

**Structure-viscosity relationship of exopolysaccharide
produced by *Lactobacillus fermentum* MTCC 25067**

2020

MENGI Bharat

Doctoral Program in Animal and Food Hygiene

Graduate school of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

Lactobacillus fermentum MTCC 25067 が生産する
菌体外多糖の構造粘性相関

令和 2 年

(2020)

帯広畜産大学大学院畜産学研究科

博士後期課程 畜産衛生学専攻

メンギ ブハラト

Table of contents

	Page
Abbreviations	
Chapter I. General introduction	
I-1. Introduction	
I-1-1. Exopolysaccharides	1
I-1-2. Classification of EPS	2
I-1-3. Isolation of EPS and physicochemical characterization	3
I-1-4. Rheological analysis of EPS	5
I-1-4.1. Simple rotational viscometer	6
I-1-4.2. Rotational rheometer	6
I-1-5. Microscopic techniques for EPS-EPS, EPS- bacterial interaction	7
I-1-5.1. Atomic force microscopy	7
I-1-5.2. Scanning electron microscopy	9
I-1-6. Gene knockout strategies used in lactic acid bacteria	10
I-2. Significance of the study	11
I-3. Objectives of the study	13
Chapter II. Comparative analysis of EPSs to study the changes in physicochemical properties at different cultivation times	
II-1. Introduction	19
II-2. Materials and methods	20
II-2-1. Bacteria strains and reagents	20
II-2-2. Growth profile	19
II-2-3. Preparation of EPS	21
II-2-4. Measurement of viscosity	21
II-2-5. Comparative analysis of molecular weight distribution in EPS using gel permeation chromatography	22
II-2-6. Side chain structure using ¹ H-NMR analysis	22
II-3. Results and discussion	
II-3-1. Cultivation profiles	22
II-3-2. Measurement of viscosity	23
II-3-3. Molecular weight distribution of the EPS	23
II-3-4. Side chain structure using ¹ H-NMR analysis	25

V-3. Results	
V-3-1 Construction of plasmids for antibiotic cassette insertion/ insertional mutagenesis	57
V-3-2. Electroporation of pTRK669 plasmid into <i>L.fermentum</i> for gene knockout using pORI based temperature sensitive system.	57
V-4. Tables and figures	59
Chapter VI. Summary and conclusions	66
References	68
Acknowledgements	87
Japanese abstract	89

ABBREVIATIONS

3D,	three dimensional;
AF4,	asymmetrical flow field fractionation;
AFAM,	atomic force acoustic microscopy;
AFM,	atomic force microscopy;
BSE,	backscattered electrons;
CRISR-Cas,	clustered regularly interspaced short palindromic repeats / crispr associated proteins;
D ₂ O,	deuterium oxide;
DNA,	deoxyribonucleic acid;
EI-MS,	electron ionization mass spectrometer;
EPS,	exopolysaccharide;
HePS _{144h} ,	EPS isolated at 144 hours of growth;
EPS _{48 h} ,	EPS isolated at 48 hours of growth;
EU,	european union;
eV,	electron volts;
FDA,	food and drug administration;
FFF,	field flow fractionation;
G' ,	storage modulus;
G'' ,	loss modulus;
GC,	gas chromatography;
GIT,	gastrointestinal tract;
GRAS,	generally recognized as safe;
HDC,	hydrodynamic chromatography;
HePS,	heteroexopolysaccharides;
HMW,	high molecular weight;
¹ H-NMR,	proton nuclear magnetic resonance;
HOPS,	homoexopolysaccharides;
HPAEC,	high performance anion exchange chromatography;
HPSEC,	high performance size exclusion chromatography;

HPSEC	high performance size exclusion chromatography;
MALLS,	multi-angle laser light scattering;
kHz,	kilohertz;
LAB,	lactic acid bacteria;
LMW,	low molecular weight;
MRS,	de Man, Rogosa and Sharpe;
MW,	molecular weight;
nm,	nanometer;
NMR,	nuclear magnetic resonance;
PAD,	pulsed amperometric detection;
QPS,	quality presumption of safety;
SE,	secondary electrons;
SEM,	scanning electron microscopy;
SPM,	scanning probe microscopy;
TEM,	transmission electron microscopy;
TR,	torsional resonance;
US,	United States;
USD,	United States dollar.

CHAPTER I. General introduction

I-1. Introduction

Bacteria belonging to *Lactobacillus* species have an age-old history of safe use and are generally recognized as safe (GRAS) by Food and Drug Administration (FDA); with 35 species bearing Quality Presumption of Safety (QPS) status by European Food Safety Authority (EFSA) (London *et al.*, 2014). Till date, over 200 species and 27 subspecies have been reported for *Lactobacillus* (Euzéby, 1997). As such, lactobacilli are very diverse in nature and originate mostly from rich, carbohydrate-containing niches such as plants, human and animal intestine, silage and milk (Hammes and Vofel, 1995; Bernardeau *et al.*, 2008). They also play an important role in fermentation of foods (Wood, 1997). Besides production of lactic acid, flavouring compounds and bacteriocin like substances, several lactic acid bacteria (LAB) strains have been found to secrete extracellular polysaccharides in favourable environments such as milk (Sikkema and Oba, 1998; Cerning and Marshall, 1999; Ricciardi and Clementi, 2000). Some of them have even been reported to contribute to human health by production of bioactive compounds (Mitsuoka, 2014; Ruas-Madiedo *et al.*, 2006). Of these, many strains have been reported to produce exopolysaccharides (EPS) (De Vuyst and Degeest, 1999); which show a highly diverse structures and physicochemical properties (Salazar *et al.*, 2009).

I-1-1. Exopolysaccharide

The term exopolysaccharide (EPS) describes the extracellular polysaccharide either attached as capsule with bacterial cell wall or liberated into the medium as ropy polysaccharide (Sutherland, 1972) produced during metabolism of microorganisms (Amjres *et al.*, 2015). Apart from their physiological role to protect cells from detrimental environmental conditions; such as dehydration, macrophages, antibiotics, and bacteriophages, to sequester essential cations, and to be involved in adhesion and biofilm formation (Looijesteijn *et al.*, 2001). EPS have been used in the food industry.

EPS act as food stabilizer, viscosifier, emulsifier, gelling agent or natural thickener, playing an important role in the improvement of the physical properties of fermented milks (Ruas-Madiedo and Reyes-Gavilan, 2005). Examples of EPS produced by LAB include dextran (*Leuconostoc mesenteroides*), mutan (*Streptococcus*

mutans) and fructan (*Strep. salivarius* subsp. *thermophilus*) (Montiville *et al.*, 1978; Cerning, 1990).

Xanthan, acetan and gellan are produced by gram negative bacteria, *Xanthomonas campestris*, *Acetobacter xylinum* and *Sphingomonas paucimovilis*, respectively, in larger quantities than EPS produced by food grade LAB; and are commercially available as food additives (Harvey and McNeil, 1998). However, they are not preferred, being having a non-food grade, non-GRAS origin and also due to high recovery costs (De Vuyst *et al.*, 2001). Also, adding purified EPS into food product may have different effects compared to in situ production of EPS (Doleyres *et al.*, 2005).

The use of polysaccharide producing LAB strains is not new in the manufacture of fermented milk products. The EPS producer LAB have been used traditionally in the Scandinavian fermented dairy products to achieve desirable texture and rheological properties (Macura and Townsley, 1984). The products made with ropy strains are smooth, highly viscous and have lesser syneresis compared to those made with non-ropy ones (Wacher-Rodarte *et al.*, 1993). Several bacterial EPSs have also been reported to have health benefits such as anti-tumor, anti-ulcer, anti-obesity, and immune-modulating effects (Fanning *et al.*, 2012; Nagaoka *et al.*, 1994; Wang *et al.*, 2014; Zhang *et al.*, 2016; Harris and Ferguson, 1993; Cummings and Englyst, 1995; Kitazawa *et al.*, 1998; Chabot *et al.*, 2001; Dal Bello *et al.*, 2001). Recently, several researches have even reported recovering toxic compounds such as heavy metal ions from the environment using EPS (Gupta and Diwan, 2017; More *et al.*, 2014).

I-1-2. Classification of EPS

EPS from LAB are much diversified and could be classified following different criterion. According to the most classical one, being based on compositional monomers i.e. repeating monosaccharide units, EPS of LAB are classified broadly into two groups; namely homoexopolysaccharides (also denoted sometimes as HoPS) and heteroexopolysaccharides (denoted as HePS). HoPS contain only one type of monosaccharides; fructose or glucose e.g. dextran, mutan and levan (Barker and Ajongwen, 1991; Monsan *et al.*, 2001). HoPS are synthesized by anchored or secreted transglycosylases, which catalyze the transfer of corresponding glycosyl moieties

(Monsan *et al.*, 2001). On the contrary, HePS are made up of different monosaccharide repeating units, the most common being glucose and galactose; and also some amounts of rhamnose, mannose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid (Cerning, 1990; Van den Berg *et al.*, 1995; Stingele *et al.*, 1996; 1997; Grobben *et al.*, 1997). Heteropolysaccharides contain backbone of repeated unbranched or branched subunits at positions C2, C3, C4 or C6; consisting of three to eight monosaccharides (De Vuyst *et al.*, 2003). Unlike HoPS, the HePS are synthesized intracellularly at the cytoplasmic membrane using sugar nucleotide precursors for the assembly of polymer chain (Cerning, 1995). The key enzymes for the biosynthesis of the EPS repeating unit are the glycosyltransferases; which catalyze the transfer of sugar moieties from donor molecules to specific acceptor molecules, leading to the formation of glycosidic bond. Several types of HePS are secreted by mesophilic as well as thermophilic lactic acid bacteria with varied sugar composition and molar masses ranging from 10^4 to 6.0×10^6 (Cerning, 1995; De Vuyst and Degeest, 1999). In general, the thermophilic LABs produce HePSs in larger amounts compared to mesophilic LAB (Mozzi *et al.*, 2001).

I-1-3. Isolation of EPS and physico-chemical characterization of EPS

The structural analysis of any bacterial polymer starts with the isolation of purified EPS. The most important concern in this, is to avoid EPS contamination with microbial growth medium components, usually mannan in yeast extract. Generally, the EPS isolation protocols include (i) removal of cells by centrifugation or filtration, (ii) EPS precipitation by the addition of chilled ethanol or acetone (usually two to three volumes depending on the type of polymer released), (iii) dialysis and drying of the precipitated EPS; and lastly, a reprecipitation and dialysis (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Sometimes, purification of EPS also includes membrane-filtration, anion- exchange and/or gel permeation chromatography (Sanz and Martinez-Castro, 2007). During EPS isolation from culture media having high-protein content (e.g., dairy products), removal of proteins is done either using trichloroacetic acid precipitation or hydrolysis with proteases or a combination of both. EPS production by dairy LAB varies within species between 25 to 9800 mg/l (Behare *et al.*, 2009).

In general, rheological properties of EPSs are defined by their molecular mass distribution, monosaccharide composition, linkage between sugar monomers, and

presence of side chains (Shene, 2008). Bacterial EPS have been found to influence the texture and rheology of fermented milk products at extremely low concentration (Duboc and Mollet, 2001). The understanding the nature and molecular characteristics of the polymer helps determine its effect on functional properties (Kleerebezem *et al.*, 1999; Tuinier *et al.*, 1999 a; Tuinier *et al.*, 1999 b). Usually, EPS contributes to the thickening of the final product, depending on its viscofying ability. The viscofying ability of EPS is further determined by parameters such as intrinsic viscosity, specific volume occupied by dispersed particle and also dispersed polymer concentration (Tuinier *et al.*, 1999b). The specific volume of EPS in solution is determined by its molecular mass and its “radius of gyration” (size of the polymer in solution). It has been reported that in order to obtain higher intrinsic viscosity, the molecular characteristics, either the molar mass (chain length) or the stiffness of the polysaccharide must increase (Laws and Marshall, 2001).

Exopolysaccharides concentration is usually estimated as the neutral carbohydrate content; usually determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956); or by weighing the dry matter of polymer (Vanangelgem *et al.*, 2004). In addition, EPS concentration can be determined by means of high-performance size exclusion chromatography coupled with refractive index (RI) detection (HPSEC-RI); EPS concentration is calculated by the integration of the RI signal using calibration curves obtained with known molecular weight dextrans (Sánchez *et al.*, 2006).

Molecular weight of a polysaccharide is generally measured on the basis of retention time of the polysaccharide eluted by HPSEC-RI; recently, HPSEC-MALLS is more commonly used for this purpose (Picton *et al.*, 2000). Field flow fractionation (FFF) and hydrodynamic chromatography (HDC) can also be used for determining the average MW of ultrahigh-molecular mass polysaccharides with higher specificity and in more complex samples (Cave *et al.*, 2009; Isenberg *et al.*, 2010; Galle *et al.*, 2012b). Asymmetrical flow field fractionation (AF4) has been successfully implemented for separating starch-like glucans. Both, HPSEC and AF4, alongwith multiple inline detection of scattering intensities and mass profiles, provide a distribution of apparent molecular weight and radius of gyration of parated fractions (Rolland-Sabate *et al.*, 2007; Juna *et al.*, 2011).

The monomer composition can be determined using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) following total acid hydrolysis (Cataldi *et al.*, 2000). Alternatively, methanolysis and per-trimethylsilylation can also be used to provide samples for analysis by gas chromatography (GC). The monosaccharide analysis can be further used to determine the carbohydrate content and verify the sample purity. The D- or L- configuration can be established by GC of the corresponding (–) 2- butyl glycosides (Gerwig *et al.*, 1979). The linkage pattern of the monosaccharide constituents can be determined by polymer hydrolysis after methylation of all free hydroxyl groups, and reduction of monomers to alditols using sodium borodeuteride. Subsequent acetylation provides deuterated partially methylated alditol acetates that can be analyzed by GC coupled with EI-MS (Ciucanu and Kerek, 1984). The ratio (percentages) of terminal, internal, and branched glucose units determined by the methylation analysis provides an insight into the polymer structure. ^1H and ^{13}C NMR spectroscopy can then be used to obtain additional information about the structural features of the EPS. High-resolution NMR spectroscopy provides detailed information on the type of constituent monosaccharides, ring size, and anomeric configurations, and the position of glycosidic linkages (Damager *et al.*, 2010); and is considered the most powerful method for the unambiguous identification of carbohydrate chains.

I-1-4. Rheological analysis of EPS

Rheological characterization of a sample can be done using different types of viscometers and rheometers. Dynamic rotational rheometers are becoming increasingly important in the food industry because of reasons like reduced destruction of samples, short time required for measurement and determination of G' and G'' for determining the viscoelasticity. The relative magnitude of elasticity and viscosity for a material depends on the deformation over a scale of observation time. A material may appear more viscous, if the rate of deformation is very slow. On the contrary, if the rate of deformation is fast, it behaves more like an elastic fluid. Thus, a material can be characterized by determining its mechanical response over wide range of deformation rates. (Ikeda and Foegeding, 2003).

In dynamic rotational methods, the test fluid is continuously sheared between two surfaces, one or both of which are rotating. Rotational methods have also been used to carry out oscillatory and normal stress tests to characterize the viscoelastic properties. Rotational measurements can be categorized as stress-controlled or strain-controlled. In stress-controlled measurements, the resulting rotation speed is determined upon application of a constant torque to the measuring device for generating rotation. This rotation speed can be converted into a corresponding shear rate by using a well-defined tool geometry. In strain-controlled measurements, a constant rotation speed is maintained and a stress-sensing device (a torsion spring or strain gauge) is used to measure torque generated by the sample.

There are different types of rheometers present commercially; Simple Brookfield type (rotational viscometer) and rotational rheometer.

I-1-4.1. Simple rotational viscometer

It is the least expensive commercially available type of controlled-rate rotational viscometer and is commonly referred to as a "Brookfield type" viscometer (Fig. 1). It measures viscosity of the fluid at a known rotation speed by driving a measurement spindle, immersed in the fluid to be tested, through a calibrated torsion spring. The viscous drag of the fluid against measurement tool causes the spring to deflect, which is correlated to torque. The calculated shear rate depends on the rotation speed, the geometry of the measurement device, and sample container (size and shape). The viscosity is calculated from the determined torque using conversion factors which are pre-calibrated for specific device and container geometries. The torque is directly proportional to the product of viscosity and rotational speed, for Newtonian fluids: however, this proportionality is not seen for non-Newtonian fluid.

I-1-4.2. Rotational rheometer

This is a high-precision and continuously-variable shear instruments in which the fluid sample is sheared between rotating cylinders, plates or cones under controlled-stress or controlled-rate conditions. It typically consists of four parts: (i) a measurement tool with a well-defined geometry, (ii) a device for application of a constant torque or rotation speed to the tool over a range of shear stress or shear rate values, (iii) an

instrument to determine the stress or shear rate response, and (iv) a temperature control unit.

The relative rotation about a common axis of tool geometries is used in rheometers. The three most commonly used geometries are concentric cylinder, cone and plate; and parallel plates (Fig. 2).

In concentric cylinder or couette or coaxial geometry, the inner or outer, or both cylinders may rotate. The fluid to be tested is constrained in the annulus between the cylinder surfaces. This type of geometry is typically used for analyzing fluid suspensions. The cone and plate geometry consists of an inverted cone (usually with an angle less than 4°) in near contact with a lower plate. Depending on the instrument, either the upper or lower surface may rotate. The parallel plate geometry is a version of the cone and plate geometry having a 0° angle cone. The test sample is maintained in the narrow gap between two surfaces. Cone and plate; and parallel plate geometries are most commonly used for highly viscous samples, gels and concentrated suspensions.

I-1-5. Microscopy techniques for EPS-EPS, EPS- bacterial interaction

The ability to observe structures at magnified levels has always helped in understanding of biology. Emergence of novel information often results as a result of advances in technology. The developments in scanning microscopy technologies have made it possible to capture extracellular polysaccharide images under aqueous conditions resembling the physiological state more closely than other ultramicroscopy imaging techniques. More recently, the emergence of nanoscience and nanotechnology offers new insights into the morphology of EPS at a nano-scale. EPS morphology and its interaction with its producer strains has been extensively studied by microscopic techniques like atomic force microscopy (AFM) and scanning electron microscopy (SEM) (Ikeda *et al.*, 2019; Liu *et al.*, 2014; Wang *et al.*, 2015; Dertli *et al.*, 2013; Su *et al.*, 2012).

I-1-5.1. Atomic force microscopy

AFM has evolved as a powerful tool compared to other microscopy techniques and has been reportedly applied in studying biomolecules, including DNA, proteins, polysaccharides; bacteria; and also their interactions. AFM is a scanning probe microscopic (SPM) technique invented by Gerd Binnig, Calvin Quate and Christoph

Gerber in 1986 (Binnig *et al.*, 1986; Binnig and Rohrer, 1986). SPM revolutionized the field of microscopy and uses a probe for scanning a surface to give information on morphology based on the interactions between the surface and the probe. AFM has a unique ability to capture high-resolution images of any surface in air, vacuum or liquids with extraordinary topographic contrast; direct height and force measurements; and 3D features.

Commercially available AFM instruments generally consist of a cantilever, a piezo scanner, mirror, photo-detector, a laser diode, and a feedback controller (Fig. 3). Laser beam reflected from the cantilever back into the mirror hits the photo-detector. The position of the laser on the photo-detector changes due to deflection of the cantilever resulting from the interactions between the tip and the sample. The feedback controller collects surface information and forms an image on a computer screen

The choice of cantilever, tip geometry and the sample surface decide the performance and resolution of AFM images. AFM tips are generally made of Si or Si₃N₄, and specialized activated tips are constructed for specific purposes, such as tips with carbon nanotubes. An ideal tip has a well-defined shape and a sharp apex. In fact, the tip often becomes blunt after long use. A large range of tip shapes and geometries are commercially available with cantilever spring constants ranging from 0.001 to 2000 N/m; and cantilever coatings like aluminium or gold for imaging without causing damage to delicate matter.

The force between tip and sample causes deflection of cantilever upon bringing the tip into proximity of sample surface, according to Hooke's law. AFM can be used to measure mechanical contact forces, van der Waals forces, capillary forces, chemical bonding, electrostatic forces as well as hydration forces.

Further, AFM can be operated in static and dynamic imaging modes. Static modes include contact mode and friction force microscopy (FFM, or lateral force microscopy, LFM). Whereas, dynamic AFM modes include three modes; 1) non-contact tapping mode, 2) force modulation mode, atomic force acoustic microscopy mode; 3) torsional resonance mode; 4) lateral excitation mode (Song *et al.*, 2008).

Further, the contact mode can be run with constant height or constant force. In constant force mode, the laser beam measures deflection of the cantilever while images are collected on the basis of feedback to a piezoelectric scanner. AFM probes with low

spring constant are used for running contact mode. Forces between the tip and samples can be measured using different tips and surfaces. Contact mode is used for high resolution imaging. However, its limitations include tip contamination caused by attached particles on a surface and damage to the surface upon application of strong force while scanning of delicate samples.

In tapping mode, the tip is used to sense the sample surface with minimal contact at a given amplitude for dynamic and intermittent contact imaging. The tip oscillates with fixed amplitude (in nanometer) and a typical frequency of the tip of 50-400 kHz.

The non-contact being quite similar to the tapping mode mode, differs in the cantilever oscillating slightly higher than the resonance frequency; without the tip actually touching the surface of samples (remaining 5-10 nm away). This mode is more suitable for 'soft' matter including bacteria and proteins. Table 1 shows the analysis of different modes.

There are multiple advantages of using AFM over other microscopy techniques such as scanning and transmission electron microscopy (SEM and TEM) and fluorescent or confocal laser scanning microscopy. AFM provides quantifiable and accurate three-dimensional topographies, down to the Angstrom level. Measurements and images can be captured by AFM in air, aqueous, or vacuum at different temperatures. The sample preparations are much more easier compared to SEM/TEM since it only requires basic sample preparation (e.g., no dehydration or labeling with fluorescent dyes or antibodies, or surface coating) (Stylianou, 2013; 2016). After imaging, AFM offers a combination of qualitative and quantitative information based on characterization of mechanical, electrical or magnetic property of sample surface.

I-1-5.2. Scanning Electron Microscope

SEM is a powerful tool to witness the invisible worlds of microspace and nanospace. Scanning electron microscopy has been used to investigate the interaction between live cells and their metabolites or surrounding medium and also for atomic resolution of thick specimens and three dimensional appearance of the specimen image. SEM has the ability to do super high magnifications for complementing the information available from optical images. SEM consists of following components (Fig. 4): a) an

electron gun or emitter to generate high energy electrons b) a column for electrons to travel through two or more electromagnetic lenses, c) a deflection system consisting of scan coils, d) an electron detector for backscattered and secondary electrons, e) a specimen chamber and f) a computer system with viewing screen to display the scanned images and keyboard to control the electron beam.

The details and complexity can be revealed by SEM using the process well described by Goldstein *et al.* (2003). The analysis is done by applying a beam of high energy electrons (100-30,000 eV) emitted by a thermal source. SEM is equipped with electromagnetic lenses to compress the spot size (< 10 nm) and direct the focused electrons on the specimen to generate a sharp image. The signals generated by the interaction and penetration (1 μ m depth) of electrons collected from the final lens with the sample are used to produce an image. Point by point images of the specimens are obtained due to the movement of electron beam caused by movement of the scan coils. The electron beam moves to discrete locations as straight lines until a rectangular raster is produced on the specimen surface. In the case that a higher magnification is desired, the scan coils deflect the electron beam to cross a smaller area. Electron detector detects the emitted electrons (signals) from the scanned specimen. For SEM image production, both secondary electrons (SE) as well as backscattered electrons are taken into account. Both SE and BSE are collected when a positive voltage is directed to the collector screen. However, if negative voltage is applied on the collector screen, only BSE can be collected. Scintillator detector detects both the secondary and backscattered electrons. The detected signals are then displayed on a viewing screen; where brightness and the intensity is controlled until a reasonably clear image can be seen.

The partial 3-D image obtained from SEM depends on the number of BSE and SE, which affect the visualization of the topography (shape, size and surface texture) of the specimen. These nanoscopic imaging techniques are also being used to compare differences in the mutant and the wild strains (Sletmoen *et al.*, 2010).

I-1-6. Gene knockout strategies used in lactic acid bacteria

Different strategies are being used for the production of genetically altered strains for studying the gene functions as well as to improve the strains. One such approach is the ability to transform exogenous DNA into cells to modify and

genetically engineer their genome. For this, various strategies have been utilized, although they are usually strain-dependent. Some of these tools which have been developed and successfully applied in *Lactobacillus* species include Cre-lox (Lambert *et al.*, 2007), bacteriophage integrases (Auvray *et al.*, 1997), introns (Sasikumar *et al.*, 2016), and two-plasmid integration systems (Ruseel, 2001; Van Pijekren *et al.*, 2006). For making DNA insertions or deletions after Campbell-like integration, upp-encoded uracil phosphoribosyltransferase has been reportedly used for selection of cells in which the second homologous recombination event has occurred (Goh *et al.*, 2009). These tools are indispensable to the LAB research community, but gene engineering approaches with high-throughput genome are still limited until now.

After the discovery of clustered regularly interspaced short palindromic repeats /crispr associated proteins (CRISPR-Cas) in 2007 (Barrangou, 2007), different research groups have used it as a genome editing tool (Mali *et al.*, 2013; Cong, 2013). The authors of recent comparative genomics study revealed that CRISPR-Cas systems are widely distributed in the genus *Lactobacillus* and suggested this probably due to phage predation, horizontal gene transfer, and the extensive genome remodelling, which collectively contribute to genetic diversity of this genus (Sun *et al.*, 2015; Makarova *et al.*, 2006; Canchaya *et al.*, 2006). However, CRISPR-Cas systems are reportedly present in about 46% of the total bacterial genomes in CRISPR databases, the *Lactobacillus* genus encodes them in approximately 63% of the sequenced genomes (Sun *et al.*, 2015). This huge wealth of CRISPR-Cas systems in lactobacilli offers researchers an opportunity for using native Cas enzymes, alongwith with user-defined CRISPR arrays, for selecting recombinant genotypes (Selle, 2015).

I-2. Significance of this study

With an increase in health awareness and consumer demands for natural low calorie dairy products without any additives, there is call for making product lines as natural as possible. But sometimes, these products develop undesirable textural, rheological and sensorial defects which have been attempted to be controlled by incorporating additives like stabilizers. However, they don't support the consumer's view of a natural food without artificial additives. The use of viscous EPS producing cultures find a suitable place as safe-food additives or natural functional food ingredients as a possible replacement or for reducing the use of external hydrocolloids

(Giraffa, 2004; Tieking *et al.*, 2005a; Leemhuis *et al.*, 2013a). Although the structural characteristics of EPS and the GRAS status born by most EPS producer LAB strains allow consideration of *in situ* production of texture modifying and/or biologically active EPS (Badel *et al.*, 2011) due to their very low yields, no EPS from LAB has yet been commercially exploited as food additive except for dextran (produced by *Leuc. mesenteroides*) (Monsan *et al.*, 2001).

In such a scenario, novel microbial biopolymers may fill this gap in the market available polymers and/or may even replace traditional products in terms of improved rheology and stability characteristics. Dahi is a popular fermented milk product of India resembling yoghurt (Behare and Prajapati, 2007). *Lactobacillus fermentum* MTCC 25067 was previously isolated from *Dahi*, as a producer of a neutral HePS (Leo *et al.*, 2007). Solutions of the purified HePS showed high viscosities comparable to a commercially available viscosifier, xanthan gum (produced by *Xanthomonas campestris*), at lower concentrations of approximately 100 mg/L, suggesting that the HePS is a potential candidate for a novel viscosifier for the food industry. Its chemical structure consists of a main chain of 1,3-glucan, composed of α -glucose and β -glucose residues appearing alternately, and a disaccharide subunit of glucose- α -1,6-galactose- α -1,2- substituted at the C-2 position of the α -glucose (Gerwig *et al.*, 2013). It has been confirmed that the average molar ratio of the terminal D-Glcp to the 3-substituted D-Glcp to the 2,3-disubstituted D-Glcp to the 6-substituted D-Galp is 1.3:1.0:1.1:1.1 using proton nuclear magnetic resonance (^1H -NMR) spectroscopy (Gerwig *et al.*, 2013). The non-integer ratio is considered to reflect the heterogeneity of the distribution of side chains along the main chain. Recently, the formation of a supramolecular network has been suggested as a major reason for the high viscosity of the HePS solution (Ikeda *et al.*, 2019).

Moreover, the structural diversity of EPS among different probiotic lactobacilli may contribute to the strain-specific probiotic properties. EPS produced by probiotic lactobacilli has been reported to be associated with their probiotic action such as stress resistance, persistence in the gut ecosystem (Fanning *et al.*, 2012) and interactions with specific receptors and effectors of the host defence system (Welman and Maddox, 2003). The properties of purified EPS differs considerably from that produced *in situ* (Duboc and Mollet, 2001), the latter being more desirable. The *in situ* EPS production

may play useful role in the manufacture of a variety of cultured dairy products such as yoghurt, drinking yoghurt, cheese, cultured cream and milkbased dessert (Crescenzi, 1995; Cerning, 1995; Bouzar *et al.*, 1997; Christiansen *et al.*, 1999).

I-3. Objectives of the study

The study was conducted with the following objectives:

1. Comparative analysis of EPSs to study the changes in physicochemical properties at different cultivation times,
2. Rheological and structural characterization of EPS,
3. Structural insights into Bacterial-EPS interactions using Atomic Force Microscopy and Scanning Electron Microscopy,
4. Construction of plasmids and preparation of cells for gene knockout systems in *L. fermentum* MTCC 25067 to study the role of EPS related genes in the biomechanism of EPS production.

Table 1. Comparitive analysis of different scanning modes. Adapted from Song and Bhushan, 2008.

Operation Mode	Contact Mode	Non-contact Mode	Tapping Mode
Tip loading force	Low-high	Low	Low
Contact with sample surface	Yes	No	Periodical
Manipulation/ damage to sample	Yes	No	Yes
Contamination of AFM tip	Yes	No	Yes
Insight	Good High scan speeds. “Atomic resolution” is possible. Easier scanning of rough samples with extreme changes in vertical topography.	Not good Low force is exerted on the sample surface and no damage is caused to soft samples.	Fine Higher lateral resolution (1nm to 5nm). Lower forces and less damage to soft samples in air. Almost no lateral forces.
Disadvantages	Lateral forces can distort the image. Capillary forces from a fluid layer can cause large forces normal to the tip-sample interaction. Combination of these forces reduces spatial resolution and can cause damage to soft samples.	Lower lateral resolution, limited by tip-sample separation. Slower scan speed to avoid contact with fluid layer. Usually only applicable to extremely hydrophobic samples with a minimal fluid layer.	Slower scan speed than in contact mode.

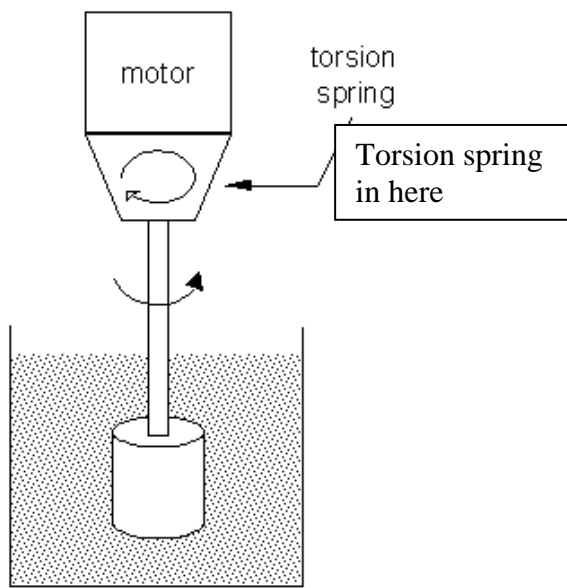


Fig. 1. Schematic diagram of a brookfield type viscometer. Adopted from <https://ciks.cbt.nist.gov/~garbocz/SP946/node14.htm>.

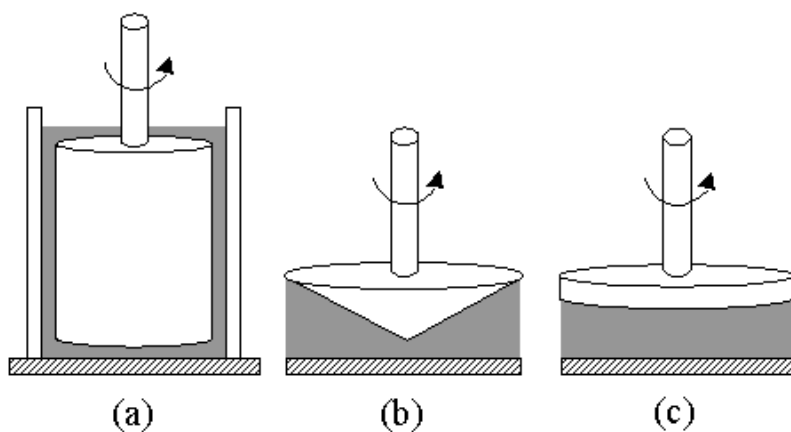


Fig. 2. Schematic diagram of basic tool geometries for the rotational rheometer..

(a) concentric cylinder, (b) cone and plate and (c) parallel plates. Adopted from

<https://ciks.cbt.nist.gov/~garbocz/SP946/node14.htm>.

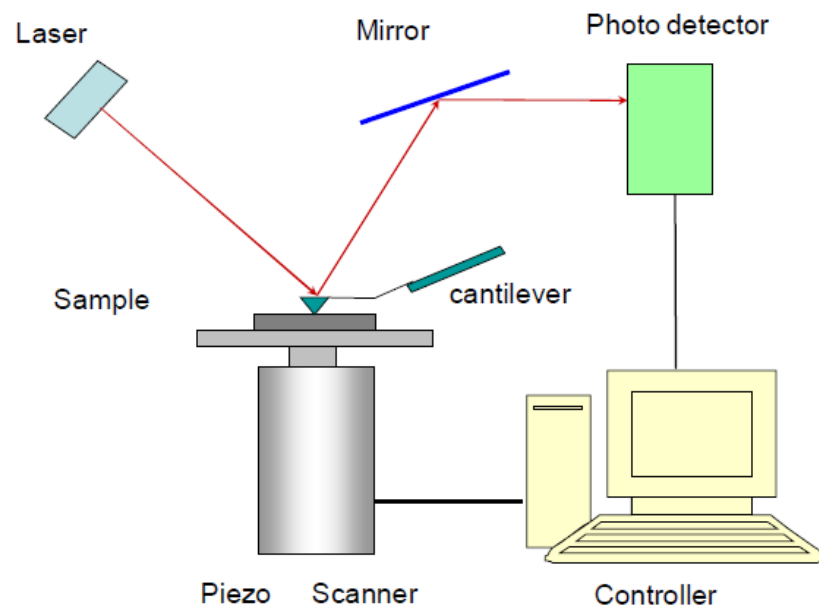


Fig. 3. Schematic diagram of working of AFM. Adopted image (Agilent systems).

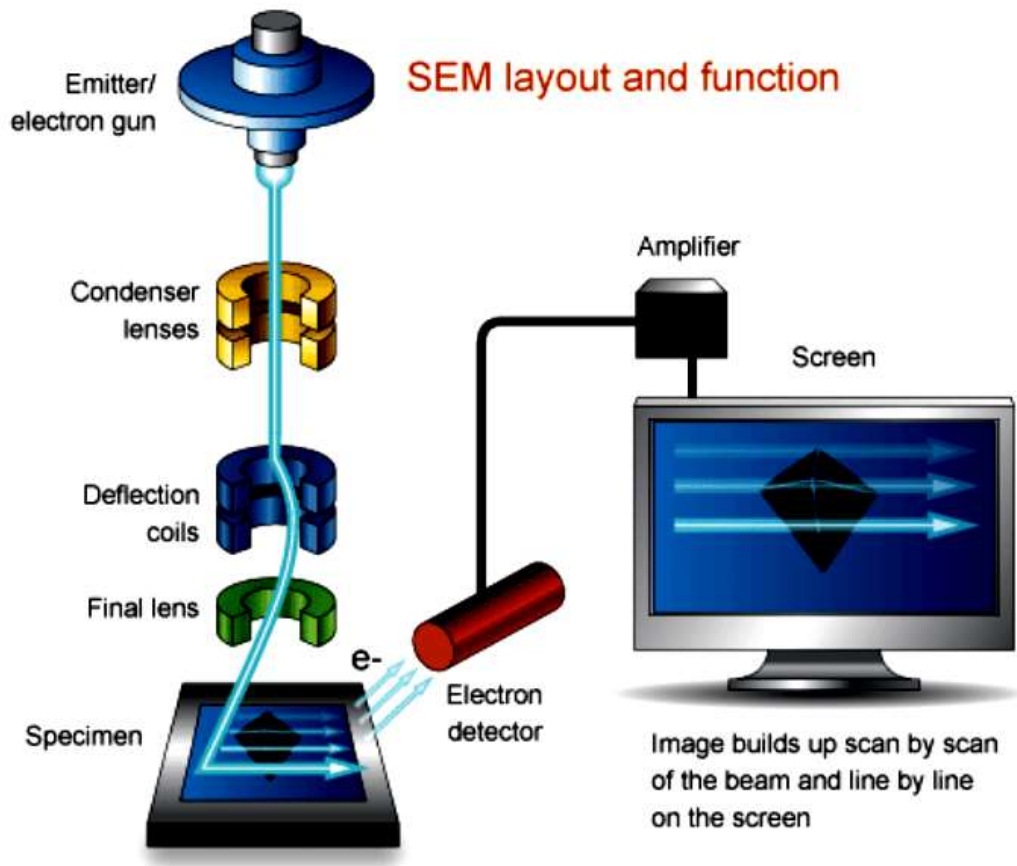


Fig. 4. Image adopted from <https://myscope.training/legacy/images/sem/layout-and-function.png>.

CHAPTER II. Comparative analysis of EPSs to study the changes in physicochemical properties at different cultivation times

II-1. Introduction

LAB are in use for producing fermented foods, such as yogurt, kimchi, cheese, etc. since long time. LAB are non-pathogenic, gram-positive bacteria having the generally recognized as safe (GRAS) status and have been in focus of attention for industrial importance due to their potential probiotic properties (Badel *et al.*, 2011).

Industrial reports state that global market for LAB and probiotics is mainly characterized by three applications: (i) probiotics in foods and beverages [e.g. cereal, dry, dairy, non-dairy, baked food and fermented meat products], (ii) probiotic dietary supplements [e.g. nutraceuticals, single cell protein, specialty nutrients, infant formula], and (iii) animal feed probiotics (e.g. substitutes of antibiotic-based growth stimulants) (Zoumpopoulou *et al.*, 2018). In 2017, the US and EU markets for probiotic products have been estimated at over 1.8 billion and 630 million USD, respectively (<https://www.gminsights.com/industry-analysis/probiotics-market>). Owing to an increased consumer interest in health and globally increasing incomes, this market is projected to grow even more. So there is a competition between the food and feed companies to try utilizing LAB in their products to meet market demand, improving product quality, and moreover to diversify their product lines.

Several types of polysaccharides are produced by algae, plants, and microorganisms that are being used to modify texture, emulsify or viscosify foods and/or to increase the consistency and texture of the fermented dairy products. Their use catches extra attraction of the food industry when they are excreted during the manufacture of food product and constitute a new generation food thickeners (Zhang *et al.*, 2011). Therefore, there has been an increasing interest in the production of EPS by LAB.

EPS production, thickening properties, molar mass and structural conformations are greatly influenced by environmental factors (Ruas-Madiedo *et al.*, 2005). In some species, only the EPS yield is affected without any changes in monosaccharide composition (Looijesteijn and Hugenholtz, 1999).

Further, the identification of such LAB; and isolation and characterization of novel bioactive molecules is of utmost importance in the production of new value-added products (Ku *et al.*, 2016). Moreover, the first step in their commercial utilization as probiotics and drugs, as well as for metabolic engineering is the characterization of their metabolites (Jia *et al.*, 2008; Krivoruchko *et al.*, 2015 and Meadow *et al.*, 2015).

Since the relationship among the EPS amount, its composition/structure as well as functionality is not yet completely unrevealed, a rational screening for novel EPS from particular LAB strains that are characterized by a unique structure or molar mass is of outmost importance regarding possible application in food industry (Grosu- todur and Zamfir, 2013). Hassan (2008) attributed the inability to understand the functions of EPS in dairy production to two main causes; 1) major variations amongst EPS, even from the same group of microorganisms, making it difficult to apply information from one EPS to other; and 2) the lack of techniques available to observe the microstructure and distribution of highly hydrated EPS in fermented dairy products. Keeping in view the above mentioned facts, an attempt has been made to characterize the EPS production at different growth intervals which can be expected to resemble their *in-situ* commercial production.

II-2. Materials and methods

II-2-1. Bacterial strain and reagents

The bacterial strain *L. fermentum* MTCC 25067 was obtained from the bacterial collection in our laboratory at Obihiro University of Agriculture and Veterinary Medicine. The strain was stored at -80°C in de Man-Rogosa-Sharpe (MRS) broth containing 20% (v/v) glycerol until used. Bacterial culture was revived on MRS agar and cultured twice before use. MRS media was obtained from Oxoid (Cambridge, UK). DEAE-Sephadex A-50 and Toyopearl HW-55F were purchased from GE Healthcare (Little Chalfont, UK) and Tosoh (Tokyo, Japan), respectively. All the chemicals used were of analytical grade.

II-2-2. Growth profile

Bacterial culture and EPS preparation have been done according to Ikeda *et al.* (2019) with slight modifications. Viable cell numbers and pH of the culture medium were monitored at 0, 8, 16, 24, 32, 48, 96, and 144 h of cultivation as previously

reported (Fukuda *et al.*, 2010). The glucose concentration of the culture supernatant was measured at the same time intervals as above on the basis of the phenol-sulfuric acid method using glucose as the standard (Dubois *et al.*, 1956).

II-2-3. Preparation of the EPS

Each culture medium was diluted 5 times with distilled water after 48h and 144 h of growth in a 1-liter MRS broth each at 30°C, followed by removal of bacterial cells using centrifugation (17,000 g, 1 h, 4°C). Crude EPS was precipitated by adding an equal volume of ice-cold ethanol to the supernatant obtained after removal of cells. The precipitate collected by centrifugation at 17,000 g for 30 min (4°C) was dissolved thoroughly in 100 ml of ion-exchanged water. 300-mL slurry of DEAE-Sephadex A-50 equilibrated with 50 mM Tris-HCl (pH 8.7) was added to the crude EPS solutions and allowed to adsorb overnight in a jar with stirring using magnetic stirrer to remove protein contaminants. The non-adsorbed fraction was collected using aspirating filtration. The EPS was once again precipitated by adding an equal volume of ice-cold ethanol to the filtrate, collected using centrifugation (17,000 g, 30 min, 4°C), and dissolved thoroughly in 50 mL of Milli-Q water. The EPS solutions thus obtained were extensively dialyzed against Milli-Q water overnight, lyophilized, and stored in a desiccator until used.

II-2-4. Measurement of viscosity

Viscosities of the culture media collected at 0, 8, 16, 24, 32, 48, 96, and 144 h of cultivation were measured using a single cylinder-type rotational viscometer VT-03F (Rion, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 460 mL of a culture broth was placed in an outer cup of radius 46 mm for a measurement using an inner rotor of radius 30.6 mm. The apparatus was left at room temperature for 4 min before measuring viscosity at a rotational speed of 62.5 rpm. The shear rate was estimated to be 17/s using the following equation (1):

$$D = (2\pi n/60)(1 + \delta^2)/(\delta^2 - 1) \quad (1)$$

where D , n , and δ represent shear rate (1/s), rotational speed (rpm), and the ratio of the radius of the inner rotor to the radius of the outer cup, respectively. All the experiments were done in triplicate.

II-2-5. Comparative analysis of molecular weight distribution in EPS using Gel permeation chromatography

To investigate the changes in molecular weight distribution in EPS at the maximum and minimum viscosity, EPS samples were purified from the media cultivated for 48 h (EPS_{48h}) and 144 h (EPS_{144h}), respectively, and subjected to gel permeation chromatography. One mg of a purified EPS sample was dissolved in 1 mL of water, and then a 100- μ L aliquot of the HePS solution was loaded onto a TSK gel G5000PWXL column (ϕ 7.8 \times 300 mm) equilibrated with 50 mM Tris-HCl (pH 7.0). Isocratic elution was done at 40 °C and a flow rate of 1 mL/min using the same buffer. Ultraviolet absorbance was monitored using a UV-2075 Plus spectrophotometer (Jasco, Tokyo, Japan). The 20 fractions of eluate were collected every 1 min, manually. The phenol-sulphuric acid method was used to estimate the concentration of total carbohydrates in each fraction using glucose as the standard (Dubois *et al.*, 1956); and reported as the ratio of the carbohydrates in each fraction to the total carbohydrates. The ratio of high molecular weight polysaccharides content (HMW) to that of low molecular weight polysaccharides (LMW) was calculated as the ratio of summation of the relative concentrations in the fractions collected at elution times of 6–8 min to those at elution times of 9–15 min. Experiments were performed in triplicate.

II-2-6. Side chain structure using ¹H-NMR analysis

Purified EPS_{48h} and EPS_{144h} (2 mg each) were dissolved in 99.96% D₂O (Eurisotop, Saint-Aubin, France), lyophilized, and subjected to the following ¹H-NMR. ¹H-NMR spectra of EPS_{48h} and EPS_{144h} dissolved in D₂O (99.999 atom %D, Across Organics, New Jersey, USA) were recorded at 600 MHz using a Varian INOVA 600 spectrometer (Varian Inc., Palo Alto, CA) operated at 323.1 K. Chemical shifts (δ) were expressed as those relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid but were measured practically using internal acetone (δ = 2.225 ppm) as a reference. Experiments were done in triplicate.

II-3. Results and discussion

II-3-1. Cultivation profiles

Changes in the viable cell number, pH, the residual glucose concentration, and the viscosity of the culture were recorded during cultivation for 144 h (Fig. 5 A, 5B and 5C). The viable cell number decreased gradually over time during cultivation for a longer period of time after reaching a maximum at around 16–24 h of cultivation. The residual glucose concentration and pH decreased remarkably during the first 24 h of cultivation and remained constant thereafter.

II-3-2. Measurement of viscosity

The culture media viscosity increased during the first 24 h, maintained relatively high values in the subsequent 24 h, and then decreased continuously toward the end of cultivation. Although, all the three independent experiments had similar values of viable cells, pH, and the residual glucose concentration; considerable differences in the viscosity medium among three independent experiments were noticed at 24–96 h of cultivation. The maximum average viscosity of 53 mPa·s at the estimated shear rate of 17/s was observed for culture medium at 48 h. The profile (Fig. 5D) shows that the EPS production may be growth dependent. Also, the polymer may only be produced during the log phase and not until the stationary phase, probably due to an increased cell permeability (Spizizen, 1958). Similar growth associated EPS production has also been reported by Kimmel *et al.* (1998) and Yuliani *et al.* (2011). Jiang *et al.* (2016) also reported highest production of EPS by *L. plantarum* WLPL04 at during logarithmic phase at 24 h. However, some LAB strains, e.g. *Streptococcus thermophilus* ST111, producing maximum EPS (yield) at the end of fermentation (Vaningelgem *et al.*, 2004).

II-3-3. Molecular weight distributions of the EPS

The elution profiles of both EPS_{48h} and EPS_{144h} showed first peak at 7 min of the elution time (Fig. 6A). A second peak could be seen at 9–10 min for EPS_{48h}; however a second and a third peak were observed for EPS_{144h} at 9 min and 11 min, respectively. Similar elution profiles have been reported previously by Fukuda *et al.* (2010) where they also found two major peaks and a shoulder at retention times similar retention times recorded in this study. The highest peak was seen at 7 min for EPS_{48h},

or at 9 min in for EPS_{144h}. Amongst all the fractions, only the fractions at 12 min of elution time indicated significantly different relative carbohydrate concentrations between the two EPS. EPS_{48h} showed a significantly higher ratio of HMW to LMW polysaccharides as determined from the ratio of those obtained at elution times of 6–8 min to those obtained at elution times of 9–15 min, indicating more low molecular weight polysaccharides in EPS_{144h} compared to EPS_{48h} (Fig. 6B). It is noteworthy to mention the broad peaks observed suggesting a large distribution of molecular weights; a common characteristic documented for some other EPS-producing strains (Gorret *et al.*, 2003).

There is a probability of HePS-degrading enzymes such as glycosidases and 1,3-glucanases, capable of hydrolyzing the main chain of the HePS consisting of 1,3-glucan, leaking out from dead bacterial cells into the medium as also proposed by Pham *et al.* (2000). It is unlikely that polysaccharide lyases, capable of hydrolyzing uronic acid-containing polysaccharides *via* a β -elimination mechanism, participate in the degradation of the present HePS, because of the absence of uronic acid in the HePS (Linhardt, Galliher and Cooney, 1986). In the case of *L. rhamnosus* R, a prolonged period of incubation for 72 h resulted in losses of the viscosity of the culture medium up to approximately 20%, accompanied by increasing ratios of the low molecular weight (2.5×10^4 Da) fraction of the EPS (Pham *et al.*, 2000). The viscosity of the EPS also decreased when it was mixed with the cell extract and incubated. Consequently, the researchers concluded that the loss of the viscosity was attributed to enzymatic degradation of the EPS by the concerted action of glycohydrolases, such as α -D-glucosidase, β -D-glucosidase, α -D-galactosidase, β -D-galactosidase, β -D-glucuronidase, and α -L-rhamnosidase. Exploration of inhibitors against such EPS-degrading enzymes may provide a means for preventing viscosity losses of LAB-derived HePSs during cultivation.

The molecular weight analysis of EPS plays an important role in understanding its bioactivity and studying possible structure-function relationship of the EPS in fermented products (Vanningelgem *et al.*, 2004). High molecular weight EPS have been found to improve the texture of fermented milk (Hassan *et al.*, 2003). Also, the differences in molecular weight compositions of EPSs might be associated with their specific functions. High molecular weight of EPS contributes to reduced syneresis and

improved texture of fermented milk by cross-linking of the EPS into a network structure with the proteins during fermentation (Hassan *et al.*, 2003).

II-3-4. Side chain structure using ^1H -NMR analysis

Almost identical chemical shift profiles could be seen in the 6000-MHz 1D ^1H -NMR spectra of the HePS_{48h} and HePS_{144h} (Fig. 7-12). Previously, Gerwig *et al.* (2013) reported ^1H -1 chemical shifts around $\delta = 5.674$, 5.325, 4.985, and 4.731 ppm assigned to $-(1\rightarrow2,3)\text{-}\alpha\text{-D-Glcp-(1}\rightarrow$ or 2,3-disubstituted D-Glcp, $-(1\rightarrow6)\text{-}\alpha\text{-D-Galp-(1}\rightarrow$ or 6-substituted D-Galp, $\alpha\text{-D-Glcp-(1}\rightarrow$ or terminal D-Glcp, and $-(1\rightarrow3)\text{-}\beta\text{-D-Glcp-(1}\rightarrow$ or 3-substituted D-Glcp, respectively. Table 2 summarizes the ^1H -1 chemical shifts and the peak areas of the signals assigned to these four sugar residues in this study. The average molar ratio of terminal D-Glcp to 3-substituted D-Glcp to 2,3-disubstituted D-Glcp to 6-substituted D-Galp was determined to be 0.9:1.0:0.8:0.7 for EPS_{48h} and 0.9:1.0:0.8:0.6 for EPS_{144h}, as a result of calculating the ratio of the peak area of each residue to that of the 3-substituted D-Glcp. In short, these EPSs had almost identical average molar ratios of constituent sugars, suggesting that the chemical structure of side chains is preserved to a large degree during cultivation for 144 h. Earlier, Gerwig *et al.* (2013) reported the average molar ratio of terminal D-Glcp to 3-substituted D-Glcp to 2,3-disubstituted D-Glcp to 6-substituted D-Galp to be 1.3:1.0:1.1:1.1 for the HePS produced by the same strain of LAB used in this study. The differences in the molar ratio between the two studies indicate overestimation of the quantity of 3-substituted D-Glcp, due to the presence of partially overlapping contaminant peaks to the one assigned to 3-substituted D-Glcp in the present study (Figs. 7-12).

Previously, *Lactobacillus fermentum* strain V10 has been found to produce a HePS with glucose: rhamnose : galactose ratio of 1 : 13 : 1.5. The most frequently found monosaccharides found in the EPS produced by LAB strains are glucose (Cerning, 1995), rhamnose (Mozzi *et al.*, 2006; Nakajima *et al.*, 1990); and galactose (De Vuyst *et al.*, 2003; Doleyres *et al.*, 2005). Monosaccharide analysis of another purified EPS produced by *L. fermentum* strain F6 isolated from traditional dairy products in Inner Mongolia of China with an estimated molecular weight of 3.54×10^6 Da was found to have two distinct peaks in HPLC, corresponding to glucose and galactose in a molar ratio of 4:3 (Zhang *et al.*, 2011).

The monosaccharide compositions of EPS varies with the strain (Wang *et al.*, 2014; Caggianiello *et al.*, 2016); and may be closely related to the functionality of EPS (Bello *et al.*, 2001; Russo *et al.*, 2012; Das *et al.*, 2014).

The contribution of the HePS to structure/function relationships is very complex. For example, it has been postulated that stiffer chains, as the case of β -(1,4) linkages, are required for high intrinsic viscosities; eventually leading to higher consistence of EPS solutions. Further, while the degree of branching is proposed to contribute to the polymer stiffness; the viscosity of EPS solutions is influenced by the complexity of their primary structure (namely size, monomer composition, and side groups, α - and β -linkages, branching) (Tuinier *et al.*, 2001).

II-4. Tables and Figures

Table 2. H-1 chemical shifts and peak areas of sugar residues consisting of the HePSs.

Residue	HePS _{48h}		HePS _{144h}	
	δ (ppm)	Peak area (%)	δ (ppm)	Peak area (%)
-(1→2,3)- α -D-Glcp-(1→	5.679, 5.680, 5.682	3.80 ± 0.92	5.683, 5.684, 5.682	3.07 ± 0.18
-(1→6)- α -D-Galp-(1→	5.327, 5.326, 5.326	3.20 ± 0.91	5.330, 5.330, 5.329	2.51 ± 0.20
α -D-Glcp-(1→	4.979, 4.977, 4.978	4.56 ± 1.04	4.978, 4.978, 4.979	3.64 ± 0.18
-(1→3)- β -D-Glcp-(1→	4.741, 4.740, 4.743	4.88 ± 0.30	4.745, 4.754, 4.744	4.06 ± 0.03

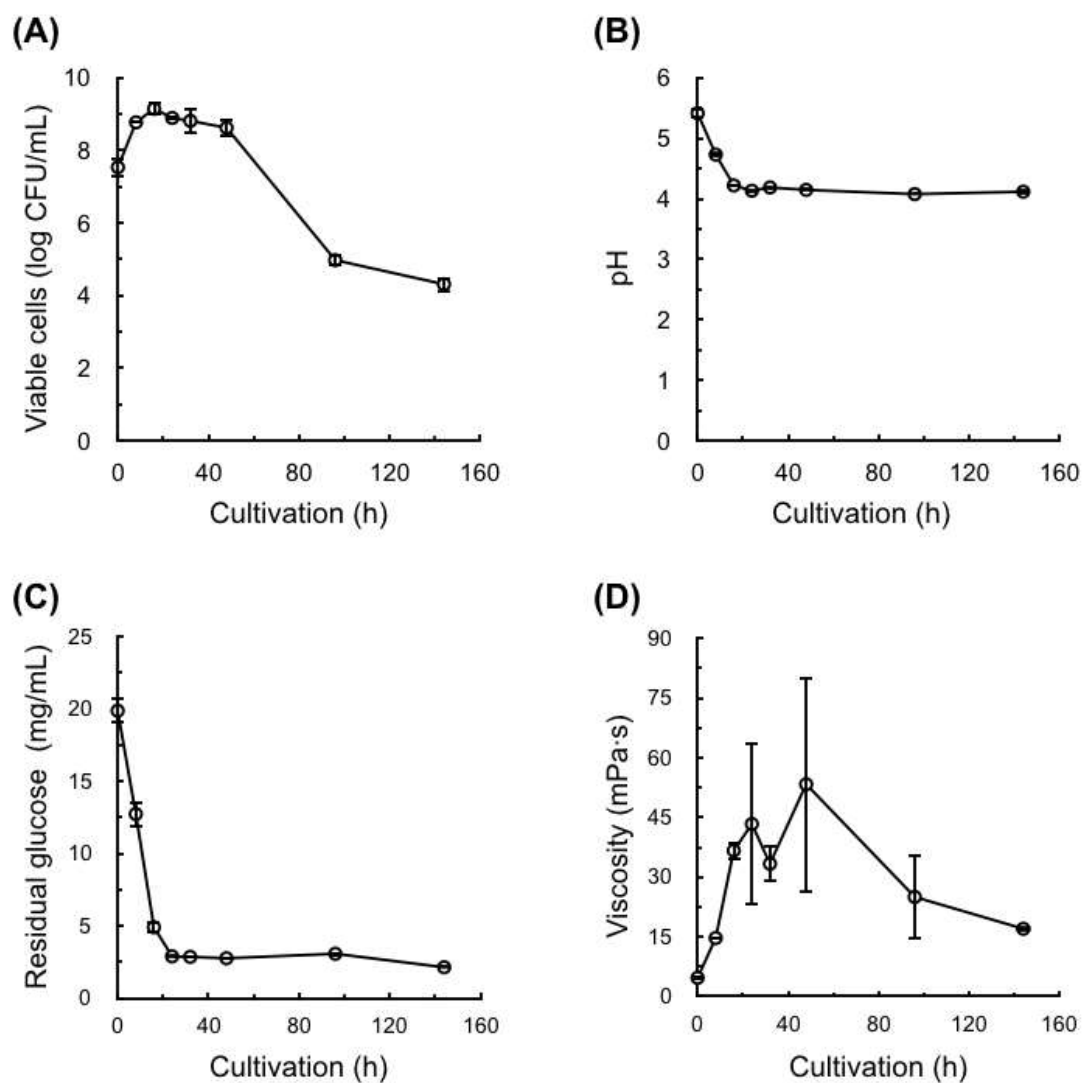


Fig. 5. Changes in (A) viable cell counts, (B) pH, (C) residual glucose concentration, and (D) viscosity in the culture medium during cultivation of *L. fermentum* MTCC 25067. The error bars indicate standard deviations.

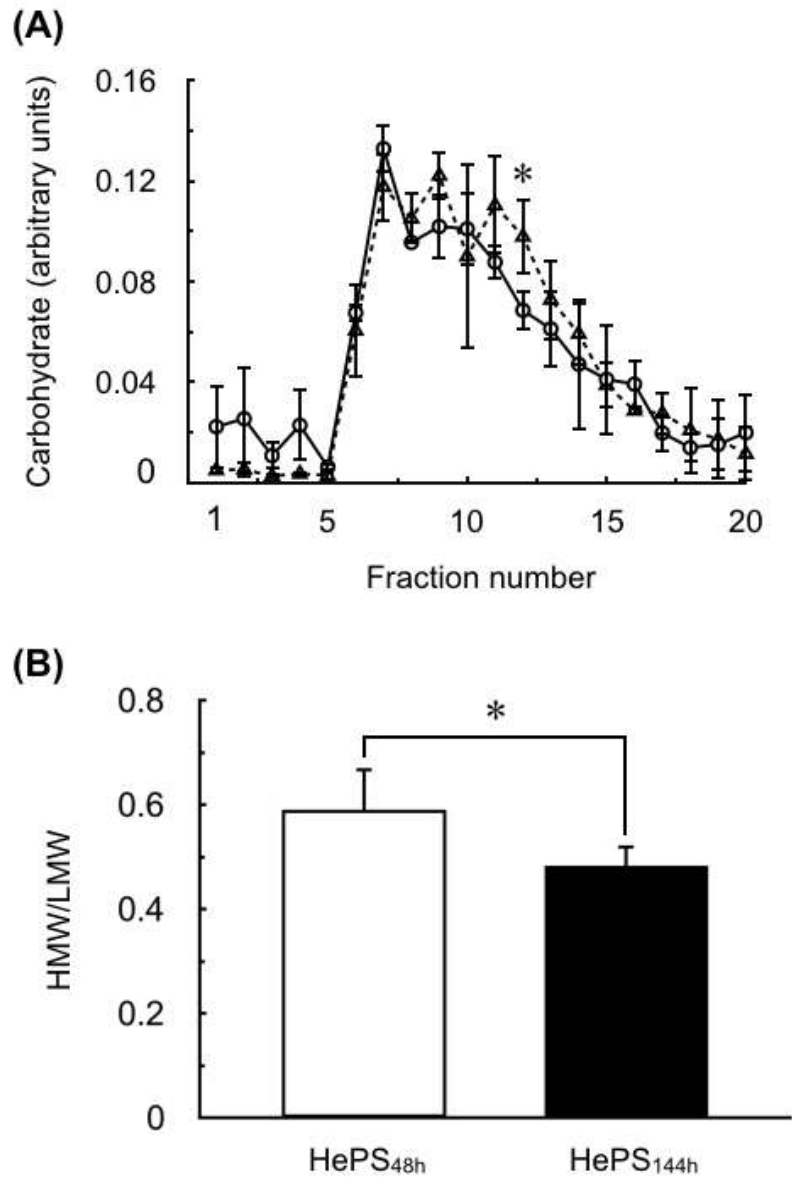
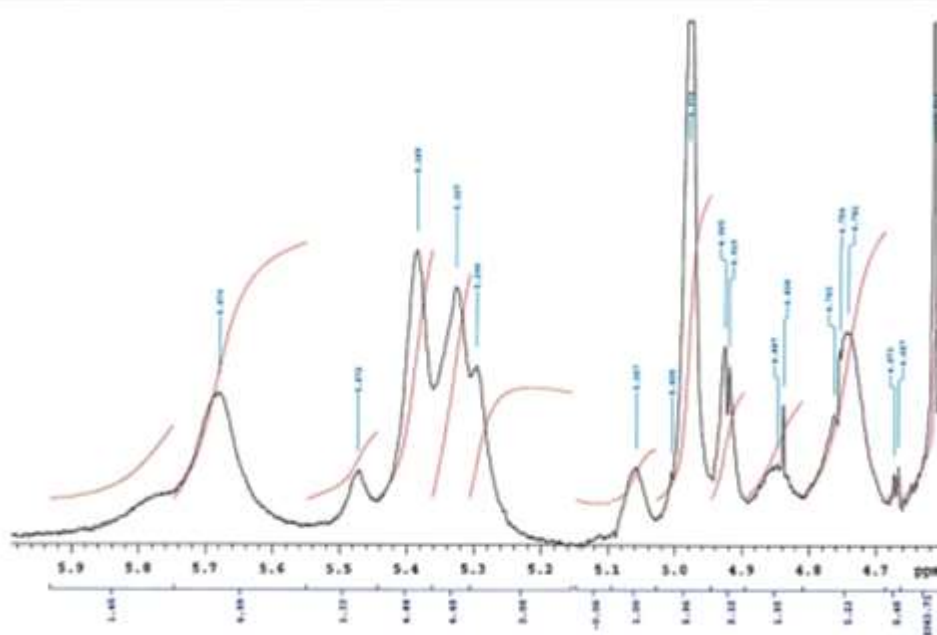


Fig. 6. (A) Gel permeation chromatography profiles of the HePS_{48h} (circles) and HePS_{144h} (triangles) and (B) comparison of the ratio of the content of high molecular weight polysaccharides (HMW) to that of low molecular weight polysaccharides (LMW) in the HePSs. The error bars indicate standard deviations. An asterisk (*) indicates statistical significance ($P < 0.05$).



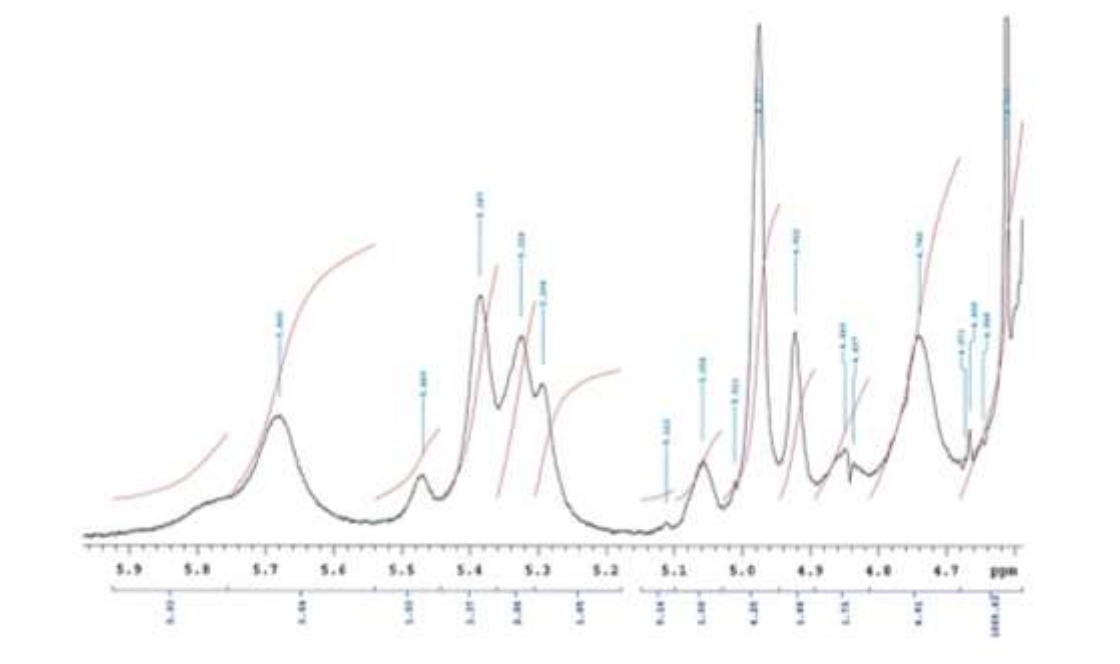
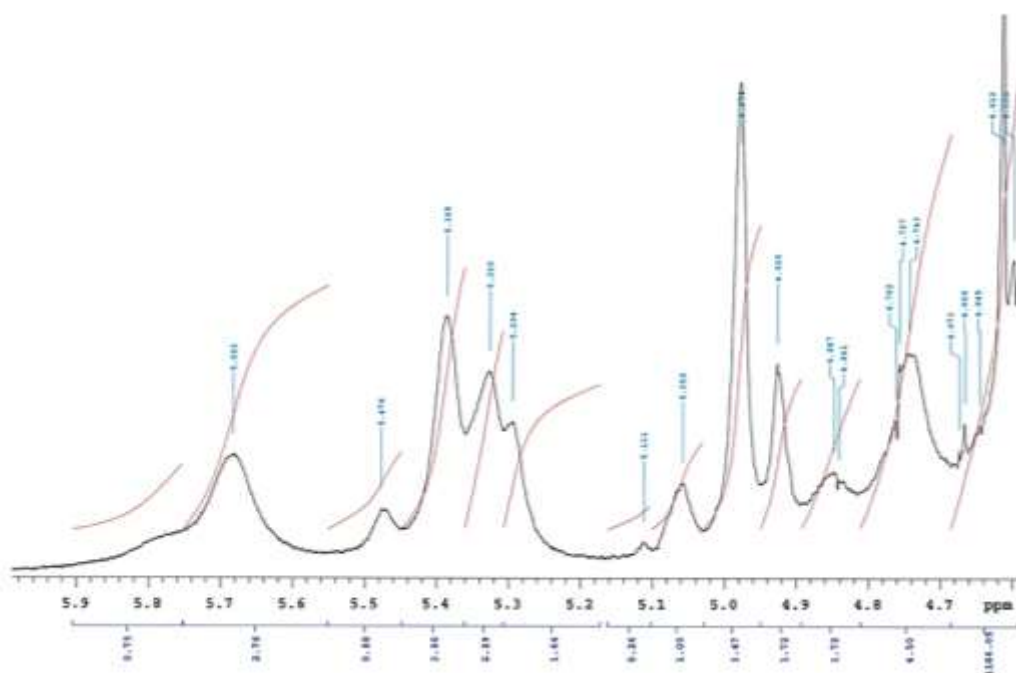


Fig. 8. Chemical shift profile using ^1H -NMR spectra of the HePS_{48h} (Replicate 2).



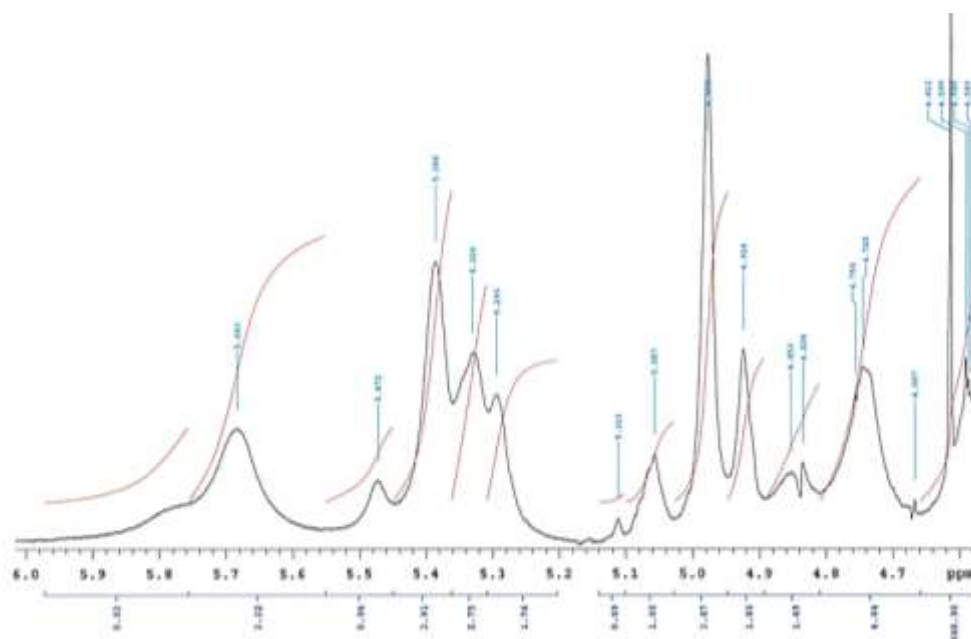


Fig. 10. Chemical shift profile using ^1H -NMR spectra of the HePS_{144h} (Replicate 1).

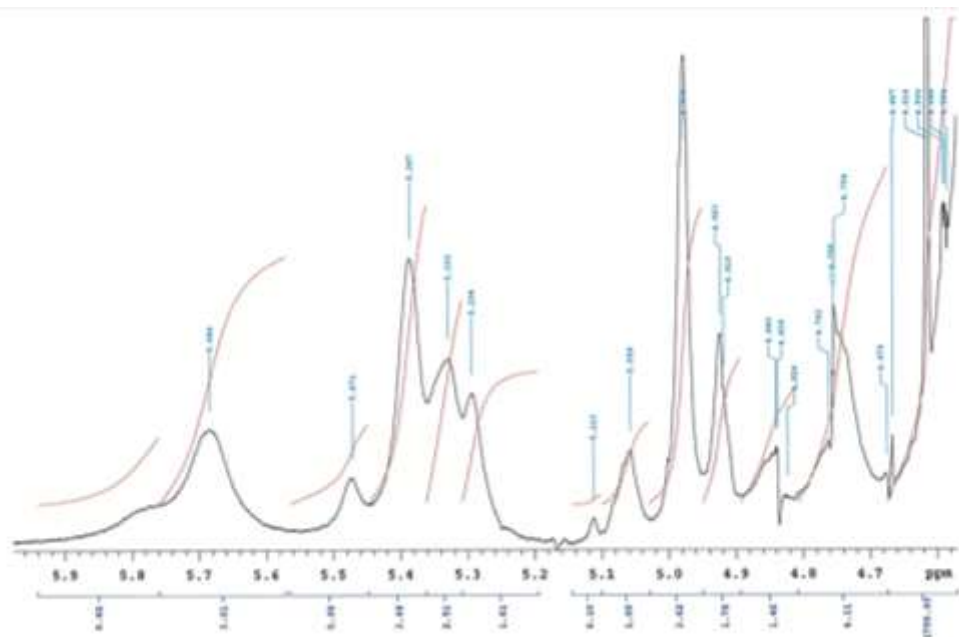


Fig. 11. Chemical shift profile using ^1H -NMR spectra of the HePS_{144h} (Replicate 2).

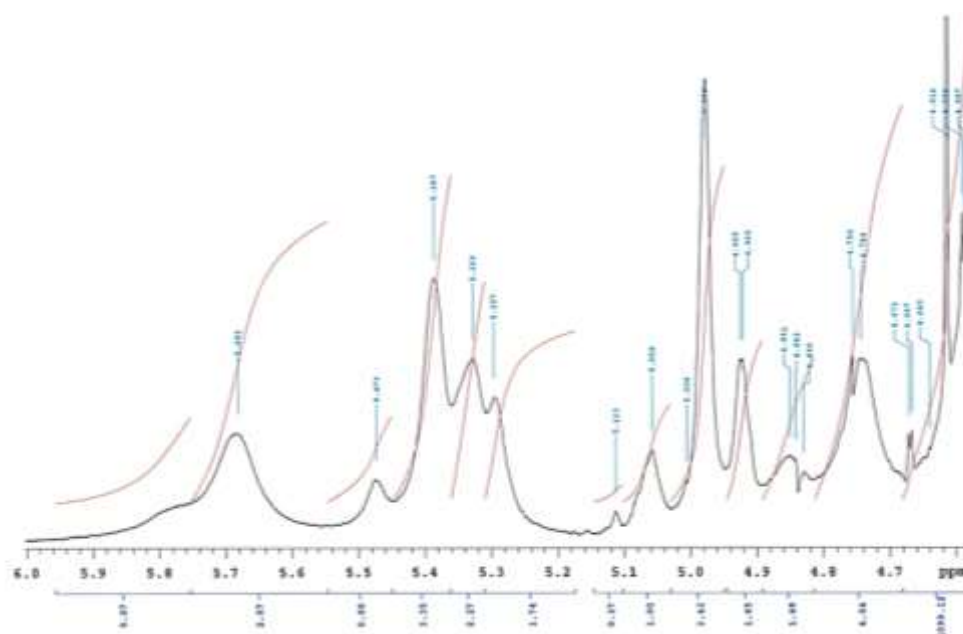


Fig. 12. Chemical shift profile using ^1H -NMR spectra of the HePS_{48h} (Replicate 3).

CHAPTER III. Rheological and structural characterization of EPS

III-1. Introduction

EPS, especially those produced by LAB are gaining importance day by day, owing to their use in improvement of physical, rheological and sensory properties of fermented dairy products viz-a-viz dahi, lassi, yoghurt, lassi, matha (cultured buttermilk) etc. (Behare *et al.*, 2009 a; Behare *et al.*, 2009 b; Behare *et al.*, 2010); and also due to their application as thickeners in processed products industry (Cerning, 1990). It also plays a vital role in product texture development, with the EPS type exerting a greater effect than the EPS concentration (Vanningelgem *et al.*, 2004). Generally, two types of EPS are being frequently assessed for their effects on product texture, namely ‘ropy’ and ‘capsular’ depending on the strain used. Moreover, some bacterial strains have even been found to produce a mixture of both the types in different proportions (Zisu and Shah, 2003; 2005). The combinations of the two different types of EPS producing cultures have been reported not only to improve the total EPS production but also affects the texture of the product (Marshall and Rawson, 1999).

LAB producing EPS are typically used as functional starter cultures that contribute to improvement of consistency and rheology of fermented products. There is an emerging trend, for incorporation of EPS-producing LAB in fermented milks, cheese and beverages (Li *et al.*, 2012; Lynch *et al.*, 2014). Recently, many EPS producer LAB species have been employed in milk fermentation for preventing syneresis and replacing stabilizers (Kailasapathy, 2006). EPS-producing bacterial species used successfully for fermented milk production with improved physicochemical and biological properties include Streptococci (Purohit *et al.*, 2009), lactobacilli (Folkenberg *et al.*, 2006), lactococci (Ayala-Hernández *et al.*, 2009) and bifidobacterium (Prasanna *et al.*, 2013). EPS production depends on the strain and other factors like growth medium composition, age of the cell, pH and temperature (De Vuyst *et al.*, 2003; Ruas-Madiedo and de los Reyes, 2005). Behare *et al.* (2009c) and Doleyres *et al.* (2005) have even reported an additive effect of *in situ* EPS production on technological properties of fermented dairy products compared to the addition of EPS powder externally.

The effects of including EPS-producing cultures as well as purified EPS on different aspects, such as functionality and microstructure of fermented dairy products have been reported in many studies (Girard and Schaffer-Lequart 2007; Gorret *et al.*, 2003; Hassan *et al.*, 1996, 2001; Ruas-Madiedo *et al.*, 2002). EPS produced by LAB have been reported to affect viscosity (Ruas-Madiedo *et al.*, 2002); gel formation and gel strength (storage modulus (G')) (Hassan *et al.*, 2002; Girard and Schaffer-Lequart, 2007; Hassan, 2008). Further, the intrinsic properties of a specific EPS determine its effect on inclusion in a dairy matrix (Hassan, 2008). Girard and Schaffer-Lequart (2007) reported the charge of the EPS and the EPS–protein interactions responsible for an increase in gel strength. However, no clear correlation has been found between EPS concentration and its effect on the rheological properties of fermented milk (Petry *et al.*, 2003).

Rheological properties of pure bacterial EPSs are diverse, reflecting wide variations in their molecular weight distributions, monosaccharide compositions, types of linkages between sugar monomers, presence of side chains, and modifications such as acylation, carboxylation, phosphorylation, pyruvation, sulfation, etc. (Lambo-Fodje *et al.*, 2007; Tuinier *et al.*, 2001; Wyatt *et al.*, 2011; Zeidan *et al.*, 2017).

However, several EPS have been reported to influence dairy products texture, and mechanisms on their interactions with milk proteins have also been proposed, there is still a gap in information on the rheological properties of EPS produced by *L. fermentum* MTCC 25067. Rheological characterization and analysis helps in elucidating the structure-function relationship of the compounds at molecular level in aqueous solvents (Tako *et al.*, 2016). Understanding rheology of bacterial exopolysaccharides aids in exploring their possible applications in fermentation industry viscosity modifiers and stabilizers, so that a final product with optimum viscosity and desired rheological characteristics can be obtained. This information could be used to predict the *in-situ* production characteristics of EPS and its use in dairy products; and, also the possible interactions of EPS with milk component(s) during fermentation that may affect the end product texture. Therefore, the current experiments were designed with the objectives to study the rheological and morphological changes to better understand the molecular basis behind the changes occurring during the course of growth.

III-2. Materials and methods

III-2-1. Measurement of steady shear viscosities

A 0.02% (w/v) solution of EPS obtained at 48h (EPS_{48h}) and 144h (EPS_{144h}) were prepared in MRS broth. 1% (w/v) EPS solutions (EPS_{48h} and EPS_{144h}) were also prepared in ion exchange water. Steady shear viscosities of both the solutions were recorded at 25°C as a function of shear rate ranging from 0.1/s to 100/s using a TA Discovery HR-2 rheometer (TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature control unit. A cup and bob assembly was used for 0.02% (w/v) EPS solutions in MRS whereas, a cone and plate attachment (diameter 20 mm; angle 1.009°; gap 65 µm) was used for 1% (w/v) EPS solutions in ion exchanged water.

III-2-1. Topographical imaging using AFM

The lyophilized EPS samples (EPS_{48h} and EPS_{144h}) were dissolved in milli-Q water to obtain 1 mg/L and 2.5 mg/l (w/v) solutions. A 2-µL aliquot of the EPS solution was deposited onto a freshly cleaved mica surface and left to dry for 30 min at room temperature. Topographical images were taken under air using a BioScope Catalyst atomic force microscope (Bruker, Santa Barbara, CA, USA) operated in peak force tapping mode using a NanoScope V controller (Bruker). The images obtained were flattened using NanoScope Analysis software version 1.40 (Bruker).

III-3. Results and discussion

III-3-1. Viscosities of the purified EPSs

A typical shear-thinning behavior in response to increasing shear rate was seen in 1% (w/v) aqueous solutions of both EPS_{48h} and EPS_{144h} in ion exchanged water (Fig. 13 A, 13B, 13C and 13D). The shear thinning behavior of EPS, mainly results from the breakdown of structural units in the EPS by hydrodynamic shear forces, and is considered important in yielding desired sensory properties like mouthfeel and flavor release properties; and also in processing operations such as stirring, pouring, pumping, spray drying (Zhou *et al.*, 2014). The steady shear viscosity (η) were significantly different between the two HePSs at the shear rate range lower than 25/s. The EPS under study is typically produced approximately at the rate of 100 mg/l during a single batch of cultivation. To simulate the in-situ production, the EPS_{48h} and EPS_{144h} were dissolved in MRS broth to give a polysaccharide concentration of 0.02% (w/v) and then

subjected to the viscosity measurements. Both of the solutions exhibited typical Newtonian fluid behavior (Fig. 13B). The steady shear viscosities of HePS_{48h} and HePS_{144h} at a shear rate of 15.8/s, which was close to the one used in the viscosity measurement using the single cylinder-type rotational viscometer (Fig. 5D), were 1.6 and 1.2 mPa·s, respectively. Differences in the viscosity between the two HePSs were significant, except at a shear rate of 63/s. No reliable data could be obtained from measurements at shear rates equal to or lower than 1/s because of too low signal-to-noise ratios. The viscosities of the HePSs dissolved in the MRS broth were significantly lower compared to that in the culture medium during bacterial growth.

Similarly, Costa *et al.* (2012) found significant differences in the size of the EPS aggregates were detected between the purified and the native, the native being bigger in size than the purified one. They suspected the likelihood of EPS forming large aggregates in the native form; but after purification and filtering steps, EPS aggregates become considerably reduced.

III-3-2. Topographical imaging using AFM

AFM is a powerful tool that can be employed to characterize three-dimensional surface structures; morphology and conformation of the individual macromolecules, the dynamics, and super molecular structures of polysaccharides at the molecular level (Ahmed *et al.*, 2013). The microstructure and surface morphology of the HePS_{48h} deposited from a 1 mg/L solution onto the mica surface and air-dried prior to imaging showed a network structure similar to that of the same type of EPS reported previously (Ikeda *et al.*, 2019) (Fig. 14A and 14B). The average length and height of the HePS_{48h} fibres were found to be 1.29 μm and 1.25 nm, respectively. HePS_{144h} also showed a similar network structure, but the population of shorter (0.36 μm on average) and thinner (1.09 nm on average) fibres appeared to be greater than HePS_{48h} (Fig. 14A and 14B). The possibility of unknown enzymes degrading HePS *via* a β -elimination mechanism such as those similar to α -glucan lyases cannot be excluded (Lee *et al.*, 2003). Aggregates of EPS molecule could be seen probably due to an indirect effect of dehydration during sample preparation. Moreover, the short chains of aggregates in EPS under study may also be due to the neutral charge of the EPS (Leo *et al.*, 2007). Camesano and Wilkinson (2001) observed EPS xanthan in 0.01M KCl using AFM and

reported repulsion of negatively charged individual chains as the reason for prevention of aggregate formation and observation of long chains.

Several shapes and structures of EPS produced by different organisms have been reported. Li *et al.* (2015) found rounded to spherical lumps of different sizes in EPS of *Lactobacillus helveticus*. Ahmed *et al.*, 2013 reported the reticulate shape of EPS produced by *Lactobacillus kefiranofaciens* ZW3. Recently, formation of a network structure has been suggested as a major reason for the high viscosity of the HePS produced by *L. fermentum* MTCC 25067 (Ikeda *et al.*, 2019). Similar structural property has also been reported by Shang *et al.* (2013) for an EPS produced by *Bifidobacterium animalis* RH; and postulated to be associated with inter- or intra-molecular aggregation (or both) of polysaccharide chains and branches, and further formation of an interconnecting network structure. However, AFM performed to elucidate the surface roughness and morphology of the EPS from *B. amyloliquefaciens* GSBa-1 revealed spherical lumps with variable heights (0.3-0.7 nm), comparable with the height of a single polymer chain (0.1 to 1 nm) indicating no intra- and /or inter- molecular aggregation in the EPS solution (Zhao *et al.*, 2018).

Polysaccharides usually adopt a secondary structure or suitable conformation which is governed by its monomer composition and their linkages. The most common is the helical conformation, where helices may further aggregate together forming a network-like structure. However, ordered conformation is not exhibited by all polysaccharide systems in solutions. Moreover, some degree of order is also induced by the ions of appropriate valency and charge present in the system. Most polysaccharides undergo a transformation, induced thermally or by removal of specific ions, from an ordered to a disordered state in solution. Galactoglucan produced by *Rhizobium meliloti*, is highly flexible and always found to exist in a disordered conformation. Lack of intra-chain hydrogen bonds and location of anionic pyruvic ketal along with acetyl groups on the periphery of the helical structure of the polysaccharide are responsible for the absence of an ordered conformation (Wingender *et al.*, 2012). Hence, the solution properties of the polysaccharides are defined by the molecular conformation and flexibility of the polymer, its chemical structure and composition, molecular weight, presence of ions in the system, nature as well as the number of intra- and intermolecular interactions.

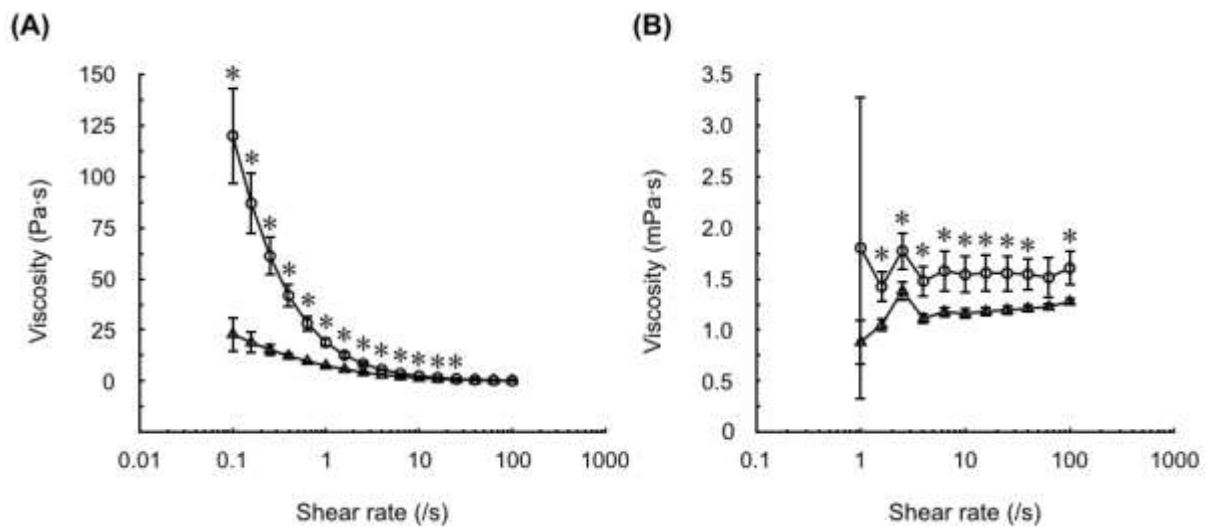


Fig. 13. Steady shear viscosities of (A) 1% (w/v) HePSs dissolved in water and (B) 0.02% (w/v) HePSs dissolved in MRS broth. Circles and triangles represent the HePSs sampled at 48 and 144 h of cultivation, respectively. The error bars indicate standard deviations. An asterisk (*) indicates statistical significance ($P < 0.05$).

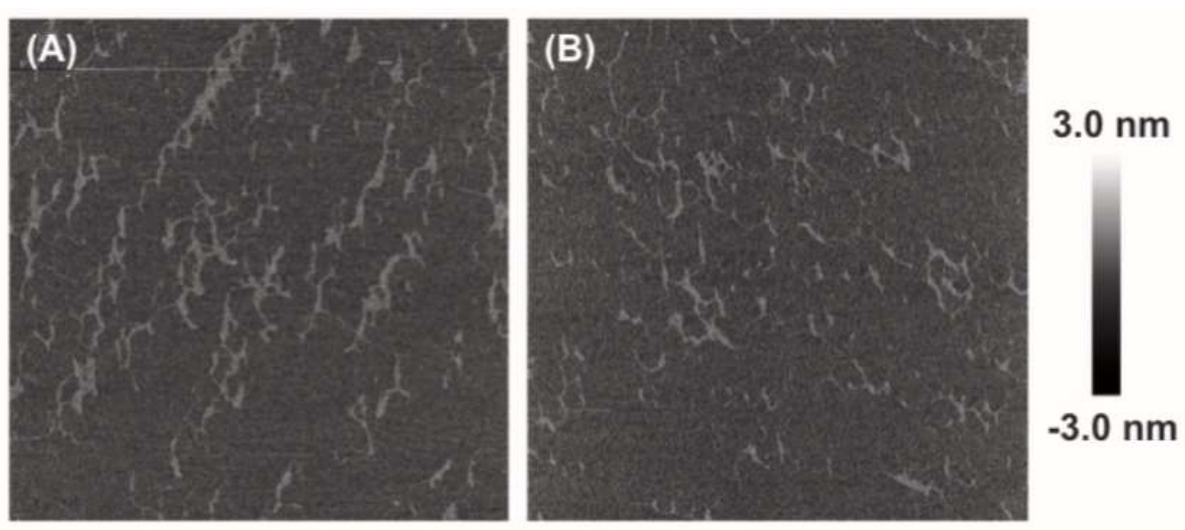


Fig. 14. Topographical AFM images of (A) HePS_{48h} and (B) HePS_{144h}. The scan size is $3\ \mu\text{m} \times 3\ \mu\text{m}$ (A, B).

CHAPTER IV. Structural insights into bacterial-EPS interactions using atomic force microscopy and scanning electron microscopy.

IV-I. Introduction

A microbial cell is a highly dynamic system with its cell wall components constantly interacting with their environment. The functions of cell surface are arbitrated by a complex dynamic assembly of specific macromolecules, such as proteins, polysaccharides and lipids. Although much progress has been made in understanding the composition and biosynthesis of EPS, a little is known about the organization and interactions between live cells and the EPS.

Whereas classical microbiology provides information on populations of cells, single-cell microbiological assays make it possible to analyze the behavior and heterogeneity of single cells, thereby enabling researchers to investigate cellular properties and interactions in a way that was impossible before (Brehm-Stecher *et al.*, 2004); Examples of single-cell technologies include fluorescence assays, flow cytometry techniques, microspectroscopic methods, mechanical, optical, and electrokinetic micromanipulations, microcapillary electrophoresis, biological microelectromechanical systems, and atomic force microscopy (AFM). Recently, AFM-based techniques have been used increasingly for the multiparametric analysis of microbial cell surfaces, and consequently providing novel insights into their structure-function relationships. The main advantages of AFM for microbiologists is its ability to capture images of cellular structures at molecular level and under physiological conditions (i.e., in buffer solution); the ability to monitor *in situ* the structural dynamics of cells with the surroundings as well as the capability to measure the localization, adhesion, and mechanics of single cell.

The potential ultramicroscopic tools for characterization of exopolysaccharides (EPS) and their interactions with live cells are getting wealthier than ever with advancement in the light microscopy having resolution beyond the far field diffraction limits, non-linear methods, and combinations of the various imaging modalities. Their application range from imaging of isolated microbial polysaccharides, structures involved in polyelectrolyte complexes, aspects of enzymatic degradation, as well as imaging of cell surface localization of polysaccharides secreted by the organism. These

aspects illustrate the expansion in imaging technologies relevant for EPS characterization supporting their characterization of functional and structural aspects.

Polymers are present abundantly in microbes and are responsible for a variety of specific functions. Extracellular polysaccharides, or exopolysaccharides (EPS), secreted by the bacteria constitute a group of carbohydrate based polymers; representing an interface to the environment. Generally, EPSs are believed to be excreted from the bacterial surface variably alongwith the surface bound polysaccharides. Several types of microorganisms produce EPS, including those existing in nature as normal commensals of GIT, in environment and/or pathogens. Moreover, some of them are also used for large scale industrial production of EPS during their biotechnological fermentation. As mentioned previously, fermented microbial EPS are applied variably in a wide range of industrial application. EPS have different functions in their natural environment; however, their functionality in commercial use is underlined by their structure, which not necessarily coincides with their functionality in the native state. Novel imaging tools can be used in the elucidation of their functional and structural relationships

A recent report of Dertli *et al.* (2013) also illustrated the use of applicability of the imaging technology for studying the capsular EPS mediated mechanical and adhesive properties of *Lactobacillus johnsonii* FI9785; and further the functional changes in EPS in genetically altered strains affecting the biosynthesis of one of the EPSs. AFM probes equipped with a lectin from *Pseudomonas aeruginosa* (PA1) using a flexible linker have been used for specific adhesion studies for comparison of adhesion mapping of the native bacterial strains and its mutants. Reduced adhesion in the genetically modified strains under aqueous solution indicated the unquestioned capability of the scanning probe tools for studying the structural related-functional relationships of exopolysaccharides (Lilledahl *et al.*, 2015).

AFM and SEM imaging of live growing cultures of *Lactobacillus* MTCC 25067 was done to gain insights into the morphological/structural interactions of EPS and their supramolecular structure with the bacterial cells in their native state (live growing cells in culture medium) which may further add to the understanding of the structural-functional relationship of EPS.

IV-2. Materials and methods

IV-2-1. Topographical imaging of live bacterial culture using AFM

To observe bacterial cells and EPS in the culture medium simultaneously, topographic imaging was done under air using a BioScope Catalyst atomic force microscope (Bruker, Santa Barbara, CA, USA) operated in peak force tapping mode using a NanoScope V controller (Bruker). A 2 μ L culture medium collected during cultivation was diluted 100 times using ion exchanged water, deposited onto a freshly cleaved mica sheet, and left to dry for 2 h in a desiccator. The images obtained were flattened using NanoScope Analysis software version 1.40 (Bruker).

IV-2-2. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis was done using a colony of *L. fermentum* MTCC 25067 harvested on an agar medium after glutaraldehyde fixation (2% in 0.1 M phosphate buffer, pH 7.4) for 30 min at ambient temperatures. After washing 3 times using the 0.1 M phosphate buffer (PB), the samples were immersed in 1% tannic acid dissolved in PB for 1 h followed by washing with 0.1 M PB and conductive staining in 1% osmium tetroxide dissolved in PB for 30 min at room temperature. The samples were then dehydrated 3 times in ascending series of ethanol (70, 80, 90, 95, and 100%) for 5 min each, transferred into 2-methyl-2-propanol, and dried in a freeze dryer ES-2030 (Koki Holdings, Tokyo, Japan). The dried samples were coated with platinum-palladium in an ion sputtering device E1010 (Koki Holdings), mounted on an aluminum plate, and examined using a field emission SEM S-4100 (Hitachi High Technologies, Tokyo, Japan) operated in SE mode.

IV-3. Results and discussion

IV-3-1. Topographical imaging of live bacterial culture using AFM

AFM height images were taken from an active colony in culture medium. The images clearly indicate that the 48 hour bacterial culture had a polysaccharide network surrounding the bacterial cell (Fig. 15A). Further, to confirm the presence of lactobacillus cells, the regions surrounded by white dotted squares in Fig. 16A and 16B (originally same as Fig. 15A and 15B) were focused with modified z-scales (-4 ~ 300 nm) as the z-scale applied in Fig. 15A and 15B was too low to visualize bacterial cell

shape. Apparently, the rod shaped bacterial cells could be seen as shown in Fig. 16 C and 16D. Bacterial cell size was found ranging from 1.5 to 2.2 micrometer at 300 nm of z-scale (Fig. 16A), which was similar to bacterial cell sizes of *Lactobacillus* species observed by AFM previously (Dean *et al.*, 2019).

The EPS in its native unpurified form extended from the bacterial surface into the surrounding growth medium. However, remarkable disintegration of the network structure could be noticed in the culture medium sampled at 144 h of cultivation, especially in the area surrounding bacterial cells (Fig. 15B). AFM images of the culture media collected at 48 h and 144 h and air-dried on the mica surface revealed that the network of HePS fibres surrounding bacterial cells in the 144-h culture medium were absent in contrast to those in the 48-h culture medium (Fig. 15A and 15B). The absence of the apparent network structure of HePS in the surrounding area of bacterial cells was observed during the late growth phase, supporting an idea that the decreases in culture viscosity during a prolonged period of cultivation were caused mainly by reduced interactions between bacterial cells and the intact supramolecular networks as a consequence of decreasing bacterial cell wall integrity and partial degradation of HePS molecules. On average, heights of certain cross-linking regions in the HePS network (white spots, some of them were indicated by arrowheads in Fig. 15B) were 1.9-fold greater than other part of the network. Such tall cross-linking regions were less in the culture medium collected at 48 h (Fig. 15A) and undetectable in the purified HePS_{144h} (Fig. 15B). The AFM imaging of the EPS produced by *Lactobacillus plantarum* WLPL04 deposited from a 10 µg/mL aqueous solution showed homogeneous rod-shaped lumps with variable heights (0.25 to 4.51 nm), significantly higher compared to single polysaccharide chain (approximately 0.1–1 nm) (Liu *et al.*, 2017). Similarly Wang *et al.*, 2015), topographical AFM images of *Lactobacillus plantarum* SKT109 EPS showed the presence of spherical lumps (10 - 50 nm diameter) with varying the height of the lumps (1-9.3 nm). The height of the spherical structures of EPS were much higher than a single polymer chain, suggesting the involvement of inter- and/or intra-molecular aggregation, which may be beneficial in improving the viscous character of the EPS when used in fermented milk. In the present study, the 48-h culture medium showed one order higher magnitude of viscosity (53 mPa at an estimated shear rate of 17/s) compared to 0.02% (w/v) solutions of HePS_{48h} dissolved

in the fresh MRS broth (1.6 mPa at a shear rate of 15.8/s). It is therefore likely that interactions between HePS fibre networks and bacterial cells and/or their metabolites contributed largely on the high viscosity of the 48-h culture medium. It may also be due to random release of HePS from bacterial cells during prolonged incubation due to partial degradation by EPS-degrading enzymes, which might have leaked out of the dead bacterial cells.

Dufrêne (2008) summarized the application of AFM based systems to obtain information related to structure, chemical composition, nature of interaction forces, and specific molecular recognition as part of analysis of microbes at the nanoscale using SPM images. Sletmoen *et al.* (2010) also summed up the examples of more recent application of these strategies. The examples combined include studies on the effects of inhibition of the biosynthesis of arabinans (a major cell wall component), differences in EPS topology between bacterial mutants and wild types, and also the effects of EPS in cellular adhesion. It is noteworthy that similar to present study, although individual EPS chains could not be clearly resolved in these studies but the consequences of gene alterations are clearly promising. The presence of polysaccharide capsules surrounding *Zunongwangia profunda* SM-A87 has been recently characterized by tapping mode AFM where overall outline of the EPS containing capsules, the fibrils, but not individual, dispersed polysaccharide were clearly revealed. (Su *et al.*, 2012). This aggregation feature of the polysaccharide molecules may be beneficial in improving the viscous behavior of the EPS from strain when used in fermented products.

IV-3-2. Scanning electron microscopy

Scanning electron micrographs of live culture of *Lactobacillus fermentum* MTCC 25067 showed rod shaped bacteria, either grouped as pairs and/or chains. The network structure that HePS forms as seen in AFM and its interaction with the bacterial cells was observed in SEM imaging (Fig. 18A). The bacterial cells were seen covered by multiple layers of polysaccharide networks at several locations in the colony (Fig. 18B). The formation of similar HePS networks has been reported in the case of the HePS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in a 10% (w/v) reconstituted skim milk (Goh *et al.*, 2005).

The EPS produced by *Streptococcus thermophiles* CC30 showed a 3-D structure with irregular lumps having variable size and a coarse surface in SEM analysis. The spherical structure and porous nature of the EPS was seen at higher magnification (Kanamarpudi and Muddada, 2017). Similarly, a porous web-like structure has been demonstrated using scanning electron microscopy for an EPS from *Lactobacillus plantarum* (Wang J, 2015). However, observation of the EPS from strain *Lactobacillus plantarum* SKT109 using SEM revealed a compact structure composed of smooth, similar sized cubes different from previous observations of other EPSs produced by LAB with the sheet-like or porous web-like structures (Wang *et al.*, 2010; Prasanna *et al.*, 2012). Moreover, EPS from strain SKT109 was found to be made of homogeneous matrix, indicating its structural integrity (Ahmed *et al.*, 2013). Yadav *et al.* (2011) found highly compact flake-like structural units of EPS from *Lactobacillus fermentum* CFR2195 using scanning electrostatic imaging. The differences in the surface morphology and topography of polysaccharides from different microorganisms are probably due to the differences in sample extraction, preparation, and purification protocols and; also owing to the differences in the physicochemical properties of the EPS.

Scanning electron micrographs obtained upon examination of surface morphology and microstructure of the EPS from *B. amyloliquefaciens* GSBa-1 revealed uniform shaped ellipsoid or globose with smooth surface. Similarly, a globose microstructure of EPS has been reported earlier for another EPS producing *B. amyloliquefaciens* LPL06the 1 strain and also by oral streptococci; including *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius* (Yang *et al.*, 2015; Bowen *et al.*, 2017).

Mouthfeel properties of a given food substance is based on the adhesiveness. EPS can also be used for improving the organoleptic properties of the product. Although EPS, itself has no taste, they are used to modify mouthfeel properties as they cause the flavor compounds in the final fermented product to stay longer in contact with the palate and taste receptors. Also, the contribution of the EPS producing strains to the textural properties is believed to be a consequence of extracellular polysaccharides secretion and also, the capability of the polysaccharides to form strands, connecting bacteria to the casein micelles (Tamime *et al.*, 1984). Thus, the

characterization of EPS bacterial interactions may be used to improve the mouthfeel and organoleptic of final products made using the strain under investigation.

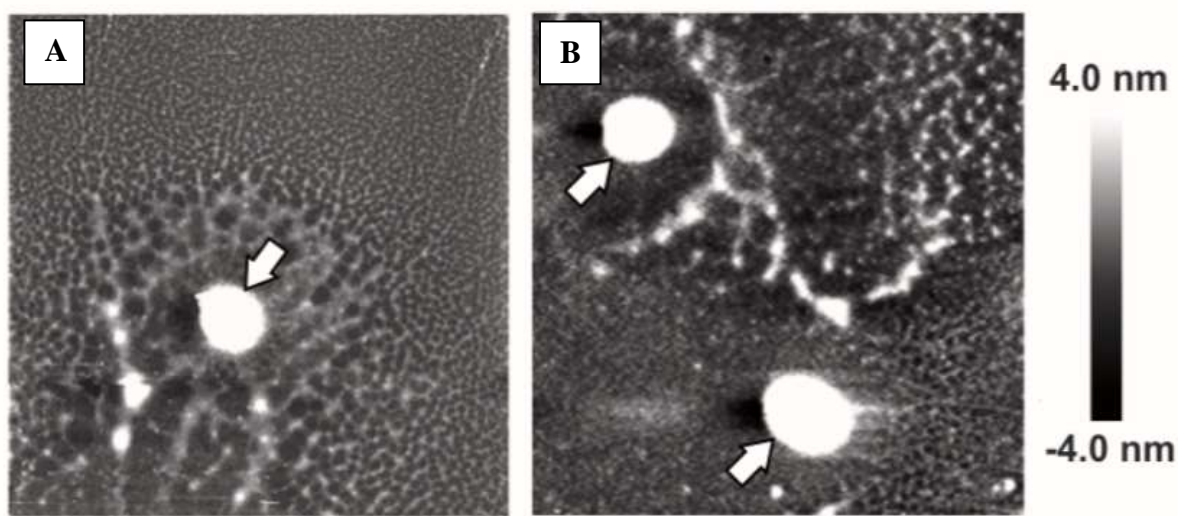


Fig. 15. Topographical AFM images of (A) LAB in the culture medium collected at 48 h and (B) 144 h of cultivation. The white arrows indicate bacterial cells. The scan size is $20\ \mu\text{m} \times 20\ \mu\text{m}$.

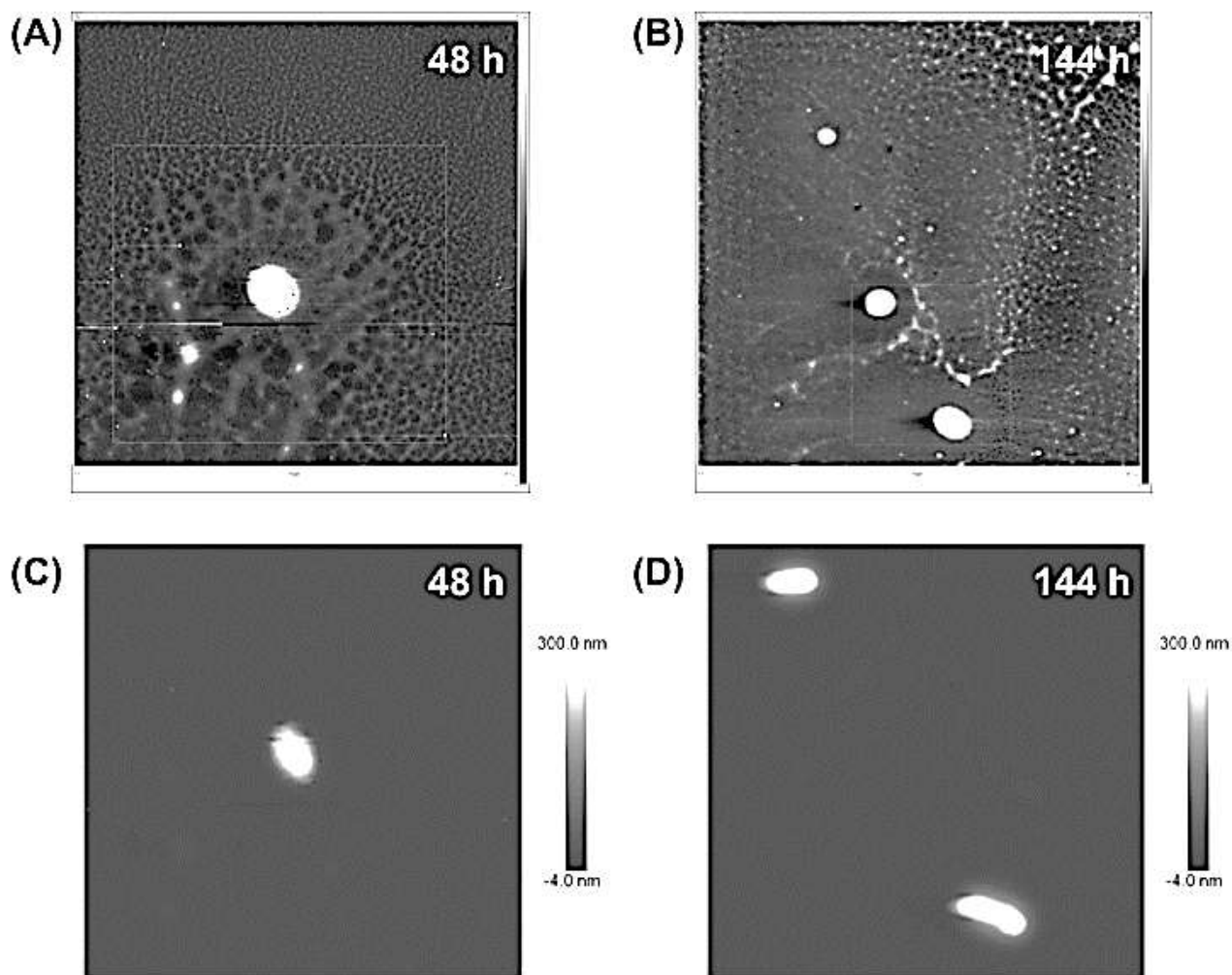


Fig. 16. Regions surrounded by white dotted squares focused (A, B; originally same as Fig. 5C and 5D). Rod-shaped bacterial cells (C, D) as observed with modified z-scales (-4 ~ 300 nm).

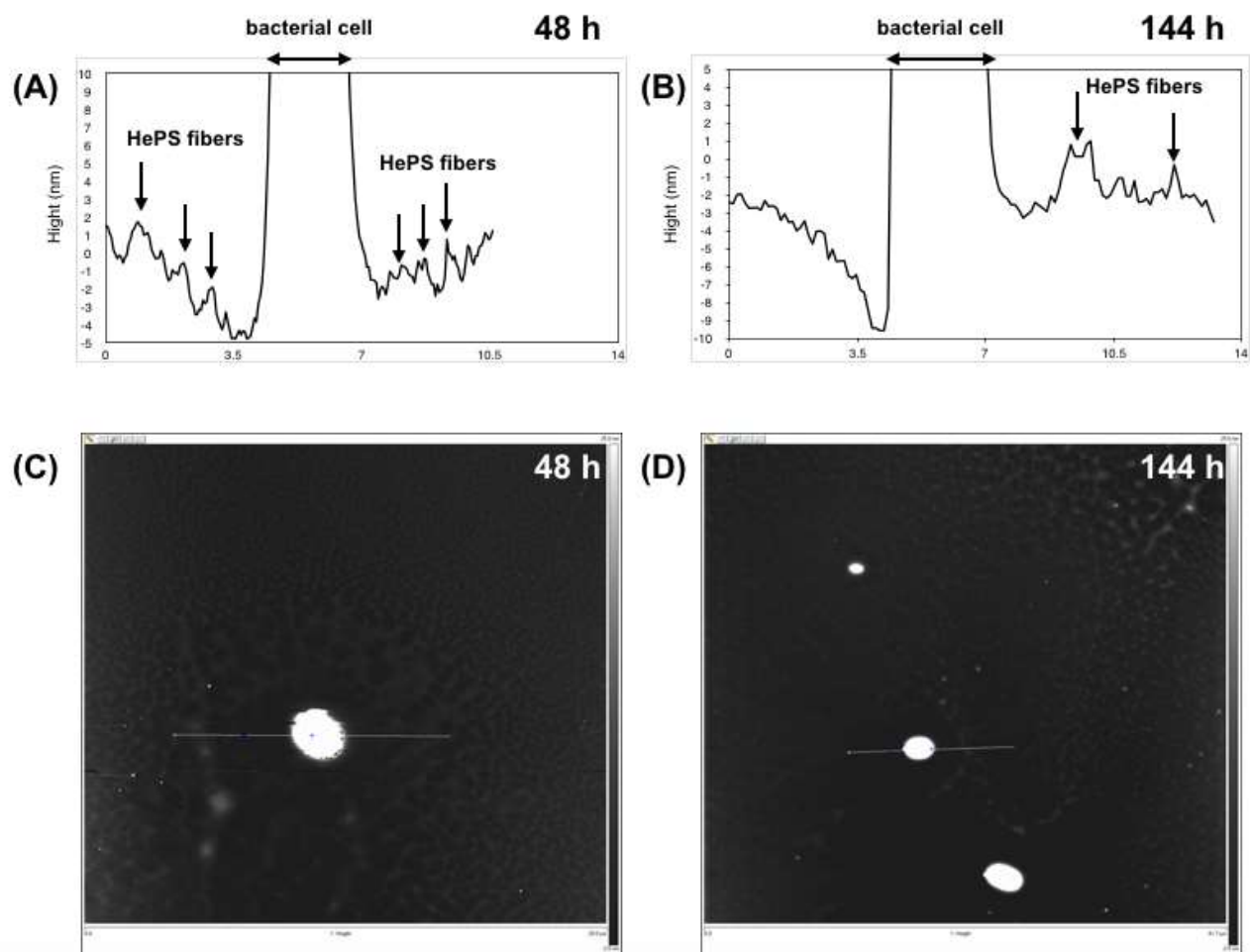


Fig. 17. Height data of 48hr and 144 hr HePS using AFM indicating the presence or absence of EPS fibers in the surrounding areas of bacterial cells.

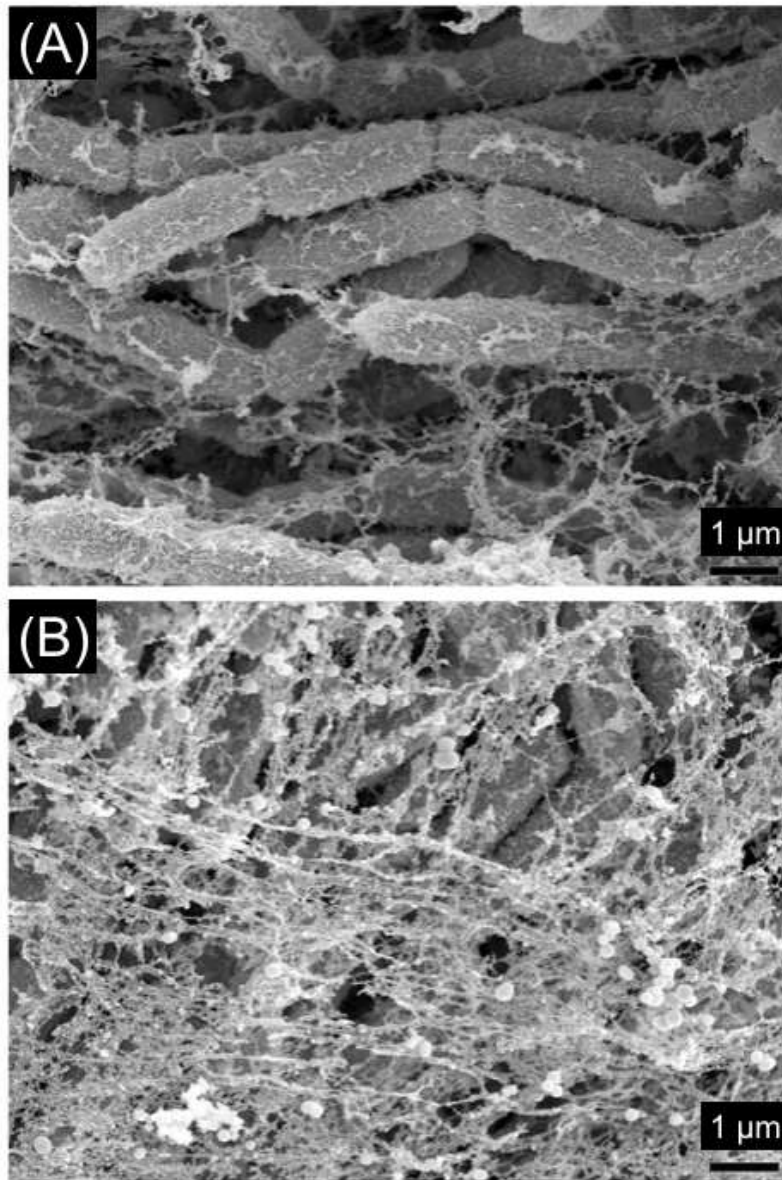


Fig. 18. SEM images of (A) bacterial cells exposed to the environment and (B) bacterial cells covered by the layers of HePS.

CHAPTER V. Construction of plasmids and preparation of cells for gene knockout systems for *L. fermentum* MTCC 25067.

V-1. Introduction

Different strains of *Lactobacillus* produce exopolysaccharides (EPS) having a huge diversity in their structure and physicochemical properties (Salazar *et al.*, 2009). It has been found in several studies that the biosynthesis of hetero-EPS is controlled by several housekeeping genes and an EPS related gene cluster which consists of functional regions for regulation of EPS production, chain length determination, biosynthesis of the repeating unit and polymerisation and export of repeating units (Laws *et al.*, 1996). *L. fermentum* MTCC25067 has been found to produce a thick type of ropy hetero-EPS (Leo *et al.*, 2007). A little is known about the mechanism of its biosynthesis. Previously, the genome of *L. fermentum* MTCC25067 was found bearing a single slime-like EPS-related gene cluster (20.25 kb) found in the chromosome harbouring genes encoding one transcriptional regulator LytR ortholog (Lebeer *et al.*, 2009), five putative proteins comprising EPS secretion machinery (a tyrosine-protein kinase, a tyrosineprotein phosphatase, Wzx, Wzy, and Wzz) (Islam and Lam, 2014), five putative glycosyltransferases, one hypothetical protein, and eight transposable elements. (Aryantini *et al.*, 2017). The five putative genes encoding glycosyltransferases in this cluster play an important role in the biosynthesis of EPS repeating units (Jolly, 2001) and transfer a sugar moiety to the activated acceptor molecule (De Vuyst, 1999; Jolly, 2002). On the basis of homology to conserved domains, EpsG gene was found specific to the strain under study and it was proposed that the glycosidic bond of EPS produced by *L. fermentum* MTCC 25067 (previously, TDS 030603) is mainly responsible for the viscosity and is responsible for a key enzyme that modifies the physicochemical properties of EPS solution (Dan *et al.*, 2009). Horn *et al.* (2013) have reported changes in accumulation levels of EPS upon alterations in the EPS cluster in derivatives of *L. johnsonii* FI9785; where an putative epsE gene deletion mutant could still produce EPS, in comparatively lower quantities, while an increased EPS production was recorded upon a spontaneous epsCD88N gene mutation.

Different strategies have been reportedly used to create geneknockout mutants in order to study the function of genes. These include the use of antibiotic cassette

insertion system (Zhang *et al.*, 2013), temperature sensitive suicidal vector based strategy, use of incompatible plasmids (Hirayama *et al.*, 2012) as well as combination of recombineering and Cre LoxP or FLP/FRT system (Xin, *et al.*, 2008).

In the preceding chapters, we characterised the physico-chemical and structural changes in EPS produced by wild type *L. fermentum* 25067. The next step is understanding the role of EPS related genes in its biosynthesis and the changes in structure and characteristics of EPS production on gene alterations (gene knockout). We have prepared the plasmids and cells required for two different gene knockout systems; namely, antibiotic insertion/insertional mutagenesis and pORI28 based temperature sensitive system.

V-2. Materials and methods

V-2-1. Bacterial cultures

Bacterial culture of *Lactobacillus fermentum* MTCC 25067 were maintained as glycerol cultures at -80 °C and grown on de Man, Rogosa and Sharpe (MRS) broth/agar. Table 5 shows the plasmids and bacterial cultures used in this study. *E. coli* cells were grown on Luria bertani broth/agar.

V-2-2. DNA isolation and transformation procedures

Plasmid DNA from *E. coli* was extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instructions. Chemically competent *E. coli* TOP10 were used for transformation.

V-2-3. Construction of plasmid for antibiotic cassette insertion system/ insertional mutagenesis

For inactivation of the 121 gene (EPS related gene) using the suicide vector pUC19, carrying an ampicillin resistance gene for insertional inactivation of gene, a 121 gene fragment was amplified using the genomic DNA from *L. fermentum* 25067 using primers 121-F and 121-R (Table 4), with a PstI restriction site at the 3' end and a EcoRI site at the 5' end. The amplified fragment was digested with PstI and EcoRI and cloned into PstI- and EcoRI-digested pUC19 plasmid to produce pUC19-121 fragment. The ligation product was transformed directly into chemically competent *E. coli* Top10 cells as per the instruction of the manufacturer (Thermo-Fischer Scientific). After

transformation, 1 ml of SOC medium was added and the mixture was incubated for 3 h for recovery. The cells were then plated on ampicillin (100 µg/ml) containing LB plates with X-gal. The white colonies were selected. The presence of 121 gene fragment was confirmed using colony PCR and restriction enzyme digestion. Erythromycin resistance marker, *erm* from pORI28 plasmid was amplified using the infusion cloning primers 121_erm_inf_fwd and 121_erm_inf_rev and introduced into the puc19_121 linearized with primers PUC19_121-LIN FWD and PUC19_121-LIN REV using In-Fusion® HD Cloning Kit w/Cloning Enhancer (Takara, Cat. no. 639633) following the instructions of the manufacturer.

V-2-4. Preparation of *L. fermentum* 25067 electrocompetent cells

Electrocompetent cells of *L. fermentum* 25067 were prepared using the following protocol followed by Wei *et al.* (1995) with slight modifications. A 1% inoculum of an overnight bacterial culture was grown in MRS broth for 2 h to reach an OD₆₆₀ of 0.1-0.2 at 37°C without shaking. Afterwards, it was diluted 5 times with ice-cold sterile PBS and the EPS was removed. The harvested cells were washed three times with ice-cold washing buffer (5 mM sodium phosphate, pH 7.4, 1 mM MgCl₂) by washing the cells thrice with ice-cold sterile PBS by centrifugation (8000 rpm for 30 minutes). The cells were then suspended in 1 ml ice-cold PBS. Lysosyme was added at the final concentration of 10 µg/ml and incubated for 1 hour. Afterwards, the cells were washed once again with ice-cold washing buffer and then suspended in ice-cold electroporation buffer (0.9 M sucrose, 3 mM MgCl₂, pH 7.4). All the steps were performed at ice-cold temperatures. Fresh bacterial cells were prepared everytime before use.

V-2-5. Electroporation of pTRK669 plasmid into *L. fermentum* cells for gene knockout using pORI based temperature sensitive system

Electroporation of pTRK669 plasmid into the *L. fermentum* cells was done using protocol described by Wei *et al.* (1995) with slight modifications. One µg plasmid DNA from pTRK669 was mixed with 50 µl of ice-cold bacterial cell suspension in a 0.2 cm Gene Pulser cuvette and held on ice for at least 2 min. The mixture was then exposed to a high voltage electric pulse (peak voltage of 2.5 kV,

capacitance of 25 μ F, parallel resistance of 400 Ω) delivering a peak field strength of 12.5 kV/cm. Afterwards, MRS broth was added immediately without any delay and the cells were incubated at 37°C for 2 hours with shaking. The cells were then plated on chloramphenicol containing plates (15 μ g/ml) at 37°C for 24 h. After 24 h incubation, potential pTRK669 transformants emerging on antibiotic plates were selected. The positive colonies were confirmed using colony PCR using RepA-f and RepA-r primers.

V-2-6. Preparation of electrocompetent EC1000

The E.coli EC1000 electrocompetent cells were prepared using the protocol described by Miller and Nickoloff (1995). Five ml of fresh overnight E.coli culture was inoculated in L-broth and then incubated with shaking at 37°C to an OD₆₀₀ of 0.6. The cells were then chilled on ice for 20 mins and then cells were harvested in ice cold centrifuge bottles using centrifuge (4000 g, 15 min, 4° C). The cells were washed three times in reducing volumes of ice cold 10% glycerol (500 ml, 250 ml, 20 ml) and finally resuspended in 1 ml of ice cold 10% glycerol. One hundred μ l aliquots of cells were made and stored at -80°C until use.

V-3. Results and discussion

V-3-1 Construction of plasmid for antibiotic cassette insertion system/ insertional mutagenesis.

The construction of 121 containing fragment pUC19_121 was confirmed using colony PCR (Fig. 19). The construct was further confirmed using single restriction enzyme digestion (Fig. 20A) and double restriction enzyme digestion (Fig. 20B). Further, the colonies emerging after infusion cloning of erm gene into pUC19_121 plasmid were screened using colony PCR for the presence of erm gene (Fig. 21) and subsequently, confirmed for the construction of suicidal plasmid puc19_121_erm using single and double restriction enzyme digestion (Fig. 22 A,B).

V-3-2. Electroporation of pTRK669 plasmid into *L.fermentum* cells for gene knockout using pORI based temperature sensitive system.

For using the pORI28 based temperature sensitive system, there is a prerequisite of a RepA harbouring host. In this study, this is achieved by transformation of

pTRK669 into the *L.fermentum* cells. After repeated attempts and trials, the successful electroporation of pTRK669 has been done in the *L.fermentum_pTRK669* cells. The successful incorporation of pTRK669 plasmid DNA was confirmed using colony PCR (Fig. 23). It is noteworthy to mention here that the highly viscous EPS produced by the *L. fermentum* strain is a hurdle in this electroporation step and it needs to be removed by extra washing steps during the preparation of electrocompetent cells.

After successful preparation and confirmation of plasmids; and standardization of protocols for electroporation of pTRK669 plasmids into bacterial cells. Experiments are now being conducted using the newly prepared pUC19_121_erm suicidal plasmid and electrocompetent *L. fermentum* MTCC25067 cells for establishing a new gene knockout system in *L. fermentum* MTCC 25067.

Table 4. Primers used for PCR amplification reactions.

Primer name	Sequence (5' to 3')
121	AAA <u>ACTGCAGG</u> TGAAAATAGATATTCTGGTCTC
121	CCGGAAT <u>TC</u> CTATTTTAAATGACTCATGAAAGT
Erm	GAA <u>AGATCT</u> ATGAACGAGAAAAATATAAAACAC
Erm	GAAAGATCTGCATCCCTTAACCTACTTATTAAAT
pORI28_121_inf f	CATGCCATGGTACCCCGTGATAGTTGTTGAACAGTAT
pORI28_121_inf r	GACGTCGACGCGTCTGCTTCAGCAAGCACCAA
pORI28_Lin f	AGACGCGTCGACGTCATAT
pORI28_Lin r	GGGTACCATGGCATGCATCG
RepA f	TTGGGCGTATCTATGGCTGT
RepA r	CTGATAATTGCCCTCAAACCA
121_erm_inf_fwd	AGTTACATACAGAGGATGAACGAGAA
121_erm_inf_rev	GGATCTTCAACCCATTTACTTATTAA
PUC19_121-LIN FWD	ATGGGTTGAAGATCCTTGGA AAAAT
PUC19_121-LIN REV	CCTCTGTATGTAACCTCGATCAGAAAT

Artificial restriction sites are underlined.

Table 5. Plasmids and bacterial cultures used in this study.

Strain or plasmid	Genotype and (or) relevant characteristics	Source
E.coli Top 10	<i>F</i> - <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(araleu)7697$ <i>galU</i> <i>galK</i> <i>rpsL</i> (<i>StrR</i>) <i>endA1</i> <i>nupG</i>	Invitrogen
pUC19	<i>Amp^r</i> , 2680bp	Addgene
pORI28	<i>Em^r</i> , 1692bp	Addgene
pUC19-121	<i>Amp^r</i> , pUC19 containing 1024 bp <i>121</i> gene insert obtained from <i>L. fermentum</i> MTCC25067	This study
pTRK669	ori (pWV01), Cmr , RepA	Addgene
<i>L. fermentum</i> MTCC 25067	Dahi isolate	OUAVM culture collection
<i>L. fermentum</i> _pTRK669	<i>L. fermentum</i> MTCC 25067::pTRK6669	This study
pUC19_121_erm	<i>Amp^r</i> , pUC19 containing 1024 bp <i>121</i> gene insert obtained from <i>L. fermentum</i> MTCC25067 and <i>Erm</i> gene from pORI28.	This study
pORI28		



Fig. 19 Colony PCR for confirmation of integration of 121 gene into pUC19 plasmid using conventional restriction enzyme cloning. Lane 1- 1kb DNA ladder, Lane 2- negative control, Lane 3-11 colonies emerging after transformation of pUC19_121 plasmid.



Fig. 20 (A) Single digestion of pUC19_121 fragment using NdeI, Lane 1. 1kb ladder, Lanes 2-6. Plasmids obtained from colonies emerging after transformation of pUC19_121 plasmid (B) Double digestion of pUC19_121 fragment using PstI and EcoRI. Lane 1- 1 kb ladder, Lane 2. NdeI digested pUC19, Lane 3. pUC19_121 plasmid, Lane 4. 121 gene.



Fig.21 Colony PCR for confirmation of integration of erm gene into pUC19_121 plasmid using conventional infusion cloning. Lane 1. 1kb ladder. Lane 2-11 colonies emerging after transformation pUC_19_121 plasmid, Lane 12. Negative control, Lane 13. Positive control pUC19_121 plasmid, Lane 14-17. Colonies emerging after transformation pUC_19_121 plasmid

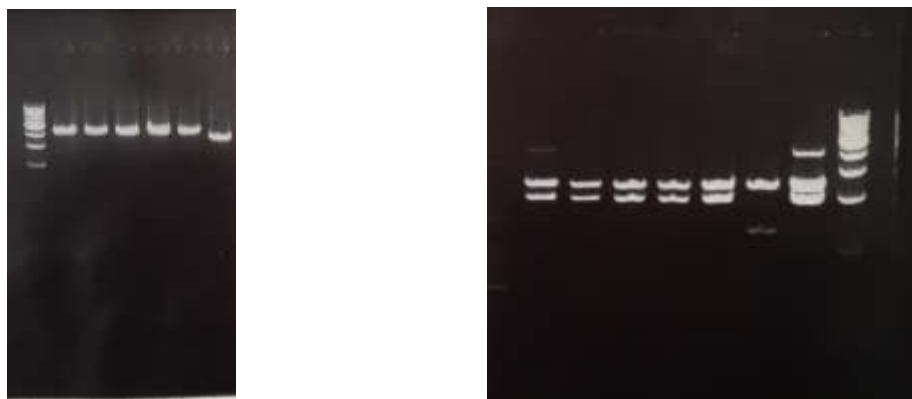


Fig. 22 (A) Single digestion of pUC19_121_erm plasmid obtained after transformation with pUC_19_121 plasmid using NdeI. Lane 1. 1kb Ladder, Lane 2-6. Plasmid obtained from transformed colonies, Lane 7. pUC19_121 plasmid DNA. (B) Double digestion of pUC19_121_erm plasmid obtained after transformation with pUC_19_121 plasmid using fragment using NdeI and PstI. Lane 8. 1kb Ladder, Lane 1-5. Plasmid obtained from transformed colonies, Lane 6. pUC19_121 plasmid DNA.



Fig.23. Colony PCR for confirmation of pTRK669 plasmid. Lane 1. 100 bp ladder. Lane 2-7. Colonies after transformation of *L.fermentum* MTCC 25067 with pTRK669 plasmid. Lane 8.- Negative control.

CHAPTER-VI. Summary and conclusions

The HePS produced by *L. fermentum* MTCC 25067 has been reported to exhibit high viscosities comparable to xanthan gum due to the formation of supramolecular networks of the polysaccharide as a result of lateral association of multiple molecular chains. In the present study, the viscosity of EPS produced by *L. fermentum* MTCC 25067 decreased significantly during the late stationary phase of growth. Up to 68% reduction was observed when the cultivation period was extended from 48 h to 144 h. previously, Fukuda *et al.* (2010) have reported a decrease in the production yield upon prolonged cultivation. The proportion of smaller molecular weight fraction of the HePS increased significantly after a prolonged period of incubation. The possibility of unknown enzymes degrading HePS are very remote due to almost negligible effects on the chemical structures of side chains upon prolonged incubation. AFM images of both HePS_{48h} and HePS_{144h} exhibited network structures. However, the population of thinner and shorter polysaccharide fibrils was larger in the HePS_{48h} compared to HePS_{144h}. This can be associated with the degradation of HePS molecules to decrease in viscosity, whereas influences of variabilities in side chains of HePS are controversial. AFM imaging of EPS purified after prolonged incubation showed a greater proportion of thinner and shorter fibrils. AFM images of the culture media collected at 48 h and 144 h and air-dried on the mica surface revealed that the HePS networks attached to bacterial cells at 48 h incubation is detached from the cells in the 144-h culture. Additionally, SEM images indicated the presence of HePS networks in the immediate proximity of bacterial cell surfaces as well as the formation of several polysaccharide layers covering bacterial cells. Therefore, the attachment of the HePS network to the bacterial cell is highly likely to be the nature of the strain. Further, in order to study the roles of genes responsible for the HePS production, plasmids for the two well-known knockout strategies used in lactic acid bacteria, antibiotic insertion/insertional mutagenesis and temperature sensitive pORI28 based system, were successfully prepared for disrupting a glycosyltransferase gene. Gene-knockout protocols were also optimized using wild type of the strain as a host.

To summarize, the difference in the viscosities of the culture medium of *L. fermentum* MTCC 25067 can be considered to arise mainly from the attachment of the

HePS network to the bacterial cell surface. During a prolonged period of cultivation, the HePS network was disintegrated as a result of partial degradation of HePS molecules by the action of EPS-degrading enzymes, which were likely to have leaked out from dead bacterial cells.

Conclusions

The present findings put a light on the importance of understanding interactions between EPS molecules and bacterial cell surfaces to manipulate the viscosity of the culture medium as well as fermented foods. The precise control of culture conditions and exploring inhibitors against EPS-degrading enzymes are suggested approaches to effectively avoid loss of the viscosity. Interactions between the HePS under study and food matrices should be a future study subject in order to assess its commercial applicability.

References

- Agilent Technologies 5500 scanning probe microscopy. User's guide, Agilent technologies. 2008.
- Ahmed, Z., Wang, Y., Anjum, N., Ahmad, A., Khan, S.T. 2013. Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir – Part II. *Food Hydrocolloids*. 30, 343–350.
- Allison, D.P., Mortensen, N.P., Sullivan, C.J., Doktycz, M.J. 2010. Atomic force microscopy of biological samples. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. 2(6), 618-634.
- Amjres, H., V. Bejar, E. Quesada, D. Carranza, J. Abrini, C. Siquin, J. Ratiskol, S. Collic-Jouault, Llamas., I. 2015. Characterization of haloglycan, an exopolysaccharide produced by *Halomonas stenophila* HK30. *International Journal of Biological Macromolecule*. 72, 117–124.
- Aryantini, N. P. D., Prajapati, J. B., Urashima, T., Fukuda, K. 2017. Complete genome sequence of *Lactobacillus fermentum* MTCC 25067 (formerly TDS030603), a viscous exopolysaccharide-producing strain isolated from Indian fermented milk. *Genome Announcements*. 5, e00091–17.
- Auvray F, Coddeville M, Ritzenthaler P, Dupont L. 1997. Plasmid integration in a wide range of bacteria mediated by the integrase of *Lactobacillus delbrueckii* bacteriophage mv4. *Journal of Bacteriology*. 179, 1837-1845.
- Ayala-Hernández, I., Hassan, A.N., Goff, H.D., Corredig, M. 2009. Effect of protein supplementation on the rheological characteristics of milk permeates fermented with exopolysaccharide-producing *Lactococcus lactis* subsp. cremoris. *Food Hydrocolloids*. 23, 1299–1304.
- Badel, S., Bernardi, T., Michaud, P. 2011. New perspectives for Lactobacilli exopolysaccharides. *Biotechnology Advances*. 29, 54–66.
- Barker, P.E., Ajongwhen, N.J. 1991. The production of the enzyme dextranucrase using nonaerated fermentation techniques. *Biotechnology and Bioengineering*. 37,703-707
- Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. 315, 1709-1712.

- Behare, P.V., Singh, R., Kumar, M., Prajapati, J.B. 2009 (b). Exopolysaccharide of lactic acid bacteria-a review. *Journal of Food Science and Technology*. 47, 1-13.
- Behare, P.V., Singh, R., Nagpal, R., Kumar, M., Tomar, S.K., Prajapati, J.B. 2009 (c). Comparative effect of exopolysaccharides produced in situ or added as bioingredients on dahi properties. *Milchwissenschaft*. 64, 369-400.
- Behare, P.V., Singh, R., Singh, R.P. 2009 (a). Exopolysaccharide-producing mesophilic lactic cultures for preparation of fat-free Dahi- an Indian fermented milk. *Journal of Dairy Research*. 76, 90-97.
- Behare, P.V., Singh, R., Tomar, S.K., Nagpal, R., Kumar, M., Mohania, D. 2010. Effect of exopolysaccharide producing strains of *Streptococcus thermophilus* on technological properties attributes of lassi. *Journal of Dairy Science*. 93, 2874-2879.
- Bello, F. D., Walter, J., Hertel, C., Hammes, W. P. 2001. In vitro study of prebiotic properties of levan-type exopolysaccharides from Lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Systematic and Applied Microbiology*. 24, 232-237.
- Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Gueguen, M. 2008 Safety assessment of dairy microorganisms: the *Lactobacillus* genus. *International Journal Food Microbiology*. 126, 278–285.
- Binnig, G., Quate, C.F., Gerber C. 1986 a. Atomic force microscopy. *Physical Review Letters*. 56, 930-933.
- Binnig, G., Rohrer, H. 1986. Scanning tunnelling microscopy. *IBM Journal of Research and Development*. 30, 355-369.
- Bouzar F., Cerning J., Desmazeaud, M. 1997. Exopolysaccharide production and texture promoting abilities of mixed-strain starter cultures in yoghurt production. *Journal of Dairy Science*. 80:2310-2317.
- Bowen, W.H., Burne, R.A., Wu, H., Koo, H. 2017. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends in Microbiology*. 26, 229-242.
- Brehm, B.F., Johnson, E.A. 2004. Single-cell microbiology: tools, technologies, and applications. *Microbiology*. 68, 538–559.
- Caggianiello, G., Kleerebezem, M; Spano, G. 2016. Exopolysaccharides produced by lactic acid bacteria: From health-promoting benefits to stress tolerance mechanisms. *Applied Microbiology and Biotechnology*. 100, 3877-3886.

- Canchaya, C., Claesson, M.J., Fitzgerald, G.F., van Sinderen, D., O'Toole, P.W. 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology*. 152, 3185- 3196.
- Cataldi, T.R., Campa, C., DeBenedetto, G.E. 2000. Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: the potential is still growing. *Fresenius' Journal of Analytical Chemistry*. 368, 739–758
- Cave, R.A., Seabrook, S.A., Gidley, M.J., Gilbert, R.G. 2009. Characterization of starch by size-exclusion chromatography: the limitations imposed by shear scission. *Biomacromology*. 10, 2245-2253.
- Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews*. 87, 113-130
- Cerning, J. 1995. Production of exopoly saccharides by lactic acid bacteria and dairy propionibacteria. *Lait*. 75, 463-472
- Cerning, J., Marshall, V.M. 1999. Exopolysaccharides produced by the dairy lactic acid bacteria. *Recent Research Developments in Microbiology*. 3, 195-209.
- Chabot, S., Yu, H.L., De Leseleuc, L., Cloutier, D., van Calsteren, M.R., Lessard, M., Roy, D., Lacroix, M., Oth, D. 2001. Exopolysaccharide from *Lactobacillus rhamnosus* RQ-595M stimulate TNF, IL- 6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-g in mouse splenocytes. *Lait*. 81, 683-697.
- Christiansen, P.S., Madeira AI, M.R., Edelstein, D. 1999. The use of ropy milk as stabilizer in the manufacture of ice cream. *Milchwissenschaft* .54, 138-140
- Ciucanu, I, Kerek, F. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*. 131, 209-217.
- Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 339, 819-823.
- Costa, N., Wang, Auty, M., Hannon, J., Mcsweeney, P. 2012. Rheological, microscopic and primary chemical characterisation of the exopolysaccharide produced by *Lactococcus lactis* subsp. cremoris DPC6532. *Dairy Science and Technology*. 92 (3), 219-235.
- Crescenzi, V. 1995. Microbial polysaccharides of applied interest: Ongoing research activities in Europe. *Biotechnology Progress*. 11:251-259

- Cummings, J., Englyst, H. 1995. Gastrointestinal effects of food carbohydrate. *The American Journal of Clinical Nutrition*. 61, 938S-945S.
- Dal Bello, F.D., Walter, J., Hertel, C., Hammes, W.P. 2001. *In vitro* study of probiotic properties of levan-type exopolysaccharides from lactobacilli and nondigestible carbohydrates using denaturing gradient gel electrophoresis. *Systemic Applied Microbiology*. 24, 232-237
- Damager, I., Engelsen, S.B., Blennow, A., Moller, B.L., Motawia, M.S. 2010. First principles insight into the α -glucan structures of starch: their synthesis, conformation, and hydration. *Chemical Reviews*. 110, 2049-2080.
- Dan, T., Fukuda, K., Sugai-Bannai, M., Takakuwa, N., Motoshima, H., Urashima, T. 2009. Characterization and expression analysis of the exopolysaccharide gene cluster in *Lactobacillus fermentum* TDS030603. *Bioscience Biotechnology Biochemistry*. 73, 2656 -2664.
- Das, D., Baruah, R., Goyal, A. 2014. A food additive with prebiotic properties of an alpha-D-glucan from *Lactobacillus plantarum* DM5. *International Journal of Biological Macromolecules*. 69, 20-26.
- De Vuyst, L., Degeest, B. 1999. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiology Reviews*. 23, 153-177.
- De Vuyst, L., deVin, F., Vaningelgem, F., Degeest, B. 2001. Recent development in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *International Dairy Journal*. 11, 687-707.
- De Vuyst, L., Zamfir, M., Mozzi, F., Adrian, T., Marshall, V., Degeest, B., Vaningelgem, F. 2003. Exopolysaccharide-producing *Streptococcus thermophilus* strains as functional starter cultures in the production of fermented milks. *International Dairy Journal*. 13, 707-717.
- De Vuyst, L., Degeest, B. 1999. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiology Reviews*. 23, 153-177.
- Dean, S.N., Leary, D.H., Sullivan, C.J. 2019. Isolation and characterization of *Lactobacillus*-derived membrane vesicles. *Scientific Reports*. 9, 877.
- Dertli, E., Colquhoun, I. J., Gunning, A. P., Bongaerts, R. J., Le Gall, G., Bonev, B. B. 2013. Structure and biosynthesis of two exopolysaccharides produced by *lactobacillus johnsonii* fi 9785. *Journal of Biology and Chemistry*. 288, 31938-31951.

- Doleyres, Y., Schaub, L., Lacroix, C. 2005. Comparison of the functionality of exopolysaccharides produced *in situ* or added as bioingredients on yoghurt properties. *Journal of Dairy Science*. 88,4146-4156.
- Dragan, M., Townsley, P.M. 1984. Scandinavian Ropy Milk — Identification and characterization of endogenous ropy lactic streptococci and their extracellular Excretion. *Journal of Dairy Science*. 67 (4), 735-744.
- Duboc, P., Mollet, B. 2001. Applications of exopolysaccharides in dairy industry. *International Dairy Journal*. 11, 759-768
- Dubois, M., Gills, K. A., Hamilton, J. K., Roberts, P. A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28, 350-356.
- Dufrêne, Y.F. 2008. AFM for nanoscale microbe analysis. *Analyst*. 133, 297-301.
- Euzeby, J.P. 1997. List of bacterial names with standing in nomenclature: a folder available on the internet. *International journal of systematic bacteriology*. 47, 590-592.
- Fanning, S., Hall, L. J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., Motherway, M. O., Shanahan, F., Nally, K., Dougan, G., van Sinderen, D. 2012. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proceedings of the National Academy of Sciences, USA*. 109, 2108-2113.
- Folkenberg, D.M., Dejmek, P., Skriver, A., Guldager, H.S., Ipsen, R. 2006. Sensory and rheological screening of exopolysaccharide producing strains of bacterial yoghurt cultures. *International Dairy Journal*. 16, 111-118.
- Fukuda, K., Shi, T., Nagami, K., Leo, F., Nakamura, T., Yasuda, K., Senda, A., Motoshima, H., Urashima, T. 2010. Effects of carbohydrate source on physicochemical properties of the exopolysaccharide produced by *Lactobacillus fermentum* TDS030603 in a chemically defined medium. *Carbohydrate Polymers*. 79, 1040-1045.
- Gadegaard, N. 2006. Atomic force microscopy in biology: technology and techniques. *Biotechnic & Histochemistry*. 81(2-3), 87-97.
- Galle, S., Schwab, C., DalBello, F., Coffey, A., Gänzle, M., Arendt, E. 2012b. Comparison of the impact of dextran and reuteran on the quality of wheat sour dough bread. *Journal of Cereal Science*. 56, 531-537.

- Gerwig, G. J., Dobruchowska, J. M., Shi, T., Urashima, T., Fukuda, K., Kamerling, J. P. 2013. Structure determination of the exopolysaccharide of *Lactobacillus fermentum* TDS030603-a revision. *Carbohydrate Research*. 378, 84-90.
- Gerwig, G.J., Kamerling, J.P., Vliegthart, J.F. 1979. Determination of the absolute configuration of mono-saccharides in complex carbohydrates by capillary G.L.C. *Carbohydrate Research*. 77, 10-17.
- Giraffa, G. 2004. Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews*. 28, 251-260.
- Girard, M., Schaffer-Lequart, C. 2007. Gelation of skim milk containing anionic exopolysaccharides and recovery of texture after shearing. *Food Hydrocolloids*. 21, 1031-1040.
- Global Market Insights. <https://www.gminsights.com/industry-analysis/probiotics-market>. Accessed 6 Mar 2019.
- Goh Y.J., Azcárate-Peril M.A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S., Klaenhammer, T.R. 2009. Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology*. 75, 3093-3105.
- Goh, K.K.T., Haisman, D., Singh, R.H. 2005. Examination of exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* using confocal laser scanning and scanning electron microscopy techniques. *Journal of Food Science*. 70, 224-229.
- Goldstein, J., Newbury, D.E., Joy, D.C., Lyman, C.E., Echlin, P., Lifshin, E., Sawyer, L., Michael, J.R. 2003. Scanning Electron Microscopy and X-Ray Microanalysis. Kluwer Academic, New York.
- Gorret, N., Renard, C., Famelart, M.H., Maubois, J.L., Doublier, J.L. 2003. Rheological characterization of the EPS produced by *P. acidipropionici* on milk microfiltrate. *Carbohydrate Polymers*. 51, 149–158.
- Grobbe, G.J., van Casteren, W.H.M., Schols, H.A., Oosterveld, A., Sala, G., Smith, M.R., Sikkema, J., de Bont, J.A.M. 1997. Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. *Applied Microbiology and Biotechnology*. 48, 516-521.

- Grosu-Tudor, S.S., Zamfir, M. 2013. Functional properties of lactic acid bacteria isolated from Romanian fermented vegetables. *Food Biotechnology*. 27, 235-248.
- Gupta, P., Diwan, B. 2017. Bacterial exopolysaccharide mediated heavy metal removal: A Review on biosynthesis, mechanism and remediation strategies, *Biotechnology Reports*. 13, 58-71.
- Hammes, W.P., Vogel R.F. 1995. The genus *Lactobacillus*. In *The Genera of Lactic Acid Bacteria*. The Lactic Acid Bacteria, eds. Wood, B.J.B, Holzapfel, W.H. Vol. 2, 19-54.
- Harris, P.J., Ferguson, L.R. 1993. Dietary fiber: Its composition and role in protection against colorectal cancer. *Mutation Research*. 290, 97-110.
- Harvey, L.M., McNeil, B. 1998. Thickeners of microbial origin. In: Microbiology of fermented foods. Wood J B (ed), Blackie Academic and Professional, Glasgow, UK. 148-171.
- Hassan, A. N., Ipsen, R, Janzen, T, Qvist, K.B. 2003. Microstructure and rheology of yogurt made with cultures differing only in their ability to produce exopolysaccharides. *Journal of Dairy Science*. 86, 1632-1638.
- Hassan, A.N. 2008. ADSA Foundation Scholar Award: Possibilities and challenges of exopolysaccharide-producing lactic cultures in dairy foods. *Journal of Dairy Science*. 91, 1282-1298.
- Hassan, A.N., Corredig, M., Frank, J.F. 2001. Viscoelastic properties of yogurt made with ropy and non-ropy exopolysaccharides producing cultures. *Milchwissenschaft*. 56, 684-687.
- Hassan, A.N., Corredig, M., Frank, J.F. 2002. Capsule formation by non ropy starter cultures affects the viscoelastic properties of yogurt during structure formation. *Journal of Dairy Science*. 85, 716-720.
- Hassan, A.N., Frank, J.F., Schmidt, K.A., Shalabi, S.I. 1996. Rheological properties of yogurt made with encapsulated nonropy lactic cultures. *Journal of Dairy Science*. 79, 2091-2097.
- Hirayama, Y., Sakanaka, M., Fukuma, H., Murayama, H., Kano, Y., Fukiya, S., Yokota, A. 2012. Development of a Double-Crossover Markerless Gene Deletion System in *Bifidobacterium longum*: Functional Analysis of the α -Galactosidase Gene for Raffinose Assimilation. *Applied and Environmental Microbiology*. 78 (14), 4984-4994.

- Horn, N., Wegmann, U., Dertli, E., Mulholland, F., Collins, S. R., Waldron, K. W., Bongaerts, R. J., Mayer, M. J., Narbad, A. 2013. Spontaneous mutations reveals influence of exopolysaccharide on *Lactobacillus johnsonii* surface characteristics. *PloS One*. 8, e59957.
- <https://ciks.cbt.nist.gov/~garbocz/SP946/node14.htm>
- <https://ciks.cbt.nist.gov/~garbocz/SP946/node14.htm>
- <https://myscope.training/legacy/images/sem/layout-and-function.png>
- Ikeda, S., Foegeding, E.A. 2003. Measurement of Gel Rheology: Dynamic Tests. *Current Protocols in Food Analytical Chemistry*. 7 H3.2.1-H3.2.9.
- Ikeda, S., Murayama, D., Tsurumaki, A., Sato, S., Urashima, T., Fukuda, K., 2019. Rheological characteristics and supramolecular structure of the exopolysaccharide produced by *Lactobacillus fermentum* MTCC 25067. *Carbohydrate Polymers*. 218, 226–233.
- Isenberg, S.L., Brewer, A.K., Côté, G.L., Striegel, A.M. 2010. Hydrodynamic versus size exclusion chromatography characterization of alternant and comparison to off-line MALS. *Biomacromology*. 11, 2505-2511.
- Islam, S.T., Lam, J.S. 2014. Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Canadian Journal of Microbiology*. 60, 697-716.
- Jia, W., Li, H., Zhao, L., Nicholson, J.K. 2008. Gut microbiota: a potential new territory for drug targeting. *Nature Reviews Drug Discovery*. 7, 123.
- Jiang, M., Zhang, F. Wan, C., Xiong, Y., Shah, N.P., Wei, H., Tao, X. 2016. Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk. *Journal of Dairy Science*. 99, 1736-1746.
- Jolly, L., Newell, J., Porcelli, I., Vincent, S. J., Stinglele, F. 2002. *Lactobacillus helveticus* glycosyltransferases. From genes to carbohydrate synthesis. *Glycobiology*. 12, 319-327.
- Jolly, L., Stinglele, F. 2001. Molecular organization and functionality of exopolysaccharide gene clusters in lactic acid bacteria. *International Dairy Journal*. 11, 733-745.
- Juna, S., Williams, P.A., Davies, S. 2011. Determination of molecular mass distribution of amylopectin using asymmetrical flowfield-flow fractionation. *Carbohydrate Polymers*. 83, 1384-1396.

- Kailasapathy, K. 2006. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. *LWT-Food Science and Technology*. 39, 1221-1227.
- Kanamarlapudi, S.L.R.K., Muddada, S. 2017. Characterization of exopolysaccharide produced by *Streptococcus thermophiles* CC30. *BioMed Research International* doi:10.1155/2017/4201809.
- Kimmel, S.A., Robert, F.R., Gregory, R., Ziegler. 1998. Optimization of exopolysaccharide Production by *Lactobacillus delbrueckii* subsp. *bulgaricus* RR grown in a semidefined medium. *Applied and Environmental Microbiology*. 64 (2), 659-664.
- Kitazawa, H., Harata, T., Uemura, J., Saito, T., Kaneko, T., Itoh, T. 1998. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus*. *International Journal of Food Microbiology*. 40,169-175.
- Kleerebezem, M., van Kranenburg, R., Tuinier, R., Boels, I.C., Zoon, P., Ellen, L., Hugenholtz, J., de Vos, W.M. 1999. Exopolysaccharides produced by *Lactococcus lactis*: from genetic engineering to improved rheological properties?. *Antonie Van Leeuwenhoek*. 76, 357-365.
- Krivoruchko, A., Zhang, Y., Siewers, V., Chen, Y., Nielsen, J. 2015. Microbial acetyl-CoA metabolism and metabolic engineering. *Metabolic Engineering*. 28, 28-42.
- Ku, S., Park, M., Ji, G., You, H. 2016. Review on *bifidobacterium bifidum* BGN4: functionality and nutraceutical applications as a probiotic microorganism. *International Journal of Molecular Sciences*. 17, 1544.
- Lambert, J.M., Bongers, R.S., Kleerebezem, M. 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Applied and Environmental Microbiology*. 73, 1126-1135.
- Lambo-Fodje, A. M., Leeman, M., Wahlund, K.G., Nyman, M., Öste, R., Larsson, H. 2007. Molar mass and rheological characterisation of an exopolysaccharide from *Pediococcus damnosus* 2.6. *Carbohydrate Polymers*. 68, 577-586.
- Laws, A., Gu, Y., Marshall, V. 2001. Biosynthesis, characterization, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnology Advances*. 19 (8), 597-625

- Laws, A.P., Marshall, V.M. 2001. The relevance of exopolysaccharides to the rheological properties in milk fermented withropy strains of lactic acid bacteria *International Dairy Journal*. 11,709-722.
- Lebeer, S., Verhoeven, T.L., Francius, G., Schoofs, G., Lambrichts, I., Dufrêne, Y., Vanderleyden, J., De Keersmaecker, S.C. 2009. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. *Applied Environmental Microbiology*. 75, 3554-3563.
- Leemhuis, H., Dijkman, W.P., Dobruchowska, J.M., Pijning, T., Grijpstra, P., Kralj, S. 2013. 4,6- α -Glucanotransferase activity occurs more widespread in *Lactobacillus* strains and constitutes a separate GH70 subfamily. *Applied and Environmental Microbiology*. 97, 181-193.
- Li, W., Mutuvulla, M., Chen, X., Jiang, M., Dong, M. 2012. Isolation and identification of high viscosity-producing lactic acid bacteria from a traditional fermented milk in Xinjiang and its role in fermentation process. *European Food Research and Technology*. 235, 497-505.
- Lilledahl, M., Bjørn B.T.S. 2015. “Novel imaging technologies for characterization of microbial extracellular polysaccharides.” *Frontiers in microbiology*. 6, 525-28.
- Linhardt, R. J., Galliher, P. M., Cooney, C. L. 1986. Polysaccharide lyases. *Applied Biochemistry and Biotechnology*. 12, 135-176.
- Liu, L., Qin, Y., Wang, Y., Li, H., Shang, N., Li, P. 2014. Complete genome sequence of *Bifidobacterium animalis* RH, a probiotic bacterium producing exopolysaccharides. *Journal of Biotechnology*. 189, 86-87.
- London, L., Price, N., Ryan, P. , Wang, L. , Auty, M. , Fitzgerald, G. , Stanton, C. Ross, R. 2014. Characterization of a bovine isolate *Lactobacillus mucosae* DPC 6426 which produces an exopolysaccharide composed predominantly of mannose residues. *Journal of Applied Microbiology*. 117, 509-517.
- Looijesteijn, P. J., Hugenholtz, J. 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. cremoris NIZO B40 and optimization of its synthesis. *Journal of Bioscience and Bioengineering*. 88,178-182.

- Looijesteijn, P.J., Trapet, E., Vries, D., Abee, T., Hugenholtz, J. 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. *International Journal of Food Microbiology*. 64, 71-80.
- Lynch, K.M., McSweeney P.L.H., Arendt, E.K., Uniacke-Lowe, T., Galle, S., Coffey A. 2014. Isolation and characterisation of exopolysaccharide-producing *Weissella* and *Lactobacillus* and their application as adjunct cultures in Cheddar cheese. *International Dairy Journal*. 34, 125-134.
- Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D. 2006. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences, USA*. 103, 15611-15616.
- Marshall, V. M., Rawson, H. L. 1999. Effects of exopolysaccharide-producing strains of thermophilic lactic acid bacteria on the texture of stirred yoghurt. *International Journal of Food Science and Technology*. 34, 137-143.
- Masakuni, T., Seiko, K., Takuya, Y., Keiko, U., Masayuki, O., Yukihiro, T., Shuntoku, Uechi. 2016. Structure-Function Relationship of a gellan family of polysaccharide, S-198 Gum produced by *Alcaligenes* ATCC31853. *Advances in Biological Chemistry*. 6, 55-69.
- Meadow, J.F., Altrichter, A.E., Bateman, A.C., Stenson, J., Brown, G.Z., Green, J.L., Bohannon, B.J.M. 2015. Humans differ in their personal microbial cloud. *Peer J*. 3, 1258.
- Mitsuoka T. 2014. Development of functional foods. *Bioscience of Microbiota, Food and Health*. 33, 117-128.
- Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot, R. M., and Remaud-Siméon, M. 2001. Homopolysaccharides from lactic acid bacteria. *International Dairy Journal*. 11, 675-685.

- Montiville, T.H., Cooney, C.L., Sinskey, A.J. 1978. *Streptococcus mutans* dextransucrase: A review. *Advances in Applied Microbiology*. 24, 55-84
- More, T., Yadav, J.S.S., Yan, S., Tyagi, R.D., Surampalli, R.Y. 2014. Extracellular polymeric substances of bacteria and their potential environmental applications. *Journal of Environmental Management*. 144, 1-25.
- Mozzi, F., Rollan, G., Savoy de Giori, G., Font de Valdez, G. 2001. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87. *Journal of Applied Microbiology*. 91, 160-167.
- Mozzi, F., Vaningelgem, F., Hebert, E.M. 2006. Diversity of heteropolysaccharide producing lactic acid bacterium strains and their biopolymers. *Appl Environ Microbiol*. 72, 4431-4435.
- Nagaoka, M., Hashimoto, S., Watanabe, T., Yokokura, T. 1994. Anti-ulcer effects of lactic acid bacteria and their cell wall polysaccharides. *Biol. Pharm. Bull.* 17, 1012- 1017.
- Nakajima, H., Toyoda, S., Toba, T. 1990. A novel polysaccharide from slime forming *Lactococcus lactis* subsp. cremoris SBT 0495. *Journal of Dairy Science*. 73, 1472-1477.
- Petry, S., Furlan, S., Waghorne, E., Saulnier, L., Cerning, J., Maguin, E. 2003. Comparison of the thickening properties of four *Lactobacillus delbrueckii* subsp. bulgaricus strains and physicochemical characterization of their exopolysaccharides. *FEMS Microbiology Letters*. 221, 285-291.
- Pham, P. L., Dupont, I., Roy, D., Lapointe, G., Cerning, J. 2000. Production of exopolysaccharide by *Lactobacillus rhamnosus* R and analysis of its enzymatic degradation during prolonged fermentation. *Appl. Environ. Microbiol*. 66, 2302-2310.
- Picton, L., Bataille, I., Muller, G. 2000. Analysis of a complex polysaccharide (gumarabic) by multi-angle laser light scattering coupled on-line to size exclusion chromatography and flow field flow fractionation. *Carbohydrate Polymers*. 42, 23-31.
- Prasanna, P.H.P., Grandison, A., Charalampopoulos, D. 2012. Screening human intestinal Bifidobacterium strains for growth, acidification, EPS production and viscosity potential in low-fat milk. *International Dairy Journal*. 23.
- Prasanna, P.H.P., Grandison, A.S., Charalampopoulos, D. 2013. Microbiological, chemical and rheological properties of low fat set yoghurt produced with exopolysaccharide (EPS) producing *Bifidobacterium* strains. *Food Research International*. 51, 15-22.

- Purohit, D.H., Hassan, A.N., Bhatia, E., Zhang, X., Dwivedi, C. 2009. Rheological, sensorial, and chemopreventive properties of milk fermented with exopolysaccharide-producing lactic cultures. *Journal of Dairy Science*. 92, 847-856.
- Ricciardi, A., Clementi, F. 2000. Exopolysaccharides from lactic acid bacteria: Structure production and technological applications. *Italian Journal Food Sciences*. 12(1), 23-45.
- Rolland-Sabate, A., Colonna, P., Mendez-Montecalvo, M.G., Planchot, V. 2007. Branching features of amylopectins and glycogen determined by asymmetrical flowfield-flow fractionation coupled with multiangle laser light scattering. *Biomacromolecules*. 8, 2520-2532.
- Ruas-Madiedo, P., Alting, A. C; Zoon, P. 2005. Effect of exopolysaccharides and proteolytic activity of *Lactococcus lactis* subsp cremoris strains on the viscosity and structure of fermented milks. *International Dairy Journal*. 15, 155-164.
- Ruas-Madiedo, P., De los Reyes-Gavilán, C. 2005. Invited Review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by Lactic Acid Bacteria. *Journal of Dairy Science*. 88, 843-856.
- Ruas-Madiedo, P., de los Reyes-Gavilán, C.G. 2005. Methods for the screening, isolation and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Sciences*. 88, 843-856.
- Ruas-Madiedo, P., Gueimonde, M., Margolles, A., de los Reyes-Gavilán, C.G., Salminen, S. 2006. Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. *Journal of Food Protection*. 69, 2011-2015.
- Ruas-Madiedo, P., Tuinier, R., Kanning, M., Zoon, P. 2002. Role of exopolysaccharides produced by *Lactococcus lactis* subsp. cremoris on the viscosity of fermented milks. *International Dairy Journal*. 12, 689-695.
- Russell, W.M., Klaenhammer, T.R. 2001. Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. *Applied and Environmental Microbiology*. 67, 4361-4364.
- Russo, P., Lopez, P., Capozzi, V., Palencia, P.F., Duenas, M.T., Spano, G., Fiocco, D. 2012. Beta-glucans improve growth, viability and colonization of probiotic microorganisms. *International Journal of Molecular Sciences*. 13, 6026-6039.

- Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G., de Los Reyes-Gavilan, C.G. 2009. Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. lactis IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH controlled batch cultures. *International Journal of Food Microbiology*. 135, 260-267.
- Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G., de Los Reyes-Gavilan, C.G. 2009. Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. lactis IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *International Journal Food Microbiology*. 135, 260-267.
- Sánchez, J.I., Martínez, B., Guillén, R., Jiménez-Díaz, R., Rodríguez, A. 2006. Culture conditions determine the balance between two different exopolysaccharides produced by *Lactobacillus pentosus* LPS26. *Applied and Environmental Microbiology*. 72, 7495-7502.
- Sanz, M.L., Martinez-Castro, I. 2007. Recent developments in sample preparation for chromatographic analysis of carbohydrates. *J. Chromatog. A*. 1153, 74-89.
- Sasikumar, P., Paul, E., Gomathi, S., Abhishek, A., Sasikumar, S., Selvam, G.S. 2016. Mobile group II intron based gene targeting in *Lactobacillus plantarum* WCFS1. *Journal of Basic Microbiology*. 56, 1107-1116.
- Selle, K., Klaenhammer, T.R., Barrangou, R. 2015. CRISPR-based screening of genomic island excision events in bacteria. *Proceedings of the National Academy of Sciences, USA*. 112, 8076-8081
- Shang, N., Xu, R., Li, P. 2013. Structure characterization of an exopolysaccharide produced by *Bifidobacterium animalis* **RH**. *Carbohydrate Polymers*. 91, 128-134.
- Shene, C., Canquil, N., Bravo, S., Rubilar, M. 2008. Production of the exopolysaccharides by *Streptococcus thermophilus*: Effect of growth conditions on fermentation kinetics and intrinsic viscosity. *International Journal of Food Microbiology*. 124 (3), 279-284.
- Sikkema J, Oba. 1998. Extracellular polysaccharides of lactic acid bacteria. *Snow Brand R&D Report*. 107,1-31.
- Sletmoen, M., Klein, D. C. G., Stokke, B. T. 2010. Single-molecule characterization of microbial polysaccharides,” in *Microbial Glycobiology*, eds Moran, A. P., Holst, O., Brennan, P. J., von Itzstein, M. (San Diego, CA: Academic Press). 253-268.

- Song, Y., Bhushan, B. 2008. Atomic force microscopy dynamic modes: modelling and applications. *Journal of Physics: Condensed Matter*. 20, 1-29.
- Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences, USA*. 44(10), 1072-1078.
- Stingele F, Neeser, J.R., Mollet, B. 1996. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* SFI6. *Journal of Bacteriology*. 178, 1680-1690.
- Stingele, F., Lemoine, J., Neeser, J.R. 1997. *Lactobacillus helveticus* Lh 59 secretes an exopolysaccharide that is identical to the one produced by *Lactobacillus helveticus* TY1-2. *Carbohydrate Research*. 302, 197-202.
- Stylianou, A., Stylianopoulos, T. 2016. Atomic force microscopy probing of cancer cells and tumor microenvironment components. *BioNanoScience*. 6 (1), 33–46.
- Stylianou, A., Yova, D., Alexandratou, E. 2013. Nanotopography of collagen thin films in correlation with fibroblast response. *Journal of Nanophotonics*, 7 (1), Article 073590.
- Su, H. N., Chen, Z. H., Liu, S. B., Qiao, L. P., Chen, X. L., He, H. L. 2012. Characterization of bacterial polysaccharide capsules and detection in the presence of deliquescent water by atomic force microscopy. *Applied Environmental Microbiology*. 78, 3476-3479.
- Sun, Z., Harris, H.M.B., McCann, A., Guo ,C., Argimón, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O’Sullivan, O., Ritari, J., Douillard, F.P., Paul Ross, R., Yang, R., Briner, A.E., Felis, G.E., de Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H., O’Toole, P.W. 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications*. 6, 8322.
- Sutherland, I.W. 1972. Bacterial exopolysaccharides. *Adv Microb Physiol*. 8, 143-212.
- Tamime, A.Y., Kaloh, M., Davies, G. 1984. Microstructure of set-style yoghurt manufactured from cows milk fortified by various methods. *Food Microstructure*. 3, 83-92.
- Tieking, M., Ehrmann, M.A., Vogel, R.F., Gänzle, M.G. 2005. Molecular and functional characterization of a levansucrase from the sour dough isolate *Lactobacillus sanfranciscensis* TMW1.392. *Applied Microbiology and Biotechnology*. 66, 655-663.

- Tuinier, R., Van Casteren, W. H. M., Looijesteijn, P. J., Schols, H. A., Voragen, A. G. J., Zoon, P. 2001. Effects of structural modifications on some physical characteristics of exopolysaccharides from *Lactococcus lactis*. *Biopolymers*. 59, 160-166.
- Tuinier, R., Zoon, P., Olieman, C., M Cohen-Stuart, M.A., Fler, G.J., Kruif, C.G. 1999 b. Isolation and physical characterization of an exocellular polysaccharide. *Biopolymers*. 49, 1-9.
- Tuinier, R., Zoon, P., Stuart, M., Fler, G., Kruif, C.G., Kees. 1999 a. Concentration and shear-rate dependence of an exocellular polysaccharide. *Biopolymers*. 50, 641-6.
- Van den Berg, D.J.C., Robijn, G.W., Janssen, A.C., Giuseppin, M.L.F., Vreeker, R., Kamerling, J.P., Vliegthart, J.F.G., Ledeboer, A.M., Verrips, C.T. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. *Applied Environmental Microbiology*. 61, 2840-2844.
- van Pijkeren J-P, Canchaya C, Ryan KA, Li Y, Claesson MJ, Sheil B, Steidler L, O'Mahony L, Fitzgerald GF, van Sinderen D, O'Toole PW. Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. *Appl Environ Microbiol*. 2006; 72:4143–4153. [PubMed: 16751526]
- Vaningelgem, F., Zamfir, M., Adrian, T., Vuyst, D. L. 2004. Fermentation conditions affecting the bacterial growth and exopolysaccharide production by *Streptococcus thermophilus* ST 111 in milk-based medium. *Journal of Applied Microbiology*. 97, 1257-1273.
- Vaningelgem, F., Zamfir, M., Mozzi, F., Adrian, T., Vancanneyt, M., Swings, J., De Vuyst, L. 2004. Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. *Applied and Environmental Microbiology*. 70, 900-912.
- Vuyst, De.L., Zamfir, M., Mozzi, F. 2003. Exopolysaccharides producing *Streptococcus thermophilus* strains as functional starter cultures in the production of fermented milks. *International Dairy Journal*. 13,707-717.
- Wacher-Rodarte, C.M., Galvan, M.V., Farres, A., Gallardo, F., Marshall, V.M.E., Garcia-Garibay, M. 1993. Yoghurt production from reconstituted skim milk powders using different polymer and non-polymer forming starter cultures. *Journal of Dairy Research*. 60, 247-254.

- Wang, J., Zhao, X., Tian, Z., He, C., Yang, Y., Yang, Z. 2015. Isolation and characterization of exopolysaccharide-producing *Lactobacillus plantarum* SKT109 from Tibet Kefir. *Polish Journal of Food and Nutrition Sciences*. 65(4), 269-279.
- Wang, K., Li, W., Rui, X., Chen, X., Jiang, M., Dong, M. 2014. Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810. *International Journal of Biological Macromolecules*. 63, 133-139.
- Wang, Y.C., Li, Liu, P., Ahmed, Z., Xiao, P., Bai, X. 2010. Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet kefir. *Carbohydrate Polymers*. 82,895-903.
- Wei, M-Q., Rush, C.M., Norman, J.M., Hafner, L.M., Epping, R.J., Timms. P. 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. *Journal of Microbiological Methods*. 21(1), 97-109.
- Welman, A.D., Maddox, I.S. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends in Biotechnology*. 21, 269-274.
- Wingender, J., Neu, T., Flemming, H. 2012. Microbial extracellular polymeric substances: Characterization, structure and function. *Springer-Verlag Berlin Heidelberg*. 10, 60147-7.
- Wood, B.J.B. 1997. Microbiology of fermented foods. Blackie Academic Professionals, London.
- Wood, P.J. 1997. Oats as a functional food for health- the role of β -glucan. *Canada Chemistry News*. 53, 17-19.
- Wyatt, N.B., Gunther, C.M., Liberatore, M.W. 2011. Increasing viscosity in entangled polyelectrolyte solutions by the addition of salt. *Polymer*. 52, 2437-2444.
- Yadav, V., Prappulla, S.G., Jha, A., Poonia, A.A. 2011. Novel exopolysaccharide from probiotic *Lactobacillus fermentum* CFR 2195: Production, purification and characterization. *Biotechnology, Bioinformatics and Bioengineering*. 1, 415-421.
- Yan, X., Yu, H-J., Hong, Q., Li, S-P. 2008. Cre/lox System and PCR-Based Genome Engineering in *Bacillus subtilis*. *Applied and Environmental Microbiology*. 74 (17), 5556-5562.
- Yang, H., Deng, J., Yuan, Y., Fan, D., Zhang, Y., Zhang, R. 2015. Two novel exopolysaccharides from *Bacillus amyloliquefaciens* C-1: antioxidation and effect on oxidative stress. *Current Microbiology*. 70, 298-306.

- Yuliani, E., Imai, T., Teeka, J., Tomita, S., Yogi, S. 2011. Exopolysaccharide production from sweet potato- shochu distillery wastewater by *Lactobacillus sakei* CY1. *Biotechnology and Biotechnological Equipment*. 25, 2329-2333.
- Zeidan, A.A., Poulsen, V.K., Janzen, T., Buldo, P., Derkx, P.M.F., Øregaard, G. 2017. Polysaccharide production by lactic acid bacteria: from genes to industrial applications. *FEMS Microbiology Reviews*. 41, S168-S200.
- Zhang, T., Zhang, C., Li, S., Zhang, Y., Yang, Z. 2011. Growth and exopolysaccharide production by *Streptococcus thermophilus* ST1 in skim milk. *Brazilian Journal of Microbiology*. 42(4), 1470-1478.
- Zhang, X., Shang, N., Zhang, Xu, Guia, M., Li, P. 2013. Role of plnB gene in the regulation of bacteriocin production in *Lactobacillus paraplantarum* L-XM1. *Microbiological Research*. 168, 305-310.
- Zhang, Z., Zhou, Z., Li, Y., Zhou, L., Ding, Q., Xu, L. 2016. Isolated exopolysaccharides from *Lactobacillus rhamnosus* GG alleviated adipogenesis mediated by TLR2 in mice. *Scientific Reports*. 6, Article number 36083.
- Zhao, W., Zhang, J., Jiang, Y-Y., Zhao, X., Hao, X.N., Li, L., Yang, Z-N. 2018. Characterization and antioxidant activity of the exopolysaccharide produced by *Bacillus amyloliquefaciens* GSBa-1. *Journal of Microbiology and Biotechnology*. 28 (8), 1282-1292.
- Zhao, X., Tian, Z., Yang, Y., Yang, Z. 2015. Characterization of an exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibet Kefir. *Carbohydrate Polymers*. 125, 16-25.
- Zhou, F., Wu, Z., Chen, C., Han, J., Ai, L., Guo, B. 2014. Exopolysaccharides produced by *Rhizobium radiobacter* S10 in whey and their rheological properties. *Food Hydrocoll*. 36, 362-368.
- Zisu, B., Shah, N. P. 2003. Effects of pH, temperature, supplementation with whey protein concentrate, and adjunct cultures on the production of exopolysaccharides by *Streptococcus thermophilus* 1275. *Journal of Dairy Science*. 86, 3405-3415.
- Zisu, B., Shah, N. P. 2005. Textural and functional changes in low-fat Mozzarella cheeses in relation to proteolysis and microstructure as influenced by the use of fat replacers, pre-acidification and EPS starter. *International Dairy Journal*. 15, 957-972.

Zoumpopoulou, G., Kazou, M., Alexandraki, V., Angelopoulou, A., Papadimitriou, K., Pot, B., Tsakalidou, E. 2018. Probiotics and prebiotics: an overview on recent trends. Probiotics and prebiotics in animal health and food safety. Berlin: Springer. 1–34.

Acknowledgements

“If you can imagine it, you can achieve it. If you can dream it, you can become it.”

William Arthur Ward

Upon completion of this thesis, I would like to take this humble opportunity to express my sincere and deep sense of gratitude and heartfelt indebtedness to my esteemed supervisor Dr. Kenji Fukuda, Associate Professor, Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan for his keen painstaking interest, intellectual guidance, constant motivation and support as well as critical suggestions during different phases of planning, commencement and during the course of study until ultimate completion. With all sincerity, I cannot express my appreciation and gratitude to him, for his proficient planning, supervision, help and above all, for indoctrinating a sense of scientific research in me. No words of mine can fully acknowledge my earnest regards for him. Trying to write down my feelings I would place him next to God and I can never repay the countless debits for everything single thing he has taught me, throughout my life. It is all because of his open mindedness, objective thoughts, deep imminence in research that has helped me to accomplish this PhD. Infact, I feel lucky to be his student and blessed with a supervisor like him.

I also express special gratitude and feel highly esteemed to thank honorable members of my advisory committee, Dr. Tadasu Urashima, Dr. Kazutaka Umetsu, Dr. Shinya Ikeda for their time, valuable suggestions and support throughout the course of my study. It is my honor to have these renowned scientists in my advisory committee. The work would have never been completed without their ever available support and help. I would also like to thank Dr. Hiroki Bochimoto, Dr. Haruki Kitazawa and Shinpei Matsumoto san, for the help in conducting various experiments and publication of the manuscript.

Once more, I would like to thank President, OUAVM for giving me an opportunity to study in this university. I would also like to thank President, GAMRC for giving me an opportunity to go to University of Winsconsin, during my internship cum study abroad program as a J1 scholar to learn new techniques and skills which will help me throughtout my life.

I feel happy to express a sense of gratitude to my seniors, Ni Putu Desy Aryantini and Khuukhenbaatar Ganzorig, for their moral and technical help during the PhD. I would also like to thank all the labmates, Yuri san, Sato san, Kanbe san, Tsiamidy san, Tanaka san, Saruwatari san and also all the lab members, whom I couldn't name here, for their help and encouragement throughout the course of study. I would also like to thank Dr. Hassan, Dr. Daiki Maruyama and Miss Vivian for the help in experiments in Wisconsin University.

I extend my sincere thanks to Ishiyama san, Chiba san, Hara san and all the staff of international students section, academic and financial sections of OUAVM which I couldn't name here for their ever ending help and support during the three years of PhD.

Last but not the least, I would like to bring on record with immense pleasure, my sincere thanks to my wife, Dr. Shveta Sharma for her understanding nature to overcome difficult times and also in editing my PhD thesis. It would have been impossible to complete this task without her cooperation. I am also thankful to my family for their wholehearted support, constant inspiration and their timely help making my work comfortable.

インド産伝統的発酵乳「ダヒ」から単離された乳酸菌 *Lactobacillus fermentum* MTCC 25067 株が生産する菌体外多糖 (HePS) はキサンタンガムと同等の高い粘性を示す。近年、同菌株の培養液が示す高粘性は複数の当該 HePS 分子が会合し超分子ネットワークを形成することに起因すると示された。予備実験として生菌数、pH 値、培養上清中の残存グルコース濃度、培養液の粘性を培養 144 時間にわたり測定した。その結果、生菌数は培養開始後 16 時間から 24 時間で最高値を示したのち漸減した。pH 値と残存グルコース濃度は培養開始後 24 時間で大幅に減少したのち一定値を示した。同菌株の培養液が示す粘性は培養開始後 48 時間で最大となり、その後、定常期の間、少なくとも培養開始後 144 時間までは低下した。生菌数、pH 値、残存グルコース濃度は 3 反復行った実験間での誤差は非常に小さい値であったのに対し、培養液粘性に関しては培養開始後 24 時間から 96 時間にかけて大きく変動した。そこで本博士論文は、同 HePS が示す構造粘性相関と、その物理化学的性質の培養期間中における経時変化を明らかにすることを目的とした。まず、同菌株の 48 時間培養上清と 144 時間培養上清から精製した HePS (以下それぞれ HePS_{48h} および HePS_{144h} と略す) の特徴を比較した。HePS_{48h} および HePS_{144h} の分子量分布パターンをゲル濾過クロマトグラフィーとフェノール硫酸法による糖濃度測定により見積もったところ、HePS_{48h} および HePS_{144h} はほぼ同様のピークパターンを示したが HePS_{144h} にのみ第 3 のピークが観察されたことから、HePS_{144h} がより多くの低分子画分を有することが示された。次に、600-MHz 1D ¹H-NMR を用いて HePS 分子の側鎖構造を解析した。3 位置換 D-Glcp のピーク面積に対する比として計算したところ、末端 D-Glcp、3 位置換 D-Glcp、2,3 位置換 D-Glcp、6 位置換 D-Galp のピーク面積比は HePS_{48h} と HePS_{144h} でほぼ同様であった。従って、HePS_{48h} および HePS_{144h} の側鎖構造に相違はないと結論した。次に、レオメーターを用いて HePS_{48h} および HePS_{144h} の 1% 水溶液の 25°C における 0.1/s to 100/s の範囲における定常ずり粘度を求めたところ、ずり速度の上昇に伴いずり応力が低下する非ニュートン流体に典型的なパターンを示した。そのような特徴を示す物質として大きな回転半径を有する直鎖状高分子が挙げられる。一方、低ずり速度における定常ずり粘度は HePS_{144h} 水溶液と比較して HePS_{48h} 水溶液は有意に高い値を示した。従って、HePS_{144h} 水溶液の粘性低下は HePS 分子の切断による低分子化が一因であると考えられた。さらに、原子間力顕微鏡 (AFM) を用いた HePS_{48h} および HePS_{144h} の微細構造観察の結果、両 HePS 共にネットワーク構造が観察されたが、HePS_{48h} とは対照的に HePS_{144h} は細く短い HePS 繊維が大きな割合を占めていることが示された。従って、これも HePS_{144h} 水溶液の低ずり速度における定常ずり粘度低下の一因であると示唆された。二次代謝産物や培地成分などバクテリア周囲に存在する物質と菌体との相互作用も培養液の物性に大きな影響を与えることが推定される。そこで菌体を含む培地成分と HePS 分子との相互作用が粘性に与える影響を調べるため、0.02% の HePS_{48h} または

HePS_{144h}を MRS 培地に溶解し粘性を測定した。その結果、両 HePS 溶液ともニュートン流体の挙動を示し、菌体培養液と比較して有意に低い粘性を与えた。また、AFM により培養液そのものを用いて菌体の形状解析を実施したところ、48 時間培養では菌体周辺部に明瞭な HePS 超分子ネットワークが観察されたが 144 時間培養ではこれが消失していることが示された。さらに、144 時間培養液中で形成された HePS ネットワークには網の目の交点が他の部分よりも 1.9 倍高い構造が観察され、理由は不明であるが 48 時間培養液中で同様の構造は観察されなかった。これらの発見により、長時間培養後、死菌体の細胞壁の脆弱化により菌体表層と HePS 超分子ネットワーク間の相互作用が低下し、その結果として培養液の粘性が低下したとする仮説を得た。また、同菌株のコロニーの走査型電子顕微鏡 (SEM) の結果、菌体周辺に幾層にも重なった明瞭な HePS 超分子ネットワークの存在を認めたことから、超分子ネットワーク形成は AFM に使用した試料調製過程 (室温での乾燥) により生じたアーティファクトではなく、当該 HePS 分子が元来有する性質であることが支持された。以上から、正確な培養条件の制御と HePS 分解因子の探索が効果的に培養液粘性の低下を防ぐ方法であると示された。また、産業利用のためには当該 HePS と食品成分との相互作用を明らかにすべきである。さらに、当該 HePS 生産に関連する遺伝子の働きを明らかにする必要がある。本研究では、乳酸菌の遺伝子破壊株作製に汎用される二手法、すなわち抗生物質耐性遺伝子をマーカーとして用いるプラスミドと温度感受性ベクター pORI28 プラスミドを使用し、当該菌株の糖転移酵素遺伝子 LF25067_00121 (GenBank ID: AP017973.1) 遺伝子破壊株作製用プラスミドを構築し、同菌株の野生型を宿主として用いた HePS 生合成関連遺伝子破壊株を取得するため方法を最適化した。その結果、電気穿孔によるコンピテントセルへのプラスミド導入は、菌体の十分な洗浄による HePS 除去が重要であることが示された。以上、本研究で得られた知見を適用することで、培養上清や発酵食品の粘性を操作するために必要な当該 HePS 分子と菌体表層との相互作用の理解に関して重要な知見を得ることが今後期待される。