristics of the final product (Zannini et al., 2018). Despite the fact that LAB normally produces modest amounts of EPS, their use may still be economically viable if production parameters are improved through the use of cheap substrates and affordable fermentation techniques (Patel et al., 2011; Korcz and Varga, 2021). A surplus of nutrients, carbohydrates, and low temperature are often favourable conditions for the production of EPS. Limiting nitrogen, carbon, and other trace elements may have an impact on the yield and

composition of the EPS and altering at the genetic level can also make changes in EPS production can be increased (Vidhyalakshmi and Nachiyar, 2011).

Table. 1.1. EPS production titre of different lactic acid bacteria

Organism	Production (mg/L)	References
Lactobacillus plantarum SKT109	58-140	(Sørensen et al., 2022; Wang et al., 2015)
Lactobacillus rhamnosus 9595M	85	(Dupont et al., 2000)
L. plantarum MK O2	208	(Adebayo-Tayo et al., 2017)
L. rhamnosus E/N	210	(Polak-Berecka et al., 2015)
L. lactis subsp. *cremoris*	520	Looijesteijn and Hugenholtz, 1999)
L. helveticus MB2-1	658	(Li et al., 2014)
Lactobacillus delbrueckii subsp. *bulgaricus*	830	(Shene and Bravo, 2007)
L. rhamnosus ŁOCK 0935	900	(Oleksy-Sobczak and Klewicka, 2020)
Lactobacillus plantarum NTMI05 *and* NTMI20	956	(Imran et al., 2016)
L. rhamnosus ZY	2498	(Ng and Xue, 2017)
Lactobacillus kefiranofaciens	2580	(Cheirsilp et al., 2018)

LAB genus and species, culture medium type, environmental factors that affect their growth and activity in the environment such as pH, water activity, oxidation/reduction potential, the presence of antimicrobial compounds, nutrient source, and effective external factors such as temperature, relative humidity, the composition of atmospheric gases, and temperature, are just a few of the variables that affect the amount and type of EPS derived from probiotics. Although probiotics are capable of producing EPS regularly, adjustments to any of the aforementioned

parameters may have a substantial impact on their production. For instance, altering process conditions such as temperature and pH may result in increased levels of EPS (Pourjafar et al., 2022).

LAB are capable of producing relatively low amounts of EPS. The highest yields are observed for the HoPS-producing *Weissella* and *Leuconostoc* species, which can convert sucrose to dextran in high proportions (Aman, Siddiqui, & Qader, 2012). To date no LAB-based HePS is known that could reach economically significant levels. Therefore, current challenges are to increase the productivity of EPS formation by LAB. The engineering of improvements in these properties will depend on a deep understanding of the EPS biosynthetic metabolism and genetic regulation, e.g. possible pathways for overexpression of responsible genes. The synthesis of HePS can be limited by the fact that it is an energy-intensive process, and the production has to compete for common enzymes with several household routes (Audy et al., 2010; Degeest et al., 2001; Korcz and Varga, 2021).

1.3.2.2. EPS as Postbiotics

Postbiotics are as soluble metabolites released by food-grade microorganisms during the growth and fermentation in complex microbiological culture, food or gut. It is rich in high and low molecular weight biologically active metabolites. Thus, postbiotics of probiotic cultures can be cell metabolites and cell wall-derived substances and EPS can be considered a potential postbiotic. Along with EPS, teichoic acids polar lipids, peptides like bacteriocins, organic acids, vitamins, enzymes etc are considered to be postbiotic compounds from probiotic bacteria. Like the aforementioned substances, postbiotics are functional fermentation by-products that can be utilised in conjunction with dietary supplements to improve health. Several health benefits of EPS have been described, and moreover it has been suggested that using postbiotics could be an

attractive alternative for other '-biotics' in critically ill patients, young children and premature neonates To evaluate the safety and health-promoting effectiveness of various kinds of microbial EPS, however, human clinical trials are necessary (Wegh et al., 2019).

Probiotic EPS (EPS derived from probiotics) can be thought of functionally as clean-labelled multifunctional polysaccharides with postbiotic properties. The ability to withstand bile salts, low pH, oxalate breakdown, and the formation of EPS were found to be positively correlated, suggesting that this property may be crucial in the selection of probiotic strains. Additionally, an *in-silico* analysis of Lactobacilli identified the same gene locus for EPS synthesis, cell aggregation, and bile resistance (Cai et al., 2019). There are several strains of lactic acid bacteria that have been effectively employed for EPS extraction, including *Lactobacillus plantarum* OF101, *L. plantarum* YO175, *Weissella confusa* WS90, *W. confusa* OF126, and *Pediococcus pentosaceus* OF31 etc. (Fusconi and Godinho, 2002; Korcz and Varga, 2021; Pourjafar et al., 2022).

1.3.3. Biosynthesis of EPS in LAB

1.3.3.1. Classification of EPS based on composition and their general biosynthesis

EPS production in LAB is a complex process with the involvement of different cassettes of genes and their gene products. The complexity of EPS biosynthesis is in direct correlation to the structural diversity and intricacy of the EPS backbone; greater the complexity, more the number of genes involved (Suresh Kumar et al., 2007). The whole mechanism, its genetics, and the detailed implication of each of the proteins and regulation of the process is still a hot topic to be explored. Many researchers around the globe still work on it to solve the mystery in this regard. The general mechanism of exopolysaccharide biosynthesis involves the assembly of the repeating units of monosaccharides on to a lipid carrier from sugar nucleotides by specific group

of enzymes called glycosyltransferases (GTFs) and then the retrospective polymerization of these repeating units to form polysaccharides and their export outside the cell (Van Kranenburg et al., 1999). The lactic acid bacterial EPS gene clusters might be localized in the plasmids they carry or in the chromosome itself. One such most widely studied EPS plasmid is *Lactococcus lactis* subsp. *cremoris* NIZO B40. It carries a 40kb plasmid, pNZ4000, with a 12-kb region consisting of 14 genes that encode capsular polysaccharide, lipopolysaccharide, and teichoic acid biosynthesis in the organism. Plasmid-encoded EPS gene cluster makes it easy to transfer the genes to other bacteria and is convenient to study the EPS biosynthetic pathway and further metabolic engineering for better yield and production of tailor-made EPS (Van Kranenburg et al., 2000, 1999). EPS is classified into two based on their backbone composition- homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

1.3.3.1.a. Homopolysaccharides

EPS from LAB are generally categorized into two groups: homopolysaccharides, HoPS and heteropolysaccharides, HePS, of which homopolysaccharides again consists of four sub-groups, namely (a) α-D-glucans, i.e., dextrans (*Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum*), mainly composed of α-1,6-linked glucose residues with variable (strain specific) degrees of branching at position 3, and less frequently at positions 2 and 4, and alternan (*Leuc. mesenteroides*) and mutans (*Streptococcus mutans* and *Streptococcus sobrinus*), both composed of α-1,3- and α-1,6-linkages; (b) β-D-glucans composed of β-1,3-linked glucose molecules with β-1,2-branches, produced by *Pediococcus* spp. and *Streptococcus* spp.; (c) fructans, mainly composed of β-2,6-linked D-fructose molecules, such as levan with some β-2,1-branching where sucrose is the main carbon source. (d) others, like polygalactan, composed of structurally identical repeating units with different glycosidic

linkages. The main enzyme that steps-in in the production of dextrans by *Leuconostoc* spp., is dextransucrase, or glycosyltransferase (Donot et al., 2012). Glycosyltransferases (GTF) and fructosyltransferases (FTF) are the major enzymes that take part in the synthesis of the homopolysaccharides which in most cases happen outside the bacterial cells. These enzymes can either be extracellular, intracellular or can be found embedded in the cell membrane as in some bacteria. Fig. 1.2 is the pictorial representation of the structure of dextran polysaccharide.

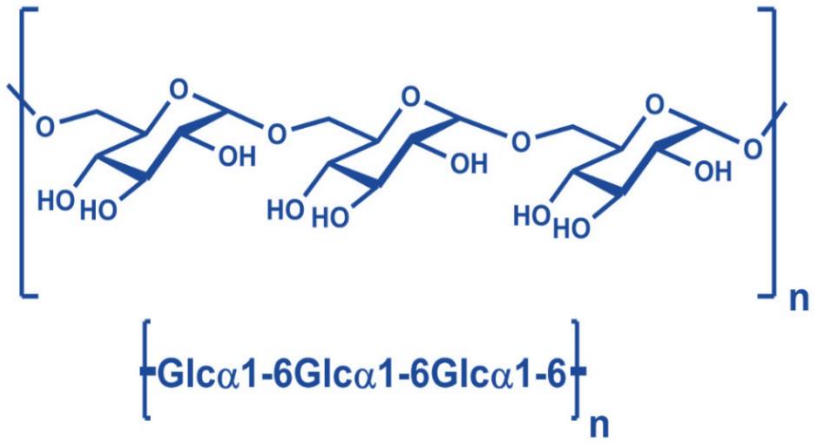


Fig.1.2. Structure and linkage of Dextran homopolysaccharide

1.3.3.1.b. Heteropolysaccharides

Heteropolysaccharides, in contrast to HoPS, for the formation requires the intracellular scope with two or more different repeating units of monomers in addition to lipids such as isoprenoid glycosyl that participate in HePS development (De Vuyst and Degeest, 1999a; Zannini et al., 2016). D-galactose, D-glucose and L-rhamnose are the frequently seen sugar moieties in the backbone of hetero EPS but in varied ratios. Table 1.2. summarizes some of the LAB HePos with their composition.

Table. 1.2. Lactic acid bacterial heteropolysaccharide composition with their average molecular weights

Organism	Monosaccharide Composition & Functional Groups Involved	Mol. Weight of EPS	References
Bacillus sp. S-1	Galactose, glucose, and mannose. Hydroxyl groups	1.765×10^4 Da	(Hu et al., 2019)
Weissella cibaria SJ14	Mannose, glucose, galactose, arabinose, xylose, and rhamnose Hydroxyl, carboxyl and acetyl groups	7.12×10^4 Da	(Zhu et al., 2018)
Weissella confuse	Galactose, mannose, glucose, fructose, rhamnose, arabinose, xylose and ribose. Hydroxyl and carbonyl groups	Not Available	(Kavitake et al., 2020)
Lactobacillus delbrueckii ssp. *bulgaricus* SRFM-1	Galactose, glucose with hydroxyl, sulfate and carboxyl groups	3.97×10^5 Da, 3.86×10^5 Da	(Tang et al., 2017)
Lactobacillus plantarum KX041	Arabinose, mannose, glucose, galactose with hydroxyl, carbonyl and amide groups	38.67 kDa	(Yuanmei Xu et al., 2019)
Bacillus amyloliquefaciens GSBa-1	Glucose and carboxyl, carbonyl, hydroxyl groups	54 kDa	(Zhao et al., 2018)
Streptococcus thermophilus GST-6	Glucose and galactose Sulfate groups	Not tested	(J. Zhang et al., 2016a)
Lactobacillus plantarum ZDY2013	Galactose and xylose	51.7 kDa	(Z. Zhang et al., 2016)
Lactobacillus sp. *Ca*6	Glucose. Carboxyl, hydroxyl and amide groups	53.23 kDa	(Trabelsi et al., 2015)
Enterobacter cloacae Z0206	Fucose, glucose, galactose, and glucuronic acid	1.1×10^6 Da	(Jin et al., 2014)
Bacillus velezensis MHM3	Glucose, fructose, rhamnose and glucuronic acid	6.89×10^{20} kDa	(Mahgoub et al., 2018)
Lactobacillus plantarum TMW 1.1478	Glucose, rhamnose and galactose, traces of the amino sugar glucosamine (GlcN)	2×10^6 Da	(Velasco et al., 2021)
Lactobacillus kefiranofaciens	Glucose, galactose	7.6×10^5 Da	(Fuso et al., 2023)
Lactobacillus pentosus	Glucose, rhamnose	Not Available	(Saif and Sakr, 2020)
Streptococcus thermophilus S-3	N-acetyl-galactosamine, galactose, glucose	5.7×10^5 Da	(Xu et al., 2021)

Lactic acid bacterial strains like *Lb. acidophilus* LMG 9433, *Lb. helveticus* TYl-2, *Lb. helveticus* NCDO 766, *Lb. rhamnosus* C83, *S. thermophilus* Sfi20, *S. thermophilus* Sfi32 (Stingele et al., 1996) and *S. thermophilus* LY03, *S. thermophilus* BTC and *S. thermophilus* 48 lack rhamnose, *S. thermophilus* OR 901 EPS contains repeating units of galactose and rhamnose alone and *Lb. sake* EPS consists of glucose and rhamnose repeating units. In contrast, the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 contains glucose and fructose in a ratio of 1:2 (Manca et al., 1996) and the polymer produced by *S. thermophilus* MR-1C consists of an octameric basic repeating unit composed of D-galactose, L-rhamnose and L-fucose in a 5:2:1 ratio.

Apart from the sugar molecules, other residues, such as glycerol- 3-phosphate, N-acetyl-amino sugars, and phosphate and acetyl groups are also seen in the HePS chain and altogether they results in the high molecular weight polysaccharides (De Vuyst and Degeest, 1999a; Rodríguez-Carvajal et al., 2008). The formation of the hetero EPS is a bit more complex than the HoPS as there involves the participation of numerous enzymes and/or proteins in their biosynthesis and secretion. Sugar-1-phosphates play a vital role in the biosynthetic pathway where it participates in the sugar activation necessary of monosaccharide polymerization and sugar interconversions (like epimerization, decarboxylation, dehydration). Sugar activation and in-built modification of the polysaccharides go hand in hand with the these enzyme using their building blocks resulting in the final EPS (Petry et al., 2000). The main differences between homopolysaccharides and heteropolysaccharides have been briefly described in Table 1.3 (Abarquero et al., 2021; Bajpai et al., 2016; Daba et al., 2021a; Jurášková et al., 2022; Korcz and Varga, 2021; Zaheer et al., 2020; Zhou et al., 2019).

Table. 1.3. Classification and characteristics of exopolysaccharides on basis of monomeric units

Characteristics	Homopolysaccharide	Heteropolysaccharide
Type of monosaccharide	Contains only one type	Contain two or more type
Main monosaccharide	Glucose or fructose	Glucose, galactose, rhamnose
Type of link	α/β link present	α/β link present
Structure	Typically, Linear / branched	Typically branched
Molecular mass	$>10^6$ Da	10^4- 10^6 Da
Mainly produced genera	*Lactobacillus, Leuconostoc, Oenococcus and Weissella*	*Bifidobacterium, Streptococcus, Lactobacillus, Lactococcus*
Biosynthesis precursors	Produced extracellularly from sucrose or starch	Produced from intracellular intermediates
Production level	Usually in gL^{-1}	Usually in mgL^{-1}
Presence of non-carbohydrate	Absence	Presence
Charge	Typically carries no charge	Can contain charged groups
Health benefits	Associated with prebiotic capacity	Associated with immune modulation, antioxidant, antibacterial properties

1.3.3.2. Role of glycosyltranferases in EPS synthesis

There are a wide variety of exopolysaccharides and the organisms that produce, among which lactobacilli itself have diverse classes of EPSs that are known to exhibit strain-specific properties that include probiotic action and other health benefit effects and textural properties. However, the knowledge on the diversity in these EPSs within the strain, and among other organisms remains unclear. Many studies on biosynthesis and the genetics behind the synthesis are being explored and all are under the maturing stage and currently limited. Different

biosynthesis pathways have been claimed and proved for EPS production of which the role of glycosyltransferases remains highlighted (Lebeer et al., 2009; Welman and Maddox, 2003). Table 1.4 summarizes some of the bacterial exopolysaccharides reported from different genera with their biosynthetic pathway along with the composition and applications.

After decades of research and investigational studies, scientists all over the globe could find that the EPS production by bacteria can take place by either of the four general mechanisms: (i) the Wzx/Wzy-dependent pathway; (ii) the ATP-binding cassette (ABC) transporter-dependent pathway; (iii) the synthase-dependent pathway and (iv) the extracellular synthesis by use of a single sucrase protein.

In the first three pathways, the production of activated sugars/sugar acids, which are the key precursors molecules of the polysaccharides, are evolved as an outcome of the numerous enzymatic transformations within the bacterial cell (Fig 1.3). Whereas for the extracellular synthesis pathway involves the direct addition of the monosaccharides obtained because of the cleavage of di- or trisaccharide.

The Wzx/Wzy dependent pathway involves the presence of a Wzx protein, a so called flippase enzyme. Here the monomers of the polymer are formerly linked to an undecaprenol diphosphate anchor (C55) at the inner membrane, are assembled with the help of several glycosyltransferases and translocated across the cytoplasmic membrane by the Wzx protein. Polysaccharide co-polymerase (PCP) and the outer membrane polysaccharide export (OPX) family proteins are the additional proteins that are involved in the transport of the finally polymerized polysaccharides from the periplasm to the cell surface. EPS produced by this pathway have an extremely diverse sugar pattern may be up to four or five sugars within the

chemical backbone and therefore most of them fall into the heteropolysaccharides for this reason where xanthan gum is an example. The bacterial strains that produces EPS by this pathway carry genes for flippases (Wzx) and polymerases (Wzy) in EPS operons in their genome (Oleksy and Klewicka, 2018).

The synthase dependent pathway, third pathway, is the one independent of a flippase enzyme for the translocation of the polymers across the membranes and the cell wall. A single synthase protein takes the responsibility of polymerization and translocation of the polysaccharide wherein most cases are a subunit of the envelop- spanning the multiprotein complex (Rehm, 2015). The EPSs produced by synthase dependent pathway are mostly homopolysaccharides where the biosynthesis of curdlan is an example, which is a polymer of β-(1-3)-linked glucose in its backbone.

Extracellular biosynthetic pathway of EPS is another mode of polysaccharide biosynthesis where the precursor production happens inside the cell, polymerization and secretion appears to happen in the cell envelope. EPSs like dextran or levan takes place *via* this biosynthetic pathway with the incorporation and active enzymatic reactions by glycosyltransferases which are either secreted out of the cell or are covalently bound on to the cell surface.

Table 1.4. Bacterial exopolysaccharides with their biosynthetic pathway, composition, and applications

EPS Type	Bacterial Strains	EPS Biosynthetic Pathway	EPS Composition	Properties & Applications	References
Dextran	*Leuc mesenteroides* *Leuc. mesenteroides subsp. dextranicum*	Extracellular synthesis	Glucose	• As gelling, viscosifying & emulsifying agent food industry. • Used as blood flow improving agent as blood plasma extender • As cholesterol lowering agent, in human and veterinary medicine • As matrix of chromatography column in separation technology • For enhanced oil recovery & biomaterials • As anticoagulant	(Bajpai et al., 2016; Caligur, 2008; Schmid et al., 2015a; Suresh Kumar et al., 2007b)
Levan	*S. salivarus* *Lb. sanfranciscenis* *Zymomonas mobilis*	Extracellular synthesis	Fructose	• Is an ecofriendly adhesive • As prebiotic supplements • Exhibits antitumor properties • Used as hypo-cholesterolaemic agent • As bio-thickener in food products	(Bajpai et al., 2016; Ni et al., 2018; Schmid et al., 2015; Sengupta et al., 2018; Suresh Kumar et al., 2007)
Xanthan	*X. campestris,* *X. phaseoli,* *X. malvacearum,* *X. carotae,*	Wzx/Wzy dependent pathway	Mannose, Glucose, Glucuronic acid	• Constituents in cosmetics, pharmaceuticals, pesticides, paints, detergent formulations, as viscosity controlling agents in printing inks • In food, as a stabilizing and thickening agent, mostly used along with guar gum • Application in crude-oil recovery	(Bajpai et al., 2016; Kleerebezem et al., 1999; Rehm, 2015; Suresh Kumar et al., 2007)

Pullulan	*Aureobasidium pullulans*	Extracellular synthesis	Glucose, Maltotriose	• As a thickener, viscosity stabilizer or an adhesive and coating agent for many applications. • It is non-toxic & hence finding applications in biotechnological & pharmaceutical industries	(Bajpai et al., 2016; Suresh Kumar et al., 2007b)
Gellan	*Sphingomonas paucimobilis*	Wzx/Wzy dependent pathway	Rhamnose, Glucose Glucuronic acid	• As thickening agent, gelling agent & stabilizer • For studying marine microorganisms, it is used as solidifying agent for culture media preparation	(Bajaj et al., 2007; Bajpai et al., 2016; Du et al., 2019; Kumar et al., 2007; Sengupta et al., 2018)
Curdlan	*Agrobacterium sp., Paenibacillus polymyxa, Pseudomonas sp. QL212*	Synthase dependent pathway	Glucose	• As a gelling agent, & immobilization matrix • Heavy metal removal • Along with zidovudine (AZT), it displays high antiretroviral activity (anti-AIDS-drug) • Food, Medicine, Cosmetics • Used in biomedical applications such as antithrombotic activity	(Bajpai et al., 2016; Kumar et al., 2007; Monchois et al., n.d.; Schmid et al., 2015; Sengupta et al., 2018)
Mutan	*Streptococcus mutans*	Extracellular synthesis	Glucose	• Adjunct culture in cheese	(Bajpai et al., 2016; Schmid et al., 2015)

Kefiran	*Lb. delbrueckii subsp. bulgaricus* *Lb. kefirgranum* *Lb. parakefir* *Lb. kefiranofaciens* *Lb. plantarum*	Wzx/Wzy dependent Pathway	Glucose, Galactose	• As prebiotics, substitute of fat in food products • Improves visco -elastic properties of acid milk gels • Exhibits antimicrobial & wound healing properties • Increased gut mucosal immunity • Cholesterol lowering in serum, to retard tumor growth, enhance immunity of gut • Increase in viscosity and texture improvement of fermented milks & beverages	(Bajpai et al., 2016; Cheirsilp et al., 2018; Van Kranenburg et al., 1999)
Inulin	*Lb. johnsonii* NCC 533 *Leuc. citreum* CW28 *Lb.reuteri LB 121* *Lb gasseri* *S. mutans* JC2	Extracellular synthesis	Fructose	• Prebiotics, nourishes gut mucosal cells and inhibits pathogens, • For targeted drug delivery against colon cancer, • Substitute of fat in food products	(Bajpai et al., 2016; Schmid et al., 2015a)
Reuteran	*Lb reuteri* LB121 *Lb. reuteri* ATCC55730 *Lb. reuteri* 35-5	Extracellular synthesis	Glucose, Fructose	• Used in Bakery	(Bajpai et al., 2016; Pijning et al., 2012)

Lb- Lactobacillus, Leuc-Leuconostoc, S- Streptococcus, X-Xanthomonas

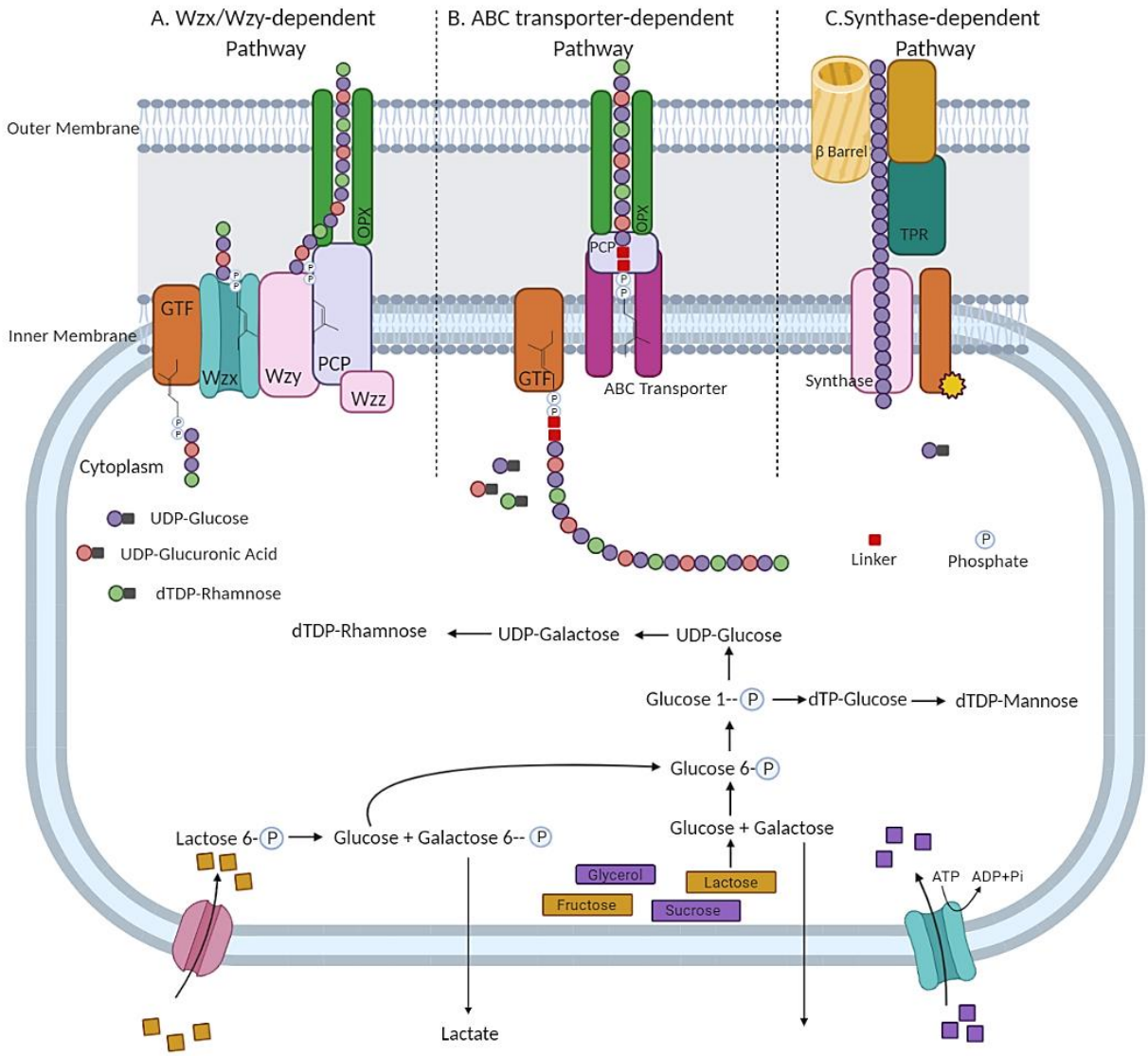


Fig. 1.3. Biosynthetic Pathways of Exopolysaccharide (EPS) Production in Lactic Acid Bacteria (LAB): Sugar uptake and transportation of sugars into the cytoplasm; Synthesis of sugar nucleotides; Polymerization, synthesis and export of EPS subunits through (A) Wxz/Wxy – dependent pathway; (B) ABC Transporter-dependent pathway; (C) Synthase-dependent pathway involves the action of various enzymes including the glycosyltransferases (Bajpai et al., 2016; Kleerebezem et al., 1999b; Schmid et al., 2015a).

UDP-Uridine diphosphate; dTDP-deoxy Thymidine diphosphate; Lactose-6-P- Lactose 6 phosphate; Glucose-6-P- Glucose 6 phosphate; GTF- Glycosyltransferases; PCP-Polysaccharide polymerase protein; OPX- Outer membrane polysaccharide; TPR-tetratricopeptide repeat

Capsular polysaccharides (CPS) are another class of polysaccharides produced by bacteria which are often seen attached on to the cell surface rather than expelled out of the cell. The second class of polysaccharide biosynthesis, ABC transporter dependent pathway mainly focuses on the biosynthesis of such capsular polysaccharides. Similar to the former case of Wzx/Wzy dependent EPS, the CPSs synthesized by ABC transporter dependent pathway, employs the glycosyltransferases (GTFs) at the cytoplasmic face of the inner membrane, where an operon with a single GTF efficiently produces the homopolysaccharides while in case of hetero EPS, operons with multiple GTFs participate in the assembly of the polymers. The polysaccharide export and their translocation across the membrane is complex process and involves a tripartite efflux pump. This efflux pump is a complex with ABC- transporters that spans into the inner membrane along with proteins of PCP and OPX family proteins that are similar to those seen in the Wxz/Wzy pathway. One of the main differences that is found between Wxz/Wzy and ABC transporter dependent pathways is that the CPSs carry a phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonicacid (Kdo) linker at the reducing terminus which is a conserved glycolipid (Ovchinnikova et al., 2016).

1.3.3.2.1. Role of GTF in homopolysaccharide synthesis

Dextrans are homopolymers of α-(1-6) linked glucose residues formed as result of the enzymatic activity of dextransucrases (generally glucansucrases). They fall into the category of glycosyltransferases (GTF, E.C. 2.4.x.y) which are considered as glycoside hydrolase family 70 (GH70) of enzymes (Cantarel et al., 2009). These GTFs are further classified, based on the final polysaccharides they synthesize, into transglucosidases (E.C. 2.4.1.y) and transfructosidases (E.C. 2.4.1.y or 2.y), Transglucosidases, encoded by these GTFs includes glucan-synthesizing dextransucrases, mutansucrases, and reuteransucrases (E.C. 2.4.1.5) (De Vuyst and Degeest,

1999). Basically, the glucansucrases catalyse the transfer of glucose molecule onto a growing chain of α-glycosidic linked oligo- and/ polysaccharides from sucrose. Linkages formed to each of the free hydroxyl groups of the sugar moiety depends on the specificity of the glucansucrases in action. These specificities and differences in linkages along with different degrees of possible branching at hydroxyl groups results in a wide variety of EPSs with not merely the α-(1-6) linked dextran, but dextrans containing a small amount of α-(1-3) or even α-(1-2) linkages, other EPSs such as mutan with mostly α-(1-3) linkages, alternan with strictly alternating α-(1-3) and α-(1-6) linkages and reuteran consisting mainly of α-(1-4) linkages that are interspersed with α- (1-6) linkages (Leemhuis et al., 2012). *Leuconostoc* sp., of lactic acid bacteria are known for their synthesis of dextrans where the only intervening enzyme is glycosyltransferase, specifically called as dextransucrases (Donot et al., 2012).

Dextransucrases mostly exhibit their enzymatic action outside the cell as they are seen extracellularly or found anchored on to the cell wall. These enzymes having an average molecular weight of 110-160 kDa, are multidomain enzymes of GH70 family proteins whose three-dimensional structures are currently available and the structural elucidation revealed different structures than that was expected from the sequence alignments (Vujicic-Zagar et al., 2010). There are three domains for the catalytic core of the glucansucrases with two extra domains attached to the core domains (Leemhuis et al., 2012). The enzymatic mechanism of glucansucrases was under debate over the years specifically concerning the chain initiation and elongation of the polysaccharide chain. But to the currently available knowledge, the scientists conclude that elongation occurs at the reducing end, which led to the proposal of two nucleophilic sites being involved in the process, where the growing chain remains covalently bind to the enzyme and is transferred from site one to the glucose moiety bound at site two and

vice versa. According to this mechanism it explains the high processivity of the enzyme as polymer length is inversely proportional to the number of enzymes.

On the other hand, Leemhuis et al., (2012) could establish the crystal structure of glucansucrases and proposed a much simpler mechanism according to which the sucrose is hydrolysed during elongation, resembling the action of a retaining glycosyltransferase where a covalent β-glycosyl enzyme intermediate is formed *via* a carboxylic acid residue of the enzyme and this step retains the high energy of the glycosidic bond of sucrose as well. The enzyme does not favour the hydrolysis of this intermediate formed, even when the simultaneous release of glucose is possible. In fact, it transfers the glucose onto a hydroxyl group and elongation of the polysaccharide occurs at the non-reducing end of the polysaccharide (Leemhuis et al., 2012).

The general mechanism of glucan synthesis can be illustrated and is represented in Fig. 1.4 The second class, fructan-catalyzing transfructosidases which includes levansucrases (E.C. 2.4.1.10) and inulosucrases (E.C. 2.4.1.9) are encoded by FTF (fructosyltransferase) genes and their mechanism of catalysis is similar to GTFs and can be represented in Fig 1.5.

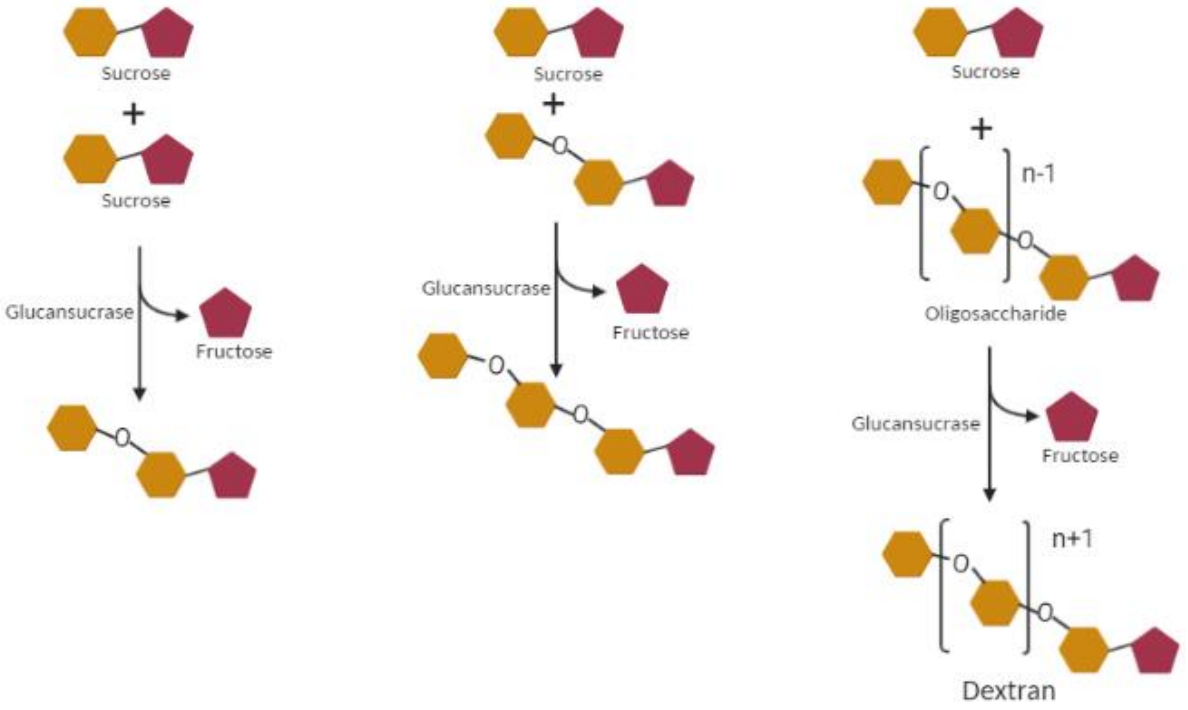


Fig. 1.4. Mechanism of glucansucrases (dextransucrase) in dextran biosynthesis

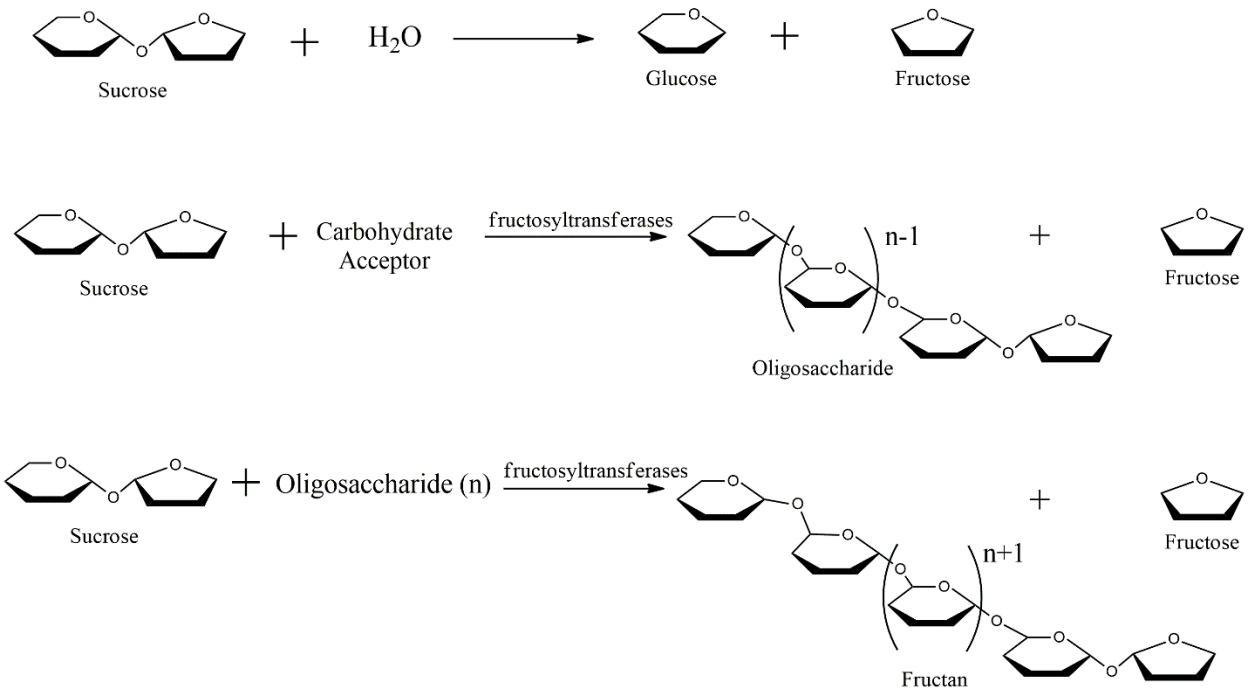


Fig. 1.5. Mechanism of fructosyltransferases in fructan biosynthesis

1.3.3.3. Significance of glycosyltransferases in different Lactobacilli

Since decades, scientists worldwide have been working on the molecular aspects of the EPS biosynthesis to find the genes and the gene clusters that participate in the EPS production in lactic acid bacteria *like Streptococcus thermophilus, Lactobacillus helveticus, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus rhamnosus, Oenococcus oeni, Weissella cibaria , Lactobacillus plantarum* and many other LAB strains (Table 1.5) (Kleerebezem et al., 1999; Stingele et al., 1999). All these LAB species have a similar way of EPS biosynthesis where the sugar monomers are assembled on to a membrane-bound undecaprenylphosphate carrier from the activated sugar donors, produced inside the cell, *via* different glycosyltransferases (Van Kranenburg et al., 1999).

L. plantarum WCSF1 serves as a model organism for probiotic lactic acid bacteria especially lactobacilli whose genome is sequenced completely and is widely used for all molecular studies as a reference among lactobacilli species. Studies with *L. plantarum* WCSF1 and its biosynthesis of polysaccharides were studied by construction of *L. plantarum* WCSF1 strains that are cps-cluster deficient mutants, include single (Δcps1A-I, Δcps2A-J, Δcps3A-J, Δcps4A-J), triple (Δcps1A-3J), and quadruple (Δcps1A-3J, Δcps4A-J) deletion mutants. The function of cps cluster mutation and its consequences were studied in surface polysaccharide fraction of the mutants in comparison to the wild-type strain. Transcriptome profiling and its analysis were carried out to determine the function of each of the gene clusters. These studies revealed that each clusters had influence on the surface polysaccharide synthesis specifically the amount, molar mass and chemical composition (Remus et al., 2012).

Table. 1.5. Exopolysaccharides lactic acid bacteria with their monomeric units, linkages, and the class of glycosyltransferases responsible for the synthesis

Organism	EPS	Monomers &Linkage	Enzyme on Action	References
Le. mesenteroides	Alternan	• α-(1,3) & α-(1,6) glycosidic linkages associated with α-(1→3), (1→6) branches	Alternansucrase	(Bajpai et al., 2016)
Lb reuteri LTH5448, *S. salivarius* SS2, *Le. mesenteroides* NRRL B512F, *Lb. sanfranciscensis* LTH2590	Levan	Fructose • β-(2→6) linkages associated withβ-(2→1), (2→6) branches	Levansucrase	(Gupta and Diwan, 2017; Oleksy and Klewicka, 2018)
Lb. reuteri 121	Reuteran	Glucose, Fructose • α-(1,4) & α-(1,6) glycosidic linkageassociated withα-(1→4), (1→6) branches	Reuteransucrase	(Kralj et al., 2005)
Lactobacillus spp. G77 *Lb. suebicus* CUPV221	β-D-glucans	Glucose • β-(1,3)-glycosidic linkages	Glucansucrases	(Ibarburu et al., 2010; Kralj et al., 2003)
Lb. fermentum, *Lb. sakei*, *Lb. hilgardii*, *Lb. parabuchneri*, *Lb. curvatus*.	Dextrans	Glucose • α-(1,6) linkages associated with a low amount of α-(1→3) linkages and α-(1→3), (1→6), α-(1→2), (1→6), α-(1→4), (1→6) branches	Dextransucrases	(De Vuyst and De Vin, 2007; Leemhuis et al., 2012)
Lb. reuteri ML1 *Lb. reuteri* GTFML1	Mutans	Glucose • α-(1,3)-glycosidic bonds	Mutansucrase	(Kralj et al., 2004a; Wenham et al., 1981)
Le. citreum CW28 *S. mutans* JC-1 *S. mutans* BHT	Inulin- Type	Fructose • β-(2,1) associated with β-(2→6), (2→1) branches	Inulosucrase	(Bajpai et al., 2016; Mozzi et al., 2006)
S. thermophilus S-3		N-acetyl-galactosamine, Galactose and Glucose	Galactosyltransferase & priming glycosyltransferase	(Xiong et al., 2019; Xu et al., 2021)
L.. mesenteroides ATCC 8293	Dextran	Glucose • α-(1→3) linkages	Glycosyltransferases	(Lee et al., 2021)
L. fermentum SH2	β-D-glucans	Glucose 1-3,1-6)- β-glucans	Glucansucrases	(Allaith et al., 2022)

Lb., Lactobacillus; *Le., Leuconostoc*; *S., Streptococcus*

Their genome analysis shows that they have two regions with genes coding (cps genes) for surface-associated polysaccharide biosynthesis (capsular polysaccharides). The first region which is of 49kb size that consists of three cps gene clusters – *cps1*, *cps2* and *cps3*. Of these three cps gene clusters, *cps1* and *cps2* are unique and is seen only in WCSF1 whereas the *cps3* gene is conserved to most of the lactobacilli species like ST-III and ATCC 14917. Region two of 14kb size has *cps4* gene cluster which remains conserved in other lactobacilli as well. *Cps2* and *cps4* structurally resembles that of Wzy-dependent polymer gene cluster (Siezen and van Hylckama Vlieg, 2011). The genes *cps2E* and *cps4E* encode the priming glycosyltransferases that catalyses the very first step in the biosynthesis of polysaccharides by transferring the sugar-1-phosphate from UDP-sugar nucleotide to the undecaprenyl-phosphate. Apart from these two genes the cps2 and cps4 clusters have genes (*cps2FGJ* and *cps4FGI*) that encodes for other glycosyltransferase enzymes that have predominant role in the biosynthesis of the capsular polysaccharides produced by *L. plantarum* WCSF (Remus et al., 2012). From the literature search and available data, Table 1.6 shows some of the reported glycosyltransferase genes.

A study by Van Kranenburg et al., (1999) reported a 40kb plasmid in *Lactococcus* NIZO B40 (pNZ4000) and its role in EPS biosynthesis (Fig.1.6). There exists a gene cluster with 14 genes in the order *epsRXABCDEFGHIJKL*and is transcribed into a single polycistronic mRNA of ~12kb in size. The disruption of *epsD* gene from the cluster abolished the ability of EPS production by the organism and thus enlightened the role of this gene in the pathway.

Table. 1.6. EPS-producing lactic acid bacterial strains with their corresponding *gtf* genes and enzymes

EPS & LAB Strains	Enzyme	Gene Name / GenBank Acc. No	References
Glucan *Leuc. reuteri* strain 121 *Leuc. mesenteroides* NRRL B-512F *Leuc. mesenteroides* NRRL B-1299 *S. mutans* LM7	Glycosyltransferases Glucansucrases Glucansucrases Glucansucrases	*gtfA* dsr-A dsr-B gtf-C	(Kralj et al., 2002) (Monchois et al., n.d.)
Lc. lactis subsp. *cremoris*	Priming glycosyltransferases	*epsD*	(Di Cagno et al., 2006)
L. rhamnosus GG	Priming glycosyltransferase	*welE* *welF* to *welJ*	(Lebeer et al., 2009)
Alternan *L. citreum* NRRL B-1355 *L. citreum* KM20 *L. citreum* LBAE C11	Alternansucrase	AJ250173 NC_010471 NZ_CAGF01000008	(Holt, 2017)
S. thermophilus Sfi6	Priming glycosyltransferase	*epsE*	(Stingele et al., 1999a)
L. plantarum MC5	Lipopolysaccharide biosynthesis glycosyltransferase Priming glycosyltransferase	*eps1E* *eps2E*	(X. Zhao et al., 2023)
L. fermentum NCC 2970	Glucanotrasnferase	*4,3-α-GTase*	(Chen et al., 2021)
L. paraplantarum, L. sanfranciscensis, W. paramesenteroides	Levansucrase	*lev*	(Guérin et al., 2020)
W. confusa LBAE C39-2	Dextransucrase	*DSR-C39-2*	(Yu et al., 2022)
Latilactobacillus curvatus TMW *1.624*	Glycosyltransferase	*Gtf1624*	(Yu et al., 2022)
β-glucans *Lactobacillus plantarum*	Glycosyltransferase	*epsD/E*	(Milanovic et al., 2020)
α-glucan *Lactobacillus casei* AG	Priming glycosyltransferase / undecaprenyl-phosphate glycosyl-1-phosphate transferase	*epsE*	(Maajid et al., 2022)

L- Lactobacillus, Lc-Lactococcus, Leuc-Leuconostoc, S- Streptococcus

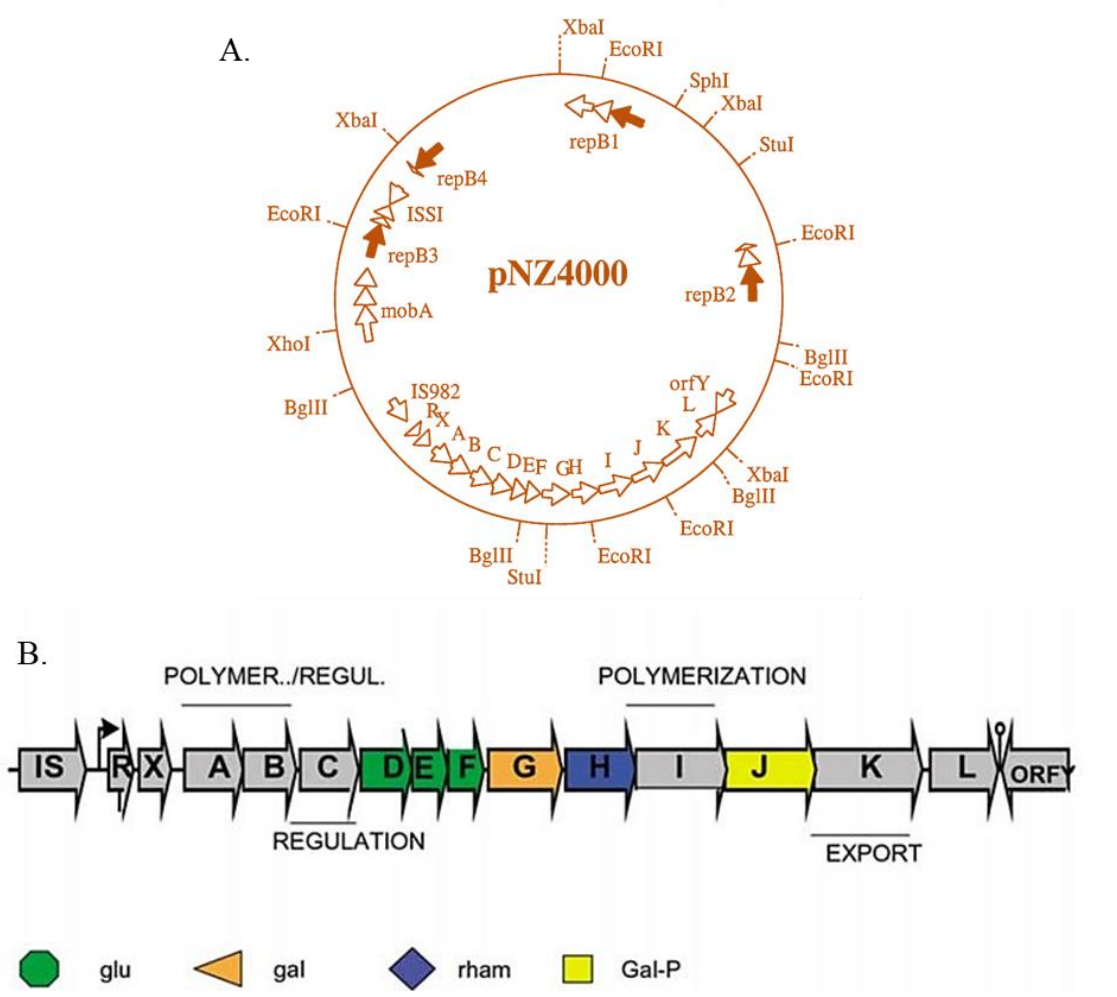


Fig. 1.6. Schematic representation of (A) *L. lactis* NIZO B40 plasmid and (B) its EPS biosynthetic gene cluster

In *L. rhamnosus* GG, along with the genes required for EPS biosynthesis, their genome is equipped with genes for the regulatory proteins. Glycosyltransferases produced inside the cell catalyse the synthesis of blocks of repeating units in most cases linked to a lipid carrier molecule at the cytoplasmic site of the inner membrane. The EPS locus of *L. rhamnosus* GG consists of six genes welE to welJ which encodes putative glycosyltransferases with each enzyme exhibiting relevant enzymatic reactions in the biosynthetic pathway. From the available databases and bioinformatic analysis of these data we see that the protein encoded by welE gene shows 61%

identity with similar protein YP_001271961 that encodes for a galactose phosphotransferase from *Lactobacillus reuteri* F275.But after several experiments and detailed study, it is seen that welE gene of *L. rhamnosus* GG successfully encodes for a priming glycosyltransferase where the enzyme has the prime role in the transfer the first sugar moiety of each EPS molecule and hence help in the priming of the growing EPS repeating unit of the entire polysaccharide backbone. This reaction is the first and foremost reaction in the biosynthetic pathway as well. Rest of the five genes, welF to welJ in the pathway also codes for glycosyltransferases which probably transfers the next sugars of the EPS backbone thus facilitating the formation of glycosidic linkages to form a perfect frame of EPS. From different structural characterization it was found that the *L. rhamnosus* GG produces a galactose-rich heteropolysaccharide EPS and further genomic analysis shows that the EPS subunits are transferred across the cell membrane by Wzx-type exporter and the polymerization of the EPS.

1.3.3.4. Structure and reaction mechanism of glycosyltransferases

Glycosyltransferases, GTFs, mediate the synthesis of high molecular weight polysaccharides using sugars as monomeric units and catalyse the formation of the glycosidic linkages between them. These enzymes have been reported and classified in glycoside hydrolase family 70, GH70, in the CAZy database (http://www.cazy.org/fam/GH70.html) and this is based on the similarity in the amino acid sequences. The catalytic residues and the mechanism of action of these enzymes remain conserved within the GH family. The common characteristic of these enzymes is that they cleave the glycosidic linkages between glucose moiety and another sugar moiety like fructose, glucose using a catalytic (β/α)8 barrel domain. The enzyme is structured in such a way that is has a signal peptide, a variable N-terminal region, a catalytic core domain

where the transfer of the glycosidic linkages takes place and a C- terminal glucan binding domain (Fig. 1.7).

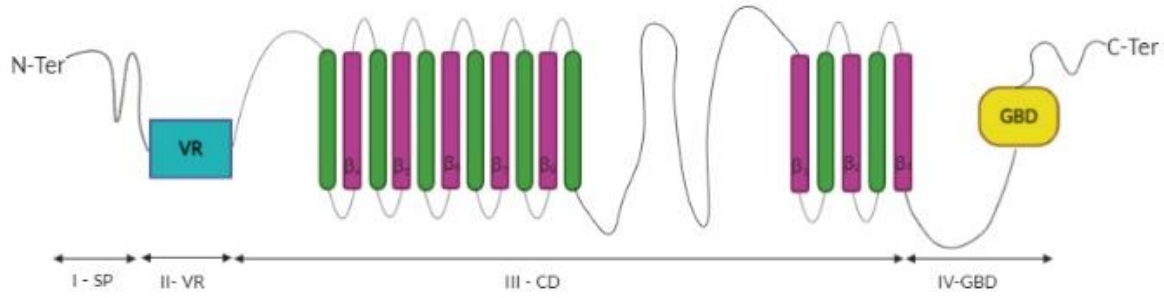


Fig. 1.7. Schematic Structure of GTF gene of lactic acid bacteria. The numerical at the top represent the amino acid residue

I – Signal peptide**; II –** Variable Region**; III –** N- terminal Catalytic Domain**; IV –** Glucan Binding Domain at C-terminal

It has also been noted that the GH70 family of enzymes has close relation with that of the GH13 family enzymes (α-amylase family, contains starch modifying or hydrolysing enzymes) and to the GH77 family (amylomaltases), especially in the catalytic domain. So, for more convenience, the GH70, GH13 and GH77 family of enzymes are grouped together as GH-H clan or α-amylase super family. The amino acid residues involved in substrate binding and catalysis share a high sequence similarity and hence these enzymes are believed to share common reaction mechanism which involves a covalent β-glucosyl enzyme intermediate, with the retention of the α-anomeric configuration in the product (Pijning et al., 2008).

Many investigations have been carried out to study the nature, structure, and molecular mechanism of glycosyltransferases enzymes and for this purpose the studies have been done in, glucansucarse GTF180 from *Lactobacillus reuteri* 180. It produces glucans with α-1,6 and α-1,3 glycosidic linkages catalysed by the glucansucrases of 198kDa (Kralj et al., 2004). Attempts for

crystallisation of both the full length GTF180 protein and of 117kDa fragment (lacks the N-terminal variable domain, GTF180-ΔN) of the enzyme have been carried out. For this to carry out, the full-length enzyme GTF180 (MG-39-1772-His6, lacking signal sequence) and the GTF180-ΔN were over-expressed in *E. coli* BL21 (DE3) and was purified before crystallization experiments. The purpose of deletion was to avoid the protein flexibility and thereby enhance the possibility of crystallization. The end result was surprising that the truncated enzyme showed completely active and produced the α-glucan with similar linkage and mass as that of the full length glucansucarse, which shows that the N-terminal deletion did not significantly affect the enzyme activity. All these experiments revealed the 3D-structures of first GH70 GTFs and they became available. Apart from glucansucarse structures of GTF180-ΔN from *L. reuteri* 180, GTF-SI from *S. mutans* and the ΔN123-GBD-CD2 of the α (1 → 2) branching GS DSR-E from *L. mesenteroides* NRRL B-1299 were some among the characterized GTFS in the recent times. But in the crystallization experiments, only the truncated enzymes formed crystals and only they could be studied (van Leeuwen et al., 2008) and the crystal structures of the complete GTF enzymes are yet to be reported.

The average amino acid sequence residue of glucansucrases is approximately 1600-1800 and is comparatively larger enzymes than the GH13 and GH77 family (~500–600 amino acids). But the sequence similarity of these enzymes with the GH13 α-amylases and the GH77 amylomaltases, glucansucrases are believed to exhibit an α-displacement mechanism for the catalysis reactions to take place. Primarily, the α-1-2 glycosidic bond of sucrose is cleaved and releases the fructose. This yields a glucosyl-enzyme intermediate, where the glucosyl unit is covalently attached to a catalytic nucleophile *via* a β-glycosidic linkage. The next step is the transfer of the covalently attached glucosyl unit to the either the reducing or the non-reducing

end sugar acceptor residue of the growing polysaccharide chain and the reformation of the α-glycosidic bond. An alternative mechanism of action is the transfer of the glucosyl moiety to a low molecular mass acceptor like maltose or isomaltose or to a water molecule. It is also proposed that the mechanism requires two nucleophilic residues with one catalytic site or two closely attached catalytic sites where the nucleophilic residues form covalent glucosyl-enzyme intermediates which alternatively transfers the glucosyl moiety to the C1 atom to the other covalently bound glucose there by freeing the nucleophile for the next round of reaction cycle (Vujicic-Zagar et al., 2010).

With the evidence and revealing of the crystal structures of GTF180-ΔN, the molecular mechanism of the GH70 enzyme and its interactions became clearer and more evident. This explains the mechanism in contrast to the previous hypothesis and states that only an single active site is present and there is no space for a second covalently bound glucose or glucan polymer. It restates the hypothesis that there exists no double active-site/double-nucleophilic insertion mechanism. Along with this, the key amino acid residues involved in the substrate binding and the catalysis in GH13 and GH70 enzymes share similarities and the action and mechanism of of glycosidic bind cleavage by GH70 glucansucrases is in close connection with the α-amylase super family enzyme (Vujicic-Zagar et al., 2010). A similar kind of study about the crystal structure of *L. reuteri* 121 GtfB-ΔNΔV also suggests that the architectural design of the active- site of GTF intermediates between GH13 α-amylases and GH70 glucansucrases, having a binding groove just like that of the α-amylases but partly covered by loops forming a tunnel (Bai et al., 2017).

Studies on any enzymes relays on validation of its catalytic mechanism and quantification of the catalytic rate of the reaction. There are several studies that reports several of these assays

by which this has been carried out with glycosyltransferases. Literature reports are available that clearly explains the principle and mechanism of with assays with crude glucansucrases, dextransucrases where the basis of all these assays is the detection of the fructose, which serves as the by-product of the reaction. The end-product detection can be either of the three ways- chemical, enzymatical or chromatographical way. The easy way is chemical detection by measuring the reducing power of the carbohydrate released during the hydrolysis activity of the GTFs and quantify the glucose or fructose released during the process. It is mostly expressed in terms of per unit of the enzyme such as one glucansucrase unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose and/or glucose per minute at 30 °C, under specific buffering conditions (pH 5.4) and in the presence of specific concentration of sucrose. Likewise, his-tag-purified GTF enzymes from *E. coli* extracts and their culture supernatants of Lactobacillus strains grown on MRSs were also used as a source of enzyme for the enzyme assays as well. The GTF total activity measurement remains the same that of the previous case by determining the release of fructose from sucrose under specific enzymatic conditions (Van Geel-Schutten et al., 1999).

Alternate way is by the quantification of the end product- i.e., to quantify the amount of polysaccharides produced, weighing after extraction and purification of the α- glucan or dextran produced or by degradation of the polysaccharides to its monomers (glucose). Another method to quantify is by labelling the sucrose. Using ^{14}C-labelled sucrose as substrate for the production of α- glucan can help easily quantify the amount of glucan produced (Leemhuis et al., 2012).

1.3.4. Physiochemical Properties of EPS from LAB

1.3.4.1. Structure-Function Relationships of Exopolysaccharides

LAB produces a wide variety of heteropolysaccharides at levels of 10-1000mg/L, which is comparatively low to homopolysaccharide production, but with high molecular masses generally above 10^6 g/mol (Parente and Ricciardi, 1999). Some of the recent research studies on EPSs give an idea that the structure of heteropolysaccharides is strain specific. They often differ by their monosaccharide composition, linkages between units, presence of repeated sidechains and substitutions, length and composition of branching etc and all these strongly affects the rheological properties since it affects the compactness of the EPS produced (Jacques-Edouard et al., 2001). Even at low concentrations, the rheology and texture of fermented products are influenced by EPS as they have good thickening ability. But there is no specific correlation between EPS concentrations and apparent viscosities of the fermented products, since the polysaccharides of different LAB greatly vary in composition, charge, spatial arrangement, rigidity, and ability to interact with proteins, However, some trends have been traced. To obtain a high viscosity, the molar mass should be high and the chain should be relatively stiff (Jolly et al., 2002). Some general features on the relation between polysaccharide chemistry and chain stiffness can also be outlined.

Sidechains can also affect the chain stiffness and hence the thickening properties. Moreover, it is observed that the β-(1,4) linkages result in stiffer chains than α-(1,4) or β-(1,3), as well as the much more flexible α-(1,6) and β-(1,6) linkage (Tuinier et al., 2001).

1.3.4.2. Functional Properties of Exopolysaccharides

To achieve the industrial application and acceptance for commercialization, it is of great importance to study the relationship between chemical structures and physicochemical properties

of EPS. These acquired knowledge is made useful to interpret the evidence of HePS from LAB on human health benefits including blood cholesterol-lowering, antioxidant, antitumor, anti-ulcer, and immunomodulating activity, in regard of their structure/function relationships. Several EPSs, especially the homopolysaccharides, have already been commercialized.

Certain EPSs produced by LAB are also claimed to have beneficial physiological effects. The residence time of fermented milk, after ingestion, in the GI tract showed low levels of degradability due to the high viscosity of the EPS in those food products. This in turn helps in the colonization of the probiotic microflora (Ruijssenaars et al., 2000). A further example of a suggested health benefit of some EPSs is the generation of short-chain fatty acids (SCFAs) upon degradation in the gut by the colonic microflora. SCFAs provide energy to epithelial cells and some have been claimed to play a role in the prevention of colon cancer (Zhu et al., 2020). *In vivo* studies by oral administration of EPSs will be crucial to clearly demonstrate the different health beneficial properties that have been mentioned in the literature so far (e.g., antitumor, antioxidant, antiulcer, immunomodulating or cholesterol-lowering activities). LAB also shows antioxidant activity due to some compounds it produces in its basic metabolic pathway. Major contribution for the antioxidant property of LAB is due to their exopolysaccharide production which itself has antioxidant properties and have been proved and reported in the past decades. EPS from LAB have been found to have significant antioxidant and anti-tumor activities, and they have drawn attention recently. The increasing concerns over the conventional cancer therapies like surgery, chemotherapy and radiotherapy which exhibit serious side effects, such as multidrug resistance of tumor cells, toxicity to normal cells, and immune injury, it is critical to develop novel drugs with low side effects for cancer patients. Some EPSs from LAB strains have been studied for their antitumor activities (Gezginç et al., 2022; Jiang et al., 2020; Riaz Rajoka et

al., 2019; Wei et al., 2019). The anticancer activity of polysaccharides are mediated by activating specific immune system cells, primarily T- and B-lymphocytes, macrophages, and inducing NK cells to release interleukin (Khalil et al., 2022; Rahbar et al., 2019). Moreover, exopolysaccharides exhibit apoptotic and anti-angiogenic effects due to their effects on the c-Myc, c-Fos, and vascular endothelial growth factor expression indicate their potential broad bioactivity as anti-cancer adjuvants. The antitumor property of the polysaccharide could be mainly attributed by the 1,3-linkages in the EPS initiated by the binding of glucans to β-glucan receptor, such as dectin-1 of immune cells and they cooperates with Toll-like receptors and many other surface receptors for the recognition of different microbial products (Ismail and Nampoothiri, 2013; Jiang et al., 2020; Rahbar et al., 2019; Sheng et al., 2022). Chemical modifications of the EPS by oxidation, reduction, transglycosylation, acetylation or sulphonation can decrease the IC50 and improve the antitumor properties. Even though the mode of action of the EPS is through apoptosis, further *in vitro* and *in vivo* studies are required to reveal the detailed mechanism behind the action (Jiang et al., 2020; Wu et al., 2021). This potentiality of EPS from LAB is of great importance and a hot topic for exploitation and exploration. LAB have been known to contribute to the modulate of intestinal homeostasis by displaying antagonistic and antioxidant activities (Li et al., 2012). Therefore, their probiotic properties and functional capacities are crucial for their application as probiotics (Y. J. Yu et al., 2018).

Similarly, *L. plantarum* MA2 exhibited antioxidant effects *via* free radical scavenging and expression of antioxidant enzymes- such as SOD, NADH peroxidase, and glutathione reductase. Exogenous probiotic supplementation is recommended to modulate the oxidative stress, owing to the limited endogenous antioxidant capacity (Y. J. Yu et al., 2018). Thus, these isolates may contribute to the improvement of the intestinal antioxidant EPS obtained from the

cell-free supernatant of LAB exhibit antioxidant activities. EPS produced by *L. plantarum* C88 was confirmed to be involved in the antioxidant activity of this strain since the purified EPS exhibited strong *in vitro* radical scavenging activity and antioxidant activity against H2O2-induced injury in Caco-2 cells (Li et al., 2013).

Recently researchers determined the structure of a polysaccharide produced by *Streptococcus macedonicus* Sc136 that contains the trisaccharide sequence β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp. This corresponds to an internal EPS backbone of lacto-N-tetraose and lacto-N-neotetraose. In fact, the same trioses have been identified in the structure of several human milk oligosaccharides that are important for infant nutrition. Deleting and inserting new genes coding for glycosyltransferases can be one way to engineer functional EPSs, based on an existing template such as the EPS produced by *S. macedonicus* Sc136, similarly to the approach based on LPS in *Escherichia coli* that led to *in vivo* neutralization of the Shiga toxin in mice. The use of EPS-producing probiotics to display *in situ* health beneficial polysaccharides is a very promising approach for a new generation of functional food. Low amounts of EPS produced by the LAB reduce their potential use as food-grade ingredients. However, EPSs offer new opportunities to targeted applications for improved consumer health benefits going in the direction of consumer-specific nutrition (Jolly et al., 2002b).

The beneficial properties of certain LAB may be related to the biological activities of EPS which was explained earlier. It is a fact that most of the commercialized probiotics are known to produce EPSs of its kind. Increased growth, mucosal adhesion, and prebiotic activity were observed in trials with EPS producers (Hongpattarakere et al., 2018).

Modification of the innate EPS either chemically, or genetically, for enhanced biological activity is one of the alternate threshold areas where researchers work on these days. Sulphonation, carboxymethylation, acetylation and phosphorylation are some of the reported chemical modifications that positively affect the physiochemical nature of the EPS. Specially, sulfonation is most widely used and confirmed to remarkably enhance the biological activities of polysaccharides, including anticoagulant and anti-inflammatory activities (Zhang et al., 2017). The chemical modifications and its effects on EPS biological properties have been schematically represented in Fig. 1.8 (Andrew and Jayaraman, 2020).

Modified EPS usually finds applications in pharmaceutical and food industries. Introduction of suitable ionic groups with appropriate degree of substitution can significantly improve the bioactivity of polysaccharides, such as antioxidant, antitumor, antiviral and immunomodulating activities. However, chemical modifications of exopolysaccharide from Lactobacillus have been scarcely reported. Therefore, it is of great interest to investigate the methods to increase the EPS yield and subsequently enhance the bioactivity of exopolysaccharides from Lactobacillus *via* chemical modifications (Chen et al., 2015; Chouchane et al., 2020; Fujiike et al., 2022; Hernandez-Tenorio and Giraldo-Estrada, 2022; Jin et al., 2014; Li et al., 2021; Liu et al., 2021; Salimi and Farrokh, 2023; Xiao et al., 2020a) and ultimately their application in food products.

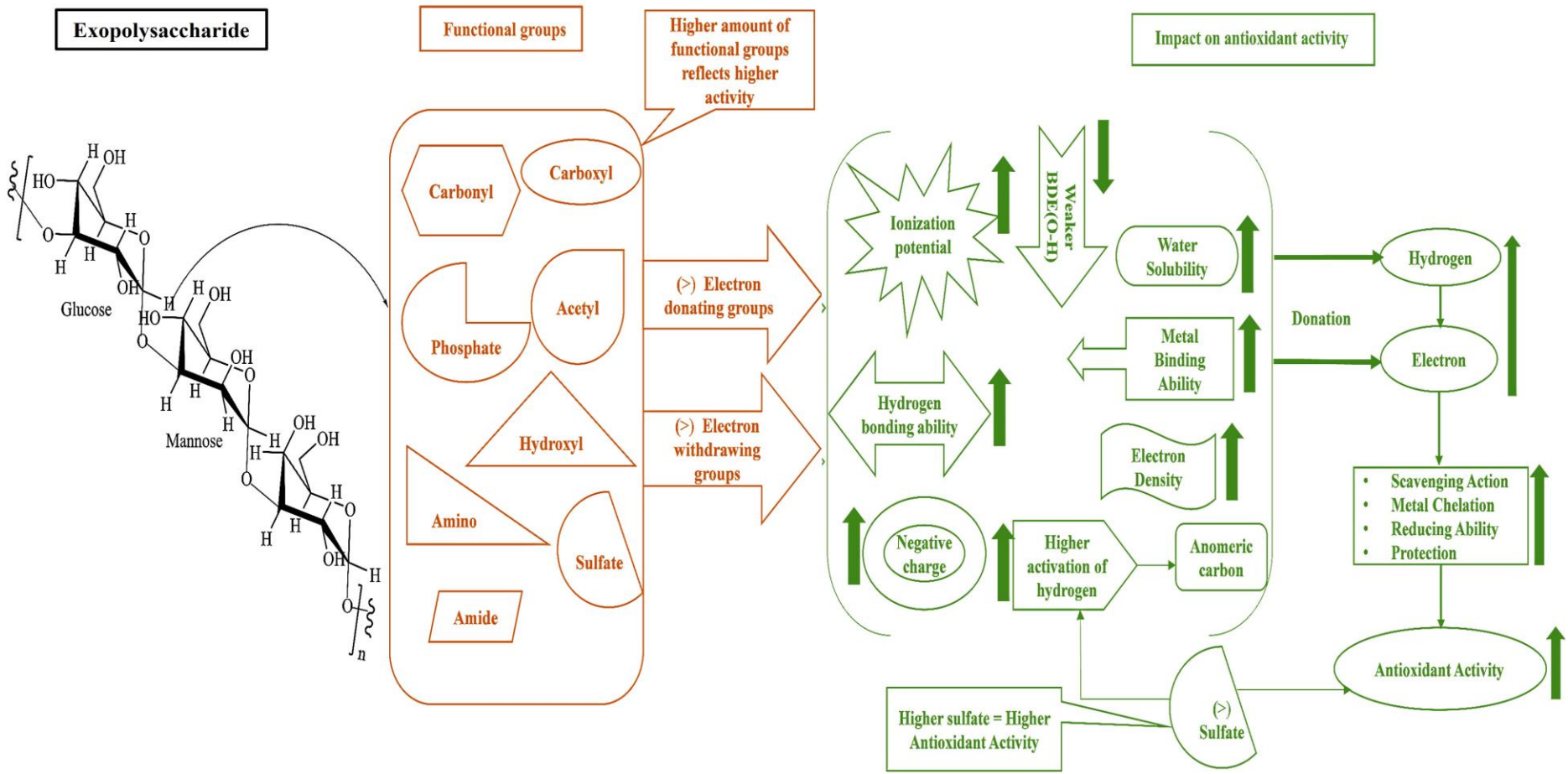


Fig. 1.8. Schematic representation of chemical modifications of EPS and its effects on biological properties of EPS

1.3.5. Commercial Potential and Applications of Exopolysaccharides

Considering all the functional properties of EPSs, there lies a huge opportunity to exploit their properties for human benefits in different fields. LAB-derived EPS offer a natural alternative to commercial food additives because of their physicochemical characteristics. The use of EPS in food production can have many advantages because they may impart desirable rheological changes in the food matrix such as increased viscosity, reduced syneresis, and improved texture, in addition to having emulsifying, thickening, and stabilizing properties. Moreover, EPS provide desirable body-texture, firmness, creaminess, and mouthfeel, or could even be applied in the preparation of food packaging (Korcz and Varga, 2021). The application of LAB-derived EPS in food could reduce or avoid the use of additives, which means not only production optimization, but it can also satisfy customer needs. The use of commercially available hydrocolloids must be labelled on food packaging, which is contrary to consumer expectations that foods should contain fewer additives. However, the use of EPS formed *in situ* by LAB does not require labelling. EPS are an alternative class of bio-thickeners used widely in the food industries and have been proven to provide many other techno-functional properties. To understand the diversity of this group of polymers and to be able to use them effectively, it is important to understand the mechanism of microbial biosynthesis of EPS.

Due to their structure, Lactobacillus polysaccharides have rendered a multitude of physicochemical characteristics with extensive roles in numerous applications, including bio-thickeners, viscosifiers, stabilizing agents and emulsifying agents, as well as ion exchange resins, hydrophobic molecular hosts, heavy metal removal agents, bioflocculants and biosorbents. The functional applications of Lactobacillus polysaccharides based on their physicochemical characteristics are reported in Table 1.7.

Table. 1.7. Composition, functional properties, and application of LAB - EPS

Organism	EPS Composition	Functional Properties & Applications	References
Lactobacillus delbrueckii subsp. *bulgaricus* DGCC291	Glucose, galactose	Increasing viscosity and decreasing syneresis in yogurt	(Surber et al., 2021)
Lactobacillus helveticus MB2-1	Glucose, mannose, galactose, rhamnose, arabinose	Antioxidant and prebiotics	(Xiao et al., 2020b)
Lactobacillus gasseri FR4	Glucose, mannose, galactose, rhamnose, fucose	Antioxidant and viscosity-enhancing agent	(Rani et al., 2018)
Lactiplantibacillus plantarum	Arabinose, mannose, glucose, galactose	Improving texture and rheological properties	(Huang et al., 2022)
Lactobacillus plantarum BR2	Glucose and mannose	Cholesterol-lowering and antioxidant	(Sasikumar et al., 2017)
Lactiplantibacillus plantarum C88	Glucose, galactose	Antioxidant and prebiotics	(Garcia-Gonzalez et al., 2021)
Lactiplantibacillus plantarum WLPL04	Xylose, glucose, galactose	Functional food ingredient	(Kumari et al., 2023)
Streptococcus thermophilus AR333	Galactose, glucose, galactosamine	Improving quality of fermented milk	(Zhang et al., 2018)
Streptococcus thermophilus CC30	Glucose, galactose	Emulsifier	(Kanamarlapudi and Muddada, 2017)
Streptococcus thermophilus NIZO 2104	Galactose, ribose, glucose, N-acetyl-galactosamine	Increasing viscosity and elastic modulus and decreasing syneresis in yogurt	(Gentès et al., 2013)
Streptococcus thermophilus S-3	N-acetyl-galactosamine, galactose, glucose	Interacting with milk proteins and decreasing syneresis in yogurt	(Xu et al., 2021)
Lactiplantibacillus plantarum JLAU103	Arabinose, rhamnose, fucose, xylose, fructose, galactose, glucose, mannose	Antioxidant and functional additive	(Gao et al., 2021)
L. delbrueckii ssp. *bulgaricus*	Glucose, galactose	Production of B-group vitamins e.g., folic acid	(Tang et al., 2020)

Rawson and Marshall, (1997) revealed the ability of polysaccharide producing *Lactobacillus delbrueckii* in improving the ripeness of yogurt. (Perry et al., (1997) used the exopolysaccharides from *Lactobacillus delbrueckii* and *Streptococcus thermophilus* as the starter culture of cheese. Results demonstrated that the exopolysaccharide-producing starter cultures were able to increase the moisture content and melt in the products compared with starter cultures unable to produce exopolysaccharide.

Furthermore, the polysaccharides derived from lactic acid bacteria also have the potential to enhance rheology, texture, and mouthfeel of fermented milk products. In addition, according to Abid et al., (2018) and Saravanan et al.,(2016) the polysaccharides produced from Lactobacilli exhibits high thermostability and mechanical stability. This is conducive to the plasticity of polysaccharides and the productive process of related probiotic products (Bai et al., 2016). It was already stated that the function of EPS is greatly influenced by its structure. The presence of β-(1, 4) linkages in HePs from lactic acid bacteria enables the EPS solution with increased consistency and high intrinsic viscosities. As well as the degree of branching directly affects the stiffness and the presence of functional group to the polysaccharide backbone gives EPSs the potentiality of promoting health benefits.

Lactobacillus delbrueckii subsp. *bulgaricus*, and *Streptococcus thermophiles*, both produce *in situ* heteropolysaccharides in yoghurt with an average production of 60-150mg/L and 30-890mg/L respectively. It is observed that these two bacteria grow synergistically where *L. delbrueckii* grows first and secretes metabolites that promote the growth of *S. thermophilus*. *S. thermophilus* produces formic acid and carbon dioxide that stimulates the growth of *L. delbrueckii*. The EPS producing starter cultures not only improved the water retention in

yoghurts thereby reducing the syneresis, but also improved the rheological properties like viscosity and elasticity.

At present, lactic acid bacteria EPS has been widely used as a new type of natural food additive in some European and American countries. The food technology characteristics of lactic acid bacteria EPS are as follows: 1) Improving the texture and tissue state of the fermented product EPS produced by lactic acid bacteria can improve the water holding capacity and yield of low-fat Mozzarella cheese; It was found that EPS produced by thermophilic lactic acid bacteria is in the processing and production of fermented dairy products, it can prevent the precipitation of whey, improve the texture and tissue state of the product, so that the product does not need to add any stabilizer, enhance the food safety and nutritional value, and prolong the shelf life of the product 2) improve rheological properties EPS produced by lactic acid bacteria is a natural thickener, can make the yogurt products smooth, increased viscosity, improve shear resistance, improve the rheological properties of fermented dairy products. A lower concentration of lactic acid bacteria EPS can increase the viscosity and does not form a gel; a small shear force can increase its fluidity, and when the shear force is removed, the viscosity can be quickly recovered. It has an important improvement effect on the sensory characteristics and intrinsic quality of fermented dairy products (Zhu et al., 2020).

Another example is the low – fat cheddar cheese, which is a main food component in European countries are fermented using *Lactococcus lactis* that produces EPS taking into advantage of improving water desorption rate and cheese moisture content increased upto 9.5% (Daba et al., 2021b). “Kefir” is another dairy beverage that is sour and slightly carbonated developed by some LAB and yeasts. EPS and some peptides are the by-products formed after fermentation of kefir grains developed with the help of some LAB and yeasts. EPS along with

few peptides are formed after fermentation and the EPS is a HePs known as Kefiran which contains hexa- or hepta-saccharide repeating units (glucogalactan), with the same amount of glucose and galactose.

Apart from all these applications in food industry, there are other areas where exopolysaccharides find significant role to play. That includes preparation of bio-based films, interpenetrating polymer networks, anti-cancer drug targeting, tissue engineering to name a few. Their ability to form hydrogels and film forming units makes them eligible for drug delivery or as drug carriers. Some EPSs being insoluble can hold large amounts of water inside the cross-linked 3D structural network. These gels can entrap drugs inside and also absorb/adsorb molecules outside the surface as well. Further *in vivo* studies have to be carried out to support EPS as drug delivery agents. EPS properties can be manipulated by different methods. One is by mixing it with other biopolymers. A mixture of galactomannan with xanthan is developed to form a gel structure and is an example of synergism. Another method is chemically modifying the EPS structural backbone by the addition of different functional groups to change the nature of the EPS. These chemical reactions with other constituents and biopolymers help to form cross links to achieve polymeric structures with enhanced functionality. Due to the non – Newtonian behavior and high viscosity in aqueous solutions, they find applications as thickening agents, stabilizers, and binding agents. Recent developments were seen in this field exploiting the ability of EPS to form novel structures like micro/nanospheres, polymer beads and capsules that are edible so as to carry vitamins, antioxidant molecules, probiotics, and prebiotics etc.

Exopolysaccharides like bacterial alginate, gellan gum, xanthan gum, FucoPoI and GalactoPoI have the capability to establish physical and chemical intermolecular interactions that can result in forming a cohesive polymer matrix thus forming a film that are edible and eco-

friendly. These films in turn have many attracts as it can be used as food packaging material, drug delivery coatings as discussed earlier, including scaffolds, tissue engineering, wound dressing and materials. This results in the development of bacterial EPS in pharmaceutical importance. Their inherent biological activity boosts this application of EPS being used as film forming units. FucoPoI exhibits high anti-inflammatory, anti-tumour, and immune enhancer drugs as a result of high fucose contents. Xanthan, sulphated dextran, sulphated dextran shows antiviral and anti-cancer properties. Glucomannan EPS produced by *L. plantarum* shows cholesterol lowering ability along with antioxidant properties.

All these applications and the commercial importance marks the future to flourish in this area of research. Enhancing the EPS productions with low cost, sustainable raw materials aids to the study. Fermentative production of EPS by genetic engineering the metabolic pathways of the EPS biosynthesis is yet another area to work on so as to increase the yield and diversity of EPS produced to get tailor-made EPS with varied applications and commercial relevance and significance.

1.3.5.1. Food Industry

LAB fermentations are regarded as a potential technology for the release of certain bioactive compounds from natural resources. Simultaneously, fermentation processes involve initial digestion of foods to improve absorption thereof in the human body. Moreover, they enrich food with such ingredients as proteins, sugars, vitamins, and amino acids. In food, EPS may serve as viscosifying, stabilizing, emulsifying, gelling, or water-binding agents. A significant portion of the polysaccharides included in food come from plants. The majority of them including cellulose, starch, pectin, alginate, and carrageenan, have undergone chemical or

enzymatic modifications in order to enhance their rheological properties. Even at low concentrations, EPS produced by microorganisms exhibit unique rheological properties because of their capability of forming very viscous solutions and their pseudoplastic nature. Numerous EPS-producing bacteria have intriguing industrial uses, including xanthan, pullulan, dextran, and gellan (Donot et al., 2012; Silva et al., 2019). First industrial polysaccharide to be commercialized by lactic acid bacteria is dextran. Dextran can be used in confections to increase viscosity, moisture retention, and to prevent sugar crystallization. It serves as gelling agents in gum and jelly candies. It prevents the production of crystals in ice cream, and it gives pudding mixtures the desired consistency and mouthfeel (Kumar Singha, 2012).

Levans, a category of fructans, have also been utilized in the food, pharmaceutical, and cosmetic industries, with a focus on their usage as food and feed additives when combined with prebiotics. Additionally, xanthan has demonstrated its strong potential for usage in the food industries as a viscosifier, thickener, emulsifier, or stabilizer. Microbial EPSes are well known for having a wide range of rheological characteristics, which makes them good candidates for use in the food industries particularly in a variety of yoghurts with a high purity rate (Tieking et al., 2005). Exopolysaccharides and fructose-oligosaccharides can shield bacteria from harmful stress conditions. Thus, they could be used in the food processing industries to preserve starter cultures and safeguard the health-promoting lactic acid bacteria in fermented food items (Tieking et al., 2005). The beneficial role of EPS formed from LAB in bread and dough, influences their technological characteristics in a number of ways, including loaf volume, staling, and water absorption. EPS have also been utilised successfully in baking applications to enhance the metabolic characteristics, flavour, texture, and shelf life of bread (Bajpai et al., 2016).

The food industry uses EPS generated from LAB as emulsifiers, stabilisers, thickeners, gelling agents, moisture retainers, for affecting rheology, hardness, and syneresis, and to enhance texture, sensory characteristics, and mouthfeel. They are employed because of their physicochemical characteristics, non-Newtonian behaviour, and high viscosity in aqueous systems. The LAB EPS with potentially technological impacts in addition to their antioxidant and antibacterial effects, expands their potential for use in the food sectors particularly in the fermented dairy, non-dairy, bread, and meat industries (Korcz and Varga, 2021).

The dairy industry makes extensive use of LAB-derived EPS generated *in situ.* Dairy starter strains that can produce appropriate quantities of EPS have attracted research's attention in recent years. As stabilisers, EPS can improve the firmness of the casein network by binding water and interacting with other milk components, such as proteins and micelles, to increase the final product's viscosity and act as thickeners and texturizers by thickening and texturizing the final product. Additionally, EPS can reduce syneresis, which is a beneficial feature in the processing of dairy products (Daba et al., 2021b; Hassan and Amjad, 2010; Mendes et al., 2016). Many fermented dairy products, including yoghurt, kefir, cultured cream, milk-based sweets, and cheese, are incorporating EPS during their production. This improves the textural and sensory properties of various foods, especially in the case of reduced- or low-fat varieties, where reducing fat content negatively affects product characteristics. The shelf life and consumer acceptance are decreased by quality flaws like syneresis. The issue can be resolved by making the casein-lactoglobulin complex more hydrophilic and increasing the water-binding capacity. Yogurt syneresis can be avoided by adding stabilisers, improving the protein level, and keeping the mineral content low and another simple fix is to use starter strains that produce EPS to control syneresis. As a result, it is possible to enhance organoleptic qualities of yogurt, even at

low protein concentrations, without sacrificing other aspects of the product (Jayarathna et al., 2020). According to Low et al. (1998), to improve the moisture retention of low-fat mozzarella, *Streptococcus thermophilus* MR-1C is used where the organism produces EPS composed of galactose, rhamnose, and fucose and were responsible for the increased moisture content of cheese samples. The use of an EPS-synthesizing *Lactococcus lactis* subsp. *cremoris* starter in the production of half-fat Cheddar cheese resulted in textural and melting properties similar to those reported for full-fat Cheddar due to increased levels of primary proteolysis, moisture retention, and diluted intact casein (Costa et al., 2012). Similar results were obtained with set-type probiotic yogurts prepared from caprine milk and an EPS-producing starting culture. These yogurts displayed an increased apparent viscosity, a reduced level of syneresis, and better sensory qualities. Dextran is utilized in the production of ice cream as a stabilizer and to increase viscosity. In a research conducted, EPS generated *in situ* by *Streptococcus thermophilus* strains had impact on the physicochemical, molecular, microstructural, rheological, and sensory characteristics of ice cream and was useful in preparation of fermented ice cream that wouldn't need chemical stabilizer addition (Dertli et al., 2016). There are numerous instances of using *ex situ* produced LAB EPS in dairy products. low-fat yogurts supplemented with pure EPS at concentrations of 73 mg/L and 146 mg/L had higher hardness values than did control and they persisted until the end of the study period (Elisa C Ale et al., 2016; Korcz and Varga, 2021).

The use of LAB in the production of meat extends back to the prehistoric era and has spawned a vast array of regional cuisines around the world. Currently, lactic acid and acetic acid production, direct nutritional competition, and the generation of bacteriocin make *Lactobacillus* and *Pediococcus* the most often utilized LAB genera to promote food safety by lowering the quantities of native bacteria in raw meat products. The moisture content, texture, and colour of

meat products are also influenced by these processes (Tsuda et al., 2012). EPS can provide protection for the cells and compared to their lyophilized equivalents; some data suggests that fermented meat as a matrix may protect lactobacilli strains as they move through the digestive tract. In meat matrices, such as a traditional German spreadable fermented raw sausage, LAB was able to produce EPS *in situ*. The qualitative characteristics of the products were modified by the in situ produced EPS, which also enhanced the spreadability of these fat-reduced raw sausages. Thus several attempts have been made to reduce or replace fat as consumer demands are high for fat-reduced meat products. Before EPS-synthesizing LAB is widely used in industry, there are still a lot of challenges that must be overcome. For instance, only if EPS generation by LAB is kept within a specific, well-defined concentration range a uniformly high food quality cab be ensured (Korcz and Varga, 2021).

The ability of EPS to bind water and create a network with various dough components is what gives baked foods their beneficial effects. Therefore, EPS enhances the rheology, structure, and volume of bread, leading to a reduction in staling rates and ensures a prolonged shelf life. Bran as a fibre source reduces the hydration of the gluten and, as a result, prevents the formation of the gluten network, which has a detrimental effect on the baking, particularly for bread. *Weisella confusa* is employed to address this issue because of it produces dextran in wheat bran, as the EPS enhances the quality of high-fiber wheat bread (Daba et al., 2021b). Additionally, EPS can enhance dough rheology for fermented foods made from cereal-legume. *Pediococcus parvulus, W. confusa, L. mesenteroides,* and *W. cibaria* all produces EPS that enhance rheological characteristics, texture, mouth-feel, flavour perception,, and the consistency of fermented foods like idli batter (Daba et al., 2021b; Kavitake et al., 2016).

Despite the fact that LAB can normally synthesize modest amounts of EPS, there are several advantages to using them that should be taken into account. However, a major difficulty is that the EPS production of LAB strains varies greatly in terms of both quantity and quality. This problem can be eliminated by prudent strain selection and optimization of production parameters. The specific manufacturing procedures and the properties of food products must be taken into account when determining the type and quantity of EPS produced as well as the ideal growth conditions for the microorganism utilized. Further developments in their applications requires a deeper knowledge of the structure-function link of EPS for its applications in food industries. However, in order for their industrialization and commercialization to be appealing, more work must be put into polymer characterization and the proof-of-concept of their application in high-value pharmaceutical, food, and cosmetic areas, where product quality and functional properties are significantly more important than production cost. Therefore, those high-value market niches have the potential for creation of novel microbial EPS (Silva et al., 2019).

1.3.5.2. Other Applications

Other than food industries, lactic acid bacterial EPS finds applications in various other sectors including pharmaceuticals, cosmetics, packaging industries. Dextran has been employed extensively in pharmaceutical and biological investigations as well as a fluorescence detecting material for drug administration Additionally, the lactic acid bacteria-derived inulin has been employed in targeted drug delivery systems to prevent colon cancer. According to some recent research, Lactobacillus polysaccharides were highly feasible for the administration and regulated release of probiotics and other bioactive substances. However, further research is still needed on the practical uses and commercial production of these polysaccharides. LAB EPS are also

significant sources of hydrogel materials for use in food, agriculture, chemicals, the environment, and pharmaceuticals (Liao et al., 2020).

The use of microbial biopolymers as packaging materials is the subject of recent research since they are biodegradable and made from renewable resources and hence EPS finds its way in these applications. Pullulan, kefiran, cellulose, and gellan are some examples of EPS that can be formed into films that are intended for packaging applications. Several of the EPS mentioned can be formed into thin films or used as coating over foods, particularly fruits and vegetables. These films can prolong the shelf life of fruits, vegetables, confections, meat products, and seafood by preventing surface browning, dehydration, oxidation, rancidity, and oil diffusion. The mechanical properties of a film made with microbial EPS can be greatly influenced by the structure of the film, and the presence of different sugars (such as glucose and mannose) can significantly alter how stiff the films are (S. Li et al., 2020; Vivek et al., 2021). Cross-linking can significantly alter the adhesive properties of the films as well as the mechanical characteristics of the film when ester bonds are formed between polysaccharide hydroxyl groups and carboxylic groups (or acid anhydride created by the reaction between two carboxylic groups). According to Vuddanda et al., (2017) partially purified lysozyme is added to crude pullulan to create antimicrobial pullulan films. The films with the highest enzyme activity recovery (65–70%) were made by adding lysozyme at 260 mg/cm^2 (1409 U/cm^2), and they were successful in preventing *E. coli* growth. Also, commercial pullulan films are utilized in breath fresheners or oral hygiene products.

As it is said to have antibacterial and wound-healing characteristics, the ability to lower blood pressure and lower serum cholesterol, and the ability to slow tumor growth, kefiran has the potential to be used commercially in the pharmaceutical sector (Vinderola et al., 2006). At a

concentration of 1%, kefiran forms an excellent film; however this film is hard, brittle, and has a high elastic modulus. It also has a low deformation at break. Designing and creating new properties based on requirement through controlled synthesis can lead to improvement in properties of existing exopolysaccharides and create novel biopolymers of great commercial interest and value for wider applications. The advancements ought to be directed at making bio-based packing materials competitive with currently available petroleum-based polymers. Research should concentrate on a comprehensive strategy for creating affordable, biodegradable packaging with acceptable qualities (Vijayendra and Shamala, 2014).

Additional *in vivo* research is needed to support the use of EPS as gene delivery vectors in the context of EPS applications in the pharmaceutical and healthcare industries. Using functionalized EPS as drug-carriers to treat tumours or other neurological illnesses by crossing the blood-brain barrier is a very intriguing area that explores the new possibilities. The ability of EPS to activate the T and B defence cells against cancer cells determines how effective they are as anticancer medicines. Although having a slow action if compared chemotherapy or radiation, it is yet durable, specific, and adaptable. Therefore, immunotherapeutic EPS might be useful preventative and synergistic anticancer drugs. To overcome and hasten their activity, more research is needed (Daba et al., 2021b).

1.4. Summary

Recent research on lactobacilli mainly focuses on their biosynthetic pathway engineering, strain improvement and development of industrially relevant strains for better and enhanced production of the value-added compounds. The food-grade status of the organism, its occurrence and availability, and most importantly the ability of the organism to synthesize the vast variety of

value- based compounds make lactic acid bacteria most aptly called as a microbial cell factory of industrial importance. Glycosyltransferases, being the major enzyme, mediated exopolysaccharide production by various lactic acid bacteria has a critical role in engineering aspects for better and improved production of EPS. The genes and the gene clusters responsible for EPS biosynthesis are seen enrooted in their chromosomes or in plasmids or might be distributed in both. From the available data and literature, we conclude that the EPS biosynthesis cannot take place in the absence of glycosyltransferases enzymes especially priming GTFs. Thus, engineering these enzymes in the pathway may be milestone to produce tailor-made EPS. Bacteria with GTF enzymes are used extensively in industry for a variety of applications. The polymer dextran is one prominent example of a universally used sugar chain. It is fermented at commercial scale for uses in veterinary medicine, separation technology, biotechnology, the food industry for gelling, viscosifying, and emulsifying, in human medicine as a prebiotic, cholesterol-lowering agent or blood plasma expander, and more. Characterization of these novel GTF enzymes and their exopolysaccharide products will provide a greater insight into the evolution (i.e., phylogeny and conserved sequence motifs), biochemistry (pH and temperature-optima; hydrolysis vs. trans-glycosylation activities), structure/function relationships of these enzymes contributing to, or determining, substrate/product specificities.

More information on the genome of the microorganism will enable to develop strategies to successfully enhance production rate and to engineer EPSs properties by modifying composition and chain length. In microbial EPS production, a better understanding of biosynthesis mechanism is a significant issue for optimization of production yields, improvement of product quality and properties, and for the design of novel strains. Understanding how to

manipulate EPS production can also have importance in the biological performance of probiotics.

Exopolysaccharides of lactic acid bacteria is a vast area of research as there is diversity in structure and functions of the EPS that it produces. Some of this diversity is seen in the monomeric compositions, linkage bonds and associated conjugates while the functions could be summarized as intrinsic and applied. The intrinsic functions, including morphological, structural and protective functions while applied is seen in human usage, medical, cosmetics, pharmaceutical, dairy products and other forms of industrial and environmental applications. Although a myriad of application is available for the constellation of exopolysaccharides produced by bacteria, it is vital with respect to human usage that the exopolysaccharides meet GRAS (generally regarded as safe) status or at least have a cost effective. The major limiting factor is its production cost due to its low levels of yield. However, the search for bacteria with high exopolysaccharides and techniques to increase the production with the manipulation of fermentation conditions, genetic and metabolic engineering as well as the exploration of cheap fermentation substrates for their production includes genome shuffling, protoplasmic fusion, alternate C-source etc., are suggested tools for improving the chances of commercial scale production and field application of these EPSs, and these are the subject of on-going investigations.

Chapter 2

Chapter 2

General Materials and Methods

2.1. Introduction

This chapter provides the detailed methodologies of the general assays and techniques invariably used throughout the thesis. Only very specific methodologies are described in the individual chapters. Standard protocols of microbial techniques, analytical methods and recombinant cloning techniques widely used in the thesis are described in this chapter by providing appropriate procedures and references.

2.2. General Materials

2.2.1. Bacterial Strains and Plasmids

The entire study was carried out with the *Lactobacillus plantarum* BR2 culture, a facultative anaerobe isolated from rotten jackfruit and capable of producing exopolysaccharides. It was identified using the 16S rRNA sequencing technique, and an entry with the accession number MN176402 has been made in the NCBI database.

Escherichia coli MC1061, the chemically competent *E. coli* strain prepared for heat shock transformation, obtained from Thermo Fischer USA, is used in overexpression studies. MC1061 is a recombinant positive strain (recA+) provided for cloning and amplification of plasmid DNA (Silva-Bea et al., 2022) of diverse Gram-positive bacteria, e.g., plasmids for expression in *Lactococcus lactis* or *Bacillus subtilis*.

Streptococcus thermophilus CUD3 (NCBI accession number MT176494), an indigenous isolate from sour curd is used as the starter culture in EPS application studies. Its starter culture properties and probiotic nature has already been reported by Suresh and Nampoothiri, (2022).

The plasmid pNZ8148 was maintained and propagated in *E. coli* MC1061 cells. These plasmids were used for the cloning of genes for transformation into *L. plantarum* BR2. These plasmids with pSH71 rolling circle replicon can replicate in many Gram-positive bacteria such as *Lactobacillus plantarum* and *Streptococcus thermophilus* and in Gram-negative *E. coli,* but require a recA+ strain like MC1061 while a recA- strain, like DH5α, cannot be used for their replication (Silva-Bea et al., 2022). Thus, this plasmid functions as a shuttle vector between *E.coli* and *Lactobacillus plantarum*. Another vector used in the study is pNZ9530 which is a regulatory plasmid. The replication genes from the plasmid pNZ9530 originally came from the *Enterococcus faecalis* plasmid pAMß1. This plasmid can only replicate in Gram-positive host strains, for instance, *Lactococcus lactis*, *Lactobacillus plantarum*, etc. (Simon and Chopin, 1988; Kleerebezem, 1997). This is employed for nisin controlled expression studies in *Lactobacillus plantarum* BR2. Annexure II depicts the vector maps of pNZ8148 and pNZ9530 used in the study.

2.2.2. Culture Media

MRS (de Man, Rogosa and Sharpe) agar or broth was used for *Lactobacillus* species and M17 medium for *Streptococcus* and *E. coli* cells were cultured in Luria Bertani (LB) medium. All the general media components were obtained from Hi-media Laboratories, Mumbai. The media compositions are provided in Annexure I.

2.2.3. Chemicals, Reagents and Kits

The chemicals such as glucose, lactose, sucrose, galactose, yeast extract, beef extract, tryptone, ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium citrate, sodium nitrite, potassium nitrate, sodium acetate, di-potassium hydrogen phosphate, magnesium sulphate, manganese sulphate tween 80 etc used in media for exopolysaccharide production were purchased from Hi-media Laboratories, Merck (India) and SRL Pvt. Ltd.(India). Other chemicals like deuterium oxide for Nuclear Magnetic Resonance (NMR) spectroscopy, potassium ferricyanide and ascorbic acid for antioxidant activity were procured from Sigma-Aldrich, USA. The stains used for microscopy such as crystal violet, safranin, and ruthenium red were procured from Hi-media Laboratories Pvt Ltd (Mumbai, India). Dextran standards for gel permeation chromatography were procured from Sigma-Aldrich, USA

Solvents such as ethanol, phenol-chloroform-isoamyl alcohol, acetone, and chemicals like phenol and concentrated sulphuric acid were purchased from SRL Pvt. Ltd. (India) for the extraction and purification of exopolysaccharides.

Genomic DNA isolation kits, Plasmid isolation kits, PCR clean-up kits, and Gel extraction kits were obtained from Qiagen, Germany. Restriction enzymes (BamHI, EcoRI, HindIII, XbaI), 2X Taq Master mix, Q5 High Fidelity Master mix, T4 DNA ligase, and DNA markers were purchased from New England Biolabs, USA. Nisin, lysozyme, bovine serum albumin (BSA), antibiotics like chloramphenicol, erythromycin were procured from HiMedia, India. Molecular biology grade chemicals like Agarose, Polyethylene glycol (PEG) MW 4000, Tris-base, glycine, ethylenediaminetetraacetic acid (EDTA), glycerol were obtained from Sigma-Aldrich, USA. Analytical grade solvents like dimethyl sulfoxide (DMSO), trifluoroacetic acid

(TFA), and glacial acetic acid were purchased from Merck, Germany. Other chemicals used in the study are procured from Hi-media Laboratories, Merck (India) and SRL Pvt. Ltd. (India).

2.3. Online Tools and Software

The various software along with other genome analytic tools employed during the study are listed in Table 2.1.

Table. 2.1. Different online tools and software used in the study

Softwares	Application	Source
BioRender	To create and draw diagrams, and illustrations	https://www.biorender.com
ChemDraw	To draw chemical structures and reactions as well as biological objects and pathways	https://chemdrawdirect.perkinelmer.cloud/js/sample/index.html
SnapGene	Enables DNA sequence visualization, sequence annotation, sequence editing, cloning, protein visualization, and simulating common cloning methods	https://www.snapgene.com/
OriginPro 8.5	Scientific graphing and data analysis	https://www.originlab.com/origin
BLAST	Sequence homology search	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Clone Manager 9	For primer designing	https://scied.com/dl_cm9.htm
ClustalW	Multiple sequence alignment	https://www.genome.jp/tools-bin/clustalw
Expasy ProtParam tool	Physicochemical properties of protein	https://web.expasy.org/protparam/
Image Lab	Gel documentation (Nucleic acid and protein)	www.biorad.com
LC solution	HPLC chromatograms	www.shimadzu.com
IBM SPSS 20.0	IBM SPSS Statistics version 20.0	https://www.ibm.com/spss

2.4. General Microbiology

2.4.1. Culture Growth Conditions and Maintenance

Lactobacillus plantarum BR2 and *Streptococcus thermophilus* CUD3 were generally grown at 37°C and 30°C respectively under static conditions. *E. coli* cells were routinely grown and propagated at 37°C providing appropriate shaking conditions. The growth of the organisms was monitored by determining the OD at 600 nm in a UV spectrophotometer and further serial dilution and plating in their respective agar plates and incubation overnight for CFU count. All microorganisms were sub-cultured in their appropriate medium (Annexure I) as mentioned in 2.1.1 and were maintained in agar plates at 4 ºC for immediate use and sub-cultured every two weeks. For long time preservation they were maintained in 20 % glycerol stock and stored at -80 ºC.

2.4.2. Inoculum Preparation

For EPS production, a loop full of *Lactobacillus plantarum* BR2 culture from fresh agar plates was used to inoculate 50 mL of MRS medium in 250 ml Erlenmeyer flasks, which were then incubated at 37°C for 18 hours. Unless stated, 10^9 CFU/mL of culture was employed as the inoculum. The culture absorbance was measured at 620 nm.

2.4.3. Fermentation and Extraction of EPS

Unless otherwise mentioned, exopolysaccharide production was done by submerged fermentation (SmF) in 250 mL Erlenmeyer flasks with 100 mL exopolysaccharide production medium under static condition and at 37°C (Annexure I). A more description on fermentation and extraction is given Chapter 3 (3.2.4) that deals with the characteristics of EPS producing *L. plantarum* BR2.

2.5. Molecular Methods

For most of the molecular biology methods such as genomic and plasmid DNA isolation, PCR amplification, cloning, competent cell preparation of *E. coli* and transformation were carried out as per standard protocol described by Sambrook et al., (2012) with necessary modifications and are explained in detail in this section. In addition, in the experiments wherever the kits are used, the manual instructions were properly followed.

2.5.1. Isolation of genomic DNA

The genomic DNA of *L. plantarum* BR2 was isolated from overnight static culture by cell lysis, phenol/chloroform extraction, and ethanol precipitation with minor modifications. The overnight culture of *L. plantarum* BR2**,** was centrifuged (grown in 10 mL MRS broth) for 5 min at 6000 rpm. Poured off the supernatant and freezed the cell pellet at -20°C for 30-60 min (or overnight at -70°C). Re-suspended the cell pellet in 250 µL of fresh 10 mg/mL lysozyme in TE buffer (Annexure I) and transferred equally to micro-centrifuge tubes. Incubated the mixture at 37°C for 1.5-2.5 hours with gentle shaking. Further, added 50 µL of 0.5M EDTA, 50 µL of 10% SDS, 50 µL of 5M NaCl and mixed gently. Then added 1µL RNAse (1mg/mL) and incubated at 37°C for 60 min. Added 10 µL of freshly prepared 20 mg/mL proteinase K and incubated at 37°C for another 1 hour. The complete lysis of cells can be confirmed based on the lucidity of the suspension. Consequently, the DNA was extracted with 1 volume (equal volume) of phenol: chloroform: isoamyl alcohol (25:24:1), by mixing it well and then centrifuged in micro-centrifuge tubes at 13,000 rpm for 15 min. Again, extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), by mixing it well and then centrifuged in micro-centrifuge tubes at 13,000 rpm for 10 min. To the aqueous phase added 1 volume isopropanol, and centrifuged the sample for 10 min at 10,000 rpm for 30 min.

(In this step while adding isopropanol DNA might be visualized as threads in the clear solution)**.** Washed the DNA with 500 µL of 70% ethanol by centrifuging at 10,000 rpm for 15 min, dried and re-suspended in 50 µL of nuclease free water. The DNA was visualized under UV in 1% pre-stained (EtBr) agarose gel (0.8 %) and was quantified using a UV-spectrophotometer, ND1000, Nanodrop instrument, (USA). The DNA was stored for further use at -20°C with proper labelling.

2.5.2. Isolation of Plasmid DNA

Plasmid DNA was isolated from *L. plantarum* BR2 and E. *coli* strains using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, prepared the culture by inoculating it in 100 mL LB broth with 10 µg/mL of the desired antibiotics as per the plasmid used (chloramphenicol for pNZ8148 and erythromycin for pNZ9530) and incubated overnight at 37ºC. Transferred 100 mL of the overnight grown culture into the centrifuge tubes and centrifuged at 6000g for 15min at 4ºC. Discarded the supernatant and re-suspended the pellet in 4 mL of resuspension buffer P1 (provided in the kit). Added 4 mL of lysis buffer P2, mix thoroughly by vigorously inverting 4-6 times and incubated at RT for 5 min. Added 4 mL of pre-chilled neutralization buffer P3, mix thoroughly by vigorously inverting 4-6 times and incubated in ice for 15 min. After this step the mixture turns into a turbid form. After incubation, centrifuged at 14,000-18,000 g for 10 min at 4ºC. Re-centrifuged if the supernatant is not clear. Again, centrifuged at 20,000 g for 30 min at 4ºC. Re-centrifuged at the same rpm for 15 min at 4ºC. Equilibrated the Qiagen tip (column) with 4 mL of equilibration buffer QBT and empty it by gravity flow. Added the supernatant after centrifugation to the column and allowed it for gravity flow. Washed the column (twice) 2X 10mL with wash buffer QC. Allowed it to drain it by gravity flow. Eluted

the DNA with 5 mL of elution buffer QF into a clean 15 mL centrifuge tube. (For constructs larger than 45kb pre-warming the elution buffer to 65ºC may help to increase the yield). Precipitated the DNA by adding 3.5 mL (0.7 volumes) isopropanol to elute the DNA mix. Washed the DNA pellet with 2mL 70% ethanol and centrifuged at 15,000 g for 10 min. Carefully discarded the supernatant. Air dried the pellet for 5-10 min and re-dissolved the DNA in a suitable volume of nuclease free water. The presence and quality of the isolated plasmid was confirmed by performing an agarose gel electrophoresis (AGE).

2.5.3. Quantification of DNA

To determine the quantity of DNA in a sample, a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) was employed. Based on its absorbance at 260 nm, DNA can be quantified using the Beer-Lambert equation: $\boldsymbol{A = ecl,}$ where *A* is the absorbance at 260 nm, *e* is the extinction coefficient, *c* is the concentration, and *l* is the path length. Double-stranded DNA appears to have an extinction coefficient of 0.02 $ngmL^{-1}$ cm^{-1}. The quotient A260/280 or A260/230, which ranges between 1.8 and 2.0, was used to gauge the DNA's degree of purity. Lower values of DNA were contaminated by proteins or polysaccharides.

2.5.4. Agarose Gel Electrophoresis

Genomic DNA, plasmids, and PCR amplicon fragments were separated and visualized on 0.8 % -1 % agarose gel containing 0.5 µg/mL EtBr using 1X TAE (Annexure I) as running buffer for agarose gel electrophoresis. Samples were mixed with 6X loading dye (Thermo fisher scientific) and separated at a voltage of 70-100 V for 1 hour depending on the varied gel size and were visualized under far UV (320 nm) illumination using Chemi, Biorad, USA. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules

through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel.

2.5.5. Sequencing of DNA

Using heat-denatured bacterial biomass extracted from a single colony that included crude DNA as a template, colony PCR was carried out. Sanger's (dideoxy termination) Genetic Analyzer 3500 and SeqScape® Software v2.7 from Applied Biosystems were used for the sequencing of the cloned vector. The device makes use of a high-resolution capillary electrophoresis technique for fluorescence-based DNA analysis. The standard operating procedure and the supplies needed for this technique were provided by the manufacturers. Specific primers were employed for gene amplification when the gene source was genomic DNA, whereas M13 F'/R' primers were utilized when the gene was cloned in the pNZ8148 vector (Annexure II). In accordance with the protocol further described in Chapter 5 (5.2.4), 10 µL PCR reactions were set up with the relevant forward and reverse primers. Gel extraction was used to purify the PCR- amplicon of the genes and subsequently 50 ng of high purity DNA (A260/A280 ratio between 1.7 and 1.9) was used for sequencing employing QIAquick Gel Extraction Kit (Qiagen, Germany).

2.5.6. Restriction Digestion

The cut eluted gene and the isolated vector after gel extraction to which the gene needs to be cloned were double digested with the specific restriction enzymes. To clone the gene into pNZ8148 vector, the vector and the *BR2gtf* gene were restricted using XbaI and Hind III restriction enzymes from New England Biolabs with their corresponding buffers, at 37°C for 1 hour and then cut eluted for ligation purposes.

2.5.7. Ligation

Ligation cloning was done using the T4 DNA Ligase enzyme (New England Biolabs, USA) and buffer according to the user's manual. Briefly, for a 20 µL reaction, 3 µL of double digested vector mixed with 9µL of double digested PCR purified (cut eluted) *BR2gtf* gene to which 1µL of 10X ligation buffer, 1 µL Ligase enzyme and 5µL of Nuclease free water is also added. Preparation was kept for overnight incubation at 16°C.

2.5.8. Preparation of Competent cells and Transformation

2.5.8.a. Competent cell preparation of E. coli

Inoculated 2 mL of LB broth with single colony of *E. coli* MC1061 in micro centrifuge tube and incubate at 37ºC for overnight with shaking (200 rpm). Aseptically add 1 mL of overnight grown culture to sterile 100 mL LB broth and again incubate at 37ºC with shaking for 2-4 hours or until OD_{600} = 0.4. Centrifuged the culture to obtain the pellet and to the pellet, added pre-chilled TSS buffer (Annexure I), vortexed slightly to suspend the pellet and incubated in ice for 15 min. After the incubation, aliquot 200 µL of cells to fresh sterile micro centrifuge tubes and stored at -80ºC until use.

2.5.8.b. Transformation into competent cells of E. coli

After ligation, 20 µL of ligated product was mixed with 100 µL of freshly thawed *E. coli* MC1061 competent cells (prepared by TSS method, mentioned earlier in 2.4.7.1) and incubated on ice for 30 min. A heat shock at 42°C for 45 sec (water-bath) was given after the incubation. 900 µL of LB broth/SOC medium (Annexure I) was added immediately after the heat shock and incubated at 37°C at 200 rpm for 1-1.5 hours. After incubation, pelleted the cells by centrifugation at 6000 rpm for 10 min and resuspended the pellet in 100 µL of SOC medium

(Annexure I). This 100 µL of the *E. coli* MC1061 was spread plated on LB agar plate supplemented with chloramphenicol (Cmp) (10 µg/mL) and incubated at 37°C for 24 hours.

2.5.8.c. Preparation of electrocompetent cells of L. plantarum BR2

The preparation of electrocompetent cells and electroporation of *L. plantarum* BR2 was finalized after several optimizations and modifying different protocols reported earlier (Landete et al., 2014; Mason et al., 2005; Openwetware, 2018; Palomino et al., 2010; Teresa Alegre et al., 2004). For this, *L. plantarum* BR2 was cultured overnight in MRS medium supplemented with 2.5% glycine and 0.5M sucrose (modified MRS, (mMRS)) at 37ºC under static conditions. Inoculated 100 mL mMRS medium with 1% culture and incubated at 37°C until OD_{600} becomes 0.5-0.8. After the incubation, cells were harvested at 4000 rpm at 4ºC for 10 min. Supernatant was discarded and the pellets were washed with ice-cold 0.5 M sucrose containing 10% glycerol. Resuspended the pellet in ice-cold 0.5 M sucrose containing 10% v/v of glycerol and 50mM EDTA and incubated in ice for 15-20 minutes followed by centrifuged at 4000 rpm at 4ºC for 10 min. Cells were then suspended in 1/100 culture volume of fresh 0.5M sucrose containing 10% v/v of glycerol. The electrocompetent cells were then aliquoted and stored at -80ºC until use maximum of up to 3 months.

2.5.8.d. Electroporation into L. plantarum BR2

The electro-competent cells were thawed in ice for 30 min. 1 mL regeneration medium containing MRS broth with 2 mM $CaCl_2$, 20 mM $MgCl_2$ and 0.5M sucrose was taken in a microfuge tube and kept at 27°C for pre-warming. 150-300 ng/µL each of the plasmid DNAs (cloned pNZ8148 and pNZ9530) was transferred to a fresh pre-chilled microfuge tube and mixed with 100 µL of the electro-competent cells and kept in ice for 15 min. 100 µL of the mixture was transferred to pre-chilled fresh and sterile electroporation cuvette (2 mm) BioRad, USA, and

placed in ice for 5-10 min. The cells were electroporated at a voltage of 2.1kV, capacitance of 25µF, and resistance of 200 Ω in a Gene Pulser electroporator (Bio-Rad, CA, USA). 1mL of pre-warmed (at 37°C) MRS-broth containing 2mM $CaCl_2$ + 20mM $MgCl_2$ + 0.5M sucrose was added immediately to the cuvette after electroporation and was incubated at 37°C for 2 hours for recovery. The contents were immediately transferred under aseptic conditions to warm regeneration media and incubated at 37°C for 2 hours. 100 µL of the cell suspension was plated on MRS medium supplemented with 5 µg/mL of chloramphenicol and erythromycin antibiotics. The transformants appeared as pure white colonies after 16-24 hours of incubation at 37°C. The transformants were confirmed by colony PCR and the positive clones were sub cultured and used further in the study. The transformation efficiency was calculated using the following equation:

$$\text{Transformation efficiency} = \frac{\text{No. of Transformants (colonies) x Final volume at recovery}}{\text{µg of plasmid DNA x Volume plated (mL)}}$$

2.6. Analytical Methods

This section of the chapter deals only with the general and broad analytical procedures. The specific methods/assays have been described in corresponding chapters when and where required.

2.6.1. Phenol-Sulphuric Assay for Estimation of Carbohydrates

The phenol-sulfuric acid method is a quick and easy colorimetric method to ascertain the total amount of carbohydrates in a sample and here exopolysaccharides are estimated as total carbohydrates by (Dubois et al., 1951) this method. The concentrated sulfuric acid can break down all exopolysaccharides into monosaccharides by hydrolysis of its glycosidic linkages and the resulting pentoses (5-carbon sugars) are later dehydrated to furfural and the hexoses (6-carbon sugars) to the hydroxymethyl furfural. These compounds then react with phenol, resulting

in an orange-gold hue measured at 490 nm. The color produced by this reaction lasts for a long time and the precision of the procedure is within ± 2% in appropriate conditions.

2.6.1.a. Reagents

- 5 % Phenol (w/v)
- Concentrated sulphuric acid (35.7 N)

2.6.1.b. Procedure

To 1 mL of lyophilized EPS sample (diluted 20 times), 1 mL 5 % (w/v) phenol was added followed by 5 mL concentrated sulphuric acid and allowed the reaction to take place. The mixture was incubated at room temperature for 20 min. after incubation, kept the tubes in ice and then allowed to cool down to room temperature and further the absorbance was read at 490 nm. Glucose was used as the standard in the range of 0-100 µg concentration from 1 mg/ml stock solution. A standard graph (Fig. 2.1) was plotted with absorbance at 490 nm against concentration of glucose. A blank was also prepared in the same way.

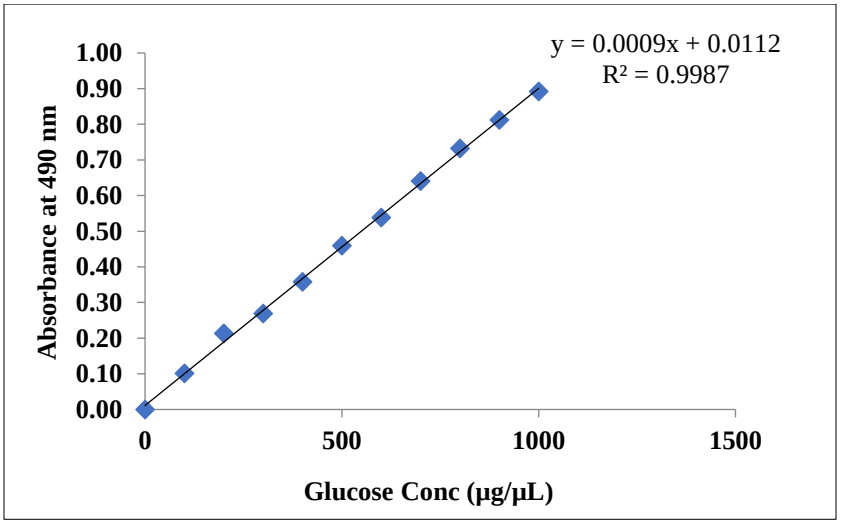


Fig. 2.1. Standard graph for phenol-sulphuric Acid assay

2.6.2. Bradford Assay for Protein Estimation

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The anionic form of the dye is stabilized by both hydrophobic and ionic interactions, which results in a discernible color change. The experiment is effective because a dye-albumin complex solution's extinction coefficient is constant over a concentration range of 10-fold. Standard protocols of Bradford et al., (1976) were used for the assay with minor modifications.

2.6.2.a. Reagents

- Bradford's Reagent (contains Coomassie Brilliant Blue G-250, phosphoric acid and methanol)
- Protein samples dissolved in appropriate buffer

2.6.2.b. Procedure

Protein samples were quantified spectrophotometrically using Infinite 200 PRO microplate reader at 595 nm along with Bradford reagent. Bovine serum albumin (BSA) was used as the standard. 10 µL of the sample was mixed with 300 µL of Bradford reagent and incubated for 5 min at RT. A standard curve was plotted against BSA concentration within a range of 0.1-1 mg/mL (Fig. 2.2). All the readings were taken in triplicates in a 96-well microtiter plate.

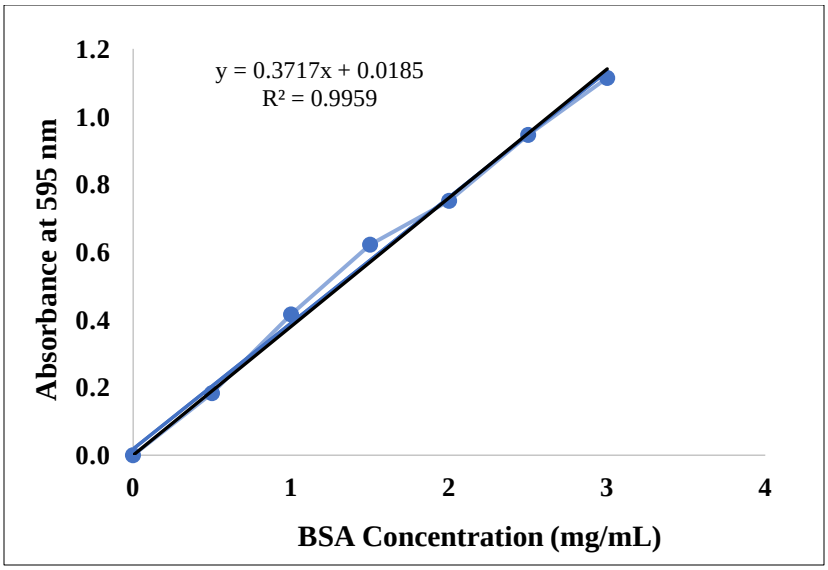


Fig. 2.2. Standard graph for Bradford assay

2.6.3. DNS Assay for Estimation of Reducing Sugars

3,5-Dinitrosalicylic acid (DNS) is used extensively in biochemistry for the estimation of reducing sugars. It detects the presence of free carbonyl group (C=O) of reducing sugars. This involves the oxidation of the aldehyde functional group (in glucose) and the ketone functional group (in fructose). During this reaction DNS is reduced to 3- amino 5-nitrosalicylic acid (ANSA) which under alkaline conditions is converted to a reddish-brown colored complex which has an absorbance maximum of 540 nm. The assay was followed based on the standard DNS procu+edure by Miller (1959) with appropriate modifications.

2.6.3.a. Reagents

- NaOH – 19.8g
- 3,5, DNS (3,5-dinitrosalicylic acid) – 10.6g
- Sodium potassium tartarate tetrahydrate – 306g
- Phenol – 7.6mL
- Sodium metabisulphate – 8.3g
- Distilled water – 1416 mL

19.8g NaOH and 10.6g of 3,5 DNS were dissolved in 1416mL of distilled water. heat at 50ºC, till a clear solution was obtained. Sodium potassium tartarate tetrahydrate (306g) was added followed by the addition of 7.6mL of phenol and 8.3g sodium metasulphate.

2.6.3.b. Procedure

Freshly prepared suitable aliquots of standard solution (100-1000µL) were transferred to to test tubes. The volume of test tubes was made to 1 mL with distilled water. 1 mL of distilled water alone served as the blank. Further 2 mL of DNS was added to each test tubes. The test tubes were kept in the boiling water bath for 5 min. The reaction was terminated by keeping the test tubes in ice after 5 min of incubation in the water bath. 0.5 mL of reaction mixture was transferred from each test tube to 4.5 mL distilled water in another set of test tubes. Read the absorbance at 540 nm in a UV spectrophotometer. 2 mg/mL of glucose was used as the standard. Fig 2.3 depicts its standard curve. The same was done for the samples to quantify the amount of reducing sugars present in it.

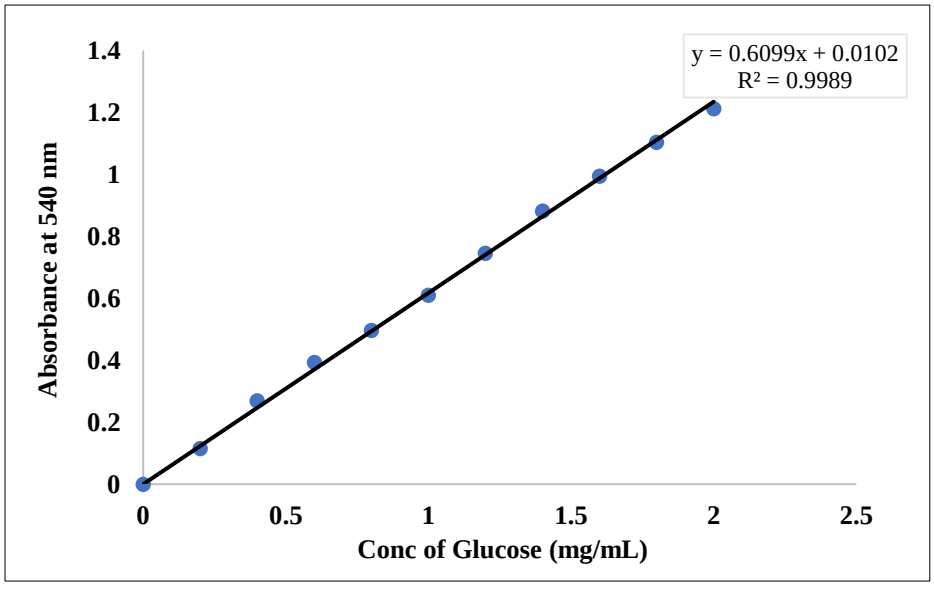


Fig. 2.3. Standard graph for DNS Assay

2.7. Instrumentation

The major equipment used during this study is listed in Annexure III. Much of the work was carried out availing the facilities at CSIR-NIIST, Trivandrum and some of the cell line studies and microscopic observations were done at Biogenix Research Center, Center for Molecular Biology and Applied Science, Trivandrum, Kerala.

2.8. Summary

This chapter provides an overview of all the general materials and methods including biological, molecular, and analytical protocols adhering to all standard procedures with appropriate references. However, certain specific protocols will be covered in the relevant working chapters. The chapter also included three annexures, at the end of the thesis, such as the media and buffer compositions, vector maps, and the major instruments used for the study.

Chapter 3

Chapter 3

Characterization of Exopolysaccharide Producing *Lactobacillus plantarum*

3.1. Introduction

Exopolysaccharides are high molecular weight long chain carbohydrate polymers produced by microorganisms to carry out various cell functions and secreted from the cell to encompassing environment or biomacromolecules seen attached to the cell membrane. Depending on the strain, conditions and substrate used for the production, they can be homo polysaccharides or heteropolysaccharides (Xiao et al., 2020b). EPS production does not provide energy to the microorganism but protects from extreme conditions such as osmotic imbalance, drought, phagocytic attacks, and penetration of heavy metals and helps in cell adhesion. Researchers have already reported the production of diverse types of EPS from various LAB including the *Lactobacillus, Pediococcus, Bifidobacterium, Streptococcus, Leuconostoc and Weissella* (Du et al., 2017) isolated from milk, cheese and other fermented food products. Due to their GRAS status, LAB varieties are more explored than other bacteria considering the safe, natural end products.

As compared to some other bacteria, Lactobacillus spp. are not very efficient producers of exopolysaccharides. For instance, around the globe over 2,000 tons of xanthan (used mostly as a thickening agent) are produced annually using the soil bacteria *Xanthomonas campestris*. Interest in EPS from Lactobacillus strains is largely attributable to the fact that these bacteria are deemed safe and confer health benefits (Liu et al., 2010). So far, approximately thirty EPS-producing Lactobacillus species have been identified, with the best-known ones being *L. casei,*

L. acidophilus, L. brevis, L. curvatus, L. delbrueckii subsp. bulgaricus, L. helveticus, L. rhamnosus, L. plantarum, and *L. johnsonii*. These bacteria are cultured on mineral-rich media such as MRS (Man, Rogosa, Sharp), milk, or milk derivatives at 30°C or 37°C, depending on the strain (Oleksy and Klewicka, 2018).

Functional properties of EPS vary according to their monosaccharide units, type of linkages, branched or unbranched structure, presence/absence of some carbonyl or other functional groups, structural and confirmational changes they acquire under different environmental conditions. Exploring the advantages and physiochemical properties of EPS, elevates the demand of EPS in textiles, cosmetology, wastewater treatment and in food industry (Mende et al., 2016; Sengupta et al., 2018b). EPS used as antioxidants, anticoagulants, flocculating, viscosifying, emulsifying and stabilizing, gelling agents in various industrial fields (Korcz and Varga, 2021; Viñarta et al., 2006; Wu et al., 2022). The potential to improve the rheological, textural and mouthfeel properties are also explored in food. EPS have signifying health-promoting advantages such as antioxidant, anti-tumour, immunomodulating, cholesterol-lowering, colonizing probiotic microbiota in intestine (Jolly et al., 2002b; Kim et al., 2021; Nampoothiri et al., 2017). Bacterial EPSs are considered to be new sources of natural polymers, which could meet the increasing demands for many industrial applications, including food, cosmetic and medicine (Jiang and Yang, 2018).

The major highlight of this chapter includes, molecular confirmation of the indigenously isolated *Lactobacillus plantarum* BR2 by 16S rRNA sequencing, *L. plantarum* BR2 growth analysis and its probiotic characterization, Exopolysaccharide production using synthetic medium, sugar utilization studies, optimization of EPS production process conditions, EPS extraction from fermented broth, purification, and characterization of EPS.

3.2. Materials and Methods

3.2.1. Bacterial Strains and Growth Conditions

The maintenance, growth, and inoculum preparation of *L. plantarum* BR2 has been described in chapter 2 (2.3.1, 2.3.2 and 2.3.3).

3.2.2. Chemicals, Reagents, and Instrumentation

Media and other components like De Man Rogosa Sharpe agar and broth (MRSA, MRSB), Yeast Extract, K_2HPO_4, $MgSO_4$, $MnSO_4$, Tween 80, ammonium sulphate, ruthenium red, crystal violet, safranin, iodine solution was purchased from Hi-Media, India. Chemicals like, hydrogen peroxide, sodium acetate, phenol and phenol-chloroform-isoamyl alcohol were procured from Sisco Research Laboratories Pvt Ltd (India). Ethanol, acetone, and other solvents were purchased from Spectrochem Pvt Ltd (India). Dialysis membrane and dextran standards were purchased from Sigma Aldrich (USA).

The instruments used in the study were, FTIR Spectrophotometry (Nexus-870 FT-IR, Thermo Nicolet Corporation, Madison, WI, USA), cooling centrifuge (Kubota, Korea), lyophilizer (Christ Alpha 1-2 LD Plus, Germany), HPLC (Shimadzu, Japan), Rezex RPM Monosaccharide Pb+2 300 x7.8 mm column for carbohydrate analysis, Microplate Reader, Tecan Nano Quant Infinite M200 Pro, (Switzerland), spectrophotometer (UV-1601 UV Visible Spectrophotometer, Shimadzu, Japan), TGA (SII Nano-technology Inc., Japan), NMR Bruker Avance II-500 Spectrometer (Switzerland), SEM (Zeiss EVO 18 cryo-SEM, Germany).

3.2.3. Probiotic Characterization of L. plantarum BR2

The probiotic characterization of *L. plantarum* BR2, involves the identification of its fermentation type, tolerance to acid, salt (NaCl) and bile salt, cell surface hydrophobicity, proteolytic activity, and antibacterial activity.

Lactobacillus plantarum BR2 culture was added to sterile MRS broth containing 0.005% bromocresol green (pH indicator dye) along with Durham's tubes and incubated undisturbed at 37°C up to 24 hours. The presence of gas in Durham's tube and the colour shift of the broth from red to yellow suggest the fermentative type of bacteria (Ali et al., 2023).

Tolerance of *Lactobacillus plantarum* BR2 to an acidic environment was checked on the basis of their survival at lower pH. Acid tolerance capability of isolate was checked as mentioned by Obioha et al., (2021) with minor modifications. 1% (v/v) with OD_{600} of 0.9-1.0 of the activated culture was inoculated in MRS broth adjusted with various pH such as, 2, 2.5, 3, 3.5 and 4 and were incubated at 37°C. Further the cell viability was determined after 2 and 4 hours by enumerating colonies present in the MRS agar plate after serial dilution and plating and was compared with the control. MRS broth with pH 6.5 served as control.

The bile salt tolerance test of the culture was performed on the basis of their survival in different bile salt concentrations and was done as per the earlier reports (Melese, 2023; Obioha et al., 2021) with slight modifications. MRS broth prepared with varying concentrations of bile salt, 0.3% ,0.5%, and 0.8%, were inoculated with *Lactobacillus plantarum* BR2 and was incubated at 37°C for 24 hours. The MRS broth without bile salt served as the control. Survival rate of the organism was analyzed enumerating the colonies by plate count method on MRS agar plates after incubation at 37°C for 24–48 hours. The survival percentage was calculated by CFU of

each bile salt concentration compared to the control CFU. Each experiment was done in duplicates

Survival percentage = {(Log CFU Test)/ (log CFU control)}*100

Saline tolerance of the isolate was identified similarly by growing it in MRS broth with different NaCl concentrations (2%, 4%, and 6%) for 24 hours at 37°C and MRS broth without NaCl served as control. After incubation, the cell viability was measured by growth appearance on MRS agar by plate count method. Colony counts were manually performed and expressed as log CFU/mL. The survival percentage was calculated by CFU of each test compared to control (Melese, 2023; H.-Z. Zhao et al., 2023).

To check the proteolytic activity of *Lactobacillus plantarum* BR2, MRS medium supplemented 5% skim milk (w/v) was prepared and to the skim milk MRS agar plates 10 µl of overnight cultures were spotted and the plates were dried and further incubated at 37ºC for 24 – 48 hours and checked for clear zone or haloes.

Cell surface hydrophobicity was determined against different organic solvents as mentioned by Özkan et al., (2021) and Zhao et al.,(2023) with minor modifications. The bacterial cells, grown in MRS broth at 37°C for 16 hours, were harvested by centrifugation at 5000 rpm for 10 mins. The cells were washed and resuspended in PBS buffer with the cell density made up to 0.5 at 600 nm. For the hydrophobicity analysis (affinity to hydrocarbons), an equal volume of *Lactobacillus plantarum* BR2 cell suspension and various hydrocarbons (hexadecane, hexane and ethyl acetate) were mixed by vortexing for 3 min and incubated at room temperature for 1 hour. The absorption of the aqueous phase was measured at 600 nm using a UV

spectrophotometer (Tecan Nano Quant Infinite M200 Pro, Switzerland). Hydrophobicity was expressed as adhesion percentage according to the formula:

Cell Surface Hydrophobicity = $[(A_0 – A_t)/A_0] \times 100$

where A represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0

Agar well diffusion method was performed to determine the antibacterial activity of the isolate against five different microbes such as *S. aureus* MTCC 96, *S. enteric* NCIM 5256, *E. coli* MTCC 443, and *L. monocytogens* NCIM 5277 and *Fusarium moneleforme* NCIM 1100 as described by Guimar et al., (2019) and Salihu et al., (2015). Briefly, these cultures were spread on Muller Hinton agar plates with wells created by metallic well puncture and 100 µL of concentrated cell free supernatant was used to fill the wells. The plates were left to dry for 30 mins followed by incubation at 37°C for 24-48 hours after which the zone of inhibition was measured.

3.2.4. Exopolysaccharide Production by L. plantarum BR2

3.2.4.1. Optimization of EPS Production Conditions

The medium used for EPS production was the one reported earlier by Ismail et al., (2010) and Sasikumar et al.,(2017), with minor modifications, and has been described in Annexture I. The EPS production was tested with different lactose concentrations (w/v) (1%, 2%, 3% and 4%) with three different incubation times (24, 48 and 72 hours) and the EPS was extracted after each incubation time. 100 mL of the EPS production media were prepared with different carbon sources (mentioned above) and inoculated with 1% (v/v) of the culture inoculum with OD_{600} 0.9-1.0. The flasks were incubated at 37°C under static condition for 72 hours.

There after the growth of *L. plantarum* BR2, its sugar utilization and the EPS production under static and shaking incubation conditions were also evaluated. The production media inoculated with 1% (v/v) of the overnight grown culture was incubated under static and shaking conditions (200 rpm) at 37°C for 72 hours. The growth of the organism was evaluated by determining the optical density and viable cell count (VCC) of the samples withdrawn at different time intervals of time and the sugar utilization was determined by estimating the residual sugar in the medium at different time intervals by DNS method described in chapter 2 (2.4.1). The lactic acid produced by the organism at different time intervals were estimated by High Performance Liquid Chromatography (HPLC, Shimadzu, Japan) equipped with refractive index (RI) detector using Phenomenex Rezex-ROA Organic acid column (300 x 7.8 mm) with column temperature at 65 °C and 0.01N H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The samples were centrifuged at 8000 rpm for 5 min, and then filtered using Nylon 6,6 membranes (pore size 0.22 mm) prior to analysis (Vivek et al., 2018).

3.2.4.2. Batch cultivation of EPS in Bioreactor

EPS production media was prepared with optimized medium components as already mentioned (Ismail and Nampoothiri, 2010). The batch fermentation was carried out in a 2.5 L stirred tank glass bioreactor with 1 L working volume (Minifors, Infors HT, Switzerland) with 1% (v/v) inoculum having an OD_{600} rage of 0.9-1.0. The fermenter was equipped with a sparger, four blade impeller, four baffles, pH probe, temperature probe, dissolved oxygen probe, cooling jacket and foam probe. Fermenter parameters suitable for the production was maintained with temperature 37°C, aeration 1.25 vvm, impeller speed 100 rpm and dissolved oxygen maintained as 100%. Initial pH of the medium was 6.9 ± 0.5. Fermentation was carried out for 72 hours

without making significant differences in the fermenter parameters. The EPS produced was extracted and purified further.

3.2.5. Extraction and Purification of EPS

After the fermentation period, the bacterial cells were pelleted out by centrifugation of the culture broth at 11,000 rpm for 10 min and the EPS was extracted and precipitated from the supernatant by the addition of double volumes of chilled ethanol, followed by overnight incubation at 4°C. The precipitated EPS was recovered by centrifugation at 3000 g for 20 min. Further purification was done by treating crude EPS with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1, (v/v/v)). Double volume of chilled ethanol was added to purified EPS and incubated overnight. Precipitated EPS was then recovered by centrifugation at 3000 x g for 20 minutes. Finally, the extracted EPS was dialyzed against deionized water with two changes of water for 24 hours at 16°C with mild and continuous agitation and after which, the EPS was lyophilized and weighed. The UV spectrum of the purified fraction was studied at 280 nm using a UV spectrophotometer to investigate the presence of protein. These EPS purification steps were followed based on the previous research of Ismail et al., (2010) and Vasanthakumari et al., (2015).

3.2.6. Characterization of EPS

3.2.6.1. Monosaccharide Composition Analysis

The monosaccharide composition of EPS was determined by high performance liquid chromatography (HPLC, Shimadzu, Japan), with RI detector using Phenomenex Rezex RPM Monosaccharide Pb+2 (300 x7.8 mm) carbohydrate analysis column (Biorad, USA). Mobile phase used was deionized water at a flow rate of 0.6 mL/min. The EPS samples for

monosaccharide analysis were prepared by acid hydrolysis under controlled conditions in an oil bath followed by neutralization using 1M NaOH (Ismail and Nampoothiri, 2010).

3.2.6.2. Fourier Transform-Infrared (FT-IR) Spectroscopy

The functional groups present in the exopolysaccharide's backbone were resolved using FT-IR (IR Tracer-100, Shimadzu, Japan) analysis. 1 mg of purified EPS samples were placed on ZeSe crystal plate and analysis at a range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

3.2.6.3. Gel Permeation Chromatography (GPC)

To determine the molecular weight of EPS, gel permeation chromatographic technique (GPC) was used with a refractive index detector. The calibration curve of dextran was prepared to calculate the molecular weight of purified EPS. EPS sample was prepared at a concentration of 15 mg/mL in distilled water and analysed in Shodex SB-804 GPC column (USA) using deionized water as mobile phase with a flow rate of 1 mL/min.

3.2.6.4. Thermogravimetric Analysis (TGA) of EPS

Thermal stability of the exopolysaccharide sample was analyzed by TGA using the TA Q50, thermogram instrument (USA). The sample preparation procedure was followed as mentioned in our previous study (Sasikumar et al., 2017) with minor modifications, 35 mg of sample was heated at the range of 30 to 400°C at a rate of 10°C/min. under continuous supply of nitrogen gas.

3.2.6.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

To determine the molecular characteristics of *L. plantarum* BR2 EPS one dimensional (1D)–Nuclear Magnetic Resonance (NMR) spectroscopy was performed at the room temperature using a Bruker Avance II 500 spectrometer (Germany). Both ^{1}H and ^{13}C NMR and 2D NMR

spectroscopic studies were performed. The sample was prepared at a concentration of 15 mg/mL with purified EPS and (99.96%) deuterium water. Chemical shifts were analyzed in parts per million (ppm).

3.2.6.6. Scanning Electron Microscopy

The surface morphology of EPS was analysed by scanning electron microscopic (SEM) images of the purified EPS samples. For this, Zeiss EVO 18 cryo-SEM, (Germany) was employed where the lyophilized EPS samples were placed in aluminum stubs.

3.3. Results and Discussion

3.3.1. Probiotic Characterization of L. plantarum BR2

Pure culture of isolate had an off-white colour with slimy, ropy nature on MRS agar plates (Fig. 3.1. (A)). The culture was catalase negative and stained crystal violet with rod-shaped cells with rounded ends in morphology which confirmed the Gram-positive nature of the culture strain. The preliminary confirmation of EPS production was confirmed by white ropy colonies present in the MRS agar plate containing ruthenium red dye after 24 hours of incubation at 37°C. *L. plantarum* BR2 was identified as homofermentative (Fig. 3.1.(B)) as it fermented glucose to lactic acid and did not show gas production in the Durham's tube, whereas those that ferment glucose with lactic acid, acetic acid, ethanol, and carbon dioxide (CO_2) can form gas in the inverted Durham's tube (Melese et al., 2023).

The critical factors that may affect commercial probiotics are the high acidity and the high concentration of bile components in the proximal intestine. Therefore, being tolerant to acidic conditions is an important criterion to be considered during the selection of potential

probiotic isolates to assure their viability and functionality. Moreover, probiotic bacteria show variable resistance to acidic conditions, and this characteristic is species and strain dependent.

The survival ability and resistance to acidic environment is an important factor to ensure the functional ability of the bacteria in GI tract. *L. plantarum* BR2 showed viability at pH values of 3, 3.5, and 4; it demonstrated > 60% cell viability at a lower pH 3 and this is depicted in Fig. 3.1.(C) and this implied that the isolates would survive being introduced into the harshly acidic state of the host stomach. Probiotics must have the capacity to endure an acidic environment in the gut as they go through the stomach and intestines and colonise there (Gupta et al., 2023).

In case of bile salt tolerance, the organism showed >80% cell viability even with the highest concentration (0.8%) of bile salt used (Fig. 3.1.(D). In a healthy human intestine, the concentration of bile salt will be 0.3%. Depending on the food that we intake, the concentration of bile salt varies in the intestine (dos Santos et al., 2020) and hence a probiotic strain should be resistant to the varying bile concentration to survive in the intestine. Additionally, the culture demonstrated greater than 60% cell viability and salt tolerance up to 8% (Fig. 3.1.(E)).

Cell surface hydrophobicity (CSH) of the culture with ethyl acetate was found to be 45.3% and for hexane it was 34.9% (Fig. 3.1.(F)). In order to overcome the surface charges and to adhere on to the walls of the tissues probiotic strains should have predominant CSH. It has been established that the findings of the other researchers are comparable. The capacity for co-aggregation among *L. plantarum* and *L. delbrueckii* ssp. *bulgaricus* strains varies depending on the strain. The high hydrophobicity of the probiotic strains indicates greater interaction with the gastrointestinal tract's epithelial cells, which suggests improved pathogen exclusion (Hoxha et al., 2023).

The clear zones or halos around the *L. plantarum* BR2 on MRS agar plates supplemented with skim milk depicts its positive proteolytic activity. The organism breaks down the proteins into smaller polypeptides or amino acids thus producing a halo around it Fig.3.1. (G).

Table 3.1 and Fig 3.1.(H) depicts the antimicrobial activity of *L. plantarum* BR2. Five different bacterial strains, including *S. aureus* MTCC 96, *Listeria monocytogenes* NCIM 5277, *S. enterica* NCIM 5256, *E. coli* MTCC 443 and *Fusarium monileforme* NCIM 1100, are all effectively inhibited by *L. plantarum* BR2 thus exhibiting its antimicrobial activity. It showed maximum zone of inhibition against *S. aureus* (2.1 ± 0.1 cm), *L. monocytogens* (2.85 ± 0.05 cm). In similar studies conducted with another strains of probiotics cultures of *L. plantarum* has been reported to have efficient antibacterial activity against broad spectrum of bacteria including clinical isolates *S. aureus* and *E. coli via* direct cell competitive exclusion as well as production of acids or bacteriocin like inhibitors. The overall probiotic characteristics of the isolate are depicted in Fig. 3.1.

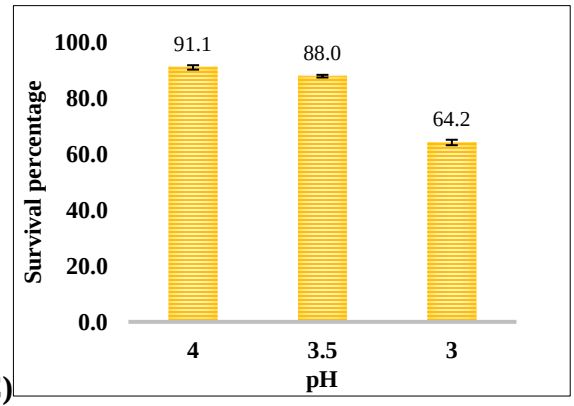
Table. 3.1. Antimicrobial Activity of *L. plantarum* BR2

Indicator strains	***Zone inhibition Diameter (cm)***
Staphylococcus aureus MTCC 96	2.1± 0.1
Escherichia coli MTCC 443	1.65 ± 0.05
Salmonella enterica NCIM5256	1.7 ± 0.05
Listeria monocytogenes NCIM 5277	2.85 ± 0.05
Fusarium monileforme NCIM 1100	1.3 ± 0.1

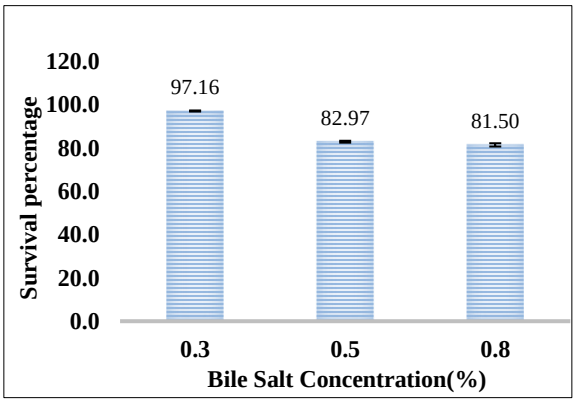
(A)

(B)

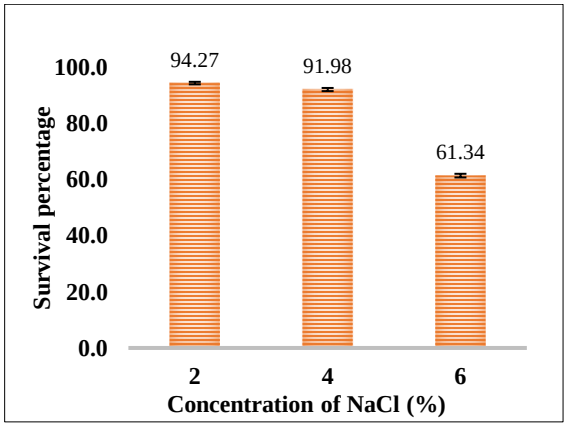
(C)


Survival percentage
100.0
80.0
60.0
40.0
20.0
0.0
91.1
88.0
64.2
4
3.5
3
pH

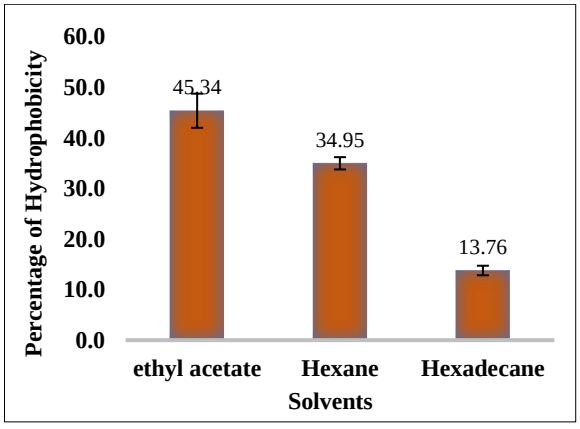
(D)


Survival percentage
120.0
100.0
80.0
60.0
40.0
20.0
0.0
97.16
82.97
81.50
0.3
0.5
0.8
Bile Salt Concentration(%)

(E)


Survival percentage
100.0
80.0
60.0
40.0
20.0
0.0
94.27
91.98
61.34
2
4
6
Concentration of NaCl (%)

(F)


Percentage of Hydrophobicity
60.0
50.0
40.0
30.0
20.0
10.0
0.0
45.34
34.95
13.76
ethyl acetate
Hexane
Hexadecane
Solvents

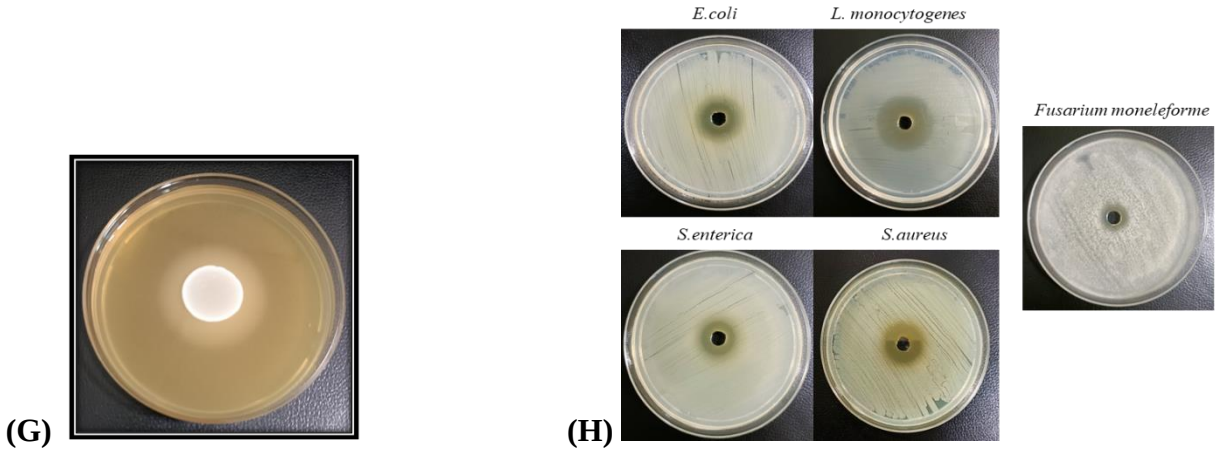
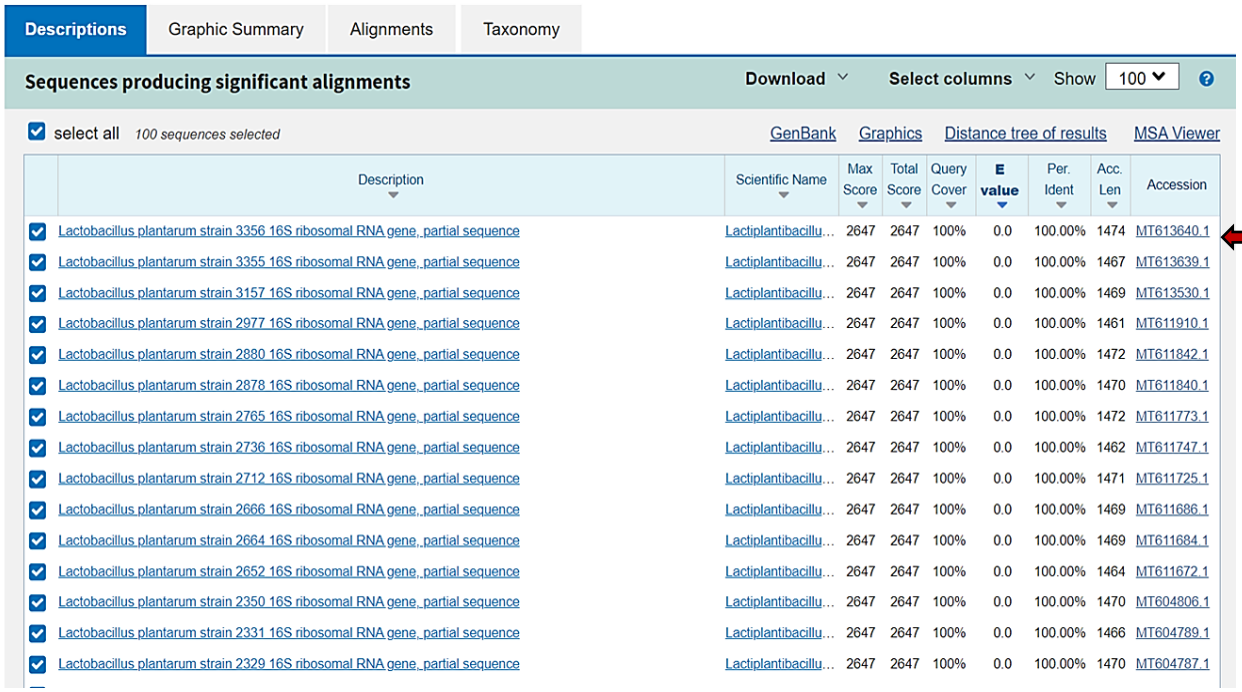


Fig. 3.1. Probiotic Characterization of *L. plantarum* BR2

(A) EPS producing *L. plantarum* BR2 on ruthenium red MRS Agar plates with ropy white colonies (**B)** *L. plantarum* BR2 being homofermentative shows no gas production inside Durham's tube but the colour of the broth was changed from green to yellow after 16 hours indicating the production of acid **(C)** pH tolerance **(D)** Bile salt tolerance **(E)** NaCl Tolerance **(F)** Cell Surface Hydrophobicity (**G)** Proteolytic activity **(H)** Antimicrobial activity of *L. plantarum* BR2 against different microbes.

The 16S rRNA sequence of *L. plantarum* BR2, was submitted in the NCBI database and obtained the NCBI Accession Number as MN176402 and the sequence comparison showed >98% similarity with the existing 16S rRNA sequences of *L. plantarum* and is showed in Fig 3.2.

(A)



(B)

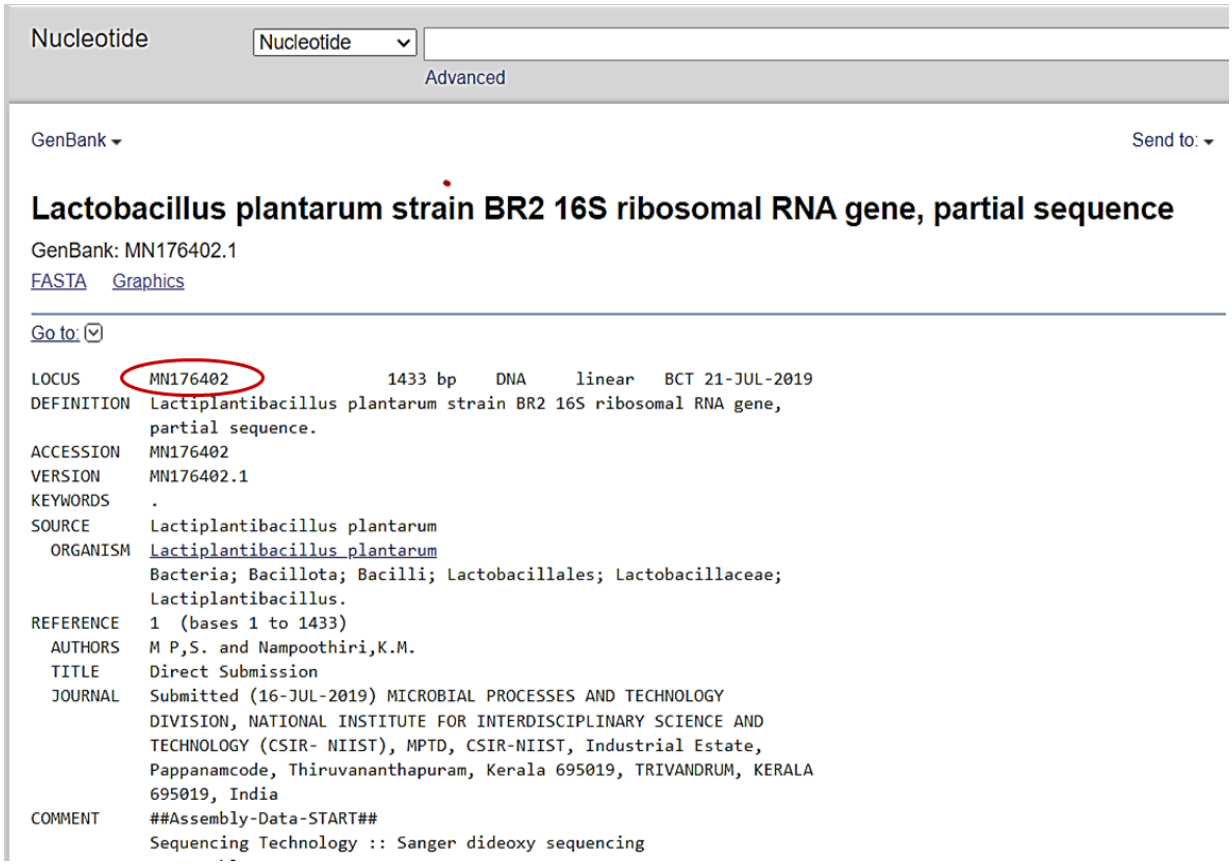


Fig. 3.2. (A) *L. plantarum* BR2 16s rRNA sequence BLAST and (B) Sequence submission in NCBI database

3.3.2. Exopolysaccharide Production by L. plantarum BR2

3.3.2.1. Optimization of EPS Production Conditions

From the previous studies by our research group (Ismail and Nampoothiri, 2010; Sasikumar et al., 2017) on exopolysaccharides, it was observed that lactose served as a best C-source for Lactobacillus EPS production. Here we attempted to optimize the lactose concentration in the production medium with different concentrations of lactose (1%, 2%, 35 and 4%) at different incubation time intervals (24, 48 and 72 hours) for fermentation by *L. plantarum* BR2 (Fig. 3.3.(A)) gave highest EPS production with 4% lactose after 72 hours of incubation.

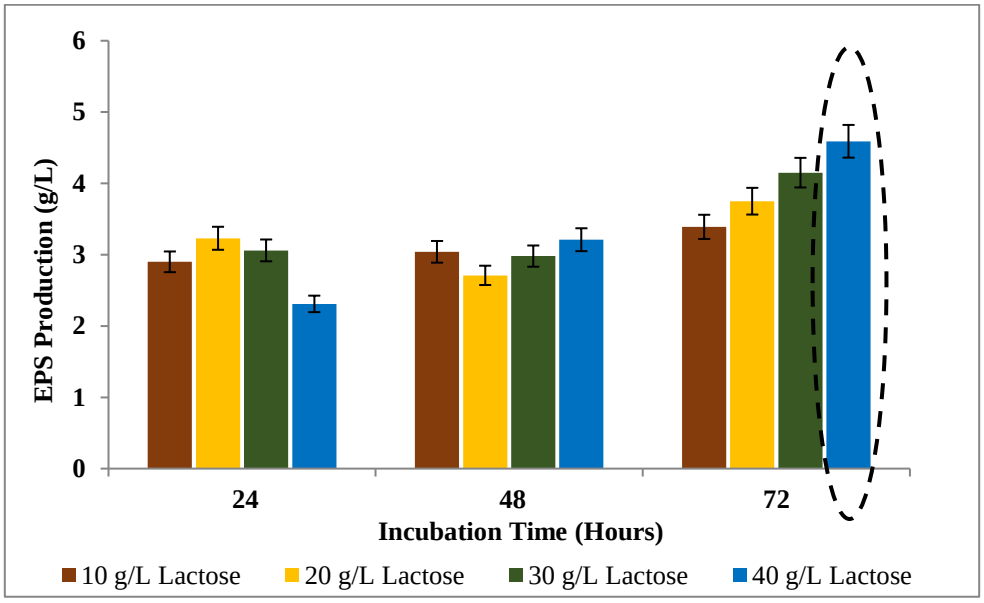


Fig. 3.3. (A) Optimization of lactose concentration and incubation time for EPS production by *L. plantarum* BR2

The lactose utilization by the bacteria during EPS production is illustrated in Fig.3.3.(B). It shows that at a lower lactose concentration (10g/L), there is complete utilization of the sugar in the medium. With increasing lactose concentration, the residual sugar left over in the medium after 72 hours of incubation also increased. A maximum EPS production of 4.5±0.5 g/L was obtained and in the fermented broth, 11±0.5 g/L residual sugar was observed at the end of 72

hours indicating that 73% of the total lactose was consumed by the organism during fermentation.

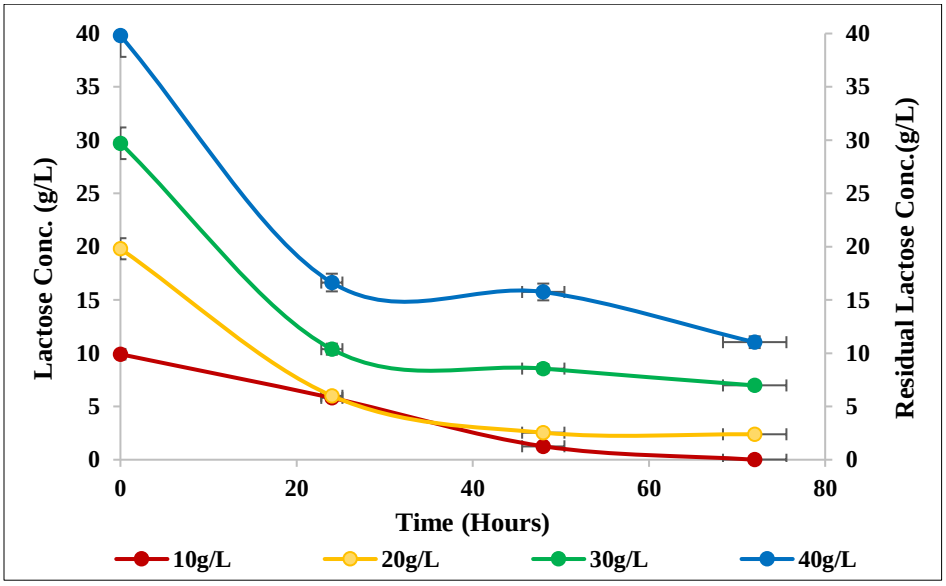


Fig. 3.3. (B) Lactose utilization by *L. plantarum* BR2 during EPS production

The EPS production and sugar utilization were analyzed under static and shaking incubation conditions and the results showed that there were no significant variations in the EPS production with a change in incubation conditions. Under both the incubation conditions provided, the growth and lactose utilization followed a similar pattern and is depicted in Fig.3.4. The growth of organism followed an initial lag phase at 0-1 hour of incubation and picked up its growth and entered to its log phase from 2 hours – 12 hours, followed by a stationary phase with a viable cell count of 9 log CFU/mL in both shaking and static incubation conditions. The sugar utilization pattern in both the incubation conditions remained same, attaining 70-73% of total sugar utilization at the end of fermentation. As the organism can grow, utilize the sugars, and produce EPS in both conditions, for further EPS production with *L. plantarum* BR2, static incubation conditions at 37°C were chosen since it is more feasible and energy efficient.

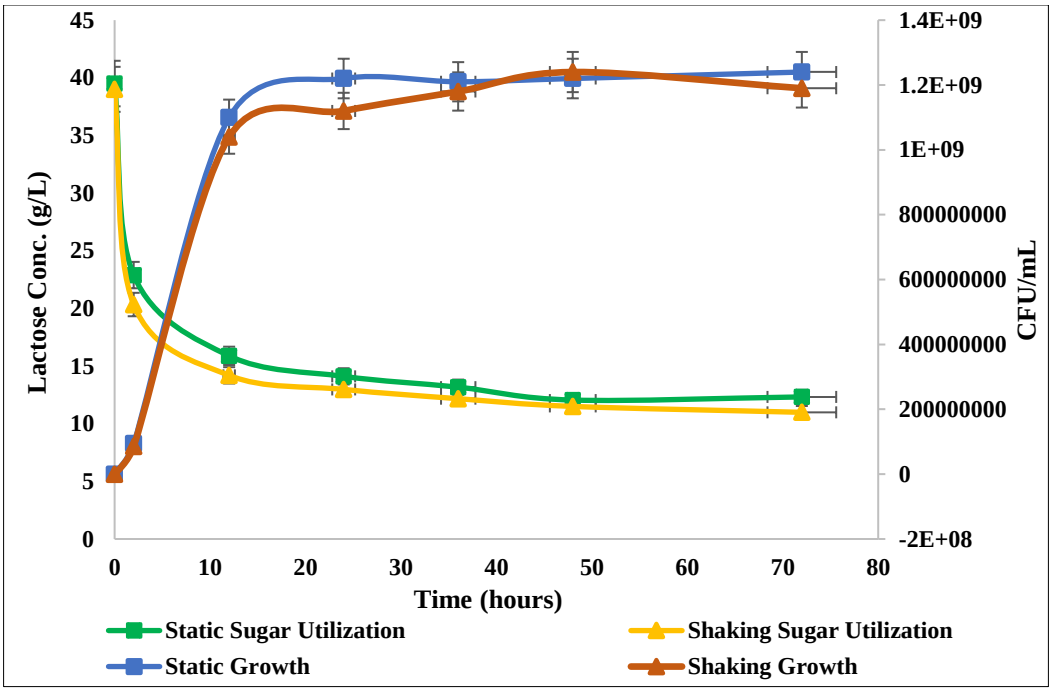


Fig. 3.4. Growth and sugar utilization of *L. plantarum* BR2 under static and shaking incubation conditions

3.3.3. Extraction and Purification of EPS

The scale-up of EPS production was done in glass fermenter having 1 L working volume with the optimized lactose concentration and incubation conditions. The EPS extraction and purification steps have been depicted in Fig.3.5. After the first step ethanol precipitation, lyophilized EPS was weighed and was observed to be 4.59 ±0.5g/L. Further purification using phenol:chloroform:isoamyl alcohol and acetone and lyophilization gave a recovery of 2.8±0.5 g/L of purified EPS. Ethanol being an antisolvent, addition of adequate amount of chilled ethanol to cell free culture supernatant disrupts the screening of charges by water and thus the dissolved EPS gets precipitated. Additionally, studies indicate that when employing ethanol for purification, just one polymer precipitates, most likely the polymer with the highest molecular weight (Ziadi et al., 2018).

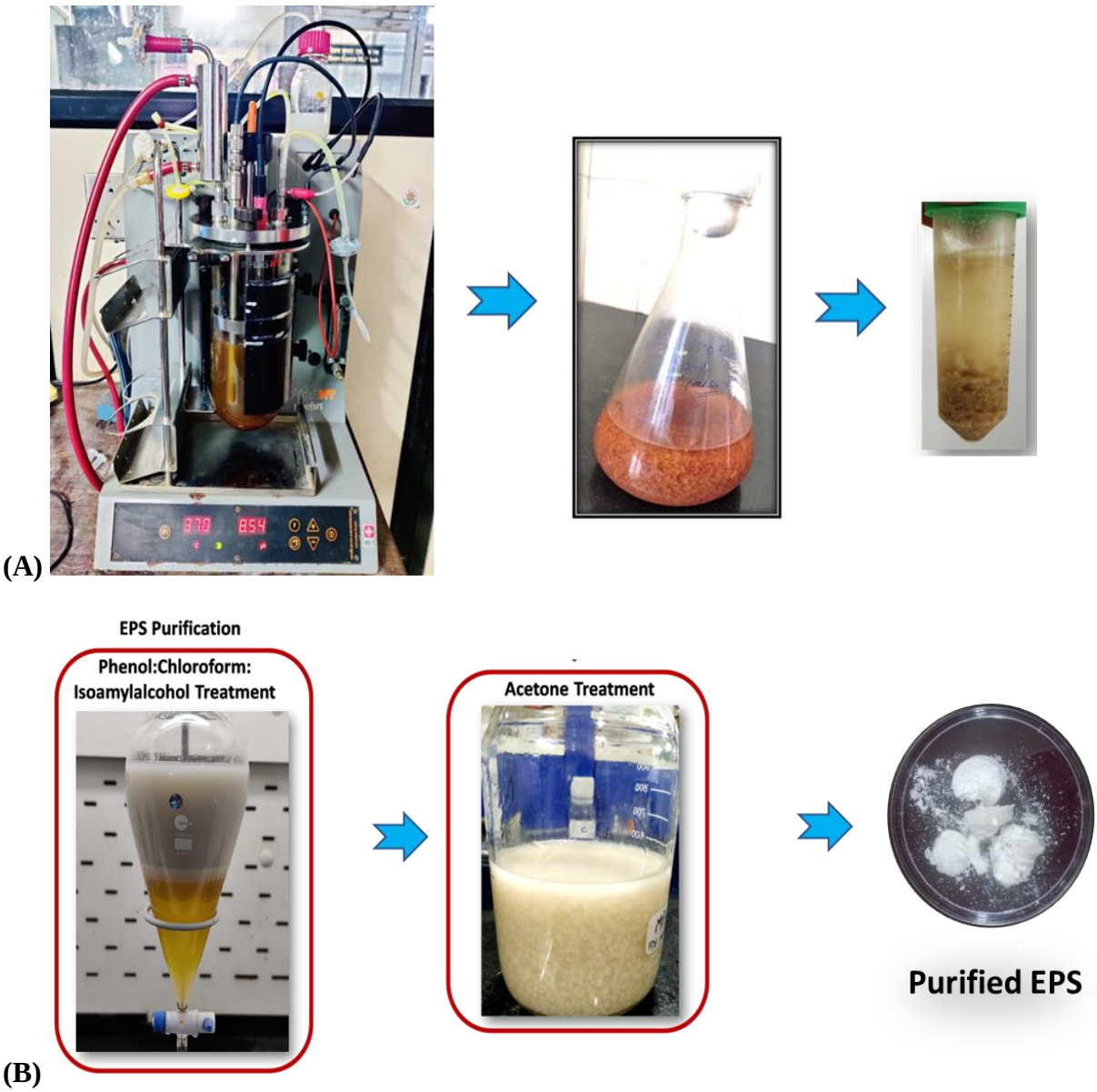


Fig. 3.5. Recovery, extraction and purification of *L. plantarum* BR2 EPS

(A) EPS production in fermenter and ethanol precipitation of EPS after incubation

(B) two-step EPS purification and recovery of lyophilized EPS

The nucleic acids and protein content quantified using a UV-spectrophotometer, ND1000, Nanodrop instrument, (USA) after the initial ethanol precipitation was observed to be 1523.9 ± 0.047 ng/µL and 16 ± 0.05 mg/mL respectively. Further treatment with phenol:chloroform:isoamyl alcohol removed the nucleic acid components from the mixture

removing the impurities. In the second step, EPS precipitated using chilled acetone aids in the removal of other impurities. There was a decrease of 75% in the nucleic acid and protein contents after this stage suggesting the removal of these impurities. The concentrated EPS extracted by centrifugation and redissolution in water upon dialysis removes salts and smaller molecules. This process traps only the high molecular EPS, enabling purification.

The fermenter scale production of EPS showed that the production of EPS made the broth viscous and there by effective aeration and agitation becomes slightly difficult. Agitation can maintain the effective oxygen transfer throughout the medium but inappropriate speed of agitation can results in poor oxygen transfer (Bandaiphet and Prasertsan, 2006). Production can be significantly decreased by heterogeneous mixing, heat transfer to the broth, and cell rupturing that can occur when the agitation speed is increased. Thus, we maintain the lowest possible agitation speed and aeration during scale-up.

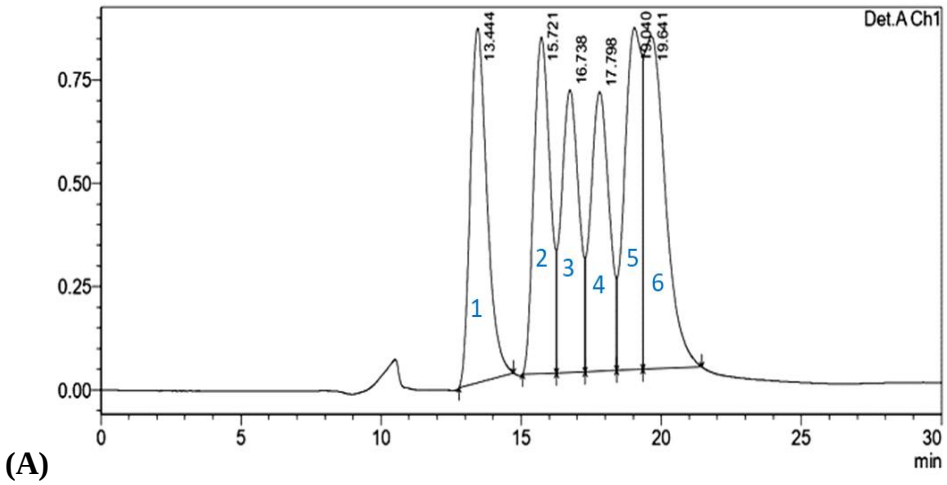
3.3.4. Characterization of EPS

3.3.4.1. Monosaccharide composition

The monosaccharide composition of the EPS backbone was determined by acid hydrolysis of the purified EPS followed by HPLC analysis. The results were then compared with the chromatogram of the standard sugars (represented in Fig 3.6.(A). The HPLC chromatogram of *L. plantarum* BR2 gives heterogenous peak of glucose and mannose when hydrolyzed and this signifies that the backbone of exopolysaccharides consists of glucose and mannose as monomeric units (Fig.3.6.(B)).

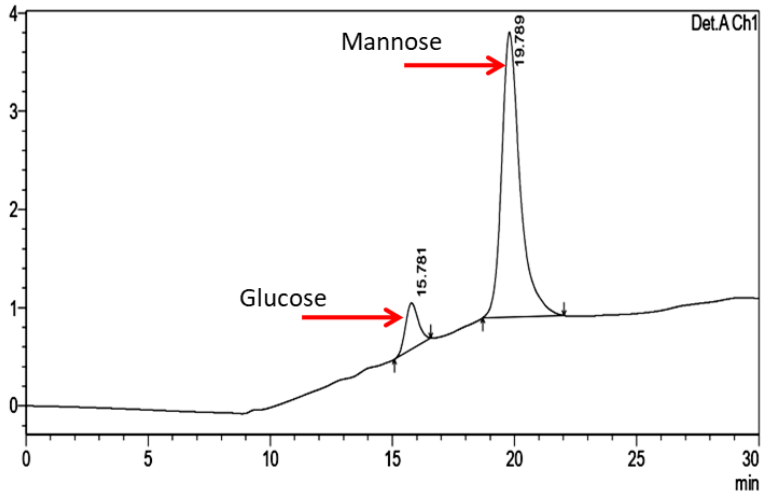
The monosaccharide determination is necessary to understand the functional properties and to categorize the EPS. Determination of monosaccharide composition by HPLC analysis of

L. plantarum BR2 EPS confirmed that the backbone of EPS contains glucose and mannose molecules, and they fall into the category of heteropolysaccharides with a glucomannan nature of EPSes.



(A)

(1) Cellobiose-13.4; **(2) Glucose**-15.7; **(3) Xylose**-16.7; **(4) Galactose**-17.7; **(5) Arabinose**-19.04; (6) **Mannose**-19.64



(B)

Fig. 3.6. HPLC Chromatogram of (A) Standard sugar mixture **(B)** *L. plantarum* BR2 EPS

3.3.4.2. Fourier transform- infrared spectroscopy

To analyze the exopolysaccharide and to identify the functional groups FT-IR spectroscopy was done. FT-IR helps to identify the structural and functional molecules of EPS

which processes specific vibrational nodes that is compared with the vibration of atoms at corresponding frequencies. FT-IR spectra of the purified EPS shown in Fig.3.7 reveals the broad stretching between 3200-3600 cm^{-1} that corresponds to stretching vibration of the hydroxyl (-OH) groups of carbohydrates. The peak around 2800-2900 cm^{-1} attributes to aliphatic C-H bond of methyl group. The absorption peak at around 1640-1700 cm^{-1}indicates the stretching vibration of C=O; considered to be the characteristic absorption peaks of polysaccharides. Stretching at 900-1200 cm^{-1} which is considered as the fingerprint region of exopolysaccharides (C-O-C/C-O) indicates the strong presence of carbohydrates. The intense peak at 1024 cm^{-1} indicated the existence of alpha-(1/6) glycosidic bond. In the anomeric region peaks between 950-700 cm^{-1} corresponds to the existence of mannose thus substantiating the glucomannan nature of the heteropolysaccharide.

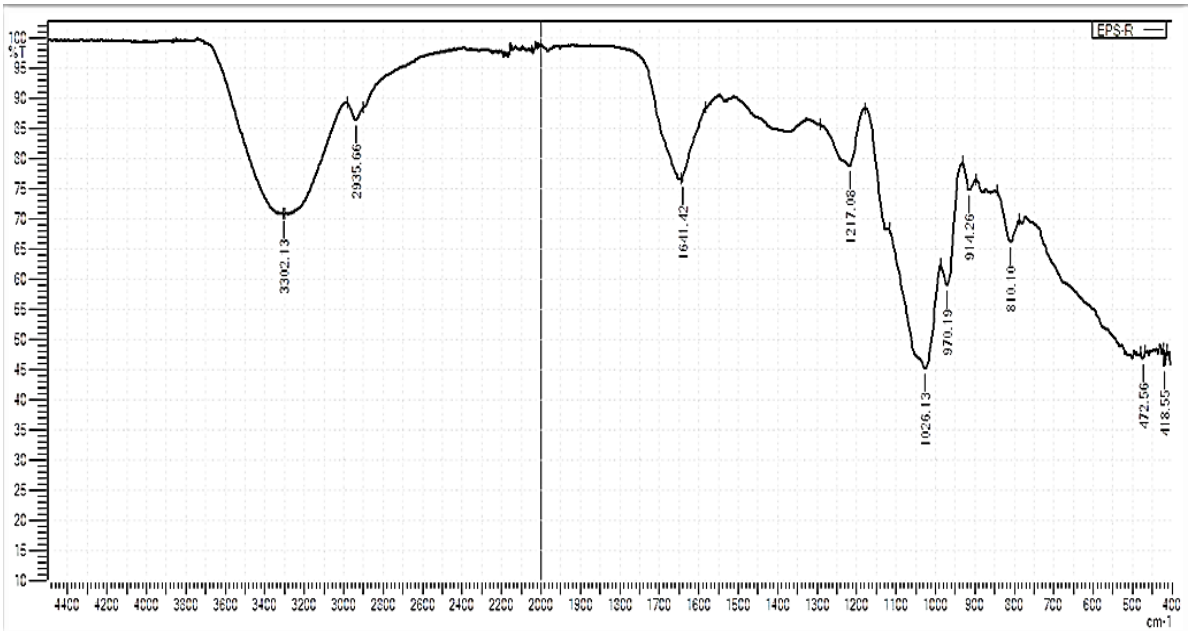


Fig. 3.7. FT-IR spectrum of *L. plantarum* BR2 EPS

3.3.4.3. Gel Permeation Chromatography (GPC)

Molecular weight of the purified EPS is determined by comparing the calibration curve plotted for commercially available dextran from Sigma-Aldrich. The molecular weight was

calculated from the plotted standard graph. Molecular weight plays a key role in solubility and rheological properties of the EPS thus contributing a lot in food applications. The weight average and the number average molecular weight of the EPS was found to be 2292 kDa and 216.5 kDa respectively as calculated from the calibration curve plotted for standard dextran EPS (Fig. 3.8). The weight average molecular weight is the ratio of the weights of all the polysaccharide chains by the total number of chains and the number average molecular weight is the total weight of polymer divided by the total number of molecules. Lower molecular weight EPS are generally more soluble as they can incorporate large amount of water and can easily form hydrogels (Elsa, 2021). This molecular weight is higher than the molecular weight of EPS produced by *L. plantarum* CIDCA 8327 that is 1000 kDa (Gangoiti et al., 2017). Determination of molecular weight has a crucial role in future applications of exopolysaccharides.

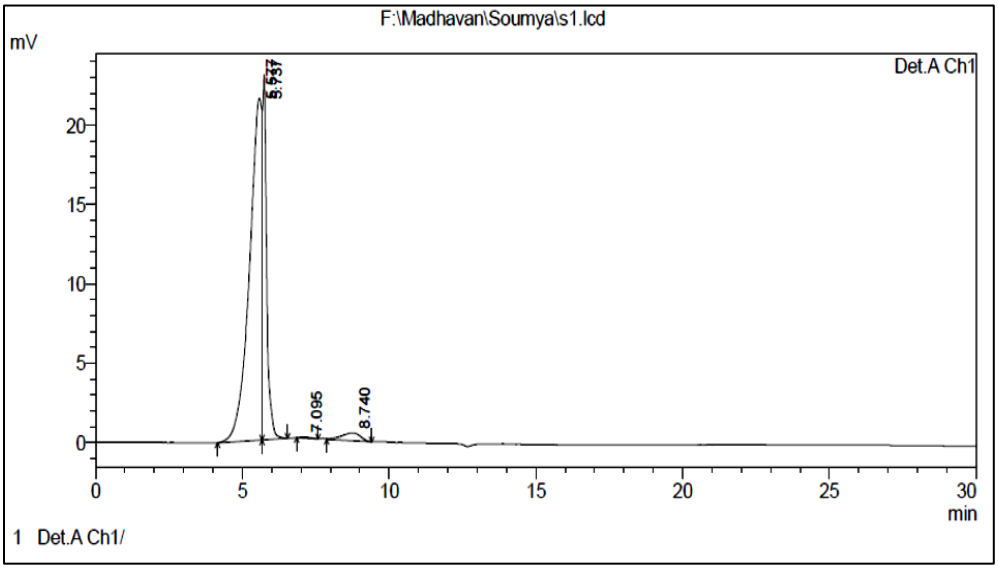


Fig. 3.8. Gel permeation chromatogram of *L. plantarum* BR2 EPS

3.3.4.4. Thermogravimetric analysis

The applicability of an EPS is crucially dependent on its thermal behavior. The temperature withstanding ability of purified EPS was studied by thermogravimetric analysis.

From Fig. 3.9. it is evident that the EPS degradation happens at two different temperature ranges. 10-15% weight loss was observed at first 30 - 75°C after that there is no significant weight loss till 260°C. From 260°C – 285°C the 50% reduction in weight is observed. The initial endothermal degradation of EPS that happened at a rate of 10-15% may be due to the loss of surface bound water from EPS (Vasanthakumari et al., 2015) and heating the polysaccharide caused moisture to escape, indicating that the EPS was not entirely anhydrous.

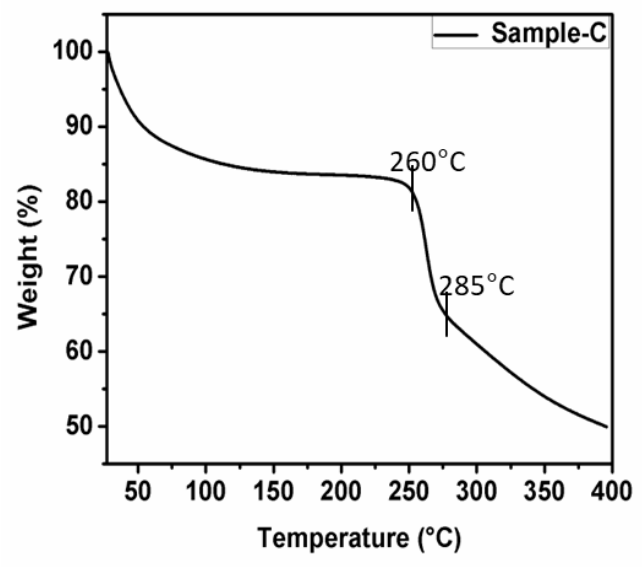


Fig. 3.9. Thermogravimetric spectrum curves of EPS

The second degradation occurred at 285–300 °C was due to degradation of the sample at higher temperature which is quite comparable to that of the previously available data. Another strain of *L. plantarum* produced an EPS with a degradation temperature of 279.59°C which is higher than that of locust gum and lower than that of xanthan gum (Xu et al., 2010). EPS from *L. plantarum* YW32 could tolerate temperature up to a high 283.5°C (J. Wang et al., 2015b). The melting point of the EPS was found to be 97.3°C and the enthalpy was obtained as 396.5 J/g by DSC analysis. The ability to withstand higher temperatures makes EPS strong enough to tolerate the processing procedures in the food industry.

3.3.4.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is a high throughput method to know the molecular characteristics of a compound. ^{1}H NMR spectrum of EPS consists of mainly three regions, anomeric regions, ring proton region and alkyl region depicted in Fig.3.10. In the spectrum, the EPS signals between 4.1 and 5.2 ppm corresponds to anomeric protons of the monosaccharides of the EPS and are signatures for differentiating complex carbohydrate structures (Ismail and Nampoothiri, 2010).

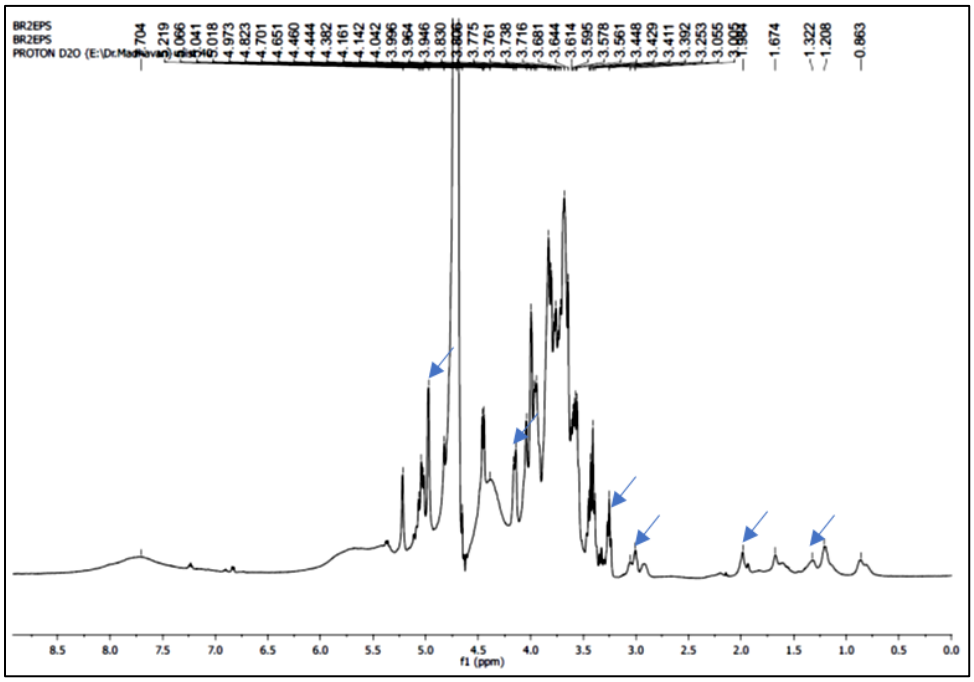


Fig. 3.10. ^{1}H NMR spectrum of *L. plantarum* BR2 EPS

The arrows represent different signals explained in the text

The anomeric proton of α-(1-3) of glycosidic linkage is indicated by a chemical shift at 5.308 ppm. The signals obtained in the spectrum between 3.2 and 4.1 ppm represent the ring proton region considered to be the fingerprint region of EPS. The signals at 1.2 - 2.1 ppm mark the alkyl region of EPS backbone and the strong signal in the spectrum at 5.04 and 4.7 ppm

corresponded to C-4 and C-5 of α-D-glucose and β-D-glucose. The chemical shift at 5.2 indicates the presence of anomeric proton of D-mannose.

3.7.6. Scanning Electron Microscopy

Scanning electron micrographs of the EPS is represented in Fig. 3.11. It shows a unique structure of EPS with a sheet-like compact morphology. A higher magnification revealed a porous web-like structure of EPS.

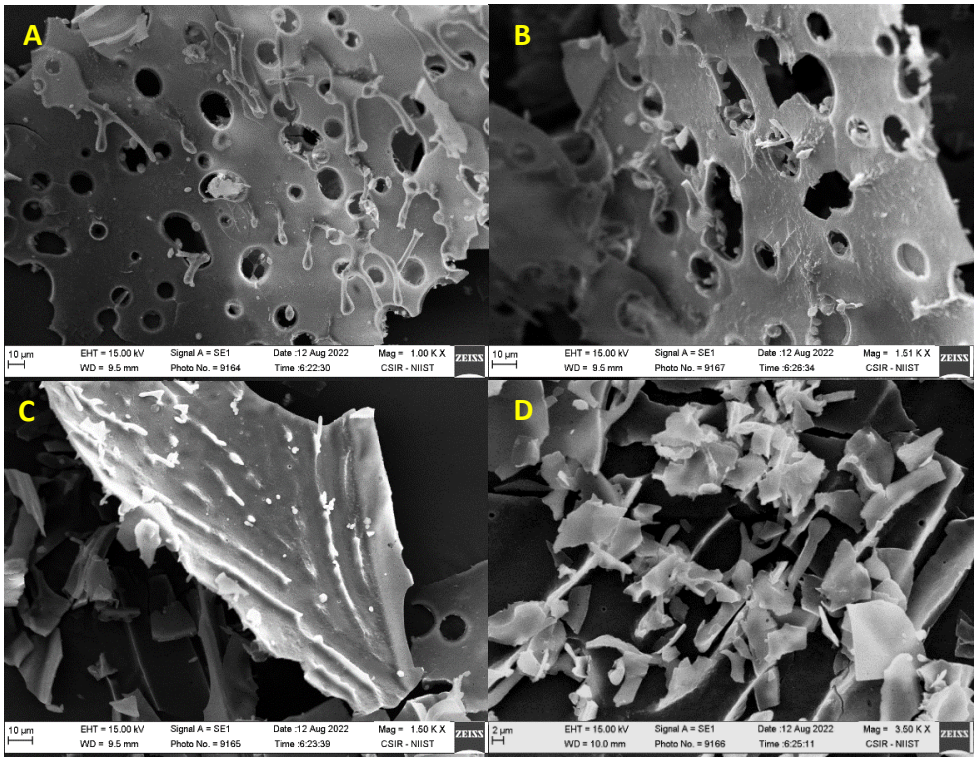


Fig. 3.11. Scanning electron micrograph showing the microstructure and surface morphology of the purified *L. plantarum* BR2 EPS under A)1000X **B)** 1500X **C)**1500X **D)** 3500X **magnifications**

This microstructure of the EPS supports its potential roles in the improvement of physicochemical properties of food products. Similarly in an earlier report, EPS produced by *Leuconostoc kimchii* strains showed hollow porous structure with a polymer matrix (Saravanan and Shetty, 2016). The difference in morphology & topography of different EPSes were caused

by the difference in sample extraction, preparation, purification & by the difference in the physicochemical properties of the EPS (Kanamarlapudi and Muddada, 2017).

3.4. Summary

L. plantarum BR2 is a homofermentative LAB exhibiting significant probiotic properties like bile salt tolerance up to 0.8%, acid tolerance up to lower pH of 3, salt tolerant up to 6% and exhibits cell surface hydrophobicity which is an important property for colonization. The culture also shows antibacterial properties and possess proteolytic activity. *L. plantarum* BR2 exhibiting these probiotic properties also produces exopolysaccharides from the optimized EPS production medium and fermentation conditions with a maximum titer of 4.5 ± 0.5 g/L with 40g/L lactose as the carbon source. Structural and physiochemical characterization of secreted exopolysaccharide reveals the glucomannan nature with a high molecular weight 2292 kDa, EPS had a high thermostability of 260°C, which can be explored in food industries as strain shows moderately good probiotic behaviour. From the FTIR spectra, absorption peak at around 1640-1700 cm^{-1} indicates the stretching vibration of C=O which are characteristic absorption peaks of polysaccharides and stretching between 900-1200 cm^{-1} is considered as the fingerprint region of exopolysaccharides (C-O-C/C-O) which indicates the presence of carbohydrates. The topological images elaborate as the potential biopolymer encompasses with a porous web-like morphology with the compact homogeneous smooth microstructure proposing it as a potent candidate with promising rheological and textural properties, which can be exploited by food industries. The water soluble, adhesive, and pseudoplastic nature of the EPS needs to be explored in the future to transform this bioprocess to the next level of research in a productive manner where it finds different applications in a sustainable manner.

Chapter 4

Chapter 4

Exploration of Alternate Carbon Sources for Exopolysaccharide Production by *Lactobacillus plantarum* BR2

4.1. Introduction

To our knowledge, there are more than 30 different lactic acid bacteria (LAB) recognized as EPS producers most of which affect the texture, stability, and rheological properties of certain fermented products. Furthermore, bacteria can produce unique EPSs with specific properties and due to this several researchers undertake the task of identifying and analysing these EPSs. With the introduction of different varieties of EPS comes the necessity of optimizing its production conditions in an economically feasible fashion. Moreover, Other EPSs such as xanthan and carrageenan are already produced at large scales thus creating the need for a competitive alternative. Fermentation optimization, exploration, and exploitation of cheaper raw materials for production and use of a biotechnological approach for a metabolically engineered strain (Y. Yu et al., 2018) would be the three main possibilities for ameliorating the large-scale production and application that pays the way for the commercial production and industrial attention of microbial polysaccharides. Streamlining to lactic acid bacteria, specifically Lactobacillus, they never compromise in their carbon and nitrogen sources as they always need a highly rich medium for their growth. Several studies demonstrate that LAB can adapt to different media and drive EPS production and molecular composition depending on the medium used. *L. delbrueckii* subsp. *bulgaricus* NCFB grown in media containing glucose or lactose produced larger amounts of EPS compared to media with fructose (De Vuyst and Degeest, 1999b). In another study by Cheng and his team, the EPS production by *L. plantarum* LPC-1 hiked when

grown in a medium with sucrose as the C-source (Cheng et al., 2019). These results highlight the possibilities to modulate EPS production, amount, structure and thus, properties of the EPS by feeding specific LAB strains with different sugars (Fuso et al., 2023). However, more relevant data are required to understand this complex relationship between LAB strain, carbon source and EPS production.

As far as any microbial fermentation is concerned, the major cost affecting factor determining the yield of a particular product is the carbon and nitrogen sources (raw material) for the microbe. They remain as the major expensive medium components for any fermentation process and hence, identification of an alternate cheaper carbon source is favoured. One of the major challenges to bridle in this area is to increase EPS production which must be remunerative and not idealistic. To make EPS production cheaper and cost-effective, EPS yield enhancement is one of the strategies that can be attained through optimized culture conditions, strain improvement, or a sustainable carbon source.

The utilization of agro residues in bioprocess technology for the production of value-added products has received greater attention in recent years. The large availability of these materials, along with their low costs, makes them alternative sources of substrates that could be used for bacterial cultivations (Coghetto et al., 2016). There are several reports where many agro-industrial wastes like potato starch, sweet potato waste, cashew juice, mature coconut water, maize, beet molasses, peat hydrolysate, deproteinized whey, molasses, brewery wastes, olive oil waste effluents, wheat and rice bran are used as substrates for pullulan and other bacterial exopolysaccharide production (Cheirsilp et al., 2018; Göksungur et al., 2011; Hayek et al., 2019; Mladenović et al., 2018; Ogidi et al., 2020; Sakr et al., 2021; Sharmila et al., 2013).

Among these wide ranges of low-cost substrates reported so far, cassava, (*Manihot esculenta*) or Brazilian arrowroots that solemnly store starch are considerably cheap, readily available and a sustainable source of fermentable glucose syrups (Ruiz et al., 2011) and can be a potential alternative carbon source for EPS production. It also contains small amounts of calcium, phosphorous and vitamin C, but is poor in protein and other nutrients with a moisture content of 10.38% (Oladunmoye et al., 2014). The thickening properties with the ability to form homogenous viscous pastes having high purity, low cost and availability make cassava starch widely acceptable. Direct enzymatic hydrolysis of starch below gelatinization temperature is desirable considering the energy costs and effective utilization of natural resources. Starch hydrolysed by α-amylase together with glucoamylase to obtain porous starch as the final product and the use of this hydrolysate for fermentations has gained attention in recent years (Chen et al., 2011). Some work has already been reported with cassava bagasse and cassava flour to produce microbial polysaccharides by yeast-like fungus *Aureobasidium pullulans* such as pullulan and xanthan by the bacterium, *Xanthomonas campestris* (Ray and Swain, 2011). But there are less or no reports of cassava hydrolysate for EPS production from LAB strains.

Jackfruit (*Artocarpus heterophyllus* Lam) seed is another sustainable C-source which is readily available and contributes to 10-15 % of the total fruit mass having high carbohydrate and protein content. Recently, the demand for jackfruit seed powder has increased considering its nutritional and health benefits. Also, there are evidence for the successful usage of jackfruit seed powder as carbon source for the production of microbial pigments (Sharmila et al., 2013).

The aim of the work was to understand whether the variation of the carbon source supplied can affect strain growth, EPS production and its chemical structure. This chapter deals with the exploration of a reliable alternate carbon source for *L. plantarum* BR2 EPS production

in batch culture. Low-cost substrates like hydrolysates of cassava starch and jackfruit seed powder were prepared by enzymatic hydrolysis and the hydrolysate with the maximum sugar yield was further utilized for media formulation for EPS production. Preparation and optimization of the hydrolysate and single parameter production optimizations along with growth comparison of *L. plantarum* BR2 in hydrolysate medium were studied. In short, process development from flask level to fermenter scale (3L) and standardization of downstream process and characterization of the purified EPS are the major highlights of this chapter.

4.2. Materials and Methods

4.2.1. Chemical Reagents and Instrumentation

Media and other components like De Man Rogosa Sharpe agar and broth (MRSA, MRSB), were purchased from Hi-Media, India and have been described in detail in Annexure I. Chemicals like sodium acetate, DNS, sodium potassium tartarate, phenol and phenol-chloroform-isoamyl alcohol were procured from Sisco Research Laboratories Pvt Ltd India. Enzymes used for saccharification were α-amylase, Termamyl 120 L, 5000 IU/mL, and glucoamylase, AMG 300, 2000 IU/mL, from Novo Industries, Bagsvared, Denmark. Ethanol, acetone, and other solvents were purchased from Spectrochem Pvt Ltd (India). Dialysis membrane and Dextran Standards were purchased from Sigma Aldrich. Cassava starch powder was purchased from local market. The instruments used in the study are stirred tank bioreactor (Minifors, Infors HT, Switzerland), FTIR spectrophotometry (Nexus-870 FT-IR, Thermo Nicolet Corporation, Madison, WI, USA), cooling centrifuge (Kubota, Korea), Lyophilizer (Christ Alpha 1-2 LD Plus, Germany), HPLC (Shimadzu, Japan), Rezex RPM Monosaccharide Pb+2 300 x7.8 mm column for carbohydrate analysis, microplate reader, Tecan Nano Quant Infinite M200 Pro, (Switzerland), spectrophotometer (UV-1601 UV Visible spectrophotometer, Shimadzu, Japan),

TGA (SII Nano-technology Inc., Japan), NMR Bruker Avance II-500 Spectrometer (Switzerland), and rotational viscometer Anton Paar ViscoQC 300 (Europe), SEM (Zeiss EVO 18 cryo-SEM, Germany).

4.2.2. Hydrolysis of cassava starch and jackfruit seed powder

Cassava starch and jackfruit seed powder slurries were prepared separately in distilled water and was gelatinized by autoclaving at 121°C at 15 lbs. pressure for 15 minutes after adjusting the pH to 4.8-5. Enzymatic hydrolysis was done with α-amylase and reaction was carried out at 90°C shaking water-bath (120-150 rpm) for 40 minutes. The resulting solution was then saccharified with a glucoamylase at 46°C in a shaking (120-150 rpm) water bath for 60 minutes. The slurry was then centrifuged at 8000 rpm for 20 minutes at 4°C. The resulting supernatant was filtered using a muslin cloth and the final glucose concentration of the hydrolysate was determined by HPLC analysis using Rezex RPM Monosaccharide Pb+2 300 x 7.8 mm column with degassed MilliQ water with a flow rate of 0.6mL/min as mobile phase and an oven temperature of 80°C.

4.2.3. Production, Recovery and Purification of EPS

Production medium, already reported in Chapter 3 (3.2.4), was used initially where the lactose 40 g/L was replaced with cassava hydrolysate with equal sugar concentration. After inoculation, (10^9 CFU/mL) the flasks were incubated for 72 h, under static conditions, at 37°C. After collecting the culture supernatant, the EPS was precipitated by addition of double volume chilled ethanol and stored at 4 ºC overnight. Precipitated EPS was recovered by centrifugation at 2500g for 20 min at 4 ºC. This was then lyophilized and analyzed. Purification of the recovered EPS was performed according to Dabour and Lapointe (2005) and the purified EPS was lyophilized and stored at room temperature.

After flask level optimization studies, the fermentation was carried out in 2.5 L fermenter (Infors HT, Switzerland) keeping intact all the parameters like incubation temperature of 37°C, an initial pH of 7.3, aeration rate of 0.1vvm, and impeller speed of 100 rpm (Bandaiphet and Prasertsan, 2006; Wang et al., 2019). The EPS produced was then extracted and purified further.

4.2.4. Growth and sugar consumption in hydrolysate medium

Overnight grown culture with a minimum OD_{600} of 0.8 (6.5 x 10^8 CFU/mL) was inoculated to 100 mL of cassava hydrolysate-based medium and incubated at 37°C under static flask conditions. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm and viability of *L. plantarum* BR2 was enumerated by using the spread plate method. Serial dilutions of the fermented broth taken at different growth phases (time intervals) were prepared up to 10^8 using sterile distilled water under aseptic conditions. Required dilutions (10^4 ,10^5 ,10^6 and, 10^7) were spotted on the MRS agar plates and kept undisturbed for drying. All plates were incubated at 37°C for 24 hours and the viable cell count was calculated as follows:

$$\text{CFU/mL} = \frac{\text{Number of colonies formed X dilution factor of sample}}{\text{1 mL of sample}}$$

The sugar utilization was recorded at desired time intervals by subjecting the culture supernatant to HPLC analysis with degassed milliQ water as the mobile phase with a flow rate of 0.6 mL/min, and RPM monosaccharide column detected using RID detector.

4.2.5. Optimization of Different EPS Production Conditions

To optimize the sugar concentration in the hydrolysate medium, different volumes of cassava hydrolysate was used to get sugar levels of 10, 20, 40, 80 and 100 g/L and the corresponding production titre was checked. Also, to study the efficacy of nitrogen sources, different organic nitrogen sources (meat extract, peptone, tryptone and yeast extract (control), 40

g/L) and inorganic nitrogen sources (sodium nitrate, ammonium nitrate, ammonium dihydrogen orthophosphate and ammonium sulphate (control), 5.5 g/L) were used independently and checked the EPS production. Similarly, optimum pH also set after checking a wide range of initial medium pH (4.5, 5.5, 6.5, 7.3, and 8.5). Tween 80, a surfactant, with different concentrations (w/v %, 0.1, 0.4, 0.5, 0.8 and 1) was provided in the cassava hydrolysate medium to see its effect in EPS production. Also, optimized the inoculum percentage after studying a range (v/v %, 0.5, 1, 2, 3 and 4) of inoculum. In order to study the effect of hydrogen peroxide and calcium chloride in EPS production, *L. plantarum* BR2 was cultured in a medium supplemented with a) 3mM H_2O_2 b) 10mM $CaCl_2$ and c) 3mM H_2O_2 and 10mM $CaCl_2$ and the one without any of these supplementations served as control.

4.2.6. Characterization of Exopolysaccharides

4.2.6.1. Monosaccharide Analysis

30mg/mL of the purified EPS (CSH EPS and Control EPS) dissolved in 1M H_2SO_4 in round bottom flasks under nitrogen environment and were kept in boiling oil bath (100°C) for 2-3 hours. The samples were neutralized to pH between 5.5-6.5 and centrifuged which was further syringe filtered using 0.22µM filter and was analyzed by HPLC along with the standard sugars with degassed milliQ water as the mobile phase with a flow rate of 0.6 mL/min, and Rezex RPM monosaccharide Pb+2 300 x7.8 mm column detected using RID detector (Ismail and Nampoothiri, 2010).

4.2.6.2. FTIR Analysis

The purified EPS samples from cassava hydrolysate medium and the control medium (lactose as carbon source) were analyzed by Fourier transformed infrared (FTIR) spectroscopy

for the identification of functional groups as a preliminary conformation. 1mg of the finely ground EPS samples were directly used for FT-IR measurement in the frequency range of 4400-400 $cm^{-1,}$ at a resolution of 4 cm^{-1}.

4.2.6.3. Thermogravimetric Analysis

The thermal stability of the EPS from cassava hydrolysate was determined by thermogravimetric analysis (TGA). 20mg of the purified EPS was subjected to a temperature range of 25- 400°C at a rate of 10°C/min.

4.2.6.4. Molecular Weight Determination

The molecular mass of the purified EPS was determined by gel permeation chromatography (GPC) with 15mg/mL of the sample dissolved in deionized water. Analysis was performed with Shodex SB-804 GPC column with a RI detector. Mobile phase used was deionized water with a flow rate of 1mL/min.

4.3. Results and Discussion

4.3.1. Hydrolysis of cassava starch and jackfruit seed powder

Cassava starch powder is insoluble in water and is difficult to hydrolyze under mild conditions (Ruiz et al., 2011). Direct enzymatic hydrolysis of cassava starch at a temperature below the gelatinization temperature is the most preferable method while considering the energy prerequisite and for the complete usage and conversion of the raw material (Chen et al., 2011) into utilizable sugar slurry that can straight away be used in the media as an alternate carbon source. The cassava starch hydrolysate was golden brownish in color with viscosity depending on the concentration of the sugar released. Lower the sugar released, the higher the viscosity due to the presence of the unhydrolyzed/partially hydrolyzed starch remnants (Fig. 4.1).

The HPLC analysis of the hydrolysates prepared showed prominent glucose peaks along with maltodextrin and maltose peaks. The concentration of glucose, maltose, cellobiose, maltodextrin, maltooligosaccharides in the hydrolysate slurry depends on the efficiency of the cassava starch hydrolysis. Optimum biomass load for the enzymes resulted in comparatively higher glucose release with minimal amount of the partially hydrolyzed starch residues.

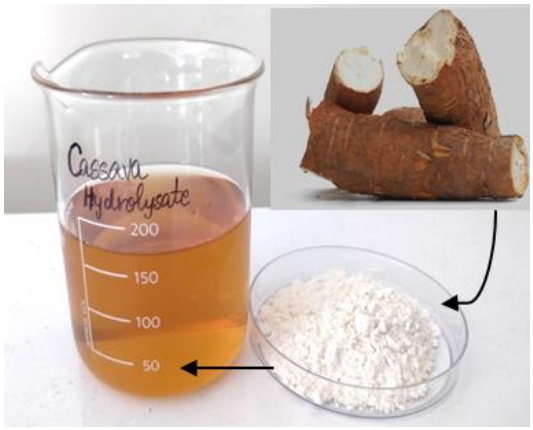


Fig. 4.1. Cassava starch hydrolysate

From the HPLC analysis of jackfruit seed hydrolysate it was evident that it gave lower glucose levels upon hydrolysis. The percentage conversion of starch to glucose was only 36% from 9% of jackfruit seed powder which is very minimal considering it as a source of carbon for fermentation purposes. Recent reports state that jackfruit seed offers a comparatively high amount of resistant starch and hence the availability of digestible sugars is minimal (Charoen et al., 2020; Kittipongpatana and Kittipongpatana, 2015). That being the case here, cassava starch hydrolysate was chosen over jackfruit seed powder hydrolysate for the future studies and EPS production.

Of the various concentrations (6, 7.5, 9, 10 and 15 % w/v) of the cassava starch hydrolyzed, 9% cassava substrate gave a hydrolysate with 76.7 g/L sugar equivalent to a conversion of 85 % (Table 4.1).

In a similar study conducted by Wu et al., (2018), they hydrolysed the raw cassava starch waste to produce curdlan from *Alcaligenes faecalis* ATCC 31749 using a thermostable α-amylase from *Thermococcus* sp. HJ21. Upon hydrolysis they yielded three hydrolysates with different polymerizations, namely CSWHs-1,CSWHs-2 and CSWHs-3 and they reported the highest yield of curdlan production with CSWHs-2 which contained glucose, maltose, isomaltose, maltotriose and traces of other maltooligosaccharides detected by HPLC and presented a polymerization degree of 4–7. The bacteria growing in a hydrolysate medium might be capable of utilizing these sugar mixtures more efficiently and this strongly suggests the reason for higher EPS production from cassava hydrolysate than from solid sugars like glucose or lactose as the sole C-source (Wu et al., 2018).

Table. 4.1. Percentage conversion of different concentrations of cassava starch to glucose after enzymatic hydrolysis

Cassava Starch (%)	*Glucose Conc. (g/L)*	*Starch to Glucose Conversion (%)*
6.0	47.5	79.1
7. 5	61.8	82.4
9.0	**76.7**	**85.2**
10.0	79.1	79.1
15.0	112.8	75.2

4.3.2. Growth comparison and optimization of culture conditions

The time course study for growth and sugar utilization conveys that the *L. plantarum* BR2 can effectively utilize the sugars in the cassava hydrolysate medium (Fig. 4.2). The growth of the bacteria in the lactose control media and the cassava hydrolysate media when compered

showed similar growth and cell count with 8.88±0.04 and 8.93±0.02 log CFU/mL, respectively, at the middle of the exponential phase of growth.

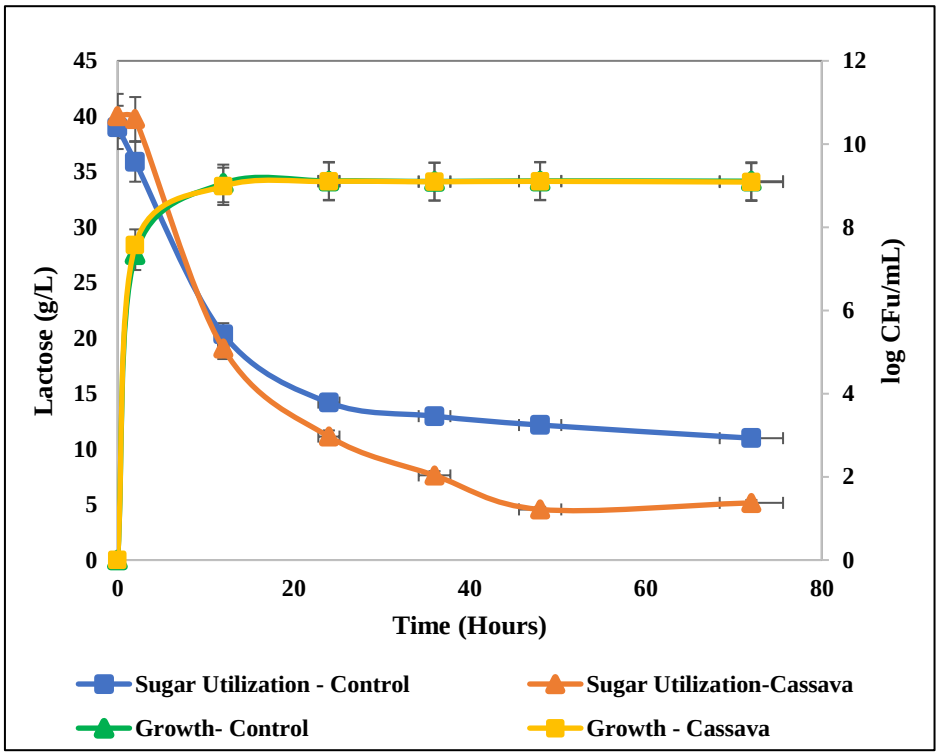


Fig. 4.2. Growth and sugar consumption by *L. plantarum* BR2 in cassava hydrolysate medium

At the end of fermentation in 72 hours, the cell count reached a maximum of 9.10±0.05 and 9.08±0.08 log CFU/mL in lactose and cassava media, respectively. The growth and cell count in the media is also supported by the sugar utilization analysis with a residual sugar concentration of 15-20% of the total sugar provided in the medium. This is in favor with the earlier data shown by the bacteria in lactose control medium. From the experimental data, the optimum sugar concentration of the hydrolysate was found to be 40 g/L. At higher cassava hydrolysate concentrations (>8%), a significant decrease in the EPS production was observed with higher residual sugar in the media and hence it is not appreciated. Excessive sugar presence

in the culture medium usually increases EPS production in LAB. Increased EPS synthesis under stress of high sugar concentration is explained by osmosis, unlimited supply of sugar building blocks and high energy availability. In some cases, it was also shown that the presence of excess sugar in the medium has a deteriorating effect on EPS production such as in *L. casei* and *L. rhamnosus*, as the growth is apparently decreased. Thus, under higher cassava hydrolysate concentrations, the growth of *L. plantarum* BR2 is affected thus resulting in higher residual sugar consequently decreasing the EPS production (Nguyen et al., 2020). The overall process optimization is mentioned in Table 4.2.

As reported earlier in one *L. plantarum* study (Ismail and Nampoothiri, 2010), the yeast extract and ammonium sulphate served as the best nitrogen source for *L. plantarum* BR2 as well in the cassava hydrolysate based medium. Availability of carbon and nitrogen sources highly influences the yield during fermentation. The maximum EPS production occurs at specific range of these factors.

Presence of 0.4 % Tween 80 in the medium favored the EPS production. Being a surfactant it permeabilizes the cell membranes and promotes migration of nutrients into the cells (Qi et al., 2009). pH of the medium is another factor to be considered. Optimum pH was found to be 7.3 and is in fact the actual pH of the medium and thus no pH adjustment was required. Extremely lower or higher than the normal pH of the media resulted in lower EPS production. From this we can again validate that the incubation time of 72 hours is reliable beyond which the media becomes acidic due to the lactic acid production by the bacteria itself and hence it is of no use in prolonging the fermentation time considering the EPS yields.

Table. 4.2. Process optimization for EPS production by *L. plantarum* BR2

Set Variables	*Exopolysaccharide (g/l)*				
Cassava Starch hydrolysate Conc. (Incubation time 72 h)	1% CSH	2% CSH	**4% CSH**	8% CSH	10% CSH
	3.8±0.01	4.95±0.01	**5.72±0.05**	3.81± 0.08	3.23± 0.08
Inorganic nitrogen source (Carbon source CSH: 40 g/l, Incubation time 72 h)	Sodium nitrate	Ammonium nitrate	Ammonium dihydrogen orthophosphate	**Ammonium sulphate**	
	2.92± 0.08	3.7±0.02	3.73±0.02	**5.92±0.01**	
Organic nitrogen source (Carbon source CSH: 40 g/l, Incubation time 72 h)	Meat extract	Tryptone	Peptone	**Yeast extract**	
	3.48±0.01	3.59±0.01	2.29±0.01	**5.6±0.01**	
Tween 80 (Carbon source CSH: 40 g/l, Incubation time 72 h)	0.1	**0.4**	0.5	0.8	1.0
	4.64± 0.08	**5.39± 0.08**	4.88± 0.08	5.05± 0.08	4.86± 0.08
pH (Carbon source CSH: 40 g/l, Yeast extract: 40g/l, Ammonium sulphate 5.5g/l)	4.5	5.5	6.5	**7.3**	8.5
	3.6±0.01	4.22±0.12	4.5±0.02	**5.2±0.01**	4.3±0.01
H_2O_2 and $CaCl_2$ (Carbon source CSH: 40 g/l, Yeast extract: 40 g/l, Ammonium sulphate 5.5 g/l**,** pH 7.3)	3mM H_2O_2	10mM $CaCl_2$	**3mM H_2O_2 + 10mM $CaCl_2$**	Control	
	5.11±0.04	4.68±0.05	**5.64±0.02**	4.4±0.03	
Inoculum Percentage (Carbon source CSH: 40 g/l, Yeast extract: 40 g/l, Ammonium sulphate 5.5 g/l**,** pH 7.3)	0.5%	1%	**2%**	3%	4%
	4.98±0.05	5.78±0.04	**6.79±0.02**	6.69±0.02	6.08±0.01

When the medium was supplemented with 3mM H_2O_2 and 10mM $CaCl_2$, it enhanced the EPS production as well. According to Ng and Xue et al., (2017), hydrogen peroxide and calcium chloride had a positive effect on EPS production in *L. rhamnosus* ZY (Ng and Xue, 2017). They justified the high levels of EPS production in presence of H_2O_2 and $CaCl_2$ was because the cells became more hydrophobic, which can be explained by EPS being intrinsically non-hydrophilic and thus altering the contact angle. The greater degree of hydrophobicity determined by contact angle measurements was consistent with the increase in EPS caused by calcium and hydrogen peroxide, and the tendency to store energy under stress. When the same was tried with *L. plantarum* BR2, it also showed a positive effect for a medium containing 3mM H_2O_2 and 10mM $CaCl_2$.

With the different inoculum sizes with an OD_{600} equivalent to 0.9-1.0, a maximum EPS of 6.79±0.02 g/L was obtained with 2 % inoculum (v/v). This inoculum concentration yielded the highest EPS which gradually declined at higher or lower level of these factors. Inoculum concentration has shown more influence on EPS yield as compared with nitrogen sources.

After these parameter optimizations, the EPS production was carried out with the best results obtained and the total EPS yield in lactose medium and cassava hydrolysate medium were compared and analyzed.

4.3.3. Production and purification of EPS

The double step ethanol precipitation of the fermented broth of the cassava hydrolysate medium resulted in slightly brownish crude EPS with a dry weight of 6.79 ± 0.02 g/L, while the lactose medium (control) gave only 4.4 ± 0.5 g/L of EPS. However, after the phenol: chloroform: isoamyl alcohol (25: 24: 1) treatment and acetone precipitation followed by dialysis, yields were 2.2 ± 0.5 g/L for EPS from cassava sugar and was 2.8 ± 0.5 g/L for control with lactose. This

indicates the probability of some impurities from hydrolysate medium in the initial ethanol precipitation step. What is interesting is the high EPS production with a relatively low cost alternate carbon source in comparison with other lactobacilli reports, 0.599 g/L by *L. plantarum* KX041 (Wang et al., 2017), 1.5 g/L by *L. plantarum* (Dilna et al., 2015a), 0.2 g/L from *L. paracasei* HCT (Xu et al., 2010), 0.26 g/L from *L. plantarum* YML009 (Seo et al., 2015), 0.4 g/L from *L. rhamnosus* (Madhuri and Prabhakar, 2014).

In fermenter, the production was carried out with a minimum impeller rotation of 50 rpm and the initial DO was 92 % and the production medium pH of 7.2, immediately after inoculation. Sugar utilization and growth of the bacteria was tantamount with that of the fermentation carried out in flask levels. Towards the end of fermentation, the medium pH dropped down to 3.8 and the media being more viscous and denser due to the presence of the cell biomass and the EPS produced (Fig. 4.3).

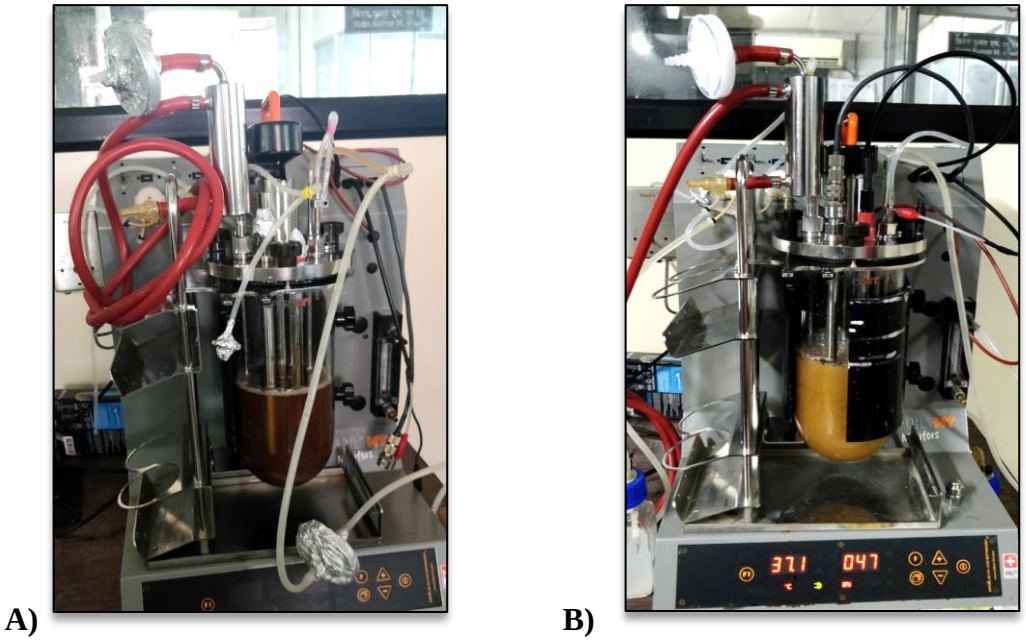


Fig. 4.3. EPS production in 2.5 L fermenter with optimized cassava hydrolysate media components A) 0th hour **B)** after 72 hours of incubation

After downstream processing, the fermenter scale production of EPS with cassava starch hydrolysate in 2.5L fermenter resulted in 6.08 ± 0.5 g/L of crude lyophilized EPS after first step ethanol precipitation. The minimal loss of EPS during extraction and downstream is inevitable for an EPS with a water-soluble nature. Even then, there is an increase in the EPS production when an alternate carbon source is used.

4.3.4. Characterization of Exopolysaccharides

The monosaccharide analysis of the cassava EPS revealed that it contains glucose and mannose as the monomers and is hence proven to be a glucomannan type EPS which is same as that of the EPS from the lactose (control) medium. This also states that the monosaccharide composition did not change with a change in the sole carbon source for EPS production (Fig. 4.4). A baseline shift was observed in all the EPS hydrolyzed samples, and it is due to the highly acidic conditions used during the process of hydrolysis.

FT-IR being a potent tool for observing structural and functional groups in EPS, the spectrum of cassava EPS exhibited many peaks in the range of 4400-400 cm^{-1}. The broad peaks between 3600-3200 cm^{-1}relates to the –OH (hydroxyl group), band around 2900-2800 cm^{-1} indicates the aliphatic C-H bond (Sasikumar et al., 2017). The peak around 1700-1600 cm^{-1} denotes the vibrational stretching of C=O group which is considered characteristic for polysaccharides. Few peaks at 950-1200 cm^{-1} corresponds to the broad stretch of C-O-C and C-O and is considered to be the fingerprint region of EPS along with the indication of α-1-4 glycosidic linkages (Zhu et al., 2019). All these data and peaks were in perfect co-relation with that of the control EPS. This revealed that there was no major change in the characteristic of the EPS produced in cassava hydrolysate medium (Fig. 4.5).

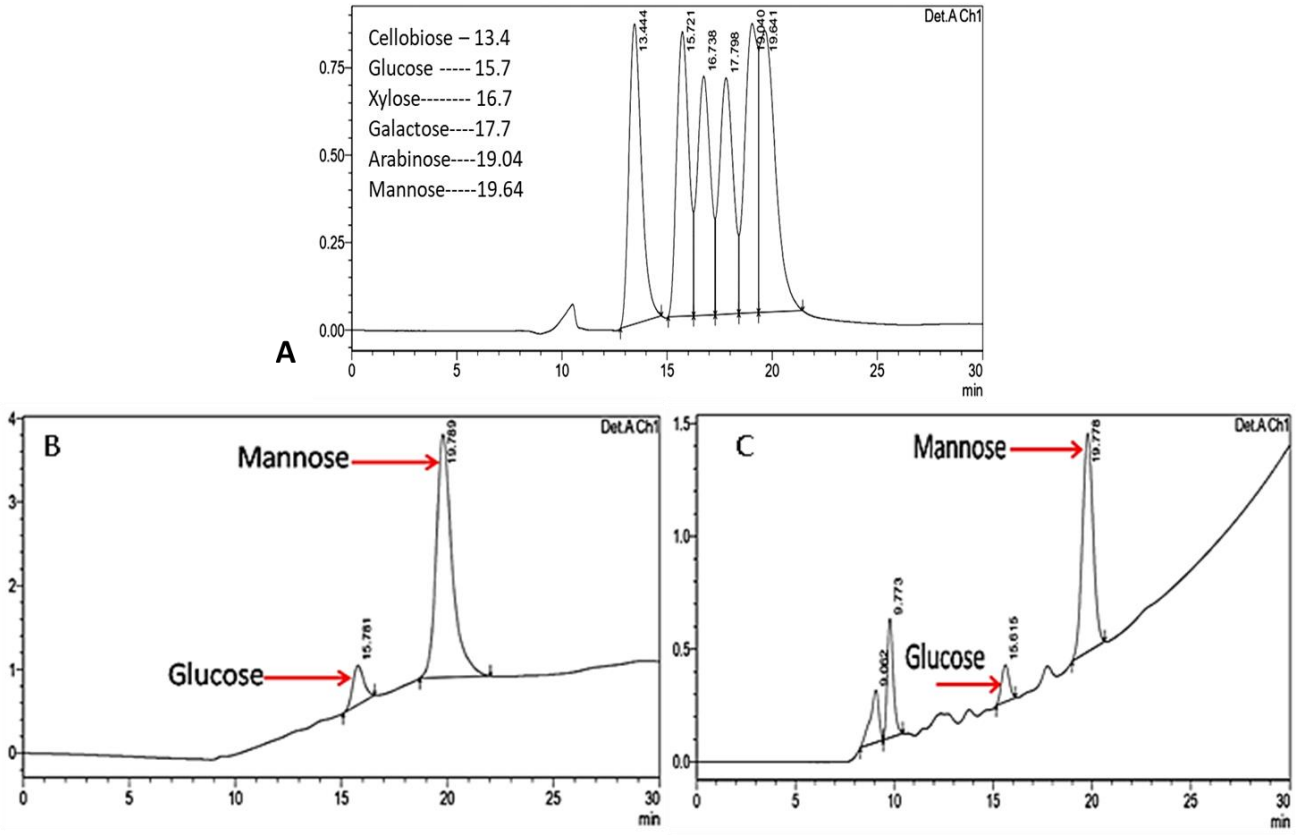


Fig. 4.4. HPLC analysis for the Monosaccharide composition of *L. plantarum* BR2

(A) Chromatogram of standard sugars **(B)** control EPS and **(C)** Cassava EPS

(Retention time – Glucose- 15.7; Mannose – 19.7)

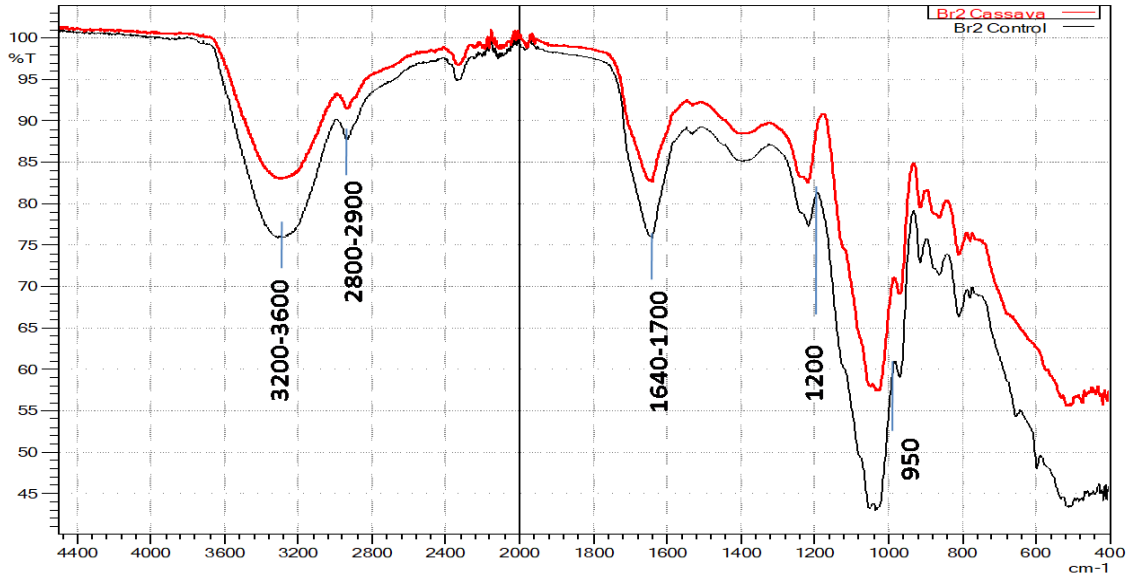


Fig. 4.5. FT-IR spectra of EPS produced by *L. plantarum* BR2 in CSH medium and in lactose medium (control)

The thermogravimetric analysis of the EPS revealed (Fig. 4.6) that degradation occurs at two stages. The first phase of decomposition occurs with a 10-14% weight loss at a temperature of 25-100°C, may be due to the moisture content in the sample. Above this temperature, weight of EPS remains constant and stable until a fast degradation takes place between 250-300°C. Beyond 300°C there is a significant decrease in weight up to a loss of 60%. Another strain of *L. plantarum* RS20D is also stable up to 250°C (Zhu et al., 2019) which is lower than that of 289.01exhibited by *L. plantarum* KX041 (Wang et al., 2017).

The ability to withstand higher temperatures makes EPS strong enough to tolerate the processing procedures in the food industry. It is safe to be used in the dairy industry at processes temperature seldom overpasses at 150 °C. The food industry benefits greatly from EPS's thermostability, particularly for enhancing rheological properties during production and processing of numerous food preparations are carried out at higher temperatures (Saravanan and Shetty, 2016).

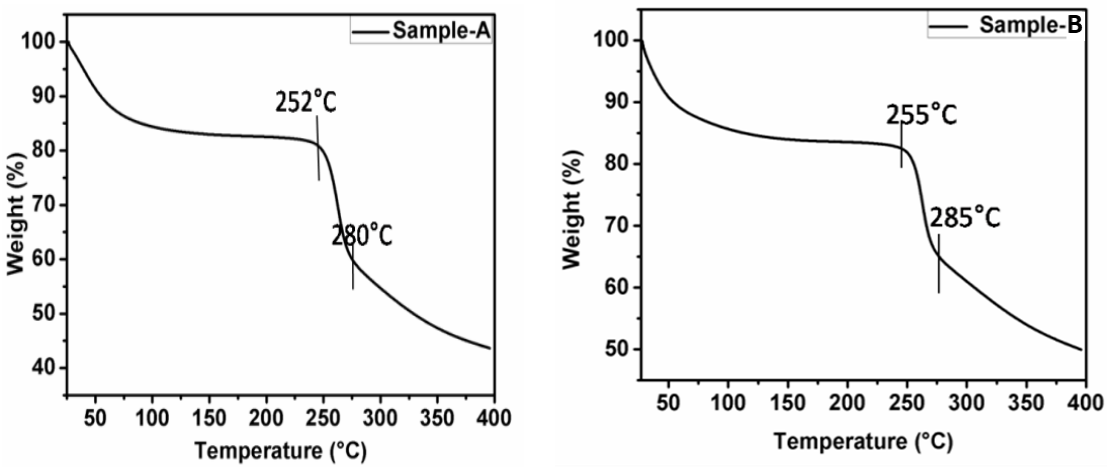


Fig. 4.6. TGA Analysis of *L. plantarum* BR2 EPS produced in (A) CSH medium and **(B)** Lactose medium (control)

From the calibration curve plotted (Fig. 4.7) for the purchased dextran standards (1000, 5000, 12000, 25000, 50000, 80000, 170000, 250000) from Sigma-Aldrich the weight average molecular weight of the cassava EPS was determined (from the corresponding retention time determined from standard, Table 4.3) to be 2200.6 kDa while that of the control lactose medium EPS was 2291.8 kDa. There were least differences in the molecular weight of the EPSes even after an alteration in the C-sources used. EPS produced by *L. plantarum* CIDCA 8327 has a molecular weight of 1000 kDa (Gangoiti et al., 2017) which is quite low in comparison to *L. plantarum* BR2. Also, with or without Se enriched EPS, the EPS from *Lactobacillus plantarum* BC-25 was found to have molecular weights of $1.83x10^4$ Da and $1.33\ x10^4$ Da, respectively (Zhou et al., 2016).

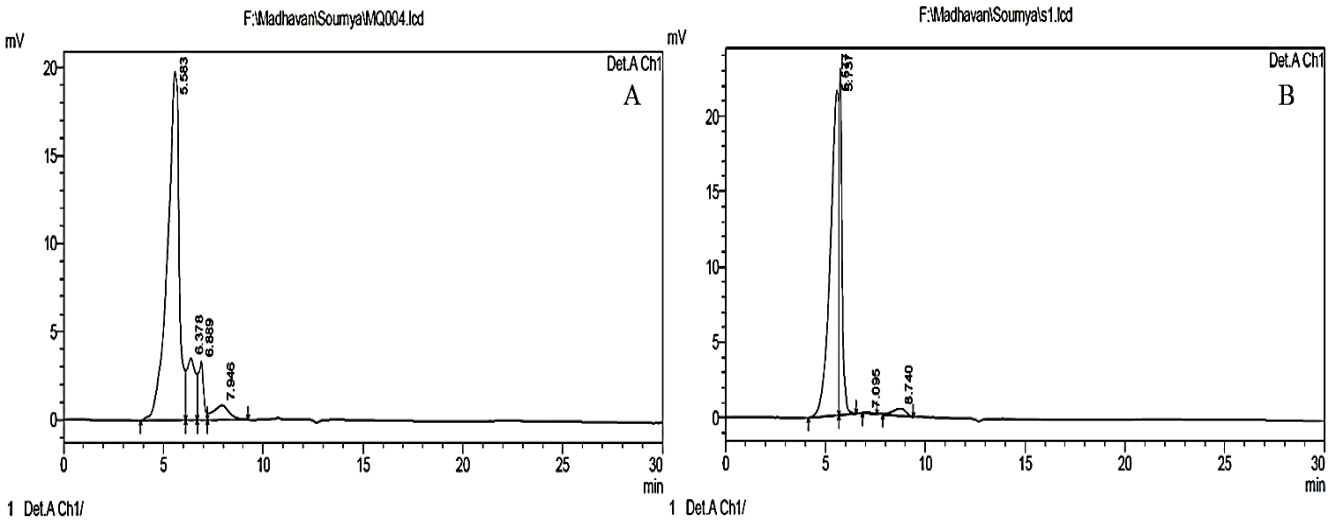


Fig. 4.7. Gel permeation chromatogram of *L. plantarum* BR2 EPS from (A) CSH medium **(B)** Lactose control medium

Table. 4.3. The retention time of dextran standards with different molecular weights

Sl. No	Retention Time (min.)	Mol. Weight (Da)
1	6.577	250000
2	6.944	170000
3	7.376	80000
4	7.698	50000
5	8.144	25000
6	8.648	12000
7	9.036	5000
8	9.665	1000

Thus, even after replacing the carbon source for EPS production, the characterization data of cassava EPS versus control EPS like the monosaccharide composition, thermal stability and molecular weight revealed that the molecular structure and properties of the EPS remains unchanged. Thus, exploiting the property of microbial fermentation of the carbohydrate contents in cassava starch hydrolysate into value added products including industrial biopolymers can be an alternative for the alarming increase in the cost of production of EPS with the synthetic media.

4.4. Summary

In this chapter we worked with the saccharification of cassava starch using commercial enzymes: α-amylase and glucoamylase under controlled pH, temperature and substrate/enzyme ratios. Cassava starch hydrolysate (CSH), containing fermentable sugars proved to be a potential alternative for EPS production by *L. plantarum*. Enzymatic hydrolysis of 9% (w/v) cassava

starch resulted in nearly 85 % conversion of it to the sugars which can be directly assimilated by the bacterium. Culture medium (pH 7.3) having cassava sugar (40 g/L) , yeast extract (40 g/L) and ammonium sulphate (5.5 g/L) along with tween 80 (4 g/L), calcium chloride (10mM) and hydrogen peroxide (3mM) found to be very effective for EPS production. From the scale up studies in a 2.5L fermenter with 1L working volume, a maximum titer of 6.79 ± 0.02 g/L EPS was obtained after the initial ethanol precipitation with 2% (v/v) inoculum, incubated at 37°C for 72 hours under static conditions. The physico chemical properties such as monosaccharide composition, thermal stability, molecular weight etc. remained unchanged irrespective of the carbon source used.

Depending on the purposes, the goal of EPS production engineering is to increase the volumetric productivity for a cost-effective production of EPS using sustainable raw materials and thus ensuring the availability of EPS for different applications in various industries.

Chapter 5

Chapter 5

Molecular Identification, Cloning and Overexpression of Glycosyltransferase (*gtf*) Gene from *Lactobacillus plantarum* BR2

5.1. Introduction

Exopolysaccharides are diverse in their composition, charge, spatial arrangements, rigidity, molecular mass, chain length and hence show significant variations in texture, viscosity, thermal stability, and rheology extending their influence in the biological properties as well. They are known to have many biological properties such as mouthwatering flavors, antioxidant activity, cholesterol-lowering and antimicrobial activities. This needs to be explored for better titer and improved biological properties, where strain improvement by genetic engineering has a major role in making tailor-made EPS. The genetic overview of the EPS production by LAB is an auxiliary area of interest as the process and the biosynthetic pathway involve numerous genes and their proteins (Schmid et al., 2015b; Soumya and Nampoothiri, 2021). The promising applications of EPS in industries point out the necessity to develop new strategies to enhance production in a sustainable and economic way. For this, investigation of sustainable and economic source of production, strain improvement for high yield, optimization of fermenter parameters and process parameters, sharpening knowledge on biosynthesis pathway for the production of EPS has to be focused (Soumya and Nampoothiri, 2021).

Many studies on exploring the biosynthetic cascade, genes involved in the pathway and the function of each gene are explained. From those studies, it is evident that glycosyltransferase

enzymes are responsible for the production of exopolysaccharides. The mechanism of production of EPS still needs clarity, but overall process involves the addition of nucleotide sugar units to lipid carrier and assembling this monosaccharide by glycosyltransferase enzymes which further polymerize with in the intra cellular space and export to outside environment (Xiao et al. 2021, Soumya and Nampoothiri 2021). The genes responsible for this mechanism can be found in the chromosome or as seen embedded in the plasmid. The biosynthesis of EPS is very complex process in which so many genes are involved in the cascade of expression of the genes, the function and complete regulation of the synthesis is continuing as mystery.

Nisin Controlled Expression System (NICE) developed from *Lactobacillus lactis*, is one of the successfully studied, easy to operate auto regulated gene expression system by the induction of lactococcal bacteriocin nisin which can be transferred to low GC Gram-positive bacteria (Mierau and Kleerebezem, 2005). Nisin is a 34-amino acid lantibiotic, previously used as preservative in food industries. Exploring the biosynthesis pathway of nisin discovered the 11 genes responsible for nisin synthesis. In those genes**,** nisK and nisR was identified as regulators for expression of target gene present at the downstream of the promoter nisA. NisK codes for a histidine protein kinase present at the cytoplasmic membrane which can act as a receptor for nisin molecules. This binding of nisin to receptor activates the response regulator nisR through autophosphorylation. Activated nisR induces the transcription of genes from the promoter region nisA (Mierau and Kleerebezem, 2005).

This auto regulated system was exploited by isolating the nisK and nisR genes and embedded to suitable host like *L. lactis subsp. cremoris* MG1316 (which is, plasmid free nisin negative bacteria) and developed a new strain NZ9000. When a gene of interest is incorporated downstream to the promoter nisA, by induction of the sub-inhibitory concentration of nisin, this

system can be regulated, and gene of interest is transcribed and expressed. pNZ9520 and pNZ9530 are the two other plasmid constructed (having high copy number and low copy number respectively) which allows the transfer of NICE system to other species (Mierau and Kleerebezem 2005). Some of the major benefits of this system includes induction specificity, if nisin is not induced, activation of signal transduction and expression not taking place, The expression of the system takes place >1000 induction fold, which is completely depends on the nisin concentration, protein expression after induction being greater than the total intracellular protein, while transformation of this dual plasmid to various strains same efficiency (Wu et al., 2014). The production of reuteran from *Lactobacillus reuteri* shows an increase in production and increase in the protein expression, after the introduction of nisin expression system (Wu et al., 2014). The expression developed was utilized not for gtf gene expression but also for expression of various other biomolecules such as antigens, cytokines, vitamins, antimicrobials proteins and membrane proteins (Guan et al. 2022, Landete 2017). β-Galactosidase is an industrially important enzymes, and it was over expressed in in recombinant *L reuteri* food vector in *Lb. plantarum* to meet the wide industrial applications using this expression system. (Nguyen et al., 2015).

Plasmid construction and its utilization in metabolic engineering of microbes for desired gene expression is very common in biotechnological applications. One such widely accepted and commercially explored plasmid is pNZ8148, a food grade vector. This plasmid have a canonical NcoI site around ATG start codon, facilitates the cloning of gene fused with nisA codon (Mierau and Kleerebezem, 2005). As discussed in Chapter 1 (1.3.3), NIZO B40 is one of the successfully studied and exploited gene expression system. The 12 kb gene cluster of *L. lactis* NIZO B40 specifically transcribes EPS biosynthetic genes where the gene cluster contains 14 coordinately

expressed genes, *epsRXABCDEFGHIJKL*. Each gene has its specific function of which some are still undiscovered. The function of EPS was analyzed previously with heterologous expression system and homologous expression system. In NIZO B40 expression system, biosynthesis of polysaccharide starts from extension of UDP glucose attached to lipid carrier done by several genes including priming glucosyltransferase (Kleerebezem et al., 1999a). These pNZ8148 vector has advantages and can easily clone any gene fused with nisA codon (Boels et al., 2001; Mierau and Kleerebezem, 2005). This broad host range vector with nisin induction was introduced to *E.coli* for expression of target genes (Wu et al., 2006) and for expression of the genes in gram-positive bacteria especially LAB, requires another vector pNZ9530 for the establishment of the NICE system for over expression of protein. But this dual plasmid system shows a growth retardation in the organism; and to overcome this in bacteria like *L. plantarum* and *L. gasseri* the nisKR gene get integrated along with the chromosome and nisA promoter on another plasmid, which also reduces the transfer complication (Pavan et al., 2000).

As mentioned, despite the nutritional and health benefits, the major limitation of LAB EPS are their production levels. The two main strategies to improve this is (a) use of sustainable media components for fermentation, which has been discussed in the previous chapter and (b) metabolic engineering approaches to increase the production of EPS, remains the focus of this chapter. The homologous expression of a glycosyltransferase gene under a nisin controlled expression system in *L. plantarum* BR2 for the EPS overproduction has been successfully demonstrated in this chapter.

5.2. Materials and Methods

5.2.1. Bacterial strains, media, and culture conditions

The bacterial strains and plasmids used in this study with their characteristics and growth conditions are listed in Table 5.1. *L. plantarum* BR2 propagation and EPS production was carried out as explained in chapter 2, (2.2.1 and 2.4.3) and *E. coli* MC1061 cells were propagated in LB medium. After genetic manipulations, the bacteria were grown in medium supplemented with appropriate antibiotics as and when required. For the recombinant strains, nisin served as the inducer for gene expression.

Table. 5.1. Bacterial strains and plasmids used in this study

Strains and Plasmids	Characteristics	Growth Conditions	Source or Ref
Bacterial Strains			
***L. plantarum* BR2**	EPS producing strain	MRS/ EPS production medium; 37°C; Static conditions	This study
***Lactococcus lactis* NZ9000 containing pNZ8148**	Chloramphenicol resistant strain	M17+0.5% glucose + 10 µg/mL chloramphenicol; 30°C; Static	NICE Expression System
***Lactococcus lactis* NZ9000 containing pNZ9530**	Erythromycin resistant	M17+0.5% glucose+10 µg/mL erythromycin; 30°C; Static conditions	NICE Expression System
***Escherichia coli* MC1061**	MC1061 is a recombinant positive strain ($recA^+$) provided for cloning and amplification of plasmid DNA of diverse Gram-positive bacteria, e.g., plasmids for expression in lactic acid bacteria	LB Medium	MoBiTec

Escherichia coli* MC1061 with pNZ8148*BR2gtf	Recombinant MC1061 cells for cloned plasmid propagation and maintenance; chloramphenicol resistance	LB Medium with 10 µg/mL chloramphenicol; 37°C; shaking at 200 rpm	This study
***L. plantarum* BR2OE4**	Recombinant *L. plantarum* BR2 with over-expressed *gtf* gene; Nisin-controlled dual plasmid system	MRS/ EPS production medium; with 10 µg/mL chloramphenicol & 10 µg/ml erythromycin 37°C; Static conditions	This study
Plasmids			
pNZ8148	Broad-host-range food-grade shuttle vector; Chloramphenicol resistant (*CmR)*; Nisin A promoter (*PnisA*); Contains a terminator after the MCS	More details are provided in Annexure II	MoBiTec, Maischberger et al., (2010); Nierop Groot and Kleerebezem, (2007)
pNZ9530	Low copy plasmid with pAMβ1 origin of replication, which carries the *nisR* and *nisK* genes. For cloning in lactic acid bacteria genera that do not have the regulatory genes integrated into the chromosome. In this case for nisin-induced expression a dual plasmid system is used: e.g., pNZ9530 (*nisRnisK*) + pNZ8148 (+insert) (Kleerebezem *et al.*, 1997).	More details are provided in Annexure II	MoBiTec, Pavan et al., (2000)
pNZ8148*BR2gtf*	Recombinant vector with *BR2gtf* gene incorporated into the MCS; Chloramphenicol resistance		This study

5.2.2. General DNA Manipulations

Genomic DNA, plasmids and recombinant DNA techniques were performed as per the standard methods described by Sambrook and Russell's molecular biology laboratory manual

(2014 Ed.) as described in Chapter 2 (2.4). The DNA isolated was visualized in 1% prestained (EtBr) agarose gel and quantified by spectrophotometer (NanoDrop 2000, USA) and finally, the whole genome analysis of *L. plantarum* BR2 was done for detailed examination of the genome for identification of *gtf* genes.

5.2.3. Whole Genome Sequencing of *L. plantarum* BR2

High-quality genomic DNA of *L. plantarum* BR2 was extracted using XcelGen Bacterial gDNA mini kit (Xcelris genomics, India) as per manufacturer's instructions. The *L. plantarum* BR2 genome was sequenced (outsourced with Bionivid Technology Pvt Ltd, Bangalore, India) using Illumina HiSeq 4000 platform with paired ends of 101 base pairs read length and having >= 80% high quality reads with phred score >= 30 (extracted using NGSQC toolkit). The high-quality reads were assembled using Velvet v.1.2.10. SSPACE v.3.0 was used to construct the scaffolds of the assembled reads. *De novo* genome validation and quality control for the final scaffolds were performed using Bowtie2 v.2.2.2 and Aragorn v1.2.36 and RNAmmer 1.2 software programs were employed to identify tRNA and rRNA genes respectively. Genome annotation and functional characterization were carried out using Rapid Annotation Subsystem Technology (RAST V 2.0) server. The screening for non-core genomic elements like plasmids and bacteriophage identification was performed by Plasmid Finder V 1.3 and PHASTER respectively.

5.2.4. Construction of Recombinant plasmid and Transformation

From the whole genome annotated data, all the glycosyltransferase genes were identified and primers specific for the *gtf* gene annotated as exopolysaccharide biosynthetic glycosyltransferase (2.4.1) (*BR2gtf)* were designed using CloneManager_V9 software. The PCR

amplification of *BR2gtf* gene was carried out using the following primers (with XbaI and HindIII restriction sites underlined) *gtf* For' GCGGCGTCTAGAGTGAAGATTGTTTACATCATTAC and *gtf* Rev' GCGGCGAAGCTTCTAGGCCAAGCAACGCAAATAG and with the Q5® High-Fidelity Polymerase (NEB, US) according to the supplier's recommendations, to avoid the addition of a polyA tail at the end of the PCR products.

Optimized PCR reaction mixture includes: 1 µg of total gDNA of *L. plantarum* BR2, 10 nmol $^{-1}$ of dNTPs, 10 µmol^{-1} of each primer and 2-5 U of Q5 DNA polymerase in 50 µL. The amplification was performed using a T100 Thermal Cycler (BioRad, USA) following the steps with: initial denaturation of 95°C for 3 min; followed by a denaturation of 95°C for 30 s, annealing at 55.4°C for 1 min 30 s and extension at 72°C for 2 min; and final extension at 72°C for 20 min. PCR product was visualized under UV gel documentation system, (Gel Doc XR+ Biorad, USA) after electrophoresis in 1% agarose gels containing ethidium bromide. The amplified PCR product and pNZ8148 vector were then digested using the respective restriction enzymes (New England BioLabs, USA) and then ligated using T4 DNA ligase and buffer from (NEB, USA) according to the user's manual.

The *E.coli* MC1061 competent cells, prepared by TSS method (Transformation and Storage Solution, composition described in Annexure I), were further transformed by heat shock with the ligated vector under controlled conditions. The transformed colonies were selected from LB- chloramphenicol (10 µg/mL) agar plates, and incubated overnight at 37°C and this *E. coli* transformations were done to ensure cloned pNZ8148-*BR2gtf* plasmid propagation and easy maintenance. From the multiple colonies produced, transformants were further confirmed by colony PCR with the *gtf* gene specific primers and from those positive clones, recombinant plasmid DNA, pNZ8148-*BR2gtf,* was extracted using QIAprep Miniprep Kit (Qiagen, Germany)

followed by double digestion and insert release from the cloned plasmid was checked for the clone confirmation.

5.2.5. Development of NICE Expression System in L. plantarum BR2

To enable the homologous expression of glycosyltransferase enzymes in *L. plantarum* BR2, pNZ8148-*BR2gtf* was constructed. For its expression to happen, there is a need to electroporate the recombinant pNZ8148-*BR2gtf* plasmid along with regulatory plasmid pNZ9530 to the *L. plantarum* BR2 host cell which serves as the regulatory plasmid for nisin expression system. Following the earlier protocols of lactobacillus electroporation (Jin et al., 2012; Landete, Arqués, Peirotén, Langa, & Medina, 2014; Teresa Alegre, Carmen Rodríguez, & Mesas, 2004) with slight modifications *L. plantarum* BR2 electrocompetent cells were prepared as explained in Chapter 2 (2.4.8.c). A volume of 80 µL of cells were electroporated immediately or aliquoted and stored at -80°C for future use within six months. *L. plantarum* BR2 cells (80 µL) were electroporated at low temperatures, 0-4°C, in Gene Pulser cuvette and the detailed procedure is mentioned in Chapter 2 (2.4.8.d). Following the electroporation, the *L. plantarum* BR2 cells, having the dual plasmid system was plated on chloramphenicol (10µg/µL) and erythromycin (10µg/µL) containing MRS agar plates for 16 to 24 hours for selection and proliferation of recombinant cells. The recombinant positive clone was further named as BR2OE cells.

5.2.6. Growth Analysis of Recombinant L. plantarum BR2

The growth of recombinant BR2OE cells were analyzed by determining the viable cell count of the bacteria at different time intervals grown in MRS broth containing appropriate antibiotics for selection of the respective plasmids. In brief, bacterial culture samples were withdrawn at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 hours of time intervals from the freshly

inoculated broth and serial dilutions were prepared up to 10^8 using sterile distilled water under aseptic conditions. The viability of BR2OE cells were enumerated by using the spread plate method. Required dilutions (10^4 ,10^5 ,10^6 and, 10^7) of the samples were spotted on the MRS agar plates containing antibiotics and kept undisturbed for drying. All plates were incubated at 37°C for 24h and the viable cell count was calculated as follows:

CFU/mL = Number of colonies formed X dilution factor of sample/Vol. of sample

*CFU: colony-forming unit

5.2.7. Optimization of Nisin Concentration

Nisin induction for recombinant *L. plantarum* BR2 cells were done as described in the previously available reports (Mierau et al., 2005; Wu et al., 2006) with modifications according to the observations obtained from the laboratory experiments. Nisin (Merck, New York) for induction was prepared in 0.05% acetic acid at a concentration of 40 mg/mL. The prepared stocks were aliquoted, at a concentration of 20 mg/mL and stored at -20 °C. To optimize the concentration of nisin for attaining the highest EPS production, different concentrations of nisin (10-80 ng/mL) were used to induce in the EPS production medium containing chloramphenicol (10 µg/µL) and erythromycin (10 µg/µL) as antibiotics when OD_{600} of the BR2OE cells reaches 0.4. After fermentation and completion of incubation, the EPS was recovered by ethanol precipitation and the final production yield was estimated. The concentration at which the highest EPS production was observed served as the optimized nisin concentration for induction in the future fermentation experiments.

5.2.8. GTF Enzyme Recovery and Assay

Ammonium sulphate precipitation of the culture supernatants of the wild-type and the recombinant *L. plantarum* BR2 strains were done to precipitate and recover the total proteins including GTF protein and further to estimate their enzyme activities. Bacterial cells were grown in the optimized MRS-sucrose EPS production medium with all the essential nutrients at 37 °C under static conditions. For the recombinant strain, in addition to the above-mentioned media, containing antibiotics and optimized concentration of nisin as inducer. After incubation for desired intervals of time (24, 48 and 72 hours), the culture broth was centrifuged at 8000 rpm for 15 min at 4°C. After fermentation, to the cell free supernatant, added different concentrations of ammonium sulphate to precipitate the proteins. Proteins were precipitated at 4°C with different ammonium sulphate concentrations (0-10%, 10-50% and 50-90%) to obtain different protein fractions until complete saturation is reached. The precipitated protein fractions were resuspended in 20mM sodium acetate buffer with 1mM $CaCl_2$, pH 5.4. Finally, we obtained three protein fractions precipitated at three different above mentioned ammonium sulphate concentrations. These protein fractions were then dialyzed against 50mM sodium acetate buffer pH 5.4 for 24 hours at 16°C. After estimating the total protein concentration by Bradford assay (as mentioned in Chapter 2 (2.5.2)), these protein fractions served as crude enzymes for glycosyltransferase assay, and they were stored at -20°C for further use.

Gtf enzymatic activity was assayed in triplicates by measuring the release of reducing sugars from sucrose by 3,5-dinitrosalicylic acid method (DNS) (Chapter 2, 2.5.3) (Bounaix et al., 2009). One GTF unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose and/or glucose per minute at 37°C, in 50 mM sodium acetate buffer (pH 5.4) in the

presence of 100 mM sucrose. The incubation time for the maximum enzymatic activity was also optimized.

5.*2.9. EPS Production by Recombinant L. plantarum BR2 in bioreactor*

EPS production, extraction, and purification by the recombinant *L. plantarum* BR2 strain was carried out as discussed in Chapter 3 (3.2.4). The addition of chloramphenicol (10 µg/µL) and erythromycin (10 µg/µL) antibiotics and nisin for induction at appropriate time to the medium were mandatory during BR2OE4 EPS fermentations. The EPS was finally recovered from the broth and further purified by ethanol precipitation and lyophilized. The EPS yield was compared with that of the wild type. To check the stability of the recombinant culture, production was also tested in 5L capacity bioreactor (Minifors 2, Infors HT, Switzerland) by controlling all parameters (37 °C temperature, initial pH of 7.3, aeration rate 0.1vvm, and agitation of 100 rpm).

5.3. Results and Discussion

5.3.1. Genome insights of L. plantarum BR2

Genome sequencing data of *L. plantarum* BR2 revealed that it has a chromosome of 3,253,735 bp in size with 44.55% of G + C content. The 16S rRNA gene sequence of *L. plantarum* BR2 obtained by Sanger sequencing and draft genome shared 100% homology. The genome predicted to encode 3242 genes which included 3167 protein coding genes. A total of 2076 genes (65.55%) were predicted to have known functions while rest of the genes were annotated as hypothetical proteins. There were no plasmids present in the genome. The genome properties and the subsystem distribution have been summarized in Table 5.2.

Table. 5.2. Genome properties and subsystem distribution of *L. plantarum* BR2 genome

Features	Data
Taxonomy Identification Method	NCBI blastn 16s rRNA database,MEGA7
Putative Species (as per sequence homology)	***Lactobacillus plantarum*** **(**GenBank Accession No: MN176402)
Total No of Contigs	47
Total Bases	3,253,735 (3.25Mb)
GC Content	44.55%
Plasmids	No
Number of genes predicted	3242
Number of protein coding genes	3167
Number of non-protein coding genes	75
Number of characterized proteins	2076
Number of hypothetical / putative protein	1032

The detailed analysis and further annotation of the genome predicted the presence of 12 glycosyltransferase genes (Table 5.3) of which one is specially annotated as exopolysaccharide biosynthesis glycosyltransferases further named as *BR2gtf* with a gene size of 1091 bp. The 363 AA length protein sequence of the *BR2gtf* gene with the ProtoParam tool reveals that the protein is of 40.14 kDa with a theoretical pI of 9.06. The TMHMM results show that this glycosyltransferase enzyme is extracellularly produced as there is neither the presence of transmembrane region nor cytosolic signals (Fig. 5.1). Homologous expression of this gene under nisin induction was attempted to enhance the overall EPS production.

Table. 5.3. Glycosyltransferase genes identified in *L. plantarum* BR2 genome and its characteristics

Sl. No	Name	START	STOP	Strand	Gene Size
1	Glycosyltransferase	891333	890599	-	734
2	Putative glycosyltransferase	949008	947680	-	1328
3	Glycosyltransferase (putative)	1404538	1403234	-	1304
4	Glycosyltransferase	1584955	1586307	+	1352
5	**Exopolysaccharide biosynthesis glycosyltransferase (EC 2.4.1)**	**1621419**	**1622510**	**+**	**1091**
6	Glycosyltransferase	1622526	1623554	+	1028
7	Glycosyltransferase	1624810	1625778	+	968
8	Uncharacterized glycosyltransferase YkoT	2032565	2031570	-	995
9	Glycosyltransferase LafB, Responsible for the formation of Gal-Glc-DAG	2222844	2221822	-	1022
10	Glycosyltransferase	2271823	2271047	-	776
11	Glycosyltransferase	2280007	2279114	-	893
12	Glycosyltransferase	2284953	2284219	-	734

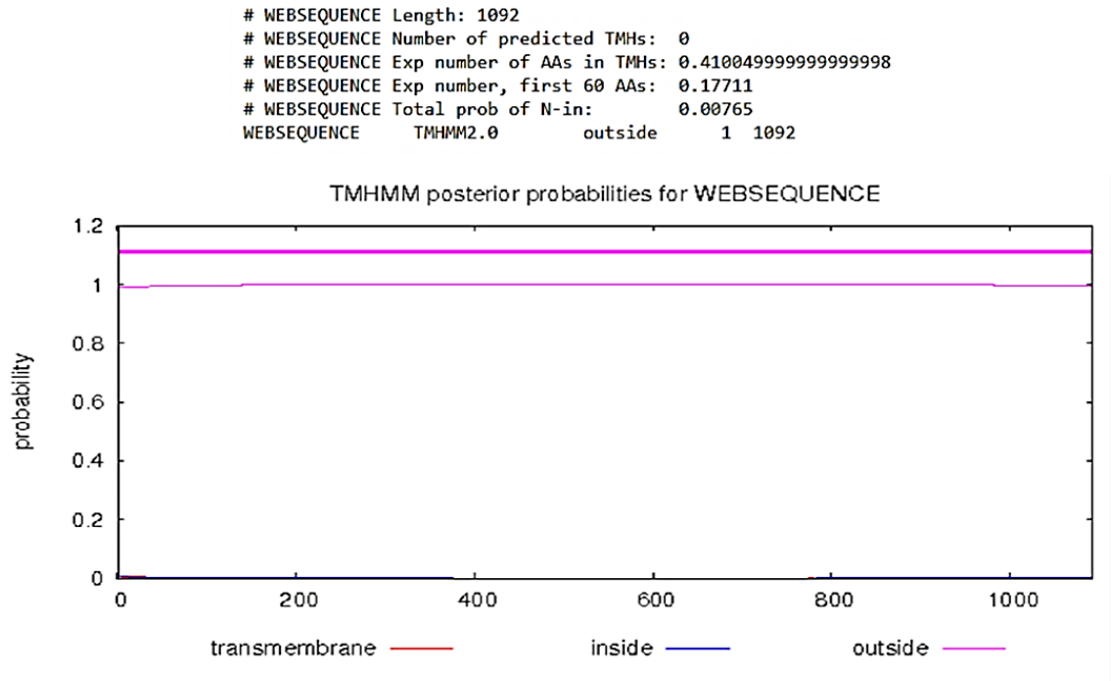


Fig. 5.1. TMHMM results of EPS biosynthetic glycosyltransferase (E.C.2.4.1) enzyme

5.3.2. BR2gtf cloning and construction of NICE system in L. plantarum BR2

The PCR reaction after several optimizations on annealing temperature and other PCR conditions with *BR2gtf* gene specific primers using *L. plantarum* BR2 genomic DNA as template gave specific bands at the expected size (Fig. 5.2). The length of the PCR amplified product including the restriction sites sequence inserted in pNZ8148 vector was 1116 bp and was confirmed by colony PCR (Fig. 5.3.(a)), double digestion and insert release (Fig. 5.3.(b)) and by gene sequencing suggesting the successful cloning of *BR2gtf* gene into pNZ8148 shuttle vector.

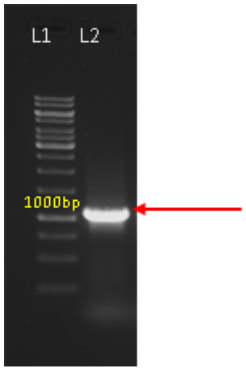


Fig. 5.2. *BR2gtf* gene amplification from *L. plantarum* BR2 genomic DNA
(L1:1kb DNA ladder, L2: *BR2gtf* amplicon of size 1116 bp)

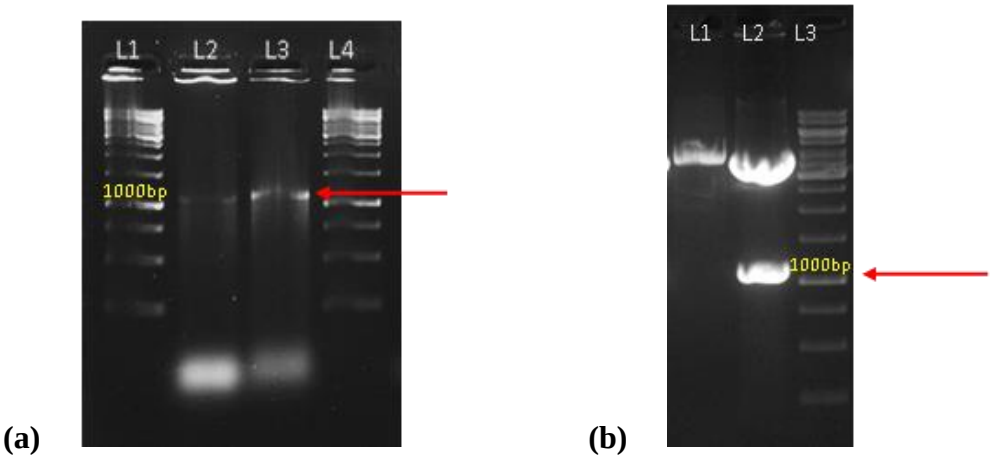


Fig. 5.3. Clone confirmation of pNZ8148-*BR2gtf*

(a) colony PCR amplified product of *BR2gtf* gene cloned to pNZ8148 vector and transformed to *E. coli* MC1061 cells (L1 & L4:1kb DNA ladder, L2: *BR2gtf* from clone 1, L3- *BR2gtf* from clone 2 of size 1116bp) **(b)** Restriction digestion profile of empty pNZ8148 and recombinant pNZ8148-*BR2gtf* plasmids with restriction enzymes XbaI and HindIII. Lane1: Native pNZ8148 double digested, Lane 2: pNZ8148-*BR2gtf* double digested, Lane 3:1 kb DNA ladder

The recombinant pNZ8148 vector carrying the *BR2gtf* gene along with the regulatory plasmid, for nisin controlled expression, pNZ9530 were then electroporated into the wild type *L. plantarum* BR2 for over-expression of glycosyltransferase enzyme. The recombinant cells carrying dual plasmids with chloramphenicol and erythromycin as selection markers was able to grow in these antibiotics containing MRS medium (Fig. 5.4). The recombinants were again confirmed by colony PCR for the amplification (Fig. 5.5) of *BR2gtf* gene with the plasmid specific primers and the positive *L. plantarum* BR2 strain with recombinant pNZ8148 and pNZ9530 was further named as BR2OE4.

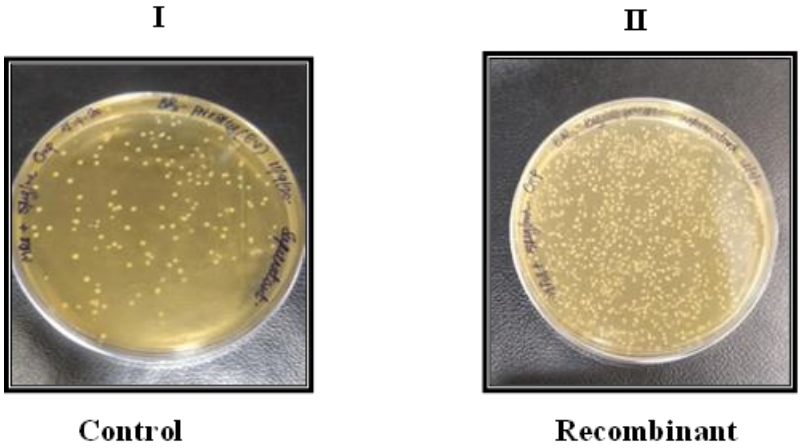


Fig. 5.4. MRS-antibiotics plates showing the recombinants with dual plasmids

(I) Control plate contains empty pNZ8148 vector and pNZ9530 **(II)** Recombinant plate contains clones electroporated with dual plasmids, pNZ8148 carrying BR2gtf gene and pNZ9530

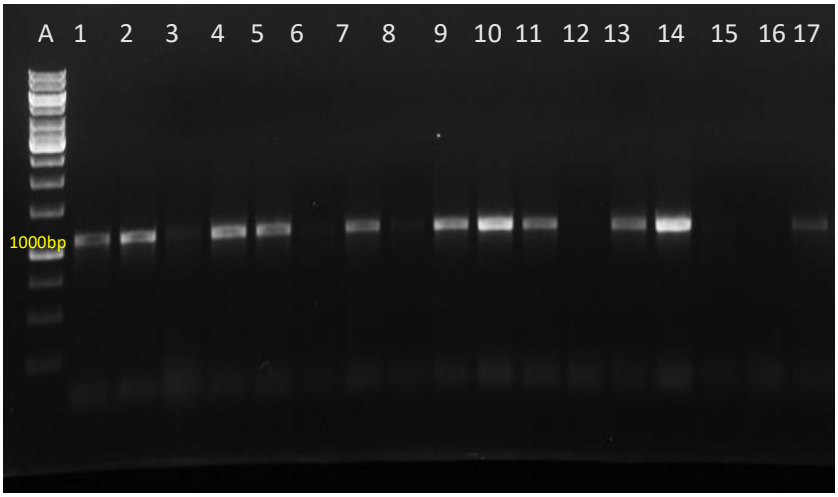


Fig. 5.5. Amplification of *BR2gtf* gene for the selection of recombinants from colony PCR with plasmid-specific primers (L & A: 1kb DNA Ladder A1-17: colony PCR amplicon of clones amplified with *BR2gtf* F' and *BR2gtf* R' with primers

5.3.3. Growth analysis of BR2OE4

The initial growth of BR2OE4 was slow when compared to the wild type *L. plantarum* BR2. The reduction in growth of BR2OE4 at their lag phase can be explained by the presence of two antibiotics, erythromycin (10 µg/mL) and chloramphenicol (10 µg/mL), in the MRS media for the propagation of recombinants. However, with prolonged incubation time, the BR2OE4 cells picked up growth within 72 hours, by the end of fermentation, both the wild-type and the recombinants showed similar CFU/mL and is summarized in Fig. 5.6.

The sugar utilization graph shows that 10-12 g/L of sugar remains unutilized, the same as the case with the wild type *L. plantarum* BR2 as explained in chapter 3 (3.3.2).

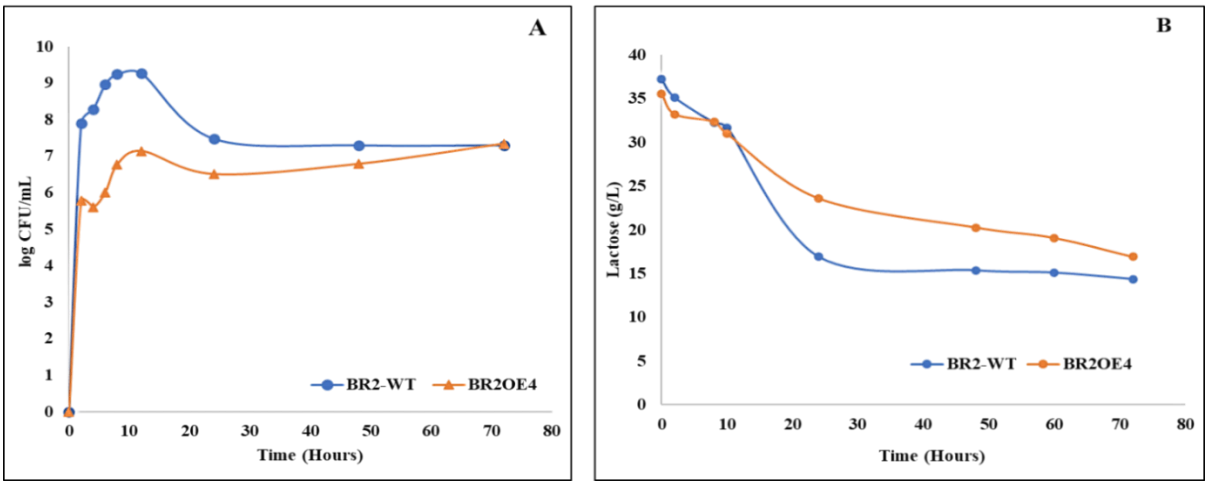


Fig. 5.6. (A) Growth and (B) Sugar utilization of recombinant BR2OE4 and wild-type *L. plantarum* BR2 in EPS production medium

5.3.4. Nisin concentration optimization and EPS production by BR2OE4

The optimum nisin concentration for the maximum gene expression in BR2OE4 was resolved by determining the EPS produced with different concentrations of nisin within a range between 10-80 ng/mL where the maximum EPS production of 7 ± 0.5 g/L was attained when 40 ng/mL of nisin was used (Fig. 5.7). There was an increase in the EPS yield with an increase in nisin concentration until 40 ng/mL after which the EPS yield started declining. The effect of nisin induction and thereby *BR2gtf* gene expression is again validated by the difference in EPS production of wild type *L. plantarum* BR2 and un-induced BR2OE4 which was lower when compared to the optimally induced BR2OE4 recombinant strain. Thus, the maximal EPS production of our system, BR2OE4 by the over-expression of *BR2gtf* gene on nisin controlled expression was calculated to be 44.79 % increase when compared with that of the wild type *L. plantarum* BR2.

Nisin controlled gene expression is a well-studied system in lactic acid bacteria consisting of an expression vector, pNZ8148 and a regulatory plasmid, pNZ9530 that has been

derived from two compatible broad host range replicons are used in this study (Silva-Bea et al., 2022). In this homologous gene over-expression system constructed in *L. plantarum* BR2, for increased EPS production, nisin acts as the inducer for the gene expression. Nisin induction is most preferred when the cell density of the culture attained an OD_{600} of 0.4-0.5 while the cells are in their exponential phase (Mierau et al., 2005).

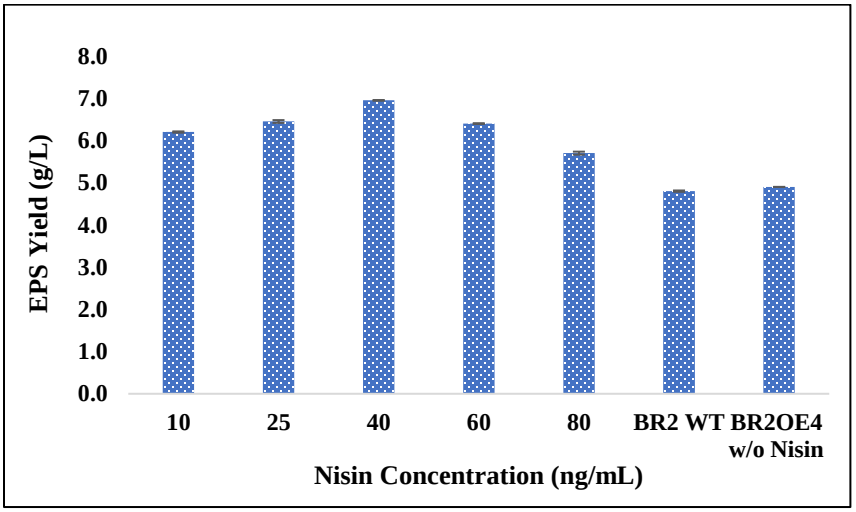


Fig. 5.7. EPS production by BR2OE4 under different concentrations of nisin compared with the uninduced BR2OE4 and the wild-type strain.

Values are expressed as mean ± standard deviation, where n = 3

Mierau et al., (2005), stated that the media composition, cell density at the time of induction and the amount of nisin shares strong correlation for a maximum gene expression to happen. Nisin induction being a dynamic process preferably needs higher density of cells in their log phase so as to ensure successful induction to occur and for the subsequent gene expression to proceed instantaneously. Nisin induction concentration and thereby product formation varies within microbes engineered. Optimum induction showed less variations in production in lactobacilli and when compared with the optimum nisin concentration *Lb. gasseri* required 50

ng/mL, while for *Lb. casei* the nisin concentration was found to be 10 ng/mL and *Lactococcus lactis* was 1-5 ng/mL, which is 10 -50 times lower.

This also supports our data that BR2OE4 gave maximum EPS production with a nisin concentration of 40 ng/mL.

5.3.5. Bioreactor studies

EPS production evaluated with the optimized fermentation conditions in a 5L fermenter (Fig.5.8) with 0.1 vvm aeration and agitation of 100 rpm resulted in 23.5 ± 0.5 g/L of EPS by the BR2OE4 which reports a highest production of EPS from a *Lactobacillus plantarum* strain in a 5L bioreactor scale.

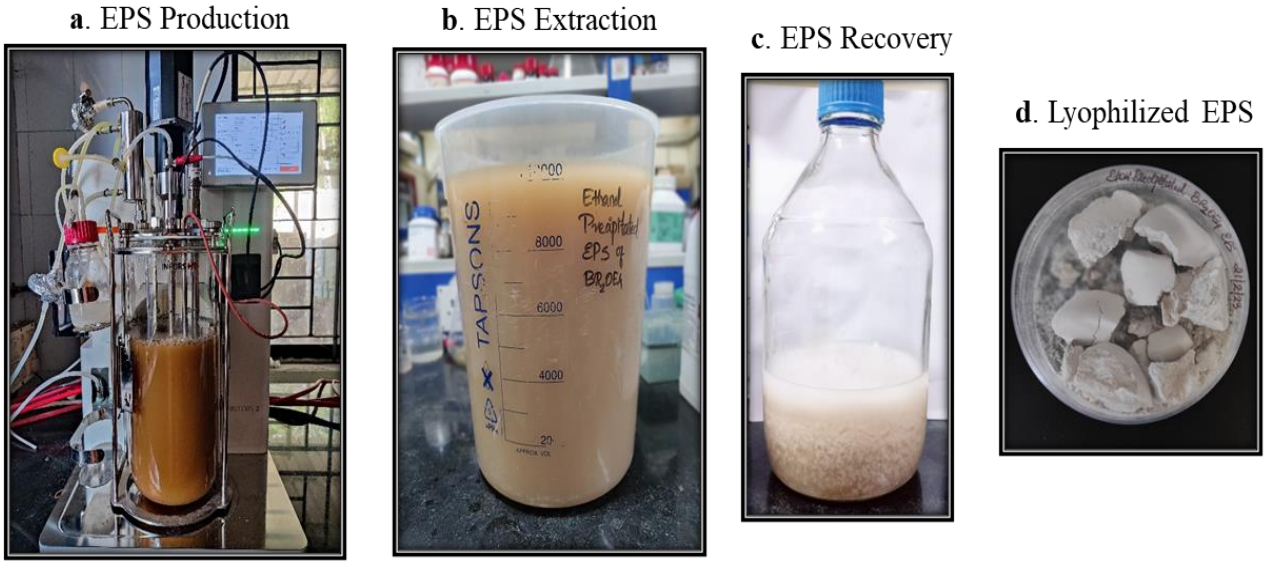


Fig. 5.8. (a) EPS production in 5L Fermenter (b) EPS extraction by ethanol precipitation (c) EPS recovery and (d) Lyophilized EPS

The fermenter scale production of EPS showed that the production of EPS makes the broth viscous and there by effective aeration and agitation is necessary. Agitation can maintain

the effective oxygen transfer throughout the medium but inappropriate speed of agitation can results in poor oxygen transfer (Bandaiphet and Prasertsan, 2006). While increasing the agitation speed there can be heterogeneous mixing, generation of heat transfer to broth, cell rupturing can happen which drastically reduces the production. So, in this condition we maintain minimum agitation speed and aeration. The rate of production of EPS under this minimum condition was seen higher for any reported Lactobacillus strain.

5.3.6. Glycosyltransferase enzyme activity assay

Glycosyltransferase enzymes plays an important role in EPS biosynthetic pathway and their role in LAB EPS is being explored by scientists all over the globe. It is proposed that extracellular glycosyltransferase activity is responsible for the synthesis of soluble EPS from a variety of C-sources (Bejar et al., 2013). Many patents have been filed on the heterologous gtf expression and development of recombinant strains in LAB taking into consideration the vast area of its applications. All these studies unanimously proved the key role of glycosyltransferases on varying polysaccharide production with wide range of applications (Soumya and Nampoothiri, 2021)**.**

Extracellular glycosyltransferase activities of over-expressed BR2OE4 and wild-type *L. plantarum* BR2 were compared (Fig. 5.9) by using their total proteins isolated by ammonium sulphate precipitation. The optimum ammonium sulfate concentration that gave maximum precipitation of proteins at 24, 48, and 72 hours of fermentation were found to be 10-50% and 50-90%.

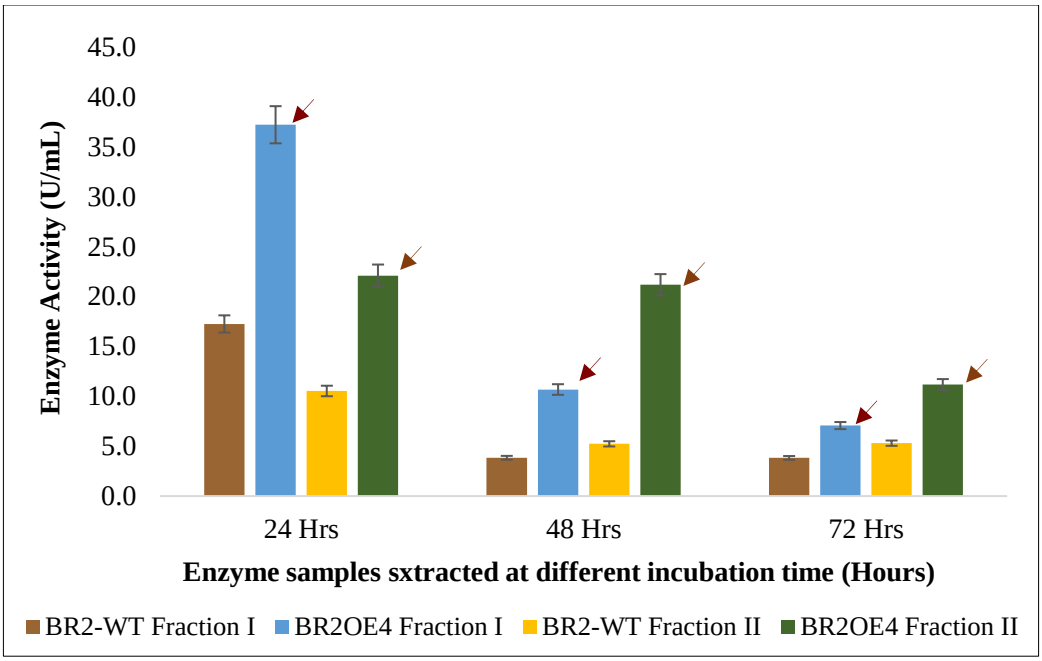


Fig. 5.9. Glycosyltransferase activity of wild-type *L. plantarum* BR2 and BR2OE4

Fraction I and Fraction II represents the crude protein samples precipitated by salting out with 10-50% and 50-90% of ammonium sulphate respectively. The arrows indicate the highest GTF enzyme activity exhibited by BR2OE4. Values are expressed as mean ± standard deviation, where n = 3.

The gtf enzyme activities of BR2OE4 determined at 24, 48 and 72 hours precipitated samples gave an enzyme activity of 37.25 U/mL, 10.69 U/mL, and 7.07 U/mL, respectively, for 10-50% ammonium sulphate precipitated fraction and 22.12 U/mL, 21.21 U/mL, 11.18 U/mL, respectively for 50-90% fraction. Whereas the level of gtf activity of the wild-type *L. plantarum* BR2 at different time intervals ranged between 17.27 U/mL, 3.84 U/mL and 3.83U/mL at 10-50% ammonium sulphate fraction and 10.55 U/mL, 5.24 U/mL, and 5.31 U/mL for their second fraction. Considering the specific activity of the enzymes, it was observed that at all time intervals BR2OE4 showed a higher gtf activity than the wild-type strain with a maximum specific activity of 26.59 U/mL with the 24 hours precipitated fraction. The maximum specific activity exhibited by the wild-type strain was only 10 U/mL. Overall, the glycosyltransferase

enzyme specific activity of BR2OE4 (Table 5.4) was 3.9-fold increased than that of the wild-type *L. plantarum* BR2 due to over-expression of the EPS biosynthetic glycosyltransferase gene.

Table. 5.4. Glycosyltransferase specific enzyme activity of WT- *L. plantarum* BR2 and BR2OE4

Specific Activity of GTF Enzymes (U/mL)						
Incubation Time (hours)	**BR2-WT**	**BR2OE4**	**Fold Increase (Fraction I)**	**BR2-WT**	**BR2OE4**	**Fold Increase (Fraction II)**
	10-50%	**10-50%**		**50-90%**	**50-90%**	
24	7.07	13.81	1.98	10.83	26.59	2.49
48	1.28	5.08	**3.9**	6.36	21.50	**3.49**
72	1.43	3.36	2.09	4.75	15.29	3.15

Fold increase is calculated between BR2-WT and BR2OE4 gtf activities of fraction I and II separately at each time intervals

The assay conditions such as incubation time (Fig. 5.10) and enzyme concentration for the maximum gtf activity was determined to be 24 hours and 10 µg/mL of the precipitated protein fractions respectively for the optimized assay conditions.

Similar work has been done where the over-expression of an enzyme involved in UDP-glucose synthesis, UDP-Glucose pyrophosphorylase (GalU), under the control of a nisin inducible promoter, increased the enzyme specific activity in *Lactococcus lactis* by 20-fold, which in turn increased both UDP-glucose and UDP-galactose synthesis by eight-fold. A study in *Sphingomonas* where the increase in the expression of biosynthetic gene cluster, especially glycosyltransferase enzyme activity enhanced the production of EPS by 20% (Ruffing and Chen, 2006).

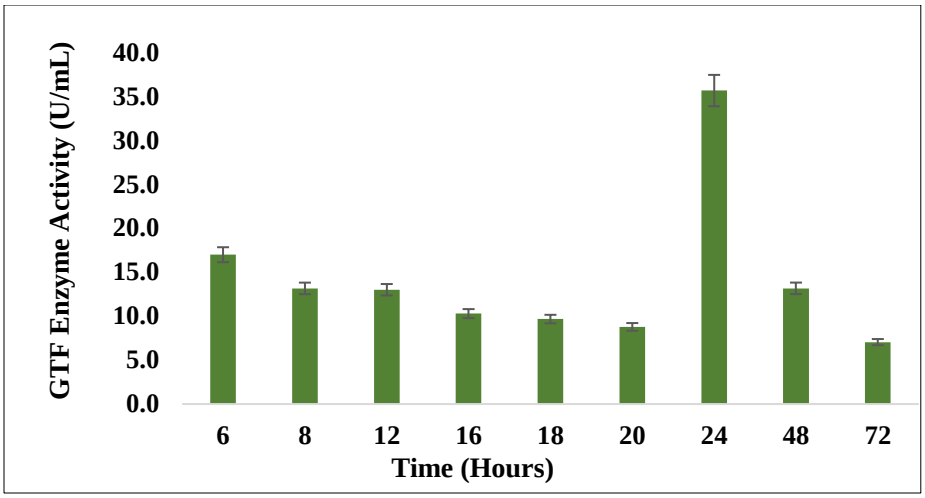


Fig. 5.10. Optimization of incubation time for glycosyltransferase enzyme assay of *L. plantarum* BR2OE4

Lactococcus lactis NIZO B40, a well-studied organism for EPS biosynthesis harbouring a 42 kb EPS plasmid, pNZ4000 contains a 12 kb *eps* operon carrying all the EPS biosynthetic genes. A study demonstrated a 15% increased EPS production with the over production of NIZO B40 priming glycosyltransferase enzymes. The elevated *eps* gene expression resulted in the higher EPS yields and this furthermore suggests the correlation of the role glycosyltransferase enzymes in EPS production (Boels et al., 2003; Nierop Groot and Kleerebezem, 2007).

The result from the above data demonstrates that the mechanism of gtf induced enhancement of EPS production by the over expression of *BR2gtf* gene in *L. plantarum* BR2 and shows a 3.9-fold increase of glycosyltransferase enzyme activity (from Table 5.4) compared to the wild-type strain and it resulted in a 54.4% increased EPS production (Fig. 5.11). The future challenges and scope of LAB EPS production lie in the EPS production bottlenecks for construction of modified or tailor-made EPS altering its composition to desirable and novel characteristics for target applications.

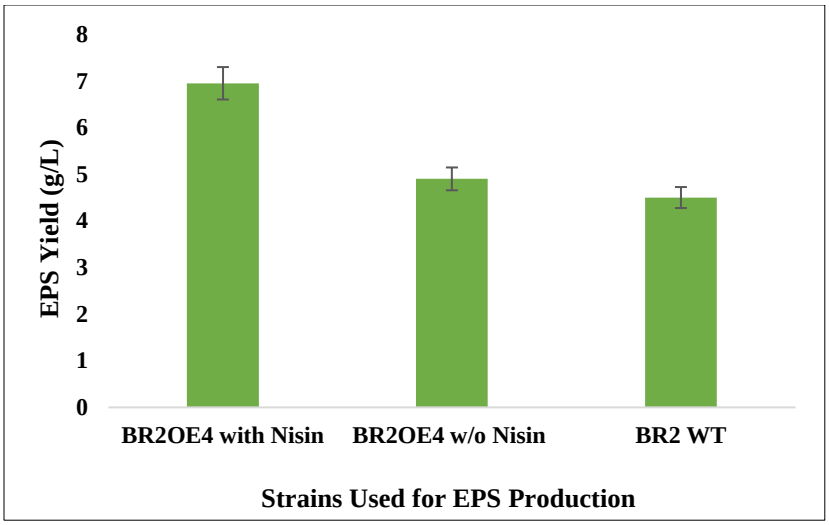


Fig. 5.11. Comparison of EPS production between wild type and recombinant *L. plantarum* BR2 with optimized fermentation conditions

This work clearly demonstrated a molecular approached strategy for increased EPS production in an indigenous probiotic *Lactobacillus plantarum* BR2 strain through nisin expression system. The NICE system is widely used for a multitude of various LAB fermentations especially in homologous and heterologous gene expression. The success rate of molecular engineering approaches with this system lies in its high expression levels (De Vos and Hugenholtz, 2004) Here in this study, we describe the implementation of the homologous over-expression of *BR2gtf* gene in *Lactobacillus plantarum* BR2 that has a pivotal role in its EPS biosynthesis depicted in Fig. 5.12. This auto-induction mechanism of nisin has been exploited for gene expression with a dual plasmid system incorporating the vectors pNZ9530 expressing the nisK and nisR genes and the plasmids such as pNZ8148, (pNZ8149, pNZ8151, pNZ8152 etc) expressing the gene of interest (*BR2gtf* in this study) placed downstream of an inducible promoter PnisA. It enables the expression of genes in Gram-positive bacteria especially in low GC-Lactobacillus without compromising the expression levels (Kazi et al., 2022).

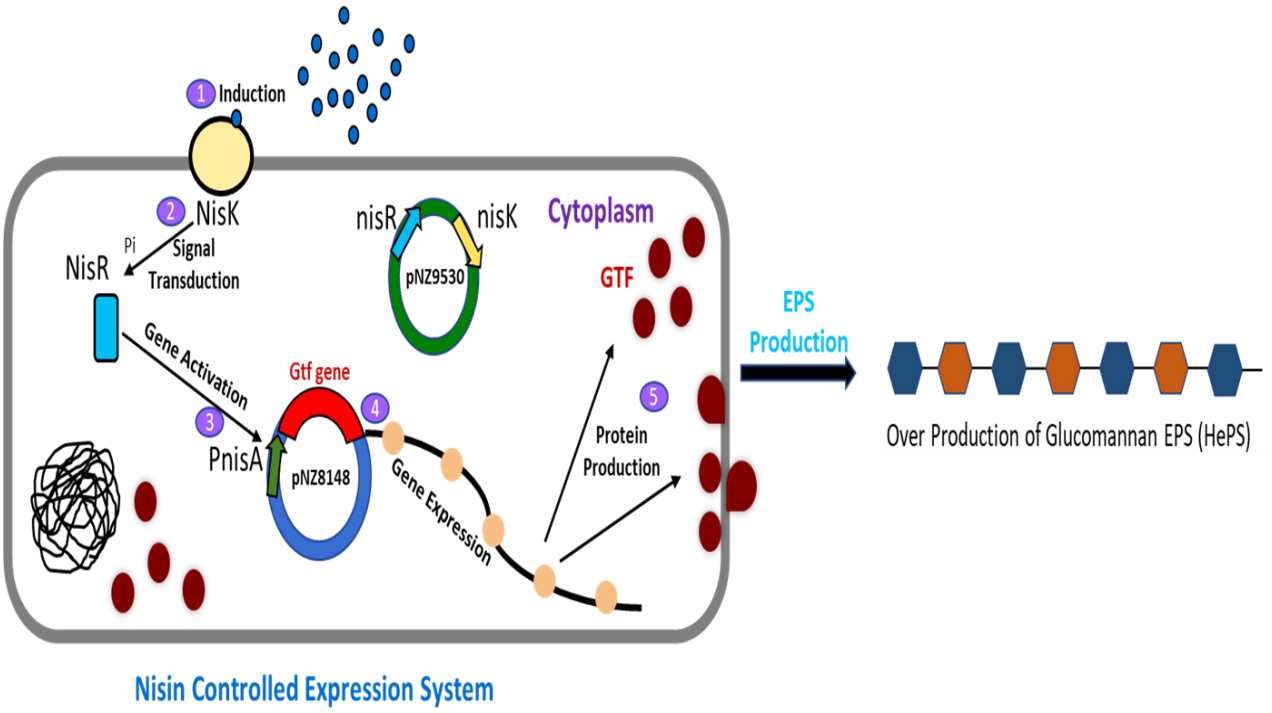


Fig. 5.12. Illustration of Nisin Controlled Expression System in *L. plantarum* BR2OE4 cell

(1) Upon nisin induction, it binds to nisK and activates the **(2)** signal transduction of nisR that **(3)** subsequently activates the PnisA promoter in pNZ8148 vector **(4)** which further enables *BR2gtf* gene expression placed downstream of the cloned vector **(5)** Gtf protein expression from the genomic DNA and the cloned plasmid results in its overexpression and is secreted out of the cell which helps in EPS overproduction

The health-promoting properties and the GRAS status of the LAB enforce them for the development of gene expression, though genetic engineering tools and therapeutic delivery systems to produce various value-added products such as antibiotics, vitamins, oligosaccharides, exopolysaccharides, cytokines and bioactive molecules (Guan et al., 2022). *Leuconostoc lactis* NZ6091 and *Lactobacillus helveticus* CNRZ32 were developed to express β-glucuronidase through this heterologous dual plasmid system employing nisin induction (Bron and

Kleerebezem, 2011) thus functionally implementing the system in LAB other than *Lactococcus lactis*.

5.4. Summary

In microbial EPS production, a better understanding of biosynthesis mechanism is a significant issue for optimization of production yields, improvement of product quality and properties, and for the design of novel strains. Understanding how to manipulate EPS production can also have importance in the biological performance of probiotics. The genome analysis of *L. plantarum* BR2 showed twelve glycosyltransferase genes, of which the gene designated as *BR2gtf*, is annotated as an EPS biosynthetic glycosyltransferase. The gene was amplified and the 1116 bp PCR product was cloned into the pNZ8148 vector. The recombinant pNZ8148 plasmid was electroporated along with plasmid pNZ9530, having a regulatory system for the nisin expression, to the host Lactobacilli. Furthermore, the glycosyltransferase activity of the recombinant and the wild-type strains were analysed. Comparing the EPS produced by the wild-type and recombinant strains, the EPS production in 5 L bioreactor using the recombinant (BR2OE4) strain over expressing *gtf* gene, showed a 54.4% increase with a maximum production titer of 23.2 ± 0.5 g/L after 72 hours of fermentation. This study shows the way an effective molecular strategy possibly be adopted in lactic acid bacteria to enhance exopolysaccharide production. To our knowledge, this is the first report on the homologous over expression of a single glycosyltransferase gene showing a significant impact on EPS production in *Lactobacillus plantarum*. To further enhance the EPS production, a complete knowledge of all the biosynthetic genes and their role needs to be explored in detail along with pathway engineering and that remains the future scope of this study.

Chapter 6

Chapter 6

Chemical Modifications to Improve the Biological Properties of Exopolysaccharides

6.1. Introduction

Most of the LAB exopolysaccharides are known to exhibit various bioactivities. Several kinds of research reveal the capabilities of bacterial EPS exhibiting antioxidant, anti-tumour, anticoagulant, anti-viral, anti-cancer and immunoregulatory activities. These diversities in the biological properties encourage EPSes in food and pharmaceutical applications for the development of functional foods and therapeutics in recent years. However, these activities are normally weak and need further improvement to be used in food and pharma industrial applications. An alternative strategy to enhance the biological activities of exopolysaccharides is through chemical modifications. The biological activities of the EPS are strongly correlated to the molecular structure they possess. This strategy of engineering EPS aims for the development of tailor-made EPS derivatives with desirable properties owing to huge industrial acceptance. Several chemical modifications have been reported based on substitutions of chemical moieties altering the backbone of the polysaccharide chain (Freitas et al., 2017; Schmid et al., 2015a).

Interestingly, there is a close relationship between the biological properties and the molecular weight, functional groups, and their degree of substitution of different chemical groups to the exopolysaccharide chain. The introduction of suitable ionic groups with an appropriate degree of substitution (DS) can significantly improve the bioactivity of polysaccharides, such as antioxidant, antitumor, antiviral and immunomodulating activities. Studies provide experimental evidence that acetylation, carboxymethylation, phosphorylation and sulphonation are some of the chemical modifications reported earlier by researchers around

the globe as an effective way to improve the bioactivity of exopolysaccharides. Reports on the antioxidant and *in vitro* antitumor properties indicated that these activities of EPS can be greatly enhanced after being modified (Jin et al., 2014; Van Casteren et al., 1998; Xie et al., 2020). However, chemical modifications of exopolysaccharide from *Lactobacillus* are limited with very few reports (Wang et al., 2015) and are not fully explored. Therefore, it is of great interest to investigate alternations of bioactivity of exopolysaccharides from *Lactobacillus via* chemical modifications.

Chemical modifications through acetylation have a greater impact on the scavenging action for hydroxyl radicals. The -OH groups present in the EPS units on acetylation gets converted into acetyl groups by esterification reaction. Ensuring proper acetylation can reduce the hydrogen bonds and thus the hydrogen atoms on anomeric carbon gets activated (Wang et al., 2015). Modification by carboxymethylation involves the substitution of carboxymethyl residues into the hydroxyl groups of EPS backbone. This consecutively makes conformational changes thus enhancing the solubility and eventually affects the bioactivities of the exopolysaccharides.

Sulphated EPS derivatives formed through sulfonation contains sulfone/sulphate groups usually introduced at the available hydroxyl groups present at C-1,2,3,4, and/or 6 of the exopolysaccharides. Numerous sulfonation mechanisms are reported incorporating the use of chlorosulfonic acid-pyridine, sulfuric acid, and sulfur trioxide-pyridine complex etc. for derivatizations (Yong Xu et al., 2019). The structural characteristics of modified EPSes are considerably different from its precursor polysaccharide, which may lead to enhanced bioactivities.

Major factors that influence the number of functional groups introduced into the EPS backbone includes the chemical properties of polysaccharides, type and configuration of glycoside bonds, molecular weight, and other structural factors. Moreover, the addition of functional groups even within the same class of exopolysaccharide largely depends on the modifying/reacting agents used, medium, temperature and reaction time as well as on the molar ratios of the reaction components (Li et al., 2017).

The antioxidant activities of the EPS derivatives following respective chemical modifications are determined by quick biochemical assays like DPPH radical scavenging assay, hydroxyl radical scavenging assay, reducing power and total antioxidant capacity assays. Various studies demonstrated that the presence of different functional groups in the EPS increased their DPPH radical-scavenging activities. The molecular weight and structure of the EPS also have great association with the scavenging activity of free radicals (Radhakrishnan et al., 2016).

Structural alterations of the native *L. plantarum* BR2 EPS through chemical modifications and comparison of the bioactivities of the native and modified EPSes are the major subject and aim of this chapter. Through appropriate methods of EPS derivatizations, it favours to alter the physicochemical properties and in consequence its bioactivities. Thus, this chapter describes a fundamental way to improve the bioactivities of the EPS through three chemical modifications: acetylation, carboxylation and sulfonation and subsequently characterization of the derivatives via FTIR, NMR, TGA and other physio-chemical techniques. Eventually, evaluation of various biological properties of the native and modified EPSes were performed foreseeing its use as an additive in food industry, and even have great potential to be applied in the pharmaceutical industry. This clearly states that the area of focus is relevant and has many

applications enabling the use of EPS in identification of new ways to formulate novel foods with the addition of various potential health-promoting probiotic strains.

6.2. Materials and Methods

6.2.1. Chemicals and Reagents

Formamide, N-bromosuccinimide, propionic anhydride, chlorosulphonic acid, pyridine, dimethyl sulfoxide (DMSO) 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 1,10-phenanthroline, hydrogen peroxide (H_2O_2), phosphoric acid (H_3PO_4) and ascorbic acid, were purchased from SRL Pvt. Ltd. and HiMedia Chemicals (Mumbai, India). Sulfur trioxide pyridine complex (SO_3.Py), N,N- dimethylformamide, dialysis tubing cellulose membranes (14 kDa) were procured from Sigma Aldrich (USA). All chemicals, reagents and solvents used in the study were of analytical grade.

6.2.2. Chemical Modifications of EPS

6.2.2.1. Acetylation of EPS

500 mg of the lyophilized and finely powdered BR2-EPS was mixed with 25 mL of formamide at room temperature for 30 minutes using a magnetic stirrer to get a homogenous mixture. 5 mL of 5% acetylating agent, N-Bromosuccinimide dispersed in propionic anhydride (w/v) was added to the EPS mixture gently and was stirred at 60°C for another 4 hours. After 4 hours of incubation, 2 mL of deionized water was added to stop the reaction and the solution was neutralized with 2M NaOH. The EPS derivative was then dialyzed against deionized water with a 14,000 Da molecular weight cutoff membrane for 24 hours at 10-15°C and then lyophilized to obtain acetylated EPS which was further named as a-EPS and stored at room temperature for subsequent analysis (Chen et al., 2014; Wang et al.,2015). The degree of substitution (DS) of the

acetyl group in a-EPS was determined according to the method described by Chen et al., (2014); Xie et al., (2020) and is calculated by the following equation:

$$DS = \frac{1.62 \times Ac}{43 - 0.42 \times Ac}$$

where Ac = % acetyl group (expressed as percentage on dry basis).

6.2.2.2. Carboxymethylation of EPS

500 mg of the BR2-EPS was dispersed in a mixture of 5 mL 20% NaOH and 12.5 mL of isopropanol and stirred constantly in an ice bath for 3 hours. 2.63 g chloroacetic acid dissolved in 5 mL of 20% NaOH and 12.5 mL of isopropanol served as the acetylating agent. This was added dropwise to the EPS mixture and stirred continuously for 3 hours at room temperature and another 1.5 hours at 60°C to evaporate the volatile solvents in the reaction mixture. After the reaction, the mixture was neutralized with conc. HCl before it was subjected to dialysis against 2L of deionized water for 24 hours with a 14,000 Da molecular weight cutoff membrane. The final carboxymethylated derivative was obtained after lyophilization which was further named as Cm-EPS (Chen et al., 2014; K. Wang et al., 2015) and stored at room temperature. The DS was determined using the neutralization titration method (Xie et al., 2020; Zhang et al., 2022) and was calculated by the following formula:

$$A = \frac{0.01H - 0.01N}{M}$$

$$DS = \frac{0.162A}{1 - 0.058A}$$

where A is the number of millimole of NaOH required per gram of the sample (mmol/g), H was the volume of hydrochloric acid used for titration (ml), N was the volume of NaOH added (ml), and M was the purified sample mass (g).

6.2.2.3. Sulfonation of EPS

The sulphated derivative of BR2 EPS was prepared by following reported methods (Liu et al., (2019); Zhang et al., (2016); Zhang, Liu, Tao, & Wei, (2016). 200 mg of EPS was dispersed in 40 mL of dimethyl formamide and was stirred for 30 minutes at room temperature to get a homogeneous mixture. To this, added 400mg of SO_3-Py complex was added and stirred at 80°C for 3 hours. This resulted in a yellow-coloured solution which was then neutralized to pH 7.0 with 10N NaOH. After neutralization, the mixture was transferred to dialysis bags and was dialyzed against 2 L of deionized water for 48 hours with changing the water at fixed intervals. The sample was then freeze-dried to obtain sulphated EPS, s-EPS and was stored at room temperature for further analysis. The degree of substitution (DS) was determined by the barium chloride – gelatin method (Terho et al., 1971) and was estimated using the Schoniger's equation :

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%}$$

where S% is the sulfate content in the sulfated EPS; 32 is the molecular mass of one S-atom

6.2.3. Characterization of modified EPS

6.2.3.1. Solubility

The solubility of the modified EPSes was examined in different solvents. For this purpose, 5mg/mL of each modified EPS was mixed with each solvent like distilled water, ethanol, methanol, chloroform and DMSO in glass test tubes, vortexed gently for 2 min and the dissolution in each of them was noted separately.

6.2.3.2. FTIR

The EPS samples after modifications were analyzed by Fourier transform infrared (FTIR) spectroscopy (IR Tracer-100, Shimadzu, Japan) to determine the functional groups such as

acetyl, methyl, carboxymethyl, sulphonyl groups attached to the polysaccharide backbones. This serves as a confirmational technique to differentiate between the native EPS and the modified EPS. 2mg of lyophilized finely powdered EPS samples were directly used for FTIR spectra analysis with a frequency range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}.

6.2.3.3. NMR

The basic molecular confirmations of the modified EPS samples were compared with the NMR spectrum of the native *L. plantarum* BR2 EPS. One-dimensional NMR spectroscopy of the EPSes was determined by a Bruker AVANCE II – 500 spectrometers (Switzerland) operated at 25°C with a 5 mm inverse probe and the nuclei of interest were ^{1}H and ^{13}C. The purified and lyophilized EPS (25mg) samples each of a-EPS, Cm-EPS, s-EPS and BR2 EPS were dissolved in 1 mL D_2O (99.6%) and were analysed further.

6.2.3.4. Thermogravimetric Analysis

The thermal stability of the EPS after modification was determined by thermogravimetric analysis (TGA) using a thermogram instrument (TA Q50, USA). For this, 5 mg of finely lyophilized powder of native *L. plantarum* EPS and modified EPS was placed in a crucible and heated to 25–400 °C at a rate of 10 °C/min under a controlled nitrogen environment. The temperature tolerance range and the degradation of EPS were determined from the data generated.

6.2.4. Determination of Biological properties of mEPS

6.2.4.1. Antioxidant Assays

The antioxidant property of polysaccharide attributes to various mechanisms namely the decomposition of peroxides and super-oxides, chelating the transition state metal ions, chain

initiation, single electron transfer (SET), hydrogen atom transfer (HAT) and radical scavenging ability etc., (Liu et al., 2012). In this study, we focus on some of these mechanisms to evaluate the antioxidant potential of the chemically modified EPSes compared with the native BR2 EPS which is assayed spectrophotometrically.

6.2.4.1.a. DPPH Radical Scavenging Assay

The free radical scavenging activity of modified EPS was measured using 1-1-diphenyl-2-picrylhydrazyl (DPPH) radicals according to Guo et al., (2013) and Zhang et al., (2016). Briefly, 150 µL of freshly prepared 0.1mM of DPPH ethanolic solution was added to 150 µL of different concentrations (0.15, 0.25, 0.50, 0.75, 1.0, 2.0 mg/mL) of all the 3 modified EPSes (a-EPS, Cm-EPS, s-EPS). Then it was incubated in dark for 20 min. The reaction was carried out in transparent flat-bottomed 96 well plates in triplicates and native *L. plantarum* BR2 EPS served as the control. After the incubation at room temperature, the absorbance was measured at 517 nm using UV-visible Spectrophotometer (Tecan Nano Quant Infinite M200 Pro, Switzerland). Ascorbic acid served as the positive control. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = [1 - (A_{sample} - A_{blank})/A_{control}] * 100$$

The experiments were done in triplicates in two or three sets and the standard deviation was calculated from individual experiments.

6.2.4.1.b. Total Antioxidant Capacity

The total antioxidant activity of modified EPS was determined and compared with that of the control *L. plantarum* BR2 EPS (Dilna et al., 2015). The total antioxidant capacity (TAC) reagent consisted of 0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate. Briefly, 1mL of the TAC reagent was mixed with 0.1mL of various concentrations

(2, 4, 6, 8,10 mg/mL) of the modified EPSes and was incubated at 95°C water bath for 90 min. The absorbance of the aqueous solution after incubation was measured at 695 nm against blank in a UV-visible Spectrophotometer (Tecan Nano Quant Infinite M200 Pro, Switzerland). Ascorbic acid was used as the positive control. The assay was carried out in triplicates and the antioxidant activity was expressed in term of Vc equivalence.

6.2.4.1.c. Reducing Power Assay

The reducing power of chemically modified EPSes were evaluated and compared with that of the control *L. plantarum* BR2 EPS using the method by Dilna et al., (2015). The assay works on the principle of the ability of the exopolysaccharides to reduce Fe^{3+} to Fe^{2+} measured by the formation of the Perl's Prussian blue complex. A higher absorbance is an indicative of a higher reducing power of the EPS. For the reaction to happen 0.25 mL of 2mM phosphate buffer (pH 6.6), 0.25 mL of 1% (w/v) potassium ferrocyanide and 0.1 mL of 10 mg/mL concentration of different modified EPSes were mixed and the mixture was incubated at 50°C for 20 min. After incubation, 0.25 mL of 10% trichloroacetic acid was added and the reaction mixture was centrifuged at 3000xg for 10 min. To 0.25 mL of the supernatant, 0.25 mL of distilled water and 0.5 mL of 0.1% (w/v) freshly prepared ferric chloride solution were added. The absorbance of the bluish aqueous solution was measured at 700 nm using a UV- visible spectrophotometer. Ascorbic acid served as the positive control. The reducing power activity of the EPS samples were expressed in terms of Vc equivalence.

6.2.4.1.d. Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging activity of the modified EPS was compared with that of the native *L. plantarum* BR2 EPS according to the protocol mentioned by Liu et al., (2012) with minor modifications. 0.5 mL of 7.5mM 1,10-phenanthroline, 0.5 mL of 7.5mM $FeSO_4$ with 1

mL of 0.2M sodium phosphate buffer were mixed with 0.5 mL of 10 mg/mL concentration of various modified EPS. To this mixture, 0.5 mL of 0.1% hydrogen peroxide solution were added and incubated at 37°C for 60 minutes. After the reaction, the absorbance was measured at 510 nm using a UV- visible spectrophotometer. The hydroxyl radical scavenging activity of EPS was evaluated using the following equation:

$$\text{HSR\%} = \frac{H_s - H_0}{H_c - H_0} \times 100$$

Where H_s is the absorbance of the EPS samples, Hc is the absorbance of the control without H_2O_2 and H_0 is the absorbance of the blank which is distilled water. Ascorbic acid (Vc) was used as a positive control and the results were expressed as Vc equivalents.

6.2.4.2. Cholesterol Lowering Assay

The inherent cholesterol-lowering ability of *L. plantarum* BR2 exopolysaccharide after chemical modifications were studied. The total cholesterol adsorption by chemically modified EPS in *in vitro* conditions were evaluated by Soh et al., (2003) method with minor modifications. Briefly, 0.1% EPS and 30 µg of cholesterol standard purchased from Sigma were mixed and incubated at 25°C for 20 minutes. Then 50 µL of 1% CTAB (hexadecyl trimethyl ammonium bromide) was added to stop the reaction. The reaction mixture was then centrifuged at 12500g for 10 min at room temperature. The total cholesterol concentration in the supernatant was further estimated by Zak's method (Zak B., and Epstein, 1961; Tsai et al., 2014). A lower value in Zak's method is an indicative of higher cholesterol adsorption by the EPS. The cholesterol lowering activity was measured using the equation:

Cholesterol Lowering Activity % =

$$\frac{\text{Conc. of Cholesterol in Control} - \text{Conc. of Cholesterol in Test}}{\text{Conc . of Chelesterol in Control}} \times 100$$

6.2.4.3. Cytotoxicity Studies

The cytotoxicity of modified EPSes and the native BR2 EPS in normal cells were analyzed using L929 fibroblast cell line. Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, DMEM (Sigma Aldrich, USA) supplemented with 10% FBS (Gibco, US), L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: penicillin (100U/mL), streptomycin (100µg/mL), and amphotericin B (2.5 µg/mL). 100µL of this cell suspension containing $5x10^4$ cells/well was seeded in 96 well tissue culture plate and incubated overnight at 37ºC in a humidified 5% CO_2 incubator. After 24 hours, the growth medium was removed and was treated with different concentrations (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) of each EPS samples (a-EPS, Cm-EPS, s-EPS and native BR2 EPS) prepared freshly in 5% DMEM. Each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated cells were maintained as control. Afterwards MTT (3-(4,5-dimethylthiazolyl- 2)-2,5-diphenyltetrazolium bromide) assay was performed to see the effect of the polysaccharide in normal cells. Briefly, after 24 hours of incubation, the sample content in wells were removed and 30 µL of reconstituted MTT solution was added to all the test and control wells and the plates were gently shaken followed by incubation at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation, the supernatant was removed and 100 µL of MTT solubilization solution (Dimethyl sulfoxide: DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured using microplate reader at a wavelength of 540 nm (Ismail and Nampoothiri, 2013; Talarico et al., 2004). The percentage of growth inhibition was calculated using the formula:

$$\% \text{ Cell Viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

6.2.4.4. Anticancer Effect of mEPS

Along with the cytotoxicity analysis, anticancer activity of the modified EPSes and the native BR2 EPS was determined, for which MCF-7 (Human breast cancer) cell line, initially procured from National Centre for Cell Sciences (NCCS), Pune, India was maintained in Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA) under the above-mentioned conditions same as that used for cytotoxicity analysis. After cell seeding with 100 µL cell suspension containing $5x10^3$ cells/well in 96-well plates, it was incubated at 37ºC in a humidified 5% CO_2 incubator. The growth medium was removed after 24 hours and the cells were treated with EPSes, each compound freshly prepared in 5% DMEM were five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100 µL were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained. The viability of MCF-7 cells after their exposure to EPSes were evaluated by direct observation of cells by inverted phase contrast microscope (Olympus, Japan with Optika Pro 5 Camera) followed by MTT assay method. The absorbance values were measured using microplate reader at a wavelength of 540 nm (Ismail and Nampoothiri, 2013; Talarico et al., 2004). The percentage of growth inhibition was calculated using the formula:

$$\% \text{ Cell Viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

6.3. Results and Discussion

The native *L. plantarum* BR2 EPS has already been characterized and have been described in Chapter 3, (3.3.4) and is a glucomannan EPS possessing various biological properties (Sasikumar et al., 2017; Soumya et al., 2019) as the native EPS is chemically modified and evaluated further in this part of work.

6.*3.1. Characterization of modified EPS*

6.3.1.1. Solubility

The native *L. plantarum* BR2 EPS is water soluble and insoluble in all polar solvents. The modified EPSes, acetylated, a-EPS and carboxymethylated EPS, Cm-EPS are easily soluble in water but the sulphated EPS, s-EPS is soluble only after vigorous vortexing. All the modified EPSes were insoluble in all other polar solvents used: ethanol, methanol, acetone, chloroform and DMSO and gets precipitated at the bottom of the tube. The branch stretches and spatial rearrangements in the polysaccharide chain, exposes the hydroxyl groups and thus facilitates higher water solubility of acetylated and carboxymethylated EPSes (Chen et al., 2014; Yong Xu et al., 2019).

6.3.1.2. FTIR

The occurrence of chemical modifications of *L. plantarum* BR2 EPS was confirmed by detailed FTIR spectra in comparison with that of the native un-modified BR2 EPS. The FTIR spectra of a-EPS, Cm-EPS and s-EPS were similar exhibiting the prominent peaks and signal stretches of typical exopolysaccharides in the range of 4400-400 cm^{-1}. However, in the detailed investigations, there were significant differences that exhibited variations in the peaks and the signal stretches. The presence of new absorption peaks in comparison to the control EPS are shown in Fig.6.1.a.

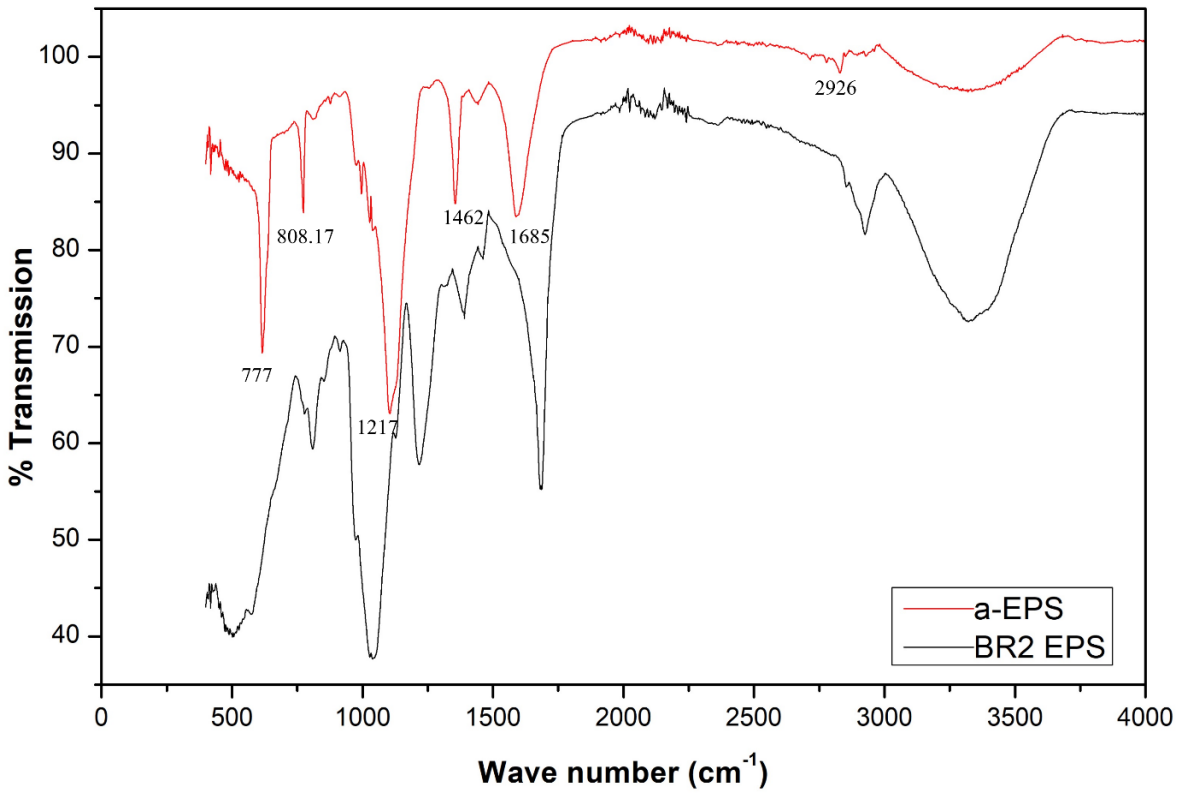


Fig. 6.1.a. FTIR spectrum of acetylated EPS (a-EPS) and native BR2 EPS from *L. plantarum* BR2

From the spectrum, peak signals at 1217 cm^{-1}, 1462 cm^{-1}, 2852 cm^{-1} and an increased signal stretch at 2926 cm^{-1} corresponds to strong C-H bending seen only in the a-EPS. The peak between 1116-1217 cm^{-1} and 1685.79 cm^{-1} coincides to a strong C=O stretching vibrations in the samples after the acetylation. These absorption intensities have been increased predominantly when compared to the control samples and this is strong evidence for the occurrence of acetylation successfully. The peaks around 777 and 808.17 cm^{-1} are attributed to 1-2 di-substituted C-H bending with strong absorbance signals also supports the previous data of acetylation (Chen et al., 2014; Xiao et al., 2020).

The strong new absorption peaks at 1323.17 cm^{-1} and 1411 cm^{-1} are a conformation for the presence of carboxyl groups of Cm-EPS shown in Fig. 6.1.b.

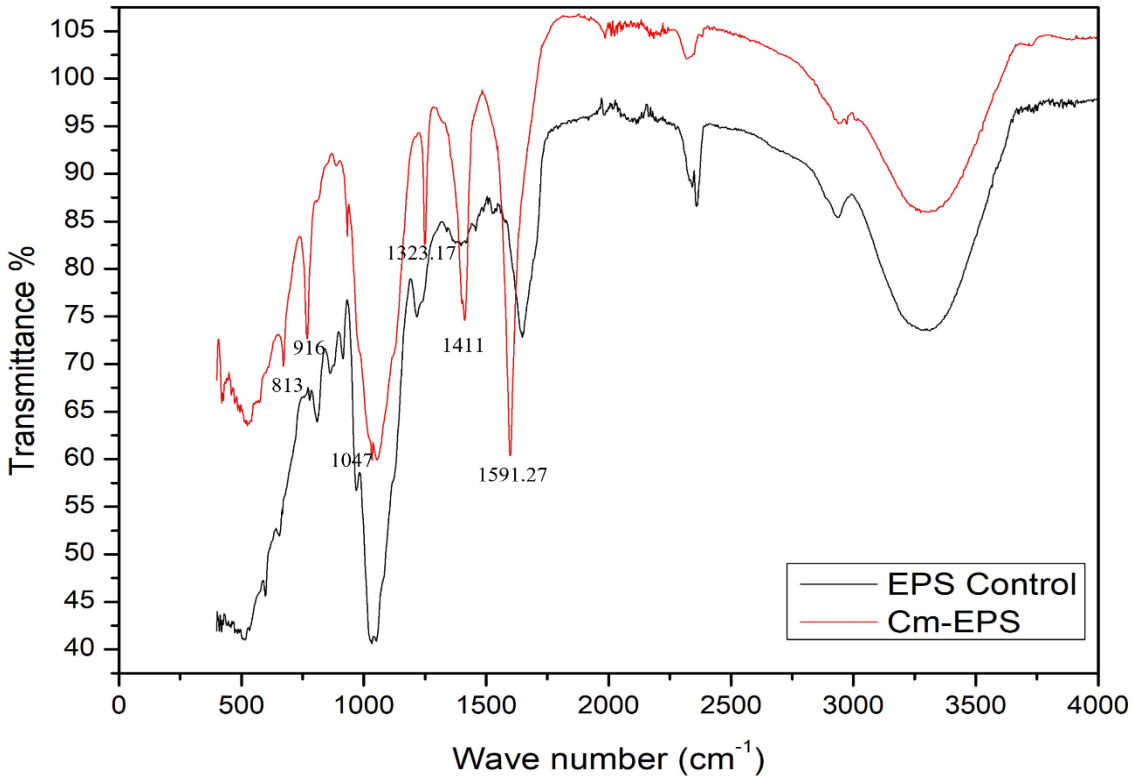


Fig. 6.1.b. FTIR spectrum of carboxymethylated EPS (Cm-EPS) and native BR2 EPS from *L. plantarum* BR2

These corresponds to the C-O-C symmetrical stretching vibrations in the spectra (Chen et al., 2014). The strong increased signal stretch at 1047 cm^{-1} 1591.27 cm^{-1} attributes to the asymmetrical stretching vibrations of C=O vibration of carboxyl groups (-COOH) and the variable angular vibration of C-H of methyl groups (-CH3) linked to the carboxyl group, respectively (Li et al., 2021). The absorption peaks at 813 cm^{-1} and 916 cm^{-1} are strong signals for C-H stretch of methyl groups.

The successful incorporation of sulphate groups was confirmed by the existence of new absorption peaks at 773 cm^{-1}, 995 cm^{-1}, 1355 cm^{-1} 1442 cm^{-1} where peaks at 773 cm^{-1} and 995 cm^{-1} attributes to the strong S=O sulfoxide stretch (De Moura Neto et al., 2011), 1355 cm^{-1} is the stretching vibrations specifically for sulfone and sulfonic acid as depicted in Fig. 6.1.c.

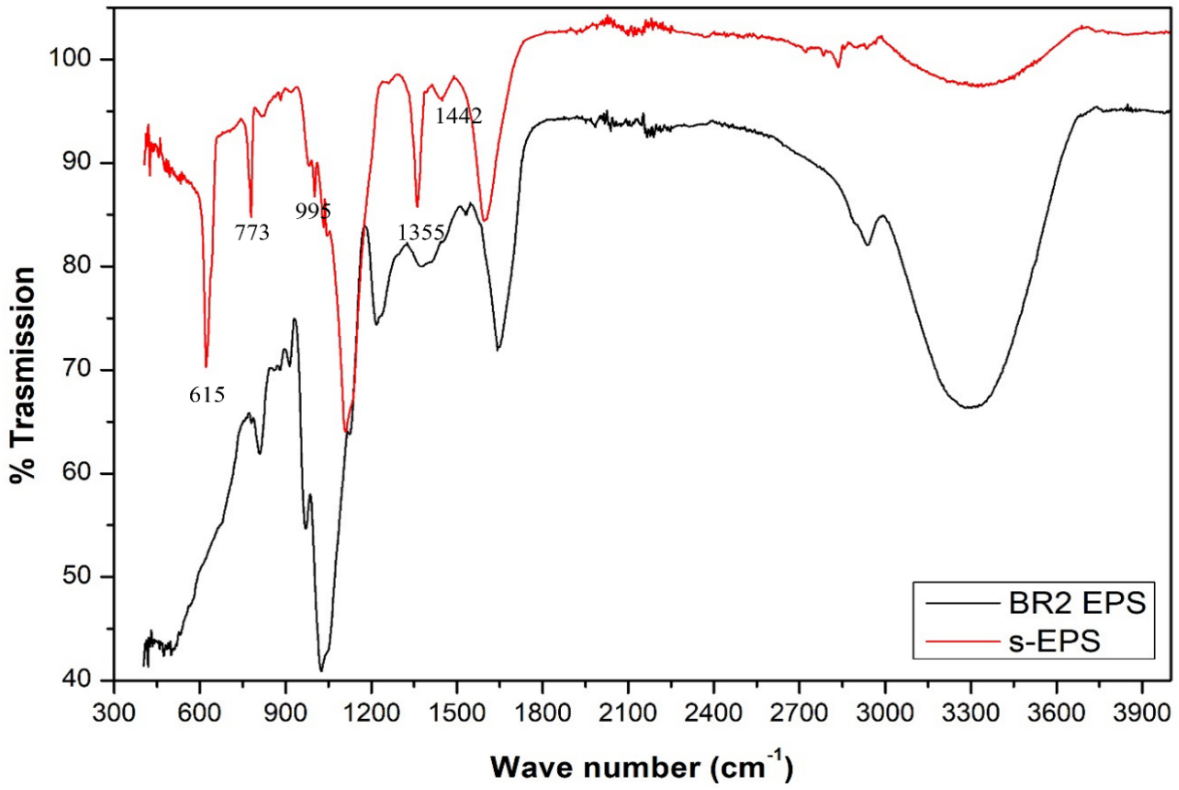


Fig. 6.1.c. FTIR spectrum of sulphated EPS (s-EPS) and native BR2 EPS from *L. plantarum* BR2

The absorption signal at 1442 cm^{-1} is another peculiar peak for S=O stretch. The strong and intense peak stretch at 615 cm^{-1} and 773 cm^{-1} corresponds to C-H bending. The broader absorption signal at around 2829 cm^{-1} signifies strong signal stretches of H-C=O. These results were in correlation with the previous reports of (Liu et al., 2019; Zhang et al., 2016) of sulphated EPSes. The degree of substitution (DS) of sulfated EPS was estimated to be 0.937 according to the calibration curve of sodium sulfate ($y = 0.0002x + 0.0451$, $R^2 = 0.9839$) by barium chloride gelatin method.

6.3.1.3. NMR

NMR spectroscopy serves as an important technique that can provide relevant information about the structure of an exopolysaccharide. When the NMR analysis depicted in

(Fig. 6.2) of the mEPSes when compared with the native BR2 EPS, mentioned in chapter 3 (3.3.4.5), showed significant variations in the spectra indicating the presence of new functional groups resulted due to chemical modifications.

The new resonance peaks at 0.9-1.2 ppm indicated the presence of R-CH_3 groups on to the EPS and the peaks at 2.1-2.5 ppm again confirmed the presence of acetyl groups after acetylation and was in line with the previous reports (Wang et al., 2015) . From the ^{1}H and ^{13}C NMR of the Cm-EPS, the presence of R-(C(=O)OH) groups at 7.1-7.5 ppm, hydroxyl groups at 6.5-6.9 ppm, and the appearance of proton signals from -$C(CH_3)=CH_2$ groups were clearly observed indicating the chemical modification. The ^{1}H NMR spectra of the s-EPS showed the presence of intense peak signals at 1.0-2.9 ppm that corresponds to R-SH groups and the peak at 3.8 ppm indicated the presence of (-C-SO_nR) sulfoxides or sulphone groups.

NMR is a high throughput method to know the molecular characteristics of a compound. ^{1}H NMR spectrum consists of mainly three regions, anomeric regions, ring proton region and alkyl region. The peak signals and their corresponding functional groups and details of the stretching vibrations of each of the modified EPSes have been summarized in Table 6.1.

Table. 6.1. ^{1}H NMR Peak signals and their corresponding functional groups of a-EPS, Cm-EPS and s-EPS

Signals	Inference
Acetylated EPS	
1.9 & 2.2 ppm	Presence of R-CH3 groups
2.1-2.5 ppm	Presence of Acetyl groups
3.5 & 4.1 ppm	Protons attached to C2-C6
Carboxymethylated EPS	
7.1-7.5 ppm	Presence of R-(C(=O)OH) groups
6.5-6.9 ppm	Presence of hydroxyl groups
5.6-6.1 ppm	the appearance of proton signals from –C(CH3)=CH2

Sulphated EPS	
1.0-2.9 ppm	Signals for R-SH groups
3.1-4.3 ppm	Corresponds to ring protons
3.8 ppm	Presence of (-C-SO_nR) sulfoxides or sulfone groups

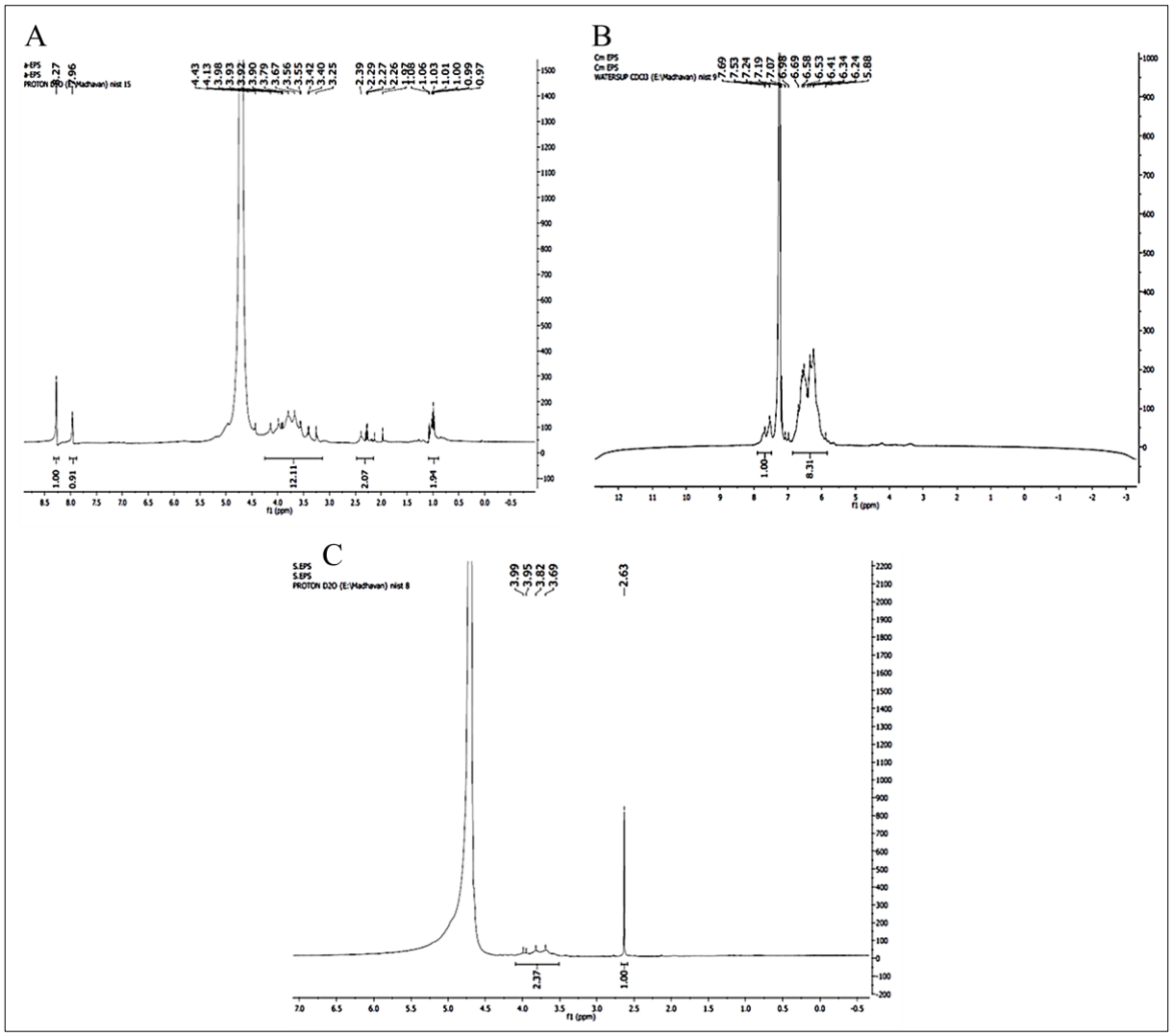


Fig. 6.2. NMR Spectrum of modified EPS A) a-EPS **B)** Cm-EPS **C)** s-EPS

Thus, considering the NMR spectroscopy of the EPSes, the specific peak signals confirmed the presence of various functional groups attached to the polysaccharide after chemical modifications.

6.3.1.4. Thermogravimetric Analysis

Taking into consideration the application of EPS in different industries, thermal stability and structural behavior plays a significant role. The thermogravimetric curves of the a-EPS, Cm-EPS, s-EPS in comparison with the native BR2-EPS shown in Fig. 6.3 revealed that the thermal decomposition of native BR2 -EPS and the modified EPS were different. The TGA curves for each of the EPS had two stages.

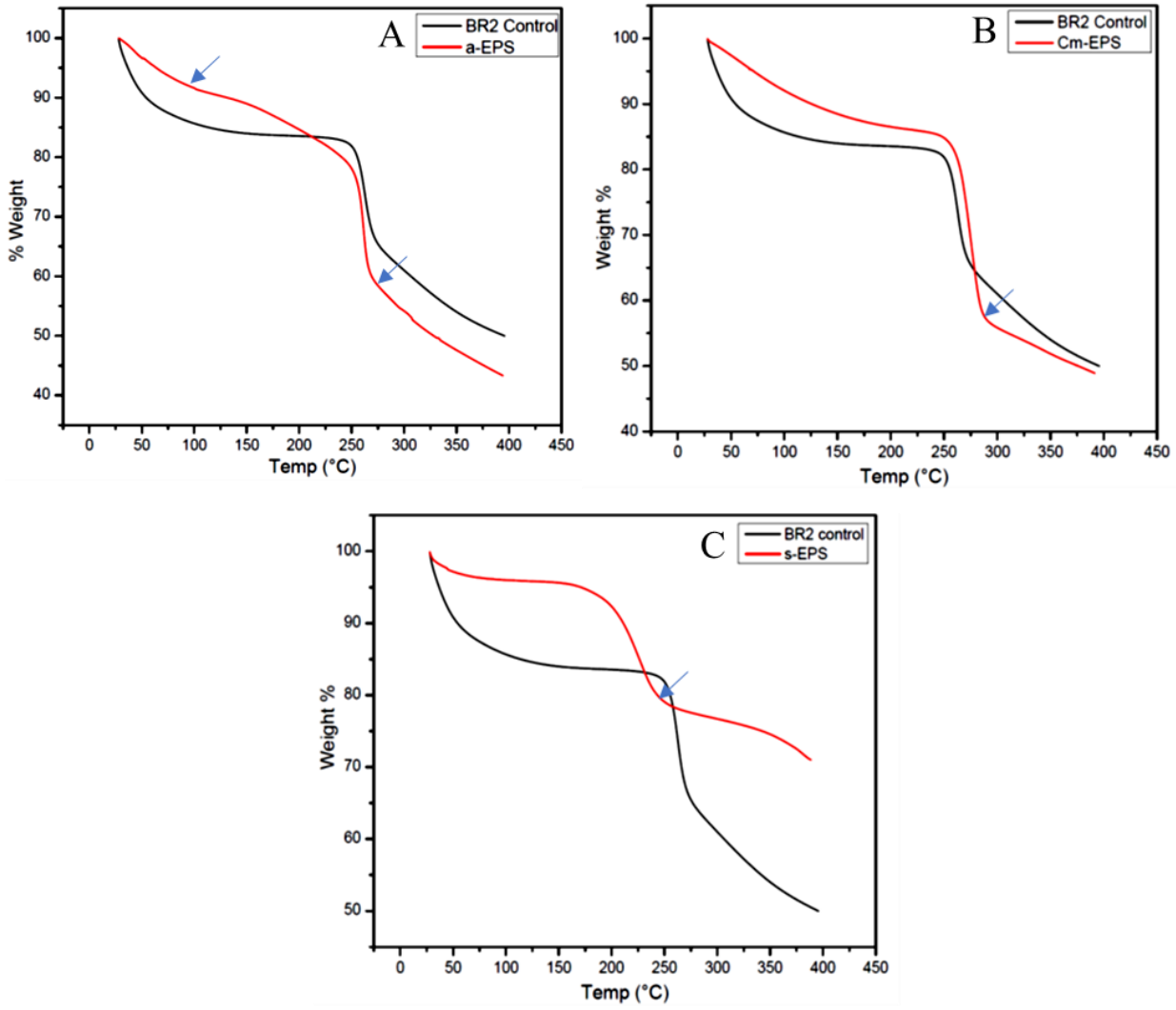


Fig. 6.3. Thermogravimetric mass loss spectrum of the BR2 EPS and its derivatives

A) a-EPS **B)** Cm-EPS **C)** s-EPS

The primary weight loss at 50°C is an indicative of water loss seen in all the samples with lesser weight loss for s-EPS at this stage. The presence of polar groups like OH groups contributes to the water absorption by the EPS thereby forming hydrogen bonds with the water molecules (Lobo et al., 2019). From our previous study we know that the native BR2 EPS is stable up to 250°C (Sasikumar et al., 2017; Soumya et al., 2019) and here we compare it with the chemically modified EPSes. The s-EPS showed an event between 200-230°C with only 10 % weight loss. Cm-EPS is stable until 270°C with only 15% weight loss whereas native BR2-EPS shows 20% degradation and a-EPS shows a 25% degradation at this temperature. For a-EPS and Cm-EPS 50% degradation occurs only after a temperature above 290°C and 300°C, respectively. This indicated a resistance of these EPSes for higher temperature which is a favorable factor for food and pharmaceutical industrial applications (Ayyash et al., 2020b). Thus, the thermal property studies of the EPS reveal that the chemical modifications contribute for a greater thermal stability than the unmodified native BR2 EPS.

6.3.2. Determination of Biological properties of mEPS

6.3.2.1. Antioxidant Assays

As discussed, acetylation, carboxymethylation and sulfonation are the major effective and widely used methods to modify natural exopolysaccharides as it can improve or even create new bioactive properties for polysaccharides. *In vitro* examination of these antioxidant capacities of EPS includes chemical reaction assays such as DPPH radical scavenging, ABTS radical scavenging, FRAP, superoxide radical scavenging, and hydroxyl radical scavenging assays (Li et al., 2017) and some of which are discussed further.

6.3.2.1.a. DPPH Radical Scavenging Assay

DPPH are organic nitrogen free radicals that accept electrons or hydrogen radicals (H.) to attain stability. The UV-vis absorption spectra for DPPH in an alcoholic solution is observed at 517nm. The DPPH radical scavenging activity of a-EPS, Cm-Eps, s-EPS and BR2 EPS at different concentrations of the EPS ranging from 0.15-2 mg/mL is shown in Fig. 6.4. The results showed that DPPH scavenging activity was concentration dependent, and a-EPS showed the greatest activity at all the tested concentrations with a maximum activity of 73.81% at 2 mg/mL. Cm-EPS and s-EPS showed their maximum scavenging activity of 63.78% and 64.65% at a concentration of 0.75mg/mL and 2mg/mL respectively. When compared to the scavenging ability of EPS produced by *Bacillus cereus* SZ1 (Zheng et al., 2016) which is supposed to have 54.0% activity at 3mg/mL, all the three modified EPSes showed a higher DPPH scavenging activity even at a very lower concentration. Usually, the scavenging activity of EPS is their hydrogen-donating ability. This increased scavenging potential of a-EPS, Cm-EPS and s-EPS is probably because of the addition of proton donors as a result of modification happened in the EPS backbone that in return contributes to the reaction with the free radicals to produce a stable diamagnetic molecule. The scavenging activity at a concentration 2 mg/mL increased in the order BR2 EPS < Cm-EPS < s-EPS < a-EPS.

Our experimental analysis on the antioxidant properties of modified EPS is in co-relation with the finding of Chen and his co-workers on *Ganoderma atrum* EPS, who reported that the incorporation of acetyl groups to the polysaccharide chain improves the scavenging activity due to the stronger hydrogen – bonding ability, polarity, conformation and charge density of the native EPS (Chen et al., 2014). Acetyl groups being a good H^+ provider, can transform the free radicals into more stable forms by inhibiting free radical chain reactions. At a concentration of 2

mg/mL, similar to our data, acetyl group addition showed a significant increase of 71.36% whereas in the unmodified EPS of *Ganoderma atrum*, the activity was 47.64%. The radical scavenging activity of s-EPS was higher than the native BR2 EPS by 1.67 fold but lower than the *Ganoderma atrum* EPS reported by Chen et al., (2015). This difference in the activity of the EPSes is a consequence of the varied physiochemical properties of the two divergent polysaccharides.

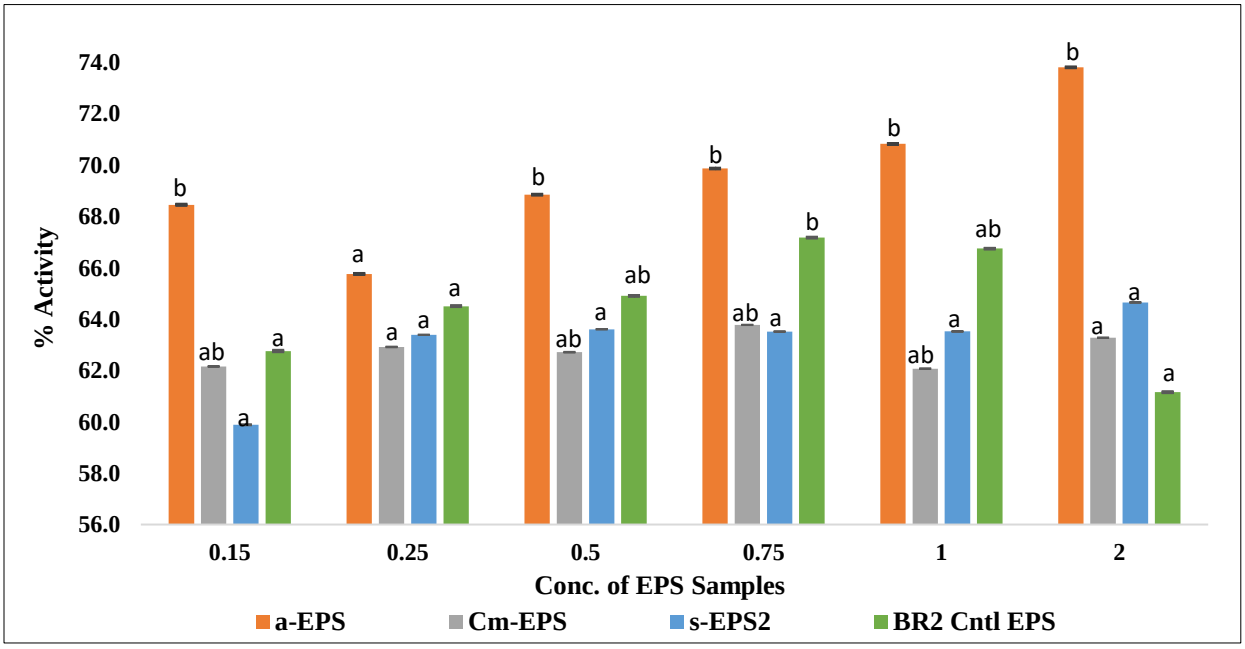


Fig. 6.4. DPPH Radical Scavenging Activity of BR2 and its derivatives

Values are expressed as mean ± standard deviation, n = 3. Values with different letters in each column are significantly different ($p < 0.05$)

A sulfated EPS produced by a bacterium designated as PRIM-30 isolated from the deep seawater collected from the offshore region of Cochin, India was studied, and this EPS showed antioxidant activities for the inhibition of DPPH and superoxide radicals. The EPS displayed a linear dose-dependent increase in total antioxidant capacity and ferric-reducing power activities (Priyanka et al., 2014). There are some promising reports on the enhancement of the biological properties of EPS by chemical modifications.

6.3.2.1.b. Total Antioxidant Capacity

The quantitative determination of antioxidant capacity of the EPS sample was measured spectroscopically at 695nm. It measures the green phosphomolybdenum complex, Mo (V), that occurs by the reduction of Mo (VI) to Mo (V) by the EPS at an acidic pH. This contemplates to an antioxidant defense system which is non-enzymatic in nature (Alam et al., 2013). Fig. 6.5 depicts the total antioxidant capacity of a-EPS, Cm-EPS, s-EPS and BR2 control EPS, expressed in Vc equivalents where one unit of ascorbic acid equals 2 mg/mL.

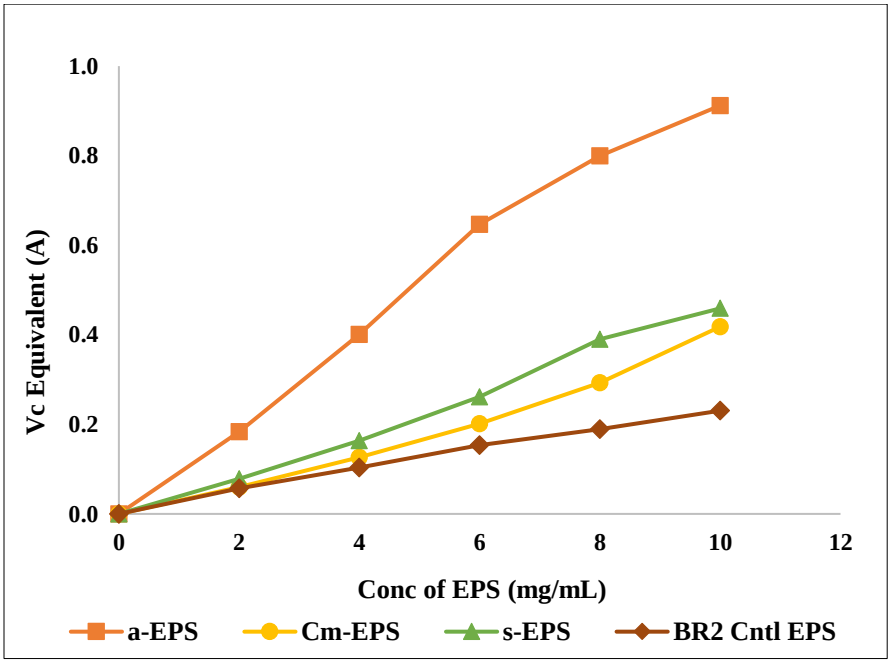


Fig. 6.5. Total Antioxidant Capacity of mEPSes in comparison with the control BR2 EPS

Data are represented as mean values with n=3

For all the EPS samples tested, the total antioxidant activity showed a dose-dependent pattern within a range of 2-10mg/mL. At the highest concentration used, antioxidant potential of a-EPS (50.78%) > s-EPS (25.56%) > Cm-EPS (23.28) > BR2 EPS (12.84) where the antioxidant activity potential of modified EPSes is higher with a-EPS exhibiting a 4-fold increase, s- EPS and Cm EPS with 2-fold increase in the total antioxidant activity than the BR2 control EPS at the

maximum concentration tested. Even at the lowest concentration used, a-EPS gave a 3-fold increase in activity with BR2 control EPS which signifies that the newly attached functional groups (acetyl groups) in the acetylated EPS contributed to this increased antioxidant activity. An antioxidant activity is associated with the breaking of the free radical chain to donate a hydrogen atom and an a-EPS allows to donate more electrons to quickly terminate the radical chain reactions. Also, the enhanced antioxidant activity of sulphated EPS is attributed to the addition of the electron-withdrawing groups to the EPS back bone. It is reported that, higher the presence of the electron-withdrawing groups like acetyl, sulphate or carboxyl groups in the EPS, lower is the energy required to dissociate the O-H bond and thus the total antioxidant capacity remains higher (Jin, Lu, Huang, Wang, & Wang, 2011). These findings validate our current observations in the study.

6.3.2.1.c. Reducing Power Assay

Among the various methods to analyze the antioxidant potential of EPS, one significant method is the reducing power assay. It investigates the reduction of Fe^{3+} to Fe^{2+} ion in the presence of the polysaccharides (Lobo et al., 2019). Fig 6.6 summarizes the reducing power potential of BR2 control EPS and all the chemically modified EPSes. It is expressed in terms of reducing power percentage calculated using Vc equivalents (ascorbic acid as standard). The reducing power of chemically modified EPSes was more pronounced in comparison with the native BR2 EPS but was lower than the ascorbic acid which is used as the reference. At 2 mg/mL concentration, the order of reducing power was observed to be s-EPS (41.39%) > a-EPS (37.43 %) > Cm-EPS (24.02) > BR2 control EPS (16%). From previous literature reports, it is evident that the reducing power attributes to the electron-donating ability. Hence the reason behind the higher activity in the present study is due to the introduction of chemical groups like sulphonyl

group, acetyl groups and carboxyl groups into the BR2 EPS and that enhances the electron-donating ability (Liu et al., 2012). The highest reducing power activity exhibited by sulphated EPS is 2.6-fold higher than the native EPS. This could be due to the electron-withdrawing abilities of the sulphate groups attached. Report suggests that sulfonation strengthens the reducing power and scavenging activities of the radicals *in vitro*, which is strongly related to the presence of $-OSO_3H$ groups and this in turn activates the H-atom of the anomeric carbon and thus increases the antioxidant activity (Li et al., 2016; Wang et al., 2015). Li and Shah in (2014) found that polysaccharides produced by *P. eryngii* and *S. thermophilus* showed higher radical scavenging activities and reducing power potential after sulphonation (Li and Shah, 2014). Polysaccharides of *Inonotus obliquus* on acetylation showed a hike in reducing power and inhibition of lipid peroxidation compared to its unmodified EPS (Li et al., 2016). Studies by Chouchane et al.,(2020) explored the possible use of a microbial carboxymethylated sulphated hetero-exopolysaccharide (CS-hEPS) as a potential anticancer agent. The investigation was carried out through antioxidant, antifatigue, and antiproliferative activities. CS-hEPS are a promising natural antioxidant, antifatigue, and antitumor harmless adjuvant materials that could be applied in human cancer therapy (Chouchane et al., 2020). Upon sulphonation, the attached sulphate groups affect the bioactivities of sulphated EPS as they actively participate in free radical scavenging reactions. For instance, in another study on an EPS from probiotic *Lactobacillus plantarum* ZDY2013, it was purified to illustrate its molecular weight, monosaccharide composition and biological activities. By sulfonation, a sulfated EPS was successfully synthesized and was confirmed using FT-IR spectroscopy. Both EPS and sulfated EPS showed radical scavenging activities, and the antioxidant activities increased after

sulfonation. Results from Liu et al.,(2019) suggested similar enhancement in antioxidant properties of *L. plantarum* WLP04 after sulphonation and was concentration dependent.

A reducing power activity of 37.43% by the a-EPS in this study is in correspondence with the antioxidant activity of the other acetylated exopolysaccharides which exhibited 30% activity as per the literature reports available. a-EPSes can donate a higher number of electrons and can rapidly terminate the radical chain reactions thus eventuating in the excessive antioxidant activity.

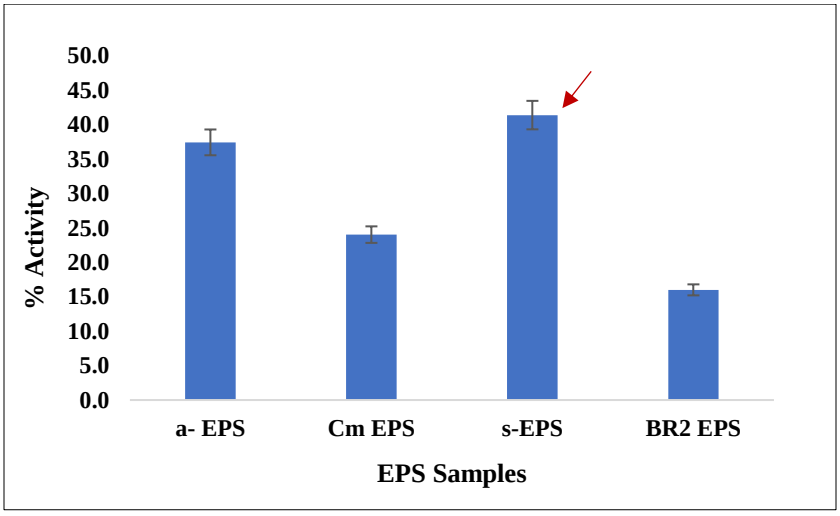


Fig. 6.6. Reducing power potential of EPS and its derivatives

Data are presented as mean values (n = 3). Arrow indicates the sample exhibiting highest reducing power potential.

6.3.2.1.d. Hydroxyl Radical Scavenging Assay

Hydroxyl radicals are known to have the potentiality of damaging nearly every biomacromolecules in the living cells. They are one of the most reactive ROS, with the ability to induce oxidative damage to cells ultimately resulting in aging, cancer, and other diseases. Therefore, abolishing these hydroxyl radicals is a crucial defensive mechanism in all living systems (Jin et al., 2011). The production of hydroxyl radical molecule is proportionate to the

concentration of metal ions (Fe^{2+}/Cu^{2+}) generated. The antioxidant molecule exhibits the ability to chelate these metal ions and thus make them inactive, shows their hydroxyl radical scavenging activity (Adebayo-tayo and Fashogbon, 2020; Li and Shah, 2014). In this study, the ability of hydroxyl radical scavenging activity of native BR2 EPS was compared with its modified EPS derivatives. From the experimental analysis depicted in Fig 6.7, the modified EPSes showed higher activity than the native BR2 EPS.

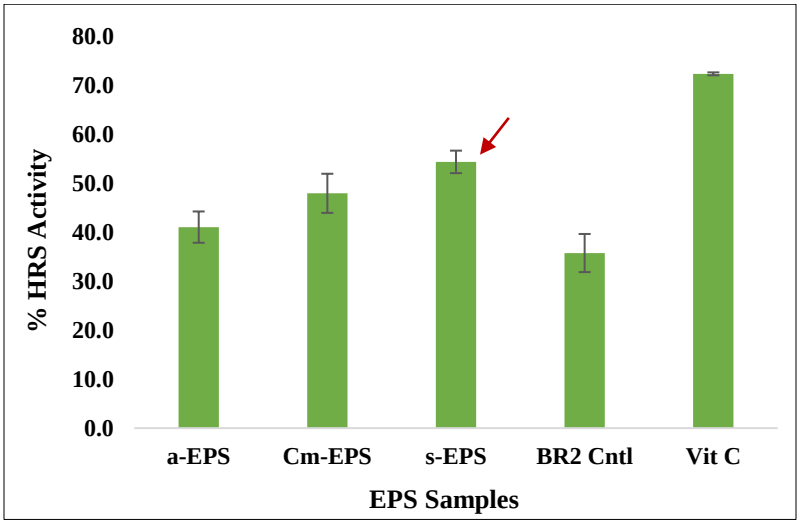


Fig. 6.7. Scavenging activity of EPS and its derivatives on hydroxyl radical

Data are presented as mean values where n = 3. Arrow indicates the sample exhibiting highest scavenging activity.

The hydroxyl radical scavenging activity was highest for the sulphated EPS (s-EPS) with 54.43%. Carboxymethylated EPS (Cm-EPS) showed 48.01% activity followed by acetylated EPS (a-EPS) 41.10%. The control, native BR2 EPS exhibited 35.80% scavenging activity. Overall, there was a 1.5-fold increase in the scavenging activity over native BR2 EPS after the sulphonyl group addition. This shows that the incorporation of the chemical groups like sulphonyl (-OSO_3H), acetyl (-CH_3CO) and carboxymethyl (-CH2-COOH) groups to the exopolysaccharide chain enhanced their ability to chelate the metal ions of Fe^{2+} or Cu^{2+}, thereby

inhibiting the generation of hydroxyl radical molecules. However, ascorbic acid used as the positive control showed the highest activity of 72.44% at a concentration of 2mg/mL. The results of s-EPS on the scavenging activity showed the significance of the sulphated group on chelation of metal ions generated during the reaction.

It was reported previously by Siqian Li and Shah, (2014) that the sulphated PEPS and sulphated ST1275 EPS showed higher scavenging activity than the non sulphated PEPS and ST1275 EPS produced by *P. eryngii* and *S. thermophilus* ASCC 1275, respectively. In another study, the EPS of *L. plantarum* ZDY2013 after sulphonation showed 49.7% hydroxyl radical scavenging activity, higher than the non- sulphated EPS (Zhang et al., 2016). The sulphated EPSes of *Enterobacter cloacae* Z0206 (SEPS -2, SEPS-3, SES-1, SEPS-8 and SEPS-6) possessed higher hydroxyl radical scavenging activity than the unmodified EPS (Jin et al., 2011). The higher activity for sulphated EPSes are correlated with their specific chelating groups within the molecule likely due to their nucleophilic character (Yuan et al., 2005).

The carboxymethylated (r-EPS1C) and acetylated EPS (r-EPS1A) of *L. plantarum* 70810 exhibited an improved scavenging activity about 1.5 fold and 1 fold respectively, than the unmodified (r-EPS1) EPS (K.Wang et al., 2015). These observations support our present study. The hydroxyl radical scavenging activity of *P. polymyxa* EJS-3 after acetylation was found to be 80.2% which was higher than what we have obtained (Liu et al., 2012) and this could be due to the number of hydroxyl groups and the variation in the composition contribute to difference in the scavenging activity of the antioxidant molecules (Zheng et al., 2016).

Specifically, sulphated modifications were effective and enhanced the antioxidant activity of polysaccharides produced by *E. cloacae* Z0206, and the sulphated derivatives of these

polysaccharides may act as potent antioxidant agents. The protective effects of sulphated polysaccharide derivatives produced by *Enterobacter cloacae* Z0206 against H_2O_2-induced oxidative damage in RAW264.7 murine macrophages as well as the possible mechanisms governing the protective effects were well studied. Sulphated polysaccharides protected RAW264.7 cells from oxidative damage and apoptosis induced by H_2O_2 by protecting the cellular structure; improving the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px); and inhibiting caspase-3 activation and DNA fragmentation (Jin et al., 2014). These results and observations showed that the chemical modifications and incorporation of specific chemical moieties to the EPS backbone could enhance its hydroxyl radical scavenging activity.

As mentioned, suitable structural and chemical modifications were found to aggrandize their antioxidant potential by boosting their water solubility, hydrogen donating ability, chelating ability, and higher electron donating capacities. Intensifying the chemical nature of EPS especially through sulfonation, carboxymethylation, selenylation, acetylation and phosphorylation can increase the physiochemical properties and could impose higher antioxidant activity through amplification of protons is schematically represented in Fig. 6.8.

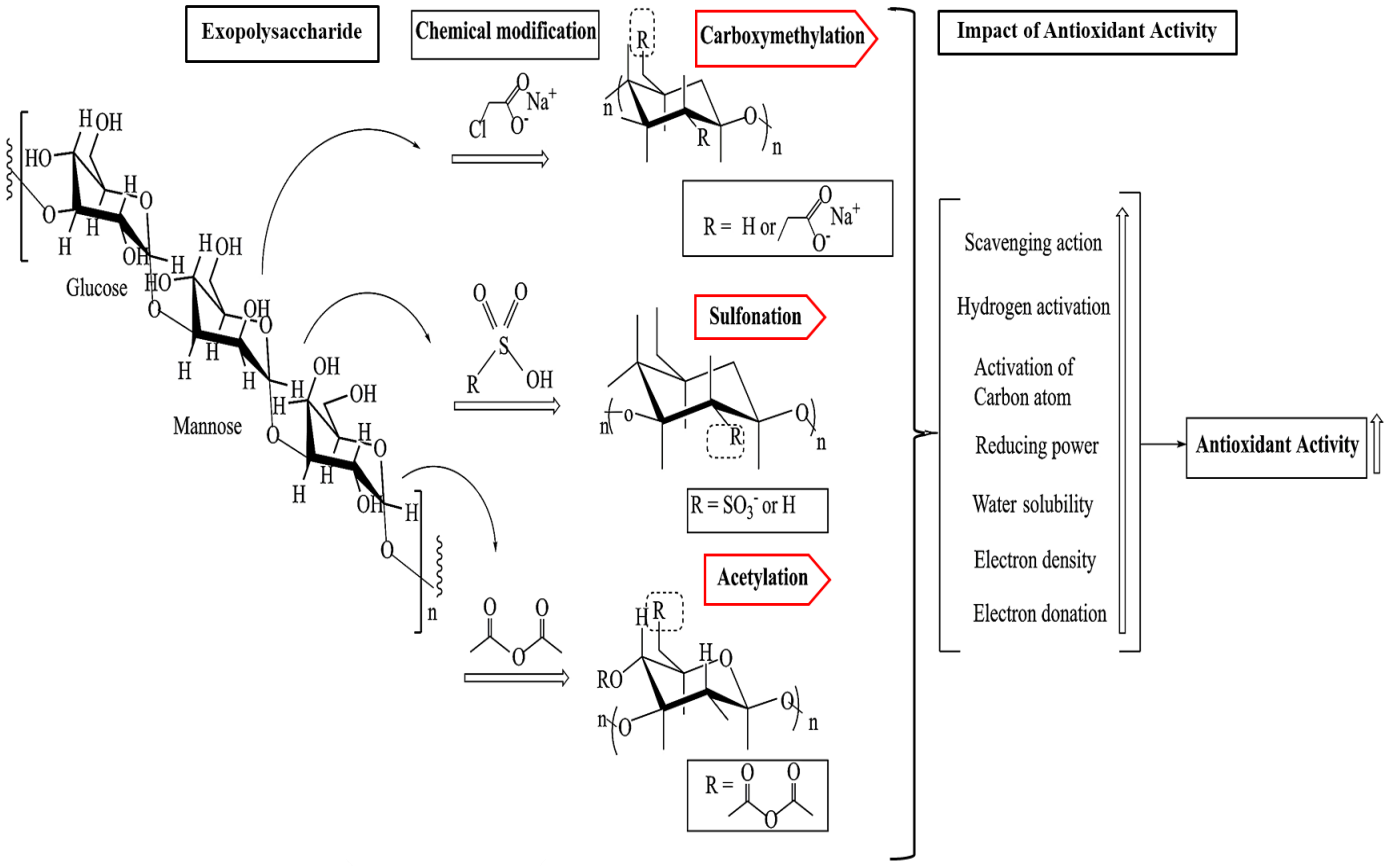


Fig. 6.8. Chemical modifications of EPS and its impact on their antioxidant activities

6.3.2.2. Cholesterol Lowering Assay

BR2 EPS as such possesses cholesterol lowering activity by adsorbing the cholesterol and reducing it in the supernatant in the above-mentioned protocol. From Fig. 6.9, the cholesterol lowering activity of native BR2 was found to be 31.6%. The effect of the cholesterol lowering property of the EPS after chemical modifications was also tested. The observations showed that the sulphated and carboxymethylated derivatives of EPS had a lowering activity of 40% and 34.5%, respectively.

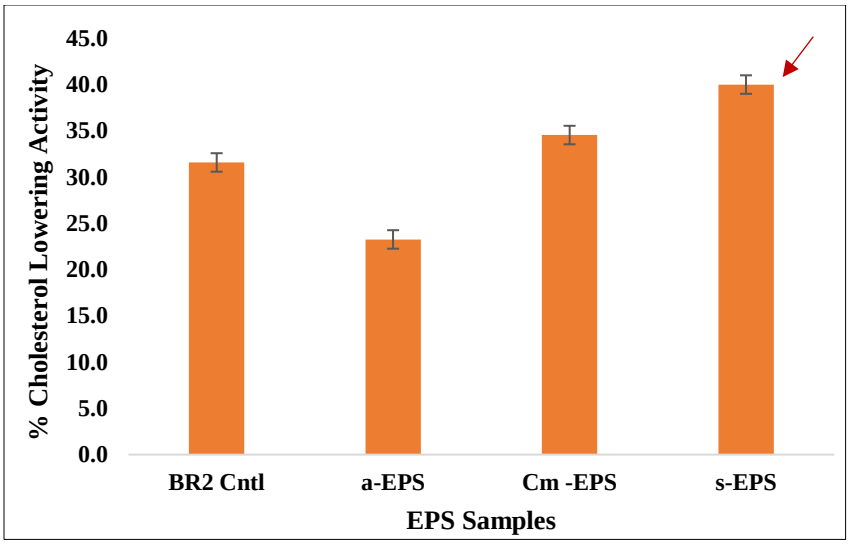


Fig. 6.9. Cholesterol lowering activity of mEPSes

Data are presented as mean values (n =3). Arrow indicates the sample exhibiting best cholesterol lowering activity.

This corresponds to a 1.3-fold and 1-fold increase in the cholesterol lowering activity than that of the control unmodified EPS. But the cholesterol lowering activity of a-EPS was 0.7-fold decreased than that of the control EPS. It is anticipated that the EPS derivatives would show variations in the cholesterol lowering activity due to the addition of different chemical groups to the native EPS backbone. This enhanced activity of the mEPS can be explored by incorporating it in functional foods to reduce the cholesterol levels as they also exhibit good antioxidant activities.

6.3.2.3. Cytotoxicity Studies

Considering food safety and application of EPS in development of functional foods, cytotoxicity analysis of EPS plays a vital role. The viability of normal L929 fibroblast cells in presence of native BR2 EPS and mEPSes were studied separately. The LD 50 value of all the mEPSes were greater than 150 µg/mL. a-EPS exhibited 177 µg/mL, Cm-EPS with 184.10

µg/mL and s-EPS with 184.10 µg/mL LD 50 values. The phase contrast images of the L929 cells after EPS treatment did not show any aggregation or elongation of the cells. The cell morphology resembles the same as that of the untreated control cells. From the data depicted in Table 6.2, overall, the cells treated with EPS and its derivatives exhibited more than 70% cell viability even at the highest EPS concentration which signifies the non-toxic behaviour of the exopolysaccharide on normal cells.

Table. 6.2. Cell cytotoxicity analysis. Expressed as the percentage cell viability of the normal L929 fibroblast cells in presence of mEPSes and native BR2 EPS (control)

EPS Concentration (µg/mL)	***a-EPS***	***Cm-EPS***	***s-EPS***	***BR2 Control EPS***
Control	100 + 0.005	100 + 0.005	100 + 0.005	100 + 0.005
6.25	95.52 + 0.003	94.05 + 0.009	91.08 + 0.007	97.67 + 0.002
12.5	91.43 + 0.008	83.89 + 0.002	90.97 + 0.012	93.22 + 0.004
25	83.24 + 0.007	76.81 + 0.010	84.91 + 0.020	91.18 +0.002
50	77.53 + 0.008	74.07 + 0.009	79.29 + 0.009	83.9 + 0.001
100	71.17 + 0.004	65.78 + 0.006	70.1 + 0.006	75.83 + 0.011

Each value in the table is mean ± SD of 3 trials.

6.3.2.4. Anticancer effect of mEPS

The anticancer activity of the control EPS was higher as the MCF-human breast cancer cell lines showed decreased cell viability with the highest concentration of BR2 control EPS and other EPS derivatives, Fig. 6.10.

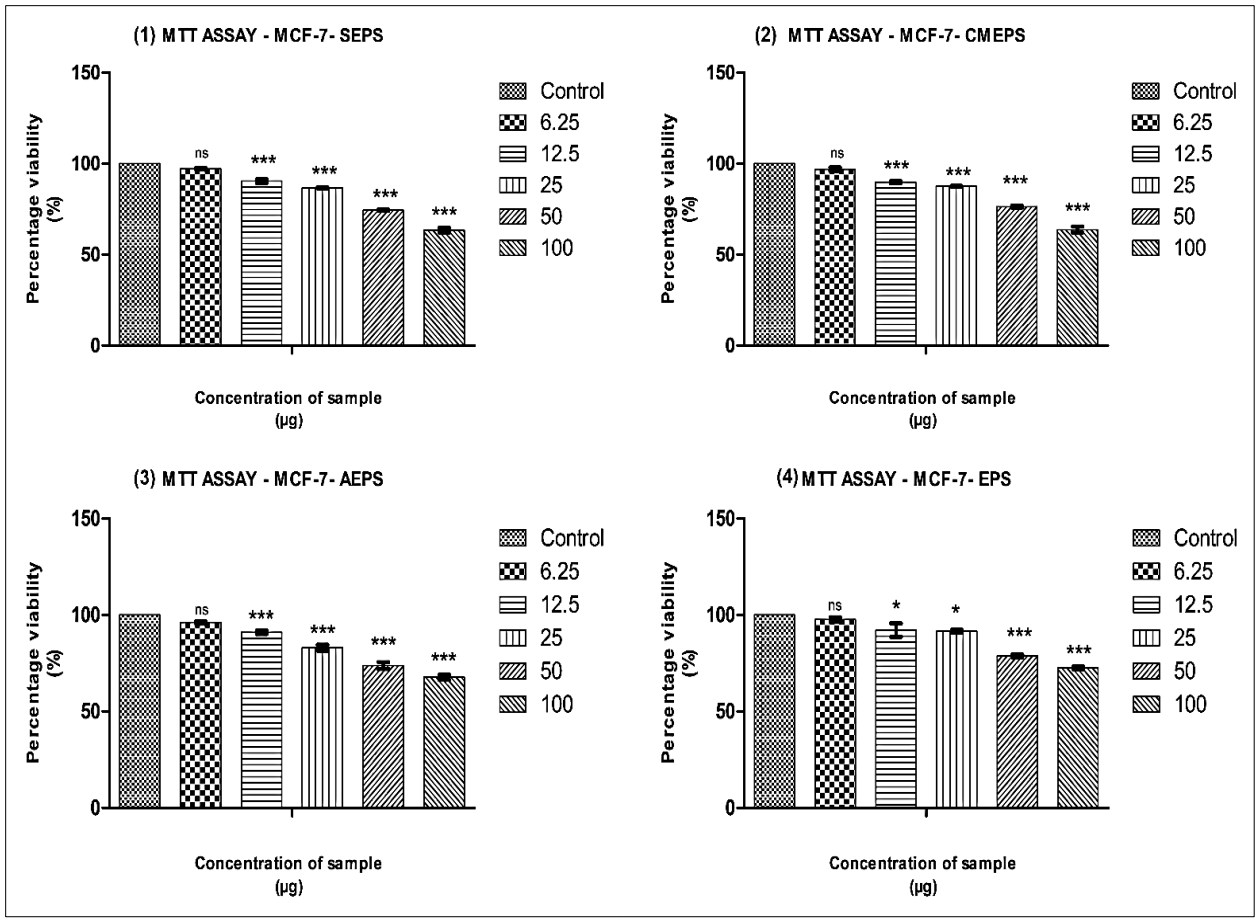


Fig. 6.10. Anticancer activity of mEPSes and BR2 control EPS on MCF-7 breast cancer cell lines. Expressed as the percentage cell viability after EPS treatment along with the control **(1)** s-EPS treated **(2)** Cm-EPS treated **(3)** a-EPS treated **(4)** BR2 control EPS treated MCF-7 cell lines. Data are mean ± SD of the triplicate assays. Data with ns = not significant and *** are significantly different at $p < 0.05$.

The LD50 (lethal dose) value was higher for BR2 EPS with 178.72 µg/mL. The LC 50 value of mEPS in comparison to the native BR2 EPS was lower and was in the order s-EPS>Cm-EPS>a-EPS. This showed that s-EPS is more cytotoxic to MCF breast cancer cell lines with a minimum concentration of 132.82 µg/mL. In general, mEPSes showed more cytotoxicity to breast cancer cell lines than the unmodified BR2 control EPS. The phase contrast images

showing the aggregation and elongation of the MCF-7 cells after mEPS treatment is shown in Fig. 6.11.

Numerous studies have revealed the improvements and changes in the biological activity of different chemically modified EPSes. The changes lead to different physicochemical properties and structural conformations. It was found that the anti-tumor activity of many polysaccharides significantly increased after the modification of polysaccharides (Fujiike et al., 2022; Wang et al., 2015; Xie et al., 2020). Acetylation, carboxymethylation and sulfonation of EPS played important roles in antitumor activity of polysaccharides. These attached functional groups can change the extension of polysaccharides chains, leading to the exposure of polysaccharides to hydroxyl groups and increasing their solubility in water.

The amount and location of these groups have significant influence on the anti-tumor activity of polysaccharides. In a study with *Lactobacillus plantarum* ZDY2013, after sulfonation, showed enhanced antioxidant activity compared to native EPS. In addition, sulfated EPS was more effective in counteracting the cytotoxicity induced by *B. cereus* enterotoxins on Caco-2 cells when compared with EPS. In summary, they proved that, sulfonation is a feasible strategy for improving the biological activities of EPS from *L. plantarum* ZDY2013 (Zhang et al., 2016). Xie et al.,(2020) explains a higher anti-tumor activity against human hepatic cancer cell line HepG2 exhibited by sulphated EPS derivatives.

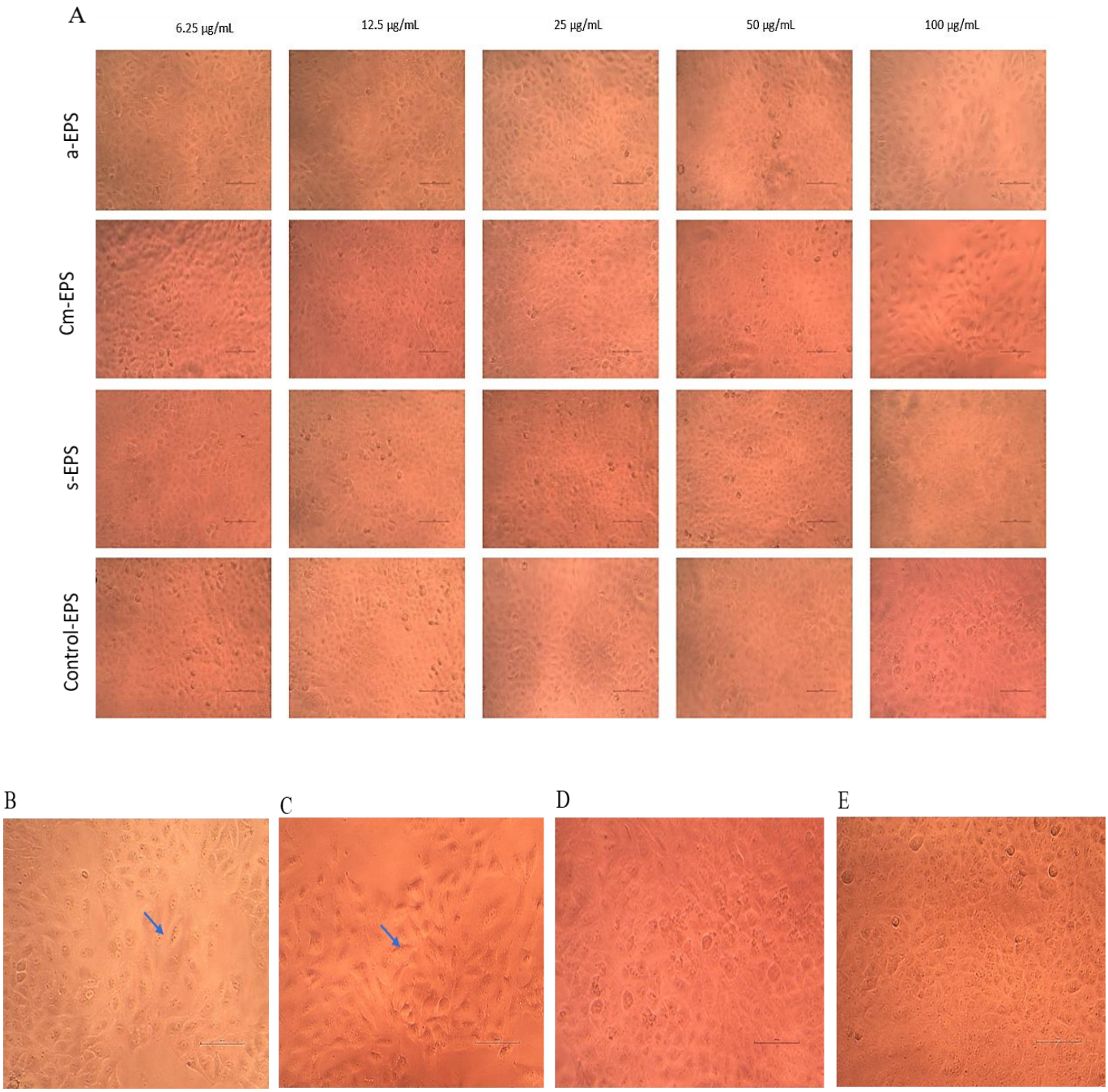


Fig. 6.11. Phase contrast microscopic (PCM) images of mEPSes and BR2 control EPS on MCF-7 breast cancer cell lines (A) at different EPS concentrations **(B)** PCM image of s-EPS (100 µg/mL) and **(C)** Cm-EPS (100 µg/mL) showing aggregation or elongation of the MCF-7 breast cancer cells (marked in blue arrows) **(D)** BR2 control EPS (100 µg/mL) treated and **(E)** MCF-7 untreated control cells showed no such aggregations

The antitumour activity of EPS from *Pediococcus pentosaceus* M41 has already been reported (Ayyash et al., 2020b) which supported our observations. Chen et al., (2014) and co-workers showed that carboxymethylated polysaccharides of *Ganoderma lucidum* inhibited the *in vitro* proliferation of Sarcoma 180 (S-180) tumor cells in a dose-dependent manner. It was also found that the acetylated levan from endophytic bacterium *Paenibacillus polymyxa* EJS-3 had strong antiproliferative activity against human gastric cancer BGC-823 cells *in vitro* (Xie et al., 2020).

Some natural polysaccharides have no antitumor activity, but their antitumor activity is induced after modification. However, at present, research on the anti-tumor activity of polysaccharides and its derivatives are still limited to superficial studies, and the mechanism of anti-tumor action need further clarifications. The antitumor activity of modified polysaccharides is closely related to the position, degree and spatial conformation of the substituents of modified polysaccharides.

6.4. Summary

This chapter describes the synthesis of three derivatives of native *L. plantarum* BR2 EPS by chemical modifications namely acetylated (a-EPS), carboxymethylated (Cm-EPS) and sulphated (s-EPS) EPSes. The structural characterization and analysis were studied by FTIR and NMR spectra. The primary structure of the EPS derivatives when compared with the control showed corresponding variations in the functional groups according to the modifications carried out. The repercussion of the chemical modifications enhanced the overall antioxidant activities of the EPS. Of the three derivatives, a-EPS exhibited the highest DPPH radical scavenging and total antioxidant activity in a dose-dependent manner. At all tested concentrations, a-EPS showed higher scavenging activity, and a maximum activity of 73.81% at 2 mg/mL. Meanwhile, s-EPS

showed the highest reducing power potential and the hydroxyl radical scavenging activities. At 2 mg/mL concentration, the order of reducing power was observed to be s-EPS (41.39%) > a-EPS (37.43 %) > Cm-EPS (24.02) > BR2 control EPS (16%) and the hydroxyl radical scavenging activity for the s-EPS was 54.43%. The highest reducing power activity exhibited by s-EPS is 2.6-fold higher and a 1.5-fold increase in the scavenging activity of native BR2 EPS after the sulphonyl group addition was observed. The increase in these activities is due to the addition of various functional groups that contributes largely to the scavenging abilities of different free radicals. Moreover, the s-EPS and Cm-EPS derivatives also exhibited an increased cholesterol lowering activity of 40% and 34.5% respectively than the native EPS. From the cell lines studies, we could conclude that the EPS and its derivatives were not cytotoxic to the normal cells with 65-70% cell viability as they did not interfere with the cell growth and viability.

Exopolysaccharides being used as food thickeners and stabilizing agents in various food preparations in recent times, the modified EPS with the enhanced biological properties can contribute greatly to the nutritional benefits of these foods and food products.

Chapter 7

Chapter 7

Development and Evaluation of EPS Fortified Probiotic Functional Yogurts

7.1. Introduction

The demand for functional meals has increased as the food industry develops and consumers' awareness of the benefits of eating wholesome foods grows. Since they are regarded as a complete food and almost entirely comprise the required nutrients, dairy products play a key role in the human diet in this regard. The most practical and affordable method to increase a product's functionality is through fermentation (Chand et al., 2021). Functional and probiotic fermented foods have become the primary choice of consumers due to the awareness occurred for their contribution to gut health and immune health. In light of this, large-scale manufacture of various milk products including fermented milk products has begun. Fermented cheese, fermented milk beverages, yogurt, fermented milk desserts, and fermented ice creams are some of the probiotic rich fermented dairy products.

Yogurt has achieved market dominance and gained popularity on the international market as a probiotic food carrier because of its high viability and the compound growth rate of yogurt products is raised to 4.8% (Chandan et al., 2017). Smooth texture, adequate viscosity, nice flavour, and fermentation acidification are all qualities that yoghurt products possess. Recently, these characteristics have been viewed as a crucial criterion for choosing a yoghurt starter (Kılıç et al., 2022). Unlike other fermented milk products, yogurt contain high soluble protein, free amino acids, non-protein nitrogen (Chandan et al., 2017) and vitamins such as riboflavin, niacin,

folate, thiamine and vitamin A due to the action of starter culture while fermentation, which help in performing additional body functions and boosting immune system. But traditional yogurt such as Greek style yogurt has high fat content of 10%. According to studies by Janahi et al., (2018) and Ji et al., (2022), dairy products can lower cholesterol, modulate the immune system, reduce diabetes, alleviate lactose intolerance, treat diarrhea, and treat inflammatory bowel illnesses.

Due to increased demand of consumers for healthy and fat-reduced products have pushed the market to use fat substitutes by adding stabilizers and additives such as pectin, starch and gelatin, carrageenan for ensuring the sensory properties of the dairy products. For every fermented milk and dairy products reducing the fat content is a big challenge because reduction in number of fat globules can alter the sensory qualities, firmness, texture and flavour of the foods especially in yogurt. Approaches to meet the demand of manufacturing all-natural, quality food products without compromising the health benefits lightened the investigation on adding EPS or EPS producing starter culture to the foods (Guler-Akin et al., 2009). To add a starter culture, adaptation ability and EPS production capacity of the culture in milk (Zhu et al., 2019) along with the antagonistic activity of culture with possible probiotic bacteria present in the gut environment and viability of the culture during fermentation and storage has to be validated (Mohammadi et al., 2012). Most commonly used strains as starter culture are *Bifidobacterium bifidum, B. longum, B. lactis, B. breve, L. reuteri, L. rhamnosus* (Mohammadi et al., 2012) *L. plantarum* (Zhu et al., 2019) and S*treptococcus thermophilus* (Feldmane et al., 2013). Exopolysaccharide produced during the fermentation produces makes the yogurt slimy or viscous gel with fermented aroma and flavor. The EPS production of each LAB varies, but on an average 144.0 to 440 mg/L during symbiotic lifecycle of starter culture (Feldmane et al., 2013).

The EPS production during the fermentation with natural culture is 13 – 170 mg/L (Mende et al., 2013), which indicates the selection of starter culture and their symbiotic life cycle is an important criteria for the yogurt quality.

Exopolysaccharides can ensure the firmness of the yogurt by elevating the viscoelastic property of diary product through their interaction with protein networks present in the yogurt and also helps to reduce wheying off in dairy products by holding water molecules to retain the moisture content and loss of soluble nutrients. EPS concentration can enhance the ropiness and extension ability of low-fat yogurt foods (Ng et al., 2022). Ng et. al (2022) proved that EPS production of *L. lactis sub. cremoris* APL15 has improved the textural properties and sticky characteristic of the low fat yogurt which in turn ensures the stabilizing potential of the EPS (Ng et al., 2022). EPS makes the yogurt more viscous and shinier with product flavor and aroma. The structural characteristics of EPS are important in their functional properties. High molecular mass EPS can enhance the thickening ability of the product. Charged EPS can increase the interaction ability of EPS with surrounding protein and also able to resist the shear stress, thereby increase the viscosity of the product. The pseudoplasticity of the EPS guarantees mouthfeel of the yogurt by improving the mixing ability and pourability. This enhances the gelling quality which in turn elevates the smoothness and flavour of the yogurt. The thermostable characteristic of the EPS does not alter the functional property of the food while processing even at higher temperature.

This chapter deals with the supplementation of EPS in the development of probiotic functional yogurts using skim milk by fermentation employing an indigenous LAB starter culture. The study looked at how adding *L. plantarum* BR2 as culture and as EPS affected the syneresis, biochemical characteristics, texture, and viscosity of yoghurts throughout the course of

a 21-day storage period. The antioxidant activity of the EPS was further examined in detail to explore the possibility of developing functional foods with defined biological role.

7.2. Materials and Methods

7.2.1. Materials and reagents

Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, gallic acid, and β-catechin, Braford reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Phenolphthalein indicator, pectin, carboxymethyl cellulose (CMC), corn starch, sodium alginate, and Skim milk used to prepare yogurt, were purchased from Hi-Media (India). All chemicals were of analytical grade.

7.2.2. Microorganisms and growth conditions

This chapter deals with the use of an indigenously isolated probiotic starter culture *Streptococcus thermophilus* CUD3 (Suresh and Nampoothiri, 2022) to ferment skim milk for yogurt preparations. As discussed in chapter 2 (2.1.1), *S. thermophilus* CUD3 and *L. plantarum* BR2, stored as glycerol stocks at -80°C, were activated by sub-culturing in M17 and MRS media respectively (Hi-media Chemicals, India) at 37°C. For fermentation experiments cultures were prepared by inoculating 1% (v/v) of each activated culture in 10 mL fresh aliquots of respective broth and incubated at 37°C under static conditions until an OD_{600} of 0.9-1.0 is attained. The total inoculum concentration for the skim milk fermentation was fixed to be 3% (v/v). The individual curdling activity of both *S. thermophilus* CUD3 and *L. plantarum* BR2 in skim milk along with their co-culturing ability were tested to ensure their non-antagonistic behavior.

7.2.3. Preparation of yogurts

Freshly prepared inoculum of *S. thermophilus* CUD3 was used as the starter culture to prepare the skim milk yogurts. 12% (w/v) skim milk prepared freshly in sterile distilled water without any lumps was heated to 95°C for 20-30 min and allowed to cool down to 40°C. Further, the pasteurized skim milk was inoculated with 3% (v/v) of overnight grown *S. thermophilus* CUD3 under aseptic conditions in sterile containers and incubated at 37°C for 6-8 hours until complete coagulation happened, and pH dropped down to 4.0. *S. thermophilus* and *L. plantarum* BR2 were combined (1:1) and inoculated (3% v/v) into skim milk for some experiments where *in-situ* production of EPS is required. Yogurt preparations were done according to Lee et al., (2021) & Zhu et al., (2019) with minor modifications and the different yogurt combinations used in the study have been mentioned in Table 7.1.

Table. 7.1. Yogurts prepared and its composition

Sl No	Yogurt Type	Inoculum used	Form of EPS Incorporated	Sample Code
1	Starter Culture Alone (Control Yogurt)	*S. thermophilus* CUD3	No EPS	Y (Control)
2	Starter culture + EPS	*S. thermophilus* CUD3	Lyophilized *L. plantarum* BR2 EPS	Y+EPS(L)
3	Starter culture + EPS producing culture	*S. thermophilus* CUD3 and *L. plantarum* BR2	*In-situ* EPS production	Y+EPS(BR2)

***Y**-Yogurt; **EPS(L)**-Lyophilized EPS; **EPS(BR2)**-EPS provided as L. plantarum BR2 culture*

Post fermentation, the yogurt samples were refrigerated at 4°C for up to 21 days and samples were withdrawn aseptically from the tubes as and when required for various physiochemical analyses.

7.2.4. Effect of EPS on syneresis of yogurts

The syneresis index of yogurt samples, were determined using the spontaneous whey separation by centrifugation method adapted by Amatayakul et al., (2006) and Nikitina et al.,(2022). Briefly, 10 g yogurt was prepared in centrifuged at 3000g for 10 min at 4°C. The resulting clear supernatant was carefully collected and weighed to determine the amount of excluded water to determine the syneresis according to the following equation:

$$\% \text{ Spontaneous whey separation} = \frac{W_i - W_f}{W_f - W_c} \times 100$$

Where W_i is initial weight (g) of the tube with yogurt and W_f is final weight (g) of the tube with yogurt after whey removal and W_c is weight (g) of the empty tube.

The experiments were done in triplicates in two or three sets and the standard deviation was calculated and expressed from individual experiments.

7.2.5. Determination of pH and titratable acidity (TA)

The pH of the skim milk before and after fermentation was measured using pH indicator paper in the range of 3.5-6.5 and 2.5-4.5 respectively. Titratable acidity (TA) of the yogurts were determined as described by Kılıç et al., (2022) with minor modifications. 1g of yogurt sample was weighed and homogenized it with 9 mL of distilled water. After adding three drops of 1% phenolphthalein (1 g of phenolphthalein in 100 mL of ethanol 95%) as the indicator, the yogurt solution was titrated against freshly prepared 0.1N NaOH until the solution become a faint pink in color constantly for 30s. The TA of each of the sample was determined by the following equation:

TA% = [(10 x V. NaOH x 0.009 x 0.1) /W] * 100

where, 10 = dilution factor, V. NaOH = volume of NaOH used to neutralize the lactic acid, 0.009 = conversion factor (1 mL NaOH (0.01 N) neutralizes 0.009 g of lactic acid), 0.1 = Normality of NaOH, and W = weight of sample

7.2.6. Extraction and quantification of EPS from yogurts

EPS extraction from yogurt were done according to procedures reported earlier with minor modifications as and when required (Huang et al., 2019; Kim et al., 2020). Briefly, 100 g yogurt was diluted twice in distilled water (200 mL) and adjusted the pH to 4.6 with 2M NaOH solution to precipitate the casein and further removed it together with the microorganism by centrifugation at 10,000 rpm for 20 min at 4°C. Consequently, the supernatant whey fraction was neutralized with additional amount of NaOH solution and heat treated in boiling water bath for 30 min and the insoluble protein was removed by centrifugation (10,000g, 4°C, 20 min). After the addition of double volume ethanol to the clear supernatant, it was incubated at 4°C overnight and the resulting precipitate was recovered by centrifugation, 2500g for 20 min at 4°C and the recovered EPS was finally lyophilized using Christ Alpha 1-2 LD Plus, (Germany).

7.2.7. Viable cell counts (VCC) of starter culture in yogurt

1mL of yogurt samples were mixed with 9 mL of sterile distilled water. Then, serial dilutions were prepared up to 10^8 using sterile distilled water under aseptic conditions (Cai et al., 2019; Weerasingha et al., 2021). The viability of the *S. thermophilus* CUD3 was enumerated by using the spread plate method. Required dilutions (10^4 ,10^5 ,10^6 and, 10^7 of the yogurt samples were spotted on the M17 agar plates and kept undisturbed for drying. In yogurts containing both *S. thermophilus* CUD3 and *L. plantarum* BR2, the serial diluted samples were plated in both

M17 and MRS agar plates to enumerate the bacterial counts. All plates were incubated at 37°C for 48h and the viable cell count was calculated as follows:

CFU/mL = Number of colonies formed X dilution factor of sample/1 mL of sample

*CFU: colony-forming unit.

7.2.8. Preparation of yogurt water extracts

For the preparation of yogurt water extracts, protocols by Shori et al., (2022) was followed, where 10 g of each yogurt samples were homogenized with 2.5 mL distilled water. Further, the pH of all the samples were acidified to a pH of 4.0 by addition of 0.1M HCl and incubated in a water bath for 10 min at 45°C. After incubation, the samples were centrifuged at 5000g at 4°C for 10 min and then the pH of the supernatants were adjusted to neutral pH using 0.1M NaOH. The samples were again centrifuged at 5000g, maintaining 4°C for 10 min and the clear supernatant obtained was the yogurt water extract, stored at 4°C and used for further analysis.

7.2.9. Determination of antioxidant activity by DPPH radical scavenging assay

150 µL DPPH reagent (0.1mM) dissolved in 95% ethanol was added to 150 µL of different yogurt water extract samples. The mixture was incubated in the dark at room temperature for 20 min. The reaction was carried out in transparent flat bottomed 96 well plates in triplicates. Absorbance at 517 nm was measured against control (150 µL of ethanol instead of the extract) using a spectrophotometer (Tecan Nano Quant Infinite M200 Pro, Switzerland) (Kim et al., 2020; Shori et al., 2022). The radical scavenging activity was calculated as below:

Scavenging activity (%) = $[1– (A_{sample}–A_{blank})/A_{control}] *100$

The experiments were done in triplicates in two or three sets and standard deviation was calculated from individual experiments.

7.2.10. Apparent viscosity

The viscosity of yogurt samples and shear rate were measured at 25°C by using a programmable rotational viscometer (Atron Paar Visco QC, Austria). The flow curves of the yogurt samples were obtained by varying the shear rate from 10-100 and 110-200 (s−30) and corresponding viscosity values (Pa-s) were measured and plotted (Ayyash et al., 2020a; Yilmaz et al., 2015).

7.2.11. Sensory evaluation

Sensory evaluation of yogurts were performed according to the previously reprts by researchers with slight modificatiosn as per our requirements (Ardabilchi et al., 2019; Kim et al., 2020; Shori et al., 2022). The sensory evaluation of the yogurt samples was done on the first day of post acidification, successfully carried out by a 17-member untrained panel committee consisting of 7 males and 10 females belonging to the age group between 25-55 years old. The panelists tested all the yogurt samples during the sensory study, which was completed within 20 minutes. During these sessions, panelists discussed and agreed on the definitions and the way to qualify the attributes in a scale using commercial natural yogurts while following the recommendations of the International Dairy Federation (International Dairy Federation [IDF], 1997. The evaluation system was mainly categorized into three:

(1) considered six parameters of the yogurt samples: texture, color, aroma, flavor, taste, and overall acceptability,

(2) texture descriptors like consistency, smoothness and creaminess. Each of these parameters were assessed based on a 10-point hedonic scale (10 = excellent, 9 = highly acceptable, 8 = good & acceptable, 7 = moderately acceptable, 6 = acceptable if improved, 5 = must improve, 4 = moderately unacceptable, 3 = unacceptable, 2 = poor & unacceptable,1 = rejected) and

(3) characteristics like syneresis, lumps presence, and flavor attributes like rancid taste, bitter taste, old taste and mouth feel were evaluated based on a 5-point hedonic scale (5 = very high, 4 = high, 3 = moderately high, 2 = no or less, 1 = very less or not at all). Test samples were presented to the panelists with a single - alphabet code in a randomized order, without revealing their original compositions for a genuine evaluation and at 10 °C after 1 and 21 days of storage at 4°C. All tests were conducted in duplicate and in a standardized room.

7.3 Results and discussion

7.3.1. Post Acidification Activity (pH and titratable acidity (TA))

For yogurt preparations, 12% skim milk (v/v) was acidified with 3% (v/v) of the starter culture *Streptococcus thermophilus* CUD3 (Genbank Accession No: MT176494) and noticed that the curdling happened after an incubation period of 6 hours at 37°C. The probiotic property and specifically the milk clotting activity of *Streptococcus thermophilus* CUD3 has been reported earlier by our research group (Suresh and Nampoothiri, 2022). The initial pH of the skim milk was in the range of 6-6.5 which is slightly acidic but more to be neutral. After fermentation, the pH of yogurt dropped down to a pH ranging between 3.5-4.5. The bacteria utilize the available sugars in the skim milk fermenting it into lactic acid and other organic acids thus resulting in a drop in pH. The accumulation of lactic acid acidifies the milk resulting in its coagulation. Starter cultures with probiotic potential will be an added advantage and the

probiotic bacteria can also influence the sensory and antioxidant properties of yogurt (Kim et al., 2020).

Skim milk inoculated with *L. plantarum* BR2 alone could not curdle the milk in 12 hours as it exhibited least curdling property and thus its use as a starter culture was significantly low. The co-culturing of both *Streptococcus thermophilus* CUD3 and *L. plantarum* BR2 did not exhibited any antagonistic activity and thus can serve as a consortium for preparation of skim milk yogurt to evaluate the in-situ EPS production.

As a preliminary analysis, the TA of yogurt with EPS, yogurt with EPS producing culture and control yogurt which contains only the starter culture, were estimated at the end of 3rd day post fermentation and the changes of pH in yogurts during storage at 4 °C. Further the TA of yogurts with a higher and a lower concentration of EPS along with the control yogurts from day 1 - 21 of post acidification was evaluated and has been depicted in Fig. 7.1.

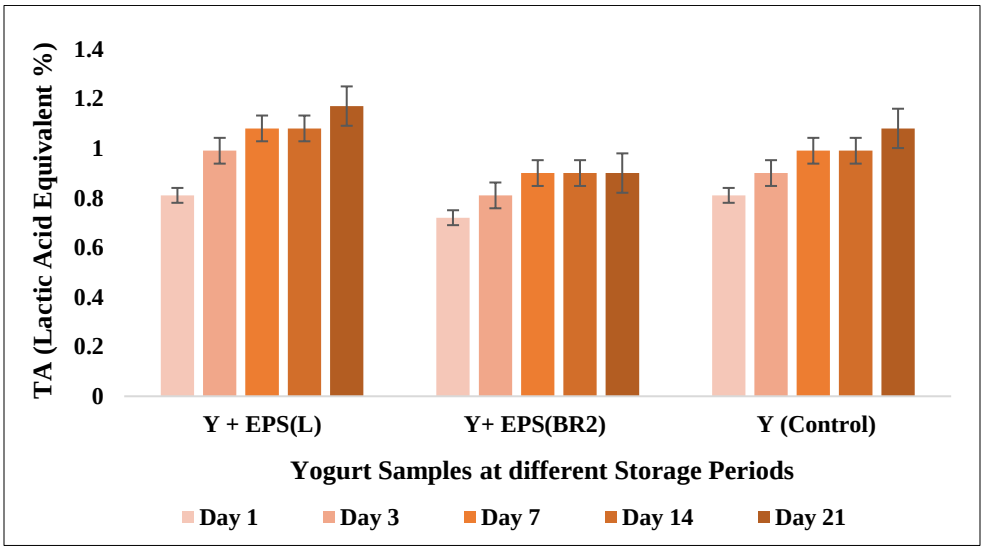


Fig. 7.1. Titratable acidity of yogurts with EPS compared with the control post acidification from Day 1 – Day 21

Y*-Yogurt;* ***EPS(L)****-Lyophilized EPS;* ***EPS(BR2)****-EPS provided as L. plantarum BR2 culture*

These results show that the EPS containing yogurts gave a higher TA than the control. The yogurts showed a sustained reduction in pH post-acidification during 21 days of refrigerated storage (3.5-4.5 ± 0.5). In contrast, TA increased during refrigerated storage enhanced more ($p > 0.05$). However, at the end of 21st day, the TA was in the order Y +1% EPS (1.2) > Control (1.0) > Y + 0.1% EPS (0.9) yogurt. The metabolic activity of the viable bacteria in the yogurt samples aided to further acidification during extended storage and refrigeration. The enzymatic hydrolysis and production of certain secondary metabolites by the LAB with the utilization of the fermentable sugars present in the skim milk during refrigeration (0-5°C) resulted in the accumulation of various metabolic by-products by the bacteria such as lactic acid, acetoin, EPS etc.

According to Behare et al., (2013) there was no discernible difference between EPS-producing cultures and non-EPS cultures in terms of titratable acidity. However, Güler-Akin et al., (2009) discovered that both EPS-producing and EPS-non producing strains had an impact on the pH value and titratable acidity of the yogurt. For an EPS-producing strain compared to a non-EsPS-producing strain, a longer period was needed to reach pH 4.5. When an EPS-producing culture is added to yogurt, it affects both the production of lactic acid and EPS. As a result, it took longer to reach pH 4.5 than the yogurt with the non-EPS generating culture. However, yogurt that has been mixed with EPS powder may have more acidity than yogurt that hasn't. Here, EPS just serves as an additional carbon source, giving the yogurt a slightly higher acidity than yogurt without EPS. EPS either directly affects the texture of the yogurt or encourages the production of other metabolites by yogurt starter cultures. Additionally, when added in the right amounts, EPS can lessen syneresis and improve the texture of fermented dairy products in addition to serving as a substrate for fermentation (İspirli et al., 2019). Thus, in our study also,

the yoghurts showed a progressive decrease in pH during storage. Y + 1% EPS showed the lowest pH after 21 days of storage followed by control yogurt. Similarly, a continuous decrease in pH was reported with probiotic yogurt containing *B. lactis B. animalis* and *B. longum* (Tiwari et al., 2021).

7.3.2. Syneresis Effect of EPS supplemented yogurts

Syneresis or whey separation occurs in coagulated milk products and is caused by the aggregation and sedimentation of protein particles during the storage period. Syneresis, which is considered as a defect in the yogurt quality, is observed to be more prominent without a stabilizer added to the yogurt matrix (Rashid and Dutta, 2020). When the stabilizers were added to yogurt, whey separation was reduced compared to that in yogurt without any stabilizer. Stabilizers usually aid to firmness and consistency of yogurt and are thus used to stabilize the structure and viscosity of yogurt. Therefore, the effect of the addition of EPS as stabilizer was studied, compared by treatment of the skim milk yogurts with different stabilizers namely, sodium alginate, pectin, carboxymethyl cellulose (CMC), sucrose, and lyophilized EPS (Fig. 7.2). From the experiment it was observed that the EPS incorporated yogurt samples gave the lowest syneresis at the given stabilizer concentration. The whey separation was lowest for EPS treated samples, and in general the yogurt samples exhibited syneresis in the increasing order of EPS < Control < CMC < sodium alginate (NaAl) < Pectin < Sucrose < Corn starch.

This may be a result of the propensity of yogurt protein matrix to bind to water in the presence of EPS (Güler-Akin et al., 2009). Casein precipitates in yogurt as a result of hydrophobic interactions and creates a continuous network with voids containing serum and bacterial cells. It is seen that the EPS surrounds the starter bacterial strains, allowing cells to adhere to the protein matrix via a web of filaments thus reducing the syneresis.

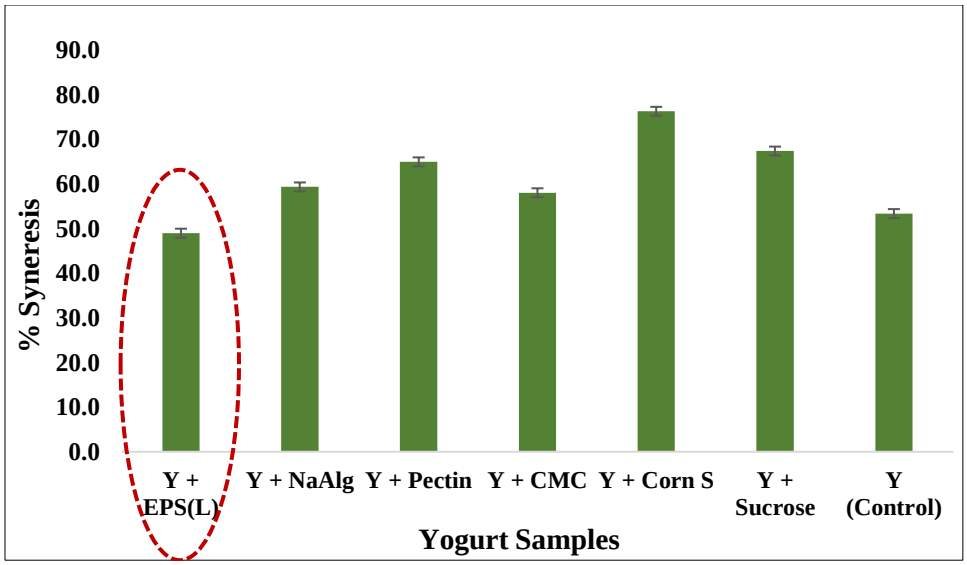


Fig. 7.2. Effect of different stabilizers in syneresis of yogurt

Y*-Yogurt;* ***Y+ NaAl*** *– Yogurt with sodium alginate;* ***Y+Pectin****- Yogurt with pectin;* ***Y+CMC****-Yogurt with carboxymethyl cellulose;* ***Y+Corn S****- Yogurt with corn starch;* ***Y+sucrose****- Yogurt with sucrose;* ***EPS(L)****-Lyophilized EPS*

The amounts of whey isolated from yogurts marginally decreased during cold storage. In set-type yoghurt studies by Güler-Akin et al., (2009) and Macit et al., (2019) found comparable outcomes. The levels of whey separation in the experimental yogurts also showed a negative connection with fat content. The low solids content may have contributed to the incidence of whey separation.

The next part of the study was to optimize the concentration of lyophilized EPS in yogurt with the least syneresis. From the experimental analysis, yogurt with 0.2% EPS (w/v) exhibited a decreased syneresis by 10.96% . As the concentration of EPS was increased, the percentage syneresis increased, which is unfavorable. The values of syneresis in the yogurts with different EPS concentration measured during storage are shown in Fig. 7.3.

The primary noticeable textural flaw of yogurt that arises during storage and has a detrimental effect on customer acceptance is syneresis (whey separation). The results of the current study showed that there was a significant ($p<0.05$) difference in syneresis among the yogurts with varying EPS concentration. This might be because of EPS's capacity to bind free water and alter the yogurt gel during the coagulation process. Syneresis typically results from the diminished capacity of yogurt gel to completely entrap the serum phase as a result of the network's thinning (Lucey, 2002). Thus, yogurt gel strengthening can lower the syneresis (Prasanna et al., 2013; Weerasingha et al., 2021).

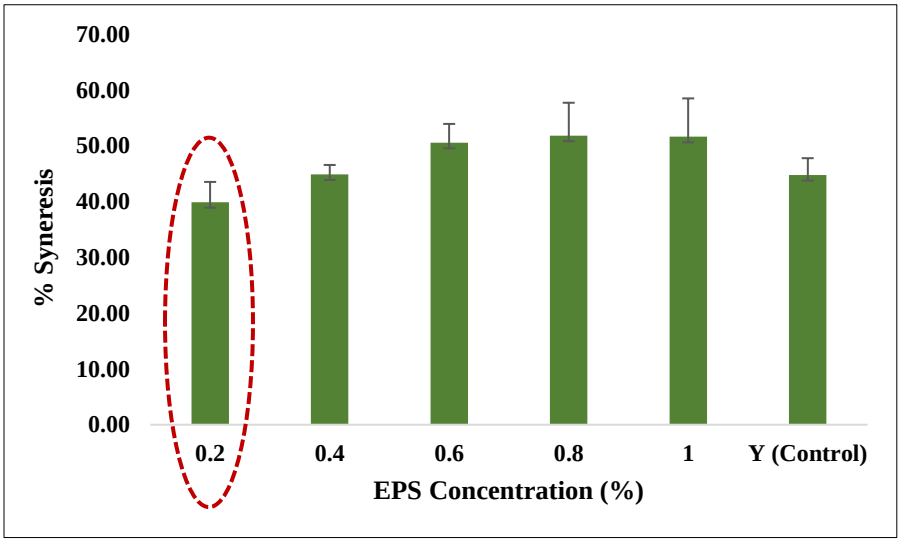


Fig. 7.3. Syneresis effect of yogurt with varying EPS concentrations

The water holding capacity of yoghurt is primarily the quantity of water carried by the protein structure and the increase in WHC means increased hydration of the protein network. The relationship between syneresis and water holding capacity are inversely proportional. Lower the syneresis, higher the water holding capacity of yogurts. The difference in WHC of the yogurts may be attributed to the properties of the different proteins present in them. Interactions

of water with proteins are very important in food systems because of their effects on the flavour and texture of foods. Intrinsic factors affecting water holding capacity of food proteins include amino acid composition, protein conformation and surface polarity/ hydrophobicity. Stabilizers have two basic functions in yogurt i.e., the binding of water and improvement in texture. Stabilizers bind with water to reduce water flow in the matrix space and some may interact with protein in the food matrix, further increase hydration behavior (Thaiudom and Goff, 2003).

In another study by Pan et al., (2022), In order to gauge the stability of the set yoghurt, water-holding capacity was examined and they observed that the WHC of set yogurt significantly increased after addition of XG5 EPS. The fact that XG5 EPS binds water into the milk base, strengthening the stability of set yoghurt, was likely the origin of this event. The level of water hydration is further increased by the interaction of XG5 EPS with the components of milk, particularly the proteins.

7.3.3. EPS quantification

For evaluating the *in-situ* EPS production by *L. plantarum* BR2, it was inoculated along with the starter culture during the yogurt preparations. Fig. 7.4 represents the EPS production post acidification at different storage period compared with that of the control yogurt. The EPS production in yogurt with *L. plantarum* BR2 was higher compared to that of the control yogurt at all time intervals with a maximum of 3.6 ± 0.14 g /L at the end of 21 days of storage which is greater than the earlier reports by Prasanna et al., (2012).

In various studies around the globe, Researchers have reported different EPS concentration in yogurt made using EPS-producing strains ranging from 40 to 400 mg/L (Güler-Akin et al., 2009; Leroy and De Vuyst, 2004). In another study, a similar level of EPS

production (2.2 ± 0.1 g/L) was obtained during batch culture of *Lb. rhamnosus* RW-9595M in a supplemented whey medium containing 11% whey solids, 1% yeast extract, and some minerals (Doleyres et al., 2005). The use of various strains and the degree of inoculation of starter cultures, variations in fermenting conditions, and the methods of isolation, purification, and quantification of EPS are a few potential explanations for the discrepancy between these investigations.

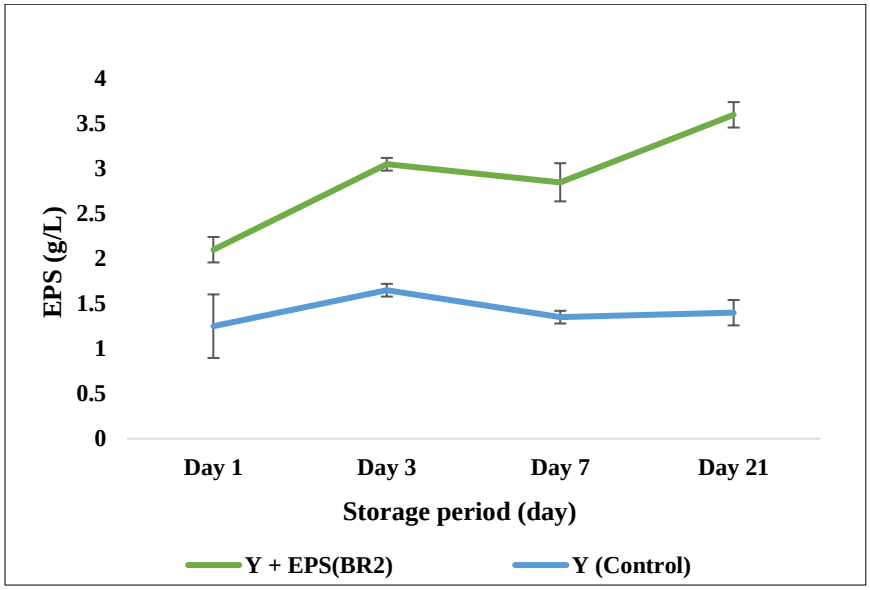


Fig. 7.4. *In-situ* EPS production in yogurts

***Y**-Yogurt; **Y+EPS(BR2)**-Lyophilized EPS*

Several interactions could take place between EPS, proteins, and yogurt protein aggregates when EPS is added to milk before fermentation, produced *in situ* by the culture, or added after the protein gel network is formed. Consequently, the *in-situ* EPS production also finds role in regulating the viscosity, rheology, and other sensorial characteristics of yogurts. Firmness, syneresis, or microstructure may be impacted by factors other than EPS synthesis in systems using in situ EPS since fermentation time and acidification rate are highly strain-dependent (Mende et al., 2016).

7.3.4. Viable cell count of the LAB

The viable cell count (VCC) of starter culture, *Streptococcus thermophilus* CUD3 was determined and was represented as a decadic logarithm of colony forming units per mL (log CFU/mL) in each of the yogurt samples (Table 7.2). The viable load of the bacteria was estimated on day 1, day 3, day 7, day 14 and day 21 of the refrigerated storage of the yogurt samples. The yogurt samples maintained a vital force of bacterial count at a level of at least 9 (log CFU/mL) along all storage period from day 1, post acidification, till the end of storage period (21 days) at 4°C in both yogurt with lyophilized EPS and the control samples.

Table. 7.2. Viable cell count of *S. thermophilus* CUD3 in Y+EPS(L) and control yogurt samples from day 1- day 21 of post acidification

Sample	Day 1	Day 3	Day 7	Day 14	Day 21
Yogurt + EPS(L)	9.78 ± 0.16	10.15 ± 0.53	9.56 ± 0.38	9.78 ± 0.23	9.73 ± 0.71
Control Yogurt	9.51 ± 0.56	9.54 ± 0.18	9.45 ± 0.09	9.62 ± 0.64	9.35 ± 0.79

This is due to the ability of *S. thermophilus* CUD3 to utilize all kinds of sugars and micronutrients found in the skim milk and it appeared as minute pinpoint colonies when plated on M17 agar plates. There was a significant difference ($p < 0.05$) between means of *Streptococcus thermophilus* CUD3 counts for yogurt samples containing EPS and the control yogurt and that was due to the ability of EPS to promote the bacterial growth in its presence. However, the extended cold storage of the yogurt at 4°C slightly reduced the VCC of the starter culture in the yogurt irrespective of the presence or absence of EPS. Chand et al., (2021),

reported a similar probiotic count of *S. thermophilus* LR_74 (9.10 ± 0.10 log CFU/mL) by the end of incubation time (8 hours) than other samples in a study on low-calorie symbiotic yogurt from indigenous probiotic culture and combination of inulin and oligofructose.

The cell viability displayed a different pattern in yogurt that contained both starter culture and the EPS-producing *L. plantarum* BR2, where in-situ EPS production occurs (Fig. 7.5). *S. thermophilus* CUD3's VCC was initially observed to be higher than *L. plantarum* BR2 with 9 log CFU/mL. The VCC of *L. plantarum* BR2 grew as storage time was extended, while the VCC of the starter culture decreased and they appeared as white ropy colonies after incubation in MRS agar plates after serial dilution and plating. This reiterates the justification for the earlier-mentioned increase in in-situ EPS production. The co-culturing of these bacteria is what caused the decline and alteration in viable cell counts. Prolonged storage to 21 days resulted in a reduction in *S. thermophilus* VCC but an increase in *L. plantarum* BR2 might be due to its ability to produce EPS at a nutrient deficient condition which the starter culture is unable to do. More investigation and study is required to understand the proteolytic activity titer of the bacterial culture in yogurt samples during prolonged storage to assure the health benefits of probiotic yogurts. As per the literature reports, a probiotic food product should be able to serve at least a minimum level requirement of viable bacterial count ranging between 10^6- 10^7 CFU/mL until the expiry of the product (Shori, 2013). This finding was reconfirmed in the present study with *S. thermophilus* CUD3 viability reached above 10^7 CFU/mL. The observed viable bacterial count differences in each of the yogurt samples could be due to several factors such as milk type, composition of the milk, dry matter content, temperature and time for pasteurization of milk, starter culture used and its properties, and storage conditions of the final yogurt products.

(A)

(B)

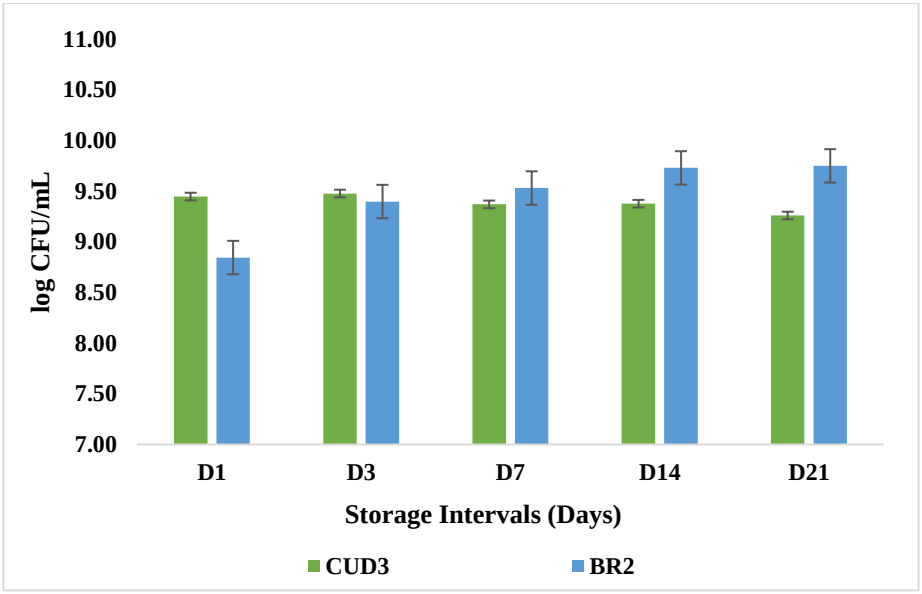


Fig. 7.5. (A) Co-culturing of *S. thermophilus* CUD3 and *L. plantarum* BR2 for antagonistic activity check (B) Viable cell count of *S. thermophilus* CUD3 and *L. plantarum* BR2 in Y+EPS(BR2). ***D****-Day;* ***CUD3*** *– Starter culture;* ***BR2****- EPS producing culture*

To deliver health benefits and improve the gut microflora, the appropriate viable load of the bacterial cultures in the yogurts is mandatory. To ensure this, there is a necessity to maintain these live bacteria to survive throughout the shelf-life period prior to being consumed. Edible ingredients such as sucrose, pectin, carboxymethyl cellulose or compound from plant (dextrose) or exopolysaccharides as prebiotics were found to enhance the viability of probiotics in dairy

products. However, the increased concentration of organic acids is one of the important factors that can dramatically affect bacterial growth (Shori, 2013). Thus, the reduction of the bacterial viable cell count observed in yogurts is associated with the post-acidification which caused further reduction in pH during extended storage. The increased viable cell count of *S. thermophilus* CUD3 during the initial days of refrigeration and storage for all the yogurt samples prepared is consensus to the previous studies reported.

7.3.5. Antioxidant activity

The DPPH radical scavenging activity of each of the yogurts were determined using their yogurt water extracts prepared from day 1 to day 21. The DPPH activity of day 1 was significantly highest for yogurt containing EPS. Nevertheless, during the extended storage, a significant reduction ($p < 0.05$) in DPPH scavenging activity of control yogurt was observed with the least radical scavenging activity observed at 21^{st} day of storage. Furthermore, the presence of EPS in the yogurt resulted in the highest DPPH scavenging activity than the control throughout the storage time (Fig. 7.6) from day 1 to day 21 with significantly less difference in their activity. The refrigerated storage had little effect on the scavenging activities of yogurt with EPS samples even though, these yogurt samples showed variations in the scavenging activity at day 3, 7, 14 and 21, their activities were similar and were not significantly different in the radical scavenging activity (RSA) percentage. Additionally, the microbial metabolic activity during fermentation and refrigerated storage, resulting in milk protein proteolysis and organic acids production also contributes to the antioxidant activities of the yogurt samples.

According to several reports, the molecular weight and content of polysaccharides may influence their antioxidant capacity. include glycosidic bonds, composition, molecular weight of EPS etc. Overall, the findings indicated that XG5 EPS may have weak antioxidant action *in vitro*

(Pan et al., 2022). In reality, the presence of whey proteins in yogurt and the resulting EPS-protein interaction molecules, coupled with EPS, can boost the product's antioxidant profile when yogurt and EPS are combined.

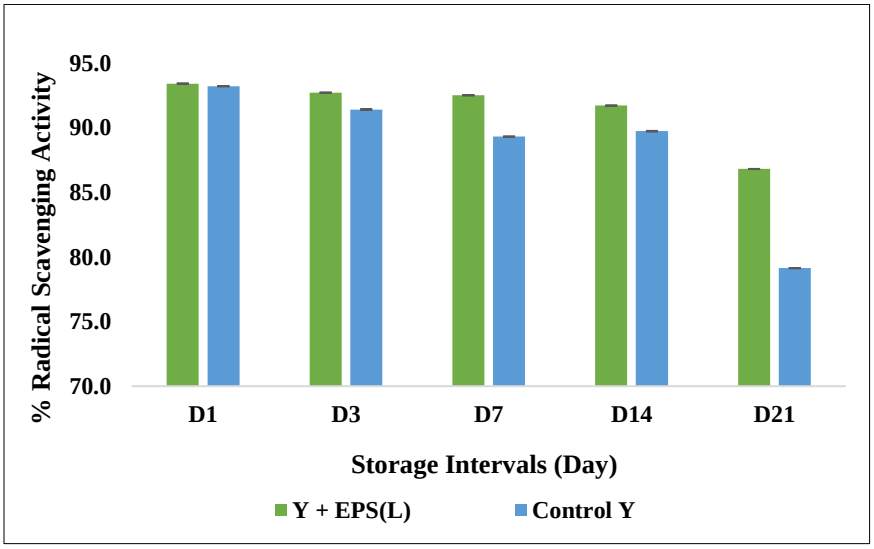


Fig. 7.6. Antioxidant activities of yogurts at different time intervals. DPPH radical scavenging activity of yogurt water extracts of Y+EPS(L) from day 1 to day 21

Data are presented as mean ± SEM with n=3. DPPH radical scavenging activity of yogurt ***D****-Day;* ***Y+EPS(L)****– Yogurt with lyophilized EPS;* ***Control Y****-Starter culture alone*

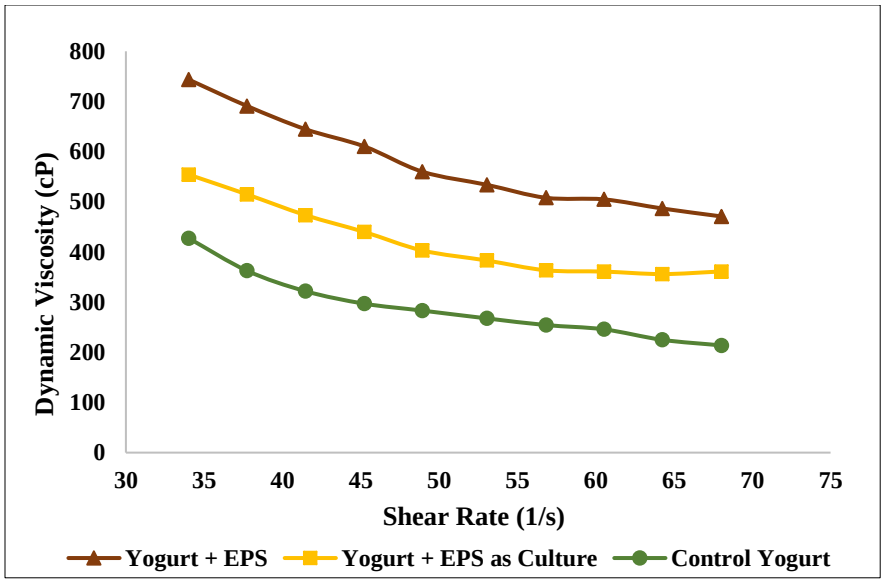
7.3.6. Viscosity analysis

One of the primary metrics used to assess fermented milk and characterize a fluid's resistance to flow is viscosity. Numerous elements, including molecule size, structure, and interactions, are known to have an impact on viscosity. A key element in the development of yoghurt texture, according to reports, is the interaction between EPS and milk proteins. Through the van der Waals force and electrostatic repulsion, interactions between milk proteins and EPS may lead to the development of a dense network structure with increased elasticity (X. Li et al., 2020).

The viscosities of the yogurts were determined using a rotational viscometer and the viscosity curves of these samples were drawn using the dynamic viscosity verses the shear rate and is depicted in Fig. 7.7. The resistance to movement of one layer of a fluid (here yogurt) over another defines its dynamic viscosity and shear rate is the rate of deformation of the liquid and the apparent viscosity (sometimes denoted η) is the shear stress applied to a fluid divided by the shear rate. From the graph it is clear that the yogurt with lyophilized EPS exhibited a greater viscosity than the other samples followed by yogurt with EPS producing culture. The control yogurts were the least viscous among the three. Thus, the order of viscosity was found to be in the decreasing order of: Y+EPS(L) > Y+EPS(BR2) > Control yogurts.

The reasons for higher viscosity can be attributed to the presence of higher EPS content present the yogurts. Thus, a direct correlation between the total amount of EPS and the viscosity and consistency of the corresponding yogurts was established. In addition to this effect, fat contents in the milk or yogurts inversely affects the viscosity of the yogurts. A proper homogenization of the milk can enable the coating of fat globules with protein molecules present in the milk which attributes the formation of gel structure during the fermentation process ensuring an increased viscosity/consistency of the yogurt samples. During the fermentation of skim milk, lactic acid bacteria aggregate the protein strands into three-dimensional structures, through isoelectric precipitation, which gives an even consistency and viscous behaviour to yogurts. Additionally, these protein fibres within the yogurt samples, formed by weak electrostatic and hydrophobic interactions, when broken down by shearing decreases the size of aggregated proteins resulting in lower viscosity of the formed yogurts (Gamli and Atasoy, 2018).

A)



B)

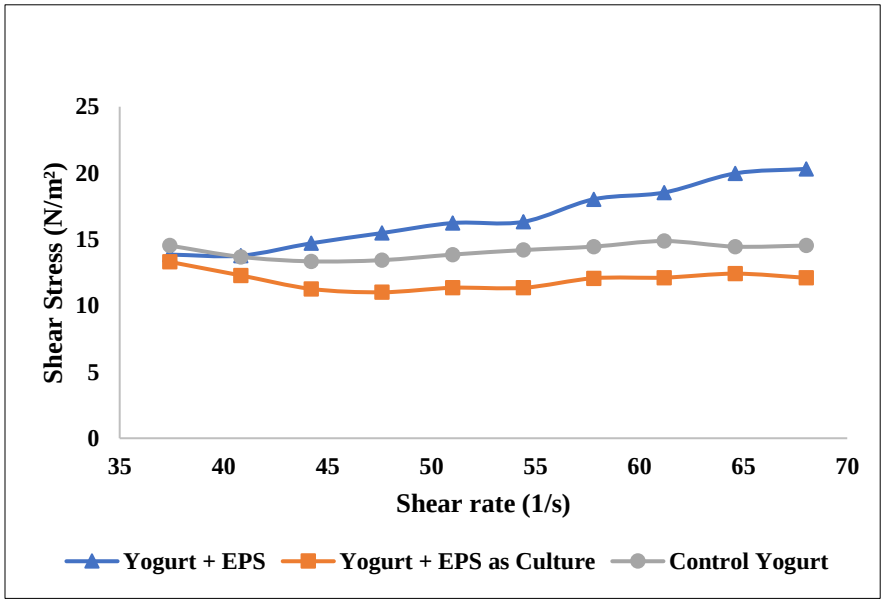


Fig. 7.7. Viscosity curves of yogurts

(A) Apparent viscosity and **(B)** Flow curve of yogurts

7.3.7. Sensory evaluation

For a food product to be acceptable by the consumers, sensory evaluation plays a vital role in the food industry and its applications as it enables to provide quality-assured products in the market. Sensory evaluation of yogurt samples was conducted by panellists where they were

asked to evaluate the consistency, smoothness, creaminess, texture, colour, aroma, flavour, taste and overall acceptability of the fresh yogurts stored at 4°C based on a 10-point hybrid hedonic scales (Ibarhim and Khalifa, 2015) and is summarized in Fig. 7.8.

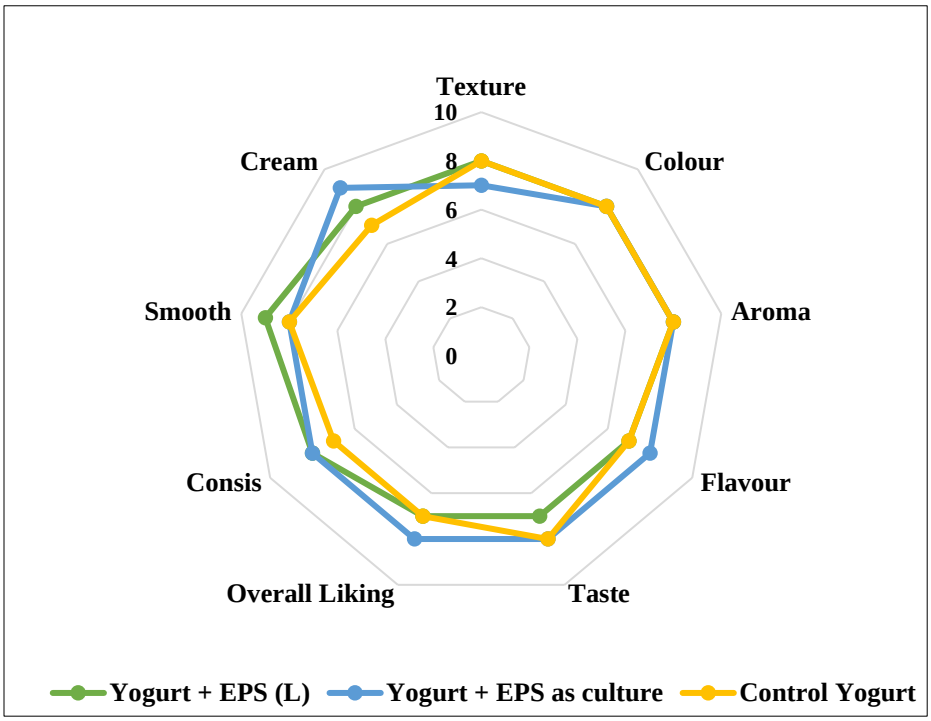


Fig. 7.8. Evaluation of organoleptic properties and texture descriptors (consistency, smoothness, creaminess) of yogurts

It was found that mean overall quality scores of yogurts were all higher than 6 (acceptable) and the texture, colour, aroma, and flavour of the yogurts obtained mean scores above 7 (moderately acceptable). Meanwhile the taste scores of all yogurts were evaluated to be equal to 8 or above. The development of EPS incorporated yogurts exhibited excellent textural and sensorial properties with enhancing the homogeneous, creamy, and white appearance of yogurts.

Considering the textural properties, consistency and smoothness, yogurt with lyophilized EPS was widely accepted by the panellists followed by yogurt with EPS producing culture. Y + EPS (BR2) yogurts achieved higher scores in creaminess, flavour and taste. Taste buds and olfactory receptors work together to sense flavour, and flavour is closely tied to food preferences and quality attributes (Kim et al., 2020). Sensory perception, particularly, taste, is the most important dimension of quality evaluation in a consumer's purchase criterion and in the current study, the best scores for taste were attained by yogurt with EPS in either form. Compared with the control. The preference of taste positively affected the overall acceptability of yogurts. The consumer liking and the overall acceptability of the yogurts differed greatly based on the type and amount of protein present in the milks. This further translated to the acceptance of the yogurts. From the detailed texture descriptor analysis shown in Fig. 7.9 and Fig. 7.10, was revealed that, EPS containing yogurts exhibited least amount of syneresis compared with the control which had the maximum syneresis. The mouthfeel of Y+EPS(L) had the highest score due to the presence of EPS.

These results are supported by the finding of Chand et al., (2021), where they demonstrated that a combination of inulin and oligofructose gave a more viscous and less syneresis effect on yogurts. The findings of GÜler-Akin et al., (2009) imply that exopolysaccharide-producing cultures may produce textures for low-fat yogurts that are superior to those produced by additions like stabilisers or fat substitutes. To best utilise EPS application in dairy products and to comprehend its behaviour in samples more resembling the commercial yogurts now on the market, additional studies based on modifications in the matrix (raising the amount of total solids in milk or fat content, for example), are required (Elisa C. Ale et al., 2016).

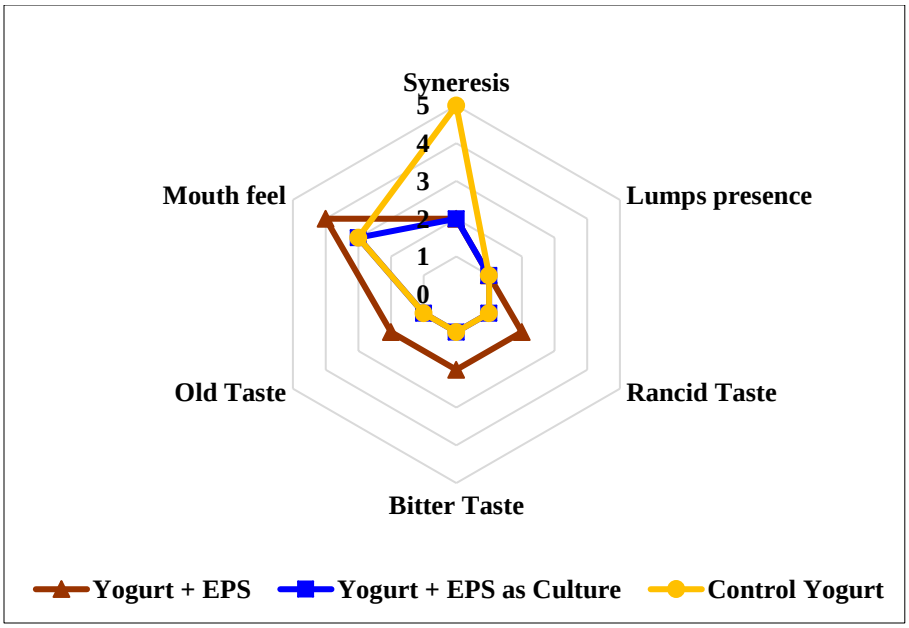


Fig. 7.9. Evaluation of weaknesses of yogurts
(Syneresis, lumps presence, mouthfeel, old, and rancid tastes)

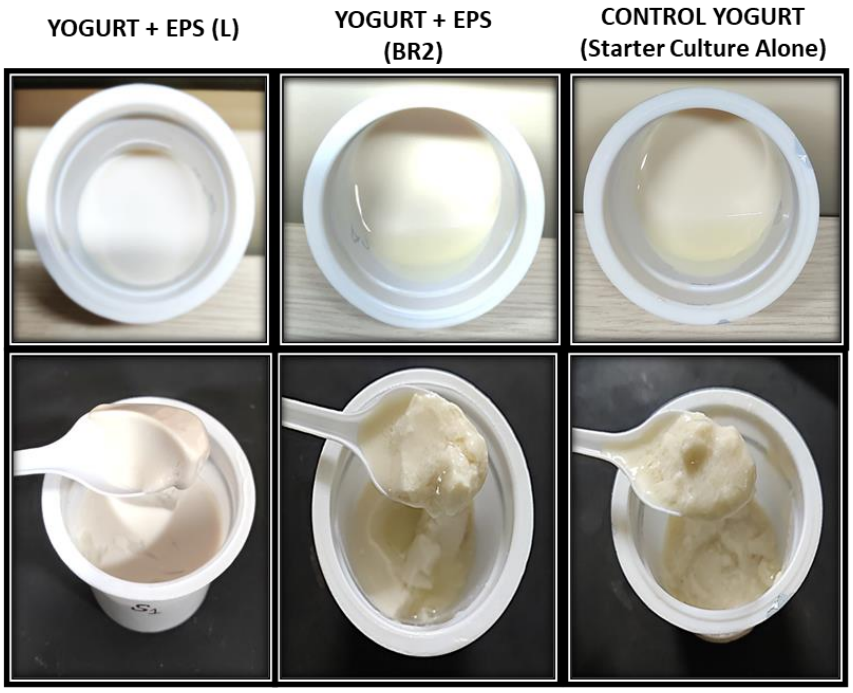


Fig. 7.10. Syneresis of yogurts with and without the addition of EPS

Yogurt +EPS(L)*-Lyophilized EPS;* ***Yogurt +EPS(BR2)****-EPS provided as L. plantarum BR2 culture;* ***Control Yogurt****- With starter culture alone*

In order to fully understand this polymer's influence on textural characteristics from a molecular perspective, future studies will validate its fundamental structure as well as how it interacts with the food matrix. All the studies done demonstrated the potential of EPS to be employed as techno functional natural element, and it should be recognised that its impact on yogurt texture improvement is being evaluated as well, with promising preliminary results. No yogurts exhibited bitter, rancid, or old taste beyond the minimum.

7.4. Summary

From the above discussed results, it is observed that skim milk with *S. thermophilus* CUD3, as the starter culture is able to acidify the skim milk in a minimum curdling time of 6 hours at 37°C. After fermentation, a higher rate of acid production, probiotic count, antioxidant activity, viscosity and *in-situ* EPS production were evaluated. The presence of the viable cells in the yogurts accelerated the reduction of pH and promotion of titratable acidity in yogurt during storage. When compared to their control samples, the incorporation of EPS boosted the probiotic count (9 log CFU/mL), textural qualities, and sensory evaluations of the yogurt. The dynamic viscosity curve of the yogurts showed an increased viscosity for EPS containing yogurts, and this attain the highest score for texture with least syneresis. Yogurt with EPS exhibited the maximum activity (>89.7%) even after 21 days of storage due to its innate antioxidant properties. The *in-situ* EPS production by the *L. plantarum* BR2 resulted in increased EPS recovery than the control yogurt samples.

Yogurts containing EPS exhibited appreciable textural properties, mouthfeel, and consistency, together with recommendations on more research to improve the overall sensory properties and the acceptability of the yogurts. The results of this study provided the indication

that EPS embodied yogurts can be safely consumed by people to avail all the health benefits provided by these probiotic functional yogurts. The development of further fermentation-based dairy products including native probiotic strains and a combination of diverse and potent postbiotics like EPS is strongly advised in light of our findings in order to take advantage of their health-promoting qualities.

In conclusion, the use of EPS from *L. plantarum* BR2 as an alternative to chemical stabilizers gave yogurt a creamy consistency, enhanced hardness, and improved water holding capacity without the presence of any undesirable flaws. According to earlier studies, all the analysis demonstrated the potential of this EPS to be employed as a food additive with immense health benefits.

Chapter 8

Chapter 8

Summary, Conclusions and Future Perspectives

Research on food-grade Lactobacilli is an ever-flourishing topic with immense scope and applications for the benefit of mankind. Probiotic Lactobacilli serves as a potential source of prebiotic and postbiotic components where these cell factories can produce lactic acid, acetic acid, sugar alcohols, antifungal molecules, oligopeptides, sugar polymers, and vitamins. The industrial relevance of these compounds is pretty high considering their market demand and applications, of which exopolysaccharides (EPS) are one such metabolite. Knowledge of the type and yield of EPS produced by varying the carbon source could be of great importance in defining the choices for EPS production for different applications.

Exopolysaccharides of lactic acid bacteria (LAB) are a thrust area of research and several reports on the LAB EPS show that they exhibit significantly improved texture and rheological properties of fermented foods. Furthermore, they also showcase many other biological properties like mouthwatering flavours, antioxidant activities, cholesterol-lowering, antimicrobial activities etc. In addition, there is increasing attention on exploiting the EPS-producing LAB, since EPS-yielding *in situ* by LAB-starters contributed to biological activities, e.g., antioxidant activity, potential immunostimulatory, enhancement of colonization of probiotic bacteria and antagonistic effect for cytotoxicity induced by bacterial toxins. Therefore, EPS from LAB has the potential for development as both food additives and fermented food ingredients. Considering their GRAS status, LAB EPS need to be explored for better production titer and improved biological properties.

The thesis discussed in detail the EPS production by an indigenous *L. plantarum* BR2 strain (Accession No: MN176402) focusing on the exploration and exploitation of cheaper raw materials, optimization of fermentation process parameters, strain improvement by employing molecular approaches for ameliorating the large-scale EPS production that pays the way for the commercial production and industrial attention of these exopolysaccharides. One of the major challenges to bridle in this area is to increase EPS production which must be remunerative and not idealistic.

The tolerance of *L. plantarum* BR2 to acidic condition (pH 3), high NaCl concentrations (6 w/v %), bile salt (0.8 w/v %), and its antimicrobial and proteolytic activity along with cell surface hydrophobicity reveals its potent probiotic nature and thus demonstrates the salient features of our indigenously isolated *L. plantarum* BR2. Using an optimized EPS production medium with lactose as the major carbon source and other fermentation conditions, EPS yield was 4.5±0.5 g/L after initial ethanol precipitation. Further, the EPS characterization by monosaccharide composition analysis (HPLC), FT-IR, TGA, GPC, SEM and NMR revealed the glucomannan nature of the EPS with a molecular weight of 2292 kDa and possessing a porous web-like morphology determined by SEM analysis. The probiotic nature of the bacteria and its EPS with these significant characteristics explored its possibility of use in various industries.

LAB-EPS production is greatly associated with bacterial secondary metabolism involving a complex biosynthetic mechanism. The structural and physio-chemical properties of the EPSes are conditioned on several variables such as production media composition, culture conditions, temperature etc., which are correlated to the amount and characteristics of the EPS produced. Carbon source being the prime factor in media engineering, its screening and optimization aids to better EPS yield with lesser cost. The potential of cassava starch hydrolysate (CSH) as an

alternative for EPS production by *L. plantarum* BR2 is demonstrated in Chapter 4. The saccharification of cassava starch and jackfruit seed powder showed that the enzymatic hydrolysis of 9 % (w/v) cassava starch resulted in 85 % conversion to sugars which can be directly assimilated by the bacterium whereas, for 9% (w/v) jackfruit seed powder, the percentage conversion of starch to glucose was only 36% which is very minimal considering it as a source of carbon for fermentation purposes. Utilization of these raw starches employing amylolytic EPS-producing cultures has further advantages in the industrial sector. Our research team recently isolated a strain of *Streptococcus lutetiensis* capable of direct utilization of starch with its inherent amylolytic and glycosyltransferase activities and produces a significantly higher amount of viscous exopolysaccharide yielding 19.92 ± 0.5 g/L (unpublished data).

A culture medium (pH 7.3) having cassava sugar, yeast extract and ammonium sulphate along with tween 80, calcium chloride and hydrogen peroxide was found to be very effective for EPS production. A maximum titer of 6.5±0.5 g/L was obtained for static flask cultures with 2% v/v inoculum at 37°C after double-step ethanol precipitation and was 4.5±0.5 g/L with the control medium with lactose as carbon source. The physicochemical properties such as monosaccharide composition, thermal stability, molecular weight etc. remained unchanged irrespective of the carbon source used.

Exopolysaccharides proved to have potential health benefits and find their applications largely in food and food processing industries. Considering the economic feasibility and market demand, EPS production must be significantly improved for their availability and constant usage in various industrial sectors. The production and yield of the LAB EPS can be improved by metabolic engineering providing the tools for the development of highly efficient microbial strains for oligo and polysaccharide synthesis. The probiotic *L. plantarum* BR2, a glucomannan

EPS producer has been engineered thereby increasing EPS production. More information from the genome of the organism enabled us to develop strategies aiming at increasing the pool of prime enzymes to enhance the carbon flux towards the final polysaccharide and over-expression of genes successfully enhance the production rate and engineer EPS properties by modifying composition and chain length.

EPS production is a complex phenomenon involving numerous genes and their gene products of which glycosyltransferases (*gtfs*) are the key enzymes involved in EPS biosynthesis. Current knowledge of gtfs of LAB and its manipulations are limited. Glycosyltransferases, being the major enzyme that mediates exopolysaccharide synthesis by various LAB, has a critical role in engineering aspects for better and improved production of EPS. Thus, engineering these enzymes in the pathway may be a milestone to produce tailor-made EPSes. This scenario can be exploited to make use of the least explored possibilities for a controlled homologous expression of these genes in Lactobacilli. Glycosyltransferases can synthesize a variety of exopolysaccharides with differing solubilities, rheology, and other properties by altering the type of glycosidic linkage, degree of branching, length, mass, and conformation of the polymers. Chapter 5 basically illustrates the over-expression of a glycosyltransferase gene (*BR2 gtf*) in *L. plantarum* BR2 thereby increasing the overall EPS production. From the whole genome data, specific primers were designed and amplified the *BR2gtf* gene, annotated as the EPS biosynthetic glycosyltransferase and were cloned into pNZ8148 shuttle vector. The recombinant pNZ8148 vector along with pNZ9530, a regulatory plasmid, were electroporated to *L. plantarum* BR2 for the over-expression of the *gtf* gene under a nisin-controlled expression system. After the optimization of nisin concentration and incubation time, EPS production (7± 0.5 g/L) increased by 54.4% in the recombinant strain (BR2OE4) compared to the wild type. Furthermore, the

recombinant strain exhibited a 3.9-fold increase in the total specific Gtf enzyme activity (21.50 U/mL) than the wild type (6.36 U/mL). This shows that in the EPS biosynthetic pathway, *gtf* plays a pivotal role in the transfer of sugar units from the high-energy sugar nucleotides and thus helps in the polymerization and formation of exopolysaccharides. An EPS production titer of 23.5 ± 0.5 g/L by the BR2OE4 in 5 L from a probiotic *Lactobacillus plantarum* strain is something remarkable and noteworthy.

Chemical modification of the innate EPS, for enhanced biological activity, is one of the threshold areas to work on. In the present piece of work, Chapter 6 describes three derivatives of native *L. plantarum* BR2 EPS that were synthesized by chemical modifications namely acetylated (a-EPS), carboxymethylated (Cm-EPS) and sulphated (s-EPS) EPSes. The structural characterization and analysis were studied by FTIR and NMR spectra. The primary structure of the EPS derivatives when compared with the control showed corresponding variations in the functional groups according to the modifications carried out. Further, the study focused on the antioxidant activities of the EPS after modifications. The subsequent evaluation of the biological properties of the modified EPS (mEPS) showed that acetylated (a-EPS) had the highest DPPH radical scavenging (73.81%) and total antioxidant capacity (50.78%) activities which were dose-dependent. Also, sulphated EPS (s-EPS) showed the highest reducing power potential (41.39%), and hydroxyl radical scavenging (54.43%) activities and it exhibited a 1.3-fold hike in cholesterol-lowering activity (40%) by adsorption as well. Hence, it's remarkable that we were able to increase the antioxidant activities of the native EPS by chemical modifications as antioxidants are key molecules involved in the degradation of oxidants, exerting protection and assault free radical generation. The necessity of a stable natural antioxidant is always in demand globally. In accordance with this fact, these mEPSes having suitable ionic groups with the

appropriate degree of substitution can significantly improve the bioactivity of polysaccharides and are a great source of natural hydrogen exhibiting non-toxic, biocompatible, thermally stable, and biodegradable molecules. Furthermore, the cytotoxicity studies acknowledged the non-toxic nature of the modified EPS ensuring its safer usage in food and food products. The mEPSes and unmodified EPS, even at their highest concentrations, hardly showed any inhibitory effect on normal cell growth and viability. Exploration and analysis of the characteristics and behaviour of these mEPS in *in-vivo* models can be the future aspects of the study along with its applications in functional foods. Elucidation of the structure-activity relationship of EPS is crucial and can provide more insight into the exact mechanism of their biological activities which can be attained through extensive investigations. As a natural biomacromolecule, microbial EPS can render more benefits in industrial and medical applications as natural antioxidants.

Exopolysaccharides secreted by LAB has been found to have unique physical and rheological properties that facilitate their most valuable application in the improvement of the rheology, texture, and mouthfeel of fermented dairy products that adds more value to these EPSes. Chapter 7 deals with the EPS application studies in the development of probiotic functional yogurts employing skim milk by fermentation using an indigenous LAB starter culture. The incorporation of the *L. plantarum* BR2 and its EPS in the yogurts made significant differences in the syneresis, biochemical attributes, texture, and viscosity of the yogurts evaluated throughout a period of 21 days of storage. From the sensory evaluation data, it was observed that the yogurts containing EPS exhibited appreciable textural properties, mouthfeel, and consistency, together with recommendations for more research to improve the overall sensory properties and the acceptability of the yogurts. The results of this study provided the indication that EPS-embodied yogurts can be safely consumed by people to avail all the health

benefits provided by these probiotic functional yogurts. Finally, the study investigated the possible utilization of EPS application in the development of a probiotic functional yogurt with improved textural properties and decreased syneresis. With 0.2% EPS, it exhibited a 10.96% decrease in syneresis of skim milk yogurts. Further, the probiotic functional yogurts showed good textural properties and with acceptable sensory characteristics. Along with the mouthfeel, improved texture quality, rheology and stability of the EPS, the higher antioxidant property adds more nutritional benefits and impacts the foods fermented with lactic acid bacteria and its EPS. Thus, with the increased EPS production and enhanced biological properties, and nutritional benefits, *L. plantarum* BR2 exopolysaccharides serve as potential postbiotics with greater applications in food and other industries.

In light of the information provided above, it would appear that exopolysaccharides from a probiotic origin possessing a "GRAS" status and safer use, offer a variety of health benefits and can greatly attract food industry applications. In addition, the futuristic applications of such EPS are immense as they can contribute to a wide range of other uses in the pharmaceutical, cosmetic, and medical fields. Through drug delivery systems (micro- and nanosystems for sustained delivery), anticancer drug-targeting, wound healing and tissue engineering, and the creation of composite biodegradable films with various applications, it can play significant roles in clinical and pharmaceutical sectors. It has been acknowledged that there is a need to promote the industrial production of EPS by exemplifying various methods described in this study while taking economic factors and other potential advantages into account. It is also crucial to develop links between the structure of exopolysaccharides, their physical characteristics, and their health advantages to enhance the application potential of these EPS in industry.

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ANNEXURE I – MEDIA and BUFFER COMPOSITION

A. MEDIA

I. EPS Production Medium

Constituents	Concentration (g/L)
Lactose	40.0
Yeast Extract	40.0
Ammonium Sulphate	5.5
Di-Potassium Hydrogen Phosphate	2.0
Sodium Acetate	5.0
Magnesium Sulphate	0.1
Manganese Sulphate	0.05
Tween 80	1.0
Agar (for solid medium)	15.0

II. de Man, Rogosa and Sharpe (MRS) Medium

Constituents	Concentration (g/L)
Proteose peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2.0
Final pH (at 25°C)	6.5 ± 0.2
Agar (for solid medium)	15.0

III. M17 Medium

Constituents	Concentration (g/L)
Casein peptone	2.5
Meat peptone	2.5
Soya peptone	5.0
Yeast extract	2.5
Meat extract	5.0
Disodium β-glycerophosphate pentahydrate	19.0
Magnesium sulphate hydrate	0.25
Ascorbic acid	0.5
Lactose	5.0
Final pH (at 25°C)	7.1 ± 0.2
Agar (for solid medium)	15.0

IV. SOC Medium

Constituents	Concentration (g/L)
Tryptone	20.0
Yeast extract	5.0
NaCl (10 mM)	0.5
KCl (2.5 mM)	0.18
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

V. Luria Bertani (LB) Medium

Constituents	Concentration (g/L)
Tryptone	10.0
NaCl	10.0
Yeast Extract	5.0
Final pH (at 25°C)	7.0 ± 0.2
Agar (for solid medium)	15.0

VI. Dulbecco's Modified Eagle's (DME) Medium

Constituents	Concentration (mg/L)
Amino Acids	
Glycine	30.0
L-Arginine hydrochloride	84.0
L-Cystine 2HCl	63.0
L-Glutamine	584.0
L-Histidine hydrochloride-H2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine hydrochloride	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serine	42.0
L-Tryptophan	95.0
L-Tyrosine disodium salt dihydrate	16.0
L-Valine	104.0
Vitamins	
Choline chloride	4.0
D-Calcium pantothenate	4.0

Folic Acid	4.0
Niacinamide	4.0
Pyridoxine hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochloride	4.0
i-Inositol	7.2
Inorganic Salts	
Calcium Chloride	200.0
Ferric Nitrate	0.1
Magnesium Sulfate	97.67
Potassium Chloride	400.0
Sodium Bicarbonate	3700.0
Sodium Chloride	6400.0
Sodium Phosphate monobasic	125.0
Other Components	
D-Glucose	4500.0
Phenol Red	15.0

B. BUFFERS

I. TSS Buffer

Constituents	Concentration (g/L)
PEG 8000	100.0
1M $MgCl_2$	30.0 mL
DMSO	50.0 mL
LB Medium	Make up the volume to 1L with LB medium

Filter sterilize (0.22 μm filter) and store the solution in -20°C

II. Tris-EDTA (TE) Buffer

Constituents	Concentration (g/L)
Tris-HCl (0.1M)	15.759
EDTA (1mM)	2.92

Adjust the pH to 8.0 and make up the volume to 1L with distilled water

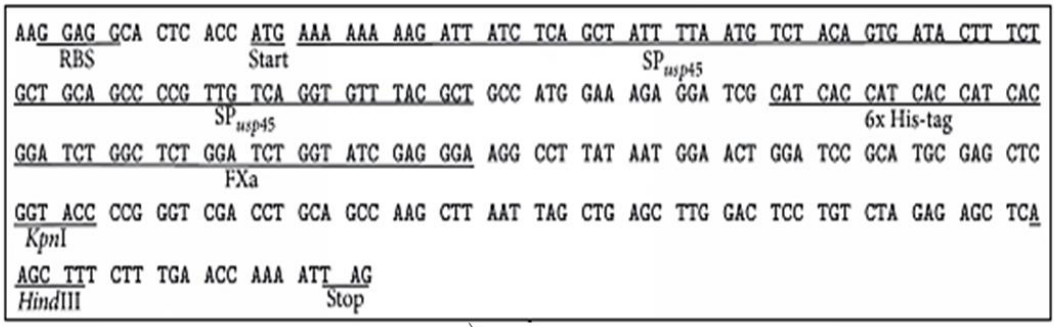
III. Tris-acetate-EDTA (TAE) Buffer- 50X

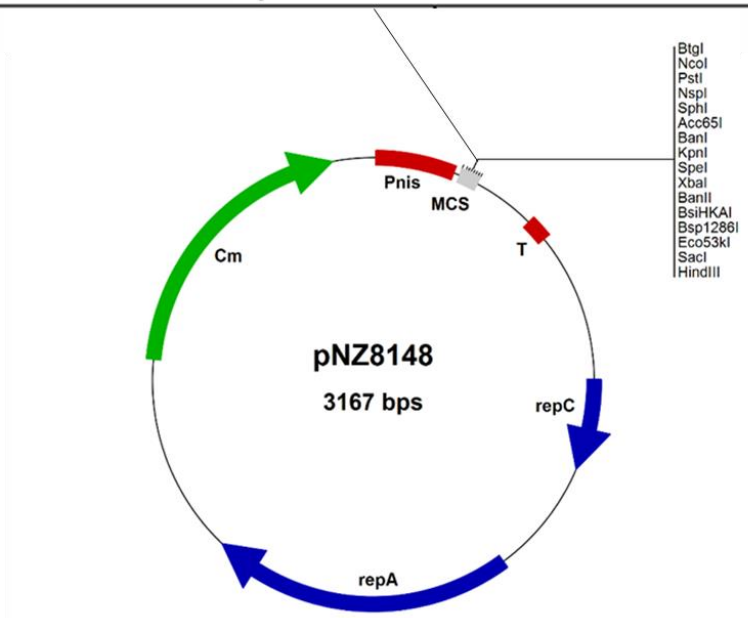
Constituents	Concentration (g/L)
Tris base	242.0
Glacial acetic acid	57.1 mL
0.5M EDTA (pH 8.0)	100.0 mL

Make up the volume to 1L with distilled water. To make the 1X TAE working buffer, add 49 parts of deionized water to 1 part of 50X TAE buffer.

ANNEXURE II – VECTOR DETAILS

1. pNZ8148 (3167 bp) (MoBiTec, Germany)





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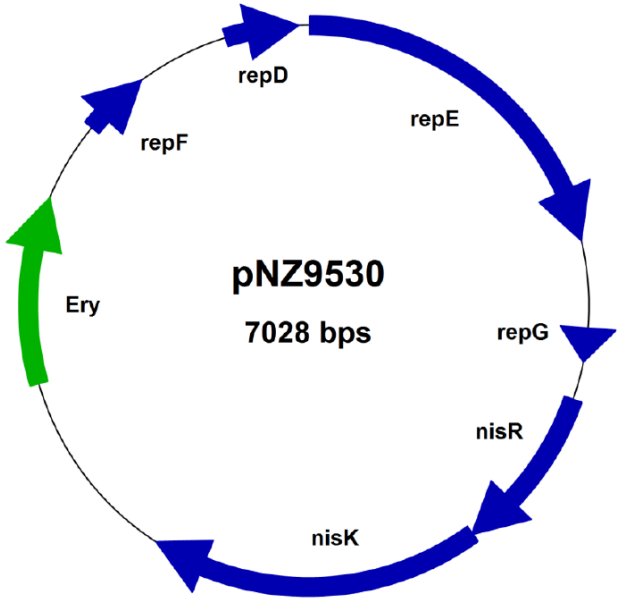
2. pNZ9530 (NIZO, Netherlands)



	Type	Start	End	Name	Description
	Gene	5	1495	*repE*	Replication gene *E*
	Gene	1845	1982	*repG*	Replication gene *G*
	Gene	2135	2821	*nisR*	Response regulator
	Gene	2814	4157	*nisK*	Histidine protein kinase
	Selectable Marker	4958	5715	Ery	Erythromycin resistance gene
	Gene	6073	6323	*repF*	Replication gene *F*
	Gene	6691	6990	*repD*	Replication gene *D*

ANNEXURE III – LIST OF INSTRUMENTS

Instruments	Model and Country
Autoclave	Labline, India
Balance	Mettler Toledo, Mumbai, India
Centrifuge	Kubota 7780, Japan; Eppendorf, Germany; MICRO CL17, Thermo Fisher Scientific, India
Cold room	Rinac Pvt. Ltd, India
Deep freezer (-80°C)	Elanpro, India; Haier, China
DNA sequencer	3500 Genetic Analyzer, Applied Biosystems, Hitachi,Japan
Electrophoresis unit	Bio-Rad, USA
Electroporator	Eppendorf, Germany
Gel documentation	ChemiDoc, Biorad, USA
Heating water bath	B20G, Lab companion, South Korea
Hot air Oven	Kemi Instruments, India
HPLC	Shimadzu, Japan
Incubator	Infors Ht, Switzerland
Laminar air flow chamber	Labline, India
Microplate reader	Infinite M200 Pro, Tecan, Switzerland,
Nanodrop spectrophotometer	ND1000, Thermo Fisher Scientific, USA
pH meter	Eutech, Thermo Fisher Scientific, India
PCR machine	MyCycler, Bio-Rad, USA; Eppendorf, Germany
NMR Spectrometer	Bruker Avance II-500, Bruker Co., USA
Scanning electron microscope	Zeiss EVO 18 cryo-SEM, Germany
Sonicator	Vibra cell, Sonics and Materials Inc., USA
Transmission electron microscope	JEM2010, JEOL, Japan
Thermostat	Eppendorf, USA

UV-Vis Spectrophotometer	UV-160A, Shimadzu, Japan
Fourier Transform-Infrared Spectrometer	Nexus-870 FT-IR, Thermo Nicolet Corporation, Madison, USA
TGA apparatus	Shimadzu H-50, Japan
Lyophilizer	Christ Alpha 1-2 LD Plus, Germany
Stirred tank bioreactor	Minifors, Infors HT, Switzerland
Rotational Viscometer	Anton Paar ViscoQC 300 (Europe

ANNEXURE IV – AcSIR Course Work

S.No.	Level & Course No.	Title	Status
	Level 100		
1.	BIO-101	Biostatistics	**Completed**
2.	BIO-102	Bioinformatics	**Completed**
3.	BIO-103	Basic Chemistry	**Completed**
4.	BIO-104	Research Methodology, communication/ ethics/ safety	**Completed**
	Level 200		
1.	BIO-NIIST-201	Biotechnology and Instrumentation	**Completed**
2.	BIO-NIIST-206	Protein Sciences and Proteomics	**Completed**
3.	BIO-NIIST-239	Basic Molecular Biology	**Completed**
	Level 300		
1.	BIO-NIIST-301	Seminar Course	**Completed**
2.	BIO-NIIST-337	Bioprocess Technology	**Completed**
3.	BIO-NIIST-369	Enzymology and Enzyme Technology	**Completed**
	Level 400		
1.		Review	**Completed**
2.		Project proposal	**Completed**
	Level 800		
1.		CSIR 800 Project	**Completed**

ABSTRACT OF THE THESIS

Name of the Student: **Soumya M P** Registration No.: **10BB17J39010**

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

AcSIR academic centre/CSIR Lab: **CSIR-NIIST, Trivandrum Kerala**

Name of the Supervisor: **Dr K. Madhavan Nampoothiri**

Title of the thesis: **Process Development, Molecular and Chemical Approaches to Improve the Production and Bioactivity of an Exopolysaccharide from a Probiotic *Lactobacillus plantarum* for Food Applications**

An exopolysaccharide producing probiotic *Lactobacillus plantarum* BR2 strain is used for this research work. The study demonstrated an improved EPS production by media engineering and substitution of refined carbon sources with sugars from starchy agro residues as raw materials. An effective downstream process was developed for the recovery and purification of EPS from the fermented broth. A nisin-controlled expression system for homologous overexpression of the glycosyltransferase gene, *BR2gtf*, resulted in a 3.9-fold enhancement of Gtf enzyme activity that subsequently enhanced the EPS production by 54.4%. The idea for incorporation of various chemical moieties into the exopolysaccharide backbone resulted in the generation of acetylated, carboxymethylated and sulphonated derivatives of the native EPS with significant improvement in the antioxidant and other biological properties without any inhibitory effect on the normal cells. The EPS food application studies for the development of probiotic functional yogurts showed good textural properties and lesser syneresis with acceptable sensory characteristics. Thus, with the increased EPS production and enhanced biological properties, and nutritional benefits, *L. plantarum* BR2 exopolysaccharides serve as a potential postbiotics with greater applications in food and other industries. To our knowledge, this is the first report on the homologous over expression of a single glycosyltransferase gene showing a significant impact on EPS production in *Lactobacillus plantarum*. The future scope of the study dwells in the metabolic engineering of the organism for tailor-made EPS production. Exploration and investigation of the characteristics and behavior of these EPS in *in-vivo* models can be the future aspects of the study along with its applications in benefiting mankind.

LIST OF PUBLICATIONS

List of Publications Emanating from the Thesis Work

Soumya M.P, Keerthi Sasikumar, Ashok Pandey, K. Madhavan Nampoothiri. "Cassava starch hydrolysate as sustainable carbon source for exopolysaccharide production by *Lactobacillus plantarum"* in *Bioresource Technology Reports 6* (2019) Pg 85-88. https://doi.org/10.1016/j.biteb.2019.02.012

Soumya MP, K. Madhavan Nampoothiri. "An overview of functional genomics and relevance of glycosyltransferases in exopolysaccharide production by lactic acid bacteria" *International Journal of Biological Macromolecules.* 184 (2021) 1014–1025. https://doi.org/10.1016/j.ijbiomac.2021.06.131

Soumya MP, Reeba Parameswaran, K. Madhavan Nampoothiri. "Nisin controlled homologous Over-expression of an exopolysaccharide biosynthetic glycosyltransferase gene for enhanced EPS production in Lactobacillus plantarum BR2". *Bioresource Technology*. 385 (2023) 129387. https://doi.org/10.1016/j.biortech.2023.129387

Soumya MP, K. Madhavan Nampoothiri. "Evaluation of Improved Biological Properties of Chemically Modified Exopolysaccharides from *Lactobacillus plantarum* BR2". Manuscript Accepted in *3 Biotech*

Soumya MP, Reeba Parameswaran, Ananadhu Suresh, K. Madhavan Nampoothiri. "Effects of *Lactobacillus plantarum* BR2 exopolysaccharide in development of probiotic functional yogurt. *(Manuscript Under Review)*

List of Publications not related to the Thesis Work

Soumya MP, Reeba Parameswaran, K. Madhavan Nampoothiri. "Direct Utilization and Conversion of raw Starch to Exopolysaccharides by a newly isolated Amylolytic *Streptococcus* sp". *Journal of Biotechnology*. 371–372 (2023) 22–32. https://doi.org/10.1016/j.jbiotec.2023.06.002

Lekshmi Sundar M S, Susmitha A, **Soumya M P**, Keerthi Sasikumar, K Madhavan Nampoothiri. "Bioconversion of xylose to D-xylonic acid by *Pseudoduganella danionis". Indian Journal of Experimental Biology (IJEB)*, 2019. Vol. 57, November 2019, pp. 821-824. http://nopr.niscpr.res.in/handle/123456789/51175

Soumya MP, Ananadhu Suresh, Reeba Parameswaran, K. Madhavan Nampoothiri. "Physiochemical and sensory evaluation of probiotic plant milk yogurts as a functional alternative for dairy yogurts. *(Manuscript Submitted & Under Review)*

List of Book Chapters

K Madhavan Nampoothiri, Nimisha R Nair, and **Soumya MP** (2019) "Ethnic fermented foods and beverages of Kerala" in *Ethnic Fermented Foods and Beverages of India: Science History and Culture*. ISBN: 978-981-15-1485-2, Prakash Tamag (Ed)

Bindhumol Ismail, **Soumya M P**, Reeba Parameswaran, K. Madhavan Nampoothiri, A Jayakumaran Nair and A Gangaprasad (2021). "Structural and functional diversities of Lactic acid bacterial polysaccharide". *Recent advances in natural polymers, green composites and green nano composites*, Amadou Belal Gueye, Sabu Thomas, Nandakumar Kalarikkal, Modou Fall (Eds) Apple Academic Press

Details of Poster Presentations in Conference Proceedings

Soumya MP, Keerthi Sasikumar & K Madhavan Nampoothiri, "Cassava Starch Hydrolysate as Sustainable carbon source for Exopolysaccharide production by *Lactobacillus plantarum* BR2". Poster presented at the international conference on Biotechnological research and innovation for Sustainable Development (BioSD-2018) & XV annual convention of the Biotech Research Society , India (BRSI), November 22-25, 2018, organized and hosted by CSIR - IICT , Hyderabad, India, IB P-20, p.258

Soumya MP, K Madhavan Nampoothiri, "Evaluation of the bioactive properties of Chemically Modified Exopolysaccharides of *Lactobacillus plantarum* BR2". Poster presented at the international conference on New Horizons in Biotechnology (NHBT-2019), India (BRSI),

November 20-24, 2019, organized and hosted by CSIR-NIIST, Trivandrum, India, FBN-12, p.296

Soumya MP, K Madhavan Nampoothiri, "Enhanced exopolysaccharide production by *L. plantarum* BR2 by the overexpression of Gtf gene, the exopolysaccharide biosynthesis glycosyltransferase", International Conference on BREEECH 2021 jointly organized by CSIR-IIP and the BRSI, India, at Dehradun, India, during December 01-04, 2021 - **Best Oral Presentation and Poster Award**

Madhavan Nampoothriri, **Soumya M P, "**Molecular and Chemical Approaches to enhance the production and biological properties of the Exopolysaccharides of a food grade *L. plantarum* BR2". At the 10th International conference on Bioprocess – IBA -IFIBiop 2022, November 20-24, 2019, organized and hosted by national Kaohsiung University of Science and Technology, Kaohsiung, Taiwan during October 27-30,2022

Workshops/ Seminars Attended

Soumya MP, Arun P M, Ananadhu Suresh, & K Madhavan Nampoothiri "Development of Fortified Yoghurt using a Probiotic Consortium". Presented a Two-day National Workshop on Bifidobacterial Probiotics: Supplementation through Fermented Foods, organized by Microbiology and Fermentation Technology Department, CSIR-CFTRI, Mysuru-570020, on 24th and 25th March 2022 – **Best Poster Award**

Bioresource Technology Reports 12 (2020) 100573

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Corrigendum

Corrigendum to "Cassava starch hydrolysate as sustainable carbon source for exopolysaccharide production by Lactobacillus plantarum" [Bioresour. Technol. Rep. 6 (2019) 85–88]

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The authors would like to apologise for any inconvenience caused.

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Cassava starch hydrolysate as sustainable carbon source for exopolysaccharide production by *Lactobacillus plantarum*

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ARTICLE INFO

Keywords:
Cassava starch hydrolysate
Exopolysaccharides
Fermentation
Lactic acid bacteria (LAB)

ABSTRACT

Cassava starch hydrolysate (CSH) proved to be a potential alternative for EPS production by *L. plantarum*. Enzymatic hydrolysis of 9% (w/v) cassava starch resulted in nearly 85% conversion of it to the sugars which can be directly assimilated by the bacterium. Culture medium (pH 7.3) having cassava sugar (40 g/L), yeast extract (40 g/L) and ammonium sulphate (5.5 g/L) along with tween 80 (4 g/L), calcium chloride (10 mM) and hydrogen peroxide (3 mM) found to be very effective for EPS production. After the double step ethanol precipitation, a maximum titer of 6.5 ± 0.5 g/L crude EPS was obtained in static flask cultures incubated at 37 °C with 2% v/v inoculum and it was 4.4 ± 0.5 g/L with the control medium having lactose as carbon source. Thermal stability and molecular weight were remained unchanged irrespective of the carbon source used. This is the first report on EPS production by a Lactobacilli using CSH as raw material.

1. Introduction

Exopolysaccharides (EPS) are high molecular weight polysaccharides produced by microbes and are secreted out of the cell to carry out various functions including cell adhesion, cell protection from external stress, and source of nutrients during shortage (Suresh Kumar et al., 2007). Since bacteria are able to produce unique EPSs with specific properties, several researchers have undertaken the task of identifying and analyzing these EPSs. With the discovery of varied varieties of EPS comes the necessity of optimizing its production. Other EPSs such as Xanthan and Carrageenan are already produced at large scales thus creating the need for a competitive alternative. EPSs from LAB which generally belongs to GRAS status play a major industrial role in the production of fermented dairy products, particularly for the production of yoghurt, cheese, fermented cream and milk-based desserts. Their presence significantly contributes to texture, mouth feel, taste perception and stability of the final products. But the major drawback of EPS produced by the LAB is that it is produced in lower amounts and this reduces their potential use as food-grade ingredients (Jolly et al., 2002). For its further exploration, an advanced approach is needed for applications of EPS in the medical or pharmaceutical field and food sectors. Nowadays, the development of prebiotic and probiotic-based dairy goods getting more and more attention and where LAB EPS can play a prime role.

Process optimization, exploration and exploitation of cheaper raw materials and use of biotechnological approach for a metabolically engineered strain are the three major possibilities for ameliorating the large scale production that pays way for the commercial production and industrial attention of microbial polysaccharides (Welman and Maddox, 2003). There are several reports where many agro-industrial wastes like potato starch, peat hydrolysate, whey, molasses, brewery wastes, olive oil waste effluents are used as substrates for microbial polysaccharide production (Göksungur et al., 2011). Streamlining to lactic acid bacteria, specifically Lactobacillus, they never compromise in their carbon and nitrogen sources as they always need highly rich medium for their growth. One of the major challenges to bridle in this area is to increase the EPS production that must be remunerative and not idealistic.

Among these wide ranges of low-cost substrates reported so far, Cassava, (*Manihot esculenta*) or Brazilian arrowroots that solemnly store starch are considerably cheap, readily available and a sustainable source of fermentable glucose syrups (Ruiz et al., 2011; Ray and Moorthy, 2007) can be a potential alternative carbon source for EPS production. It also contains small amounts of calcium, phosphorous and vitamin C, but is poor in protein with a moisture content of 10.38% (Oladunmoye et al., 2014). Considering the fact that there are no reports with cassava hydrolysate for EPS production, we explored it as a raw material for our isolate, *L. plantarum* BR2, in batch culturing.

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Available online 18 February 2019
2589-014X/

2. Materials and methods

2.1. Microorganism and inoculum

Lactobacillus plantarum BR2, an indigenous isolate from a ripened jack fruit was used and was propagated and maintained as we described earlier (Sasikumar et al., 2017). Unless it is specified, an overnight culture of 10^9 CFU/mL of cells with an approximate OD_{600} of 0.8–1.0 served as inoculum for fermentation experiments.

2.2. Hydrolysis of cassava starch

Cassava starch slurries were prepared in distilled water and was gelatinized by autoclaving at 121 °C at 15 lb pressure for 15 min after adjusting the pH to 4.8–5. Enzymatic hydrolysis was done with α-amylase (Termamyl 120 L, 5000 IU/mL, Novo Industries, Bagsvared, Denmark) and reaction was carried out at 90 °C shaking water-bath (120–150 rpm) for 40 min. The resulting solution was then saccharified with a glucoamylase (AMG 300, 2000 IU/mL, Novo industries) at 46 °C in a shaking (120–150 rpm) water bath for 60 min. The slurry was then centrifuged at 8000 rpm for 20 min at 4 °C. The resulting supernatant was filtered using a muslin cloth and the final glucose concentration of the hydrolysate was determined by HPLC analysis using Rezex RPM Monosaccharide Pb + 2300 × 7.8 mm column with degassed MilliQ water with a flow rate of 0.6 mL/min as mobile phase and an oven temperature of 80 °C.

2.3. Production, recovery and purification of EPS

Production medium reported earlier (Ismail and Nampoothiri, 2010) was used where the lactose 40 g/L was replaced with cassava hydrolysate with equal sugar concentration. After inoculation, (10^9 CFU/mL) the flasks were incubated for 72 h, under static conditions, at 37 °C. After collecting the culture supernatant, the EPS was precipitated by addition of double volume chilled ethanol and stored at 4 °C overnight. Precipitated EPS was recovered by centrifugation at 2500*g* for 20 min at 4 °C. This was then lyophilized and analyzed. Purification of the recovered EPS was performed according to Dabour and Lapointe (2005) and the purified EPS was lyophilized and stored at room temperature.

2.4. Growth and sugar consumption in hydrolysate medium

Overnight grown culture with an OD_{600} of 0.8 was inoculated to 100 mL of cassava hydrolysate based medium and incubated at 37 °C under static flask condition. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm. The sugar utilization was recorded at desired time intervals by subjecting the culture supernatant to HPLC analysis.

2.5. Optimization of different culture conditions

To optimize the sugar concentration in the hydrolysate medium, different volumes of cassava hydrolysate was used to get sugar levels of 10, 20, 40, 80 and 100 g/L and the corresponding production was checked. Also, to study the efficacy of nitrogen sources, different organic nitrogen sources (Meat Extract, Peptone, Tryptone, Yeast extract (control), 40 g/L) and inorganic nitrogen sources (sodium nitrate, ammonium nitrate, ammonium dihydrogen orthophosphate, ammonium sulphate (control), 5.5 g/L) were used independently and checked the EPS production. Similarly, optimum pH was also set after checking a wide range of initial medium pH (4.5, 5.5, 6.5, 7.3, and 8.5). Tween 80, a surfactant, with different concentrations (w/v %, 0.1, 0.4, 0.5, 0.8 and 1) was provided in the cassava hydrolysate medium to see its effect in EPS production. After checking a range (v/v %, 0.5, 1, 2, 3 and 4), optimized the inoculum percentage for EPS production. In order to study the effect of hydrogen peroxide and calcium chloride in EPS production, *L. plantarum* BR2 was cultured in a medium supplemented with a) 3 mM H_2O_2 b) 10 mM $CaCl_2$ and c) 3 mM H_2O_2 and 10 mM $CaCl_2$. The one without any of these supplementations served as control.

2.6. Characterization of exopolysaccharides

The purified EPS samples from cassava hydrolysate medium and the control medium (lactose as carbon source) were analyzed by Fourier transformed infrared (FTIR) spectroscopy for the identification of functional groups as a preliminary conformation. 1 mg of the finely ground EPS samples were directly used for FT-IR measurement in the frequency range of 4400–400 $cm^{-1,}$ at a resolution of 4 cm^{-1}. The temperature tolerance of the EPS from cassava hydrolysate was determined by thermogravimetric analysis (TGA). 20 mg of the purified EPS was subjected to a heat range of 25–400 °C at a rate of 10 °C/min. Molecular mass of the purified EPS was determined by gel permeation chromatography (GPC) with 15 mg/mL of the sample dissolved in deionized water. Analysis was performed with Shodex SB-804 GPC column with an RI detector. Mobile phase used was deionized water with a flow rate of 1 mL/min.

3. Results and discussion

3.1. Hydrolysis of cassava starch

Cassava starch powder is insoluble in water and is difficult to hydrolyze at mild conditions (Ruiz et al., 2011). Direct enzymatic hydrolysis of cassava starch at a temperature lower than the gelatinization temperature is the most preferable method while considering the energy prerequisite and for the complete usage and conversion of the raw material (Chen et al., 2011) into a sugar slurry that can be straight away used as the carbon source. 9% cassava starch powder when hydrolyzed gave a sugar concentration of 76.7 g/L with a percentage conversion of 85 which was found to be the best among the different starch concentrations (% w/v 6, 7.5,9,10 and 15) hydrolyzed.

3.2. Growth comparison and optimization of culture conditions

The time course study for growth and sugar utilization conveys that the *L. plantarum* BR2 can effectively utilize the sugars in the cassava hydrolysate for its EPS production (Fig. 1) and the optimum sugar concentration of the hydrolysate was found to be 40 g/L (Fig. 2). Like in an earlier *L. plantarum* study (Ismail and Nampoothiri, 2010) yeast extract and ammonium sulphate served as the best nitrogen source for *L. plantarum* BR2 as well in the cassava hydrolysate based medium (data not shown). Supplementing the medium with 0.4% of Tween 80

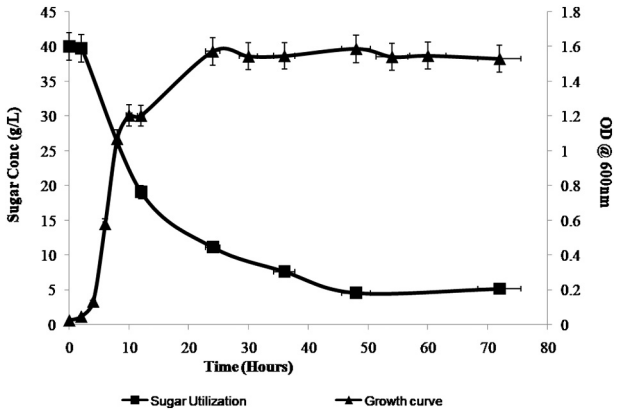

Fig. 1. Growth and sugar consumption by *L. plantarum* BR2 in CSH medium.

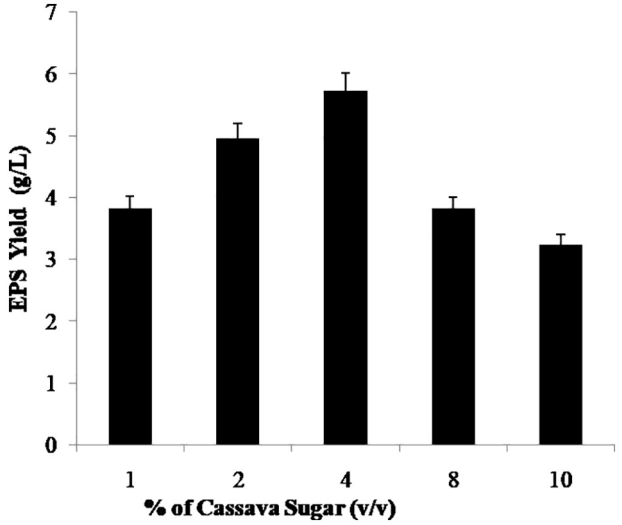

Fig. 2. Effect of cassava sugar concentration in EPS production.

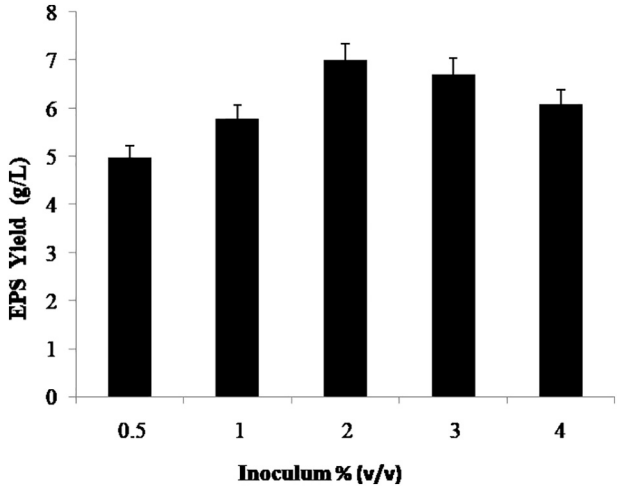

Fig. 3. Effect of inoculum percentage on EPS production in CSH medium.

favored the EPS production as it might permeabilize the cell membranes and promote the migration of nutrients into the cells (Qi et al., 2009). Optimum pH was 7.3 and is in fact the actual pH of the medium and thus no pH adjustment is required. When the medium was supplemented with 3 mM H_2O_2 and 10 mM $CaCl_2$, it enhanced the EPS production as well. According to Ng and Xue (2017), hydrogen peroxide and calcium chloride had a positive effect on EPS production in *L. rhamnosus* ZY and is found to enhance the production with *L. plantarum* BR2 as well when a combination of 3 mM H_2O_2 and 10 mM $CaCl_2$ is supplemented. 2% v/v of the inoculum served the optimum inoculum concentration with a maximum titer of 6.5 ± 0.5 g/L (Fig. 3) of EPS.

3.3. Production and purification of EPS

The double step ethanol precipitation of the fermented broth of the cassava hydrolysate medium resulted in slightly brownish crude EPS with a dry weight of 6.5 ± 0.5 g/L, while the control gave only 4.4 ± 0.5 g/L of EPS. However, after the phenol:chloroform:isoamyl alcohol (25:24:1) treatment and acetone precipitation followed by dialysis, yields were 2.2 ± 0.5 g/L for EPS from cassava sugar and was 2.8 ± 0.5 g/L for control with lactose. This indicates the probability of some impurities from the hydrolysate medium in the initial ethanol precipitation step. What is interesting is the high EPS production with a relatively low cost alternate carbon source in comparison with other lactobacilli reports, 0.599 g/L by *L. plantarum* KX041 (Wang et al., 2017), 1.5 g/L by *L. plantarum* (Dilna et al., 2015), 0.2 g/L from *L. paracasei* HCT (Xu et al., 2010), 0.26 g/L from *L. plantarum* YML009 (Seo et al., 2015), 0.4 g/L from *L. rhamnosus* (Madhuri and Prabhakar, 2014).

3.4. Characterization of exopolysaccharides

FT-IR being a potent tool for observing structural and functional groups in EPS, the spectrum of cassava EPS exhibited many peaks in the range of 4400–400 cm^{-1}. The broad peaks between 3600 and 3200 cm^{-1}relates to the −OH (hydroxyl group), band around 2900–2800 cm^{-1} indicates the aliphatic C−H bond (Sasikumar et al., 2017). The peak around 1700–1600 cm^{-1} denotes the vibrational stretching of C═O group which is considered characteristic for polysaccharides. Few peaks at 950–1200 cm^{-1} correspond to the broad stretch of C−O−C and C−O and are considered to be the fingerprint region of EPS along with the indication of α-1-4 glycosidic linkages (Zhu et al., 2019). All these data and peaks were in perfect co-relation with that of the control EPS. This reveals that there is no major change in the characteristic of the EPS produced in cassava hydrolysate medium (Fig. 4).

The thermogravimetric analysis of the EPS revealed (Supplementary Fig. 1) that degradation occurs at two stages. The first phase of decomposition occurs with a 10–14% weight loss at a temperature of 25–100 °C, may be due to the moisture content in the sample. Above this temperature, the weight of EPS remains constant and stable until a fast degradation takes place between 250 and 300 °C. Beyond 300 °C there is a significant decrease in weight up to a loss of 60%. Another strain of *L. plantarum* RS20D is also stable up to 250 °C (Zhu et al., 2019) which is lower than that of 289 °C exhibited by *L. plantarum* KX041 (Wang et al., 2017).

From the calibration curve plotted (Supplementary Fig. 2) for the purchased dextran standards (1000, 5000, 12,000, 25,000, 50,000, 80,000, 170,000, 250,000) from Sigma-Aldrich the weight average molecular weight of the cassava EPS was determined to be 2200.6 kDa and is quite comparable (2380 kDa) with the one we reported using lactose as carbon source (Sasikumar et al., 2017). EPS produced by *L. plantarum* CIDCA 8327 has a molecular weight of 1000 kDa (Gangoiti et al., 2017) which is quite low in comparison to *L. plantarum* BR2. Determination of physicochemical characterization is vital in deciding the end use application of the EPS.

4. Conclusion

Taking into consideration the GRAS status of EPS produced by lactic acid bacteria, it can fetch wide applications in food industries and the health sector. But the major limitation for LAB EPS is the yield and economic viability. In order to reduce the cost of production and improve the yield, a sustainable cheap carbon source is a must. The study indicated that the microbial fermentation of the sugar contents in cassava starch hydrolysate into value added polysaccharides could be a cost effective alternative in comparison with the synthetic pure sugars.

Conflict of interest

The authors declare that they have no conflict of interest.

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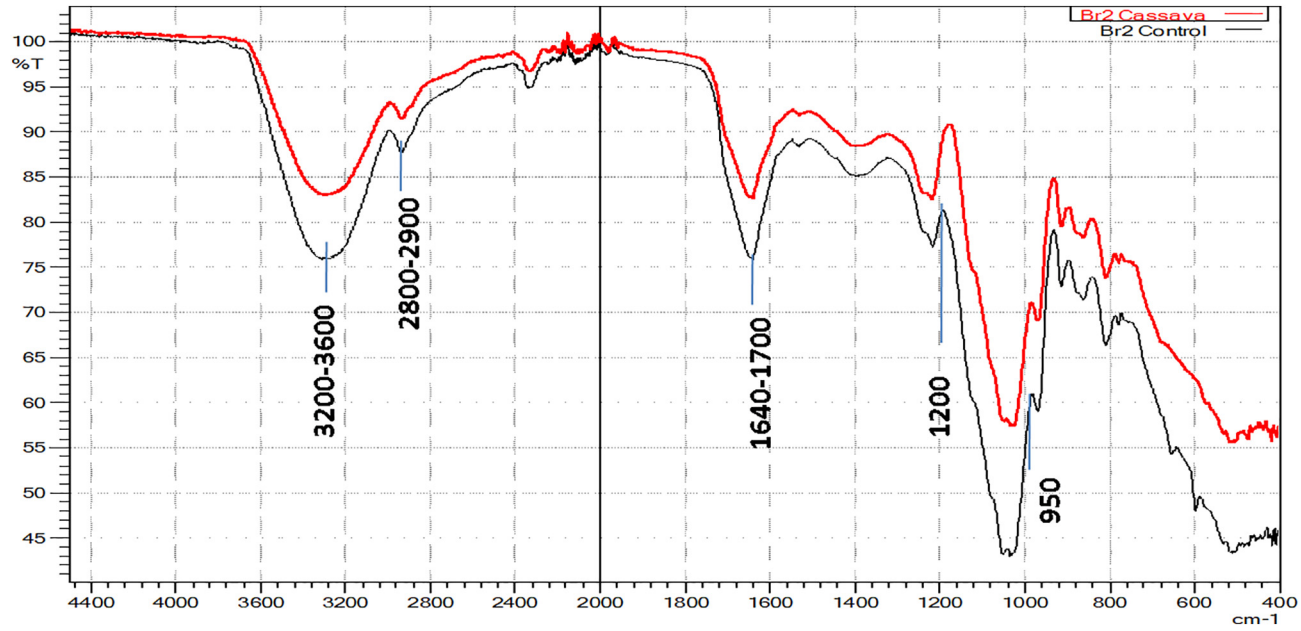

Fig. 4. FT-IR spectra of EPS produced by *L. plantarum* BR2 in CSH medium and lactose medium.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2019.02.012.

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Review

An overview of functional genomics and relevance of glycosyltransferases in exopolysaccharide production by lactic acid bacteria

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ABSTRACT

There are many reports on exopolysaccharides of lactic acid bacteria (LAB EPS) such as isolation, production and applications. The LAB EPS have been proved to exhibit significantly improved texture and rheological properties in order to prevent syneresis of fermented foods. Furthermore, they are known to have many biological properties such as mouthwatering flavors, antioxidant activity, cholesterol lowering and antimicrobial activities. Considering their GRAS status, LAB EPS need to be explored for better titre and improved biological properties, where strain improvement by genetic engineering has a major role for making tailor-made EPS. The genetic overview of the EPS production by LAB is an auxiliary area of interest as the process and the biosynthetic pathway involves numerous genes and their proteins. Among them Glycosyltransferases (gtfs) are the key enzymes involved in EPS biosynthesis. Current knowledge of gtfs of LAB and its manipulation is limited. The present review spotlights the importance of glycosyltransferases and their specific role on the biosynthesis of LAB EPS and addresses the functionality and applicability of these enzymes and their products. It enfold the available literature including some patents in recent past to underline the fact that glycosyltransferases are un-reluctantly the key proteins involved in the EPS biosynthesis.

1. Introduction

A slimy layer either seen attached on to the cell-surface or secreted out of the cell into the external environment constitutes the microbial exopolysaccharides. Exopolysaccharide production by microbes attributes to the microbial cell against desiccation, phage attack, phagocytosis, and antibiotics, facilitate their adhesion on to solid surfaces and the formation of biofilms [1]. Thereby they have roles in protection, inter and intra-microbial interactions, acts as source of nutrition during starvation and helps in virulence in case of pathogens [1,2]. Lactic acid bacteria are gram-positive, food-grade, bacteria of many genera like *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Weissella* which owns the property of lactic acid production through carbohydrate fermentation. LAB and its metabolic end products find applications in different industries as they are considered as safe organisms (GRAS). They are widely used in baking and other food industries for improvement of textural properties, flavour addition, lactic acid production and as starter cultures with varied properties for preparation of wide variety of fermented foods including dairy products, sausages, fruit juices and bakery products [3]. LAB are known to produce exopolysaccharides, which is one of the primary metabolic product the bacteria produces, which are structurally divergent and have promising physiochemical properties [4]. EPS from LAB are diverse in their composition, charge, spatial arrangements, rigidity, molecular mass, chain length and hence show significant variations in texture, viscosity, thermal stability, rheology extending their influence in the biological properties as well [5]. Biological properties that accounts for the lactic acid bacterial EPS includes anti-tumor, anti-oxidant, antiviral, anti-ulcer activities, cholesterol lowering effects, immunomodulatory properties and as pre-biotics and probiotics [6–11]. These biological properties and the GRAS status of the organism spotlight to the vast possibilities and capabilities of the LAB EPSs and increase the demand for research in this area and to be used in different industries. But, one of the major limiting factors is the amount of the polymer synthesized where the average yield still remains to be between 50 and 2000mg/L which is quite low [12,13]. The complexity of EPS biosynthesis, their structural diversity and their promising applications makes LAB as a good model for developing tailor-made, customized EPSs in the

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near future. To increase the commercial scale production of EPS, sustainable and effective strategies must be developed, followed, and implemented in the most scientific way it can be. Few of such strategies involves (i) exploration of cheap and sustainable raw materials, (ii) process development and optimization of fermentation conditions (iii) acquiring more defined knowledge on the genetic mechanism involved in the biosynthetic pathway of EPS production and last but not least (iv) utilizing this knowledge in the biotechnological aspects for genetic engineering and strain improvement.

This review focusses on the genetics of EPS production, the pathway and the genes involved and highlighting the role of glycosyltransferase enzymes and their functionality which could be effective and adds on to the knowledge in genetic engineering of LAB for better EPS production.

2. Classification of EPS based on composition and their general biosynthesis

EPS production in LAB is a complex process with the involvement of different cassettes of genes and their gene products. The complexity of EPS biosynthesis is in direct correlation to the structural diversity and intricacy of the EPS backbone; greater the complexity, more the number of genes involved [14]. The whole mechanism, its genetics, and the detailed implication of each of the proteins and regulation of the process is still a hot topic to be explored. Many researchers around the globe still work on it to solve the mystery in this regard. The general mechanism of exopolysaccharide biosynthesis involves the assembly of the repeating units of monosaccharides on to a lipid carrier from sugar nucleotides by specific group of enzymes called glycosyltransferases (GTFs) and then the retrospective polymerization of these repeating units to form polysaccharides and their export outside the cell [15]. The lactic acid bacterial EPS gene clusters might be localised in the plasmids they carry or in the chromosome itself. One such most widely studied EPS plasmid is of *Lactococcus lactis* subsp. *cremoris* NIZO B40. It carries a 40 kb plasmid, pNZ4000, with a 12-kb region consisting of 14 genes that encodes for the capsular polysaccharide, lipopolysaccharide, and teichoic acid biosynthesis in the organism. Plasmid encoded EPS gene cluster makes it easy

Table 1
Bacterial EPS showing their biosynthetic pathway, composition and applications.

EPS type	Bacterial strains	EPS biosynthetic pathway	EPS composition	Properties & applications	References
Dextran	*Leuc mesenteroides* *Leuc. mesenteroides* subsp. *dextranicum*	Extracellular synthesis	Glucose	• As gelling, viscosifying & emulsifying agent in food industries. • Used as blood flow improving agent as blood plasma extender • As cholesterol lowering agent, in human medicine and in veterinary medicine • As matrix of chromatography column in separation technology • For enhanced oil recovery & biomaterials • As anticoagulant	[44–47]
Levan	*S. salivarus* *Lb. sanfranciscenis* *Zymomonas mobilis*	Extracellular synthesis	Fructose	• Is an ecofriendly adhesive • As prebiotic supplements • Exhibits antitumor properties • Used as hypo-cholesterolaemic agent • As bio-thickener in food applications	[44–46,48,49]
Xanthan	*X. campestris*, *X. phaseoli*, *X. malvacearum*, *X. carotae*	Wzx/Wzy dependent pathway	Mannose, Glucose, Glucuronic acid	• Constituents in cosmetics, pharmaceuticals, pesticides, paints, detergent formulations, as viscosity controlling agents in printing inks • In food, as a stabilizing and thickening agent, mostly used along with guar gum • Finds application in crude-oil recovery	[27,43,44,46]
Pullulan	*Aureobasidium pullulans*	Extracellular synthesis	Glucose, Maltotriose	• As a thickener, viscosity stabilizer or an adhesive and coating agent for many applications. • It is non-toxic & hence finding applications in biotechnological & pharmaceutical industries.	[44,46,49]
Gellan	*Sphingomonas paucimobilis*	Wzx/Wzy dependent pathway	Rhamnose, Glucose Glucuronic acid	• As thickening agent, gelling agent & stabilizer • For studying marine microorganisms, it is used as solidifying agent for culture media preparation.	[44,50–53]
Curdlan	*Agrobacterium* sp., *Paenibacillus polymyxa*, *Pseudomonas* sp. *QL212*	Synthase dependent pathway	Glucose	• As a gelling agent, & immobilization matrix • Heavy metal removal • Along with zidovudine (AZT), it displays high antiretroviral activity (anti-AIDS-drug) • Food, Medicine, Cosmetics • Used in biomedical applications such as antithrombotic activity, anti	[44,45,48,50,51,54]
Mutan	*Streptococcus mutans*	Extracellular synthesis	Glucose	•Adjunct culture in cheese	[[44,45,55]
Kefiran	*Lb. delbrueckii subsp. bulgaricus* *Lb. kefirgranum* *Lb. parakefir* *Lb. kefiranofaciens* *Lb. plantarum*	Wzx/Wzy dependent pathway	Glucose, Galactose	• As prebiotics, substitute of fat in food products • Improves visco -elastic properties of acid milk gels • Exhibits antimicrobial & wound healing properties • Increased gut mucosal immunity • Cholesterol lowering in serum, to retard tumor growth, enhance immunity of gut • Increase in viscosity and texture improvement of fermented milks and beverages	[15,44,56]
Inulin	*Lb. johnsonii* NCC 533 *Leuc. citreum* CW28 *Lb. reuteri LB 121* *Lb gasseri* *S. mutans* JC2	Extracellular synthesis	Fructose	• Prebiotics, nourishes gut mucosal cells and inhibits pathogens, • For targeted drug delivery against colon cancer, • Substitute of fat in food products	[44,45]
Reuteran	*Lb reuteri* LB121 *Lb. reuteri* ATCC55730 *Lb. reuteri* 35-5	Extracellular synthesis	Glucose, Fructose	•Used in bakery	[44,57]

Lb- Lactobacillus, *Leuc-Leuconostoc*, *S- Streptococcus*, *X-Xanthomonas*.

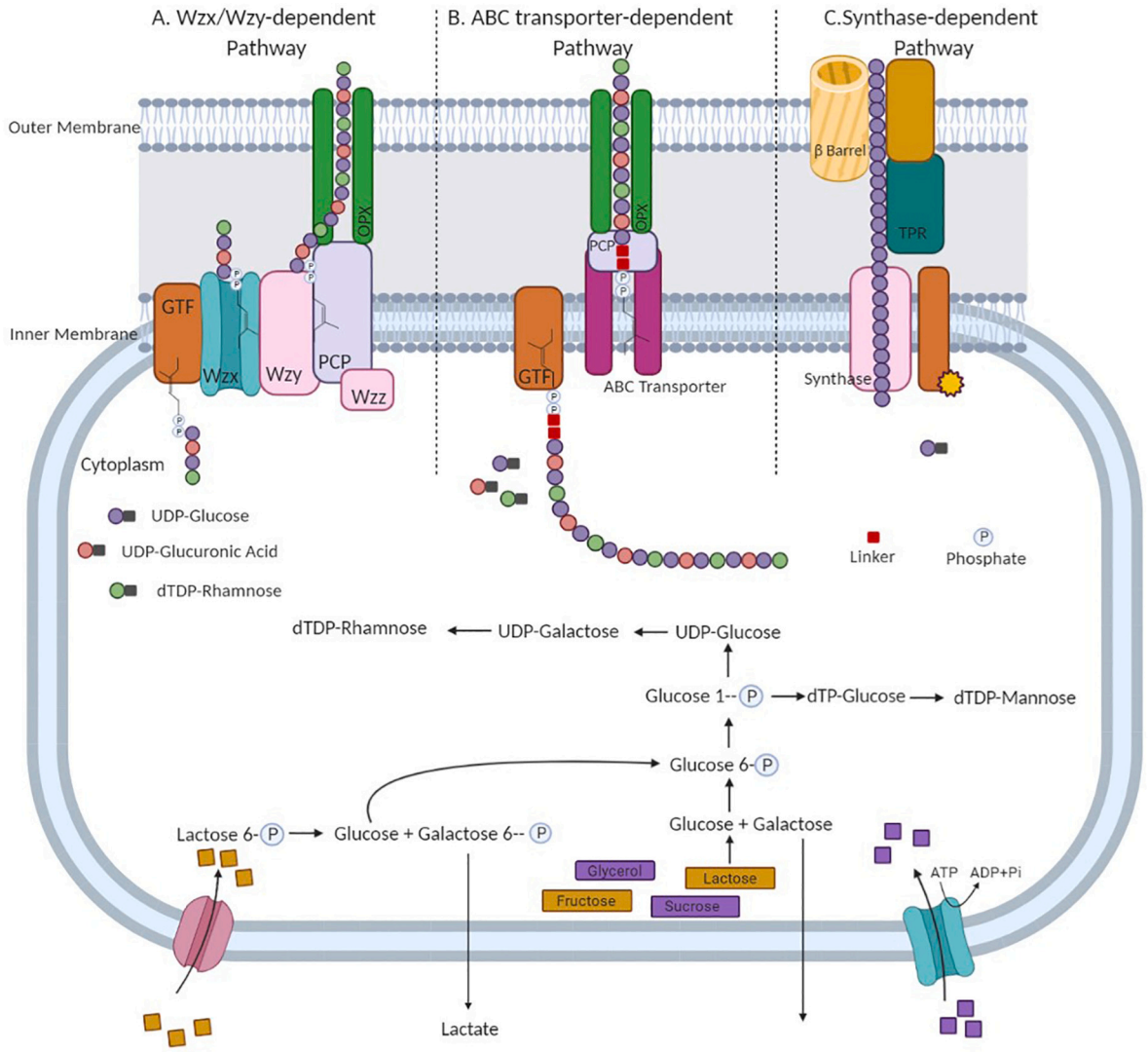

Fig. 1. An outline of biosynthetic pathways of exopolysaccharide (EPS) production in lactic acid bacteria.
Sugar uptake and transportation of sugars into the cytoplasm; Synthesis of sugar nucleotides; Polymerization, synthesis and export of EPS subunits through (A) Wxz/Wxy – dependent pathway; (B) ABC Transporter-dependent pathway; (C) Synthase-dependent pathway involves the action of various enzymes including the glycosyltransferases [43–45]. UDP-Uridine diphosphate; dTDP-deoxy Thymidine diphosphate; Lactose-6-P- Lactose 6 phosphate; Glucose-6-P- Glucose 6 phosphate; GTF- Glycosyltransferases; PCP-Polysaccharide polymerase protein; OPX- Outer membrane polysaccharide; TPR-tetratricopeptide repeat.

to transfer the genes to other bacteria and is convenient to study the EPS biosynthetic pathway and further metabolic engineering for better yield and production of tailor-made EPS [15].

EPS is classified into two based on their backbone composition - homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

2.1. Homopolysaccharides

EPS from LAB are generally categorized into two groups: homopolysaccharides, HoPS and heteropolysaccharides, HePS, of which homopolysaccharides again consists of four sub-groups, namely (a) α-D-glucans, i.e., dextrans (*Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum*), mainly composed of α-1,6-linked glucose residues with variable (strain specific) degrees of branching at position 3, and less frequently at positions 2 and 4, and alternan (*Leuc. mesenteroides*) and mutans (*Streptococcus mutans* and *Streptococcus sobrinus*), both composed of α-1,3- and α-1,6-linkages; (b) β-D-glucans composed of β-1,3-linked glucose molecules with β-1,2-branches, produced by *Pediococcus* spp. and *Streptococcus* spp.; (c) fructans, mainly composed of β-2,6-linked D-fructose molecules, such as levan with some β-2,1-branching where sucrose is the main carbon source. (d) others, like polygalactan, composed of structurally identical repeating units with different glycosidic linkages. The main enzyme that steps-in in the production of dextrans by *Leuconostoc* spp., is dextransucrase, or glycosyltransferase [16]. Glycosyltransferases (GTF) and fructosyltransferases (FTF) are the major enzymes that take part in the synthesis of the homopolysaccharides which in most cases happen outside the bacterial cells. These enzymes can either be extracellular, intracellular or can be found embedded in the cell membrane as in some bacteria.

2.2. Heteropolysaccharides

Heteropolysaccharides, in contrast to HoPS, for the formation requires the intracellular scope with two or more different repeating units of monomers in addition to lipids such as isoprenoid glycosyl that participate in HePS development [17,18]. D-galactose, D-glucose and L-rhamnose are the frequently seen sugar moieties in the backbone of hetero EPS but in varied ratios. Lactic acid bacterial strains like *Lb. acidophilus* LMG 9433, *Lb. helveticus* TYl-2, *Lb. helveticus* NCDO 766, *Lb.*

*rhamnosus*C83, *S. thermophilus* Sfi20, *S. thermophilus* Sfi32 [19] and *S. thermophilus* LY03, *S. thermophilus* BTC and *S. thermophilus* 48 lack rhamnose, *S. thermophilus* OR 901 EPS contains repeating units of galactose and rhamnose alone and *Lb. sake* EPS consists of glucose and rhamnose repeating units. In contrast, the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 contains glucose and fructose in a ratio of 1:2 [20] and the polymer produced by *S. thermophilus* MR-1C consists of an octameric basic repeating unit composed of D-galactose, L-rhamnose and L-fucose in a 5:2:1 ratio. Apart from the sugar molecules, other residues, such as glycerol- 3-phosphate, *N*-acetyl-amino sugars, and phosphate and acetyl groups are also seen in the HePS chain and altogether they results in the high molecular weight polysaccharides [18,21]. Formation of the hetero EPS is a bit more complex than the HoPS as there involves the participation of numerous enzymes and/or proteins in their biosynthesis and secretion. Sugar-1-phosphates play a vital role in the biosynthetic pathway where it participates in the sugar activation necessary of the monosaccharide polymerization and sugar interconversions (like epimerization, decarboxylation, dehydration). Sugar activation and in-built modification of the polysaccharides go hand in hand with the these enzyme using their building blocks resulting in the final EPS [22].

3. An insight into the biosynthesis of EPS and role of glycosyltransferases

There are a wide variety of exopolysaccharides and the organisms that produce, among which lactobacilli itself have diverse classes of EPSs that are known to exhibit strain-specific properties that include probiotic action and other health benefit effects and textural properties. However, the knowledge on the diversity in these EPSs within the strain, and among other organisms remains unclear. Many studies on the biosynthesis and the genetics behind the synthesis are being explored and all are under the maturing stage and currently limited. Different biosynthesis pathways have been claimed and proved for EPS production of which the role of glycosyltransferases remains highlighted [23,24]. Table 1 summarizes some of the bacterial exopolysaccharides reported from different genera with their biosynthetic pathway along with the composition and applications.

After decades of research and investigational studies, scientists all over the globe could find that the EPS production by bacteria can take place by either of the four general mechanisms: (i) the Wzx/Wzy-dependent pathway; (ii) the ATP-binding cassette (ABC) transporter-dependent pathway; (iii) the synthase-dependent pathway and (iv) the extracellular synthesis by use of a single sucrase protein. In the first three pathways, the production of activated sugars/sugar acids, which are the key precursors molecules of the polysaccharides, are evolved as an outcome of the numerous enzymatic transformations within the bacterial cell (Fig. 1). Whereas for the extracellular synthesis pathway involves the direct addition of the monosaccharides obtained because of the cleavage of di- or trisaccharide.

The Wzx/Wzy dependent pathway involves the presence of a Wzx protein, a so called flippase enzyme. Here the monomers of the polymer are formerly linked to an undecaprenol diphosphate anchor (C55) at the inner membrane, are assembled with the help of several glycosyltransferases and translocated across the cytoplasmic membrane by the Wzx protein. Polysaccharide co-polymerase (PCP) and the outer membrane polysaccharide export (OPX) family proteins are the additional proteins that are involved in the transport of the finally polymerized polysaccharides from the periplasm to the cell surface. EPS produced by this pathway have an extremely diverse sugar pattern may be up to four or five sugars within the chemical backbone and therefore most of them fall into the heteropolysaccharides for this reason where Xanthan gum is an example. The bacterial strains that produce EPS by this pathway carry genes for flippases (Wzx) and polymerases (Wzy) in EPS operons in their genome [25].

Capsular polysaccharides (CPS) are another class of polysaccharides produced by bacteria which are often seen attached on to the cell surface rather than expelled out of the cell. The second class of polysaccharide biosynthesis, ABC transporter dependent pathway mainly focuses on the biosynthesis of such capsular polysaccharides. Similar to the former case of Wzx/Wzy dependent EPS, the CPSs synthesized by ABC transporter dependent pathway, employs the glycosyltransferases (GTFs) at the cytoplasmic face of the inner membrane, where an operon with a single GTF efficiently produces the homopolysaccharides while in case of hetero EPS, operons with multiple GTFs participate in the assembly of the polymers. The polysaccharide export and their translocation across the membrane is complex process and involves a tripartite efflux pump. This efflux pump is a complex with ABC- transporters that spans into the inner membrane along with proteins of PCP and OPX family proteins that are similar to those seen in the Wzx/Wzy pathway. One of the main differences that is found between Wzx/Wzy and ABC transporter dependent pathways is that the CPSs carry a phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonicacid (Kdo) linker at the reducing terminus which is a conserved glycolipid [26].

The synthase dependent pathway, third pathway, is the one independent of a flippase enzyme for the translocation of the polymers across the membranes and the cell wall. A single synthase protein takes the responsibility of polymerization and translocation of the polysaccharide wherein most cases are a subunit of the envelop- spanning the multiprotein complex [27]. The EPSs produced by synthase dependent pathway are mostly homopolysaccharides where the biosynthesis of curdlan is an example, which is a polymer of β-(1-3)-linked glucose in its backbone.

Extracellular biosynthetic pathway of EPS is another mode of polysaccharide biosynthesis where the precursor production happens inside the cell, polymerization and secretion appears to happen in the cell envelop. The EPSs like dextran or levan takes place via this biosynthetic pathway with the incorporation and active enzymatic reactions by glycosyltransferases which are either secreted out of the cell or are covalently bound on to the cell surface.

3.1. Role of GTF in homopolysaccharide synthesis

Dextrans are homopolymers of α-(1-6) linked glucose residues formed as result of the enzymatic activity of dextransucrases (generally glucansucrases). They fall into the category of glycosyltransferases (GTF, E.C. 2.4.x.y) which are considered as glycoside hydrolase family 70 (GH70) of enzymes [28]. These GTFs are further classified, based on the final polysaccharides they synthesize, into transglucosidases (E.C. 2.4.1.y) and transfructosidases (E.C. 2.4.1.y or 2.y), Transglucosidases, encoded by these GTFs includes glucan-synthesizing dextransucrases, mutansucrases, and reuteransucrases (E.C. 2.4.1.5) [18]. Basically, the glucansucrases catalyse the transfer of glucose molecule onto a growing chain of α-glycosidic linked oligo- and/polysaccharides from sucrose. Linkages formed to each of the free hydroxyl groups of the sugar moiety depends on the specificity of the glucansucrases in action. These specificities and differences in linkages along with different degrees of possible branching at hydroxyl groups results in a wide variety of EPSs with not merely the α-(1-6) linked dextran, but dextrans containing a small amount of α-(1-3) or even α-(1-2) linkages, other EPSs such as mutan with mostly α-(1-3) linkages, alternan with strictly alternating α-(1-3) and α-(1-6) linkages and reuteran consisting mainly of α-(1-4) linkages that are interspersed with α- (1-6) linkages [29]. *Leuconostoc* sp., of lactic acid bacteria are known for their synthesis of dextrans where the only intervening enzyme is glycosyltransferase, specifically called as dextransucrases [16].

Dextransucrases mostly exhibit their enzymatic action outside the cell as they are seen extracellularly or found anchored on to the cell wall. These enzymes having an average molecular weight of 110–160 kDa, are multidomain enzymes of GH70 family proteins whose three-dimensional structures are currently available and the structural elucidation revealed different structures than that was expected from the

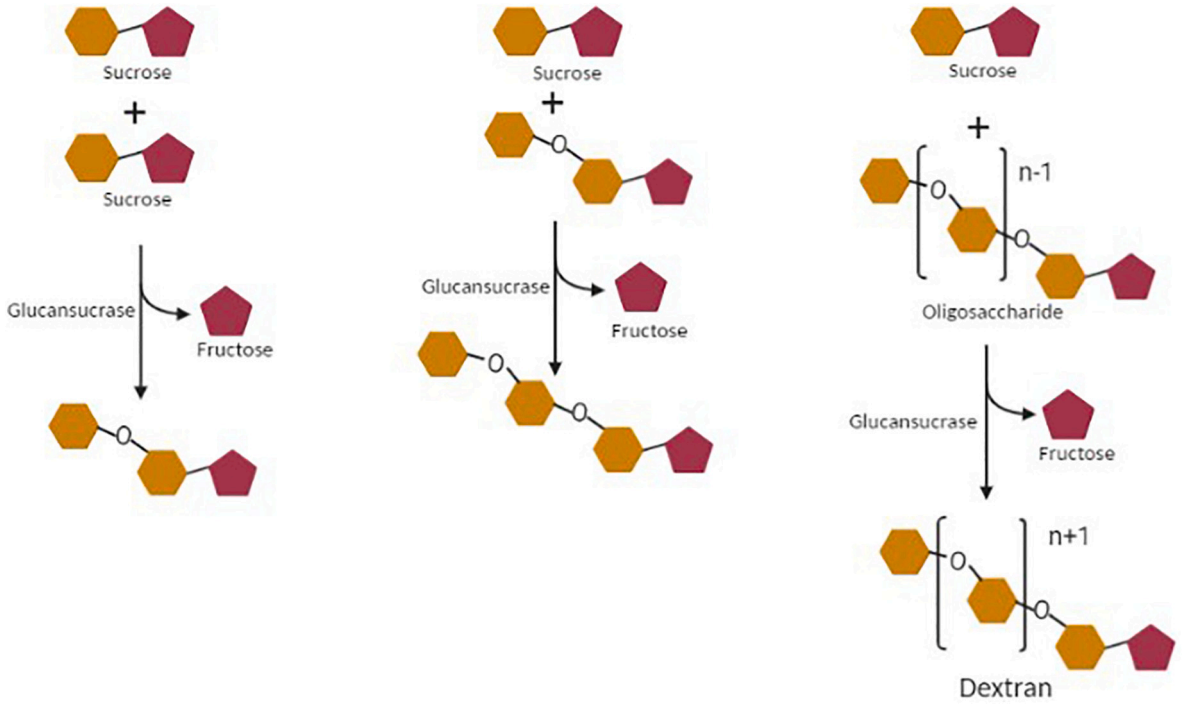

Fig. 2. Mechanism of glucansucrases (dextransucrase) in dextran biosynthesis.

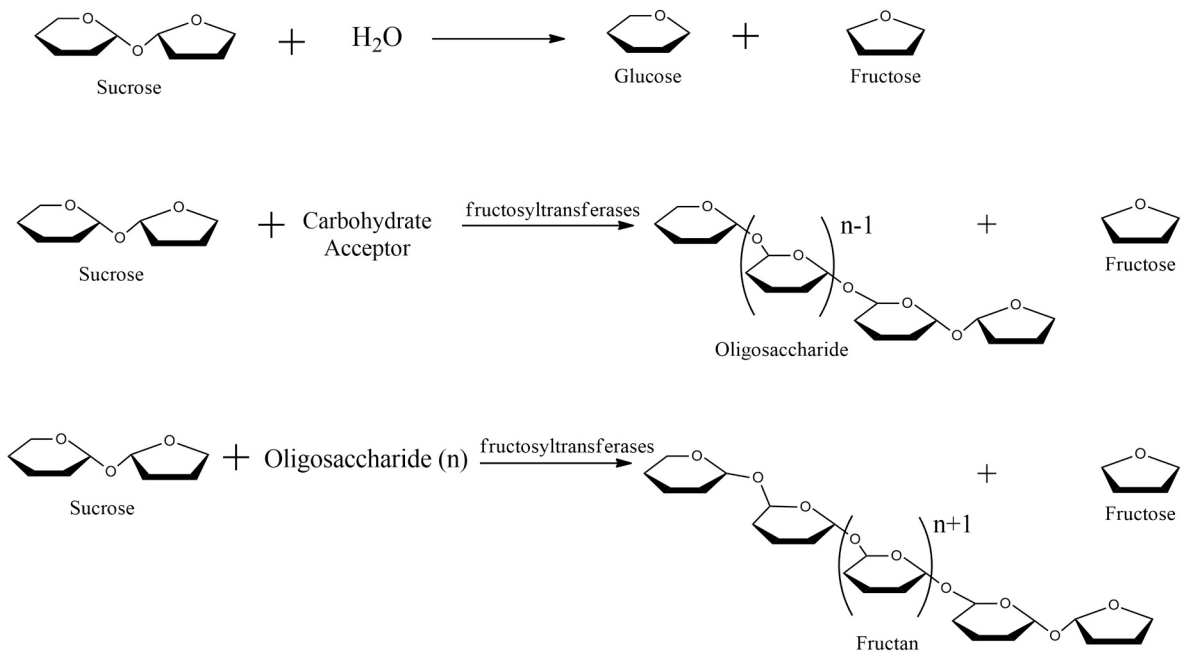

Fig. 3. Mechanism of fructosyltransferases in fructan biosynthesis.

sequence alignments [30]. There are three domains for the catalytic core of the glucansucrases with two extra domains attached to the core domains [29]. The enzymatic mechanism of glucansucrases was under debate over the years specifically concerning the chain initiation and elongation of the polysaccharide chain. But to the currently available knowledge, the scientists conclude that elongation occurs at the reducing end, which led to the proposal of two nucleophilic sites being involved in the process, where the growing chain remains covalently bound to the enzyme and is transferred from site one to the glucose moiety bound at site two and vice versa. According to this mechanism it explains the high processivity of the enzyme as polymer length is inversely proportional to the number of enzymes. On the other hand, Leemhuis et al. [29] could solve the crystal structure of glucansucrases and proposed a much simpler mechanism according to which the sucrose is hydrolysed during elongation, resembling the action of a retaining glycosyltransferase where a covalent β-glycosyl enzyme intermediate is formed via a carboxylic acid residue of the enzyme and this step retains the high energy of the glycosidic bond of sucrose as well. The enzyme does not favour the hydrolysis of this intermediate formed, even when the simultaneous release of glucose is possible. In fact, it transfers the glucose onto a hydroxyl group and elongation of the polysaccharide occurs at the non-reducing end of the polysaccharide

Table 2
LAB EPS with their monomeric units, linkages and the glycosyltransferases involved.

Organism	EPS	Monomers & linkage	Enzyme on action	References
Le. mesenteroides	Alternan	• α-(1,3) & • α-(1,6) glycosidic linkages associated with α-(1 → 3), (1 → 6) branches	Alternansucrase	[44]
Lb reuteri LTH5448, *S. salivarius* SS2, *Le. mesenteroides* NRRL B512F, *Lb. sanfranciscensis* LTH2590	Levan	Fructose •β-(2 → 6) linkages associated with β-(2 → 1), (2 → 6) branches	Levansucrase	[25,58]
Lb. reuteri 121	Reuteran	Glucose, Fructose • α-(1,4) & • α-(1,6) glycosidic linkage associated with α-(1 → 4), (1 → 6) branches	Reuteransucrase	[59]
Lactobacillus spp. G77 *Lb. suebicus* CUPV221	β-D-glucans	Glucose •β-(1,3)-glycosidic linkages	Glucansucrases	[60,61]
Lb. fermentum, *Lb. sakei*, *Lb. hilgardii*, *Lb. parabuchneri*, *Lb. curvatus*.	Dextrans	Glucose •α-(1,6) linkages associated with a low amount of α-(1 → 3) linkages and α-(1 → 3), (1 → 6), α-(1 → 2), (1 → 6), α-(1 → 4), (1 → 6) branches	Dextransucrases	[29,62,63]
Lb. reuteri ML1 *Lb. reuteri* GTFML1	Mutans	Glucose •α-(1,3)-glycosidic bonds	Mutansucrase	[62–64]
Le. citreum CW28 *S. mutans* JC-1 *S. mutans* BHT	Inulin-Type	Fructose •β-(2,1) associated with β-(2 → 6), (2 → 1) branches	Inulosucrase	[44,65]

Lb., *Lactobacillus*; *Le.*, *Leuconostoc*; *S.*, *Streptococcus*.

[29].

The general mechanism of glucan synthesis can be illustrated and represented by the following diagram (Fig. 2). The second class, fructan-catalysing transfructosidases which includes levansucrases (E.C. 2.4.1.10) and inulosucrases (E.C. 2.4.1.9) are encoded by FTF (fructosyltransferase) genes and their mechanism of catalysis is similar to GTFs and can be represented by the following diagram (Fig. 3).

4. Significance of glycosyltransferases in different lactobacilli

Since decades, scientists worldwide have been working on the molecular aspects of the EPS biosynthesis to find the genes and the gene clusters that participate in the EPS production in lactic acid bacteria like *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lactobacillus rhamnosus,Oenococcus oeni*, *Weissella cibaria*, *Lactobacillus plantarum* and many other LAB strains (Table 2) [31,32]. All these LAB species have a similar way of EPS biosynthesis where the sugar monomers are assembled on to a membrane-bound undecaprenylphosphate carrier from the activated sugar donors, produced inside the cell, via different glycosyltransferases [33].

A study by van Kranenburg et al. in 1997 [15], reported a 40 kb plasmid in *Lactococcus* NIZO B40 and its role in EPS biosynthesis. There exists a gene cluster with 14 genes in the order *epsRXABCDEFGHIJKL*and is transcribed into a single polycistronic mRNA of ~12 kb in size [34]. The disruption of epsD gene from the cluster abolished the ability of EPS production by the organism and thus enlightening the role of this gene in the pathway [15].

In *L. rhamnosus* GG, along with the genes required for EPS biosynthesis, their genome is equipped with genes for the regulatory proteins. Glycosyltransferases produced inside the cell catalyse the synthesis of blocks of repeating units in most cases linked to a lipid carrier molecule at the cytoplasmic site of the inner membrane. The EPS locus of *L. rhamnosus* GG consists of six genes welE to welJ which encodes putative glycosyltransferases with each enzyme exhibiting relevant enzymatic reactions in the biosynthetic pathway. From the available databases and bioinformatic analysis of these data we see that the protein encoded by welE gene shows 61% identity with similar protein YP_001271961 that encodes for a galactose phosphotransferase from *Lactobacillus reuteri* F275.But after several experiments and detailed study, it is seen that welE gene of *L. rhamnosus* GG successfully encodes for a priming glycosyltransferase where the enzyme has the prime role in the transfer the first sugar moiety of each EPS molecule and hence help in the priming of the growing EPS repeating unit of the entire polysaccharide backbone. This reaction is the first and foremost reaction in the biosynthetic pathway as well. Rest of the five genes, welF to welJ in the pathway also codes for glycosyltransferases which probably transfers the next sugars of the EPS backbone thus facilitating the formation of glycosidic linkages to form a perfect frame of EPS. From different structural characterization it was found that the *L. rhamnosus* GG produces a galactose-rich heteropolysaccharide EPS and further genomic analysis shows that the EPS subunits are transferred across the cell membrane by Wzx-type exporter and the polymerization of the EPS.

L. plantarum WCSF1 serves as a model organism for probiotic lactic acid bacteria especially lactobacilli whose genome is sequenced completely and is widely used for all molecular studies as a reference among lactobacilli species. The studies with *L. plantarum* WCSF1 and its biosynthesis of polysaccharides were studied by construction of *L. plantarum* WCSF1 strains that are cps-cluster deficient mutants, including single (Δcps1A-I, Δcps2A-J, Δcps3A-J, Δcps4A-J), triple (Δcps1A-3J), and quadruple (Δcps1A-3J, Δcps4A-J) deletion mutants. The function of cps cluster mutation and its consequences were studied in surface polysaccharide fraction of the mutants in comparison to the wild-type strain. Transcriptome profiling and its analysis were carried out to determine the function of each of the gene clusters. These studies revealed that each clusters had influence on the surface polysaccharide synthesis specifically the amount, molar mass and chemical composition [35].

Their genome analysis shows that they have two regions with genes coding (cps genes) for surface-associated polysaccharide biosynthesis (capsular polysaccharides). The first region which is of 49 kb size that consists of three *cps* gene clusters – *cps1*, *cps2* and *cps3*. Of these three *cps* gene clusters, *cps1* and *cps2* are unique and is seen only in WCSF1 whereas the *cps3* gene is conserved to most of the lactobacilli species like ST-III and ATCC 14917. Region two of 14 kb size has *cps4* gene cluster which remains conserved in other lactobacilli as well. *Cps2* and *cps4* structurally resembles that of Wzy-dependent polymer gene cluster [36]. The genes *cps2E* and *cps4E* encode the priming glycosyltransferases that

Table 3
LAB EPS with their corresponding gtf genes and coded enzymes.

EPS & LAB strains	Enzyme	Gene name/GenBank accession no.	References
Glucan			
Leuc. reuteri strain 121	Glycosyltransferases	*gtfA*	
Leuc. mesenteroides NRRL B—512F	Glucansucrases	dsr-A	[66]
Leuc. mesenteroides NRRL B-1299	Glucansucrases	dsr-B	[54]
S. mutans LM7	Glucansucrases	gtf-C	
Lc. lactis subsp. *cremoris*	Priming glycosyltransferases	*epsD*	[1]
L. rhamnosus GG	Priming glycosyltransferase	*welE* *welF* to *welJ*	[24]
Alternan			
L. citreum NRRL B-1355	Alternansucrase	AJ250173	[67]
L. citreum KM20		NC_010471	
L. citreum LBAE C11		NZ_CAGF01000008	
S. thermophilus Sfi6	Priming glycosyltransferase	*epsE*	[68]

L- Lactobacillus,Lc-Lactococcus, Leuc-Leuconostoc, S- Streptococcus.

catalyzes the very first step in the biosynthesis of polysaccharides by transferring the sugar-1-phosphate from UDP-sugar nucleotide to the undecaprenyl-phosphate. Apart from these two genes the *cps2* and *cps4* clusters have genes (*cps2FGJ* and *cps4FGI*) that encodes for other glycosyltransferase enzymes that have predominant role in the biosynthesis of the capsular polysaccharides produced by *L. plantarum* WCSF [35]. From the literature search and available data, Table 3 shows some of the reported glycosyltransferase genes.

4.1. Structure and reaction mechanism of glycosyltransferases

Glycosyltransferases, GTFs, mediate the synthesis of high molecular weight polysaccharides using sugars as monomeric units and catalyse the formation of the glycosidic linkages between them. These enzymes have been reported and classified in glycoside hydrolase family 70, GH70, in the CAZy database (http://www.cazy.org/fam/GH70.html) and this is based on the similarity in the amino acid sequences. The catalytic residues and the mechanism of action of these enzymes remain conserved within the GH family. The common characteristic of these enzymes is that they cleave the glycosidic linkages between glucose moiety and another sugar moiety like fructose, glucose using a catalytic $(\beta/\alpha)_8$ barrel domain. The enzyme is structured in such a way that is has a signal peptide, a variable N-terminal region, a catalytic core domain where the transfer of the glycosidic linkages takes place and a C- terminal glucan binding domain (Fig. 4). It has also been noted that the GH70 family of enzymes has close relation with that of the GH13 family enzymes (a-amylase family, contains starch modifying or hydrolysing enzymes) and to the GH77 family (amylomaltases), especially in the catalytic domain. So, for more convenience, the GH70, GH13 and GH77 family of enzymes are grouped together as GH-H clan or α-amylase super family. The amino acid residues involved in substrate binding and catalysis share a high sequence similarity and hence these enzymes are believed to share common reaction mechanism which involves a covalent β-glucosyl enzyme intermediate, with the retention of the α-anomeric configuration in the product [37].

Many investigations have been carried out to study the nature, structure, and molecular mechanism of glycosyltransferases enzymes and for this purpose the studies have been done in, glucansucarse GTF180 from *Lactobacillus reuteri* 180. It produces glucans with α-1,6 and α-1,3 glycosidic linkages catalysed by the glucansucrases of 198 kDa [38]. Attempts for crystallization of both the full length GTF180 protein and of 117 kDa fragment (lacks the N-terminal variable domain, GTF180-ΔN) of the enzyme have been carried out. For this to carry out, the full-length enzyme GTF180 (MG-39-1772-His_6, lacking signal sequence) and the GTF180-ΔN were over-expressed in *E. coli* BL21 (DE3) and was purified before crystallization experiments. The purpose of deletion was to avoid the protein flexibility and thereby enhance the possibility of crystallization. The end result was surprising that the truncated enzyme showed completely active and produced the α-glucan with similar linkage and mass as that of the full length glucansucarse, which shows that the N-terminal deletion did not significantly affect the enzyme activity. All these experiments revealed the 3D-structures of first GH70 GTFs and they became available. Apart from glucansucarse structures of GTF180-ΔN from *L. reuteri* 180, GTF-SI from *S. mutans* and the ΔN_{123}-GBD-CD2 of the α (1 → 2) branching GS DSR-E from *L. mesenteroides* NRRL B-1299 were some among the characterized GTFS in the recent times. But in the crystallization experiments, only the truncated enzymes formed crystals and only they could be studied [39] and the crystal structures of the complete GTF enzymes are yet to be reported.

The average amino acid sequence residue of glucansucrases is approximately 1600-1800 and is comparatively larger enzymes than the GH13 and GH77 family (~500–600 amino acids). But the sequence similarity of these enzymes with the GH13 α-amylases and the GH77 amylomaltases, glucansucrases are believed to exhibit an α-displacement mechanism for the catalysis reactions to take place. Primarily, the α-1-2 glycosidic bond of sucrose is cleaved and releases the fructose. This yields a glucosyl-enzyme intermediate, where the glucosyl unit is covalently attached to a catalytic nucleophile via a β-glycosidic linkage. The next step is the transfer of the covalently attached glucosyl unit to the either the reducing or the non-reducing end sugar acceptor residue of the growing polysaccharide chain and the reformation of the α-glycosidic bond. An alternative mechanism of action is the transfer of the glucosyl moiety to a low molecular mass acceptor like maltose or isomaltose or to a water molecule. It is also proposed that the mechanism requires two nucleophilic residues with one catalytic site or two

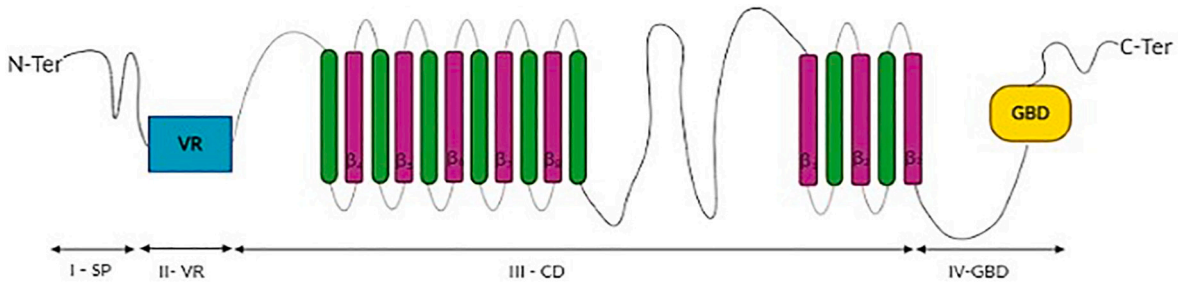

Fig. 4. General schematic structure of GTF gene of lactic acid bacteria.
The numerical at the top represent the amino acid residues. I – Signal peptide; II – Variable Region; III – N- terminal Catalytic Domain; IV – Glucan Binding Domain at C-terminal.

Table 4
Patent search on glycosyltransferases and applications.

Patent no. & year	Title	Relevant claim/field of invention	Reference
US 2018/0273919 A1 (2018)	Glucosyltransferase Enzymes for Production of Glucan Polymers	•Pertains to GTF enzymes & their poly alpha-1,3-1,6-glucans products, both of which has applications as viscosity modifiers	[69]
US 2019/0021354 A1 (2019)	Novel EPS Gene Cluster of Texturizing Lactic Acid Bacteria	• Reports on *Lactococcus lactis* subsp. *lactis*, with improved texturizing properties for making food products • Highlights on an active EPS gene cluster with genes involved in biosynthesis, regulation and modulation of EPS biosynthesis and export, including glycosyltransferases (GTFs), a polymerase and a transporter	[70]
US 2018/0305672 A1 (2018)	Modified Glucosyltransferases for Producing Branched Alpha - Glucan Polymers	•The disclosure claims to the production of branched alpha - glucans using modified glucosyltransferase enzymes	[71]
US 2018/0340199 A1 (2018)	Enzymatic Production of Alpha - 1, 3 - Glucan	•It relates to glycosyltransferase reactions comprising added oligosaccharides	[72]
US 9,644,190 B2 (2017)	Modified glucansucrases and related methods	•Pertains to a modified glycoside hydrolase enzyme comprising at least one mutation to the amino acid downstream from a transition state stabilizer site wherein the modified enzyme produces a glucan.	[73]
US 2006/0210510 A1 (2006)	Nucleic acid molecules coding for a dextran-saccharase catalysing the synthesis of dextran with alpha-1,2 osidic sidechains	•Invention pertains to DNA sequences encoding a glycosyltransferase catalysing the synthesis of dextrans or oligosides carrying α (1-2) glycosidic type linkages	[74]
US 2006/0073542 A1 (2006)	Recombinant glycosyltransferase fusion proteins	•Invention pertains to recombinant glycosyltransferase fusion proteins that catalyse the transfer of a saccharide from a donor substrate to an acceptor substrate in the enzymatic synthesis of oligosaccharide moieties of glycoproteins and glycolipid	[75]
KR101091138B1 (2011)	Glucansucrase Derived from *Leuconostoc lactis* and Method for Preparing the Same	• Invention of a novel glucansucrase from *Leuconostoc lactis* EG001 isolated from kimchi LAB • A gene encoding glucansucrase, development of a recombinant microorganism with glucansucrase activity • Application of this glucansucrase in food, pharmaceutical for synthesis of polysaccharides	[76]
US 2005/0059633 A1 (2005)	Novel Glucans and Novel Glucansucrases Derived from Lactic Acid Bacteria	• Invention is in the field of enzymatic production of biomolecules. • Deals with novel glucans derived from LAB with novel glucosyltransferases and with a process for production of new and useful glucans from sucrose	[77]
US 10, 351, 633 B2 (2019)	Enzymatic Synthesis of Soluble Glucan Fiber	•Pertains to a soluble - glucan fiber, soluble fiber compositions and methods of making and using the soluble α-glucan fiber	[78]
EP 2 427 565 B1 (2010)	Glucooligosaccharides Comprising (Alpha 1-4) and (Alpha 1-6) Glycosidic Bonds, Use Thereof, and Methods for Producing Them	• Invention relates to the field of nutritional effects of polysaccharides • In particular, it relates to the application of α- glucanotransferases in methods for preparing dietary fibers, including prebiotic oligosaccharides, and to novel oligosaccharides obtainable thereby	[79]
JP2020127403A (2020)	Alpha (1,2) Fucosyltransferases Suitable for Use in Production of Fucosylated Oligosaccharides	•Invention provides methods for engineering *E. coli* or other hosts to produce fucosylated oligosaccharides	[80]
US 10, 017, 748 B2 (2018)	Construction of New Variants of Dextransucrase DSR-S by Genetic Engineering	• Invention relates to nucleic acid sequences of truncated or mutated dextransucrases • Also, vectors containing these DNA sequences and host cells transformed by these genes encoding truncated or mutated dextransucrases	[81]
US 10, 308, 724 B2 (2019)	Very High Molar Mass Dextrans	• The subject matter of the invention is dextrans with 95% - 99% of α - 1, 6 glycosidic bonds, a weight average molar mass Mw at least equal to 0.7×10^9 g per mol^{-1} • The invention • also relates to a dextransucrase responsible for the production of these dextrans, and the method of production	[82]

(*continued on next page*)

Table 4 (*continued*)

Patent no. & year	Title	Relevant claim/field of invention	Reference
CN106434587B (2019)	A Kind of Dextransucrase and Its Application	• Invention is about a dextransucrase with its amino acid sequence (SEQ ID NO.2) • Construction of a recombinant glucansucrase plasmid • Preparation of water insoluble glucan with these dextransucrases under specific catalytic conditions	[83]
CN1692158A (2005)	Use of Lactobacillus to Produce Exopolysaccharides in Food and Pharmaceutical Compositions	• Relates to composition and preparation of food products with improved aroma, flavour, mildness, sweetness, consistency, texture, body, mouth feel, firmness, viscosity, wheying off, nutrition and health benefits • Reports a live microorganism and an enzyme that acts on the biosynthesis of an EPS.	[84]
KR100518181B1 (2005)	Mutant Gene of Dextransucrase Producing Only Oligosaccharide, Recombinant Vector Comprising the Mutant Gene, Microorganism Transformed by the Recombinant Vector, and the Dextransucrase Produced from the Microorganism	•Reports a modified dextransucrase gene, recombinant vector with the gene, a transformed microbe with the recombinant vector and production of the enzyme	[85]
US 6,867,026 B2 (2005)	Glucosyltransferases	• Pertains to a novel type of glycosyltransferase derived from *Lactobacilli* • Development of a process for recombinant production of the enzyme and for the production of useful glucans and gluco-oligosaccharides from sucrose	[86]
US 2020/0123510 A1 (2020)	Branched Alpha Glucans	• The present invention relates to the field of polysaccharides and their dietary effects. • In particular it relates to a method of producing a branched a - glucan	[87]

closely attached catalytic sites where the nucleophilic residues form covalent glucosyl-enzyme intermediates which alternatively transfers the glucosyl moiety to the C1 atom to the other covalently bound glucose there by freeing the nucleophile for the next round of reaction cycle [30].

With the evidence and revealing of the crystal structures of GTF180-ΔN, the molecular mechanism of the GH70 enzyme and its interactions became clearer and more evident. This explains the mechanism in contrast to the previous hypothesis and states that only a single active site is present and there is no space for a second covalently bound glucose or glucan polymer. It restates the hypothesis that there exists no double active-site/double-nucleophilic insertion mechanism. Along with this, the key amino acid residues involved in the substrate binding and the catalysis in GH13 and GH70 enzymes share similarities and the action and mechanism of of glycosidic bind cleavage by GH70 glucansucrases is in close connection with the α-amylase super family enzyme [30]. A similar kind of study about the crystal structure of *L. reuteri* 121 GtfB-ΔNΔV also suggests that the architectural design of the active- site of GTF intermediates between GH13 α-amylases and GH70 glucansucrases, having a binding groove just like that of the α-amylases but partly covered by loops forming a tunnel [40].

Studies on any enzymes relays on validation of its catalytic mechanism and quantification of the catalytic rate of the reaction. There are several studies that reports several of these assays by which this has been carried out with glycosyltransferases. Literature reports are available that clearly explains the principle and mechanism of with assays with crude glucansucrases, dextransucrases where the basis of all these assays is the detection of the fructose, which serves as the by-product of the reaction. The end-product detection can be either of the three ways-chemical, enzymatical or chromatographical way. The easy way is the chemical detection by measuring the reducing power of the carbohydrate released during the hydrolysis activity of the GTFs and quantify the glucose or fructose released during the process. It is mostly expressed in terms of per unit of the enzyme such as one glucansucrase unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose and/or glucose per minute at 30 °C, under specific buffering conditions (pH 5.4) and in the presence of specific concentration of sucrose. Likewise, his-tag-purified GTF enzymes from *E. coli* extracts and their culture supernatants of *Lactobacillus* strains grown on MRSs were also used as a source of enzyme for the enzyme assays as well. The GTF total activity measurement remains the same that of the previous case by determining the release of fructose from sucrose under specific enzymatic conditions [41].

Alternate way is by the quantification of the end product- i.e., to quantify the amount of polysaccharides produced, weighing after extraction and purification of the α-glucan or dextran produced or by degradation of the polysaccharides to its monomers (glucose). Another method to quantify is by labelling the sucrose. Using ^{14}C-labelled sucrose as substrate for the production of α- glucan can help easily quantify the amount of glucan produced [42].

4.2. Patent search on glycosyltransferases (gtfs) from LAB

There are so many patent reports based on glycosyltransferases. The patents put light into the relevance and the possible applications of these enzymes in a productive way rather than the mere theoretical knowledge. Most of these patents and the related inventions (Table 4) are claimed by different industries (such as DuPont) who are some of the major enzyme producers worldwide. Patents have been claimed by various researchers worldwide related to glycosyltransferase activity, EPS gene clusters of LAB, modified GTF enzymes and their related products, genetically engineered variants of dextransucrases, high molecular mass dextrans are some of them. Most of these patents focus on the EPS gene clusters and the genes involved in the biosynthetic pathway including the glycosyltransferases. Some inventions pertain to the genetically engineered or recombinant glycosyltransferases which

transfers a saccharide from a donor to an acceptor substrate and helps in the synthesis of oligosaccharides of glycoproteins and glycolipids. Heterologous expression of the glycosyltransferase genes and the development of a recombinant strain with gtf enzyme production is one of the other major patents filed. These recombinant microbes are used for the production of glucansucrases which finds applications in food, pharmaceuticals, and cosmetics industries. Patents that claim for the production of novel glucans derived from LAB with novel gtf enzymatic activity are also some of the major research areas of progress. All these studies prove the key role of glycosyltransferases on varying polysaccharide production with wide range of applications.

5. Conclusion

Recent LAB research focuses mainly on their biosynthetic pathway engineering, strain improvement and development of industrially relevant strains for better and enhanced production of the value-added compounds. The food-grade status of the organism, its occurrence and availability, and most importantly the ability of the organism to synthesize the vast variety of value- based compounds make lactic acid bacteria most aptly called as a microbial cell factory of industrial importance. Glycosyltransferases, being the major enzyme, mediated exopolysaccharide production by various lactic acid bacteria has a critical role in engineering aspects for better and improved production of EPS. The genes and the gene clusters responsible for EPS biosynthesis is seen enrooted in their chromosomes or in plasmids or might be distributed in both. From the available data and literature, we conclude that the EPS biosynthesis cannot take place in the absence of glycosyltransferases enzymes especially priming GTFs. Thus, engineering these enzymes in the pathway may be milestone to produce tailor-made EPS. Bacteria with GTF enzymes are used extensively in industry for a variety of applications. The polymer dextran is one prominent example of a universally used sugar chain. It is fermented at commercial scale for uses in veterinary medicine, separation technology, biotechnology, the food industry for gelling, viscosifying, and emulsifying, in human medicine as a prebiotic, cholesterol-lowering agent or blood plasma expander, and more. Characterization of these novel GTF enzymes and their exopolysaccharide products will provide a greater insight into the evolution (i.e., phylogeny and conserved sequence motifs), biochemistry (pH and temperature-optima; hydrolysis vs. *trans*-glycosylation activities), structure/function relationships of these enzymes contributing to, or determining, substrate/product specificities.

More information on the genome of the microorganism will enable to develop strategies to successfully enhance production rate and to engineer EPSs properties by modifying composition and chain length. In microbial EPS production, a better understanding of biosynthesis mechanism is a significant issue for optimization of production yields, improvement of product quality and properties, and for the design of novel strains. Understanding how to manipulate EPS production can also have importance in the biological performance of probiotics.

CRediT authorship contribution statement

Soumya M P: Pursuing PhD on the topic did the literature collection, Data curation and prepared the draft manuscript; Dr. K. Madhavan Nampoothiri: Provided general guidance on the content and made the critical reading and editing of the draft. Both authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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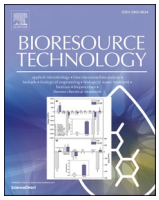

Nisin controlled homologous Over-expression of an exopolysaccharide biosynthetic glycosyltransferase gene for enhanced EPS production in *Lactobacillus plantarum* BR2

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HIGHLIGHTS

- Cloned an EPS biosynthetic glycosyltransferase gene to pNZ8148 food-grade vector.
- Developed a Nisin Controlled Expression System with dual plasmid electroporation.
- *gtf* homologous overexpression with quadrupled glycosyltransferase enzyme activity.
- *gtf* gene overexpression resulted in 54% increase in exopolysaccharide production.
- Significant exopolysaccharide production (23.5 ± 0.5 g/L) by a Lactobacillus strain.

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ABSTRACT

Glycosyltransferases synthesize a variety of exopolysaccharides (EPS) with different properties by altering the type of glycosidic linkage, degree of branching, length, mass, and conformation of the polymers. The genome analysis of an EPS-producing, *Lactobacillus plantarum* BR2 (Accession No: MN176402) showed twelve glycosyltransferase genes, and the gene *BR2gtf* (1116 bp), annotated as an EPS biosynthetic glycosyltransferase was cloned into the pNZ8148 vector. The recombinant pNZ8148 vector along with pNZ9530, a regulatory plasmid, were electroporated to *L. plantarum* BR2 for the over-expression of *gtf* gene under a nisin-controlled expression system and the glycosyltransferase activity of the recombinant and the wild-type strains were analysed. The recombinant strain showed 54.4% increase in EPS production with the maximum EPS production of 23.2 ± 0.5 g/L in a 5 L bioreactor study after 72 h of fermentation. This study shows an effective molecular strategy possibly to be adopted in lactic acid bacteria to enhance exopolysaccharide production.

1. Introduction

LAB, generally recognized as GRAS (Generally Regarded as Safe) bacterium, is widely explored in the industrial field as starter cultures for the food fermentation, production of value-added metabolites and gene expression systems due to their well-known safety status. Probiotic characteristics are added advantages with respect to their health-promoting capabilities (Nguyen et al., 2015). Live cultures of LAB are used in the fermentation of milk products to increase the bioavailability of nutrients and for obtaining beneficial effects of LAB (De Santis et al. 2019, Hsuan et al. 2022). LAB especially *Lactococcus lacis* are widely used as delivery vectors in oral and vaccine delivery systems (Hoo et al., 2020).

EPS are primary metabolites produced by bacteria as an envelope to cell to protect cells from extreme conditions without desiccating the cells. These EPS are considered as new source of polysaccharide which can meet the industrial demand and used as significant additive in food industries because of their physiochemical functions. EPS can enhance flavouring, improve textural properties, and can reduce syneresis in fermented foods such as yogurt (Korcz and Varga, 2021). The promising applications of EPS in industries points out the necessity to develop new strategies to enhance the production in a sustainable and economic way. For this, investigation for sustainable and economic source of production, strain improvement for high yield, optimization of fermenter

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parameters and process parameters, sharpening knowledge on biosynthesis pathway for the production of EPS has to be focused (Soumya and Nampoothiri, 2021). The EPS production, molecular size and structure, and its characteristics changes with respect to the microorganism used for the production. The benefits of the EPS such as, the probiotic effects, antioxidant properties, textural properties, flavour enhancing properties and other biological properties are determined by the functional units in the sidechain, organic and inorganic molecules present in the EPS, (Sasikumar et al., 2017). Thus, the potential application of EPS produced by each strain must be explored thoroughly.

Many studies on exploring the biosynthesis cascade, genes involved in the pathway and the function of each gene are explained. From those studies, it is evident that glycosyltransferase enzymes are responsible for the production of exopolysaccharides. The mechanism of production of EPS still needs clarity, but overall process involves the addition of nucleotide sugar units to lipid carrier and assembling this monosaccharide by glycosyltransferase enzymes which, further polymerize with in the intra cellular space and export to outside environment (Xiao et al. 2021, Soumya and Nampoothiri 2021). The genes responsible for this mechanism can be found in the chromosome or as seen embedded in the plasmid. The biosynthesis of EPS is very complex process in which so many genes are involved in the cascade of expression of the genes, the function and complete regulation of the synthesis is continuing as mystery.

Nisin Controlled Expression System (NICE) developed from *Lactobacillus lactis*, is one of the successfully studied, easy to operate auto regulated gene expression system by the induction of lactococcal bacteriocin nisin which can be transferred to low GC gram positive bacteria (Mierau and Kleerebezem, 2005). Nisin is lantibiotic, previously used as preservative in food industries. Nisin can induce pores in the cytoplasmic membrane causing imbalance in the potential ion concentration, which gradually leads to death. Research on the biosynthetic pathway of nisin revealed that there are 11 genes responsible for nisin synthesis. In those genes nisK and nisR was identified as regulators for expression of target gene present at the downstream of the promoter nisA. NisK codes for a histidine protein kinase present at the cytoplasmic membrane which can act as a receptor for nisin molecules. This binding of nisin to receptor activates the response regulator nisR through autophosphorylation. Activated nisR induces the transcription of genes from the promoter region nisA (Mierau and Kleerebezem, 2005).

This auto regulated system was exploited by isolating the nisK and nisR genes and embedded to suitable host like *Lactococcus lactis* subsp. *cremoris* MG1316 (which is, plasmid free nisin negative bacteria) and developed a new strain NZ9000. When a gene of interest is incorporated downstream to the promoter nisA, by induction of the sub-inhibitory concentration of nisin, this system can be regulated, and gene of interest is transcribed and expressed. pNZ9520 and pNZ9530 are the two other plasmid constructed (having high copy number and low copy number respectively) which allows the transfer of NICE system to other species (Mierau and Kleerebezem 2005). Some of the major benefits of this system includes, induction specificity, if nisin is not induced, activation of signal transduction and expression not take place, The expression of the system takes place $>$ 1000 induction fold, which is completely depend on the nisin concentration, protein expression after induction is greater than the total intracellular protein, while transformation of this dual plasmid to various strains same efficiency is illustrated (Wu et al., 2006). In production of biomolecules such as reuterin from *Lactobacillus reuteri* shows an increase in production and increase in the protein expression, after the introduction of nisin expression system (Wu et al., 2006). The expression developed was utilized not for gtf gene expression but also for expression of various other biomolecules antigens, cytokines, vitamins, antimicrobials proteins and membrane proteins (Guan et al., 2022; Landete, 2017). β-galactosidase is an industrially important enzyme, and it was over expressed in in recombinant *Lactobacillus reuteri* food vector in *Lactobacillus plantarum* to meet the wide industrial applications using this expression system (Nguyen et al., 2015).

Plasmid construction and its utilization in metabolic engineering of microbes for desired gene expression is very common in biotechnological applications. One such widely accepted and commercially explored plasmid is pNZ8148, a food grade vector. This plasmid have a canonical NcoI site around ATG start codon, advantages the ease in cloning of gene fused with nisA codon (Mierau and Kleerebezem, 2005). NIZO B40 is one of the successfully studied and exploited gene expression system which is used here. The *Lactococcus lactis* subsp. *cremoris* is known to have a plasmid NIZO B40 constructed by NIZO from *Bacillus subtilis* also (Mierau and Kleerebezem, 2005). *L. lactis* NIZO B40 contains a gene cluster of 12 kb length which specifically transcribes EPS biosynthetic genes. This 12 kb gene cluster contains 14 co-ordinately expressed genes, *epsRXABCDEFGHIJKL*. Each gene has specific function in which some of them are still undiscovered. The function of EPS was analysed with heterologous expression system and homologous expression system. In NIZO B40 expression system, biosynthesis of polysaccharide starts from extension of UDP glucose attached to lipid carrier done by epsD explained as priming glucosyltransferase. Addition of another sugar molecule to the extended bond was carried out by epsE and epsF. Addition of the third sugar unit was facilitated by epsG. The chain length of the exopolysaccharide was controlled by the combined action of epsA and epsB (Kleerebezem et al., 1999). These pNZ8148 vector has advantages and has the ease in cloning of any gene fused with nisA codon (Boels et al., 2001; Mierau and Kleerebezem, 2005). This broad host range vector with nisin induction was introduced to *E. coli* for expression of target genes (Wu et al., 2006) and for expression of the genes in gram-positive bacteria especially LAB, requires another vector pNZ9530 for the establishment of the NICE system for over expression of protein. But this duel plasmid system shows a growth retardation in the organism and to overcome this in bacteria like *Lactobacillus plantarum* and *Lactobacillus gasseri* the nisKR gene get integrated along with the chromosome and nisA promoter on another plasmid, which also reduces the transfer complication (Pavan et al., 2000).

In the present study, the homologous expression of a glycosyltransferase gene in *L. plantarum* BR2 for the EPS overproduction has been successfully demonstrated. The electroporation conditions in *L. plantarum* BR2 has also been optimized and the system works under a nisin controlled expression system.

2. Materials and methods

2.1. Chemicals, reagents, and instrumentation

Restriction enzymes, T4 DNA ligase and Q5 High-Fidelity DNA Polymerase were purchased from New England Biolabs (Massachusetts, United States). Plasmid isolations were carried out using QIAprep® Miniprep Kits from Qiagen (Germany). Culture media such as, De Man Rogosa Sharpe agar and broth (MRSA, MRSB), M17 agar and broth, Luria-Bertani broth and agar, and other components like yeast extract, K_2HPO_4, $MgSO_4$, $MnSO_4$, tween 80, ammonium sulphate, sucrose, nisin, antibiotics etc were purchased from Hi-Media (India). Chemicals like sodium acetate, DNS, sodium potassium tartrate, and phenol–chloroform-isoamyl alcohol were procured from Sisco Research Laboratories Pvt Ltd (India). Solvents such as ethanol, acetone, and others, were purchased from Spectrochem Pvt Ltd (India). Dialysis membranes and standards were purchased from Sigma Aldrich (India). Instrumentation includes BioRad Horizontal Electrophoresis Systems and Gene Pulser Xcell Electroporation Systems (BioRad, US), Microplate Reader-Tecan Nano Quant Infinite M200 Pro, (Switzerland), Spectrophotometer (UV-1601 UV Visible Spectrophotometer, Shimadzu, Japan), Cooling centrifuge (Kubota, Korea) and Lyophilizer (Christ Alpha 1–2 LD Plus, Germany).

2.2. Bacterial strains, media, and culture conditions

The bacterial strains and plasmids used are listed in Table 1. *L. plantarum* BR2 was propagated in MRS medium and *E. coli* MC1061 cells in LB medium. For EPS production studies, the bacteria were cultivated in optimized EPS production medium as mentioned in our previous reports (Sasikumar et al., 2017) at 37 °C under static conditions. After genetic manipulations, the bacteria were grown in medium supplemented with chloramphenicol (10 μg/ml) and erythromycin (10 μg/ml) antibiotics as and when required. For the recombinant strains, nisin served as the inducer for gene expression.

2.3. General DNA manipulations

Isolation of DNA, plasmids and recombinant DNA techniques were performed as per the standard methods described by Sambrook and Russell's molecular biology laboratory manual (2014). The DNA isolated was visualized in 1% prestained (EtBr) agarose gel (0.8%) and quantified by spectrophotometer (NanoDrop 2000, USA) and finally, the whole genome analysis of *L. plantarum* BR2 was done for detailed examination of the genome for identification of *gtf* genes.

2.4. Whole genome sequencing of L. plantarum BR2

High-quality genomic DNA of *L. plantarum* BR2 was extracted using XcelGen Bacterial gDNA mini kit (Xcelris genomics, India) as per manufacturer's instructions. The *L. plantarum* BR2 genome was sequenced (outsourced with Bionivid Technology Pvt Ltd, India) using Illumina HiSeq 4000 platform with paired ends of 101 base pairs read length and having $\geq$ 80% high quality reads with phred score $\geq$ 30 (extracted using NGSQC toolkit). The high-quality reads were assembled using Velvet v.1.2.10. SSPACE v.3.0 was used to construct the scaffolds of the assembled reads. Denovo genome validation and quality control for the final scaffolds were performed using Bowtie2 v.2.2.2 and Aragorn v1.2.36 and RNAmmer 1.2 software programs were employed to identify tRNA and rRNA genes respectively. Genome annotation and functional characterization were carried out using Rapid Annotation Subsystem Technology (RAST V 2.0) server. The screening for non-core genomic elements like plasmids and bacteriophage identification was performed by PlasmidFinder V 1.3 and PHASTER respectively.

2.5. Construction of recombinant plasmid and transformation

From the whole genome annotated data, all the glycosyltransferase genes were identified and primers specific for the *gtf* genes annotated as exopolysaccharide biosynthetic glycosyltransferase (*BR2gtf)* were designed using CloneManager_V9 software. The PCR amplification of *BR2gtf* gene was carried out using the following primers (with XbaI and HindIII restriction sites underlined) *gtf* For' GCGGCGTCTAGAGTGAAGATTGTTTACATCATTAC and *gtf* Rev' GCGGCGAAGCTTCTAGGCCAAGCAACGCAAATAG and with the Q5® High-Fidelity Polymerase (NEB, US) according to the supplier's recommendations, to avoid the addition of a polyA tail at the end of the PCR products. Optimized PCR reaction mixture includes: 1 μg of total gDNA of *L. plantarum* BR2, 10 nmol $^{-1}$ of dNTPs, 10 μmol^{-1} of each primer and 2–5 U of Q5 DNA polymerase in 50 μL. The amplification was performed using a T100 Thermal Cycler (BioRad, USA) following the steps with: initial denaturation of 95 °C for 3 min; followed by a denaturation of 95 °C for 30 s, annealing at 55.4 °C for 1 min 30 s and extension at 72 °C for 2 min; and final extension at 72 °C for 20 min. PCR products were visualized under UV gel documentation system, (Gel Doc XR + Biorad, USA) after electrophoresis in 1–1.5% agarose gels containing ethidium bromide. The amplified PCR products and pNZ8148 vector were then digested using the respective restriction enzymes (New England BioLabs, USA) and then ligated using T4 DNA ligase and buffer from (NEB, USA) according to the user's manual. The *E. coli* MC1061 competent cells, prepared by TSS (Transformation and Storage Solution) method, were further transformed by heat shock with the ligated vector under controlled

Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Characteristics	Growth conditions	Source or Ref
Strains			
L. plantarum BR2	EPS producing strain	MRS/ EPS production medium; 37 °C; Static conditions	This study
Lactococcus lactis NZ9000 containing pNZ8148	Chloramphenicol resistant strain	M17 + 0.5% glucose + 10 μg/mL chloramphenicol; 30 °C; Static	NICE Expression System
Lactococcus lactis NZ9000 containing pNZ9530	Erythromycin resistant	M17 + 0.5% glucose + 10 μg/mL erythromycin; 30 °C; Static conditions	NICE Expression System
Escherichia coli MC1061	MC1061 is a recombinant positive strain (recA$^+$) provided for cloning and amplification of plasmid DNA of diverse Gram-positive bacteria, e.g., plasmids for expression in lactic acid bacteria	LB Medium	MoBiTec
Escherichia coli MC1061 with pNZ8148*BR2gtf*	Recombinant MC1061 cells for cloned plasmid propagation and maintanence; chloramphenicol resistance	LB Medium with 10 μg/mL chloramphenicol; 37 °C; shaking at 200 rpm	This study
L. plantarum BR2OE4	Recombinant *L. plantarum* BR2 with over-expressed *gtf* gene; Nisin-controlled dual plasmid system	MRS/ EPS production medium; with 10 μg/mL chloramphenicol & 10 μg/ml erythromycin 37 °C; Static conditions	This study
Plasmids			
pNZ8148	Broad-host-range food-grade shuttle vector; Chloramphenicol resistant (*CmR)*; Nisin A promoter (*PnisA*); Contains a terminator after the MCS		MoBiTec, (Maischberger et al., 2010; Nierop Groot and Kleerebezem, 2007)
pNZ9530	Low copy plasmid with pAMβ1 origin of replication, which carries the *nisR* and *nisK* genes. For cloning in lactic acid bacteria genera that do not have the regulatory genes integrated into the chromosome. In this case for nisin-induced expression a dual plasmid system is used: e.g., pNZ9530 (*nisRnisK*) + pNZ8148 (+insert) (Kleerebezem *et al.*, 1997).		MoBiTec, (Pavan et al., 2000)
pNZ8148*BR2gtf*	Recombinant vector with *BR2gtf* gene incorporated into the MCS; Chloramphenicol resistance		This study

conditions. The transformed colonies were selected from LB- chloramphenicol (10 μg/mL) agar plates, and incubated overnight at 37 °C and this *E. coli* transformation were done to ensure cloned pNZ8148-*BR2gtf* plasmid propagation and easy maintenance. From the multiple colonies produced, transformants were further confirmed by colony PCR with the *gtf* gene specific primers and from those positive clones, recombinant plasmid DNA, pNZ8148-*BR2gtf,* was extracted using QIAprep Miniprep Kit (Qiagen) followed by double digestion and insert release from the cloned plasmid was checked for the clone confirmation.

2.6. Development of NICE expression system in L. plantarum BR2

To enable the homologous expression of glycosyltransferase enzymes in *L. plantarum* BR2, pNZ8148-*BR2gtf* as constructed. For its expression to happen, there is a need to electroporate the recombinant pNZ8148-*BR2gtf* plasmid along with regulatory plasmid pNZ9530 to the *L. plantarum* BR2 host cell which serves as the regulatory plasmid for nisin expression system. Following the earlier protocols of lactobacillus electroporation (Jin et al., 2012; Landete et al., 2014; Teresa Alegre et al., 2004) with slight modifications *L. plantarum* BR2 electro competent cells were prepared. Briefly, *L. plantarum* cells were grown in MRS broth containing 2.5 % glycine and 0.5 M sucrose at 37 °C until an OD_{600} of 0.6 is attained. The cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C and the cells were then resuspended and washed twice in ice-cold 10% glycerol containing 0.5 M sucrose as the washing solution. After two washes the cells were again collected by centrifugation at 4000 rpm for 10 min at 4 °C and resuspended the cells in ice-cold 10% glycerol containing 0.5 M sucrose and 50mN EDTA and was the incubate in ice for 15–20 min and was pelleted and washed further and the bacterial cells were resuspended 1:100 in the same washing solution. A volume of 80 μL or 100 μL of cells were electroporated immediately or aliquoted and stored at −80 °C for future use within six months. *L. plantarum* BR2 cells (80 μL) were electroporated at low temperatures, 0–4 °C, in Gene Pulser cuvette with an electrode gap of 0.2 cm with 8 μL of rpNZ8148 (175 ng/μL) and 8 μL of pNZ9530 (150 ng/μL) plasmid DNAs in a Gene Pulser electroporator (Bio-Rad, CA, USA). Voltage of 2.1 kV; resistance of 200 Ω; capacitance of 25μF; were the electroporation parameters followed. 1 mL of pre-warmed (at 37 °C) MRS-broth containing 2 mM $CaCl_2$ + 20 mM $MgCl_2$ + 0.5 M sucrose was added immediately to the cuvette after electroporation and was incubated at 37 °C for 2 h for recovery. Following the incubation, the *L. plantarum* BR2 cells, having the dual plasmid system was plated on chloramphenicol (10μg/μL) and erythromycin (10μg/μL) containing MRS agar plates for 16 to 24 h for selection and proliferation of recombinant cells. The recombinant positive clone was further named as BR2OE cells.

2.7. Growth analysis of recombinant L. plantarum BR2

The growth of recombinant BR2OE cells were analyzed by determining the viable cell count of the bacteria at different time intervals grown in MRS broth containing chloramphenicol (10 μg/μL) and erythromycin (10 μg/μL) as antibiotics for selection of the respective plasmids. In brief, bacterial culture samples were withdrawn at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of time intervals from the freshly inoculated broth and serial dilutions were prepared up to 10^8 using sterile distilled water under aseptic conditions. Viability of the BR2OE cells were enumerated by using the spread plate method. Required dilutions (10^4,10^5,10^6 and, 10^7) of the samples were spotted on the MRS agar plates containing antibiotics and kept undisturbed for drying. All plates were incubated at 37 °C for 24 h and the viable cell count was calculated as follows:

$$CFU/mL = Number\ of\ colonies\ formed \times dilution\ factor\ of\ sample/Vol.\ of\ sample \quad (1)$$

*CFU: colony-forming unit.

2.8. Optimization of Nisin concentration

Nisin induction for recombinant *L. plantarum* BR2 cells were done as described in the previously available reports (Mierau et al., 2005; Wu et al., 2006) with modifications according to the observations resulted from the laboratory experiments. Nisin (Merck, New York) for induction was prepared in 0.05% acetic acid at a concentration of 40 mg/mL. The prepared stocks were aliquoted, at a concentration of 20 mg/mL and stored at −20 °C. To optimize the concentration of nisin for attaining the highest EPS production, different concentrations of nisin (10–80 ng/mL) were induced in the EPS production medium containing chloramphenicol (10 μg/μL) and erythromycin (10 μg/μL) as antibiotics when OD_{600} of the BR2OE cells reaches 0.4. After fermentation and completion of incubation, the EPS was recovered by ethanol precipitation and the final production yield was estimated. The concentration at which the highest EPS production was observed served as the optimized nisin concentration for induction in the future fermentation experiments.

2.9. GTF enzyme recovery and assay

Ammonium sulphate precipitation of the culture supernatants of the wild-type and the recombinant *L. plantarum* BR2 strains were done to precipitate and recover the total proteins including GTF protein and further to estimate their enzyme activities. Bacterial cells were grown in the optimized MRS-sucrose EPS production media with all the essential nutrients at 37 °C under static conditions. For the recombinant strain, in addition to the above-mentioned media, it contained chloramphenicol (10 μg/μL) and erythromycin (10 μg/μL) antibiotics and optimized concentration of nisin as inducer. After incubation for desired intervals of time (24, 48 and 72 h), the culture broth was centrifuged at 8000 rpm for 15 min at 4 °C. After fermentation, to the cell free supernatant, added different concentrations of ammonium sulphate to precipitate the proteins. Proteins were precipitated at lower temperatures (0–4 °C) with different ammonium sulphate concentrations to obtain different protein fractions until complete saturation is reached. The precipitated protein fractions were resuspended in 20 mM sodium acetate buffer with 1 mM $CaCl_2$, pH 5.4. Finally, obtained three protein fractions precipitated at three different ammonium sulphate concentrations (0–10%, 10–50% and 50–90%). These protein fractions were then dialyzed against 50 mM sodium acetate buffer pH 5.4 for 24 h at 16 °C. After estimating the total protein concentration by Bradford assay these protein fractions served as crude enzymes for glycosyltransferase assay and they were stored at −20 °C for further use. Gtf enzymatic activity was assayed in triplicates by measuring the release of reducing sugars from sucrose by 3,5-dinitrosalicylic acid method (DNS) (Bounaix et al., 2009). One GTF unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose and/or glucose per minute at 37 °C, in 50 mM sodium acetate buffer (pH 5.4) in the presence of 100 mM sucrose. The incubation time for the maximum enzymatic activity was also optimized.

2.10. EPS production by recombinant L. plantarum BR2 in bioreactor

EPS production by the recombinant *L. plantarum* BR2 strain was carried out with the earlier reported EPS production medium (Soumya et al., 2019) and fermentation conditions with the addition of chloramphenicol (10 μg/μL) and erythromycin (10 μg/μL) antibiotics and nisin as the inducer in the fermentation media. The EPS was finally recovered from the broth and further purified by ethanol precipitation and lyophilized. The EPS yield was compared with that of the wild type. To check the stability of the recombinant culture, production was also tested in 5L capacity bioreactor (Minifors 2, Infors HT, Switzerland) by controlling all parameters (37 °C temperature, initial pH of 7.3, aeration rate 0.1vvm, 100 rpm).

3. Results and discussion

According to Sasikumar et al., 2017, the native *L. plantarum* BR2 produces exopolysaccharides with a maximum titer of 4.5 ± 0.5 g/L with 40 g/L lactose as the carbon source and under other optimized conditions. From the structural and physiochemical characterization of secreted exopolysaccharide, revealed the glucomannan heteropolysaccharide nature (consisting of glucose and mannose monomeric units) with a high molecular weight, 2380 kDa, EPS having high thermostability of 260 °C which can be explored in food industries as strain shows moderately good probiotic behaviour. The EPS also shows profound antidiabetic, cholesterol lowering and antioxidant properties (Sasikumar et al., 2017; Soumya et al., 2019). The topological images (unpublished data) of the biopolymer encompasses a porous web-like morphology with the compact homogeneous smooth microstructure proposing it as a potent candidate with promising rheological and textural properties, which can be exploited by food industries especially as a stabilizing and anti-syneresis agent (unpublished data). The water soluble, adhesive, and pseudoplastic nature of this EPS needs to be explored in the future to transform this bioprocess to the next level of research in a productive manner where it finds different applications in a sustainable manner.

As mentioned, despite the nutritional and health benefits, the major limitation of LAB EPS is their production levels. One of the main strategies to improve this is the over-expression of prime gene (*gtf*) or by metabolic engineering approaches to increase the production of EPS, and thus it remains the focus of this work.

3.1. Genome insights of L. plantarum BR2

Genome sequencing data of *L. plantarum* BR2 revealed that it has a chromosome of 3,253,735 bp in size with 44.55% of G + C content. The 16S rRNA gene sequence of *L. plantarum* BR2 obtained by Sanger sequencing and draft genome shared 100% homology. The genome predicted to encode 3242 genes which included 3167 protein coding genes. A total of 2076 genes (65.55%) were predicted to have known functions while rest of the genes were annotated as hypothetical proteins. There were no plasmids present in the genome. The genome properties and the subsystem distribution have been summarized (see Supplementary Materials).

The detailed analysis and further annotation of the genome predicted the presence of 12 glycosyltransferase genes (Table 2) of which one is specially annotated as exopolysaccharide biosynthesis glycosyltransferases further named as *BR2gtf* with a gene size of 1091 bp. Fig. 1 depicts the phylogenetic analysis of these 12 *gtf* genes (Tamura et al., 2021). The 363 AA length protein sequence of the *BR2gtf* gene with the ProtoParam tool reveals that the protein is of 40.14 kDa with a theoretical pI of 9.06. The TMHMM results show that this glycosyltransferase enzyme is extracellularly produced as there is neither the presence of transmembrane region nor cytosolic signals (see Supplementary Materials). Homologous expression of this gene under nisin induction was attempted to enhance the overall EPS production.

Table 2
Glycosyltransferase genes identified in *L. plantarum* BR2 genome and its characteristics.

Sl. No	Name	START	STOP	Strand	Gene Size
1	glycosyltransferase	891,333	890,599	–	734
2	Putative glycosyltransferase	949,008	947,680	–	1328
3	glycosyltransferase (putative)	1,404,538	1,403,234	–	1304
4	Glycosyltransferase	1,584,955	1,586,307	+	1352
5	**Exopolysaccharide biosynthesis glycosyltransferase (EC 2.4.1)**	**1,621,419**	**1,622,510**	+	**1091**
6	glycosyltransferase	1,622,526	1,623,554	+	1028
7	glycosyltransferase	1,624,810	1,625,778	+	968
8	Uncharacterized glycosyltransferase YkoT	2,032,565	2,031,570	–	995
9	Glycosyltransferase LafB, Responsible for the formation of Gal-Glc-DAG	2,222,844	2,221,822	–	1022
10	Glycosyltransferase	2,271,823	2,271,047	–	776
11	Glycosyltransferase	2,280,007	2,279,114	–	893
12	Glycosyltransferase	2,284,953	2,284,219	–	734

3.2. BR2gtf cloning and construction of NICE system in L. plantarum BR2

The PCR reaction after several optimizations on annealing temperature and other PCR conditions with *BR2gtf* gene specific primers using *L. plantarum* BR2 genomic DNA as template gave specific bands at the expected size. The length of the PCR amplified product including the restriction sites sequence inserted in pNZ8148 vector was 1116 bp and was confirmed by colony PCR (Fig. 2a), double digestion and insert release (Fig. 2b) and by gene sequencing (Fig. 2c) suggesting the successful cloning of *BR2gtf* gene into pNZ8148 shuttle vector.

The recombinant pNZ8148 vector carrying the *BR2gtf* gene along with the regulatory plasmid, for nisin controlled expression, pNZ9530 were then electroporated into the wild type *L. plantarum* BR2 for over-expression of glycosyltransferase enzyme. The recombinant cells carrying dual plasmids with chloramphenicol and erythromycin as selection markers were able to grow in these antibiotics containing MRS medium. The recombinants were again confirmed by colony PCR for the amplification (Fig. 3) of *BR2gtf* gene with the plasmid specific primers and the positive *L. plantarum* BR2 strain with recombinant pNZ8148 and pNZ9530 was further named as BR2OE4.

3.3. Growth analysis of BR2OE4

The initial growth of BR2OE4 was slow when compared to the wild type *L. plantarum* BR2. The reduction in growth of BR2OE4 at their lag phase can be explained by the presence of two antibiotics, erythromycin (10 μg/mL) and chloramphenicol (10 μg/mL), in the MRS media for the propagation of recombinants. However, with prolonged incubation time, the BR2OE4 cells picked up growth within 72 h, by the end of fermentation, both the wild-type and the recombinants showed similar CFU/mL and is summarized in Fig. 4.

3.4. Nisin concentration optimization and EPS production by BR2OE4

The optimum nisin concentration for the maximum gene expression in BR2OE4 was resolved by determining the EPS produced with different concentrations of nisin within a range between 10 and 80 ng/mL where the maximum EPS production of 7 ± 0.5 g/L was attained when 40 ng/mL of nisin was used (see Supplementary Materials). There was an increase in the EPS yield with an increase in nisin concentration until 40 ng/mL after which the EPS yield started declining. The effect of nisin induction and thereby *BR2gtf* gene expression is again validated by the difference in EPS production of wild type *L. plantarum* BR2 and uninduced BR2OE4 which was lower when compared to the optimally induced BR2OE4 recombinant strain. Thus, the maximal EPS production of the system, BR2OE4 by the over-expression of *BR2gtf* gene on nisin controlled expression was calculated to be 44.79 % increase when compared with that of the wild type *L. plantarum* BR2.

Nisin controlled gene expression is a well-studied system in lactic acid bacteria consisting of an expression vector, pNZ8148 and a regulatory plasmid, pNZ9530 that has been derived from two compatible broad host range replicons are used in this study (Silva-Bea et al., 2022). In this homologous gene over-expression system constructed in *L. plantarum* BR2, for increased EPS production, nisin acts as the inducer for the gene expression. Nisin induction is most preferred when the cell

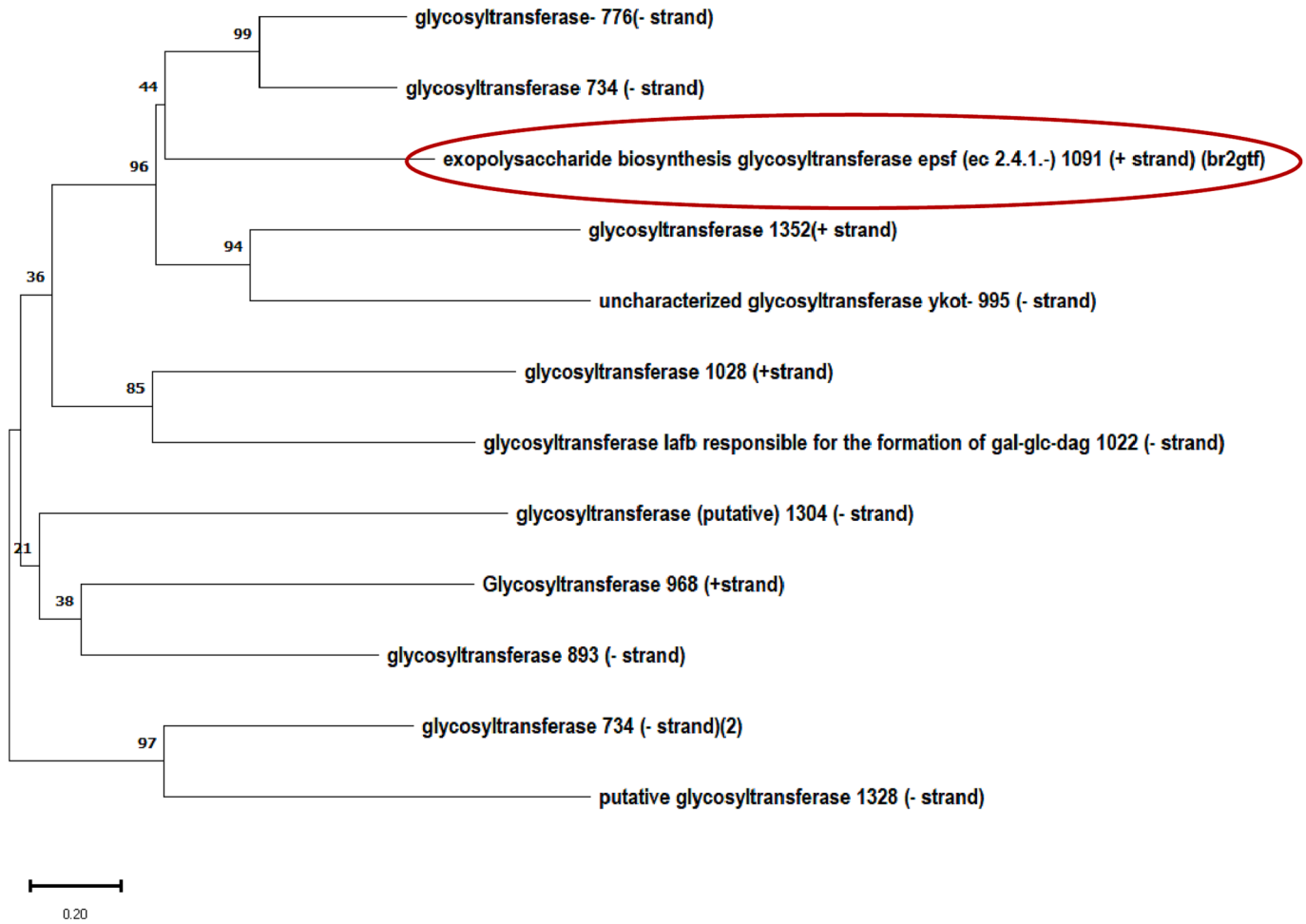


Fig. 1. Phylogenetic tree for glycosyltransferase genes of *L. plantarum* BR2 from the draft genome data. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1425 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. The gene encircled in red is the *BR2gtf* gene used in the study.

density of the culture attained an OD_{600} of 0.4–0.5 while the cells are in their exponential phase (Mierau et al., 2005).

Igor Mierau et al. (2005), states that the media composition, cell density at the time of induction and the amount of nisin shares strong correlation for a maximum gene expression to happen. Nisin induction being a dynamic process preferably needs higher density of cells in their log phase so as to ensure successful induction to occur and for the subsequent gene expression to proceed instantaneously. Nisin induction concentration and thereby product formation varies within microbes engineered. Optimum induction showed less variations in production in lactobacilli and when compared with the optimum nisin concentration *Lb. gasseri* required 50 ng/mL, while for *Lb. casei* the nisin concentration was found to be 10 ng/mL and *Lactococcus lactis* was 1–5 ng/mL, which is 10–50 times lower. This also supports the current data of BR2OE4 with maximum EPS production with a nisin concentration of 40 ng/mL.

3.5. Bioreactor studies

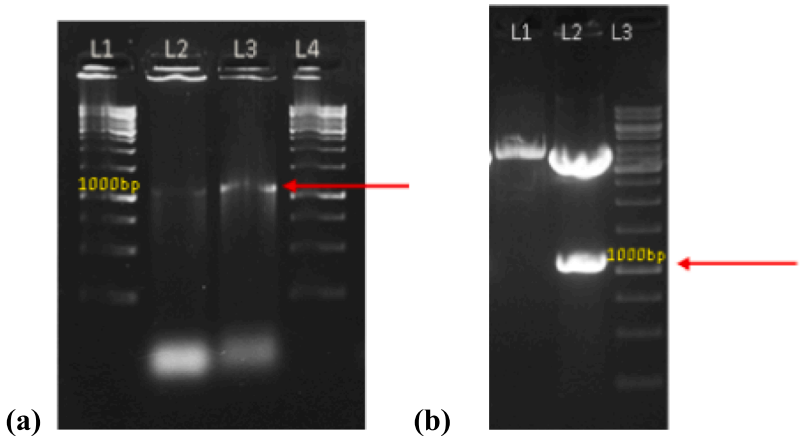
EPS production evaluated with the optimized fermentation conditions in a 5 L fermenter (see Supplementary Materials). With 0.1 vvm aeration and agitation of 100 rpm resulted in 23.5 ± 0.5 g/L of EPS by the BR2OE4 which reports a highest production of EPS from a *Lactobacillus plantarum* strain in a 5L.

The fermenter scale production of EPS showed that the production of EPS makes the broth viscous and there by effective aeration and agitation is necessary. Agitation can maintain the effective oxygen transfer throughout the medium but inappropriate speed of agitation can results in poor oxygen transfer (Bandaiphet and Prasertsan, 2006). While increasing the agitation speed there can be heterogeneous mixing, generation of heat transfer to broth, cell rupturing can happen which drastically reduces the production. So, in this condition maintain minimum agitation speed and aeration. The rate of production of EPS under this minimum condition was seen higher for any reported Lactobacillus strain.

3.6. Glycosyltransferase enzyme activity assay

Glycosyltransferase enzymes plays an important role in EPS biosynthetic pathway and their role in LAB EPS is being explored by scientists all over the globe. It is proposed that extracellular glycosyltransferase activity is responsible for the synthesis of soluble EPS from a variety of C-sources (Bejar et al., 2013). Many patents have been filed on the heterologous gtf expression and development of recombinant strains in LAB taking into consideration the vast area of its applications. All these studies unanimously proved the key role of glycosyltransferases on varying polysaccharide production with wide range of applications (Soumya and Nampoothiri, 2021).

Extracellular glycosyltransferase activities of over-expressed



(c)

Fig. 2. (a) Clone confirmation of pNZ8148-*BR2gtf* by colony PCR amplified product of BR2gtf gene cloned to pNZ8148 vector and transformed to *E. coli* MC1061 cells (L1 & L4:1kb DNA ladder, L2: BR2gtf from clone 1, L3- BR2gtf from clone 2 of size 1116 bp) **(b) Clone confirmation of pNZ8148-*BR2gtf* by Restriction digestion profile** of empty pNZ8148 and recombinant pNZ8148-*BR2gtf* plasmids with restriction enzymes XbaI and HindIII. Lane1: Native pNZ8148 double digested, Lane 2: pNZ8148-*BR2gtf* double digested, Lane 3:1 kb DNA ladder **(c) Sequence Alignment of cloned pNZ8148*BR2gtf* sequence and *BR2gtf* gene sequence.** This indicates the DNA sequence for insert and plasmid pNZ8148 overhangs (marked in red boxes) carrying BR2gtf after sequencing the cloned vector with the respective plasmid primers.

BR2OE4 and wild-type *L. plantarum* BR2 were compared (Fig. 5) by using their total proteins isolated by ammonium sulphate precipitation. The optimum ammonium sulfate concentration that gave maximum precipitation of proteins at 24, 48, and 72 h of fermentation were found to be 10–50% and 50–90%.

The gtf enzyme activities of BR2OE4 determined at 24, 48 and 72 h precipitated samples gave an enzyme activity of 37.25 U/mL, 10.69 U/mL, and 7.07 U/mL respectively for 10–50% ammonium sulphate precipitated fraction and 22.12 U/mL, 21.21 U/mL, 11.18 U/mL respectively for 50–90% fraction. Whereas the level of gtf activity of the wild-type *L. plantarum* BR2 at different time intervals ranged between 17.27 U/mL, 3.84 U/mL and 3.83U/mL at 10–50% ammonium sulphate fraction and 10.55 U/mL, 5.24 U/mL, and 5.31 U/mL for their second fraction. Considering the specific activity of the enzymes, it was observed that at all time intervals BR2OE4 showed a higher gtf activity than the wild-type strain with a maximum specific activity of 26.59 U/mL with the 24 h precipitated fraction. The maximum specific activity exhibited by the wild-type strain was only 10 U/mL. Overall, the glycosyltransferase enzyme specific activity of BR2OE4 (Table 3) was 3.9-fold increased than that of the wild-type *L. plantarum* BR2 due to over-

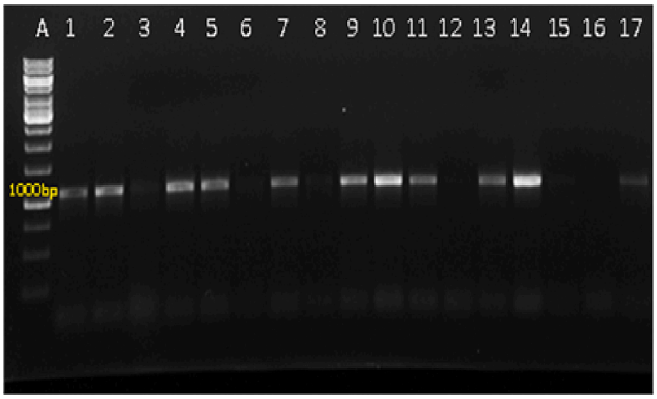


Fig. 3. Selection of Recombinants. Amplification of *BR2gtf* genes for the selection of recombinants from colony PCR with plasmid-specific primers (L & A: 1 kb DNA Ladder A1-17: colony PCR amplicon of clones amplified with *BR2gtf* F' and *BR2gtf* R' with primers.

expression of the EPS biosynthetic glycosyltransferase gene. Apart from the glycosyltransferase gene expression from the genomic DNA of *L. plantarum* BR2, the nisin induction at appropriate concentration enabled the recombinant strain to express the *BR2gtf* gene cloned into the pNZ8148 vector with the aid of the regulatory plasmid pNZ9530 for the controlled expression upon nisin induction resulting in the overall hike in the gtf enzyme activity and in turn EPS production.

The assay conditions such as incubation time (see Supplementary Materials) and enzyme concentration for the maximum gtf activity was determined to be 24 h and 10 μg/mL of the precipitated protein fractions respectively for the optimized assay conditions. According to Bounaix et al. (2009) with research on EPS producing sourdough lactic acid bacteria, mainly *Leuconostoc* and *Lactobacilli* sp. found the glycosyltransferase enzyme activity responsible for the EPS production and optimized the similar gtf assay conditions using cell free culture supernatants. The level of activity produced ranged between 0.8 and 2 U/mL (Bounaix et al., 2009). For a glucan producing *Weissella confuse* (Cab3) strain, gtf enzyme activity was performed with cell free supernatants with the enzymatic reaction performed at 30 °C for 16 h (Shukla and Goyal, 2011). In another study by Díez-Municio et al., (2012), the enzymatic reaction was carried out under optimal conditions, i.e., at 30 °C in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ at pH 5.2, and a reaction time of 48 h which is longer than the incubation period used in this study.

Similar work has been done where the over-expression of an enzyme involved in UDP-glucose synthesis, UDP-Glucose pyrophosphorylase (GalU), under the control of a nisin inducible promoter, increased the enzyme specific activity in *Lactococcus lactis* by 20-fold, which in turn increased both UDP-glucose and UDP-galactose synthesis by eight-fold. A study in *Sphingomonas* where the increase in the expression of biosynthetic gene cluster, especially glycosyltransferase enzyme activity enhanced the production of EPS by 20% (Ruffing and Chen, 2006).

Lactococcus lactis NIZO B40, a well-studied organism for EPS biosynthesis harbouring a 42 kb EPS plasmid, pNZ4000 contains a 12 kb *eps* operon carrying all the EPS biosynthetic genes. A study demonstrated a 15% increased EPS production with the over production of NIZO B40 priming glycosyltransferase enzymes. The elevated *eps* gene expression resulted in the higher EPS yields and this furthermore suggests the correlation of the role glycosyltransferase enzymes in EPS production (Boels et al., 2003; Nierop Groot and Kleerebezem, 2007).

The result from the above data demonstrates that the mechanism of gtf induced enhancement of EPS production by the over expression of *BR2gtf* gene in *L. plantarum* BR2 and shows a 3.9-fold increase of glycosyltransferase enzyme activity (from Table 3) compared to the wild-type strain and it resulted in a 54.4% increased EPS production. The future challenges and scope of LAB EPS production lie in the EPS production bottlenecks for construction of modified or tailor-made EPS altering its composition to desirable and novel characteristics for target applications.

This work clearly demonstrated a molecular approached strategy for increased EPS production in an indigenous probiotic *Lactobacillus plantarum* BR2 strain through nisin expression system. The NICE system is widely used for a multitude of various LAB fermentations especially in homologous and heterologous gene expression. The success rate of molecular engineering approaches with this system lies in its high expression levels (De Vos and Hugenholtz, 2004). Here in this study, the implementation of the homologous over-expression of *BR2gtf* gene in *Lactobacillus plantarum* BR2 that has a pivotal role in its EPS biosynthesis is depicted in Fig. 6. This auto-induction mechanism of nisin has been exploited for gene expression with a dual plasmid system incorporating the vectors pNZ9530 expressing the nisK and nisR genes and the plasmids such as pNZ8148, (pNZ8149, pNZ8151, pNZ8152 etc) expressing the gene of interest (*BR2gtf* in this study) placed downstream of an inducible promoter PnisA. It enables the expression of genes in gram-

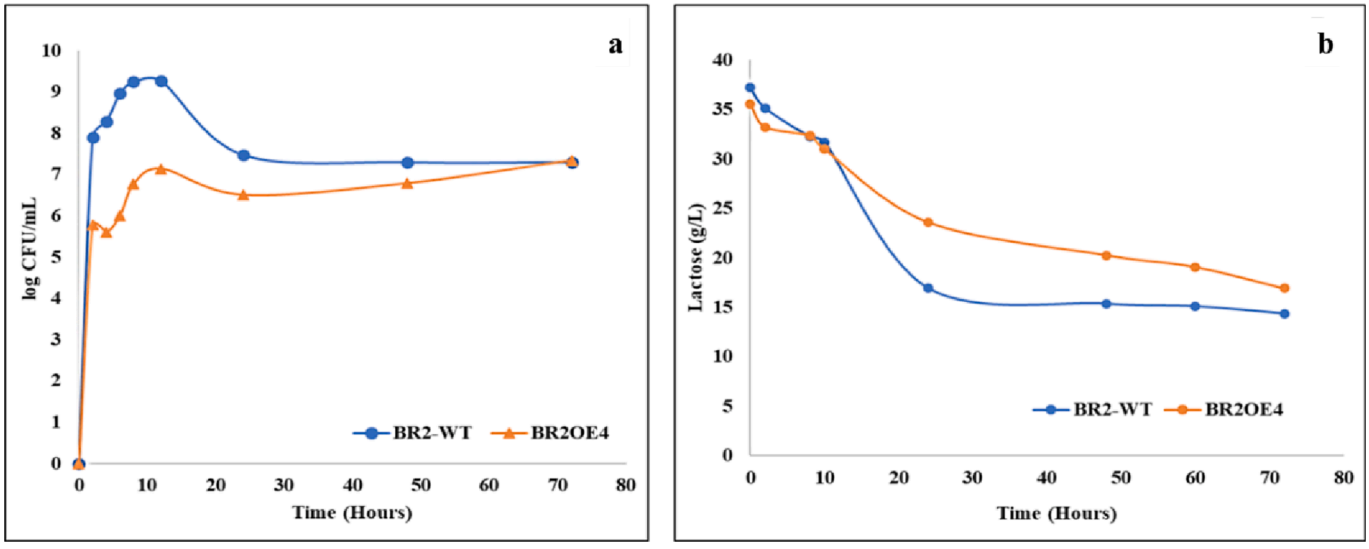


Fig. 4. (a) Growth and (b) Sugar Utilization of recombinant BR2OE4 and wild-type *L. plantarum* BR2 in EPS production medium. Values are expressed as mean ± standard deviation, where n = 3.

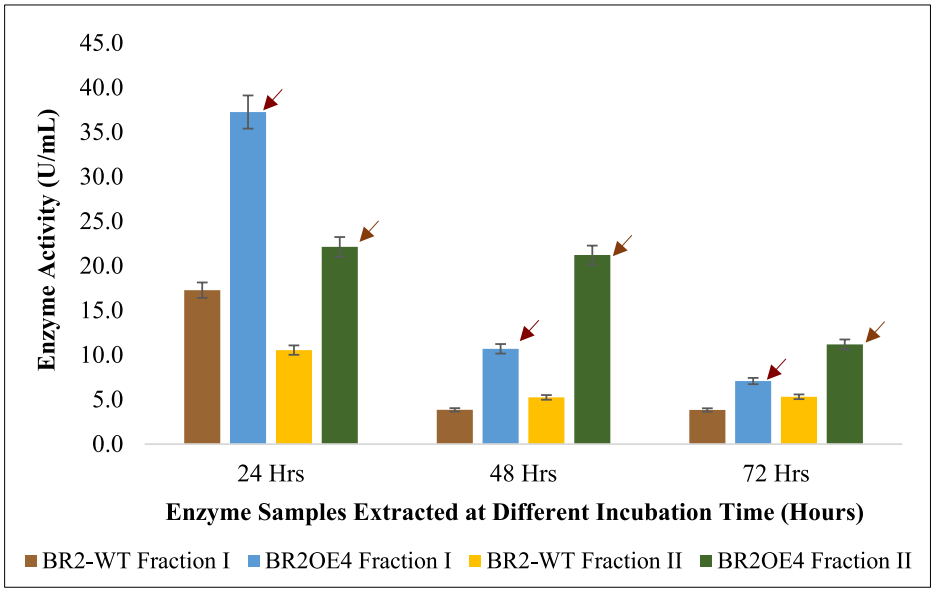


Fig. 5. Glycosyltransferase activity of wild-type *L. plantarum* BR2 and BR2OE4, Fraction I and Fraction II represents the crude protein samples precipitated by salting out with 10–50% and 50–90% respectively from ammonium sulphate precipitated fractions of BR2-WT (represented in Brown & Yellow) and BR2OE4 (represented in Blue & Green) respectively. The arrows indicate the highest GTF enzyme activity exhibited by BR2OE4. Values are expressed as mean ± standard deviation, where n = 3.

Table 3
Glycosyltransferase specific enzyme activity of WT- *L. plantarum* BR2 and BR2OE4.

Specific Activity of GTF Enzymes (U/mg)						
Incubation Time (hours)	BR2-WT	BR2OE4	Fold Increase (Fraction I)	BR2-WT	BR2OE4	Fold Increase (Fraction II)
	10–50%	10–50%		50–90%	50–90%	
24	7.07	13.81	1.98	10.83	26.59	2.49
48	1.28	5.08	**3.9**	6.36	21.50	**3.49**
72	1.43	3.36	2.09	4.75	15.29	3.15

Fold increase is calculated between BR2-WT and BR2OE4 gtf activities of fraction I and II separately at each time intervals.

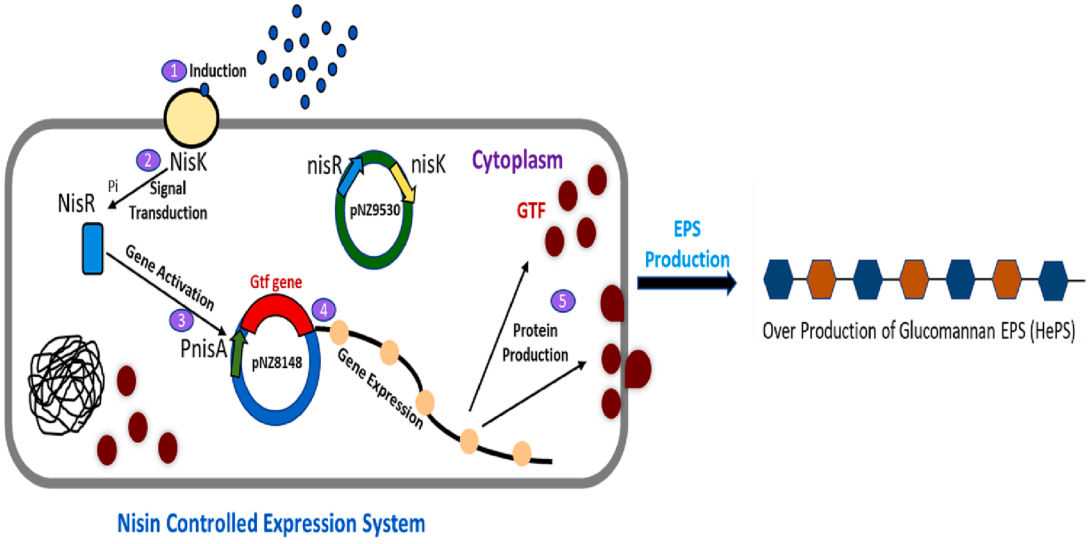


Fig. 6. Illustration of Nisin Controlled Expression System in *L. plantarum* BR2OE4 cell (1) Upon nisin induction, it binds to nisK and activates the **(2)** signal transduction of nisR that **(3)** subsequently activates the PnisA promoter in pNZ8148 vector **(4)** which further enables *BR2gtf* gene expression placed downstream of the cloned vector **(5)** Gtf protein expression from the genomic DNA and the cloned plasmid results in its overexpression and is secreted out of the cell which helps in EPS overproduction.

positive bacteria especially in low GC-Lactobacillus without compromising the expression levels (Kazi et al., 2022).

The health-promoting properties and the GRAS status of the LAB enforce them for the development of gene expression, though genetic engineering tools and therapeutic delivery systems to produce various value-added products such as antibiotics, vitamins, oligosaccharides, exopolysaccharides, cytokines and bioactive molecules (Guan et al., 2022). *Leuconostoc lactis* NZ6091 and *Lactobacillus helveticus* CNRZ32 were developed to express β-glucuronidase through this heterologous dual plasmid system employing nisin induction (Bron and Kleerebezem,

2011) thus functionally implementing the system in LAB other than *Lactococcus lactis*.

4. Conclusion

EPS production enhancement through metabolic engineering can create highly efficient microbial strains for polysaccharide synthesis to overcome the constraints of lactic acid bacterial EPS production and to satisfy market demands. Using a dual plasmid NICE expression system, a glycosyltransferase gene *(BR2gtf)* was overexpressed in EPS-producing *L. plantarum* BR2, leading to a 2-fold increase in EPS production. The recombinant (BR2OE4) strain showed 54.4% increased EPS production and exhibited a 3.9-fold increase in the total specific gtf enzyme activity. The future scope of this research dwells in through understanding of all biosynthetic genes and their functions to engineer and improve tailor-made EPS production.

CRediT authorship contribution statement

Soumya M P: Conceptualization, Validation, Data curation, Methodology, Investigation and Writing of the original manuscript; **Reeba Parameswaran:** Literature collection and Methodology; **K. Madhavan Nampoothiri:** Provided the topic and general guidance, Supervision on the experiments made, the critical reading and editing and finalizing the manuscript. Soumya M P is the first author of the paper. Reeba Parameswaran, remain as a second author to the manuscript. All the authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129387.

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Further reading

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