STUDIES ON EXOPOLYSACCHARIDE PRODUCTION BY PROBIOTIC LACTIC ACID BACTERIA

THESIS SUBMITTED UNDER THE FACULTY OF SCIENCE OF THE

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BINDHUMOL I

REG. NO. 3697

BIOTECHNOLOGY DIVISION
NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE & TECHNOLOGY–CSIR
THIRUVANANTHAPURAM 695019, INDIA

MAY 2012

“IN THE NAME OF ALLAH,
THE MOST BENEFICIENT,
THE MOST MERCIFUL”

DEDICATED TO MY FAMILY

National Institute for Interdisciplinary Science & Technology (NIIST)
Council of Scientific and Industrial Research, CSIR
Trivandrum - 695019, Kerala, India

Dr. K.Madhavan Nampoothiri
Principal Scientist
Biotechnology Division

Tel: +91 - 471-2515366, 2490674; Fax: + 91- 471-2491712, 2490186
E.mail: madhavan85@hotmail.com, m.nampoothiri@rediffmail.com

22nd May 2012

CERTIFICATE

This is to certify that the work embodied in the thesis entitled **'Studies on exopolysaccharide production by probiotic Lactic acid bacteria'** is based on the original research work carried out by Miss. Bindhumol, I under my supervision and no part of this work has been submitted previously anywhere for the award of any degree.

Dr. K. Madhavan Nampoothiri

Research Supervisor

DECLARATION

I hereby declare that the work presented in this thesis entitled **'Studies on exopolysaccharide production by probiotic Lactic acid bacteria'** is based on the original research done by me, Bindhumol I under the guidance and supervision of Dr K Madhavan Nampoothiri, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology (Formerly Regional Research Laboratory), CSIR, Thiruvananthapuram-695 019, India. I also declare that the work has not been submitted elsewhere for award of any degree.

Thiruvananthapuram **Bindhumol I**

May 2012

ACKNOWLEDGEMENTS

This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people. In this very special leaf of my thesis, it is a pleasant task to express my gratitude to all those who contributed in one or the other way to the success of this study and made it an unforgettable experience for me. I offer my blessings and regards to all those who supported me in any respect during the completion of my thesis.

Foremost, I would like to express my sincere gratitude to my supervisor Dr K Madhavan Nampoothiri, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology, for the continuous support and blessings showered by him throughout my study. His patience, motivation, enthusiasm, immense knowledge and meticulous guidance helped me in all the time of research and writing of this thesis.

I am also extremely indebted to Prof Ashok Pandey, Head of Biotechnology Division, National Institute for Interdisciplinary Science and Technology for his valuable advice, constructive criticism and unflinching encouragement throughout my research career. His innovative ideas and lectures exceptionally inspired and enriched my growth as a student and a researcher.

I gratefully place on record my gratitude towards Dr Suresh Das, Director, National Institute for Interdisciplinary Science and Technology for providing necessary infrastructure and resources to accomplish my research work. I am also thankful to Prof T K Chandrashekhar and Dr B C Pai, former Directors of the Institute, for providing me all the facilities for conducting my research in this prestigious institute.

I record my heartfelt thanks to Dr G Vijay Nair, Former Director and Dr KGK Warrier, Director-Grade Scientist, National Institute for Interdisciplinary Science and Technology for the nice moments gifted by their enthusiasm, soft-spoken manner and friendly smile.

I express my heartfelt thanks to Dr Rajeev Kumar Sukumaran, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology, for his valuable decision in the beginning of my PhD career. His suggestions and informative discussions helped me very much in moulding my research.

I gratefully acknowledge the Council of Scientific and Industrial Research, New Delhi for providing me Senior Research Fellowship to undertake this study. I also thank Department of Biotechnology, New Delhi for the financial support provided during the initial period of my study.

I owe my gratitude towards Dr T Sreelekha, Scientific Officer, Regional Cancer Research Centre, Trivandrum and Dr T Santhoshkumar, Scientist, Rajiv Gandhi Centre for Biotechnology, Trivandrum for the help and guidance offered for anti-tumour work. I also appreciate the helping hands from Mr Manu Joseph, Senior Research Fellow, Regional Cancer Research Centre, Trivandrum and Mr Vijayaprasad, Project Assistant, Rajiv Gandhi Centre for Biotechnology, Trivandrum.

I express my sincere gratitude to Dr Sarita G Bhat, Head, Department of Biotechnology, CUSAT and Expert Member of my Doctoral Committee for her valuable suggestions for improving my research studies.

I have also benefited by suggestions and discussions from Dr Binod, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology. I am also thankful to Dr Sindhu, Dr P Prema, Dr Sudheer Kumar Singh, Dr Syed Dastagar, Mrs Suganya Dorai, Er Vikram, Er Kiran Kumar,

Dr Ramesh Kumar, Dr Vijayalekshmiamma, Mr K M Prakash, Mrs Radhamony, Mrs Sasikala and Mrs Rajalekshmi for all their support.

It is my privilege to express my gratitude towards the Academic Program Committee of the Institute and the NIIST –CUSAT Research Council for the timely help during the entire course of my work and thesis submission. I also thank all the administrative and supporting staff of the Institute for their support and help rendered.

Friends are a beautiful episode of our life and it is a pleasure to express my gratitude wholeheartedly to my senior colleagues and friends. The valuable suggestions and moral support from Dr Rojan, Dr Dhanya, Dr Babitha, Dr Swetha, Mrs Sumantha, Dr Reeta, Dr Syed, Dr Sumitra, Dr Anisha, Dr, Nila, Dr Nice and Mrs Sheeja Devi helped me a lot in my career. I cherish the beautiful moments I spent with Dr Raji, Ms Divya, Mrs Sumi, Mrs Aswathy, Ms Gincy, Mrs Padma, Mr Kiran, Mr Vipin, Mr Kuttiraja, Ms Lalitha, Mrs Sajna, Ms Satya, Mr Arjun, Ms Jincy, Mrs Rekha, Mrs Himani, Mrs Deepa, Ms Sandrine C, Ms Sandrine G, Mrs Brintha, Ms Sylviya, Ms Preeti, Ms Aniesha, Mr Nishant, Mr Sai Shyam, Mr Shyam, Mr Rubex, Mrs Nisha, Mrs Anusree, Ms Anu, Mrs Sumayya, Ms Aparna, Ms Sandhya, Mr Abraham, Mr Karthik, Mrs Parvathy, Ms Gayathri, Mrs Sita, Mrs Soumya and Ms Arya. Thanks are also due to other members of Team Biotech Ms Leya, Mr Aravindh, Mrs Sabeela, Mrs Akanksha, Ms Varsha, Ms Nimisha, Ms Rajasree, Ms Deepthy, Ms Ushasree and Ms Vidya. I extend my warm thanks to my friends of other divisions in the institute who helped me at various stages of my work and stay at Trivandrum.

Family enables us to ignore the minor hurts, the opponent's pressure and the temporary failures. I would like to thank my entire family for all their love and encouragement. Special mention for my parents who raised me with a love of science and supported me in all my pursuits.

Above all, I bow my head before Allah, the Almighty, on whom ultimately we depend for sustenance and guidance. For all the blessings showered on me throughout my life.

Bindhumol I

PREFACE

The thesis entitled 'Studies on exopolysaccharide production by probiotic lactic acid bacteria' has been framed into ten chapters. Chapter 1 gives a detailed review of probiotic aspects of lactic acid bacteria, exopolysaccharides from lactic acid bacteria, their structural characteristics, production and metabolic engineering strategies adopted so far and industrial applications. Chapter 2 describes the general materials and methodology used invariably in every chapter. Isolation and probiotic characterization of more than fifteen LAB isolates from various sources has been discussed in chapter 3. The production of EPS through submerged fermentation (SmF) by *Lactobacillus plantarum* (MTCC 9510), the best EPS producing isolate, has been described in chapter 4. The factors contributing to the production of EPS have been investigated by single factor approach as well as statistical approach employing response surface methodology. Chapter 5 explains the purification and structural elucidation of EPS from *L. plantarum* with the aid of various spectroscopic and chromatographic techniques such as GC, FT-IR, 1D and 2D NMR and GPC. Encapsulation of bacterial cells, to tackle the formation of a highly viscous fermentation broth during EPS production and to ease the biomass separation and reusability, has been discussed in detail in chapter 6. Chapter 7 deals with the molecular identification of plasmid encoded genes of *Lactococcus lactis* NIZO B40, a known EPS producer and exploration of similar kind of genes in the genomic DNA of the NIIST isolate, *L. plantarum* MTCC 9510. Chapter 8 and 9 focuses on the application side of the purified EPS. Chapter 8 explores the anti-tumor activity of EPS from *L. plantarum* in human breast adenocarcinoma cell line (MCF-7) and its antioxidant activity. In food industry, the oozing out of water from fermented foods (syneresis) is of great concern. Chapter 9 focuses into the efficacy of EPS in preventing syneresis in starch. The whole work has been summarized and the conclusions derived in

Chapter 10. This is being followed by a bibliographic section. The annexures include various media compositions and list of publications made from this work.

Bindhumol I

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

$	Dollar
%	Percentage
µg	Microgram
ANOVA	Analysis of Variance
AO	Acridine Orange
ATCC	American Type Culture Collection
bp	Base Pair
$CaCl_2$	Calcium Chloride
CFU/g	Colony Forming Unit Per Gram
CFU/ml	Colony Forming Unit Per Millilitre
cm	Centimetre
$CuSO_4$	Copper Sulphate
CV	Coefficient of Variation
DNA	Deoxyribo Nucleic Acid
EB	Ethidium Bromide
EDTA	Ethylene Diamene Tetraacetic acid
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization
FBS	Foetal Bovine Serum
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
g/l	Gram Per Litre
g/dl	Gram Per Decilitre
GPC	Gel Permeation Chromatography
GRAS	Generally Recognized as Safe
GTF	Glycosyl Transferase
h	Hour
IL	Interleukins
kb	Kilo Base

kDa	Kilo Dalton
LAB	Lactic Acid Bacteria
LPS	Lipopolysaccharide
M	Molar
mg/ml	Milligram Per Millilitre
mPa.s	Milli Pascal Second
MTCC	Microbial Type Culture Collection
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
nm	Nanometre
NMR	Nuclear Magnetic Resonance
^oC	Degree Celsius
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
rRNA	Ribosomal Ribonucleic Acid
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate
SmF	Submerged Fermentation
TLR	Toll-Like Receptors
Tm	Melting Temperature
UV	Ultraviolet
w/v	Weight Per Volume
WHO	World Health Organization
τ	Shear Stress
K	Consistency Coefficient
θ	Shear Rate
n	Power- law Index
η	Apparent Viscosity

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1. Introduction

Biopolymer market is represented by a small fraction of microbial exopolysaccharides. The economic feasibility assigned with the industrial production is the main factor limiting the use of exopolysaccharides. Plants, algae and bacteria produce a variety of polysaccharides. These are adequately implied in various biological functions such as storage of energy (starch), cell wall architecture (cellulose) and cellular communication (glycosaminoglycans). The polysaccharides like cellulose, pectin, starch (plant origin), agar, carrageenan, alginate (from algae) and gums like dextran, gellan, pullunan and xanthan (from bacteria) are used commercially as food additives and also in pharmaceuticals. The only ingredients successfully incorporated into products were dietary fibre (polysaccharides) and carotenoids towards the end of twentieth century (Bidlack, 1998). The status of microbial polysaccharides over natural polysaccharides from plant and marine sources in industry was mainly due to their (i) production from well known, cheap and plentiful raw materials (ii) peculiar rheological properties and resistance to hydrolysis at different temperature and pH conditions (iii) superior ability to thicken, emulsify, stabilize, encapsulate, flocculate and swell to form colloidal suspensions, gels, films and membranes. The production and approval of dextran from *Leuconostoc mesenteroides* in 1947 and the United States Food and Drug Administration (FDA) approval of food grade xanthan from *Xanthomonas campestris* in 1969 paved the way to large scale application of microbial EPS. Phenomenal demand for natural polymers for various industrial applications has led to a vibrant interest in exopolysaccharide (EPS) production by microorganisms. Bacterial exopolysaccharides encompass a broad range of complex chemical structures and consequently different properties. The modulation of

biochemical properties of exopolysaccharide require a thorough understanding of its biosynthetic pathway and the relation between the structure of EPS and the functional effect provided by them after incorporation into the food matrix.

Commercialization of microbial EPS is often accompanied by a long and costly process of ensuring conformance to food safety to obtain approval from food legislators. Since *Lactobacilli* are food-grade bacteria known to be of the GRAS (generally recognized as safe) status and well-known probiotic strains, their EPS could be easily utilized in foods in a juridical point of view. The EPS produced by lactic acid bacteria not only be applied as natural additives but also be produced *in situ*. All these polysaccharides can be homopolymers or heteropolymers of neutral sugars (pentoses and hexoses) or anionic sugars (hexoses), substituted or non-substituted with non-sugar compounds attaining linear or ramified final conformations (Welman & Maddox, 2003; Garai-Ibabe *et al.*, 2010). Only few structural studies and functional effect of EPS produced by different strains of LAB have been reported so far.

Lactobacilli produce exopolysaccharides exhibiting a wide diversity in structure. This introductory chapter deals with literature review of recent works regarding probiotic LAB, structural diversity, molecular genetics and pertinence in food and pharmaceutics of exopolysaccharides (EPS) from lactic acid bacteria.

1.2. Objectives of the Study

i. Isolation of lactic acid bacteria having probiotic features as well as exopolysaccharide (EPS) production
ii. Bioprocess optimization for EPS production using the selected isolate
iii. Purification and characterization of EPS
iv. Molecular characterization of EPS biosynthetic genes in the isolated EPS producer

v. Application studies using crude/purified EPS such as food additive to give improved texture and better rheology and its utility related to health aspects such as anti-tumour activity or anti oxidant activity

1.3. Review of Literature

1.3.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are the most important group of bacteria inhabiting a wide variety of environmental niches. They are the habitants of mucosal surfaces, particularly the gastrointestinal tract and also seen in vegetables, plants and in food materials such as grains, cereals, pickles, milk and meat products etc (Wood & Warner, 2003). They can survive passage through stomach in an active form.

LAB is mainly involved in the fermentation of hexose sugars, yielding primarily lactic acid. Most of the LAB belong to the order *Lactobacillales*, a group of non-sporulating, gram-positive bacteria, but a few LAB species belong to the *Actinobacteria (Wood & Holzapfel, 1995)*. LAB are considered industrially important (Fig. 1.1) organisms because of their fermentative ability as well as health and nutritional benefits (Fig. 1.2). Moreover, they are generally regarded as safe (GRAS) for incorporation into food products. LAB consist of a number of diverse genera which include both homofermentative and heterofermentative LAB based on the end product of their fermentation. The ability of lactic acid bacteria to inhibit the growth of various bacteria is well known and attributed to the production of organic acids such as lactic acid and acetic acid, hydrogen peroxide, bacteriocins, bacteriocin like substances and biosurfactants.

LAB are also well-known probiotic strains. They are used as probiotics for human and animals (Salminen *et al.*, 1998). The functional properties and safety of probiotics of particular strains of *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus acidophilus* from

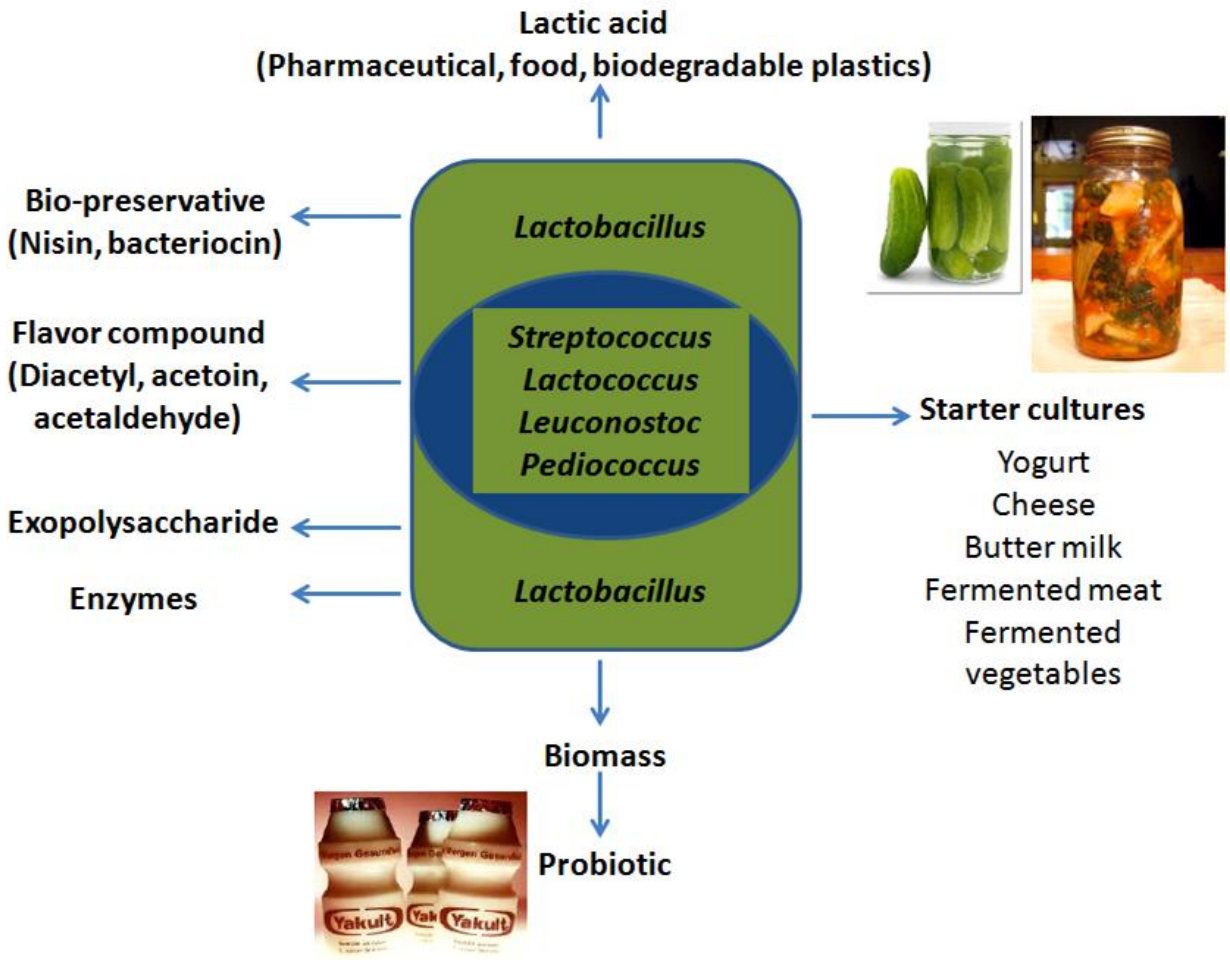


Fig. 1. 1. Lactic acid bacteria in industry

various sources have been extensively studied.

1.3.2. Lactic Acid Bacteria as Probiotic

1.3.2.1. Probiotic Properties

Nowadays, interest is being gained by foods not only in terms of taste and immediate nutritional needs, but also in terms of their ability to provide specific health benefits beyond their basic nutritional value. Presently, the largest segment of the functional food market is

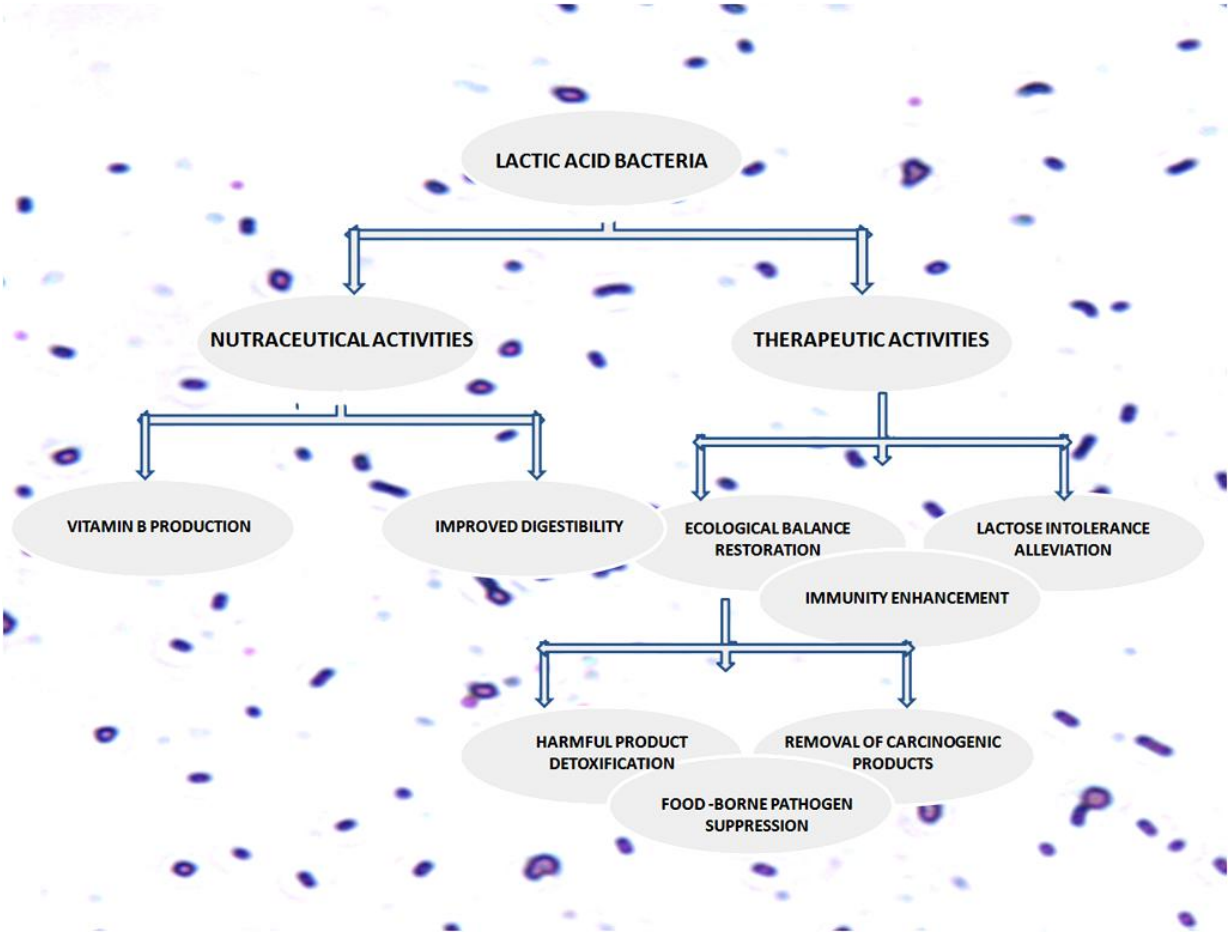


Fig. 1.2. Health benefits of lactic acid bacteria

provided by the foods targeted towards improving the balance and activity of the intestinal microflora. One of the best methods to increase the number of advantageous bacteria in the intestinal tract is the consumption of foods containing live bacteria and such bacteria are called 'Probiotics'. Probiotics are microbial cell preparations or components of microbial cells, when administered in adequate amounts, confer a beneficial effect on the health and well-being of the host (Salminen *et al.*, 1999; Holzapfel & Schillinger, 2002).

1.3.2.2. Probiotic Market

About half of the probiotic market is occupied by foods, with supplements (30-40 %) and < 10 % pharmaceuticals. Probiotic foods have the largest share of the functional food

market, which is expected to reach $17 billion in 2013, of which Japan possess the largest share of total sales, about 39% (Stanton *et al.,* 2001). In USA, the sale reached $1.1 billion in 2010, with 7.1% annual growth rate. Probiotic supplement sales in North America and Eastern Europe have a high growth rate higher than 10%, while in Asia and Western Europe the growth rate is 5 % and 8 % respectively. In 2005, the Indian joint venture between Yakult and Danone was established and awarded Emerging Company of the Year in the Indian food and beverage market from Frost & Sullivan in the year 2011 and recently Danone has made an entry in to the Chinese market. Yakult was first introduced to the US in 1999 and the firm started selling and marketing in 2007. The first bottles of Yakult will start rolling off the production line at the Japanese probiotic pioneer's first US factory in Fountain Valley, California in the fall of 2013. Now the firm has just completed the self-affirmed GRAS process for its proprietary probiotic strain *L. casei Shirota*. *L. casei* strain "Shirota" has been reported to have the longest history of safe use as a probiotic in food with proven health benefits (Goktepe *et al.,* 2006). Some of the key manufacturers of probiotic products are mentioned in Table 1.1.

Major pharmaceutical companies have become active and are devising newer drugs and probiotic products. The major drivers in the probiotic market are increasing shift towards preventive therapies, increase in disposable income, increasing shift towards self-medication, increase in health care spending, ageing population, pharma retail growth and favorable pricing environment while the major challenges are standardization, high pricing, lack of awareness and marketing and distribution.

The development and marketing of probiotic products require significant research efforts for identifying functional compounds and assessing their physiological effects. Further, a suitable food matrix has to be developed taking into account the bio-availability and potential changes during processing and food preparation. Consumer education and

clinical trials on product efficacy are important to gain approval for health-enhancing marketing claims (Kotilainen *et al.*, 2006). In other words, it is a multi-stage process, which requires contributions from commercial, academic and regulatory interests.

1.3.2..3. Common Probiotics and Characteristics

Probiotics have been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, which belong to lactic acid bacteria group. Some of the common probiotic microbes of this group are *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus GG* (Timmerman *et al.*, 2004). LAB have been extensively studied and established as valuable native inhabitants of the gastrointestinal tract (GIT). The GIT of human body encompasses a diverse range of microbial population that mediates numerous interactions with the chemical environment as the GIT represents a much larger contact area with the environment on comparison to skin surface of our body. The restoration of the GIT population, after or during antibiotic treatment, was attained with the help of pharmaceutical preparations containing live microorganisms in capsules such as probiotics (Goktepe *et al.*, 2006).

The general criteria that have been used for the selection of probiotics are tolerance to gastrointestinal conditions (acid, phenol and bile), ability to adhere to the gastrointestinal mucosa, competitive exclusion of pathogens, production of anti-microbial substances, absence of toxicity and resistance to technologic processes (*i.e.*, viability and activity in delivery vehicles) (Ouwehand *et al.*, 2002). A preliminary *in vitro* assessment is a prerequisite to assess the properties of probiotic bacterial strains.

The mucous gel layer and the underlying cell coat (glycocalix), which consists of glycoconjugates on the apical surface of the epithelium, are the interface between a

Table 1.1 Manufacturers and products in probiotic market

Manufacturer	*Brand*	*Features*
Danone, France	Actimel	Probiotic yogurt drink (*L. casei Imunitass ®)*
	Activia	Creamy yogurt (*Bifidus actiregularis* ®)
Yakult, Japan	Yakult	Fermented milk drink (*Lactobacillus casei Shirota)*
Nestlé, Switzerland	Ski BioVita:	Probiotic yoghurt containing LC1 culture
	Sveltesse Optimise 0%:	Probiotic, fat free, dairy drink with probiotic and fibre, available in strawberry and pineapple flavours
	Munch Bunch Drinky (UK)	Yogurt drink designed for children
Müller Dairy, Germany	Müller Vitality	Drinks and yogurts, uses inulin as a prebiotic
Dr Reddy's Labs, India	Becelac PB	Probiotic capsule
Orchard Maid, UK	Orchard Maid The LifeTop straw	Organic yogurt drinks (*Lactobacillus reuteri)*

Manufacturer	***Brand***	***Features***
Onken Dairy	The Onken Biopot	(*Lactobacillus acidophilus, Bifidobacterium longum*, and *Streptococcus thermophilus)*
Rowan Glen Dairy, UK	Rowan Glen	Fat-free Probiotic Yogurt
Bioferme, Finland	Yosa	Yogurt-like oat product flavoured with natural fruits and berries containing probiotic bacteria (*Lactobacillus acidophilus, Bifidobacterium lactis*)
Valašské Meziříčí Dairy, Czech Republic	Pohadka	Yogurt milk with probiotic cultures
Lifeway, USA	Soytreat	Kefir type product with six probiotics
Ingman Foods, Finland	Rela	Yogurts, cultured milks and juices (*Lactobacillus reuteri*)
Skåne mejerier, Sweden	ProViva	Refreshing natural fruit drink and yogurt in many different flavours (*Lactobacillus plantarum)*

Manufacturer	***Brand***	***Features***
H&J Bruggen, Germany	Jovita Probiotisch	Blend of cereals, fruit and probiotic yogurt
Campina, the Netherlands	Vifit	Yogurt drink with LGG, vitamins and minerals
Olma, Czech Republic	Revital Active	Yogurt and yogurt drink with probiotics
Tallinna Piimatööstuse AS, Estonia	Hellus	Dairy products (*Lactobacillus fermentum* ME-3)
Valio, Finnland	Gefilus	Wide range of LGG products
Kibow Biotech, USA	Renadyl™	Probiotic dietary supplement (*S. thermophilus* (KB-19), *L. acidophilus* (KB-27), *B. longum* (KB- 31), combined with a dietary fiber (psyllium husk))
Kibow Biotech, USA	Kibow® Flora	Probiotic immune system booster

mammalian host and microflora in the lumen. The studies related to the mechanism of non-pathogenic bacteria (lactobacilli) adhesion to mucosa is very less when compared to pathogenic strains. It has been suggested by Annuk et al (2001) that lectin-like components in surface-layered proteins of lactobacilli play an important role in the adhesion to receptors such as glycoproteins on the surface of intestinal epithelial cells. Several adhesion factors have been reported regarding the adhesion of lactic acid bacteria such as non-specific reaction by hydrophobicity, non-specific reaction by charge, lectin-like proteins, fibronectin and collagen. The attachment of probiotics to the host epithelium is an important pre-requisite in the selection criteria for probiotics, but this is not universal property of lactic acid bacteria and not necessary for successful probiosis (Fuller, 1989).

Studies by several authors showed that yogurt-producing species of lactobacilli were more sensitive to gastric juice while enteric species were more resistant. Gastric juice renders the stomach pH to approximately 2.0 (Murthy *et al.*, 2000). Hence potential probiotics should tolerate the particular pH to survive in stomach. A significant enhancement in the survival of potential probiotic dairy propionibacteria strains was observed by Huang and Adams (2004) in pH 2.0 on addition of soymilk. Corcoran et al (2005) compared the survival of lactobacilli for 90 min in the low pH conditions of human stomach using simulated gastric juice (SGJ, pH 2.0). Out of the selected strains *Lactobacillus rhamnosus* GG had the highest survival rate and maintained their initial viable cell numbers (~ 9 log CFU/ml) while the poorest survivor was *L. paracasei* NFBC 338, whose concentration declined to undetectable levels by 30 min of exposure).

Cellular stress begins in the stomach and the upper intestinal tract where bile is secreted into the gut. Bile acids are secreted in conjugated forms from gall bladder into the duodenum. Most of the human derived *Lactobacillus* and *Bifidobacterium* have the ability to tolerate the physiological concentrations of human bile (Dunne *et al.*, 2001) and gastric juice.

1.3.2.4. Probiotic preparation and viability

Probiotics can be found in capsule, liquid, powder, tablet form or as acidophilus drinks. Foods containing probiotics include yogurt or kefir, fermented and unfermented milk, miso, tempeh, and some juices and soy beverages (Probiotics added downstream of process or the food items with probiotics are subjected to mild processes that doesn't kill the probiotic bacteria). In probiotic foods and supplements, the bacteria may have been present originally or added during preparation.

But there are problems associated with low viability of probiotic strains in GIT and final products which can bring out economic loss and loss of health benefits. A recommended intake of probiotics is 10^8-10^9 viable live cells daily (Lourens-Hattingh & Viljoen, 2001) in order to get the beneficial health effects. The common techniques employed for converting probiotic cultures into concentrated powder form are spray drying, freeze drying, fluidized bed drying. The main steps involved in the production and preparation of probiotics is explained in Fig 1.3. But these techniques are not sufficient to protect the bacteria from the product environment or during their passage through the GIT as they release the bacteria completely. The problem with loss of health benefits could be overcome by the development of new technologies like encapsulation of the probiotic strain.

Encapsulation of probiotic strains (Fig. 1.4) in hydrocolloid beads protect them from bacteriophages, increase survival during freeze-drying and freezing and improve stability during storage. It was demonstrated by Hou et al. (2003) that the bile tolerance and viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* could be elevated, approximately four log units, on encapsulation within artificial sesame oil emulsions. Even after 6 months of storage at 4 °C and 21°C improved viability of probiotic organisms was observed when encapsulated in 3% v/w sodium alginate in freeze-dried yogurt (Capela *et al.*, 2006). Özer et al (2009) found

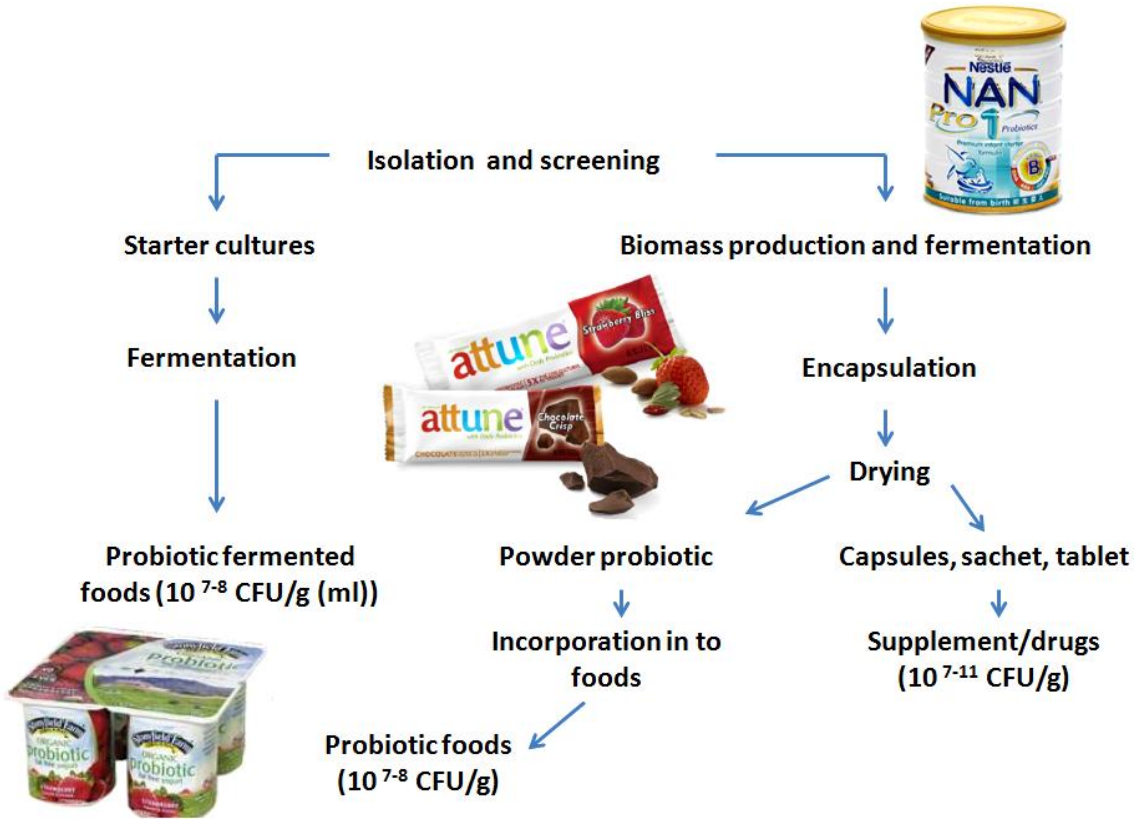


Fig. 1.3. Probiotic preparation and production

the encapsulation in sodium alginate by either extrusion or emulsion technique to be effective in keeping the number of probiotic bacteria higher than the level of the therapeutic minimum (>10^7 CFU/g), while the counts of non-encapsulated probiotic bacteria decreased approximately by 3 logs. Alginate beads are frequently used for encapsulation as it is non-toxic and an accepted food additive.

1.3.2.5. Health benefits

Probiotics can help in the balancing of intestinal bacteria, lowering of faecal enzymes and inhibition of bladder cancer. Other common health benefits attained by probiotics include immune stimulation, role as vaccine adjuvant, adherence to human intestinal cells, enhancing the protective barrier of the digestive tract, help in the production of vitamin K, prevention of diarrhoea caused by radiotherapy, antibiotic, rotavirus, acute and *Clostridium*

difficile, treatment of constipation, prevent inflammatory bowel disease, antagonistic action against carcinogenic bacteria, lower blood pressure, faecal enzyme reduction and long term survival in the intestinal tract (Andersson *et al.*, 2001; Salminen, 2001). Some studies have shown that certain strains of lactobacilli possess anti-oxidative activity and are able to reduce the risk of accumulation of reactive oxygen species in the host.

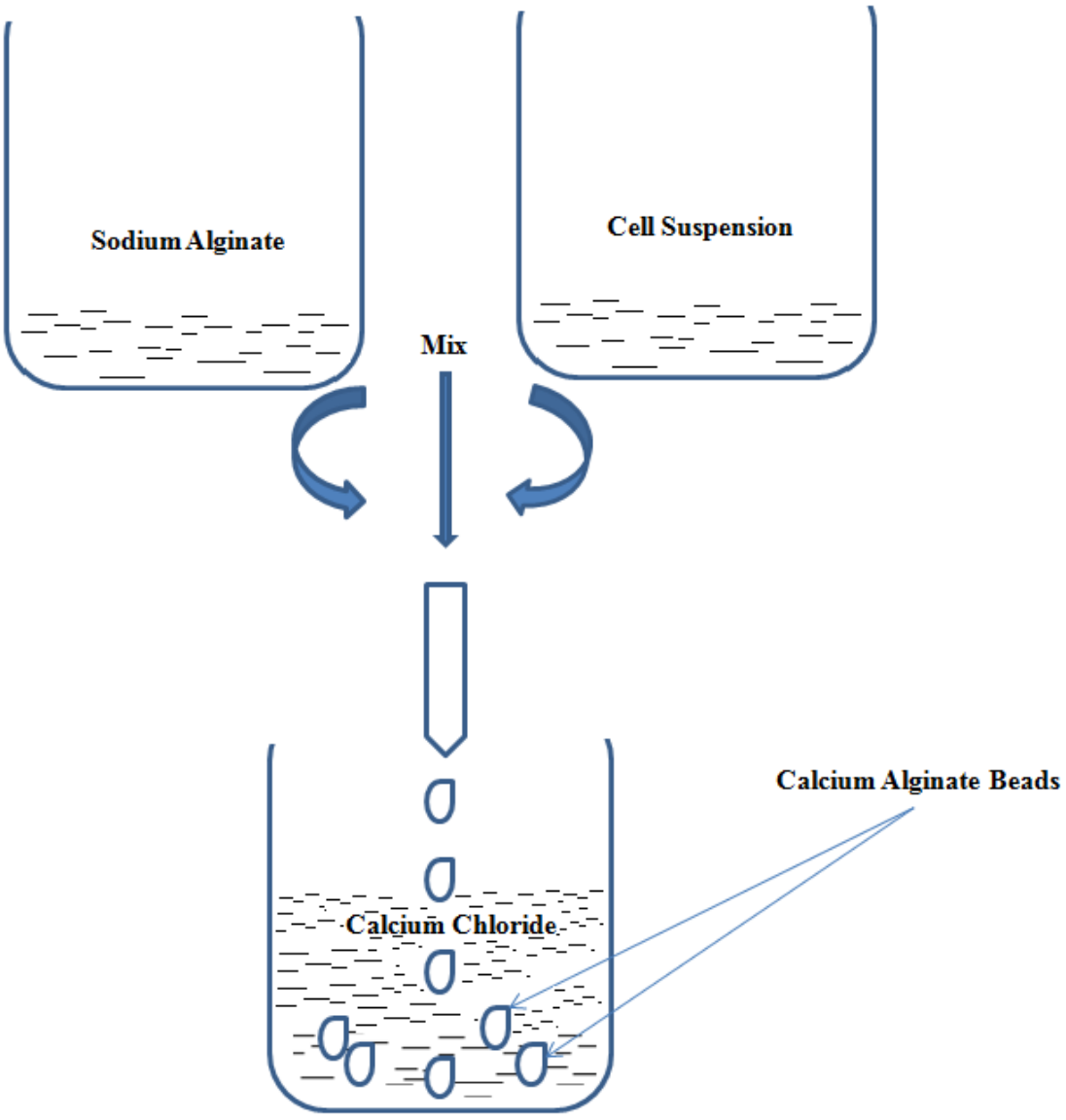


Fig. 1. 4. Encapsulation of probiotic strains using sodium alginate

Probiotics modulate inflammatory and hypersensitivity responses, an observation thought to be at least in part due to the regulation of cytokine function. They prevent reoccurrences of inflammatory bowel disease in adults as well as improve milk allergies and reduce the risk of atopic eczema in children. Immune function is improved by probiotics by increasing the number of IgA-producing plasma cells, increasing or improving phagocytosis as well as increasing the proportion of T lymphocytes and Natural Killer cells (Bodera & Chcialowski, 2009). Consumption of milk fermented with various strains of lactic acid bacteria can result in modest reductions in blood pressure because of the ACE inhibitor like peptides produced during fermentation. Two tri-peptides, valine-proline-proline and isoleucine-proline-proline, isolated from a dairy based fermentation of milk by *Saccharomyces cerevisea* and *Lactobacillus helveticus* have been identified as the active components. Probiotics can also lower serum cholesterol levels in animals presumably by breaking down bile in the gut, thus inhibiting its re-absorption (which enters the blood as cholesterol). This can bring about modest reductions in total and LDL cholesterol levels. The role of probiotics in preventing colon cancer is explained by the anti-mutagenic effects thought to be due to their ability to bind with (and therefore detoxify) hetro-cylic amines, carcinogenic substances formed in cooked meat (Rafter, 2004). They also decrease the activity of an enzyme called β-glucuronidase (which can regenerate carcinogens in the digestive system).

It was observed by Stadlbauer et al (2008) that probiotics with *L. casei Shirota* can restore neutrophil phagocytic capacity in cirrhosis by changing IL10 secretion and TLR4 expression. Another study with the same strain showed an increase in the natural killer cell activity in smokers (Reale *et al.*, 2011). Ranganathan et al (2009) reported a pilot clinical trial of an oral probiotic dietary supplement to see its biochemical and clinical effects in chronic kidney disease. The product is marketed by the trade name Renadyl™. It is proposed as a natural treatment for kidney problems as it metabolizes nitrogenous waste that has diffused

from the bloodstream into the bowel. More nitrogenous waste is consumed with the growth and multiplication of probiotics thereby effectively maintaining healthy kidney function.

1.3.2.6. Synbiotics

The use of synbiotics is another possibility of gut microflora management, where probiotics and prebiotics are used in combination. It has been observed that a combination of suitable probiotics and prebiotics enhances survival and activity of the organism. Prebiotics are non-digestible food ingredients that alter the functionality or growth of probiotic bacteria in the colon (Edina, 2007). Oligosaccharides such as lactulose, galactooligosaccharides, inulin, fructooligosaccharides and other food carbohydrates are some of the examples for probiotics. The combination (symbiotic) has synergistic effect as synbiotics not only promote the growth of existing strains of beneficial bacteria in the colon but also improve the survival, implantation and growth of newly added probiotic strains. This concept has been widely used by European dairy drink and yoghurt manufacturers such as Aktifit (Emmi, Switzerland), Proghurt (Ja Naturlich Naturprodukte, Austria), Vifit (Belgium, UK) and Fysiq (Netherlands) (Niness, 1999). It was reported by Rayes et al (2005) a synbiotic containing *Pediococcus pentoseceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei*, and *L. plantarum* with four fermentable fibres namely β-glucan, inulin, pectin and resistant starch reduced the occurrence of post-operation infections from 48 % to 13 % in 66 liver transplant patients.

1.3.2.7. Nutraceuticals from Probiotics

Apart from being a good probiotic, lactic acid bacteria are also well known for their production of nutraceuticals. The term ‘nutraceutical’ was coined in 1989 by the Foundation for Innovation in Medicine (New York, US: an educational foundation established in the US to encourage discoveries in medicine). A nutraceutical is any substance that may be considered as food or part of a food and provides medical or health benefits including the prevention and treatment of disease (DeFelice, 1992). Some examples of nutraceuticals are

exopolysaccharides, dietary fibres, polyunsaturated fatty acids (PUFA), proteins, peptides, aminoacids, anti-oxidative vitamins etc. Most of the nutraceuticals are well known for their physiological functions and biological activities.

Lactic acid bacteria produce lactic acid as a major end product during the fermentation of carbohydrates, whose level will be decreased on an increased production of nutraceuticals. Out of the various nutraceuticals produced by LAB such as vitamins, bacteriocins, low-calorie sugars, dietary fibers, exopolysaccharides etc emphasis is given to exopolysaccharides from probiotic lactic acid bacteria in the present thesis. Probiotic cultures with increased exopolysaccharide production will be an added advantage in the probiotic industry.

Exopolysaccharides are water soluble gums with novel and unique physical properties. EPS produced by food-grade microorganisms with GRAS (generally recognized as safe) status gain lot of importance as they are alternatives to the polysaccharides of plant and animal origin. Exopolysaccharides from lactic acid bacteria confer beneficial rheological and functional properties to foods (e.g. yoghurts) as natural thickening agents, giving the product a suitable viscosity and reducing syneresis. Via a better mouth-feel and longer retention time in the mouth the exopolysaccharides are believed to enhance flavour perception in viscous foods. The consistency and viscosity of the final product can be improved with the help of EPS. Some EPS have been shown to have health-beneficial properties such as, immune stimulation, antiulcer activities, antitumoral activites and cholesterol-lowering activity (Ouwehand *et al.*, 2002; Parvez *et al.*, 2006). The exopolysaccharides produced by probiotic microbes would develop an increased improvement in the properties of the final product as there will be effect from the probiotic organism as well as the EPS secreted.

It was noted in studies on probiotics as cures that any beneficial effect was usually low, a strong placebo effect often occurs and more research in the form of clinical trials is needed in order to draw firmer conclusions. But the progress in scientific understanding of probiotics and their potential for preventing and treating health conditions during the recent years is promising. The other areas of interest on probiotics are the molecular level studies of bacteria and their interaction with the body (the gut and its bacteria) to prevent and treat diseases. The advances that occurred in this area recently, throw light into the development of probiotic cultures as health cures in the future.

1.3.3. Potential Market and Production of Exopolysaccharides from LAB

1.3.3.1. Exopolysaccharide Market

India is a growing force in the international health food market (Verma, 2009). Rapid urbanization, rising incomes, changing lifestyles, dietary patterns and growing health consciousness have triggered the growth of functional foods in India. India is having a share of less than 1 %, of the global nutraceutical market of US$ 117 billion. The functional foods or nutraceutical market is currently estimated to be around US$ 1.6 billion and is expected to reach US$ 7.5 to 10 billion by 2015 growing at 25 to 30 percent compound annual growth rate. Modern biotechnology has mainly transformed the way scientists view microbes and the output materials from microbes. A vast array of novel synthetic polymers has come into play with advances in chemistry and materials science. Polysaccharides known as nature's family of sugars provide fuel or cell activity and serve as structural elements in living systems. The production of polysaccharides by selected organisms was first reported in the 1880's (Kinsella, 1988). Large scale application of polysaccharides was achieved only with the production and approval of dextran from *Leuconostoc mesenteroides* in 1947 with a market value of 2000 metric tonnes and FDA approval of food grade xanthan from *Xanthomonas campestris* in 1969. More than 20000 tonnes of xanthan are consumed each year mainly as

thickener (Sutherland, 2002). The high value market niches, such as cosmetics, pharmaceuticals and biomedicine, are paving the greatest potential to bacterial EPS as they can overcome the traditional polymers with the high degree of purity and specific functional properties.

Extracellular microbial polysaccharides as possible fermentation products received its major attention from a program of research and development initiated in 1955 at the Northern Regional Research Center of the Agricultural Research service, U.S Department of Agriculture, Peoria, Illinois. The minimum requirement of a new polysaccharide is the solubility in water, constant functional properties better or as good as the existing ones and availability on a regular basis from a stable source of supply. Traditional polysaccharides obtained from plants and sea-weeds may suffer from a lack of reproducibility in their properties, purity, supply and cost. In addition, their rheological properties may not exactly match those required. Most of the plant polysaccharides used are chemically modified to improve their structure and rheological properties. Their use is hence strongly restricted and, in the European Union, for food applications the food products need to be labelled with an E-number. Here novel polysaccharides that satisfy most of the demands of food industry, find its role as an efficient substitute for the traditional polysaccharides. The promising and noticeable aspects of exopolysaccharide from lactic acid bacteria are given in Fig. 1.5.

1.3.3.2. Identification and Production of Exopolysaccharide

Bacteria producing exopolysaccharides can be divided into groups based on their chemistry and their synthesizing conditions. A large number of lactic acid bacteria are known to produce exopolysaccharides. Table 1.2 named some of the lactic acid bacteria reported in

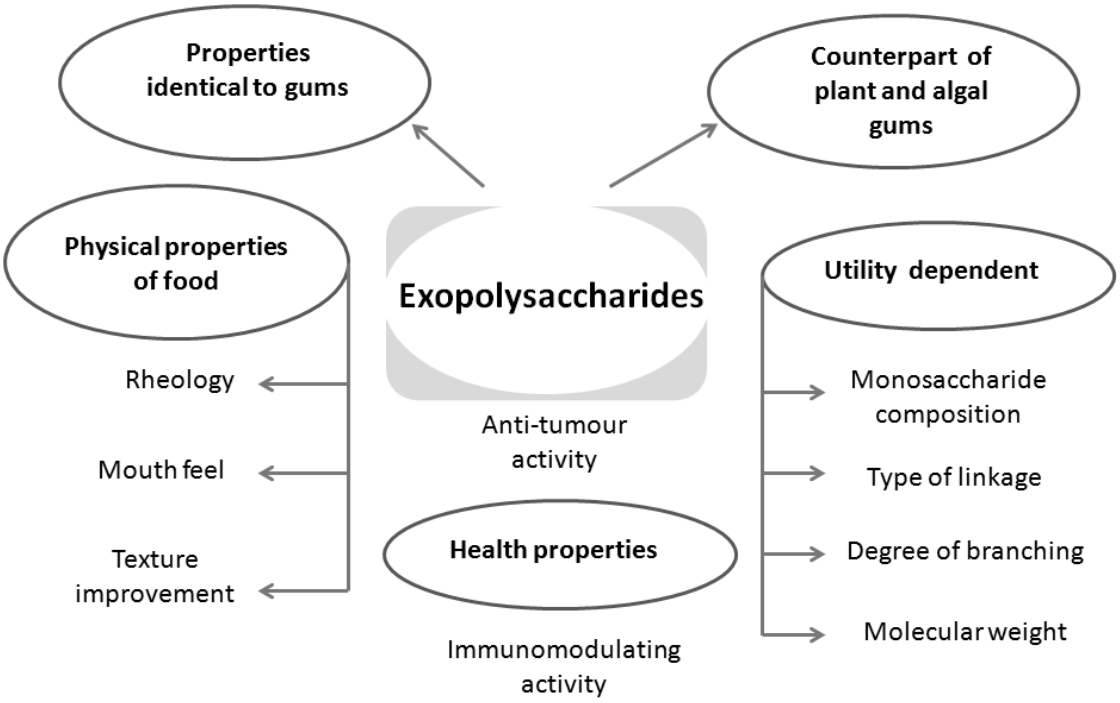


Fig. 1. 5. Wide spectrum activities of exopolysaccharides from lactic acid bacteria

literature for EPS production. Ruthenium red staining method could be used for the identification of EPS producing LAB strains (Dabour & LaPointe, 2005). The presence of EPS prevents the uptake of the stain differentiating EPS producers from non-producers by white and pink colonies. Fig. 1.6 shows an EPS producer lactic acid bacteria in ruthenium red agar plate with white colonies.

Phase-contrast microscopic technique like alcian blue staining method can be used for the qualitative analysis of cell bound as well as secreted EPS (Vicente-García *et al.*, 2004). Alcian blue stains EPS in blue colour presenting the bacterial cell and surrounding in blue in case of the presence of bound as well as secreted EPS.

Milk derivatives and MRS (de Man Rogosa Sharpe) medium are commonly employed for EPS production by *Lactobacilli*. Production of most microbial polysaccharides involves

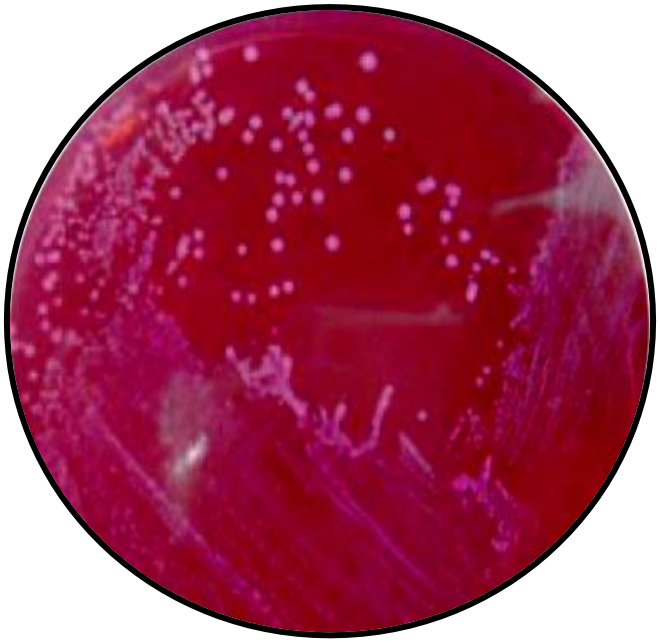


Fig. 1. 6. Exopolysaccharide producing strain in ruthenium red agar plate

growth in stirred tank fermenters using media with glucose or sucrose as the carbon and energy source. Exopolysaccharide production is favoured by a high carbon-nitrogen ratio. Literature validates that the amount of EPS depends on carbon, nitrogen sources and physico-chemical conditions for bacterial growth such as temperature, pH, oxygen rate, etc. The preferential choice of carbon substrate differs from one species to another. EPS are produced normally at temperatures just below the optimal growth temperature. Incubation temperature is often critical in EPS biosynthesis.

Mozzi et al. (2006) evaluated EPS production in milk by 31 LAB strains and observed that thermophilic strains produced more EPS than mesophilic ones, but EPS yields were generally low. Generally, the yield of production is under 1 g/l for homopolysaccharides under un-optimized culture conditions and even lesser for the majority of heteropolysaccharides. A summary of various production conditions exhibited by some of the common *Lactobacilli* were shown in Table 1.3.

Table 1.2 Lactic acid bacteria producing exopolysaccharides

Genus	*Species*
Lactobacillus	*L. casei*
	L. delbrueckii bulgaricus
	L. helveticus
	L. hilgardii
	L. rhamnosus
	L. sake
	L. kefiranofaciens
	L. reuteri
	L. sanfranciscensis
	L. johnsonii
	L. kefirgranum
	L. parakefir
	L. kefir
Lactococcus	*Lc. lactis cremoris*
	Lc. lactis lactis
Leuconostoc	*Ln. mesenteroides cremoris*
	Ln. mesenteroides dextranicum
	Ln. mesenteroides mesenteroides
	Ln. citreum
Pediococcus	*P. damnosus*
Streptococcus	*S. mutans*
	S. salivarius
	S. sobrinus
	S. thermophilus

1.3.4. Structural and Physical Properties of Exopolysaccharides from LAB

Structural variability of bacterial exopolysaccharides has been seriously discussed since many years. Most of the EPS produced by LAB are heteropolysaccharides while few produce homopolysaccharides. The sole homopolysaccharide from LAB present in industrial market is dextran synthesized by *Leuconostoc mesenteroides*. Dextran has immense applications due to the water soluble nature imparted by the presence of more than 90 % linear linkages (Purama & Goyal, 2005). The second most described homopolysaccharide is mutan, a linear EPS containing D-Glucose residues linked by α (1, 3) glycosidic bonds (more than 50 % of total linkages) associated with D-Glucose branched in α (1, 6) produced by *Lactobacillus reuteri*. Many different hetero-polysaccharides (HePS) are secreted by LAB regarding sugar composition and molecular size but they show few common structural features, which raises questions about the relationship between structure and texture. The molecular weights are typically greater than 1.0×10^6 Da (Cerning & Marshall, 1999), although a smaller molecular weight fraction of $\sim 1.0 \times 10^4$ Da has been reported. Studies on the EPS structure are crucial not only to understand their physico-chemical and biological properties, but also for the exploitation of EPS-producing microorganisms in industrial or medical applications. Primary structure of EPS is exploited by several chemical and physical techniques. The main steps involved in the recovery, purification and structural characterization of microbial EPS are shown in Fig. 1.7. The secondary and tertiary conformation of a polysaccharide is strongly dependent on its primary structure. A very small change in the primary structure may have tremendous effect on the conformation and properties of the polysaccharide.

Table 1.3 *Lactobacilli* species reported for EPS production

Microorganisms	*Media*	*Temperature (°C)*	*Time(h)*	*pH*	*EPS (g/l)*	*Reference*
L. plantarum MTCC 9510	LBM[a]	37	72	7.3	1.21	(Ismail & Nampoothiri, 2010)
L. plantarum	Supplemented Whey	25	18	-	0.15	(Tsuda & Miyamoto, 2010)
						(Dupont *et al.*, 2000)
L. rhamnosus 9595 M	BMM[b]	32-37	72	6	~1	
L. rhamnosus 9595	Whey permeate supplemented	37	24	6	2.77	(Macedo *et al.*, 2002)
L. delb. bulgaricus RR	Whey	38	24-28	5	0.09-0.11	(Briczinski & Roberts, 2002)
L. rhamnosus R	BMM[b]	37	72	6	0.50	(Pham *et al.*, 2000)
L. delb. bulgaricus	Milk	42	24	-	0.11	(Bouzar *et al.*, 1996)
L. delb. bulgaricus	MRS	40	18	-	0.26	(Aslim *et al.*, 2005)
L. rhamnosus GG	Milk	37	20	-	0.08	(Landersjö *et al.*, 2002)

Microorganisms	*Media*	*Temperature (ºC)*	*Time (h)*	*pH*	*EPS (g/l)*	*Reference*
L. delb. bulgaricus 291	Skimmed milk	37	22	-	0.08	(Faber *et al.*, 2001)
L. casei CG11	BMM[b]	25	48	-	0.13	(Cerning *et al.*, 1994)
L. helveticus	Skim milk	37	60	5	0.73	(Lin & Chien, 2007)
L. delb. bulgaricus	Whey (protein free)	37	18	6	0.80	(Shene & Bravon, 2007)
L. paracasei	BMM[b]	32-37	72	6	~0.08	(Dupont *et al.*, 2000)
L. pentosus LPS26	SDM[c]	20	72	6	0.51	(Sánchez *et al.*, 2006)
L. fermentumF6	Skim milk	37	32	6.5	0.04	(Zhang *et al.*, 2011)

[a]LBM: Lactose Based Medium; [b] BMM: Basal Minimal Medium; [c]SDM: Semi-Defined Medium

Chemical degradation and derivatization combined with chromatographic methods, often coupled to mass spectrometry (MS), are used to determine the sugar composition, together with absolute configuration, their positions of substitution and the substituent composition. The length and composition of branching strongly affects rheological properties as it affects the compactness of EPS (Vincent *et al.*, 2001). The conformation and chemical composition of polysaccharides have stronger effects on bioactivities according to the studies by many researchers (Kreisman *et al.*, 2007).

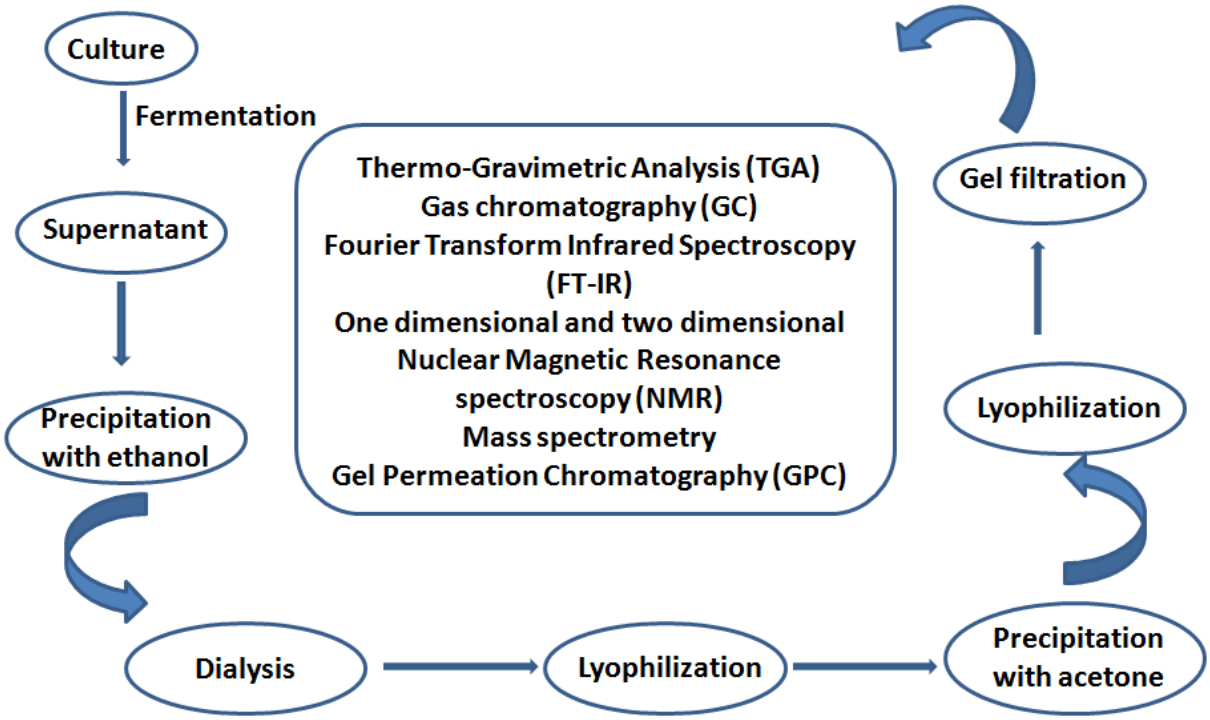


Fig. 1. 7. Steps involved in the recovery, purification and chemical characterization of exopolysaccharides

Nuclear magnetic resonance (NMR) spectroscopy provides most of the experimental data that enables the complex equilibrium of inter converting forms of reducing sugars to be unravelled (Angyal, 1992). NMR, in particular, two-dimensional (2D) ^{1}H and ^{13}C NMR, is the most powerful technique to obtain information about the nature and configuration of

sugar residues, their interconnectivity and the nature and location of substituents, which in turn is used to ultimately determine the sequence of the repeating unit. The method completely relies on the magnetic properties of nuclei. NMR experiments of polysaccharides are often performed in D_2O to reduce the complexity of the obtained NMR spectra since deuterium and protons are not observed at the same frequency. It can resolve repeat unit structures of regular and heterogeneous polysaccharides using a combination of one dimensional and two dimensional NMR techniques, including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), hetero-nuclear single quantum coherence (HSQC), hetero-nuclear multiple bond correlation (HMBC) and nuclear over-hauser enhancement spectroscopy (NOESY) (Bubb, 2003). Homonuclear COSY is useful in the identification of individual monosaccharide residues. In a hetero nuclear spectrum, like HSQC or HMQC, all signals in the spectra represent a direct correlation between a carbon and a proton. The molecular weights of exopolysaccharides are also extraordinarily heterogeneous. With incomplete stringent control over the number of subunits added to a chain (Batchelor *et al.*, 1991), long and short polymers are synthesized, although one molecular weight species predominates.

In general, EPS backbones of *Lactobacillus* spp. (*L. delbrueckii bulgaricus*, *L. rhamnosus* and *L. helveticus*) have repeating units composed of several monosaccharide units, where glucose, galactose and rhamnose are the main sugars. Some of the common lactic acid bacteria and their EPS monomers are given in the Table 1.4

Table 1.4 Lactic acid bacteria and EPS monomers

Organism	*EPS Monomers*	*Molecular Weight (Da)*	*Ratio*	*Reference*
Lactobacillus plantarum MTCC 9510	Glucose and mannose	2.68×10^5	2:1	(Ismail & Nampoothiri, 2010)
Lactobacillus plantarum	Glucose and mannose	8×10^4 Da	1:2	(Tsuda & Miyamoto, 2010)
Lactobacillus delbrueckii ssp bulgaricus NCFB 2483	Galactose, glucose, rhamnose and mannose	-	5:1:0.6:0.5	(Goh, 2004)
Lactobacillus pentosus	Glucose and rhamnose	-	-	(Sánchez *et al.*, 2006)
Lactobacillus pentosus	Glucose and mannose	-	-	(Sánchez *et al.*, 2006)
Streptococcus macedonicus Sc136	D-glucose, D-galactose and N-acetyl D-glucosamine	-	-	(Vincent *et al.*, 2001)
L. acidophilus MR1	Glucose	-	-	(Savadogo *et al.*, 2004)
L. acidophilus MR9	Glucose and galactose	-	-	(Savadogo *et al.*, 2004)
L. acidophilus MR12.	Glucose and pentose	-	-	(Savadogo *et al.*, 2004)
L. delbrueckii ssp *bulgaricus* LBB.B332	D-glucose, D-galactose and L-rhamnose	-	-	(Sánchez-Medina *et al.*, 2007)
Streptococcus thermophilus ST1	Glucose and galactose	-	-	(Säwén *et al.*, 2010)

Novel microbial biopolymers may find a position in the market-available polymers or may replace a traditional product because of its improved rheological and stability characteristics. Structural analysis combined with rheological studies has revealed a considerable variation among different exopolysaccharides. EPS may exhibit remarkable thickening and shear-thinning properties and display high intrinsic viscosities. As a reason, several slime-producing lactic acid bacterium strains and their biopolymers are being exploited towards different products, in particular natural fermented milks. The physical and rheological properties of a polysaccharide in solution are closely related to its three-dimensional structure or conformation. The relation between the chemical structure and their function is characterized by the chain stiffness.

Hydrated polymers should exhibit thixotropic or pseudo-plastic (shear-thinning) properties in order to function as a biothickener. Their rheology should decrease markedly upon shaking, stirring, or pouring, but recover completely when shear is removed. When considering processing costs, mouth-feel and consistency of food products, a drop in viscosity with increasing shear rate is important. Viscosity is highly dependent on the average molecular mass of the polysaccharide. Most of the polysaccharides are hygroscopic, containing substantial amounts of water (2-10 %) even after extensive efforts at drying (Ahmad & Muhammadi, 2007).

The production of EPS can lead to a ropy consistency, which can be detected in cultures with the formation of long strands on extension with a loop. However, not all slimy or mucoid strains are ropy. The mucoid colonies having a glistening and slimy appearance on agar plates are not able to produce strands when extended with an inoculation loop, whereas the ropy colonies form a long filament by this method. The ropy character is genetically unstable and non-ropy variants could be observed after several generations. The mere ropiness of a strain doesn't guarantee an optimal, smooth and creamy quality of a product as

texture generation involves a complex physico-chemical process (Duboc & Mollet, 2001). EPS-producing strains present higher value of elasticity and viscosity. A correlation between ropiness and amount of EPS produced from cultures could not be established as the EPS produced were similar, consisting of the same sugar residues but in different ratio. Kenji et al. (2010) found that 1 % (m/V) solution of purified EPS from *Lactobacillus fermentum* had a high apparent viscosity of 0.88 Pa·s at a shear rate of 10 s^{-1}. The consistency of EPS solution (20g/l) from *Lactobacillus delbrueckii* and *Streptococcus thermophilus* were 17.71 and 13.14 mPa.s (Canquil *et al.*, 2007).

1.3.5. EPS genes and biosynthesis in LAB

Lactococcus sp. and *Streptococcus* sp. are the most studied LAB for genome organisations. It was proved that a common operon structure and mechanism is present in EPS-producing bacteria. In mesophilic LAB, the EPS production is plasmid oriented and are regarded as a reason for EPS-producing instability in mesophilic LAB (Vescovo *et al.*, 1989). The entire thermophilic LAB appears to have *eps* genes located on the chromosome. Stingele et al. (1996) identified the eps genetic locus of *S. thermophilus* Sfi6, revealing a 15.25 kb region encoding 16 open reading frames (ORFs), within which a 14.52 kb region encodes 13 genes (*epsA* to *epsM)* capable of directing EPS synthesis. Homology searches of the predicted proteins showed a high level of homology (40–68 % identity) for *eps A, B, C, D* and *E* with the genes encoding CPS in *S. pneumonia* and *S. agalactiae*.

Van Kranenburg et al. (1997) determined that the essential information needed for the biosynthesis of EPS by *Lactococcus lactis* NIZO B40 was encoded in a single 12-kb gene cluster linked to a 40-kb plasmid (*epsRXABCDEFGHIJKL*), driven by a promoter upstream of *epsR* (Fig. 1.8). The predicted gene products of 11 of the 14 genes were homologous in sequence to gene products involved in EPS, Capsular polysaccharides (CPS),

lipopolysaccharide (LPS) or teichoic acid biosynthesis of other bacteria, and putative functions were assigned to these genes.

The eps gene cluster from *L. lactis* NIZO B40 was also found to be similar in organization to those encoding *S. thermophilus*, *S. pneumoniae* and *S. agalactiae*. The expression of the gene *epsD* in *E. coli* confirmed that its product is a priming glycosyl transferase, which links the first sugar of the repeating unit to the lipid carrier.

Glycosyl transferases (GTFs) are key carbohydrate-interacting enzymes involved in the synthesis of complex carbohydrate structures. Glycosyl transferases offer the advantages of high regio- and stereo specificities compared to a chemical approach, as well as the potential availability of many different glycosidic linkages. The diversity of EPS structures explained so far is large and provides the basis for a rapid identification of a wide variety of enzymes for the catalysis of specific reactions. All GTFs from lactic acid bacteria represent an array of putative specificities, which altogether could be defined as a 'toolbox' for the production of tailored carbohydrates.

The genetics of bacterial EPS production is shown to be linked to *eps* gene clusters encoding putative GTFs (Jolly & Stingele, 2001). The *eps* gene clusters could be of chromosomal or plasmid origin. The similarity between *eps* clusters from lactic acid bacteria is striking. Generally, they have an operon structure with a high coding density. The genes are oriented in the same direction and are transcribed as a single mRNA, while the sequence of the functions of genes in all clusters seems to be regulation, chain-length determination, biosynthesis of the repeating unit, polymerization and export of the repeating units (Jolly & Stingele, 2001).

A biosynthetic pathway (Fig.1.9) for *L. lactis* has been proposed by Van Kranenburg et al. (1997), stating that the *L. lactis* proteins of *epsE* and *epsF* may act as one glycosyl

transferase, performing the same reaction, in a similar fashion to the capsular polysaccharide gene products of *cps14F* and *cps14G* of *S. pneumonia.*

The other two glycosyl transferases involved in subsequent steps of biosynthesis were expected to be encoded by *epsG* and *epsH,* on the basis of their homology with other putative glycosyl transferases. *EpsI* is thought to encode for the polysaccharide polymerase and *epsK* involved in polysaccharide export from the cell, due to its moderate homology with *RfbX,* involved in export of the O-antigens in *Shigella flexneri* and the flippase of *Salmonella enterica*.

Fourteen putative ORFs transcribed in the same direction were observed in *L. delbrueckii* ssp *bulgaricus* (Lamothe *et al.*, 2002). Potential ribosome-binding site (RBS) sequences were found at appropriate distances from the predicted start codons. The 14 predicted gene products (*epsABCDEFGHIJKLMN*) were found to be homologous to proteins involved in the biosynthesis of various bacterial polysaccharides. A 14 kb region encoding 11 open reading frames (ORF) oriented in the same direction was identified in *L. helveticus* (Jolly *et al.*, 2002). ORF sequence analysis with the hydrophobic cluster analysis tool indicated the presence of six putative UDP-dependent GTFs falling into family 4 of retaining α-GTF (*epsF*, *epsG* and *epsH*) and family 2 of inverting β-GTF (*epsI* and *epsJ*). Five ORFs encoding for *epsK*, a polymerase; *epsL*, a glycogenin homologous protein (α-GTF); *epsM*, a UDP-galactopyranoside mutase; *epsN*, a flippase analog to Wxz and *epsO*, a putative transport protein involved in exporting the repeating unit toward the external side of the membrane.

Sun et al. (2011) reported *S. thermophilus* ND03, genome carries a unique 23.4 kb EPS gene cluster (STND_1010 to STND_1035), which contains 10 EPS related genes and six intact or truncated insertions (IS). The EPS related genes in the cluster, *epsA*, *epsB*, *epsC*, and *epsD*, were conserved between all four genomes in comparisons. The main functions of

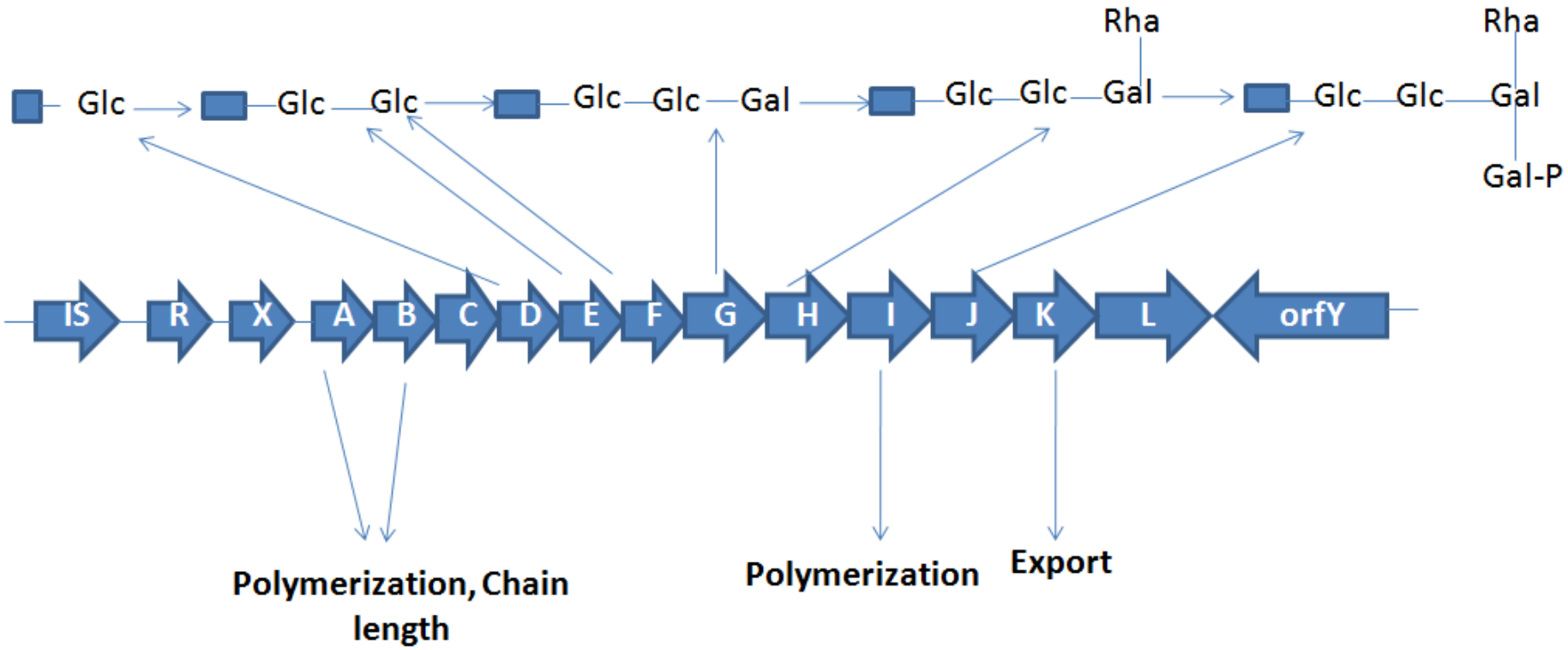


Fig. 1. 8. EPS gene cluster in *Lactococcus lactis* NIZO B40 (Source: Welman and Maddox, 2003). Gal: Galactose, Glc: Glucose, Rha: Rhamnose, Gal-P: Galactose-Phosphate

these genes are regulation, polymerization and chain length determination, and export of the EPS.The remaining six genes regarded as the key enzymes to determine the formation of a special EPS are *epsE*, *epsF*, *epsG*, *epsI*, *epsJ*, and *epsP*. These EPS genes were uniquely present in ND03. Interestingly, six copies of IS that belong to the IS*3*, IS*6*, and IS*L3* families were found in the EPS gene cluster. This increases the possibility that the unique EPS genes were imported by the transposition of these IS.

EPS biosynthetic genes in *Lactococcus lactis* ssp. *cremoris* SMQ-461 are chromosome oriented. The gene cluster is a 13.2 kb region consisting of 15 open reading frames (Dabour & LaPointe, 2005). This region is flanked by three IS*1077* related *tnp* genes (*L. lactis*) at the 5'end and *orfY*, along with an IS*981* related *tnp* gene, at the 3' end. Three (*epsGIK*) of the six predicted glycosyl transferase gene products showed low amino acid similarity with known glycosyl transferases. The structure of the repeat unit could thus be different from those known to date for *Lactococcus*. The eps locus is transcribed as a single mRNA as in other lactic acid bacteria. The function of the *eps* gene cluster has been confirmed by disrupting the priming glycosyl transferase gene (*epsD*) in *Lactococcus cremoris* SMQ-461.

Péant et al. (2005) showed that the exopolysaccharide (EPS) biosynthesis gene cluster of four *Lactobacillus rhamnosus* strains, ATCC 9595, RW-9595M, R and RW-6541M, is located in the chromosomal DNA regions of 18.5 kb encoding 17 ORFs that are highly similar among the strains (Fig. 1. 10). Fifteen genes are co-transcribed starting from the first promoter upstream of wzd. Nevertheless, five transcription start sites were identified by 5′-RACE PCR analysis, and these were associated with promoter sequences upstream of wzd, rmlA, welE, wzr and wzb. Six potential glycosyl transferase genes were identified that account for the assembly of the heptasaccharide repeat unit containing an unusually high proportion of rhamnose. Four genes involved in the biosynthesis of the sugar nucleotide

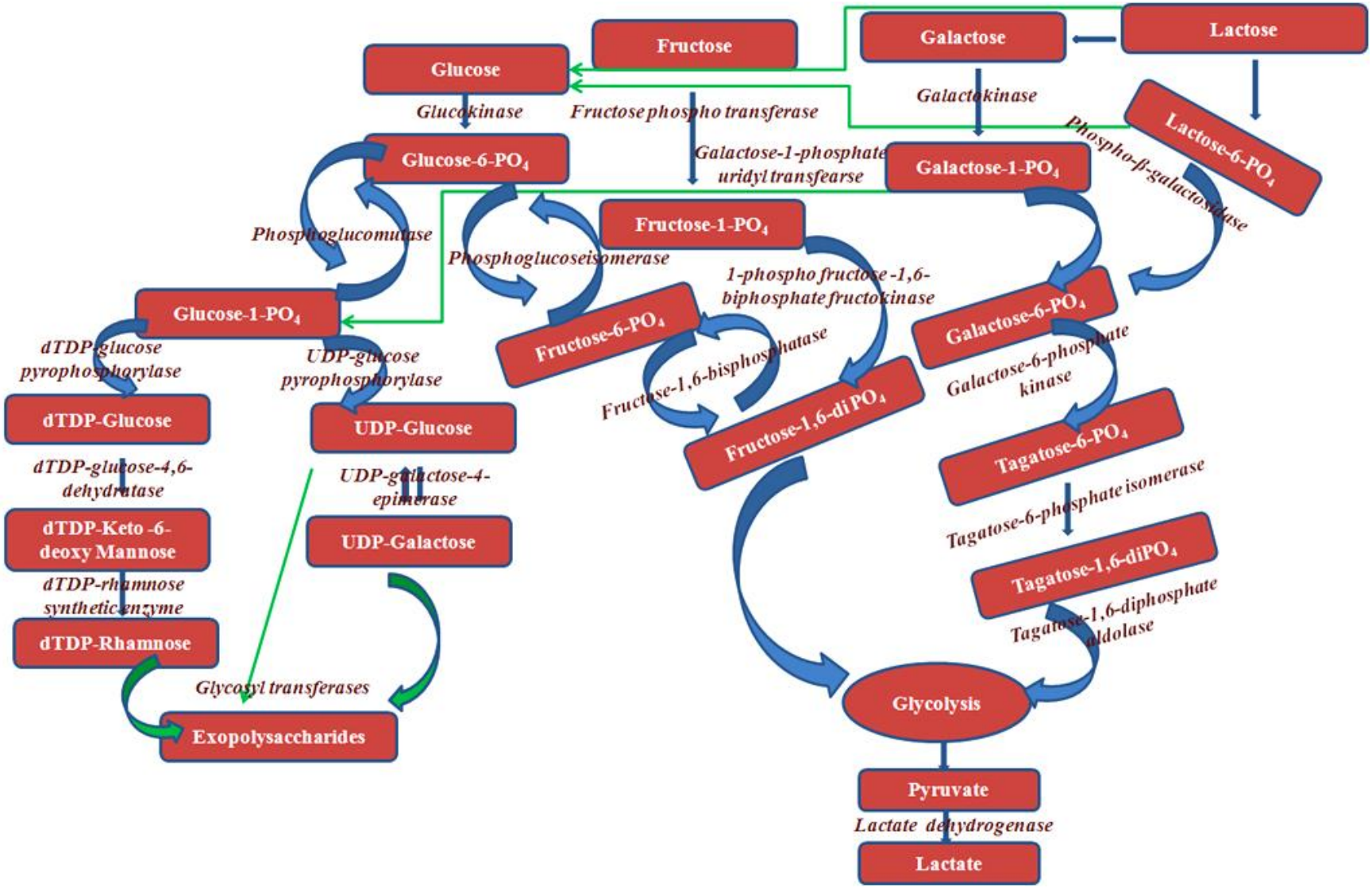


Fig. 1. 9. Biosynthetic pathway of exopolysaccharides employing *glycosyl transferases*

precursor dTDP-L-rhamnose were identified in the EPS biosynthesis locus, which is unusual for lactic acid bacteria. These four genes are expressed from their own promoter (P2), as well as co-transcribed with the upstream EPS genes, resulting in coordinated production of the rhamnose precursor with the enzymes involved in EPS biosynthesis.

Exopolysaccharide (EPS) genes in the *L. salivarius* UCC118 genome are mainly clustered in two regions, at positions 0.99–1.101 Mb and 1.62–1.65 Mb in the chromosome (Claesson *et al.*, 2006). The arrangement of the EPS biosynthesis genes in this species is in contrast to species such as *L. johnsonii* (Pridmore *et al.*, 2004), *L. acidophilus* (Altermann *et al.*, 2005) and *L. rhamnosus* (Péant *et al.*, 2005), in which most of the EPS genes are clustered in a single operon. Cluster 1 (LSL_0977–LSL_0997) contains 21 genes that include two putative chain length determinators, an oligosaccharide translocase or flippase and 12 glycosyltransferases. A homolog encoding a priming glucose phosphotransferase appears to be absent from this cluster. Cluster 1 shows low level similarity with other bacterial EPS clusters which is restricted to a minority of the glycosyl transferase genes and biosynthesis genes at either end of the cluster. Cluster 2 (LSL_1547-LSL_1574) has lower GC content (29 %) compared with the rest of the genome and comprises 21 EPS-related genes, interrupted by 7 genes of unknown or unrelated function, 14 of the genes in this cluster show significant homology to genes in the major EPS cluster of *L. plantarum*, but there is no extended synteny.

L. salivarius EPS cluster 2 includes genes for a LytR-type transcriptional regulator, a flippase and seven glycosyltransferases, including a priming glucose phosphotransferase (*EpsE* homolog; LSL1550), and, thus, represents a more complete unit than Cluster 1, relative to characterized EPS operons.

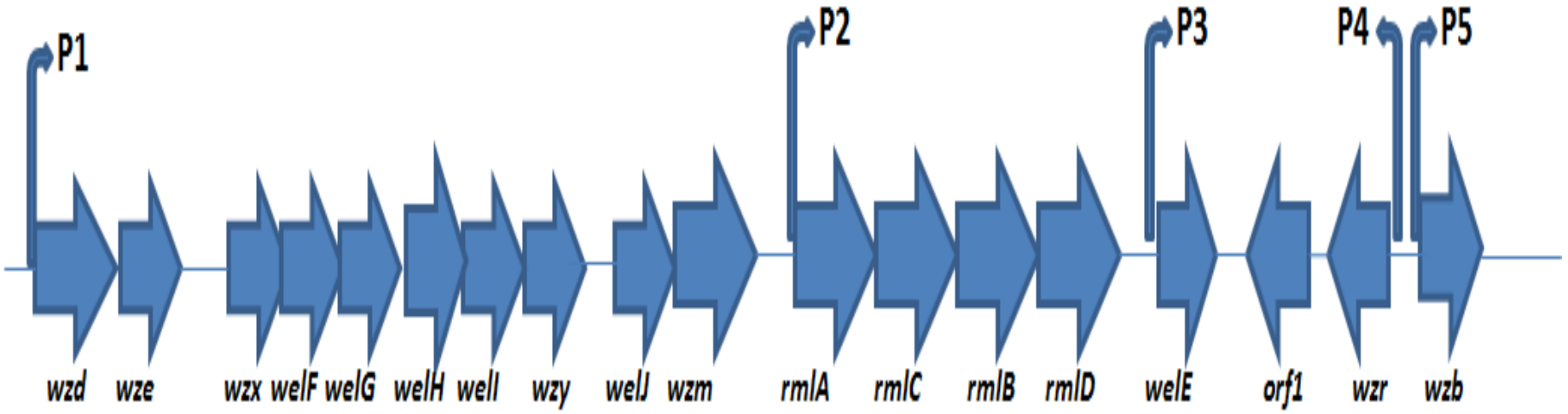


Fig. 1. 10. Organization of EPS gene cluster in *Lactobacillus rhamnosus* ATCC 9595, RW-9595M, R and RW-6541M (Source: Péant et al., 2005). P1, P2, P3, P4 and P5: Promoters

The EPS gene cluster of *L. fermentum* TDS030603 was characterized by Dan et al. (2009). The gene cluster of size 11 kb is located in the chromosomal DNA encoded with 13 open reading frames. Out of the 13 open reading frames, six were found to be involved in exopolysaccharide synthesis and five were similar to transposase genes of other *Lactobacilli* and two were functionally unrelated. One unknown gene and a putative transcription elongation factor encoding gene (*tef1*) were located upstream of *epsB*, followed by *epsC*, *epsD*, *epsE*, *epsF* and *epsG*. One transposase-like gene (*tnp1*) was present between *epsE* and *epsF*, and three transposase-like genes (*tnp2*, *tnp3* and *tnp4*) were present between *epsF* and *epsG*. Downstream of *epsG*, a transposase-like gene (*tnp5*) was found. The first transposase-like gene, *tnp1*, was identical to *tnp4*. Southern analysis using specific primers for the exopolysaccharide genes indicated that duplication of the gene cluster did not occur. The plasmid-cured strain maintained its capacity for exopolysaccharide production, confirming that the exopolysaccharide gene cluster of this strain is located in the chromosomal DNA, similar to thermophilic lactic acid bacteria.

1.3.6. Potential Applications of Exopolysaccharides from LAB

Exopolysaccharides are found to perform both ecological and physiological functions. The ecological functions of EPS produced by LAB are protection against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds, osmotic stress, metal ions, lysozymes (Looijesteijn *et al.*, 2001) and the major physiological functions falls into probiotic action, anti-tumour, anti-ulcer and immuno-modulating activity and cholesterol lowering (Chabot *et al.*, 2001). In this aspect the effect developed by EPS from probiotic strains gain more importance as they can spent more time in the gut and perform physiological functions. EPS produced by probiotics support the host strain to adhere to intestinal epithelial cells and also to interfere with pathogenic bacterial adhesion to human

intestinal mucus. This shows the important role of EPS from probiotic lactic acid bacteria in gut colonization eliminating pathogens.

Dextrans from LAB are being used in x-ray and other photographic emulsions. This result in the more economical usage of silver compounds and at the same time reduces surface gloss on photographic positives. They are used extensively in oil drilling muds to improve the ease and efficiency of oil recovery. They also have potential use in agriculture as seed dressings and soil conditioners.

1.3.6.1. Food Industry

Exopolysaccharides produced by lactic acid bacteria and many other bacteria are being widely used as viscosifiers, stabilizers, emulsifiers or gelling agents to increase the rheological properties and texture of food products. Over the past few decades, interest in using microbial exopolysaccharides in food processing has been increasing because of three main reasons: (a) their nature of polydispersity (b) water binding and low solution viscosity (c) their ease of production by fermentation and their ease of manipulation by recombinant DNA technology (Morris, 1995). Even if bacterial exopolysaccharides are limited to market niches, they could offer a better quality or a high level of purity. EPS production by diverse probiotic organisms could be significant for applications in the food industry provided they give sufficient yield and beneficial effects to the host. Probiotic strains which are facultative anaerobes would be more effective in producing energy and could overcome the problem of low oxygen level, which occurs during EPS production by obligate aerobes, *viz. X. campestris.* Agro-Food industries are always looking for new products which encourage focusing on exopolysaccharides from *Lactobacillus* species. The low yields of polysaccharide production by the majority of LAB species is the main reason of their non commercial exploitation. But, their vast structural diversity opens the way to innovations.

Yogurt manufacture remains the most important commercial application for EPS producing LAB in dairy foods, and several investigators have studied the impact of EPS producing starters on the physical properties of yogurt. Creaminess and firmness of food products are the attracting factors concerned with dairy products. This is better achieved with the incorporation of exopolysaccharides in food products or with the incorporation of probiotic strains which could produce EPS *in situ*. Problems like low viscosity, gel fracture or high syneresis (whey separation), frequently encountered during yoghurt manufacture can be solved by the use of EPS and they improve the sensory and functional properties of foods (Sikora *et al.*, 2003). EPS prevent syneresis and improve product stability firstly by increasing the viscosity and elasticity of the final product, and secondly by binding hydration water and interacting with other milk constituents, such as proteins and micelles, to strengthen the rigidity of the casein network. It is speculated that the increased viscosity of EPS-containing foods may increase the residence time of ingested fermented milk in the gastrointestinal tract and therefore be beneficial to a transient colonisation by probiotic bacteria. Adapa and Schmidt (1998) showed that less syneresis occurred in sour cream made with *S. thermophilus* strains that produced larger versus smaller capsular polysaccharides (CPS). Strains producing larger CPS also imparted a higher apparent viscosity as well as greater adhesiveness and gumminess to sour cream, which increased the "stringy" nature of the product. Research shows that ropy EPS producers and non-ropy CPS producers of *S. thermophilus* can influence the rheological properties of soft set milk products. But the effect of each strain will be different. Although the amount of polymer produced certainly affects milk viscosity, Tunier (1999) showed that the functional impact of EPS on milk products was largely determined by the polysaccharide's molar mass, monosaccharide composition, and linkage type.

EPS producing lactic cultures are also useful for stirred fermented milk products as stirred products possess a smooth, creamy texture and are made by mild homogenization of the coagulum after fermentation. The mechanical damage caused to fermented milks from pumping, blending and filling machines is reduced by the presence of EPS in stirred-type fermented milks. Mechanical processing steps also increase syneresis of the final product, but the presence of EPS produced by the lactic acid bacteria strain prevent this defect. Various investigations have been made to study the impact of EPS on fermented milks. It has been seen that the EPS produced by LAB modify the microstructure of fermented milks and these changes are then manifested as changes in rheological and other physical properties (Duboc & Mollet, 2001). The microstructure of set type fermented milks consists of a matrix of aggregated casein particles in which fat globules are embedded. The cavities of the gels are filled with serum and bacterial cells. In case of fermented milks containing EPS producing cultures, an envelope of EPS is observed surrounding the bacterial starter strains, by which ropy cells attach to the protein matrix via a web of filaments. It has been observed that the attachment of cells to the protein matrix is more pronounced in set-type fermented milks than in stirred products. Studies have also suggested that the protein gels obtained with ropy strains and non-ropy strains exhibited outstanding difference with smaller and larger cavities respectively. The larger cavities are filled with bacteria and serum (Khurana, 2006).

The consistency and texture of the final product is the result of a complex set of interactions between the different food components, which requires EPS with the right structure, conformation and properties. These polymers must be compatible with any other food components present or food processing conditions applied, *i.e.*, stability of function is often required over a wide range of pH, ionic strength and/or temperature.

EPS also finds its role in starch/hydrocolloid mixtures. Starch/hydrocolloid mixtures are widely used to modify and control the texture of foodstuffs. The addition of a

hydrocolloid strongly influences the gelatinization and retro-gradation of starch. It has been demonstrated that the structure of the hydrocolloid, including the type and number of monosaccharide backbone as well as the type, number and distribution of side units, determines its characteristics and behaviour in solutions. The four major factors influencing hydrocolloid properties include molecular weight, the monosaccharide backbone, type of side chains and distribution of side chains. Understanding such properties will lead to improvements in the formulation of starch-based foods. Texture profile analysis shows that hardness, consistency, adhesiveness, chewiness, relaxation and modulus are significantly lower in cheese fermented by EPS-producing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sp. *bulgaricus* culture (Ahmed *et al.*, 2005). Some of the applications of EPS and their relation with glycosidic linkages are listed in Table 1.5. The incorporation of EPS-producing cultures in cheese makes them smooth, creamy, moist and soft while the one made without EPS-producing strain turns dry and granular. It has been established that EPS producing cultures enhance consumer acceptability of Karish, a traditional Egyptian variety of cheese and fruit or vegetables fortified cheese by improving their spreadability and creaminess (Ahmed et al., 2005). Such products of high consumer demand are conventionally produced by adding fat, sugar, protein or stabilizers like pectin, starch, alginate or gelatine content. EPS produced by *Lactobacilli* favourably influence the properties of bread by facilitating water absorption, softening the gluten content of the dough improving the structure build-up, increasing specific volume of loaf, retarding bread staling and prolonging shelf life (Tieking & Ganzle, 2005).

Exopolysaccharides in the role of prebiotics alleviate lactose intolerance, enhance immunity against pathogens and reduce mutagenic enzymes like β-glucuronidase, nitroreductase and chologlycine hydrolase (de Roos & Katan, 2000). Prebiotic oligosaccharides derived from exopolysaccharides are non-carcinogenic, acid resistant, non-

digestible, low calorific compounds and they are used as nutraceuticals. The oligosaccharides fermented by *Leuconostoc mesenteroides* are readily catabolised by *Bifidobacteria* and *Lactobacilli*, which promotes their luxurious growth. Conversely, *Salmonella* and *Escherichia coli* are unable to utilize the oligomers and hence their proliferation is inhibited. This manifests into the domination of *Bifidobacteria* and *Lactobacilli* in the intestinal flora. Oligosaccharides are used as food additives in soft drinks, cookies, cereals, candies and dairy products.

1.3.6.2. Therapeutic Industry

Even though polysaccharides are used as emulsifying, thickening and stabilizing agents, their biological importance make them a good candidate in food market. Polysaccharides perform different tasks for microbial integrity and survival. Intracellular polysaccharides play a very important role in various biochemical reactions taking place inside cells. Healthy compounds have appeared with the production of oligo or monosaccharide syrups. Most of them possess interesting biological properties such as oligo-dextrins showing cholesterol lowering effect and anti-ulcer activity. In fact, it has been suggested that the health-promoting effect of EPS producing strains are related to the biological activities of these biopolymers. EPS might contribute to human health as prebiotics or due to anti-tumour, anti-ulcer, immunomodulating or cholesterol-lowering activities (De Vuyst *et al.*, 2001; Ruas-Madiedo *et al.*, 2002). Numerous reports have indicated that both LAB and fermented milk exert anti-cancer effects (Lee *et al.*, 2004). Data from epidemiological and experimental studies have also indicated that the ingestion of certain LAB strains or of fermented dairy products, might alleviate the risk of certain type of cancers, and inhibit the growth of tumours (Kato *et al.*, 1994).

Table 1.5 Applications of various exopolysaccharides from lactic acid bacteria

Exopolysaccharides	*Linkage*	*Applications*
Dextran	α (1, 6) glycosidic linkages in main chain and α (1, 2), α (1, 3) and α (1, 4) branched glycosidic linkages	adjuvant, emulsifier, carrier and stabilizer in food and pharmaceutical industries, plasma substitute, matrix of chromatography column, anticoagulant, paper industry, metal-plating processing, enhanced oil recovery, biomaterials
Alternan	α (1, 6) and α (1, 3) glycosidic linkages, with some α (1, 3) branching	Prebiotics, sweetener in confectionaries, low viscosity bulking agent and extender in foods
Reuteran	α (1, 4) linkage, also α (1, 6) glycosidic bonds	Used in bakery
Levan	β (2, 6) glycosidic bonds β (2, 1) linked side chains	Prebiotic, antitumor property, hypocholesterolaemic, agent, eco-friendly adhesive, bio-thickener in food industry

Exopolysaccharides	***Linkage***	***Applications***
Inulin	β (1, 2) glycosidic bonds	Prebiotics, nourishes gut mucosal cells and inhibits pathogens, targeted drug delivery against colon cancer, fat- substitute in food products
Kefiran	Glucose and galactose monomers form variable glycosidic bonds	Improve visco-elastic properties of acid milk gels, antimicrobial and wound healing properties, lower blood pressure and serum cholesterol, tumour growth retardation, enhance gut immunity
Oligosaccharides		Prebiotic, nutraceutical, alternative of antibiotics, food additives, humectant, colon cancer prevention, treatment of chronic constipation, serum lipid level reduction, skin cosmetics

Exopolysaccharides are claimed to lower blood-cholesterol. The 2005 Dietary Guidelines for Americans Index ranked cardiovascular disease as the leading cause of death among adult Americans (Fogli-Cawley *et al.*, 2007). Several studies points to the fact that the consumption of certain cultured dairy products reduce serum cholesterol (Nakajima *et al.*, 1992). Therefore, interest in the use of probiotics for lowering blood cholesterol levels has been increasing. The mechanism behind the removal of cholesterol by EPS or EPS producing cultures is still not clear. From several *in vitro* studies, a number of mechanisms have been proposed for the purported cholesterol lowering action of probiotic bacteria (Pereira & Gibson, 2002). They include the assimilation of cholesterol, cholesterol binding to the bacterial cell wall, microbial transformation of cholesterol to coprostanol and enzymatic deconjugation of bile salts (De Smet *et al.*, 1994). Gilliland et al (1985) found that certain *Lactobacillus acidophilus* strains could assimilate the cholesterol in the growth medium, thus making it unavailable for absorption from the intestines into the blood. Another plausible mechanism in the removal of cholesterol is the binding of cholesterol to bacterial cells. Nakajima et al (1992) focused on the cholesterol-lowering activity of milk fermented with an EPS-producing lactic acid bacterium. Tok and Aslim (2010) reported that EPS has a potential to interfere with the absorption of cholesterol, or of bile acids, from the intestines by binding and removing them from the body in a manner similar to the process that was reported for plant-based polysaccharides or dietary fiber.

It is also interesting to correlate prebiotic effect with short chain fatty acids production such as acetic acid, propionic acid and butyric acid secreted by several lactic acid bacteria-producing EPS (*L. delbrueckii* ssp. *bulgaricus*, *Streptococcus salivarus* ssp. *thermophilus*, *Pediococcus damnosus* and *L. reuteri*) (Lambo-Fodge *et al.*, 2006). They decrease colonic pH resulting in an increase of mineral solubility, a decreased formation of secondary bile acids and a decrease of the proliferation of unwanted pathogens. More

specifically, these short chain fatty acids have been described as energy substrate for the colonocytes, playing a role in the prevention of ulcerative colitis and cancer and as cholesterol synthesis inhibitors. It has been proved that kefiran from *L. kefiranofaciens* can reduce blood pressure, cholesterol and blood glucose rates in doses about 100-300 mg/kg on rats (Maeda *et al.*, 2004a; Maeda *et al.*, 2004b).

The first experience of application of polysaccharides as bioactive polymers is the use of a dextran-based blood-plasma substitute (Tsujisaka & Mitsuhashi, 1993). They maintain the volume of circulating blood and its osmotic pressure in cases of massive loss of blood. The therapeutic effect of these polysaccharides is determined by molecular weight and conformational characteristics of the polymeric substance. High-molecular-weight fractions with molecular weight of 200 kDa can provoke toxic reactions and preclude normalization of the blood microcirculation. Therefore, the molecular weight and concentration of polymer are adjusted so as to obtain a solution with a viscosity corresponding to that of blood (2.8–4.0). Usually, this is 6 and 10 g/dl for dextran with molecular weight of 70 and 40 kDa, respectively. Bacterial EPS could counteract the activity of bacteriophages (Durlu-Özkaya *et al.*, 2007) and of toxic compounds (Kim *et al.*, 2006).

Two main bioactivities exhibited by EPS from LAB are the anti-cancer and immunomodulatory effects. The potential broad spectrum bioactivity of this class of compounds as anti-cancer adjuvants is highlighted by the biological mechanisms such as apoptotic and anti-angiogenic effects including its effects on the c-Myc, c-Fos and vascular endothelial growth factor (VEGF) expression (Yang *et al.*, 2005). EPS produced by *Lactococcus lactis ssp. cremoris* KVS20 exhibits bioactivity, such as lymphocyte mitogenicity (Kitazawa *et al.*, 1993), macrophage cytostaticity, and cytokine (IFN-γ and IL-1β) production in macrophages (Kitazawa *et al.*, 1996).

The structure-function relationship of EPS plays an important role in its bioactivity. The bioactivity is affected by molecular weight, tertiary structure or conformation and composition. Polysaccharides in a configuration with β (1, 3), (1, 4) or (1, 6) branch chains are necessary for activity and complex branch-chained polysaccharides with anionic structures and higher molecular weights have greater immunostimulating activities (Cleary *et al.*, 1999). The stimulated immune pathway of polysaccharides from LAB, fungi, or plants is different from lipo-polysaccharides due to the above mentioned reasons. The differences in receptor affinity or receptor-ligand interaction on the cell surface can result in a bioactivity difference (Mueller *et al.*, 2000).

The antitumour effects of cell-bound exopolysaccharides (cb-EPS) from *Lactobacillus acidophilus* 606 on colon cancer cells was studied by Kim et al (2010). They found that the EPS from *L. acidophilus* developed antitumourigenic property against HT-29 colon cancer cells due to the activation of autophagic cell death directly by the induction of Beclin-1 and GRP78, as well as indirectly through the induction of Bcl-2 and Bak. Recently, Liu et al (2011) compared the antioxidant activity and immunomodulation of exopolysaccharides from *Lactobacillus paracasei* ssp. *paracasei* NTU 101 (101EP) and *Lactobacillus plantarum* NTU 102 (102EP) under *in vitro* conditions. They isolated EPS from both cultures and found that the EPS promote macrophage growth and induce the production of pro-inflammatory responses in the murine macrophage cell line, Raw 264.7. TNF-α, IL-1β, and IL-6 release from Raw 264.7 macrophages was found to increase proportionately with the dosage of EPS from LAB strains.

1.4. Conclusion

The growing concerns about health and well-being, along with an interest in consuming natural foods, have given probiotics a unique position in the food market and the benefits from probiotics and synbiotics had given an opportunity to explore the various

nutraceuticals from microorganisms. Adequate genetic tools developed during the recent years for lactic acid bacteria offers perspectives to improve the yield and properties. In the exploration of exopolysaccharides from novel sources LAB play a crucial role due to their GRAS status. Exopolysaccharides remain largely under exploited due to the lack of technological innovations. The recent structural studies and structure-function relationship of exopolysaccharides from LAB open the way for enormous research in the field of structural modification and industrial applications. However, many of the questions yet to answer regarding its therapeutic use and its interactions with the host offer a fantastic scope for future research.

CHAPTER 2
MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2. 1. Materials

2.1. 1. Microorganisms

Lactic acid bacteria isolated from different sources like sour dough, whey, fermented vegetables (cabbage, snake gourd), excreta of sheep and human baby were used for the probiotic characterization. Twenty cultures isolated were screened for probiotic characterization which comprised of four from sour dough (designated as SD1, SD2, SD3 and SD4), three from whey (designated as W1, W2 and W3), two from snake gourd (designated as SG1 and SG2), five from cabbage (designated as CB1, CB2, CB3, CB4 and CB5), one from curd (designated as MC1), three from sheep excreta (designated as G1, G2 and G3) and two from human baby faeces (designated as WJ1 and MCJ1). Subsequently, MC1 identified as *Lactobacillus plantarum* MTCC 9510, a facultative anaerobe isolated from curd was used for exopolysaccharide production.

Escherichia coli MTCC 739, *Shigella sonnei* MTCC 2957, *Shigella flexnerii* MTCC 1457 and *Staphylococcus aureus* MTCC 96 obtained from Microbial Type Culture Collection (MTCC) , Chandigarh were used for anti-microbial activity studies. *E. coli* DH5α used for transformation was obtained from Novagen Inc., CA, USA. *Lactococcus lactis* NIZO B40, an EPS producer, which harbours EPS biosynthetic genes in an operon, was obtained from NIZO Food Research, The Netherlands.

2.1.2. Culture Media

MRS (de Man, Rogosa and Sharpe) broth was used for *Lactobacillus* species and M17 broth for *Lactococcus* species. *E. coli* cells were cultured in Luria Bertani (LB) broth. *Shigella* and *Staphylococcus* strains were grown in Nutrient agar medium. All the general

media components were obtained from Hi-media Laboratories, Mumbai. Annexure I shows the composition of different media mentioned above.

2.1.3. Cell lines and maintenance

The human breast adenocarcinoma cell line (MCF-7) for anti-tumour studies and normal cell line L929 were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA). Cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, Germany) with 10 % Fetal Bovine Serum (FBS). Lymphocytes were maintained in RPMI 1640 Medium (Invitrogen, Grand Island, New York, USA) with 10 % FBS (Gemini Bio-Products, Inc. CA, USA). Both cell line and lymphocytes were maintained in a 37°C incubator with 5 % CO_2.

All tissue culture plates and other plastic wares were from Costar (Corning, New York, USA).

2.1.4. Chemicals, Reagents and kits

The chemicals used in media for exopolysaccharide production such as glucose, lactose, sucrose, galactose, yeast extract, corn steep liquor, beef extract, tryptone, ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium citrate, sodium nitrite, potassium nitrate, sodium acetate, di-potassium hydrogen phosphate, magnesium sulphate, manganese sulphate tween 80 etc were purchased from Hi-media Laboratories, Merck (India) and SRL Pvt. Ltd. Other chemicals like deuterium oxide for Nuclear magnetic Resonance (NMR) spectroscopy, potassium bromide for Fourier transform-infrared (FT-IR) spectroscopy, dithranol for Matrix-assisted laser desorption ionization (MALDI) mass spectrometry and potassium ferricyanide and ascorbic acid for anti-oxidant activity were procured from Sigma Chemical Co., USA.

Chemicals for extraction and purification of exopolysaccharide such as ethanol, phenol-chloroform-isoamyl alcohol, acetone and the chemicals like phenol and concentrated sulphuric acid were obtained from SRL Pvt. Ltd and Sd Fine Chemicals. Sephadex G-200 used for gel filtration was procured from Sigma chemicals, USA.

Trypsin/EDTA for anti-tumour activity studies was obtained from Gibco BRL (Life Technologies Inc., Grand Island, NY, USA).

The stains used for microscopy such as crystal violet, safranin, alcian blue and ruthenium red were procured from Fischer scientific chemicals. (Mumbai, India). Acridine orange (AO), ethidium bromide (EB), and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). The dye mix for the EB/AO staining was 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS.

The T vector cloning kit, plasmid mini-prep kit and gel extraction kit were obtained from Qiagen, Germany and Fermentas, USA. T4 DNA ligase and DNA polymerase were procured from New England Biolabs, MBI Fermentas and Sigma, USA. Primers and Taq DNA polymerase were procured from IDT and Sigma Co., USA respectively.

All other molecular biology grade chemicals such as agarose, Tris, SDS, EDTA, sucrose, gelatine, DL-threonine, ampicillin, chloramphenicol, erythromycin and lysozyme used were procured from either Sigma or Merck, USA or Hi-media laboratories, India.

2.2. Computer Softwares

The software Design Expert (Version 6.0.6, Stat-Ease Inc., USA) and Statistica™ (Version 7.1) (StatSoft Inc., USA) were used for statistical bioprocess analysis of EPS production. Oligoanalyzer program (www.idtdna.com) was used to analyse specific PCR

primers for their T_m values and secondary structures, prior to getting them custom synthesized.

2.3. General Microbiology

2.3.1. Microorganisms and Maintenance

All microbes except the *Lactococcus spp*. were grown at 37 ºC and the *Lactococcus* strains were grown at 30 ºC. All microorganisms were sub-cultured in their appropriate medium as mentioned in 2.1.2 and were maintained in agar slants or stabs at 4 ºC for immediate use and sub-cultured every two weeks. For long time preservation they were maintained in 30 % glycerol stock and stored at -80 ºC.

2.3.2. Preparation of Inoculum

For EPS production, an inoculum of *Lactobacillus plantarum* MTCC 9510 (MC1) was prepared in 50 ml MRS medium in 250 ml Erlenmeyer flasks by inoculating with a loop full of culture from agar stabs and incubating at 37 ºC for 18 h. The culture absorbance was noted at 620 nm and unless specified, 10^9 CFU/ml culture was used as inoculum.

2.3.3. Fermentation and Extraction of EPS

Unless otherwise mentioned, exopolysaccharide production was done by submerged fermentation (SmF) in 250 ml Erlenmeyer flasks with 50 ml exopolysaccharide production medium (Annexure I). More description on fermentation was given in the chapter dealing with submerged fermentation (chapter 4, section 4.2.2). The steps involved in the EPS extraction from lactic acid bacteria are shown as a flow chart (Fig. 2.1).

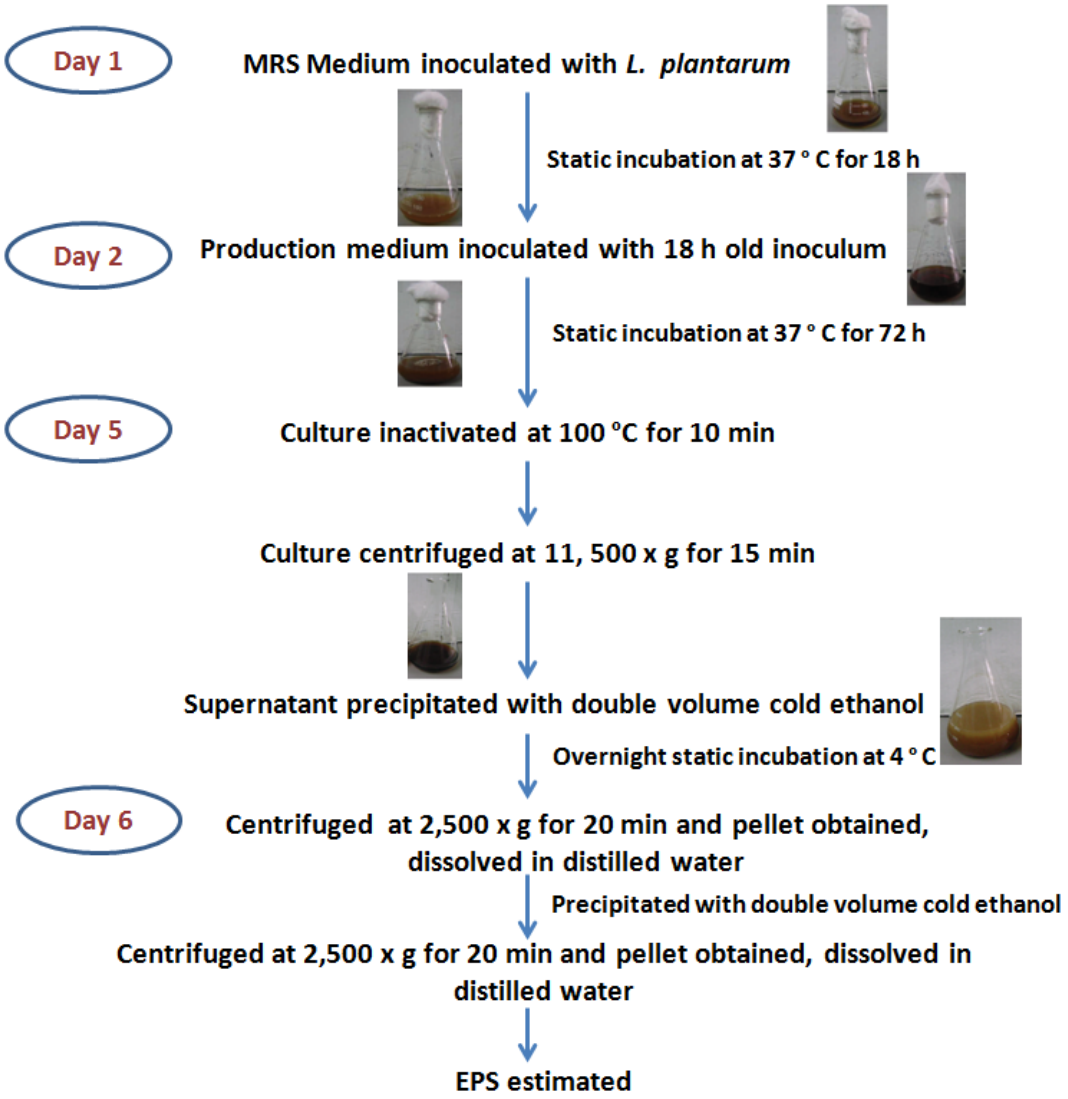


Fig. 2. 1. Steps involved in the extraction of EPS from lactic acid bacteria

2.4. Analytical Methods

For the convenience, specific methods/assays had been described in corresponding chapters. Only the general analytical methods covered in this chapter.

2.4.1. Phenol-Sulphuric Acid Method for EPS detection (Total Carbohydrate)

Exopolysaccharides were estimated as total carbohydrates by phenol-sulphuric acid method (Dubois *et al.,* 1956). Concentrated sulphuric acid causes hydrolysis of glycosidic linkages, these hydrolysed neutral sugars are then partially dehydrated with the elimination of three molecules of water to form furfural or furfural derivatives. The coloured compounds developed by the condensation of furfural or furfural derivatives with phenol are measured at 490 nm.

2.4.1.1. Reagents

Phenol - 5 % (w/v)

Sulphuric acid – Concentrated (35.7 N)

2.4.1.2. Procedure

To 1 ml of sample, 1ml 5 % (w/v) phenol was added followed by 5 ml concentrated sulphuric acid. The sample tubes were kept in ice while adding sulphuric acid. The mixture was incubated at room temperature for 20 min and the absorbance read at 490 nm. Glucose was used as the standard in the range of 0-100 µg concentration from 1 mg/ml stock solution. A standard graph (Fig. 2.2) was plotted with absorbance at 490 nm against concentration of glucose. A blank was also prepared in the same way (0 mg glucose).

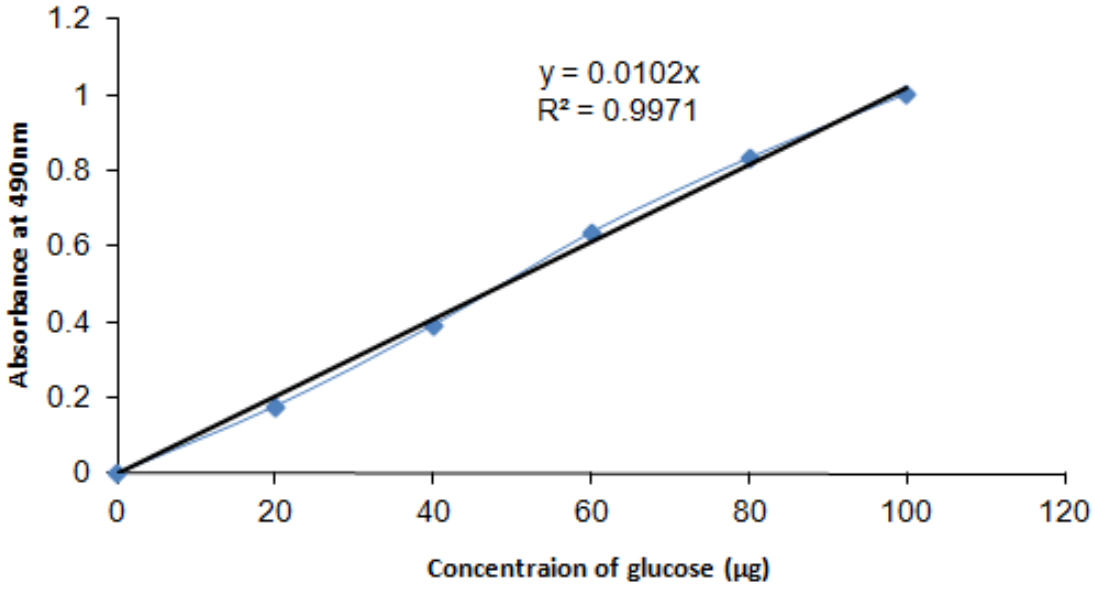


Fig. 2.2. Standard graph for phenol-sulphuric acid method

2.4.2. Lowry's Assay for Total Soluble Protein

Total soluble protein content present in the samples was estimated by Folin-Lowry method (Lowry *et al.*, 1951). The assay is based on the biuret reaction of proteins with cupric sulfate at alkaline conditions and the Folin-Ciocalteau phosphomolybdotungstate reduction to heteropolymolybdenum blue which is measured at 660 nm. The reaction is primarily due to the presence of the amino acids tyrosine and tryptophan, and to a lesser extent cystine, cysteine, and histidine.

2.4.2.1. Reagents

Reagent A – 2 % Na_2CO_3 in 0.1 N NaOH

Reagent B – 0.5 % $CuSO_4$ in 1 % Potassium sodium tartarate

Reagent C – Reagent A and Reagent B in the ratio of 50:1

Regent D – 1N Folin-Ciocalteau reagent

2.4.2.2. Procedure

To 1 ml sample, 5 ml reagent C was added and mixed well. The mixture was incubated at room temperature for 10 min. To this added 0.5 ml reagent D, mixed and kept at dark for 20 min. The resulting colour was measured at 660 nm. Bovine Serum Albumin (BSA) was used as the standard for preparing the standard graph (Fig. 2. 3).

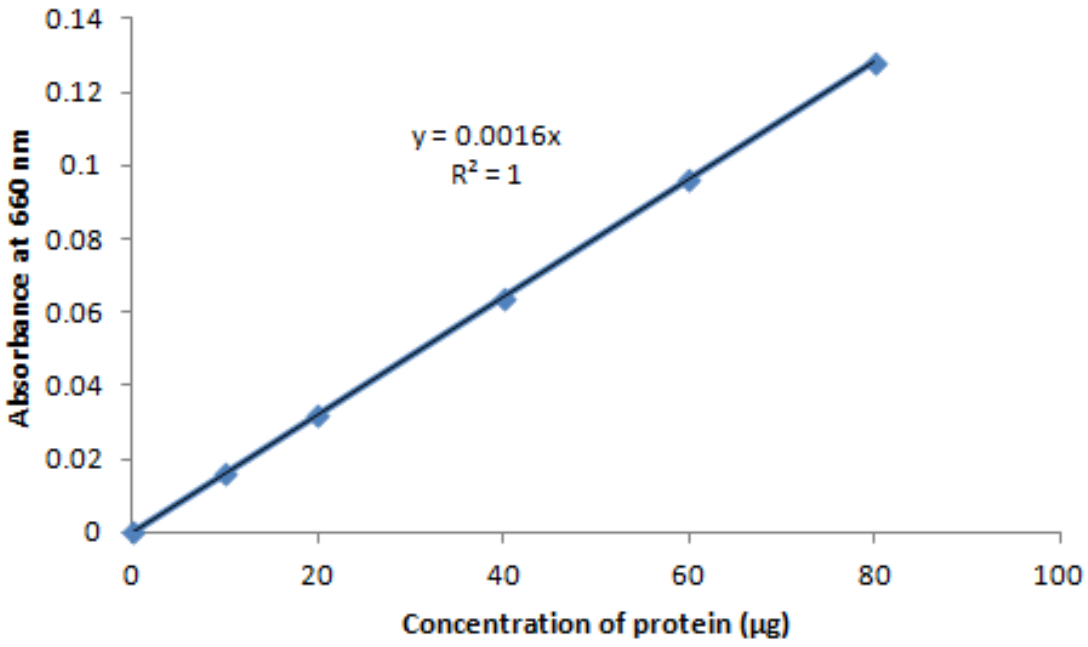


Fig. 2.3. Standard graph for Lowry's estimation of proteins

2.4.3. Barker & Summerson Assay for Lactic Acid Detection

Total lactic acid produced by the organisms was estimated by Barker & Summerson assay (1941) for lactic acid. Heating culture supernatant (containing lactic acid) in concentrated sulphuric acid produced acetaldehyde which was directly determined by the purple colour formed with p-hydroxy diphenyl in the presence of cupric copper. This blue colour was measured at 560 nm.

2.4.3.1. Reagents

Copper sulphate – 4 %

Sulphuric acid – Concentrated

p-hydroxydiphenyl – 1.5 % p-hydroxydiphenyl in 0.5 % NaOH

2.4.3.2. Procedure

To 1 ml of sample, added 0.05 ml of 4 % $CuS0_4$ mixed well and added 6 ml of concentrated sulphuric acid. The mixture incubated in boiling water bath for 10 min and added 100 µl of p-hydroxydiphenyl and kept in room temperature for 30 min. The absorbance read at 560 nm. Lactic acid (Sigma) was used as the standard in the range 0-25 µg. Standard graph plotted with absorbance at 560 nm against concentrationof lactic acid shown in Fig. 2.4. A blank was prepared in the same way (0 µg lactic acid).

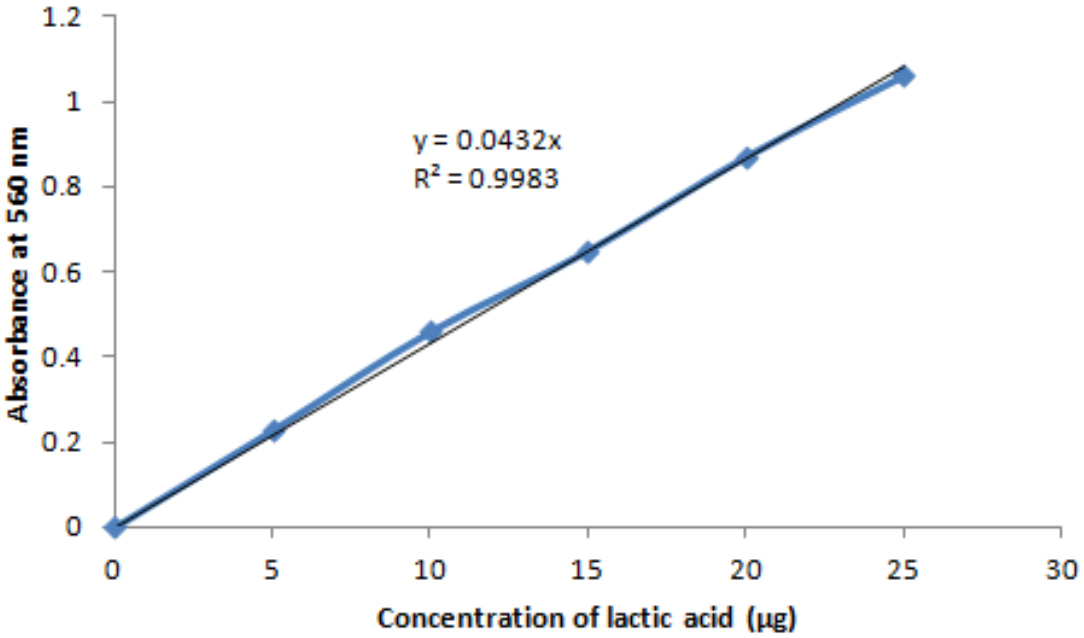


Fig. 2. 4. Standard graph for lactic acid assay

2.4.4. Dinitro Salicylic Acid (DNS) method for Reducing Sugar Analysis

The amount of reducing sugars in samples was estimated by DNS method (Miller, 1959). The aldehyde group of sugars reduces 3, 5-dinitro salicylic acid to 3-amino, 5-nitro salicylate giving rise to a brown colour measured at 575 nm.

2.4.4.1. Reagents

3, 5-Dinitro salicylic acid - 1 %

Sodium sulphite - 0.05 %

Sodium hydroxide -1 %

Phenol - 0.2 %

Rochelle salt - 40 %

2.4.4.2. Preparation of DNS

Dinitro salicylic acid and sodium hydroxide in water was dissolved gently in a water bath at 80 ºC until a clear solution was obtained and then added the remaining chemicals. Once the ingredients are dissolved, the solution is filtered and stored at room temperature in amber coloured bottles in order to avoid photo oxidation.

2.4.4.3. Procedure

To 1 ml of sample, added 3 ml of DNS reagent and incubated in boiling water bath for 5 min. The colour developed was read at 575 nm. Glucose was used as the standard for the preaparation of standard graph (Fig. 2. 5). A blank was also prepared in the same way.

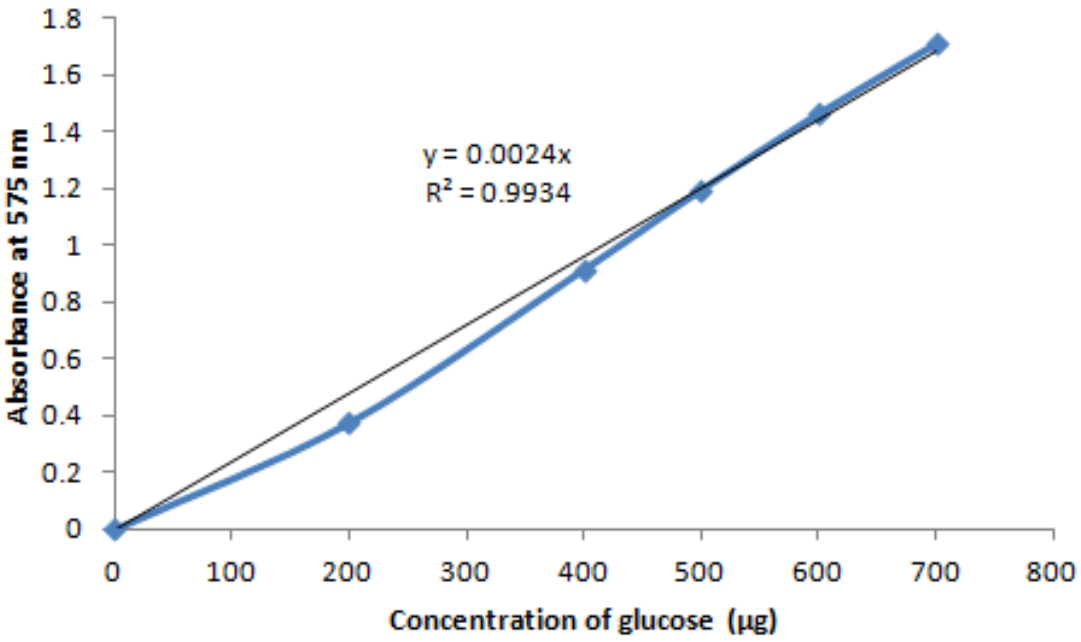


Fig. 2. 5. Standard graph for DNS method of reducing sugar analysis

2.5. Agarose Gel Electrophoresis

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. The yield and purity of genomic and plasmid DNA, estimation of size of DNA molecules, analysis of PCR products etc were done using agarose gel electrophoresis. 0.7-1 % agarose has been used with ethidium bromide to view DNA under UV light. Tris-acetate-EDTA (TAE) buffer (1X, 40 mM Tris-acetate and 1 mM EDTA, pH 8.3) was used as running buffer for agarose gel electrophoresis.

2.6. Molecular Methods

Molecular biology methods such as plasmid DNA isolation from *E. coli*, PCR amplification, competent cell preparation of *E. coli* and transformation were carried out as per standard protocol described by Sambrook et al (1989) and explained in detail in chapter 7 (section 7.2.5.1 and 7.2.5.2).

2.7. Equipments

The major equipments used for this study were listed in Annnexure I. Most of the facilities were available in NIIST, Trivandrum and some of the microscopic observations of cell lines were done in nearby institutes in Trivandrum, Rajiv Gandhi centre for Biotechnology (RGCB), Regional cancer Centre (RCC), and Sree Chithira Thirunal Institute (SCTI).

CHAPTER 3

ISOLATION OF LACTIC ACID BACTERIA AND PROBIOTIC CHARACTERIZATION

CHAPTER 3

ISOLATION OF LACTIC ACID BACTERIA AND PROBIOTIC CHARACTERIZATION

3.1. Introduction

Lactic acid bacteria (LAB) are gram-positive, non-spore forming bacteria naturally present in raw food material and in the human gastro-intestinal tract. LAB plays an important role as starter cultures for fermentation in dairy, meat and other food industries. These food-grade bacteria can improve safety, shelf life, nutritional value, flavour and quality of products. They are considered industrially important organisms because of their fermentative ability as well as health and nutritional benefits. These bacteria are also well-known for their probiotic effects.

Probiotics are live microorganisms thought to be beneficial to the host organism. According to the definition by FAO/WHO, probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". The probiotic concept is open to a large number of applications in various fields relevant to human and animal health. Developing probiotic food and feed is a key research and development area for future functional food markets. Probiotic products include exopolysaccharides (EPS), vitamins, enzymes, capsules or tablets, and some fermented foods containing microorganisms. The mechanisms of probiotic action include the production of inhibitory substances, blocking of adhesion sites, competition for nutrients, stimulation of immunity and degradation of toxin receptor (Çakır, 2003). A successful probiotic strain is expected to be of a) human origin for human usage, this criteria is important for species dependent health effects, b) acid and bile tolerant, for survival

through intestine (Holzapfel *et al.*, 2001), c) adherent to mucosal surface, to improve immune system, d) safe for food and clinical use, e) clinically validated and documented for health effects and f) possessing good technological properties (Çakır, 2003). They create a healthy equilibrium between beneficial and potentially harmful microorganisms in the gut, by competitive exclusion and by the production of organic acids, enzymes, vitamins, antioxidants, exopolysaccharides and antimicrobial compounds preventing intestinal infection (Salminen *et al.*, 1996). The employment of antibiotics in animal feeding can be substituted with the development of products with probiotic characteristics.

The present chapter describes the isolation of lactic acid bacteria from different sources like fermented vegetables, sour dough, curd, whey, sheep excreta and human baby faeces as well as decaying plant or animal matter and the investigation of their probiotic properties and finally screening their efficacy to produce exopolysaccharide (EPS). The functional and technical properties of probiotics are strain specific and thus it is necessary to screen various isolates for a variety of *in vitro* properties to obtain new probiotic candidates.

3.2. Materials and Methods

3.2.1. Bacterial Strains and Growth Conditions

Microorganism, its maintenance and inoculum preparation had been described in chapter 2 (section 2.1.1, 2.1.2, 2.3.1 and 2.3.2).

3.2.2. Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated from different sources like sour dough, whey, curd, fermented vegetables (cabbage and snake gourd), excreta of sheep and human baby

faeces. Decimal dilution of these samples was streaked in to MRS agar (Himedia, Mumbai) plates and incubated at 37 °C for 48 h under static condition. Isolated pure cultures were identified by morphology, Gram stain (Giraud *et al.*, 1991) and simple physiological tests proposed by Sharpe (1979) using morphological, phenotypic and biochemical methods. For general biochemical characterisation, the gas production from glucose in MRS broth was tested using inverted Durham's tubes. Milk agar (Himedia) plates were used for performing casein hydrolysis. After streaking the isolates, the plates were incubated at 37 °C for 24 h to check the casein hydrolysis. Catalase test was carried out by placing a drop of hydrogen peroxide to single colonies of the culture taken in a glass slide.

3.2.3. Production of Lactic Acid and Reducing Sugar Consumption

The isolates were checked for production of lactic acid in MRS broth. The amount of total lactic acid was estimated according to the colorimetric method of Barker and Summerson (1941) and was expressed as mg/ml of the fermentation medium. The amount of reducing sugar was determined by DNS method (Miller, 1959) using a UV spectrophotometer (Shimadzu, Japan) at 575nm. The detailed protocols for both the assays were given in chapter 2 (section 2.4.3 and 2.4.4).

3.2.4. Probiotic Characterization Studies

3.2.4.1. Tolerance to Inhibitory Substances

Probiotic properties of the isolates were tested by checking their tolerance to acid, salt, phenol and bile salts. Tolerance to above mentioned inhibitory substances was studied in MRS broth by inoculating a culture of cell density 10^9 CFU. Varying pH, 2.5, 3, 4 and 5 were studied. Sodium chloride: 4, 5, 8 and 12 % (w/v); 0.2-0.5 g phenol/100 ml and 0.3, 0.5 and 0.8 g sodium taurocholate /100ml were the concentration of other

inhibitory substances chosen for the study. The cultures were incubated at 37 ºC and the optical density was noted at 620 nm after 24 h.

3.2.4.2. Hydrophobicity of Strains

The degree of hydrophobicity of the strains was determined by employing the method described by Thapa et al (2004). This method was based on adhesion of cells to hexadecane droplets. Cultures were grown in 10 ml MRS broth at 37 ºC for 24 h and centrifuged at 6000 x g for 5 min to collect the cell pellet which was washed and re-suspended in 10 ml of Ringer solution (6 % NaCl, 0.0075 % KCl, 0.01 % $CaCl_2$ and 0.01 % $NaHCO_3$). The absorbance at 600 nm was measured. Cell suspension was then mixed with equal volume of n-hexadecane and mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min and absorbance at 600 nm of the lower phase was recorded. The percentage hydrophobicity of strain adhering to hexadecane was calculated using the equation:

$$\textbf{Hydrophobicity (\%)} = \frac{\textbf{OD}_{600}\textbf{ (initial)} - \textbf{OD}_{600}\textbf{ (with hexadecane)}}{\textbf{OD}_{600}\textbf{ (initial)}} \times \textbf{100}$$

3.2.4.3. Mucin Adhesion Assay

The isolates were grown at 37 ºC in Lactobacillus MRS broth supplemented with 0.1 % mucin (Sigma, USA) for 24 h to induce binding (Jonsson *et al.*, 2001). Microtitre plate wells were coated with mucin (100 µg/µl mucin in 50 mM Na_2CO_3 buffer, pH 9.7, per well) and incubated overnight at 4 ºC with slow rotation. The reaction was then blocked with PBS with 1 % Tween 20 for 1 h and washed with PBST (PBS supplemented

with 0.05 % Tween 20, pH 7.3) (Roos & Jonsson, 2002). The bacterial strains were grown as described above, washed once in PBST and diluted (A_{595} = 0.5 ± 0.02) in to the same buffer. Bacterial suspension (100 µl) was added to each well and incubated for 1 h at 30 ºC. The wells were washed with PBST and absorbance taken at 405 nm.

3.2.4.4. Antimicrobial Activity

The test materials (compounds produced by the microbial cultures having antimicrobial activity) present in the supernatant of the culture was obtained by centrifuging the culture at 20,000 x g for 15 min. The supernatant was dried under vacuum using a 45 °C water bath and a rotary evaporator, re-suspended in one-fifth the original volume of water and filtered through sterile 0.45 µm membrane filters. The test organisms used for antimicrobial activity were *Escherichia coli* (MTCC 739), *Shigella sonnei* (MTCC 2957), *Shigella flexnerii* (MTCC 1457) and *Staphylococcus aureus* (MTCC 96). Antimicrobial activity was quantitated by a ditch assay using the test organisms (Reddy *et al.*, 1983). Actively growing culture of the test organisms were mixed at a 2.5 % (2.5 x 10^7 CFU/ml) with melted nutrient agar poured in sterile petri dishes and allowed to solidify. A 0.5mm wide ditch was cut in the agar across the centre of the dish. The test material obtained from the isolated cultures was pipetted into the ditch (0.2 ml). The plates were first incubated at 4 °C for 60 min to allow the test material to diffuse in the agar and then incubated at 37 °C for 18 h. After incubation, the diameter of the clear zone was measured in millimetres.

3.2.4.5. Antibiotic Resistance Study

All the tests were performed in Mueller-Hinton agar (Oxoid). Mueller-Hinton agar was inoculated with 50 µl of the isolates of prior adjusted OD 0.6. Inoculum was spread evenly over the entire surface of the plate by swabbing in three directions.

Antibiotic discs of vancomycin (10 µg), a glycopeptide inhibitor of cell wall and erythromycin (15 µg), an inhibitor of protein synthesis were firmly applied to the surface of the agar plates dried previously. The plates were incubated at 37 °C overnight and diameters of the zone of inhibition measured.

3.2.5. Screening for Exopolysaccharide Production

3.2.5.1. Quantitative Estimation of Exopolysaccharide Production

18 h old inoculum (10^9 CFU/ml) was prepared in MRS medium by incubating in static condition at 37 °C. Exopolysaccharide (EPS) production was achieved in MRS medium incubated under the same conditions for 72 h. EPS degrading enzymes present in the culture was inactivated by heating the culture at 100 ºC for 10 min. The culture was centrifuged at 11,500 x g for 15 min at 4 °C to remove cell pellet and the supernatant precipitated with double volume-chilled ethanol was stored overnight at 4 °C. The mixture was centrifuged at 2,500 x g for 20 min and the pellet collected was dissolved in de-mineralised water, and again precipitated using double-volume cold ethanol. It was further centrifuged at 2,500 x g for 20 min to collect the pellet (Savadogo *et al.*, 2004). The total carbohydrate present in the pellet was estimated by phenol – sulphuric acid method (Dubois *et al.*, 1956) as described earlier (section 2.4.1).

3.2.5.2. Exopolysaccharide Producing Phenotype Identification by Staining Techniques

The EPS producing capacity of the culture MC1 was confirmed by the alcian blue staining technique and ruthenium red agar method.

3.2.5.2.1. Alcian Blue Staining

Alcian blue staining is a method which can be adopted to stain exopolysaccharides or capsular polysaccharides produced by bacterial cells. A bacterial

smear was prepared on a glass slide and stained using alcian blue (1 % in 3 % glacial acetic acid, whose pH adjusted to 2.5 with acetic acid) for 5 min and washed thoroughly in water. The slide was viewed under phase-contrast microscope.

3.2.5.2.2. Ruthenium Red Agar Method

Exopolysaccharide producing strains can be distinguished from the non-producing ones with the help of ruthenium red agar method. In this method, MRS agar medium containing 0.08 % ruthenium red was used. A stock solution of ruthenium red at 10 % (w/v) in water was sterilized through a 0.45 µm filter and an appropriate volume was added to the molten agar just prior to pouring it into petri plates. Spread plate of the culture was performed and incubated at 37 °C for 24 h.

3.2.6. Identification of Selected Culture by Conventional Method and 16S rRNA sequencing

The selected bacterial strain was identified by conventional physiological and biochemical tests in IMTECH, Chandigarh and molecular identification method.

For the molecular identification method, the bacterial strain was grown in MRS broth and the genomic DNA isolated as described in Chapter 7 (section 7.2.2).Multiplex Polymerase Chain Reaction (PCR) was performed in mixtures (25 µl) containing 50 ng of DNA, 1.5mM $MgCl_2$, the four deoxynucleoside triphosphates at 150 µM each, each primer (27F 5′ AGAGTTTGATCCTGGCTCAG 3′,1492R 5′ TACGGTTACCTTGTTAC ACTT 3′, *L. plantarum* specific primer 5′ TGAACAGTTACTTCAGATA 3′ (Uchida *et al.*, 2004)) at 10 pM in *Taq* buffer, and 0.5 U of *Taq* polymerase. The PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) by following the programs as described. The amplification profile consisted of one cycle at 95 °C for 3 min, then 30 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min and finally, one

cycle at 72 °C for 8 min. Amplicons were analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer. A 1 kb DNA ladder (Fermentas) was used to identify the molecular sizes of the bands.

3.2.7. Experimental Statistics

All experiments have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

3.3. Results and Discussion

3.3.1. Isolation and Characterization of Lactic Acid Bacteria

Around twenty cultures were isolated from different sources that include four from sour dough (designated as SD1, SD2, SD3, and SD4), three from whey (designated as W1, W2, W3), two from snake gourd (designated as SG1, SG2), five from cabbage (designated as CB1, CB2, CB3, CB4 and CB5), one from curd (designated as MC1), three from goat excreta (designated as G1, G2 and G3) and two from human baby faeces (designated as WJ1, MCJ1).

All the isolates were found to be Gram positive bacteria. Most of them were cocci, and the isolates from snake gourd and curd were rods. Except the isolates from goat excreta and snake gourd, all the isolates were catalase negative which showed no bubbling when hydrogen peroxide was added. Isolates, CB2, MC1, W3, G1, SD3 and SG2, produced gas bubbles in the Durham's tube. Most of the isolates did not hydrolyse casein. Based on the Bergey's manual (Holt *et al.*, 1994), most of the isolated cultures belong to the genus *Lactococcus*, which is Gram-positive cocci type whereas those from snake gourd and curd belong to *Lactobacillus.*

3.3.2. Lactic Acid Production and Reducing Sugar Consumption

Lactic acid is the major metabolic end product of carbohydrate fermentation in lactic acid bacteria and this trait has historically linked LAB with food fermentations as acidification inhibits the growth of spoilage agents. These bacteria can either be homofermentative or herterofermentative. Homofermentative lactic acid bacteria produce more than 85 % lactic acid from glucose and heterofermentative produces only 50 % lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide. The isolates were screened for the production of lactic acid and reducing sugar after 24 h of incubation. The isolates CB1, G3 and SG2 were found to produce more amount of lactic acid, 14.49, 13.95, 16.36 mg/ml respectively. The initial carbohydrate content was 20 mg/ml. After 24 h most of the carbohydrate was converted to lactic acid. The lactic acid production profile and reducing sugar consumption is shown in Fig. 3.1.

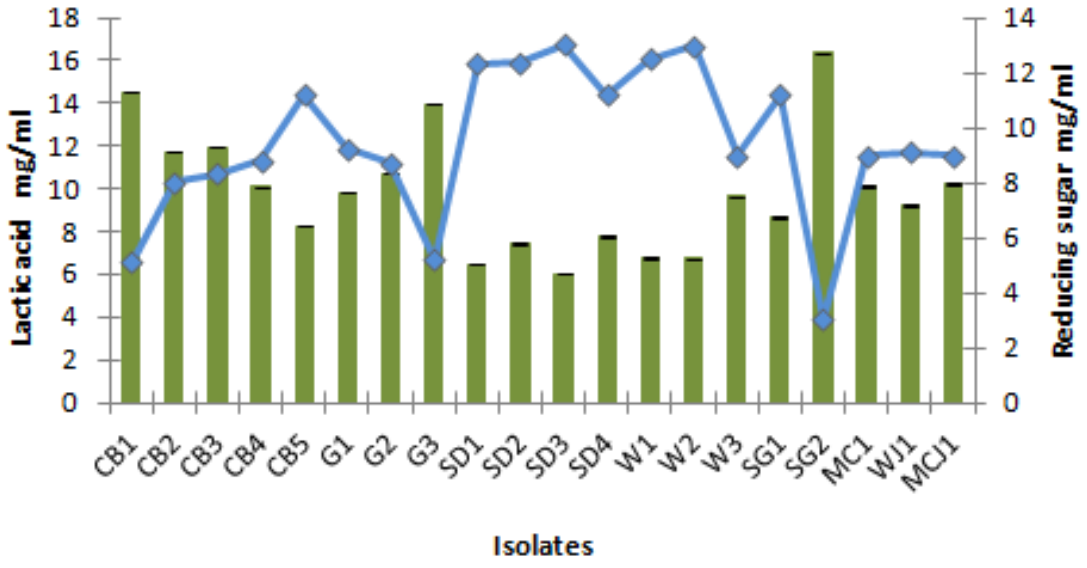


Fig. 3. 1. Lactic acid production (bar) and reducing sugar (line) consumption

3.3.3. Probiotic Characterization Studies

3.3.3.1. Tolerance to Inhibitory Substances

The main functional aspect concerning a probiotic is its survival in the gastro-intestinal tract. The survival of bacteria in gastric juice depends on their ability to tolerate low pH and high bile concentration, as the survival of the probiotic strain may be influenced by acidic stress caused by accumulation of metabolic end products of starters or by strain itself and the amount of bile produced in the tract daily. From the study it was visible that most of the isolates obtained grew at pH 5 and the isolates from cabbage, goat excreta, SG1, SG2, W3, MC1, WJ1, and MCJ1 showed growth even in pH 2.5 (Table 3.1) establishing their presence in an acidic condition even after 24 h. The growth of LAB lowers the pH due to lactic acid production and it is this acidification process which is the most desirable side-effects of their growth. More than 2 litres of gastric juice is secreted each day into the stomach (Morelli, 2000). This gastric juice renders the stomach pH to approximately 2.0 (Murthy *et al.*, 2000). The presence of food raises the pH value to the level of 3 (Erkkilä & Petäjä, 2000) low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolonged shelf life. Acidity has a negative impact on bacterial physiology by altering enzymatic activities leading to dissipation of the proton motive force and expression of acid response proteins (Champomier-Vergès *et al.*, 2002). The optimum pH for most bacteria is near the neutral point (pH 7.0). Certain bacteria are acid tolerant and will survive at reduced pH levels. Notable acid-tolerant bacteria include the *Lactobacillus* and *Streptococcus* species which play a role in the fermentation of dairy and vegetable products.

The concentration of bile to be used in the selection of probiotic species for human beings must be 0.3 % (w/v). This is so because the isolated microorganism may show tolerance to high concentrations of bile. Almost all the isolates could tolerate up to 0.5 % oxgall, where as W1, W2 from whey and WJ1 from human baby excreta showed less tolerance to 0.8 % oxgall (Table 3.1). According to Pancheniak and Soccol (2005), isolates which showed 0.3 % tolerance to bile could be used as probiotic for swine. The daily average of biliary flow is very high in swine, around two litres for each 40 kg of swine (Pancheniak & Soccol, 2005) as compared to that of an adult human (70 kg) that produces 400 to 800 ml of bile daily.

Lactic acid bacteria generally tolerate high salt concentrations. It allows the bacteria to begin metabolism, which produces acid that further inhibits the growth of non-desirable organisms. CB1, one of the cabbage isolate could tolerate up to 12 % NaCl. Some isolates such as CB4, CB5, SG1, and SG2 could tolerate 8 % NaCl shown in Table 3.1. MC1 could tolerate 8 and 12 % NaCl in a similar pattern with same viability. When bacterial cells are grown in medium with salt, they experience a loss in their turgor pressure which in turn affects the metabolism, their enzyme activity, water activity. Cells overcome this situation by regulating the pressure inside and outside of the cell by inducing osmolytes such as glycine betaine as an adaptive mechanism to withstand increased osmotic potential (Adnan & Tan, 2007).

Tolerance to phenol is a characteristic probiotic property because phenols can be formed in the intestines by bacteria that desaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins (Gilliland & Walker, 1990). Isolates from goat excreta G1, wheyW3, MC1 from curd and WJ1 from human baby faeces could tolerate up to 0.5 % phenol (Table 3.1).

3.3.3.2. Hydrophobicity of Isolates

Among the isolates, isolates of sour dough SD2, SD3 and SD4 showed hydrophobicity of 52.4, 24 and 73 % respectively, snake gourd isolates SG1, SG2 had 65 and 40 % and MC1, 23 % hydrophobicity. The result is shown in Table 3.2.

Surface characteristic is one among the *in vitro* properties which is to be studied to evaluate the potential probiotic strains. The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Kiely & Olson, 2000). Bacterial adhesion determines the colonization capability of a microorganism. Through adhesion ability and colonization of tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors (Otero *et al.*, 2004). The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely & Olson, 2000). As the hydrophobicity of the cell increases the level of adhesion also increases (Rijnaarts *et al.*, 1993).

The initial interaction of the microbe with the epithelial cells may be weak, it is often reversible and precedes subsequent adhesion processes mediated by more specific mechanisms involving cell-surface proteins and lipoteichoic acids (Roos & Jonsson, 2002). Although hydrophobicity may assist in adhesion, it is not a prerequisite for strong adhesion to human intestinal cells.

3.3.3.3. Mucin Adhesion Assay

Mucus provides protective functions in the gastrointestinal tract and plays an important role in the adhesion of microorganisms to host surfaces. The potentially important property of probiotic microbes is their ability to interact with epithelial cells in

Table 3.1 Tolerance of isolated LAB towards varying growth inhibitory substances

Isolates	*NaCl*			*pH*				*Phenol*				*Bile*		
	Growth of bacteria (OD at 620 nm)													
	4 %	*8 %*	*12 %*	*5*	*4*	*3*	*2.5*	*0.20 %*	*0.30 %*	*0.40 %*	*0.50 %*	*0.30 %*	*0.50 %*	*0.80 %*
CB1	0.11	0.033	0.56	1.749	0.056	0.012	0.103	0.085	0.061	0.067	0.066	1.514	1.316	1.489
CB2	0.24	0.04	0.065	0.781	0.46	0.002	0.11	1.047	0.31	0.116	0.046	1.475	1.407	1.45
CB3	0.98	0.04	0.065	1.754	0.06	0.002	0.133	1.603	0.378	0.176	0.06	1.815	1.34	1.433
CB4	0.8	0.8	0.051	1.709	0.076	0.017	0.122	1.724	1.597	0.53	0.063	1.455	1.867	1.802
CB5	1.147	1.497	0.041	1.773	0.029	0	0.086	1.397	0.283	0.093	0.043	1.808	1.333	1.489
G1	1.84	0.07	0.058	1.516	0.164	1.112	0.083	1.941	1.925	0.43	0.195	1.757	1.879	1.802
G2	1.614	0.044	0.045	1.492	0.176	0.166	0.165	1.655	1.73	0.156	0.081	1.813	1.841	1.775
G3	1.819	0.077	0.064	1.571	0.078	0	0.086	1.767	1.627	0.232	0.096	1.815	1.853	1.789
SD1	0.035	0.049	0.039	0.758	0.044	0.041	0.026	0.068	0.121	0.007	0.061	1.67	1.603	1.49
SD2	0.045	0.04	0.046	0.823	0.032	0	0	1.298	1.265	0.048	0.079	1.216	0.705	1.231
SD3	0.035	0.032	0.039	0.413	0.045	0.045	0.044	0.068	0	0.002	0.063	1.671	1.421	1.451
SD4	0.033	0.039	0.055	0.746	0.054	0.058	0.049	0.072	0	0.005	0.051	0.803	0.547	0.629
W1	0.538	0.026	0.042	0.003	0.068	0.007	0.017	0.061	0	0.01	0.076	0.6	0.345	0.345
W2	0.036	0.039	0.055	0.537	0.127	0.111	0.029	0.92	0.072	0.092	0.078	0.916	0.723	0.328
W3	1.359	0.035	0.036	1.48	0.33	0.111	0.136	1.961	1.924	0.623	0.325	1.871	1.854	1.846
SG1	2.158	0.806	0.061	1.631	0.027	0	0.09	1.685	0.063	0.061	0.056	1.8	1.724	1.778
SG2	2.114	0.927	0.056	1.696	0.034	0	0.089	1.632	0.122	0.069	0.054	1.843	1.765	1.753
MC1	1.012	0.09	0.076	1.62	0.156	0.132	0.12	0.921	0.441	0.211	0.124	1.755	1.582	1.771
WJ1	1.1	0.118	0.11	1.55	0.125	0.111	0.104	1.12	0.451	0.432	0.144	1.102	0.965	0.206
MCJ1	1.12	0.092	0.08	1.521	0.104	0.1	0.098	1.02	0.321	0.184	0.089	1.023	0.921	1.027

Standard deviation was found to be ± 0.05.

the intestinal tract, which may promote retention and host-bacterial communication. This study was another approach of hydrophobicity determining the interaction of microbes with epithelium. Mucus consists of a complex mixture of highly glycosylated proteins (mucins) and glycolipids which covers the epithelial cells of the intestine (Dekker *et al.*, 2002). The adhesion of the microorganisms to mucins has been reported to be mediated by proteins in most cases (Roos & Jonsson, 2002). In the humanintestinal tract, the layer of mucus may vary in thickness from about 30 to 300 μm, generally increasing in thickness from the small intestine to the rectum. The results were found to be coinciding with the previous experiment with all the isolates showing a mucin binding ability of ± 2 % of hydrophobicity. The isolate MC1 was showing 25 % mucin binding property (Table 3.2).

The low hydrophobicity and mucin adhesion of some of the isolates could be explained by the plausible reason of exopolysaccharide production by these strains such as MC1. The presence of EPS fractions will promote a significant reduction in the adhesion of the probiotic strains (Ruas-Madiedo *et al.*, 2006). EPS could directly adhere to mucus and then competitively inhibit the adhesion of probiotics. In more defined way, the EPS could stick to the probiotic surface and thereby mask bacterial molecules involved in adhesion. Sometimes it can favour adhesion as components of the pathogen surface might bind specific EPS and the bound EPS would be able to adhere to mucus (Ruas-Madiedo *et al.*, 2006).

3.3.3.4. Antimicrobial Activity

Lactic acid bacteria have been useful at promoting bacterial interference by the production of inhibitory substances like organic acids, free fatty acids, ammonia, diacetyl, hydrogen peroxide and bacteriocins. Hydrogen peroxide is produced by many of the lactics. The antimicrobial effect is based on the oxidative properties that

Table 3.2 Surface hydrophobicity and mucin adhesion of isolates

Isolates	*Hydrophobicity (%)*	*Mucin Adhesion Assay (%)*
CB1	10	10
CB2	11	12
CB3	10	12
CB4	12	14
CB5	11	12
G1	11	11
G2	12	12
G3	12	13
SD1	11	10
SD2	52.4	55
SD3	24	25
SD4	73	74
W1	4	5
W2	4	6
W3	4.2	5
SG1	65	67
SG2	40	42
MC1	23	25
WJ1	9.7	10
MCJ1	9.8	10

Standard deviation was found to be ± 0.05.

results in irreversible changes in the microbial cell membrane. Isolates from snake gourd, MC1, W3, G2, G3, and CB4 showed antimicrobial activity against potential human pathogens such as *Shigella sonnei*, *Shigella flexneri*, *Staphylococcus aureus* and *E. coli.* The zone of inhibition is tabulated in Table 3.3.

Table 3.3 Antimicrobial activity of LAB isolates

Isolates	***Zone of inhibition (mm)***			
	S. aureus	***E. coli***	***Shigella sonnei***	***Shigella flexneri***
CB1	-	5	-	-
CB2	-	5	-	-
CB3	-	6	5	-
CB4	4	7	5	-
CB5	-	10	-	-
G1	5	5	4	-
G2	-	5	-	-
G3	-	7	5	-
SD1	-	-	-	4
SD2	-	7	5	5
SD3	5	8	6	-
SD4	-	6	-	6
W1	-	6	-	4
W2	-	-	-	-
W3	6	6	6	5
SG1	6	6	5	5
SG2	5	6	6	6
MC1	6	6	6	5
WJ1	-	5	-	-
MCJ1	-	-	5	-

3.3.3.5. Antibiotic Resistance

This part of the work reports the susceptibility patterns of the LAB isolates against Gram positive spectrum antibiotics, vancomycin and erythromycin. MC1, MCJ1 and CB1 were resistant to vancomycin and the other isolates were sensitive to vancomycin. CB1, CB2, SD1 and SD2 were resistant to erythromycin. According to Klein et al (2000) the resistance of *Lactobacillus* and *Leuconostoc* spp. to vancomycin may be due to the presence of D-Ala-D-Lac as the normal dipeptide in their peptidoglycan. Table 3.4 summarizes the antibiotic resistance study of the isolates. A key requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes. Ingestion of bacteria carrying such genes is undesirable as horizontal gene transfer to recipient bacteria in the gut could lead to the development of new antibiotic-resistant pathogens (Saarela *et al.*, 2000).

Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is one of the last antibiotics broadly efficacious against clinical infections caused by multidrug-resistant pathogens. However, some LAB including strains of *L. casei*, *L. rhamnosus*, *L. plantarum*, *Pediococci* and *Leuconostoc* spp., are resistant to vancomycin. Such resistance is usually intrinsic, that is, chromosomally encoded and non-transmissible (Klein *et al.*, 1998).

3.3.4. Screening for Exopolysaccharide Production

3.3.4.1. Quantitative Estimation of Exopolysaccharide Production

Exopolysaccharides (EPS) from lactic acid bacteria contribute to specific rheology and texture of fermented milk products and finds applications even in non-dairy foods and in therapeutics. Therefore, EPS-producing strains are of commercial value for both their technological and putative probiotic properties. This aspect was taken into consideration for the screening of the LAB isolates for EPS. Microbial

polysaccharides of prominent economic interest are usually produced at the industrial level by fermentation.

Table 3.4 Performance of LAB isolates against Gram positive spectrum antibiotics

Isolates	*Diameter of zone of inhibition (cm)*	
	Erythromycin (15 µg)	*Vancomycin (10 µg)*
CB1	-	-
CB2	-	1.9
CB3	-	1.7
CB4	-	-
CB5	-	-
G1	1.0	1.0
G2	1.0	1.0
G3	3.0	0.9
SD1	-	2.5
SD2	-	2.0
SD3	-	1.5
SD4	1.5	1.5
W1	3.0	2.2
W2	3.0	2.1
W3	3.0	2.3
SG1	1.0	1.0
SG2	1.0	1.0
MC1	1.2	-
WJ1	2	1.3
MCJ1	1.1	-

EPS are primarily composed of carbohydrates. But in addition to the various sugars such as D-glucose, D-galactose and D-mannose present in EPS, there are organic

(pyruvate and acetate) and inorganic (phosphate and divalent cations) substitutents. Several amino sugars such as N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and some rare ones such as fucosamine and talosamine are also present. The study showed that one of the isolates from curd (MC1) had the maximum EPS production of 210 mg/ml (Fig 3.2).

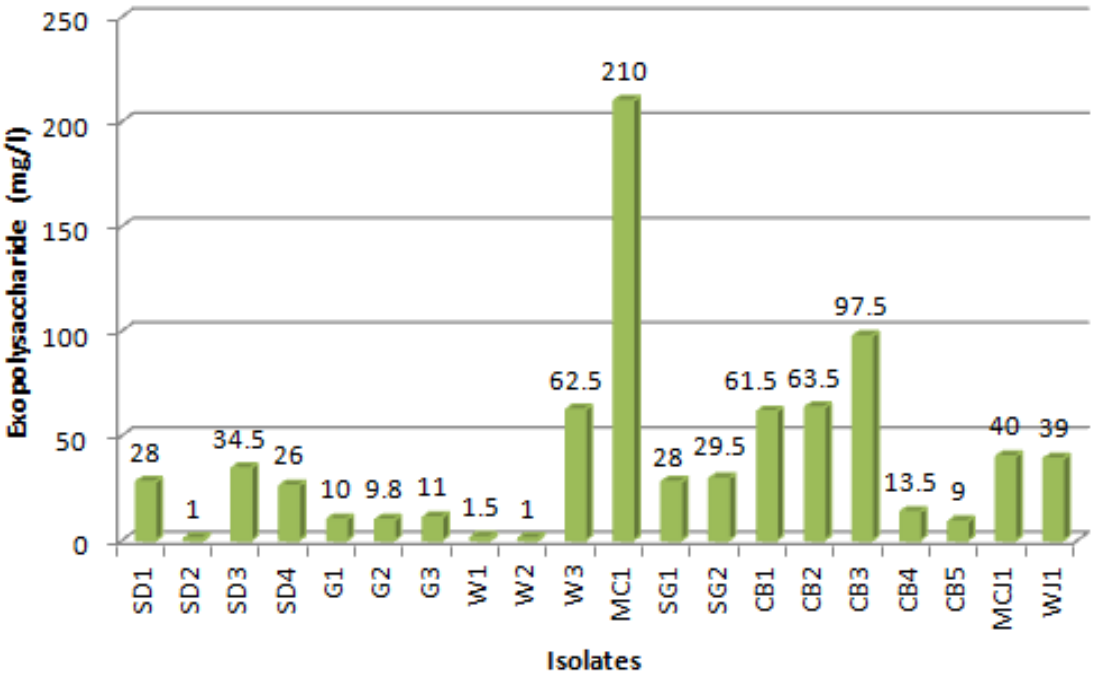


Fig. 3. 2. Exopolysaccharide production profile of LAB isolates. Standard deviation ± 0.05

3.3.4.2. Exopolysaccharide Producing Phenotype Identification by Staining Techniques

Exopolysaccharide producing phenotypes were confirmed by the below mentioned techniques.

3.3.4.2.1. Alcian Blue Staining

Alcian blue is an acidic dye which stains mucosubstances and acetic mucins in blue colour. It is a copper phthalocyanin dye and contains positively charged groups capable of salt linkage with certain polyanions. The result of staining showed the presence of exopolysaccharides on the microbial cell surface of MC1 isolate confirming its EPS producing phenotype (Fig. 3.3).

3.3.4.2.2. Ruthenium Red Agar Method

Ruthenium red is a ruthenium containing red staining dye. The dye stains bacterial cell wall in pink distinguishing the EPS producing strains with white colonies from the non-EPS producing strains. This is because the presence of EPS prevents the absorption of the stain present in the medium by the bacterial cell wall and thus producing white colonies. The MC1 strain gave white colonies on growing in ruthenium red containing MRS medium (Fig. 3.3).

3.3.5. Identification of Selected Culture by Conventional Method and 16S rRNA sequencing

The conventional physiological and biochemical studies from IMTECH Chandigarh showed that the EPS producing strain, MC1 belong to the genus *Lactobacillus* and species *plantarum*. The culture was assigned with an MTCC number 9510.

The molecular identification method by 16S rRNA sequencing by means of a multiplex PCR also coincided with the results obtained by conventional method of characterization. Two bands were obtained with the multiplex PCR reaction (Fig. 3.4), performed with the specific primers. A band of size ~1508 bp specific for eubacterial

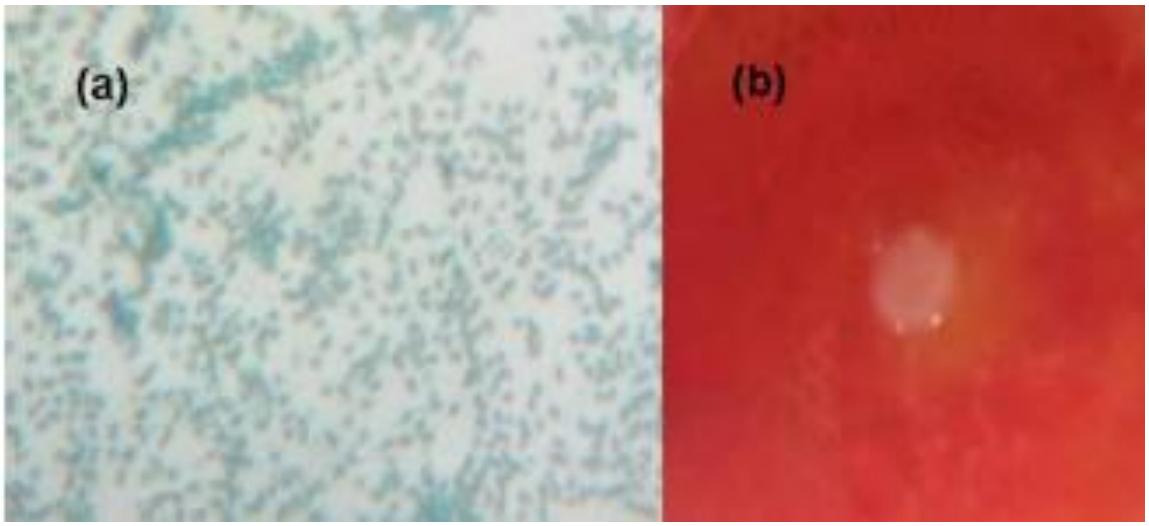


Fig. 3. 3. Phenotypic characterization by a) Phase-contrast microscopy of alcian blue staining, (b) Ruthenium red agar method

16SrRNA and a second band of ~491 bp size specific for the *plantarum* species were obtained by PCR. The 16SrRNA sequence (Fig. 3.5) was aligned and compared with other 16SrRNA gene sequences in the GenBank by using the NCBI Basic Local alignment search tools BLAST n program. The blast results of the PCR amplicons showed 100 % identity with *Lactobacillus plantarum* (Fig. 3.6). The gene sequence was submitted in GenBank and assigned with a GenBank accession number JQ809467.

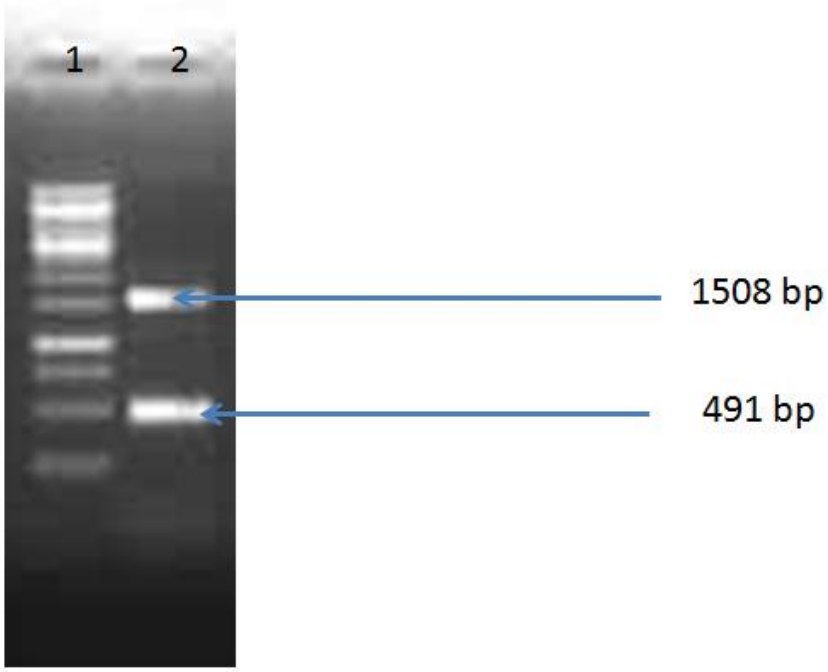


Fig. 3. 4. Multiplex PCR of 16S rRNA of MC1: Lane 1: 1 kb DNA ladder; Lane 2: Eubacterial 16S rRNA (~1508 bp) and *Lactobacillus plantarum* specific 16S rRNA (~491 bp)

TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG

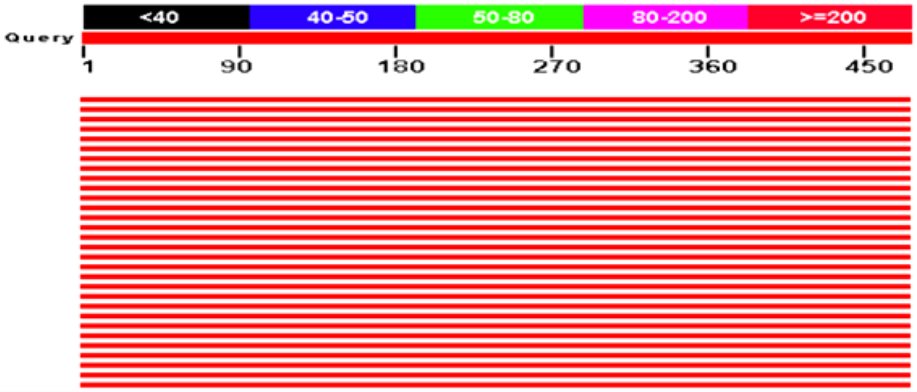
AACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGG

CGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAAC

ACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCG

AGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATT

Fig. 3. 5. 16S rRNA sequence of MC1 identified as *Lactobacillus plantarum* MTCC 9510



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AL935263.2	Lactobacillus plantarum WCFS1 complete genome	883	4402	100%	0.0	100%	
FR871789.1	Lactobacillus pentosus MP-10 draft genome, annotated contig	883	883	100%	0.0	100%	
FR745400.1	Lactobacillus plantarum 16S rRNA gene, strain C2	883	883	100%	0.0	100%	
HQ697623.1	Lactobacillus plantarum strain 34-161 16S ribosomal RNA gene	883	883	100%	0.0	100%	
GU552552.1	Lactobacillus plantarum strain KW30 16S ribosomal RNA gene,	883	883	100%	0.0	100%	
CP002222.1	Lactobacillus plantarum subsp. plantarum ST-III, complete ger	883	4369	100%	0.0	100%	
GQ988699.1	Uncultured bacterium clone O_AR_Z_G07 16S ribosomal RNA g	883	883	100%	0.0	100%	
FJ982852.1	Uncultured Lactobacillus sp. clone OHW14 16S ribosomal RNA g	883	883	100%	0.0	100%	
CP001617.1	Lactobacillus plantarum JDM1, complete genome	883	4419	100%	0.0	100%	
EU825657.1	Lactobacillus plantarum 16S ribosomal RNA gene, partial seque	883	883	100%	0.0	100%	
GQ131139.1	Lactobacillus plantarum strain IMAU70023 16S ribosomal RNA g	883	883	100%	0.0	100%	
EU675926.1	Lactobacillus pentosus strain C50-6 16S ribosomal RNA gene, p	883	883	100%	0.0	100%	
EU096230.1	Lactobacillus plantarum strain EW-p 16S ribosomal RNA gene, p	883	883	100%	0.0	100%	
EU559598.1	Lactobacillus plantarum strain ZDY78 16S ribosomal RNA gene,	883	883	100%	0.0	100%	
EU559596.1	Lactobacillus plantarum strain ZDY36a 16S ribosomal RNA gene	883	883	100%	0.0	100%	

Fig. 3. 6. Sequence identity of 16SrRNA of MC1 with *Lactobacillus plantarum*

3.4. Conclusion

A good number of lactic acid bacteria were isolated from various sources. The probiotic characteristic studies of the LAB isolates indicated that most of them were acid, bile, phenol and salt tolerant. They produced antimicrobial substances which inhibited the growth of potential human pathogens and exhibited Gram positive spectrum antibiotic resistance or sensitivity. Many of the isolated cultures were exhibiting high hydrophobicity and mucing binding properties. Considering the overall probiotic performance of the cultures, one of the isolates MC1 identified as *Lactobacillus plantarum* (MTCC 9510) with significant EPS production and prominent probiotic features was selected for further studies.

CHAPTER 4

EXOPOLYSACCHARIDE PRODUCTION BY SUBMERGED FERMENTATION (SMF) USING LACTOBACILLUS PLANTARUM MTCC 9510

CHAPTER 4

EXOPOLYSACCHARIDE PRODUCTION BY SUBMERGED FERMENTATION (SMF) USING *LACTOBACILLUS PLANTARUM* MTCC 9510

4.1. Introduction

Exopolysaccharides (EPS) produced by the food-grade Lactic acid bacteria (LAB) with GRAS (Generally Recognized as Safe) status are an important source of natural alternatives to commercial additives of plant or animal origin. EPS produced by LAB are in a great variety, depending on the type of LAB strains, culture conditions and medium composition (Looijesteijn & Hugenholtz, 1999). As both the efficiency of synthesis and the sugar composition of EPS being influenced deeply by the culture conditions it is a prerequisite to optimize the bioprocess conditions for maximum production. Production of EPS is dependent on the composition of the medium mainly carbon and nitrogen ratio as well as incubation temperature, pH of the medium, mineral and vitamin contents of the medium (Gorret *et al.*, 2001). In fact, mesophilic strains seem to produce maximum levels of EPS in suboptimal conditions for the bacterial growth, whereas EPS production appears to be growth associated in thermophilic strains (Degeest *et al.*, 2001). The main strategy of bioprocess optimization is single factor optimization, *ie*; changing one factor at a time keeping the other constant. One of the disadvantages of the method is that the interaction effect of the factors cannot be assessed. This can be overcome with the help of statistical experimental designs. These are being used for many decades and it can be applied to search for optimal conditions of a targeted response (Lee & Gilmore, 2005). The popular choices among statistical designs are Plackett-Burman design and response surface methodology with various designs (Wang

& Lu, 2005). The response surface methodology (RSM) is an empirical modelling system that assesses the relationship between a group of variables, which can be controlled experimentally, and the observed response. This methodology is applied mainly both in food science and in the optimization of fermentative processes.

The present chapter describes the single factor as well as the subsequent statistical optimization of EPS production using *Lactobacillus plantarum*.

4.2. Materials and Methods

4.2.1. Microorganism and Maintenance

Lactobacillus plantarum (MTCC 9510) is a facultative anaerobe isolated from curd and was sub-cultured, maintained and the fresh inoculum for the experiments were prepared as described previously in chapter 2 (section 2.3.1 and 2.3.2).

4.2.2. Single Factor Optimization Design and Data Analysis

Single factor optimization was employed to identify the key variables which can affect EPS production. The influence of different carbon sources on EPS production was evaluated by selecting different sugars (filter sterilized) such as lactose, glucose, sucrose and galactose (20 g/l). Each flask was inoculated with 10^9 CFU/ml of 18 h old inoculum and incubated at static condition at 37 ºC for 72 h. The effect of incubation time on EPS production was monitored by incubating the inoculated flasks at 37 ºC for different time intervals such as 24, 48, 72, 96 and 120 h. The impact of different complex nutritive sources on the EPS production was also evaluated by selecting different organic nitrogen sources like corn steep liquor, yeast extract, beef extract and tryptone (25 g/l). The combinatorial effect of all the three (yeast extract, beef extract and tryptone) organic nitrogen sources was studied in the ratio 2:2:1. Inorganic nitrogen sources (2 g/l) like ammonium sulphate, ammonium nitrate, ammonium chloride, tri-ammonium citrate, sodium nitrite and potassium nitrate were supplemented individually in to the previously optimized medium to see the influence of

inorganic nitrogen sources on EPS production. To study the effect of initial medium pH on EPS production, the medium pH was set to the range of 4-8 using 1N HCl or NaOH. The control pH was 7.3.

4.2.3. Box-Behnken Model of Design

A fractional-factorial design, Box-Behnken model was employed for the statistical optimization of production medium for EPS. The experimental design consisted of seventeen runs and the independent variables were studied at two different levels, a high level and a low level. The high level is commonly coded as +1 and the low level as -1. It is necessary to include centre points as well (in which all factors are at their central values). The response variable (EPS) was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is

$$Y_i=b_0+b_i\sum X_i+b_{ii}\sum X_i^2+b_{ij}\sum_i\sum_j X_iX_j \quad (1)$$

where Y_i is the predicted response, X_i, X_j are input variables which influence the response variable Y; b_0 is the offset term; b_i is the i^{th} linear coefficient; b_{ii} is the quadratic coefficient and b_{ij} is the ij^{th} interaction coefficient. Based on the single factor optimization, three factors were selected for the design. The three factors selected according to single-parameter optimization were yeast extract, lactose and ammonium sulphate. A design was generated with these factors, having a low level and a high level. The low level for yeast extract and lactose was 10 g/l and the high level was 40 g/l. The low level for ammonium sulphate was 1 g/l with a high level of 10 g/l. The pH of all flasks was adjusted to 7.3 and the incubation time for production was kept as 72 h based on the single-factor optimization. The ‘Design

Expert' software (version 6.0, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design (Table 4.1), regression and graphical analyses of the data obtained. The amount of EPS produced was taken as the response.

Table 4.1 Experimental design generated with Design Expert and the predicted and actual values of production of exopolysaccharides

Run order	*Yeast extract (g/l)*	*Lactose (g/l)*	*Ammonium sulphate (g/l)*	*Predicted value (g/l)*	*Actual value (g/l)*
1	25	25	5.5	0.67	0.58
2	25	10	1	0.07	0.08
3	40	10	5.5	0.44	0.56
4	40	25	10	0.5	0.4
5	40	40	5.5	1.1	1.21
6	10	25	1	0.033	0.13
7	10	25	10	0.19	0.32
8	25	25	5.5	0.67	0.78
9	25	25	5.5	0.67	0.72
10	25	40	10	0.42	0.41
11	40	25	1	0.75	0.62
12	10	10	5.5	0.2	0.09
13	25	25	5.5	0.67	0.55
14	25	10	10	0.24	0.22
15	25	40	1	0.69	0.71
16	25	25	5.5	0.67	0.71
17	10	40	5.5	0.32	0.2

Standard deviation was found to be ± 5 %

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its

associated probability $P(F)$, correlation coefficient R, determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's t-value for the estimated coefficients and associated probabilities, $P(t)$. The quadratic models were represented as response surface graphs. Validation of the experiment was also performed by selecting different combinations of the factors as recommended by the software.

4.2.4. Extraction and Quantification of EPS

EPS was extracted from the production medium as per the protocol described by Savadogo et al (2004). The culture was centrifuged at 11,500 x g for 15 min at 4 °C to remove cell pellet and the supernatant precipitated with double volume-chilled ethanol was stored overnight at 4 °C. The mixture centrifuged at 2,500 x g for 20 min and the pellet collected was dissolved in de-mineralised water and again precipitated using double-volume cold ethanol. It was further centrifuged at 2,500 x g for 20 min to collect the pellet. The total carbohydrate present in the pellet was estimated by phenol – sulphuric acid method (Dubois *et al.,* 1956) as described earlier (section 2.4.1).

4.2.5. Experimental Statistics

All experiments have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

4.3. Results and Discussion

4.3.1. Single Factor Optimization Design and Data Analysis

The medium formulation for EPS production was optimized by single factor method keeping the other factors constant. The factors that had profound influence in the EPS production were lactose, yeast extract, ammonium sulphate and incubation time (Table 4. 2). It was observed that as the growth (cell dry weight) attained a maximum at 72h, the EPS production also increased (Fig. 4.1) giving a maximum production at 72 h. The experiment

proved that the production reached the maximum towards the end of the log-phase and decreased in the stationary and declining phase. Aslim et al (2005) explained that the EPS production in *L. delbrueckii* sub *bulgaricus* at 40 °C increased during the exponential growth phase and no further production was observed in the stationary growth phase. They could attain a production level of only 0.26 g/l. A control in the case of organic nitrogen sources with a combination of peptone, beef extract and yeast extract in the same composition as in MRS medium (Annexture 1) was also used and the production obtained in it was 0.14 ± 0 g/l EPS.

A combination of organic nitrogen sources without inorganic nitrogen source didn't prove to be effective as yeast extract. Lin and Chen (2007) proved in their experiment that

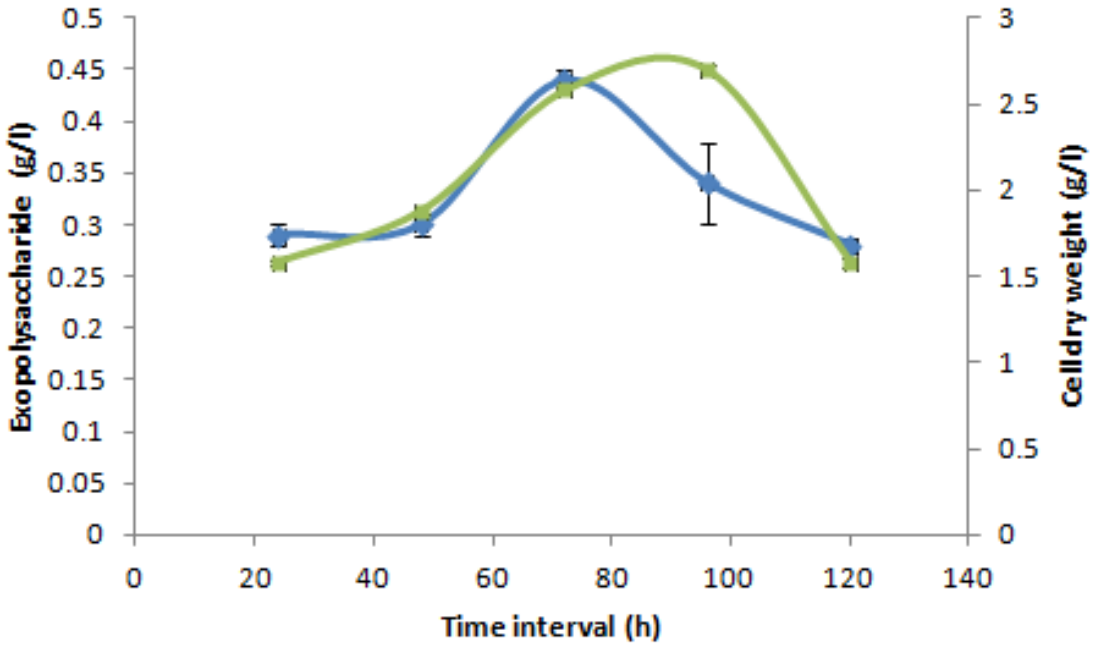


Fig. 4.1. Pre-optimized exopolysaccharide production (blue line) and growth pofile (green line) of *Lactobacillus plantarum*

yeast extract could be utilized to improve the EPS production. The composition of yeast extract includes amino-acids, peptides, carbohydrates, water soluble vitamins and salts.

However, it was noticed that the high level of EPS obtained when yeast extract was used as the nitrogen source was not due to the interference of carbohydrates present in yeast extract, as the production medium was also checked for polysaccharides by phenol-sulphuric acid assay and was found to be very negligible.

There was an improvement in the EPS yield to 1.08 g/l when ammonium sulphate (2 g/l) was used as the inorganic nitrogen source and it was confirmed experimentally that the medium pH after adding inorganic nitrogen source had no influence in the production because the pH in all cases was almost similar. Interestingly, other ammonium ion containing sources were not showing the same impact. It was observed by Majumdar et al (1999) that of the various nitrogen sources used, ammonium sulphate when used at 0.006 %, produced the highest quantities of EPS.

4.3.2. Box-Behnken model of design and purification of exopolysaccharides

In order to search for the optimum combination of the factors in the medium, the Box-Behnken model of RSM was employed. There were total seventeen runs based on the model generated by the software, each in triplicates. The experimental data were statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA) and the 3D graphs were designed. ANOVA is a statistical technique that subdivides the total variation of a set of data into component associated to specific sources of variation for the purpose of testing hypotheses for the modelled parameters. The ANOVA for the model is showed in Table 4. 3, which explains the model to be significant with a Model F-value of 6.86. There is only a 0.94 % chance that a "Model F-Value" this large could occur due to noise. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 9.587 indicates an adequate signal. The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.90) indicates the significance of the model. The coefficient of variation (CV) indicates the

degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case lower value of CV (29.77) indicated a greater reliability of the experiments performed. The *P* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. Value of Prob > *F* less than 0.05 indicate model terms are significant. Here the terms X_1, X_2 and X_3^2 are significant. That is, the linear effect of yeast extract and lactose and the quadratic effect of ammonium sulphate are significant.The polynomial equation derived on the basis of the experimental factors, quadratic effect of the factors and the interactions among the factors by the input of values in equation (1) is shown below:

$$Y = 0.67 + 0.26\,X_1 + 0.20\,X_2 - 0.024\,X_3 - 0.070\,X_1^2 - 0.083\,X_2^2 - 0.23X_3^2 + 0.13\,X_1X_2 - 0.10\,X_1X_3 - 0.11X_2X_3 \quad (2)$$

Where, Y is the response, that is, exopolysaccharides, X_1, X_2 and X_3 are the coded values of the test variables yeast extract, lactose and ammonium sulphate. The probability value 0.0015 and 0.0060 respectively for yeast extract and lactose ensures the factors to be significant in the EPS production. The interactions between the factors not found to be significant showing that the interaction between factors is not necessary for the EPS production.

Table 4.2 Single parameter experimental analysis

Set Variables	***Exopolysaccharide (g/l)***					
Carbon source (Incubation time 72 h)	Glucose 0.3±0.01	Lactose 0.44±0.01	Sucrose 0.21±0.05	Galactose 0.24±0.08		
Incubation time (Carbon source: lactose 20 g/l)	24 (h) 0.29±0.01	48 (h) 0.3±0.01	72 (h) 0.44±0.01	96 (h) 0.34±0.04	120 (h) 0.28±0.01	
Organic nitrogen source (Carbon source: lactose 20 g/l, Incubation time 72 h)	Yeast extract 0.64±0.01	Beef extract 0.24±0.01	Tryptone 0.03±0.01	Peptone 0.01±0.01	Corn steep liquor 0.06±0.01	
Inorganic nitrogen source (Carbon source: lactose 20 g/l, Incubation time 72 h, Yeast extract 25 g/l)	Ammonium Citrate 0.48±0.04	Ammonium Chloride 0.5±0.02	Ammonium Nitrate 0.68±0.02	Potassium Nitrate 0.82±0.05	Sodium Nitrite 0.83±0.01	Ammonium Sulphate 1.08±0.01
pH (Carbon source: lactose 20 g/l, Incubation time 72 h, Yeast extract 25 g/l, Ammonium sulphate 2 g/l)	4 0.52±0.00	5 0.83±0.12	6 0.81±0.02	7 0.92±0.01	7.3 1.08±0.01	8 0.79±0.04

Table 4. 3 ANOVA for response surface quadratic model

Source	*Sum of Squares*	*DF*	*Mean Square*	*F Value*	*Prob > F*
Model	1.309	9	0.146	6.856	0.0094
A	0.525	1	0.525	24.746	0.0016
B	0.312	1	0.312	14.700	0.0064
C	0.005	1	0.005	0.213	0.6587
A2	0.023	1	0.023	1.064	0.3366
B2	0.031	1	0.031	1.458	0.2664
C2	0.229	1	0.229	10.791	0.0134
AB	0.073	1	0.073	3.434	0.1063
AC	0.042	1	0.042	1.980	0.2022
BC	0.048	1	0.048	2.280	0.1748
Lack of Fit	0.106	3	0.035	3.326	0.1380

The response of the RSM was shown as 3D response surface graphs, which gives infinite number of combinations of the two factors selected keeping the other constant. The maximum amount of EPS (1.21 g/l) was produced by a combination, yeast extract 40 g/l, lactose 40 g/l and ammonium sulphate 5.5 g/l. It was noted that the EPS production increased with increase in concentration of yeast extract and lactose. The production level was maximum when both of the components were given at their maximal level. The high level value for lactose and yeast extract was chosen as 40 g/l and beyond the limit won't be feasible at an industrial level. The combined effect of carbon source (lactose) and nitrogen source (yeast extract) was investigated using the statistical approach of Box–Behnken model, which can help to identify and quantify the interaction between variables. The interaction between

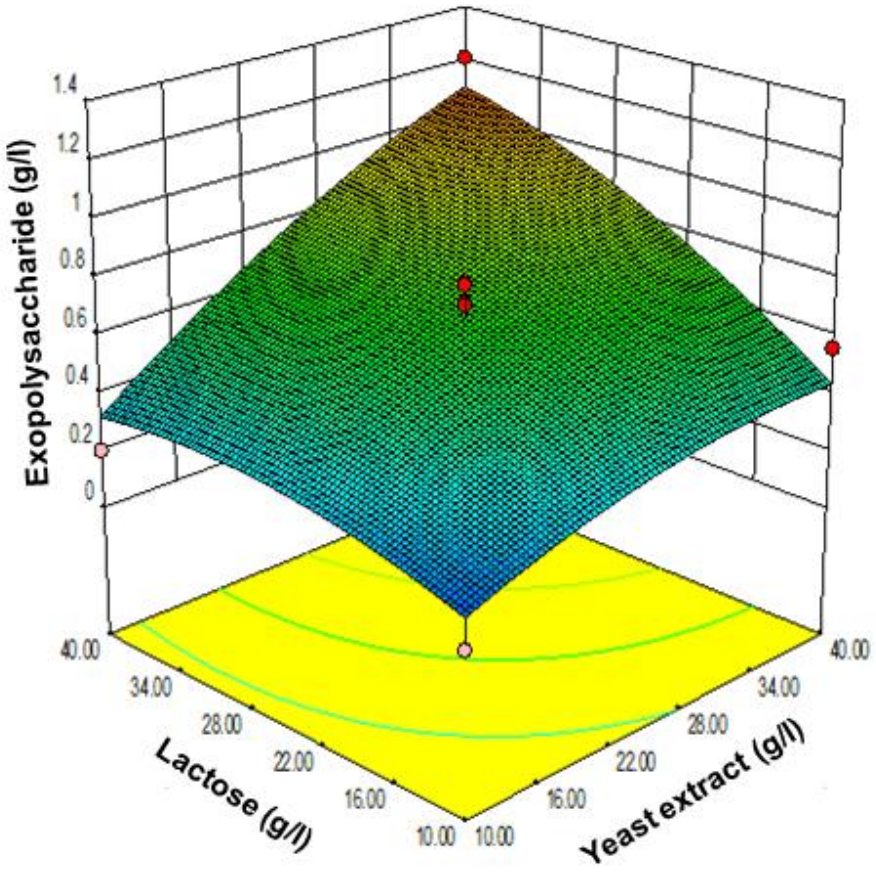
lactose and yeast extract was promising as the increase in EPS production was directly proportional to the concentration of yeast extract and lactose (Fig. 4. 2).

The production pattern was not the same while considering the effect of yeast extract and ammonium sulphate (Fig. 4.3). The production was linear for yeast extract but when the concentration of ammonium sulphate was increased the production increased first and then decreased. At higher concentrations, ammonium sulphate was found to have some inhibitory effect. The interaction between lactose and ammonium sulphate (Fig. 4.4), yeast extract and ammonium sulphate was not so significant.

The model was validated in shake flask level by the conditions predicted by the software. The deviation of actual values from the observed values plotted in Fig.4.5. It was observed that there was not much deviations in the observed and predicted values showing the significance of the model.

The maximal production observed during validation was 1.21 g/l as in the initial experiment against the predicted value of 1.10 g/l. It was observed from literature that many researchers have studied the production of EPS in varying media and production conditions. However, there has been no report on such a high titre of EPS production by lactic acid bacteria with a lactose based semi-defined medium. The only higher EPS production titre ever reported is 2.77 g/l, in whey permeate supplemented medium, using *L. rhamnosus* 9595 (Macedo *et al.*, 2002). Similarly, Tsuda and Miyamoto (2010) had attained a production of 0.15 g/l in supplemented whey using *L. plantarum*. Whey has been made use of as a production medium for EPS production using the strains *L. delbrueckii* sub *bulgaricus* RR and *L. delbrueckii* sub *bulgaricus* with production levels of 0.09-0.11 g/l and 0.80 g/l respectively (Briczinski & Roberts, 2002; Shene & Bravon, 2007). It is interesting that Shene and Bravon attained this production with a protein free medium. Sánchez et al (2006) reported a production of 0.5 g/l in a semi-defined medium using *L. pentosus* LPS26.

(a)



(b)

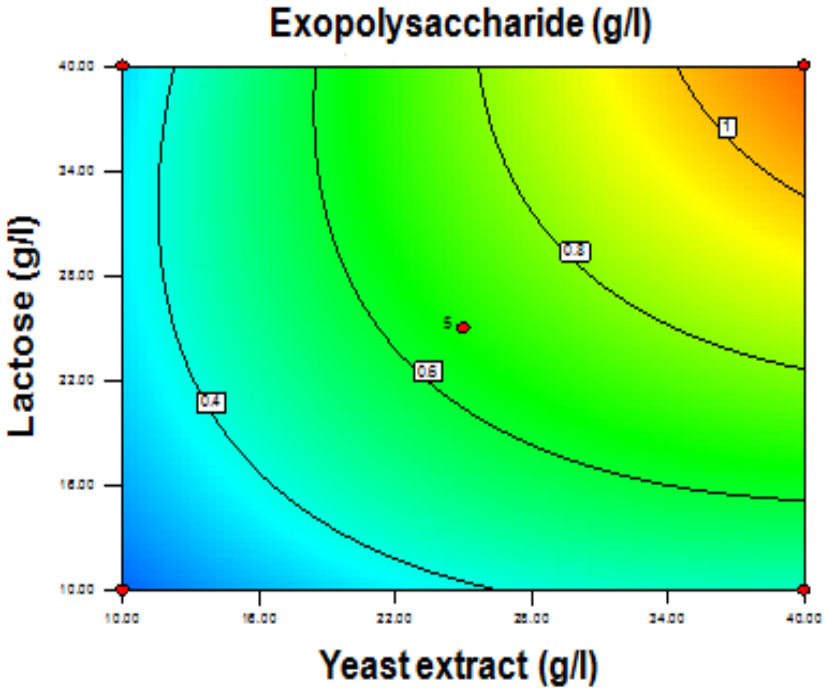
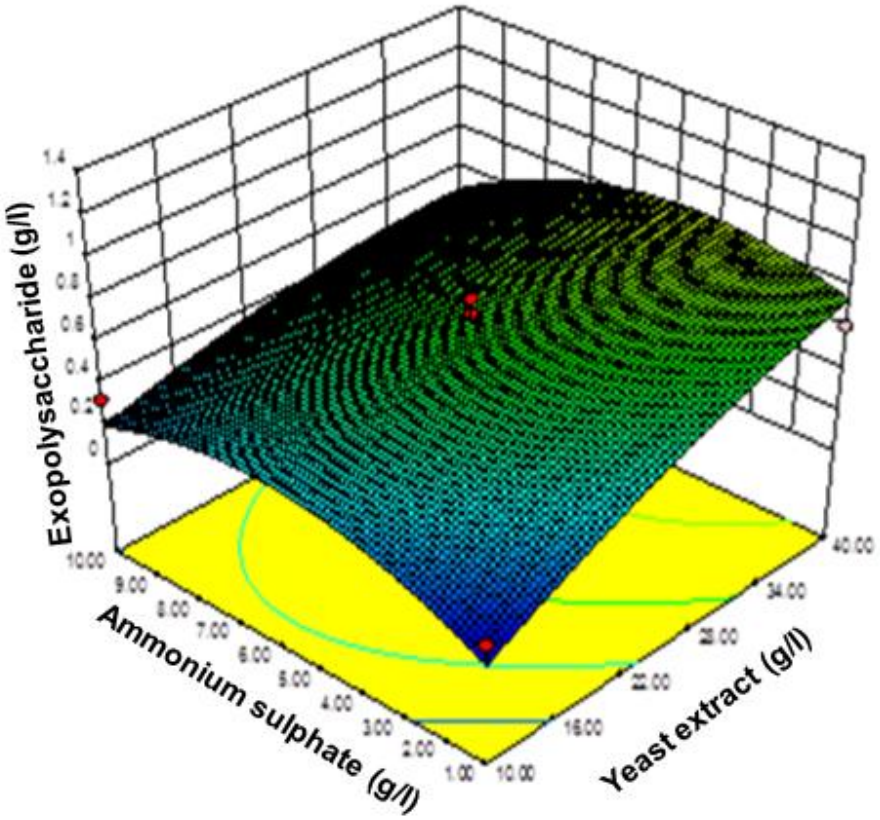


Fig. 4. 2. Interaction effect of yeast extract and lactose on production of exopolysaccharide (a) RSM graph (b) contour plot

(a)



(b)

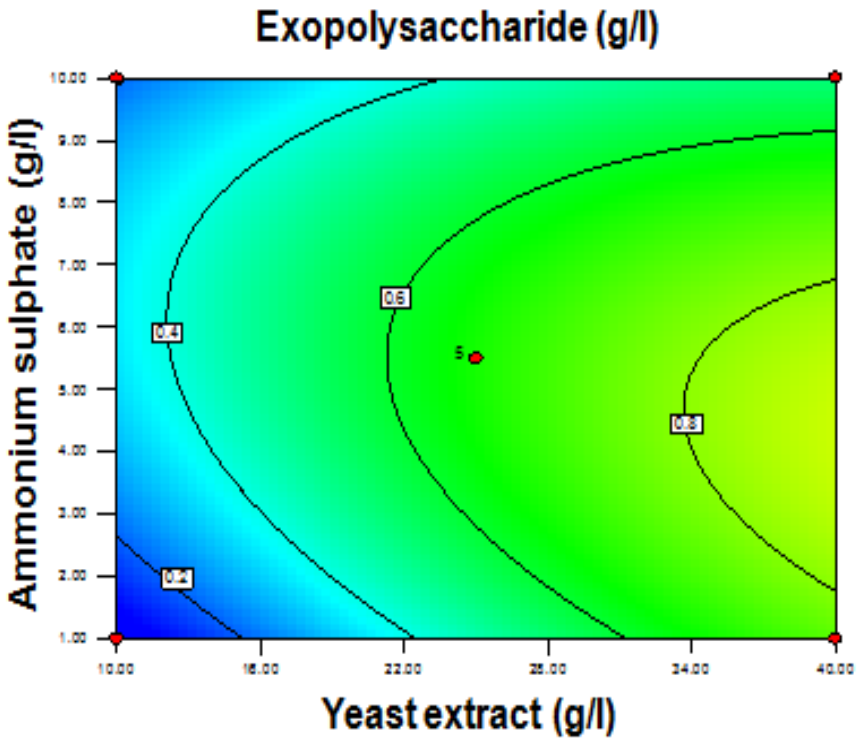
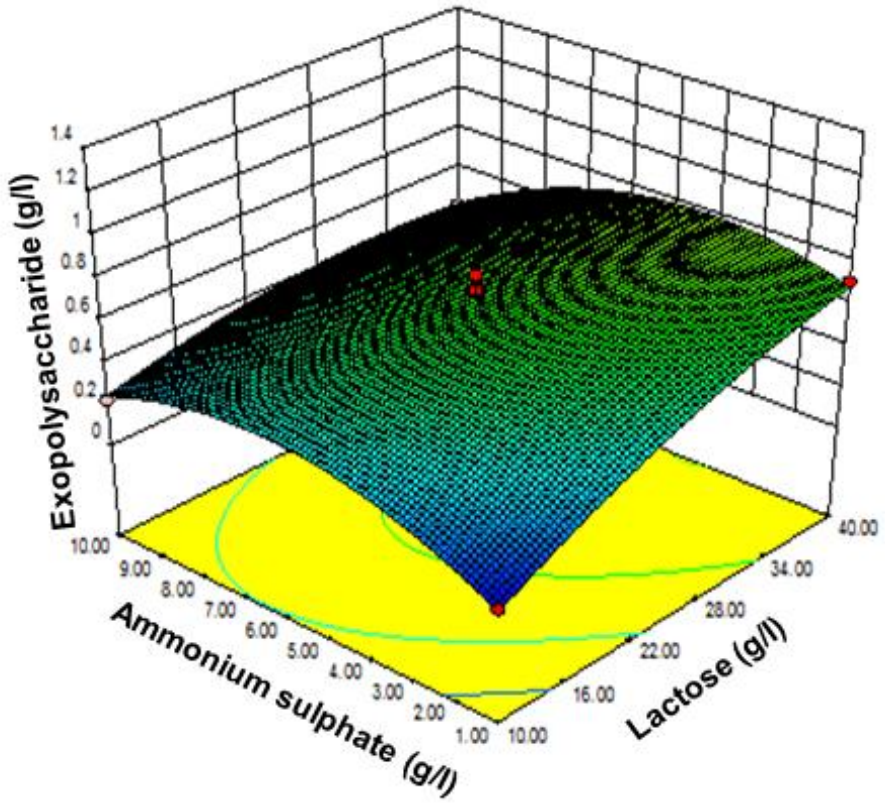


Fig. 4.3. Interaction effect of yeast extract and ammonium sulphate on production of exopolysaccharide (a) RSM graph (b) contour plot

(a)



(b)

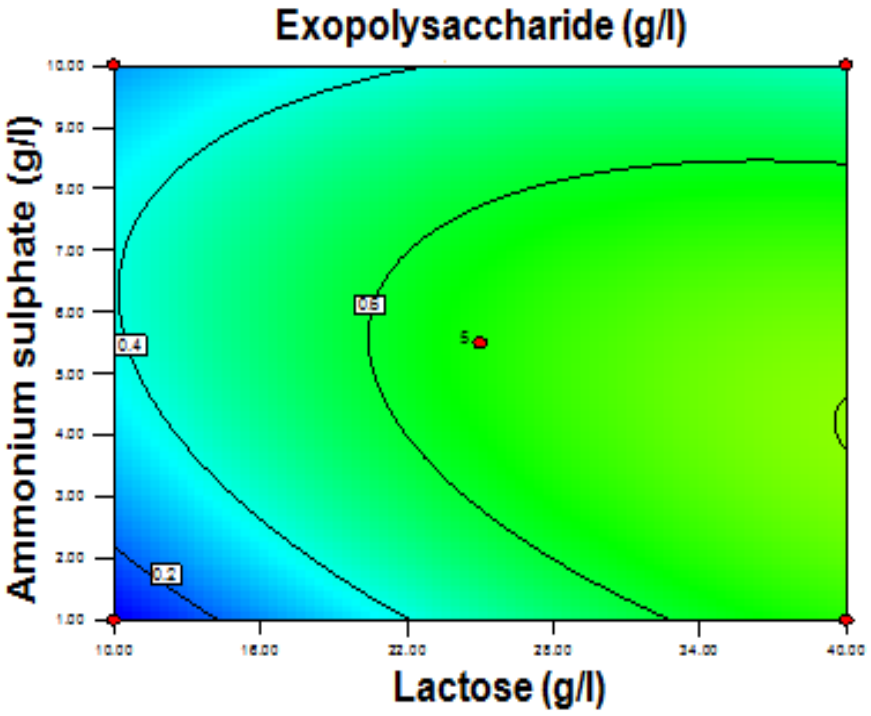


Fig. 4.4. Interaction effect of lactose and ammonium sulphate on production of exopolysaccharide (a) RSM graph (b) contour plot

Inspite of all these research, it is quite interesting that out of the various lactic acid bacteria strains reported for EPS production, only *L. rhamnosus* showed an EPS production ≥1 g/l (Dupont *et al.*, 2000; Macedo *et al.*, 2002).

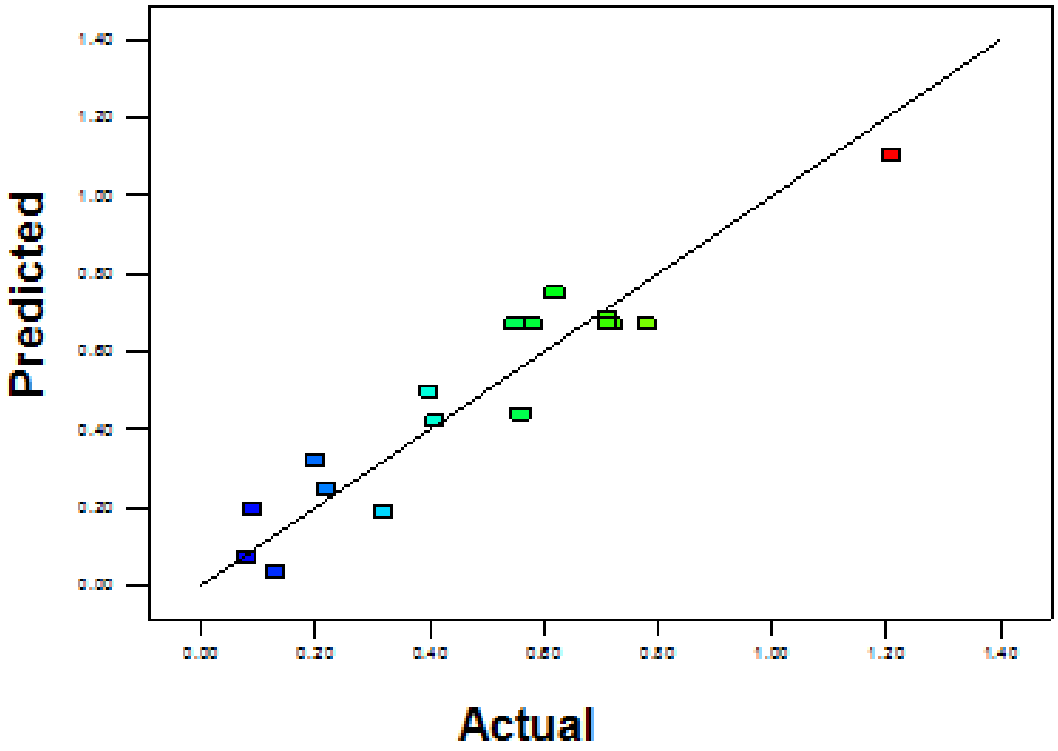


Fig. 4.5. Predicted Vs Actual values of exopolysaccharide production

4.4. Conclusion

A collection of statistical and mathematical approach used in this investigation suggested the importance of various factors at different levels for EPS production in submerged fermentation. A highly significant quadratic polynomial obtained by the Box-Behnken model was very useful for determining the optimal concentrations of constituents that have significant effects on production. From the experiments, the model was found to be significant with yeast extract and lactose as the most significant factors influencing EPS production. A maximum of 1.21 g/l EPS was produced with yeast extract 40 g/l, lactose 40

g/l and ammonium sulphate 5.5 g/l. A high similarity was obtained between the predicted and experimental values and it showed the accuracy of the response surface methodology.

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDE

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDE

5.1. Introduction

Exopolysaccharides are long-chain polysaccharides consist of branched, repeating units of sugars or sugar derivatives. The length and composition of branching strongly affects rheological properties as it affects the compactness of EPS (Vincent *et al.*, 2001). EPS produced by LAB are in a great variety, depending on the type of LAB strains, culture conditions and medium composition (Looijesteijn & Hugenholtz, 1999). The EPS often differ by monosaccharide composition, linkages between units, presence of repeated side-chains and substitutions. The conformation and chemical composition of polysaccharides have stronger effects on bioactivities according to the studies by many researchers (Surrenjav *et al.*, 2006; Tao *et al.*, 2006; Kreisman *et al.*, 2007). The structural diversity and effectiveness of exopolysaccharides among Lactic acid bacteria creates a necessary situation of detailed structural study of the molecule. NMR spectroscopy provided most of the experimental data that enabled the complex equilibrium of inter converting forms of reducing sugars to be unravelled (Angyal, 1992). The molecular weights of exopolysaccharides are also extraordinarily heterogeneous. In case of polysaccharides, long and short polymers are synthesized, although one molecular weight species predominates (Batchelor *et al.*, 1991).

The present chapter explains the purification and physico-chemical characterization of the EPS including monosaccharide composition, its sequence and linkage determination of carbohydrate backbone by employing Thermo Gravimetric Analysis (TGA), Fourier Transform Infra-red Spectroscopy (FT-IR), Gas Chromatography (GC), mass spectrometry, one dimensional NMR and two-dimensional NMR techniques like Correlation Spectroscopy

(COSY), Nuclear Over-hauser Effect Spectroscopy (NOESY), Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Quantum Coherence (HMQC). Molecular weight determination was done with the aid of Size Exclusion Chromatography (SEC) and Matrix-assisted laser desorption ionization- Time of Flight (MALDI-TOF). Since the primary structure, solution conformation and molecular weight of an exopolysaccharide play a role in immune function and performance of the food harbouring the polymer, the present characterization study becomes extremely important.

5.2. Materials and Methods

5.2.1. Inoculum

18 h old fresh inoculum of *Lactobacillus plantarum* MTCC 9510 required for the work was prepared as mentioned in chapter 2 (section 2.3.2).

5.2.2. Extraction and Purification

After fermentation in the production medium at 37 °C for 72 h, the culture was centrifuged at 11,500 x g for 15 min at 4 °C to remove cell pellet and the supernatant precipitated with double volume-chilled ethanol was stored overnight at 4 °C. The mixture centrifuged at 2,500 x g for 20 min and the pellet collected was dissolved in de-mineralised water and again precipitated using double-volume cold ethanol. It was further centrifuged at 2,500 x g for 20 min to collect the pellet (Savadogo *et al.*, 2004). The extracted EPS was dialysed using 5 KDa membranes against distilled water for 24 h with two changes of water and lyophilized.

The lyophilized EPS was purified according to a modified protocol of Dabour and LaPointe (2005). 1 % of the lyophilized powder was extracted two times with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 [v/v/v] and precipitated overnight with an equal volume of acetone. The precipitated EPS was dissolved in water, dialyzed using 5 KDa membrane for 24 h with two changes of water and lyophilized using a

SCANVAC Cool Safe^{TM} lyophilizer (Labogene, Denmark). The total carbohydrate content (Dubois *et al.*, 1956) and protein content (Lowry *et al.*, 1951) were evaluated before and after purification as per the standard protocols described in the chapter 2 (section 2.4.1 and 2.4.2). The UV spectrum of the purified fraction was studied at 280 nm using a spectrophotometer (Shimadzu UV-1601) to investigate the presence of protein.

5.2.3. Molecular Weight Determination of EPS by Gel Filtration Chromatography (GFC) and MALDI-TOF

Molecular weight of the pure exopolysaccharide was determined by Gel Filtration Chromatography and further confirmed by Matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed on an Axima CFR^{+} spectrometer (Applied Biosystems, Boston, MA) in the positive and negative modes, using a matrix of 10 mg/ml dithranol in Tetrahydrofuran (THF). Samples were desorbed with a nitrogen laser (λ 330 nm) using a detector sensitivity of 1000 mV FS. Mass spectra were recorded over an *m/z* range of 0-200 KDa and represent the summation of 200 acquisitions

Pure EPS (10 mg/ml) in de-ionized water was filtered through 0.22 µm filter membranes and loaded onto a Sephadex G-200 column (2x30 cm). The column was equilibrated using de-ionized water and the sample eluted using de-ionized water at a flow rate of 1 ml/min using a peristaltic pump. Fractions of 1 ml were collected. Total carbohydrate content of the fractions was determined by phenol-sulphuric acid method as mentioned in the chapter 2 (Dubois *et al.*, 1956). Void volume of the column was determined using blue dextran (Fluka, U.K) at a concentration of 1 mg/ml. Standard dextran (0.3 mg/ml) (Sigma, USA) with molecular weights of 1, 50 000 Da, 50 000 Da, 22 650 Da, 13 580 Da and 5, 000 Da were used to build the calibration curve. The presence of dextran was determined by measuring the spectrum between 280 and 190 nm, which gave a maximum

absorbance at 191nm. The molecular weight of the major eluted fraction was determined from the calibration curve developed using standards by GFC and confirmed by MALDI-TOF.

5.2.4. Molecular Weight and Polydispersity Index (PDI) of EPS by High Performance Size Exclusion Chromatography (HPSEC)

Weight average molecular weight (Mw), number average molecular weight (Mn) and size average molecular weight (Mz) of pure exopolysaccharide was determined by GPC. The experiment was carried out in a GPC instrument (Shimadzu, Japan), equipped with GPC data processing software. Phenogel columns 10E6A and 10E3A were connected in series for the experiment. The columns were calibrated with polystyrene standards (3 mg/ml) of 271, 000 Da, 96, 000 Da, 74, 500 Da and 12, 500 Da. Finally, the EPS (3 mg/ml) was eluted using tetrahydrofuran and operated isocratically at a flow rate of 0.6 ml/min. The injection volume was 20 µl.

5.2.5. Thermo gravimetric analysis (TGA) of EPS

Thermo gravimetric analysis of the polysaccharide was done using Shimadzu H-50 TGA apparatus using 15 mg of the test material. The TGA curve plots the TGA signal, converted to percent weight change on the Y-axis against the reference material temperature on the X-axis. The main changes of the line trend at two different temperatures, 90 °C and 260 °C were checked by Fourier Transform-Infrared Spectroscopy (Shimadzu, IR Prestige-21) of the polymer at the particular points.

5.2.6. Monosaccharide Composition Analysis

Monosaccharide composition analysis of EPS was done by employing the techniques gas chromatography, Fourier transform infrared spectroscopy, one dimensional NMR and two dimensional NMR spectroscopy and mass spectrometry.

5.2.6.1. Gas Chromatography (GC) analysis

Glycosyl compositions were determined by GC-MS analysis of the trimethylsilyl (TMS) glycoside derivatives dissolved in cyclohexane. The trimethylsilyl glycoside derivatives were obtained by hydrolysis of pure EPS with 2N trifluoroacetic acid at 110 °C for 2h. GC was performed on a Hewlett-Packard 4890A Gas chromatograph fitted with an OV1 capillary column (0.30 mmx12m) and a flame ionization detector. A GC chromatogram was obtained with standard monosaccharide sugars such as xylose, rhamnose, fructose, galactose, mannose and glucose in order for comparison.

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

FT-IR spectra of pure EPS were recorded from 450 to 4600/cm with a resolution of 4.0 cm^{-1} and 25 scans by subtracting both the background and atmospheric water at resolution. The sample pellets were prepared by mixing the fine freeze dried EPS (1 mg) with 100 mg KBr. Spectra were recorded using Shimadzu FT-IR spectrophotometer (IR Prestige-21).

5.2.6.3. One Dimensional ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy

One-dimensional (1D)-NMR spectra of the polysaccharide solution was recorded at room temperature using a Bruker Avance II-500 spectrometer. Both ^{1}H and ^{13}C NMR were performed. The polysaccharide was dissolved and analyzed in 99.96 % D_2O yielding clear solutions at 5 mg/500µl, spectra were referenced to internal Tetra methyl silane (TMS). For ^{1}H NMR spectra the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.00 s and 3.17 s respectively, whereas for ^{13}C NMR, the D1 and AQ were 2.0 and 1.1 s respectively. Chemicals shifts were expressed in parts per million (ppm).

5.2.6.4. Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed on an Axima CFR^{+} spectrometer (Applied Biosystems, Boston, MA) in the

positive and negative modes, using a matrix of 10 mg/ml dithranol in Tetrahydrofuran (THF). Samples were desorbed with a nitrogen laser (λ 330 nm) using a detector sensitivity of 1000 mV FS. Mass spectra were recorded over an *m/z* range of 0-10 KDa and represent the summation of 200 acquisitions.

5.2.7. Sequence and Linkage Confirmation of EPS by Two-Dimensional NMR Techniques

A combination of homo-nuclear and hetero-nuclear NMR experiments was carried out at 500 MHz using purified EPS. A set of homo-nuclear experiments like Correlation Spectroscopy (COSY) and Nuclear Over-hauser Effect Spectroscopy (NOESY) were performed to assign the ^{1}H resonances and sequence confirmation. ^{13}C resonances were assigned with the help of hetero-nuclear experiments like Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Quantum Coherence (HMQC). The two-dimensional NMR was recorded at room temperature using a Bruker Avance II-500 spectrometer. The polysaccharide was dissolved and analyzed in 99.96 % D_2O yielding clear solutions at 30 mg/500µl, spectra were referenced to internal Tetra methyl silane (TMS). For COSY NMR spectra, the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.4 s and 0.2 s respectively. For NOESY NMR, the D1 and AQ were 1.8 and 0.3 s respectively. Chemicals shifts were expressed in parts per million (ppm).

5.3. Results and Discussion

5.3.1. Extraction and Purification

The purified fractions (Fig. 5.1) of EPS after acetone precipitation showed negligible amount of protein and the UV absorption spectra of EPS showed no absorption at 280 nm, implying that protein was absent in this polysaccharide. There was no loss of polysaccharide in the purification process as the amount of EPS was the same before and after purification.

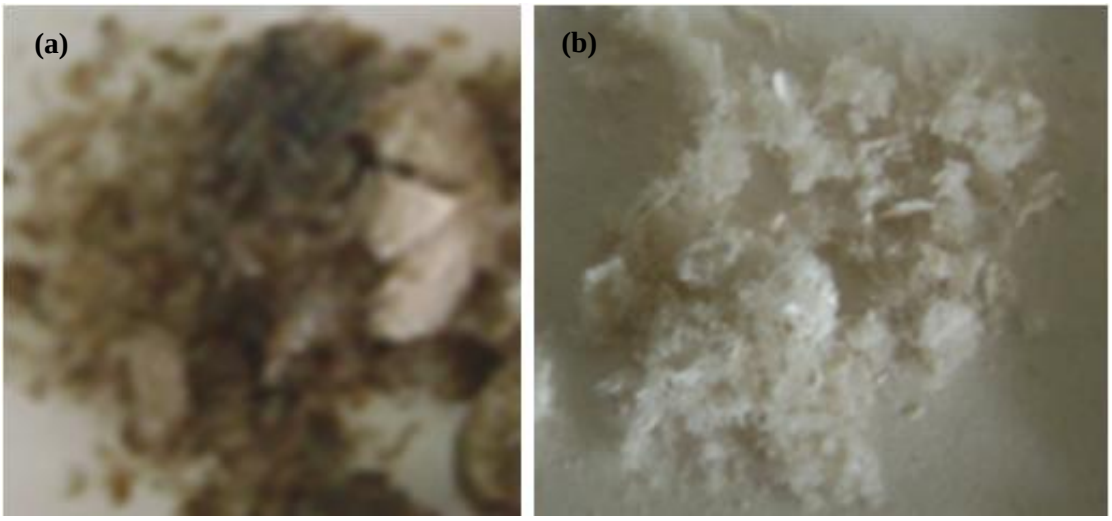


Fig. 5. 1. Exopolysaccharide after purification (a) EPS after ethanol precipitation (b) EPS after acetone precipitation

5.3.2. Molecular Weight Determination of EPS by Gel Filtration Chromatography (GFC) and MALDI-TOF

Gel Filtration Chromatography (GFC) is a chromatographic technique in which analytes are separated based on their molecular size in solution. It is used to determine molecular mass (weight) distribution (MWD) of both natural and synthetic polymers. The void volume of the column and the elution volume of the standards were obtained from graph, plotting absorbance against eluted fractions. The partition coefficient of the standards and the sample calculated based on the void volume of the column and their elution volumes as shown in Table 5.1

Table 5.1 Partition coefficient (K_{av}) of standard dextrans and *EPS from Lactobacillus plantarum*

Markers	*Elution volume (ve)*	*Void volume (vo)*	*Total bed volume (vt)*	*Kav=(ve-v_0)/(vt-v_o)*
M1150000	29	22	70	0.15
M2 50000	42	22	70	0.42
M3 22650	48	22	70	0.54
M4 13580	51	22	70	0.60
M5 5000	60	22	70	0.79
L. plantarum EPS	31	22	70	0.19

The logarithm of molecular weight of the standard dextrans as a function of partition coefficient was plotted (Fig. 5.2). The data was modelled using a third order polynomial equation as it fits to the model. The coefficient of multiple determination (R^2) was obtained as 1, which shows the perfect fit of the model developed with higher order polynomial equation.

A major fraction of molecular weight ~1.31 x 10^5 Da was obtained from the chromatographic separation, although polysaccharide fractions were obtained in a range of 204-6.5 KDa. The lower molecular weight fractions could be mainly the oligomers present in the polysaccharide. The major fraction molecular weight was confirmed by MALDI-TOF (Fig. 5.3), which gave a single peak at 132 KDa very near to the value obtained from the calibration curve. Similar to all other polysaccharides or polymers, fractions of different molecular weights were present but the major fraction was of the specified molecular weight giving a major peak after the void volume of the column.

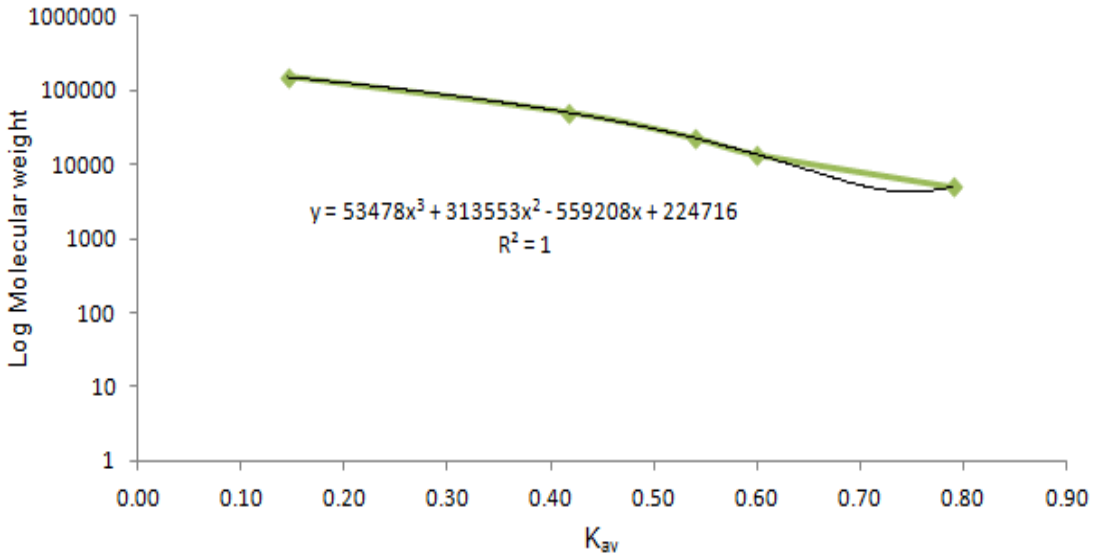


Fig. 5. 2. Graph displaying dextran standards partition coefficient vs. logarithmic molecular weight

5.3.3. Molecular Weight and Polydispersity Index (PDI) of EPS by High Performance Size Exclusion Chromatography (HPSEC)

A calibration curve was plotted with logarithm of molecular weight as a function of retention time with the polystyrene standards. Based on the third order polynomial equation developed for the model, EPS subjected to GPC was found to have a weight average molecular weight (Mw) of 2.68 $x10^5$ Da, number average molecular weight (Mn) of 2.55 $x10^5$ Da and a size average molecular weight (Mz) of 2.83 $x10^5$ Da (Fig. 5.4 a,b).

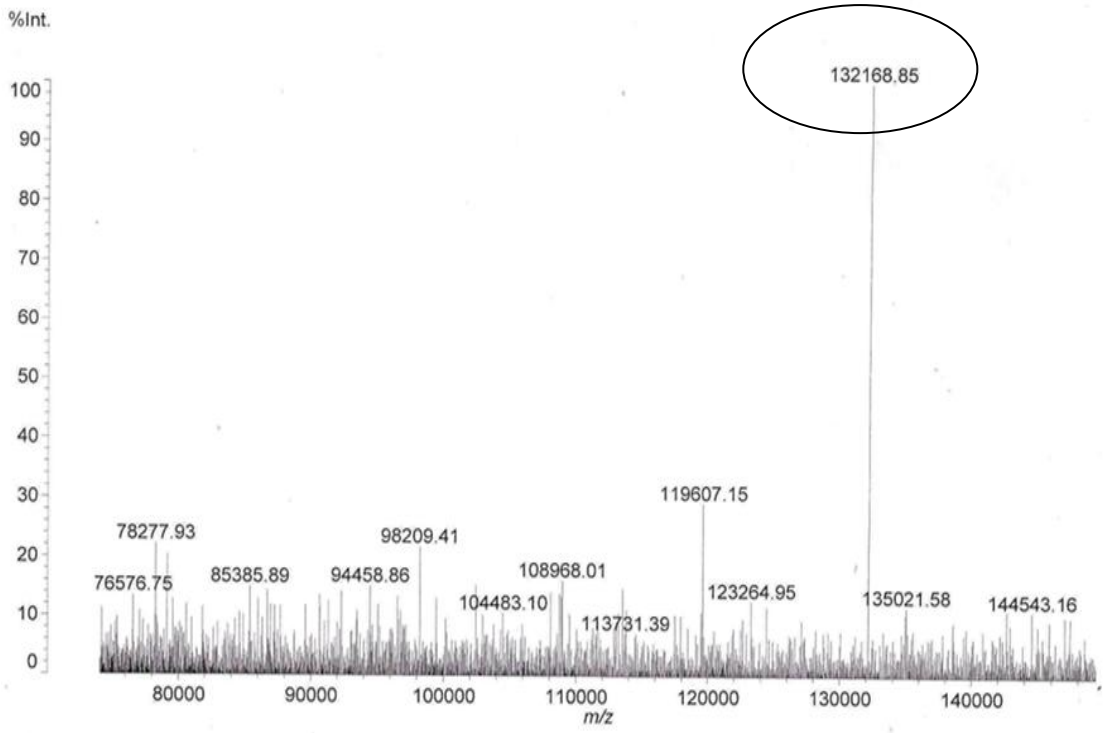


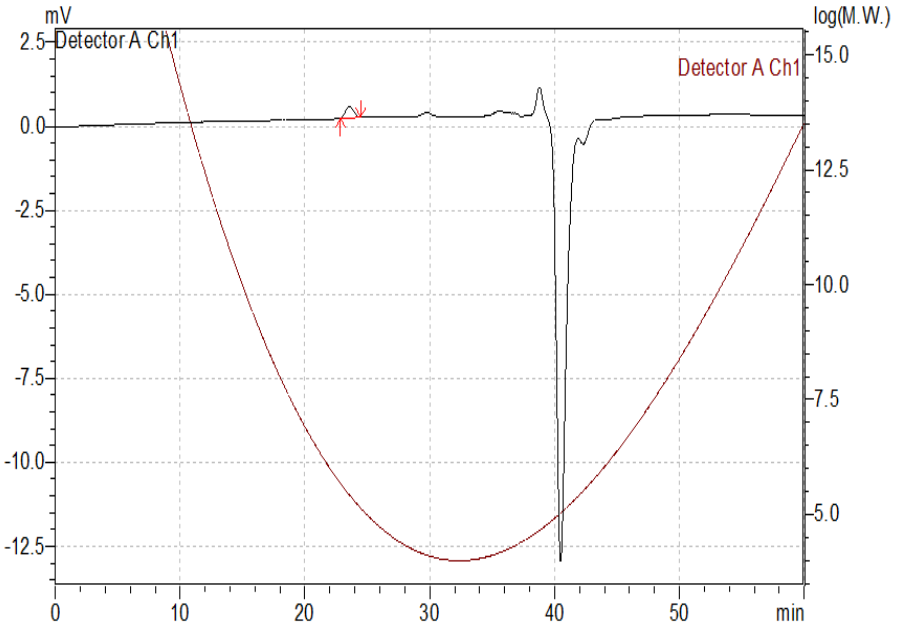
Fig. 5. 3. MALDI-TOF of the eluted fraction of *Lactobacillus plantarum* exopolysaccharide

The PDI (Mw/Mn) of the exopolysaccharide was obtained as 1.05, which is a measure of the distribution of molecular mass in the sample. The PDI has a value ≥1. Difference in molecular weight distribution (MWD) pattern was observed in the two gel permeation experiments with the same sample. This difference in the distribution could be attributed to the difference in the standards, mobile phase and detector. The sample which was soluble in water was only sparingly soluble in THF.

5.3.4. Thermo gravimetric analysis (TGA)

TGA involves the measurement of change of sample mass with change of temperature. It measures the variation of heat flux in a sample with variation of temperature.

(a)



(b)

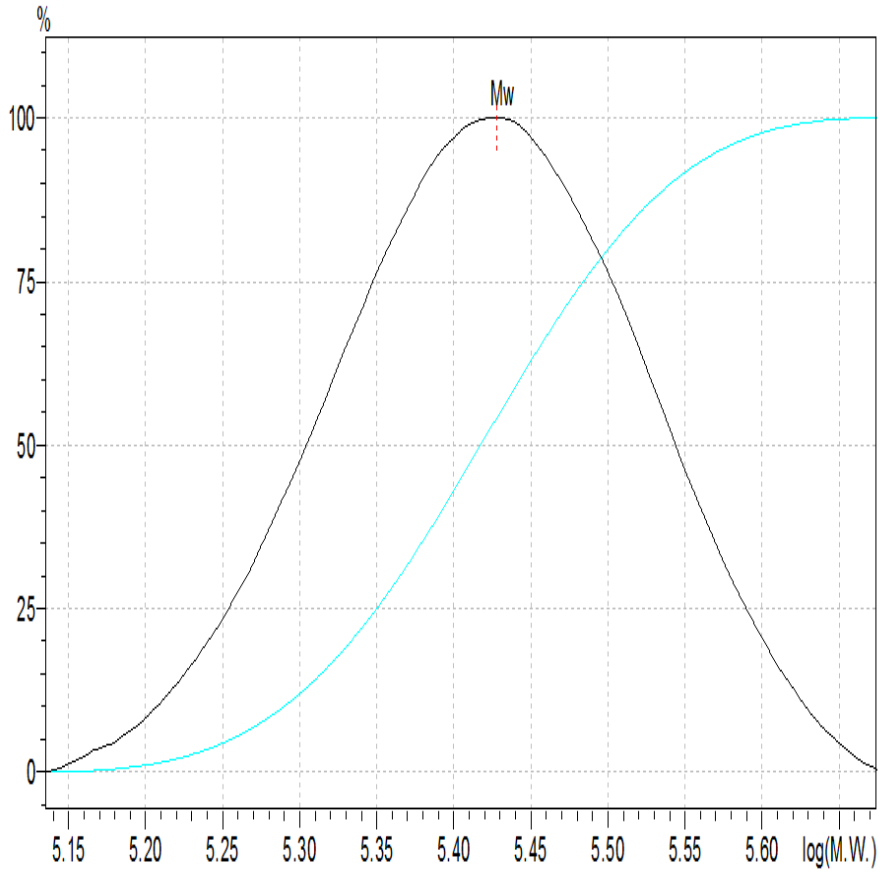


Fig. 5. 4. (a) GPC chromatogram of purified exopolysaccharide (b) Molar Mass Distribution (MMD) pattern

The test result is a graph of the TGA signal (gain converted to percent weight loss) on the Y-axis plotted versus the sample temperature in °C on the X-axis which is shown in Fig.5.5. Moisture release during heating of the polysaccharide suggested that the EPS was not truly anhydrous.

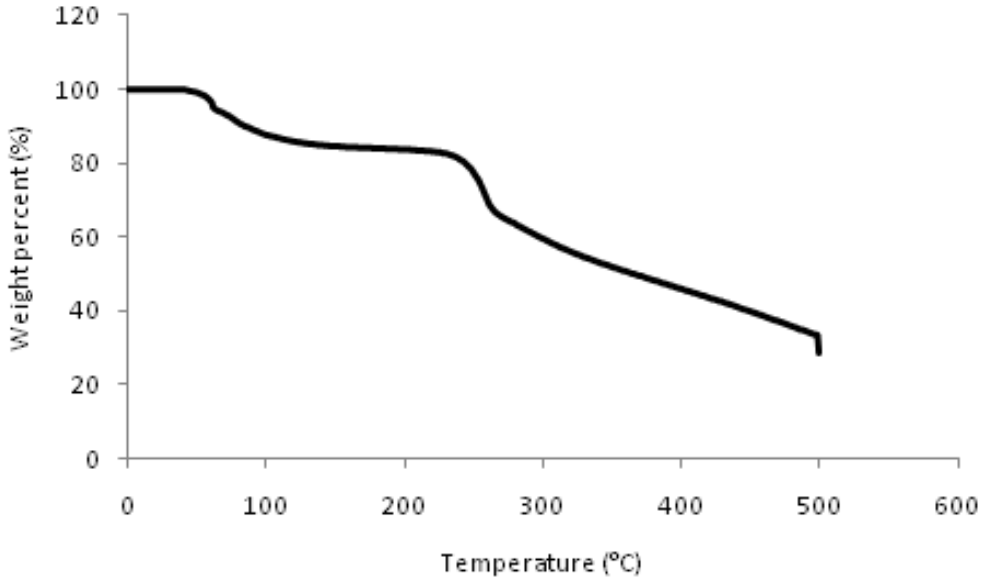


Fig. 5. 5. TGA thermogram of purified exopolysaccharide from *Lactobacillus plantarum*

A degradation temperature (Td) of 260 °C was determined from the TGA curve of the polysaccharide. About 90 % of the EPS was stable up to 238 °C. This fact implies that the material should not be submitted to temperature ranges close to 260 ºC, in order not to compromise the physical integrity of the material evaluated. The weight of polymer was dramatically lost at around 400 °C and continued gradually to decrease and the final residue was 29 %. Two main changes of the line trend were observed in the TGA thermogram at different temperatures, 90 °C and 260 °C respectively. FT-IR spectrum of the polysaccharide at the two different temperatures was analyzed for relevant changes in the structure during heating. The FT-IR spectrum (Fig. 5.6) of the polymer at 90 °C and 260 °C showed not much

structural changes. However, observed a difference in the absence of a peak corresponding to an amino group at 1531.8 cm^{-1} when subjected to 260 °C and presence of two more peaks at 912.6 and 862.2 cm^{-1} and there were some small shifts in the peaks and all these could be due to the physical transition of the polymer. The absence of the peak at 1531.8 cm^{-1} could be due to the probable reason that the amino group was denatured when heated at 260 °C.

5.3.5. Monosaccharide Composition Analysis

5.3.5.1. Gas Chromatography (GC) analysis

GC analysis of the polysaccharide on comparison with GC chromatogram obtained with a mixture of sugar standards, rhamnose, fucose, xylose, glucose, galactose and mannose revealed that the EPS was composed of glucose and mannose. From the chromatogram (Fig. 5.7), it was evident that two forms of glucose, α-D-glucose and β-D-glucose and one form of mannose, either α or β form were present.

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

FT-IR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. In comparison with the IR spectra of polysaccharides documented in literature, a characteristic absorption band appeared at 1658.78 cm^{-1} (Fig. 5.8) and was assigned to the stretching vibration of carbonyl group (C=O), while another absorption band at 2937.59 cm^{-1} was intensified and assigned to the stretching vibration of methylene group (–CH_2–), usually present in hexoses, like glucose or galactose or deoxyhexoses like rhamnose or fucose. A continuous absorption beginning at approximately the region of 3304.06 cm^{-1} is characteristic of a carbohydrate ring. Absorption peak at 1056.99 cm^{-1} was assigned to carbohydrate C–O stretching vibrations. A small peak around 1530 cm^{-1} corresponding to an amino group was also observed in the spectrum of EPS. The obvious absorption peak 879.54 cm^{-1} revealed the existence of β-glycosidic bond.

It was explained by Braissant et al (2009) that the peak at 898 cm^{-1} could be attributed to the β-glycoside linkage between sugar monomers. All the vibrational peaks were similar to the peaks obtained for a standard exopolysaccharide (dextran) confirming its nature as a polysaccharide (Fig. 5.8).

The band in the range 1030-944 cm^{-1}, with minimum at 998 cm^{-1} can be attributed to Glc (Černá *et al.*, 2003). In the anomeric region (950–700 cm^{-1}) it exhibited the obvious characteristic absorption at 812.03 cm^{-1} corresponding to the existence of mannose (Mathlouthi *et al.*, 1986). There were three peaks near 1000 – 1200, indicating that the polysaccharide contained α-pyranose (Chi *et al.*, 2007). The absorption bands at 812 and 879 cm^{-1} indicated that the EPS contained both α and β-type glycosidic linkages in its structure (Barker *et al.*, 1954). The carbohydrates show high absorbencies in the region 1200–950 cm^{-1}, that is within the so-called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide, allowing its possible identification (Filippov, 1992).

5.3.5.3. One Dimensional ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H NMR spectrum of a polysaccharide can generally be divided into three major regions: the anomeric region (δ_H 4.5 - 5.5), the ring proton region (δ_H 3.1 - 4.5) and the alkyl region (δ_H 1.2 - 2.3). In the spectrum, signals between δ5.2 and δ4.5 ppm correspond to the anomeric protons of the monosaccharide components of the EPS and these signals often serve as signatures for differentiating complex carbohydrate structures. The signals obtained in the spectrum (Fig. 5.9) between δ4.4 and δ3.2 ppm were due to protons attached to C2-C6 and were poorly resolved due to the overlapping chemical shifts. The pattern of these signals showed similarities to the spectrum of standard samples. The ^{1}HNMR spectrum showed three signals in the anomeric region corresponding to a trisaccharide repeating unit. Based on the comparison with the data published in carbohydrate research database (www.glyco.ac.ru), the

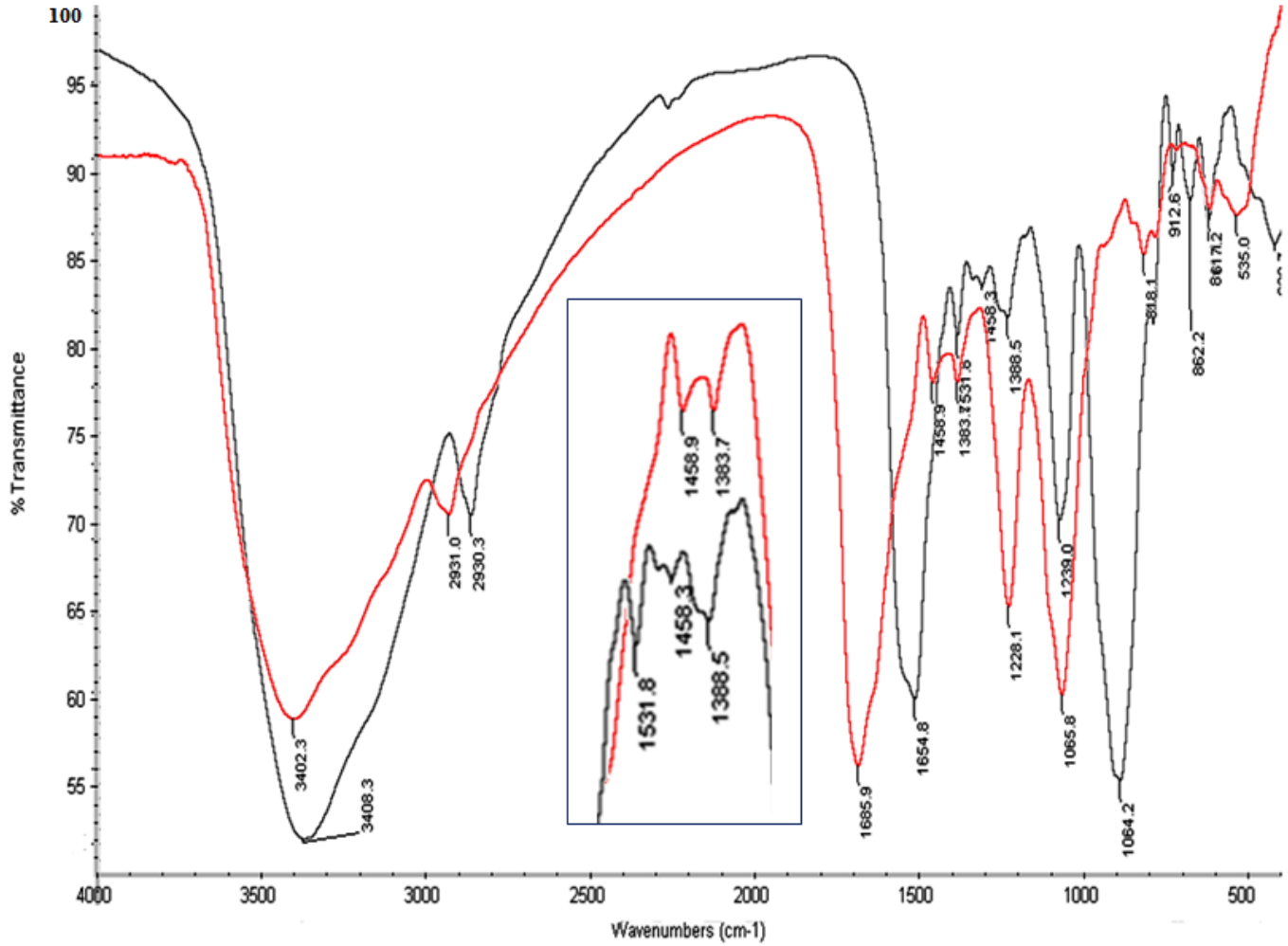


Fig. 5. 6. Fourier-transform infrared spectrum of purified exopolysaccharide at 90 °C (black line) and 260 °C (red line) (inset is the magnified portion showing difference in peak)

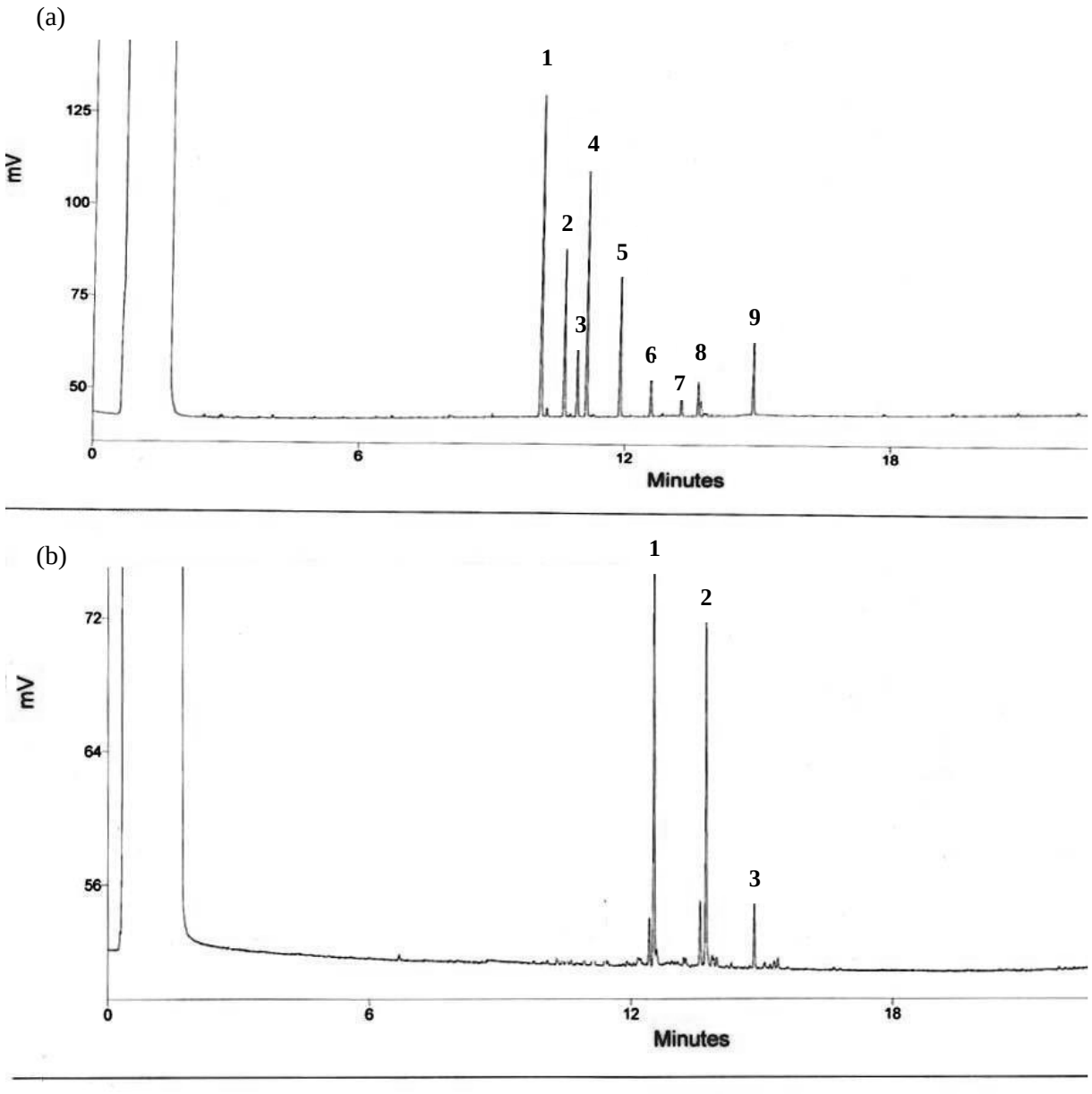


Fig. 5. 7. GC profile of a) standard sugars and b) hydrolysed polysaccharide: Peak identity: a) 1-rhamnose; 2-fucose; 3-rhamnose; 4-fucose/xylose; 5-xylose; 6-mannose; 7- galactose 8 - glucose; 9 - glucose. (b) 1- mannose; 2 & 3- Glucose (left to right)

chemical shift δ5.2 (1H, s) corresponds to the anomeric proton of α-D-mannose, δ5.02 (1H, t, 11.5 Hz) to α-D-glucose and δ4.8 (1H, d, 16.5 Hz) to β-D-glucose. Maeda et al (2004b) quoted that chemical shifts, δ 5.14 was assigned to a α-hexapyranosyl residue and δ 4.82, δ 4.68, δ 4.53, δ 4.49 were assigned to pyranose ring forms in a β-anomeric configuration. The resonances at δ_H 5.626, 5.408, 5.341, 5.322 and 5.2 ppm are typical of anomeric protons in residues with the α-anomeric configuration and the signals at δ_H 4.808, 4.608 and 4.450 ppm are typical of β-anomeric protons (Hallack *et al.*, 2010). It was also mentioned that the signal at δ5.2 corresponds to α-D-mannose and the one at δ4.8 corresponds to β-D-glucose.

Concentrated samples are required in order to obtain a good ^{13}C NMR spectrum. The ^{13}C NMR spectrum can also be divided into different regions. The anomeric carbons are found at δ_C 95 - 110, the ring carbons at δ_C 50 - 85, the alkyl carbons at δ_C 15 - 25 and the carbonyl carbons at δ_C 165 - 180. Based on the chemical shifts in ^{13}C NMR (Fig. 5.10), there were three signals in anomeric region confirming the presence of three repeating units as obtained from the GC and ^{1}H NMR result. C-1 signal at δ 100.78 was assigned to α-hexopyranosyl residue, α-D-glucose, while the other anomeric signal (δ 104.70) was assigned to a β-hexopyranosyl residue, β-D-glucose. The chemical shift of anomeric carbons occurring at approximately δ104 ppm indicated a β-configuration.

The presence of signals with a chemical shift above δ102 p.p.m. indicates β configuration of glucose (Usui *et al.*, 1973). The C-1 signal at 103.09 ppm could be assigned to an α-D-mannopyranosyl, non-reducing end-group linked (1-2) to an adjacent α-D mannopyranosyl residue with regard to Gorin (1981). Hence, the third unit of the trisaccharide could be α-D-mannose supporting the data from ^{1}H NMR.

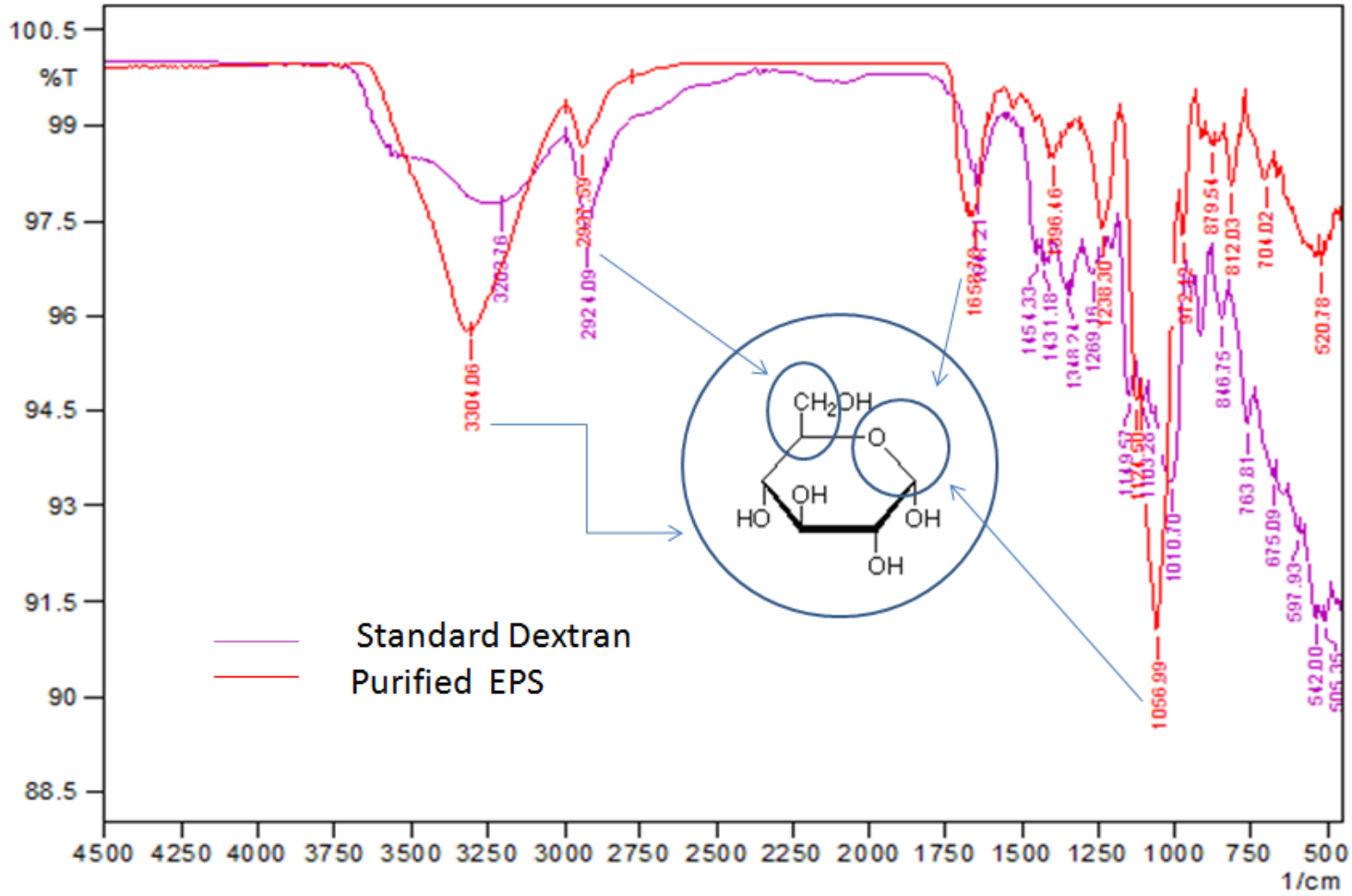


Fig. 5. 8. Fourier-transform infrared spectrum of exopolysaccharide extracted from *Lactobacillus plantarum*

The strong signal in the spectrum at 103.4, 79.9, 72.7, and 62.3 ppm were assigned to C-1, C-4, C-3 and C-6 of mannose (Chaubey & Kapoor, 2001). There was no signal in the region near 90 p.p.m. suggesting that there was no furanose ring, *i.e*., all sugars had the pyranose ring configuration. The peak at δ32.78 was assigned to internal acetone. Indeed, the signal at δ100.78 ppm corresponded to the anomeric carbon involved in α (1, 3) linkage. The presence of the (1, 3) linkage was further confirmed by the signal at δ81.23 ppm which corresponded to C-3 involved in α (1, 3) linkage. Vanhaverbeke et al (1998) assigned two downfield signals (δ110.12, δ 107.67) to two residues having β-configuration and three signals at (δ 99.73, δ 99.99 and δ 100.73) to three residues having α-configuration.

5.3.5.4. Mass Spectrometry

Mass spectra of polymers can provide a variety of information in a mass range where single polymer chains are resolved. Among this information, the most fundamental are the mass of the constituent repeating unit, the end group and the average molecular weight data *M*n and *M*w. MALDI-TOF spectrum (Fig. 5.11) having a most probable peak at 5297.7 suggests that the compound consists of repeating units with a molecular weight of 486.77.The masses of the end groups were found to be 321.26. The molecular weight of the molecule giving peak at 5297.7 was obtained to be 5189. The molecular weight of the repeat units and the end groups points to the facts that the chemical structure comprises repeating units of three hexose molecules with two hexose molecules free at the ends supporting the data obtained from the previous studies. The spectrum showing a most probable peak at 5297.7 Da points to the presence of an oligosaccharide of the respective molecular weight in the mixture. As the ionization of oligosaccharides is easier than polysaccharides, the signals obtained could be of the oligosaccharide in the mixture. It has already been explained the possibility of obtaining a better spectrum through hydrolysis of polysaccharides.

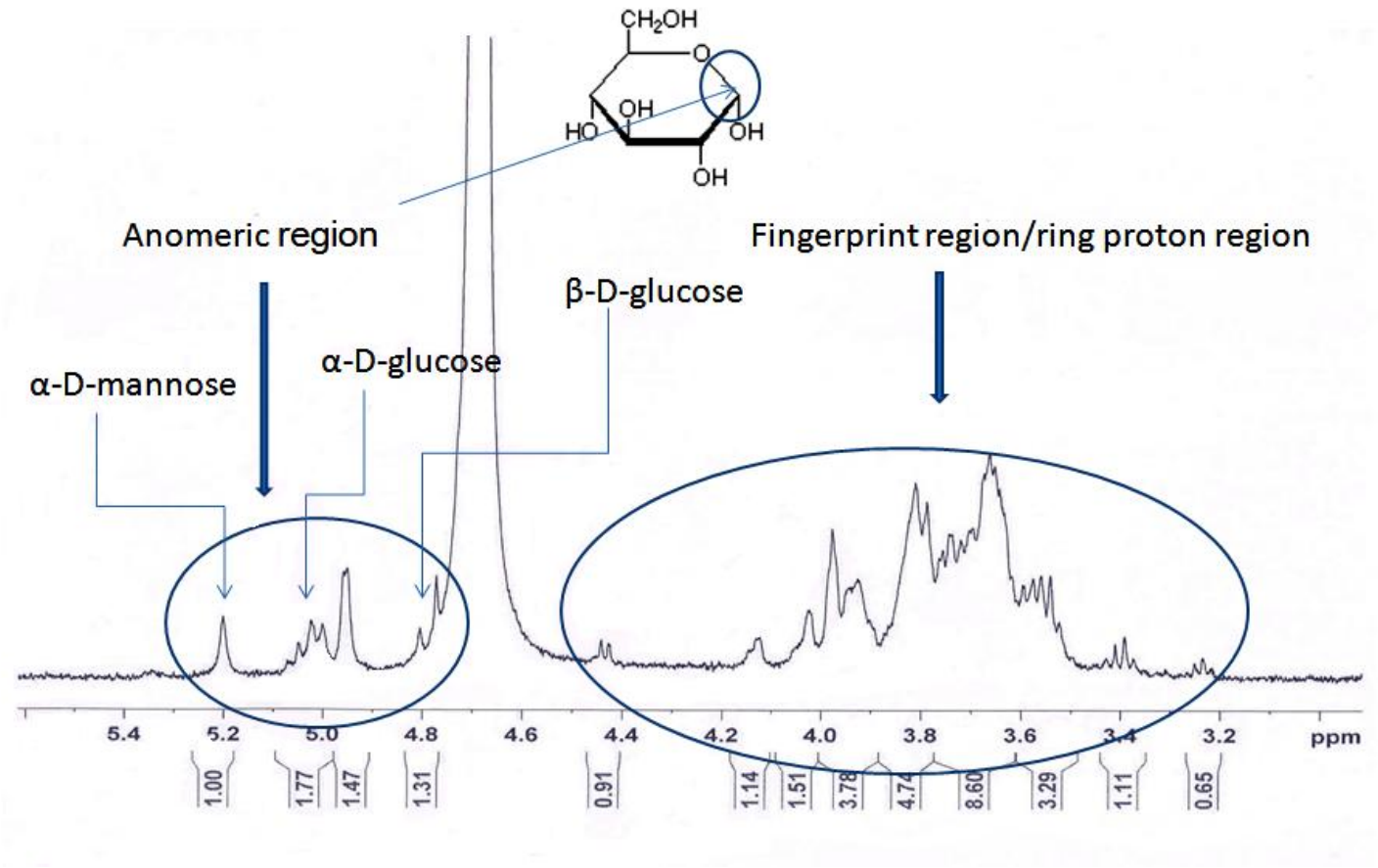


Fig. 5. 9. ^{1}H NMR spectrum of purified *Lactobacillus plantarum* exopolysaccharide

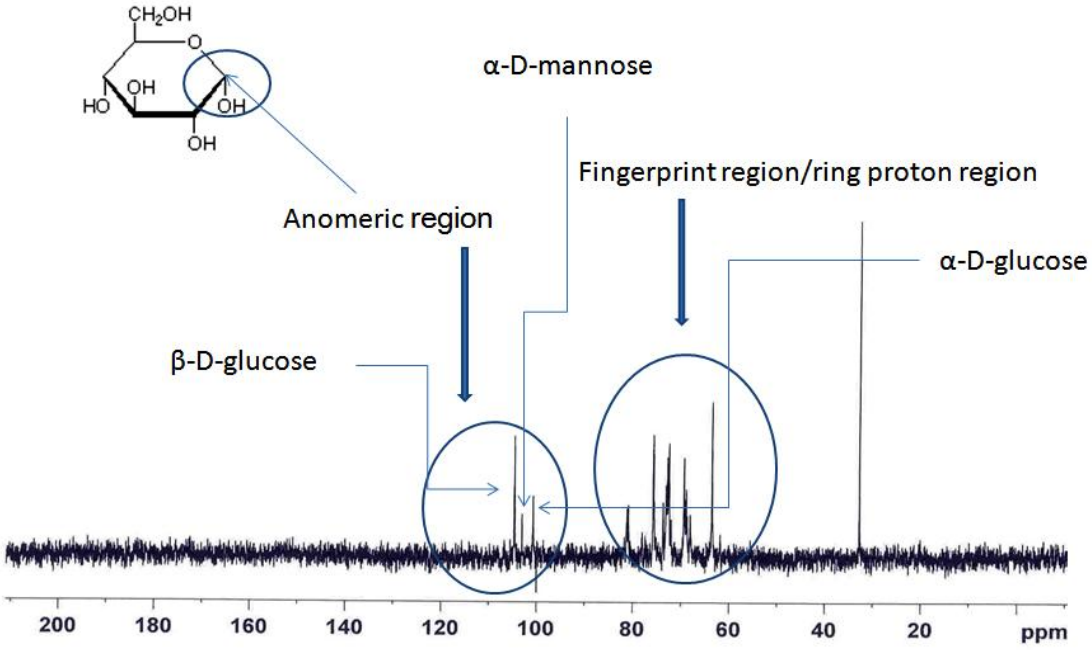


Fig. 5. 10. ^{13}C Nuclear magnetic resonance spectrum of purified exopolysaccharide from *Lactobacillus plantarum*

5.3.6. Sequence and Linkage Confirmation of EPS by Two-Dimensional NMR Techniques

The 2D-NMR COSY spectrum profiled the chemical shifts of each proton in the monosaccharide units of the exopolysaccharide. The profile of the spectrum coincided with the 1D-^{1}H NMR spectrum. By correlating the proton shifts with the carbon shifts obtained from ^{13}C-^{1}H HMQC and HSQC, the resonances of the anomeric and the non-anomeric carbons were assigned. The signals from the NOESY confirmed the signals from COSY and the sequence of monosaccharides present in the exopolysaccharide backbone. The proton-proton interactions were obtained from COSY and NOESY spectrum.

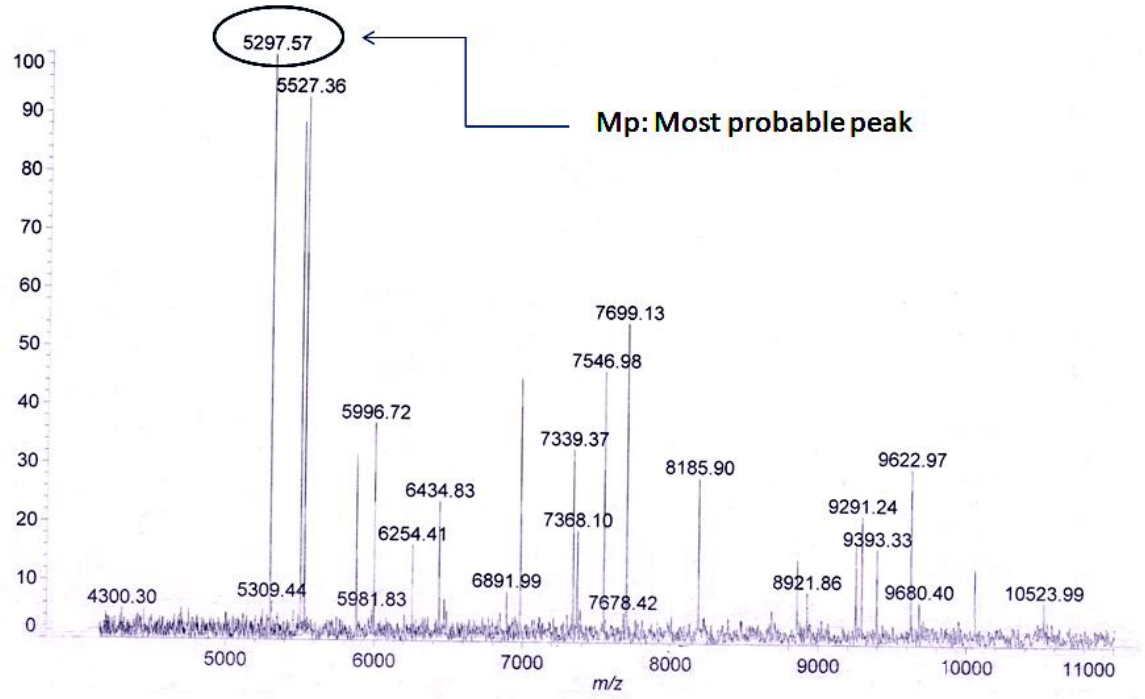


Fig. 5. 11. MALDI-TOF mass spectrum of exopolysaccharide purified from *Lactobacillus plantarum*

In one dimensionalNMR of carbohydrates as there will be peak overlap in the fingerprint region, the protonand carbon resonances could be obtained only from two-dimensional NMR techniques. The interaction of the anomeric protons of the three residues with the adjacent protons were assigned from COSY and NOESY spectrum. The one dimensional ^{1}H-NMR had three signals in the anomeric region, the correlation of this anomeric proton resonance with the adjacent proton resonance was assigned from the COSY experiment. The additional information of spatial interaction between the residues was obtained from NOESY which could lead to monosaccharide sequence determination and linkage between the residues.

COSY spectrum shows frequency correlations between nuclei that are connected by chemical bonds. This information can be used to assign the frequencies of all nuclei in the molecule, whereas NOESY experiments give signals that correspond to hydrogen atoms

which are close together in space. Structures can be derived from a collection of such signals which define distance constraints between hydrogen atoms. The cross peaks of the anomeric protons with the non-anomeric protons could be clearly obtained from Fig. 5.12.

The cross peaks δ5.0/ δ4.0, δ5.2/ δ4.1 and δ4.8/ δ4.4 were detected in ^{1}H-^{1}H COSY, since δ 5.2, δ 5.02 and δ 4.8 resonances corresponded to anomeric protons, the other resonances were assigned to H-2 of the three residues. Like-wise, the resonances of the rest of the protons of all the three residues, H-3 to H-6 were assigned. Based on the resonances from the COSY experiment (Table 5.2), the resonances of the anomeric and non-anomeric

Table 5. 2. Resonances of anomeric and non-anomeric protons of monosaccharide units of exopolysaccharide from *Lactobacillus plantarum* from ^{1}H-^{1}H COSY NMR (SD ± 0.2)

Proton-Proton signals	***Monosaccharide units***		
	α-D-Glucose	***α-D-Mannose***	***β-D-Glucose***
H1/H2	5.0/4.0	5.2/4.1	4.8/4.4
H2/H3	4.0/3.8	4.1/3.9	4.4/3.4
H3/H4	3.8/3.6	3.9/3.8	3.4/3.3
H4/H5	3.6/3.4	3.8/3.7	3.3/3.2
H5/H6	3.4/3.2	3.7/3.5	3.2/3.0

carbons (Table 5.3) were obtained from ^{1}H-^{13}C HMQC and HSQC spectra except the C-6 of β-D-glucose. The signals in ^{1}H-^{13}C HSQC and HMQC represents a proton that is bound to a carbon atom. The anomeric proton resonances δ 5.2, δ 5.02 and δ 4.8 of α-D-mannose, α-D-glucose and β- D-glucose obtained on comparison with standards, correlated to anomeric

carbon signals at δ 103.09, δ 100.78 and δ 104.7 in the HSQC and HMQC (Fig.5.13) experiments.

Table 5. 3. Resonances of anomeric and non-anomeric carbons from ^{1}H-^{13}C HMQC and HSQC (SD ± 0.2)

Carbon-Proton signals	*Monosaccharide units*		
	α-D-Glucose	***α-D-Mannose***	***β-D-Glucose***
H1/C1	5.0/100.78	5.2/103.09	4.8/104.7
H2/C2	4.0/68.87	4.1/78.77	4.4/69.40
H3/C3	3.8/78.42	3.9/81.23	3.4/80.97
H4/C4	3.6/75.63	3.8/59.40	3.3/68.87
H5/C5	3.4/76.03	3.7/73.32	3.2/74.94
H6/C6	3.2/74.94	3.5/69.61	3.0/-

These three peaks in the anomeric region are indicative of the three monosaccharide residues, α-D-mannose, α-D-glucose and β-D-glucose. The signals at δ81.23, δ78.42 and δ80.97 confirm the carbons involved in linkage. As the particular signals are of third carbon atoms, it can be concluded that linkage present in the polysaccharide is (1, 3) linkage. Carbons linked to other sugars are found between δC 75 and 85 ppm and non-substituted ring carbons usually have the chemical shifts between δC 65 and 75 ppm (Dag, 2005).

Along with the carbon and proton resonances obtained, some inter-residue and intra-residue NOE contacts (Table 5.4 and Table 5.5) were obtained with NOESY spectrum (Fig.5.14), which could lead to the conclusion of the monosaccharide sequence present in the carbohydrate backbone.

Table 5. 4. ^{1}H-^{1}H NOESY intra-residue signals in the monosaccharide subunits of exopolysaccharide from *Lactobacillus plantarum* (SD ± 0.2)

Monosaccharide Units		
α-D-Glucose	***α-D-Mannose***	***β-D-Glucose***
5.0/3.6 (H1/H4)	5.2/4.1 (H1/H2)	4.8/4.4 (H1/H2)
5.0/3.8(H1/H3)	5.2/3.9(H1/H3)	4.4/3.3 (H2/H4)
4.0/3.8(H2/H3)	5.2/3.7 (H1/H5)	
4.0/3.6(H2/H4)		

NOESY provides through-space correlations between protons that are positioned near each other and are not necessarily connected through bonds (Bubb, 2003). The inter-glycosidic NOE resonances between the protons δ5.0/δ 3.9, δ5.0/δ 3.7, δ5.2/δ3.4, δ5.2/δ3.2, δ4.8/3.8, δ3.9/3.6, δ4.4/δ4.1, δ4.4/δ 3.5, δ4.4/3.7 indicated that the sequence of carbohydrate backbone is - α-D-glucose- α-D-mannose- β-D-glucose- (Fig.5.15) linked by α (1, 3) linkages and a possibility of β (1, 3) linkage at the terminal.

The anomeric proton of α-D-glucose connected to non-anomeric protons of α-D-mannose and α-D-mannose connected to β-D-glucose non-anomeric protons through NOE inter-glycosidic resonances.

The inter-residue correlations obtained between the monosaccharide units were of the anomeric protons of each unit with the third and fifth protons of the nearby unit due to the (1,3) linkage between the units. Linkages through C1 were obtained for all the three sugars from the NOESY spectrum. The data obtained from the two-dimensional NMR techniques reveals the complexity of the exopolysaccharide structure. The probability of the presence of

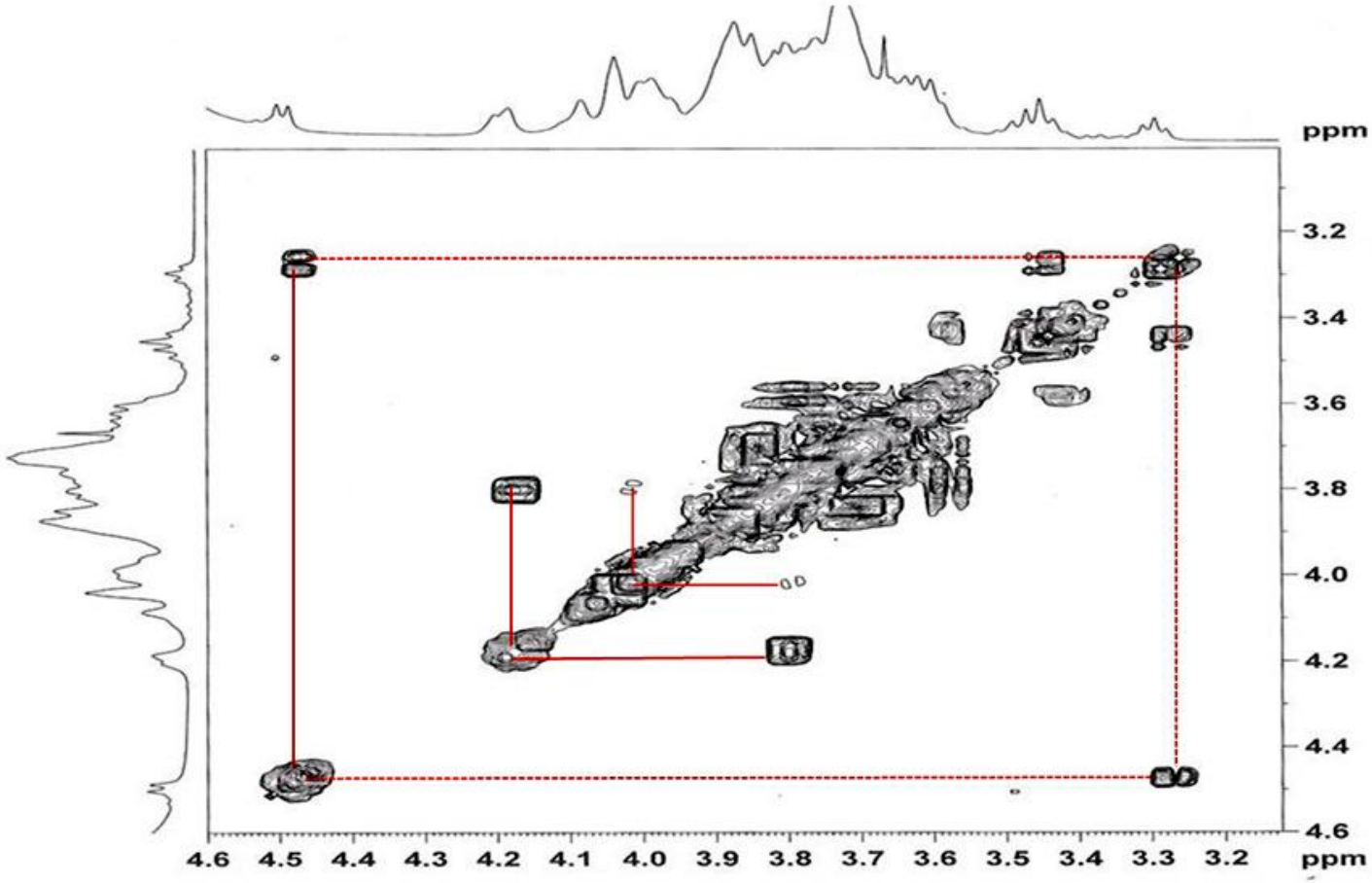


Fig. 5. 12. ^{1}H-^{1}H COSY NMR (expanded region) of purified exopolysaccharide from *Lactobacillus plantarum* (Red lines indicate the proton-proton interactions)

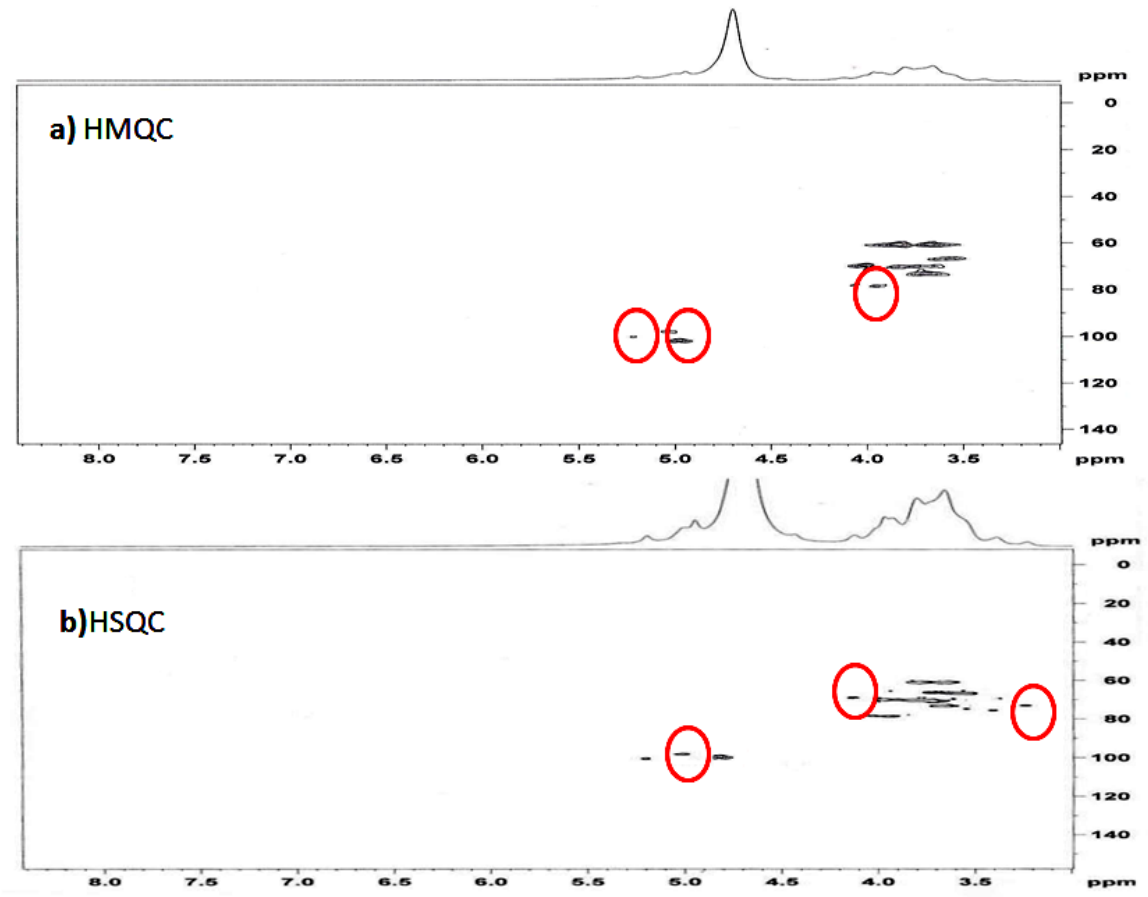


Fig. 5. 13. ^{13}C-^{1}H HMQC and HSQC of purified exopolysaccharide from *Lactobacillus plantarum* (red circles indicate the carbon-proton interactions)

an amino group in the polysaccharide was ruled out as the peak corresponding to an amino group was not present in any of the NMR experiments. Signals from nitrogen-bearing carbons are found between δ_C 50 and 60 ppm (Dag, 2005). There were no signals in the corresponding region in 1D and 2D carbon NMR.

A complementary procedure for identification of the monosaccharide constitution of complex carbohydrates is nuclear magnetic resonance (NMR) spectroscopy which is non-destructive and relies on the magnetic properties of some nuclei. NMR experiments are often

Table 5. 5. ^{1}H-^{1}H NOESY inter-residue signals in the monosaccharide subunits of exopolysaccharide from *Lactobacillus plantarum* (SD ± 0.2)

Monosaccharide Units		
***α*-D-Glucose**	***α*-D-Mannose**	***β*-D-Glucose**
5.0/3.9	5.2/3.6	4.8/3.9
5.0/3.7	5.2/3.4	4.8/3.8
5.0/3.5	5.2/3.2	4.8/3.5
5.0/3.2	3.9/3.6	4.4/4.1
5.0/3.0	3.5/3.4	4.4/3.7
	3.5/3.2	4.4/3.5

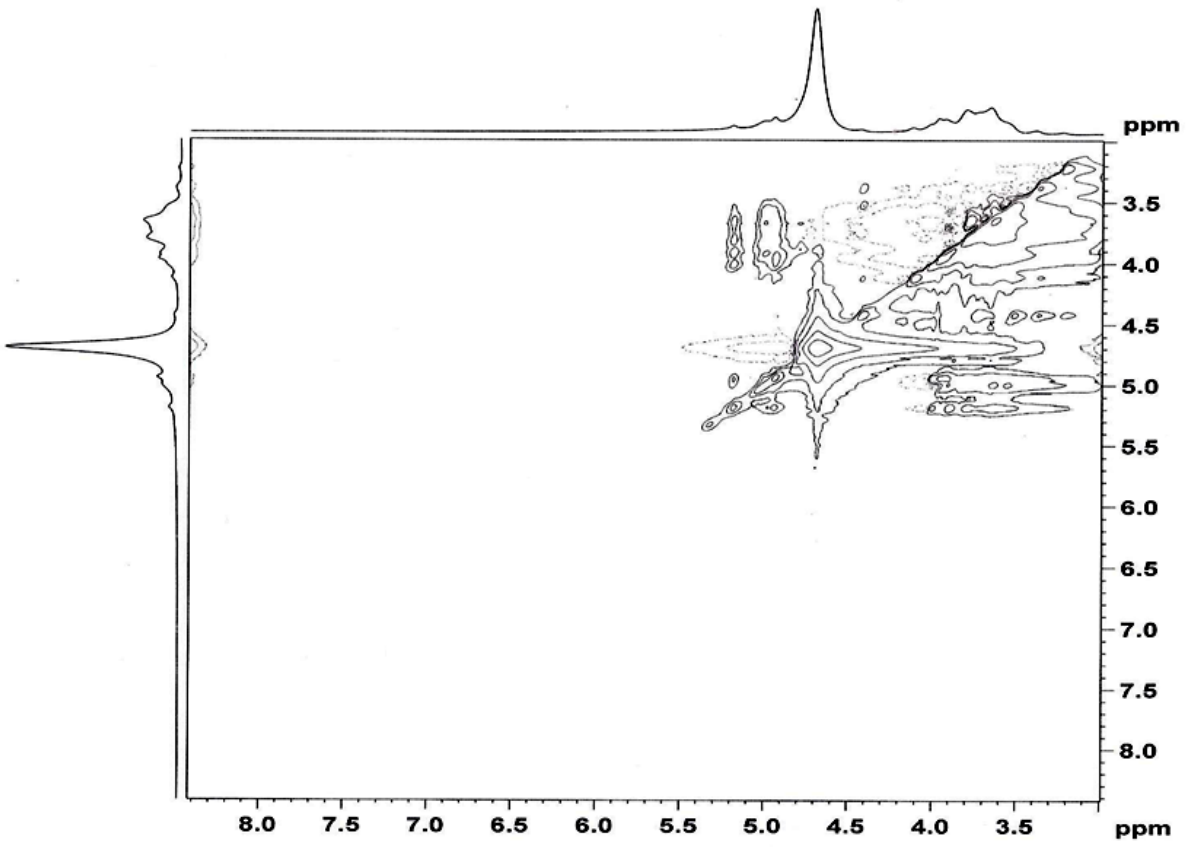


Fig. 5. 14. ^{1}H-^{1}H NOESY NMR of purified exopolysaccharide from *Lactobacillus plantarum*

performed in D_2O to reduce the complexity of the obtained NMR spectra since deuterium and protons are not observed at the same frequency. It is mainly considered for structural elucidation as nuclear magnetic resonance (NMR) spectroscopy can resolve repeat unit structures of regular and heterogeneous polysaccharides using a combination of 1D and 2D NMR techniques including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), hetero-nuclear single quantum coherence (HSQC), hetero-nuclear multiple bond correlation (HMBC) and nuclear over-hauser enhancement spectroscopy (NOESY) (Bubb, 2003). Homonuclear COSY is useful in the identification of individual monosaccharide residues. In a hetero nuclear spectrum, like HSQC or HMQC, all signals in the spectra represent a direct correlation between a carbon and a proton.

A combination of chemical and NMR analyses were decisive in establishing the structure of the EPS. These results suggest that the EPS consists of a trisaccharide backbone consisting of glucose and mannose residues with α (1, 3) linkages. Based on the analysis, one of the probable structures of the EPS from *L. plantarum* MTCC 9510 is shown in Fig. 5.15.

The location and structural complexity of these polysaccharides make them of particular interest. Production of heteropolysaccharide by lactic acid bacteria, although in very low quantities, has been reported earlier. In the present study, production of a heteropolysaccharide comprising glucose and mannose was obtained. The characterization of the exopolysaccharide indicated that the polysaccharide from *Lactobacillus plantarum* was having a trisaccharide repeating unit of glucose and mannose.

Glucose was present in both α and β forms while only α form of the mannose residue was present. Sánchez et al (2006) reported production of a low molecular weight EPS having glucose and mannose and a high molecular weight EPS with glucose and rhamnose, by *L. pentosus* LPS26. Vijayendra et al (2009) reported the production of a heteropolysaccharide with glucose and mannose from a *Lactobacillus* sp. However, there are no reports of EPS

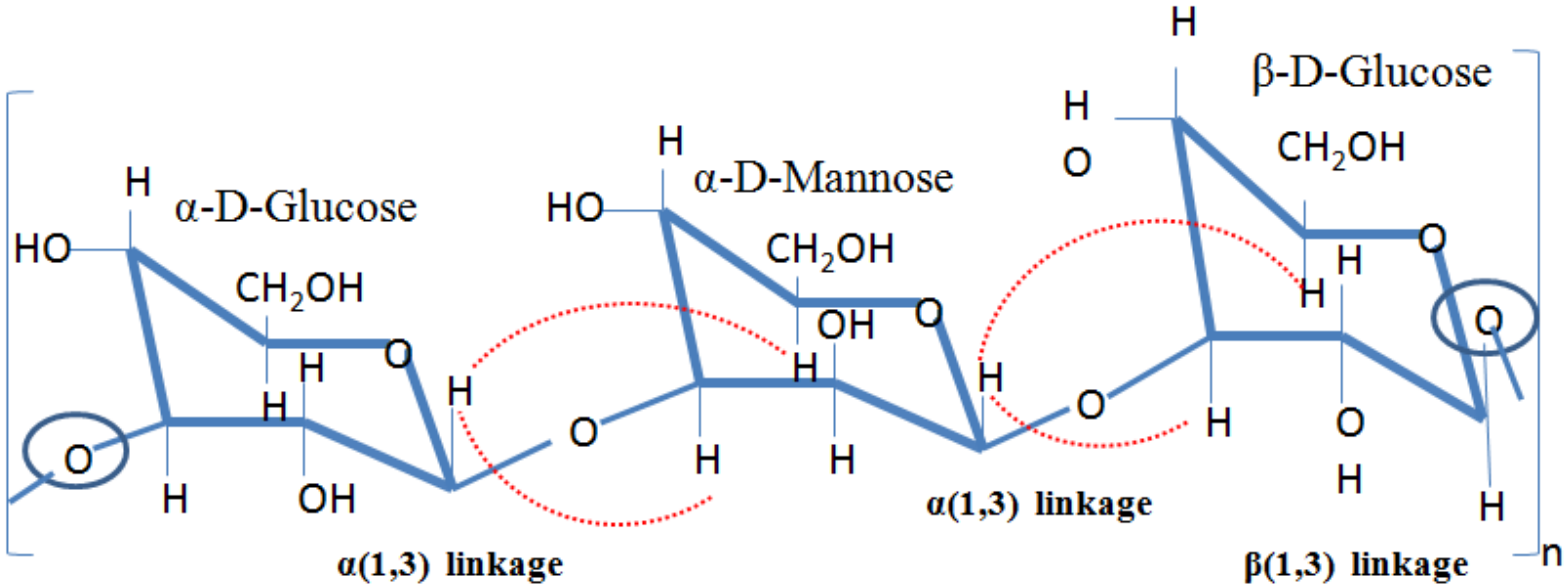


Fig. 5. 15. Illustration of a proposed structure of exopolysaccharide from *Lactobacillus plantarum* with α (1, 3) and β (1, 3) linkages (dotted lines show the inter-residue NOE signals)

from *L. Plantarum* with glucose and mannose monomers. This clearly indicates that there is a widevariation in the composition of EPS produced by different species of *Lactobacilli*, making the investigation of structure a necessary.

5.4. Conclusion

Polysaccharides present the highest capacity for carrying biological information since they have the greatest potential for structural variability. A deeper understanding of the diversity of carbohydrates is of high interest. This diversity of the molecule owes to the vast variety of interesting applications of the compound. Two-dimensional NMR spectroscopic techniques played a prominent role in the sequence determination of the hetero-polysaccharide from *Lactobacillus plantarum* MTCC 9510. The EPS produced by the isolate is a heteropolysaccharide with - α-D-glucose- α-D-mannose- β-D-glucose- trisaccharide repeats. The molecular weight and solubility of the polysaccharide in water doesn't eliminate the possibility of a branched structure for the compound.

CHAPTER 6

MICROENCAPSULATION OF LACTOBACILLUS PLANTARUM FOR EXOPOLYSACCHARIDE PRODUCTION

CHAPTER 6

MICROENCAPSULATION OF LACTOBACILLUS PLANTARUM FOR EXOPOLYSACCHARIDE PRODUCTION

6.1. Introduction

Microbial polysaccharides of economic interest are usually produced at industrial level by fermentation. Problems associated with fermentation during industrial scale up may result in inconsistent productivity, yield and quality and altogether can constitute to financial losses. This can be solved by optimising inoculation methodology and fermentation conditions. A high exopolysaccharide (EPS) production could result in the formation of a highly viscous fermentation broth making it difficult the recovery of biomass and further downstream processing (Champagne *et al.*, 2007). Microencapsulation of cells makes easier the biomass separation and the biomass reusability. Encapsulation of probiotic bacteria with a matrix can increase its survival rate by protecting from adverse conditions without affecting the production of metabolite.

Encapsulating probiotic bacteria with a matrix that is not interfering in the production of the metabolite can protect friendly bacteria against harsh conditions of stomach and upper intestine, allowing for greater delivery of these value-added ingredients. Encapsulated cells exhibit many advantages over free cells, including the maintenance of stable and active biocatalysts, high volumetric productivity, improved process control, protection against damage and reduced susceptibility to contamination. Presently, research has focused on protecting probiotics during processing and expanding the food categories available to prebiotics. Such an avenue of research has led companies like Cell Biotech from Korea to use a dual-coating, to protect probiotics against oxygen, acid, moisture and high temperatures and

for their use in emerging new product categories such as breakfast cereals and smoothies, which are marketed under the brand name Duolac™. Similarly, yogurt products containing encapsulated lactic acid bacteria have been distributed under the brand name Doctor-Capsule (Bingrae Co., Kyunggi-do, Korea) in Korea.

The main challenge in applying microencapsulation of probiotics to new foods to meet consumer interests is finding the appropriate microencapsulation technique, safe and effective encapsulating materials and potent bacterial strains. Consumers also expect the applied technology to be nature-friendly and free from hazardous chemicals. Different types of potential microencapsulation techniques are available nowadays, out of which extrusion and emulsion techniques avoid using high temperatures during encapsulation process. The main wall materials used for microencapsulation of lactobacilli and bifidobacteria are alginate, *κ*-carrageenan, κ-carrageenan plus locust bean gum, xanthan plus gellan, alginate plus corn starch, and whey proteins (Muthukumarasamy & Holley, 2007; Reid *et al.*, 2007). High survival rate of bacteria is obtained in these methods against spray drying techniques with low survival rate and stability during storage (Rokka & Rantamäki, 2010).

As synbiotics (probiotic/prebiotic combinations) may gain interest in the near future, an attempt was made in the present chapter to microencapsulate the probiotic strain isolated from curd with calcium alginate, the most commonly used matrix for immobilizing enzymes and cultures, to investigate the effect of encapsulation on production and release of EPS and the survival of the strain. Alginate has the benefits of being nontoxic to the cells being immobilized and it is an accepted food additive too (Prevost & Divies, 1988). This methodology can improve the viability of cells (since in traditional probiotic products large number of viable cells will be killed before they reach the intestine) as well as release EPS produced by the strains which can provide the benefits offered by EPS as a prebiotic or in other terms.

6.2. Materials and Methods

6.2.1. Bacterial Strains and Media

Lactobacillus plantarum MTCC 9510 is a facultative anaerobe isolated from curd and possessing many probiotic features. The organism was sub-cultured and maintained as mentioned in chapter 2 (section 2.3.1).

6.2.2. Inoculum Preparation and Encapsulation of Lactobacillus plantarum by Extrusion Method

10^9 CFU/ml of culture was inoculated into MRS broth and incubated at 37 ºC for 18 h to prepare inoculum. Inoculum was prepared from 25 ml of culture by centrifuging at 8000 x g for 10 min at 4 °C. The cells were then washed with physiological saline and re-suspended in 5 ml of sterile distilled water and mixed with 5 ml of sterile sodium alginate (1, 2 and 3 %). The mixture was dropped into 25 ml of calcium chloride (0.1, 0.5 and 1 M) using a syringe (needle size 0.70 x 32 mm) and left for curing (2, 3 and 4 h) at 4 °C depending on the experimental set up (Fig. 6.1).

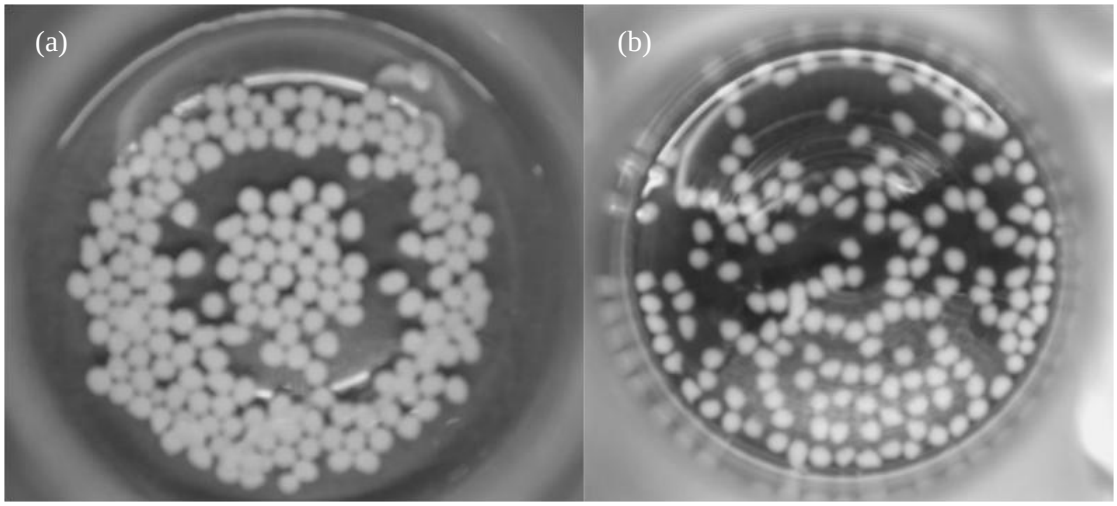


Fig. 6. 1. Microencapsulation of *Lactobacillus plantarum* using calcium alginate (a) Beads in calcium chloride solution (b) Beads in EPS production medium

6.2.3. Fermentation Conditions and Extraction of Exopolysaccharides

Fermentation was conducted in 250 ml Erlenmeyer flask using 50 ml modified EPS production medium containing (g/100 ml), yeast extract 4.0, lactose 4.0, tween 80 0.1, sodium acetate 0.5 and ammonium sulphate 0.5. The inoculated flasks were incubated at 37 ºC for 72 h under static condition. Cell leakage was checked at regular intervals by measuring the optical density of the fermented broth at 620 nm. EPS was extracted and quantified according to the protocol described in chapter 4 (section 4.2.4).

6.2.4. Box-Behnken Design and Data Analysis

A fractional-factorial design, Box-Behnken model was employed for the statistical optimization of the encapsulation conditions. The experimental design consisted of seventeen runs and the independent variables were studied at two different levels, a high level and a low level. The high level is commonly coded as +1 and the low level as -1. It is necessary to include centre points as well (in which all factors are at their central values). The response variable (EPS) was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is

$$Y_i=b_0+b_i\sum X_i+b_{ii}\sum X_i^2+b_{ij}\sum_i\sum_j X_iX_j \quad (1)$$

where Y_i is the predicted response, X_i, X_j are input variables which influence the response variable Y; b_0 is the offset term; b_i is the i^{th} linear coefficient; b_{ii} is the quadratic coefficient and b_{ij} is the ij^{th} interaction coefficient. Three factors selected for optimization were concentrations of sodium alginate and calcium chloride along with time for curing. A design was generated with these factors, having a low level and a high level. The low level of sodium alginate chosen for study was 1 % and the high level 3 %. The low and high levels for

calcium chloride were 0.1M and 1M respectively. A time of 2 h and 4 h were considered as the low and high level values for curing. The 'Design Expert' software (version 6.0, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design (Table 6.1), regression and graphical analyses of the data obtained. The maximum value of EPS produced was taken as the response.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability *P*(*F*), correlation coefficient *R*, determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's *t*-value for the estimated coefficients and associated probabilities, *P* (*t*). The quadratic models were represented as response surface graphs and contour plots. Validation of the experiment was also performed by selecting different combinations of the factors as recommended by the software. All the experiments were performed in triplicates.

6.2.5. Comparison of Free and Encapsulated Lactobacillus plantarum for EPS production

The efficiency of free and encapsulated cells in EPS production was investigated. Two different inoculum volumes were made use for the experiment, 1 x 10^9 CFU and 25 x 10^9 CFU of 18 h old inoculum. Fermentation was carried out with both 1 x 10^9 CFU and 25 x 10^9 CFU free cell inoculums and also with encapsulated cell beads. The efficiency of free and encapsulated cells of both inoculum volumes was also monitored. The flasks containing 50 ml EPS production medium were inoculated with specific inoculum and incubated at 37 °C under static condition. The EPS production by free and encapsulated cells was monitored at regular intervals of time up to 72 h. The experiment was performed to study the efficiency of cells at two extreme inoculum strength (1 x 10^9 CFU and 25 x 10^9 CFU), when

Table 6. 1 Experimental design generated with Design Expert and the predicted and actual values of production of exopolysaccharides (standard deviation: ± 5 %)

Run	*Sodium alginate (%)*	*Calcium chloride (M)*	*Curing time (h)*	*Actual EPS (g/l)*	*Predicted EPS (g/l)*
1	3	0.55	2	0.2	0.23
2	1	1	3	0.1	0.21
3	3	0.55	4	0.64	0.68
4	3	0.1	3	0.83	0.72
5	2	0.55	3	0.94	0.94
6	2	0.55	3	0.94	0.94
7	2	0.55	3	0.94	0.94
8	2	1	2	0.23	0.16
9	2	1	4	0.25	0.17
10	2	0.1	4	0.45	0.52
11	1	0.1	3	0.34	0.3
12	1	0.55	4	0.13	0.09
13	2	0.55	3	0.94	0.94
14	2	0.1	2	0.14	0.22
15	3	1	3	0.35	0.39
16	2	0.55	3	0.94	0.94
17	1	0.55	2	0.26	0.22

they remain free or encapsulated, rather than selecting a series of closely related inoculum strengths.

6.2.6. Reusability of Beads

A repeated batch study with encapsulated cells of 25 x 10^9 CFU inoculum was conducted to check the reusability of encapsulated *L. plantarum*. Repeated batch fermentation was carried out in 50 ml flasks for 72 h under the previously mentioned conditions. EPS quantified after each fermentation and cell leakage also noted at intervals.

6.2.7. Experimental Statistics

All experiments have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

6.3. Results and Discussion

6.3.1. Box-Behnken Design and Data Analysis

Three variables (sodium alginate concentration, calcium chloride concentration and curing time) were selected to investigate the optimum combination of factors suitable for encapsulating *L. plantarum* cells using calcium alginate. The Box-Behnken model of RSM was employed in this experiment. There were total seventeen runs based on the model generated by the software, each in triplicates. The experimental data were statistically analysed using the Fischer's statistical test for analysis of variance (ANOVA (Table 6.2)) and the 3D graphs and contour plots were designed. ANOVA is a statistical technique that subdivides the total variation of a set of data into component associated to specific sources of variation for the purpose of testing hypotheses for the modelled parameters. The ANOVA for the model explains the model to be significant with a Model F-value of 24.51. There is only a 0.02 % chance that a "Model F-Value" this large could occur due to noise. "Adeq Precision"

Table 6.2 ANOVA for response surface quadratic model

Source	*Sum of squares*	*DF*	*Mean square*	*F Value*	*Prob > F*	
Model	1.8	9	0.2	24.51	0.0002	significant
A	0.18	1	0.18	21.71	0.0023	
B	0.086	1	0.086	10.56	0.0141	
C	0.051	1	0.051	6.28	0.0406	
A2	0.26	1	0.26	31.63	0.0008	
B2	0.35	1	0.35	42.68	0.0003	
C2	0.62	1	0.62	76.54	< 0.0001	
AB	0.014	1	0.014	1.77	0.2255	
AC	0.081	1	0.081	9.96	0.016	
BC	0.021	1	0.021	2.58	0.1524	
Residual	0.057	7	8.15E-03			
Lack of Fit	0.057	3	0.019			

measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 12.183 indicates an adequate signal. The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.97) indicates the significance of the model. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case lower value of CV (17.81) indicated a greater reliability of the experiments performed. The *P* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. Value of Prob > *F* less than 0.05 indicate model terms are

significant. Here the terms X_1, X_2, X_3, X_1^2, X_2^2, X_3^2 and X_1X_3 are significant. That is, the linear and quadratic effects of sodium alginate, calcium chloride and curing time, and interaction effect of sodium alginate and curing time.

The polynomial equation derived on the basis of the experimental factors, quadratic effect of the factors and the interactions among the factors by the input of values in equation (1) is shown below:

$$Y = 0.94 + 0.15\,X_1 - 0.10\,X_2 + 0.080\,X_3 - 0.25\,X_1^2 - 0.29\,X_2^2 - 0.39X_3^2 - 0.060\,X_1X_2 + 0.14\,X_1X_3 - 0.073X_2X_3 \quad (2)$$

Where, Y is the response, that is, exopolysaccharide, X_1, X_2 and X_3 are the coded values of the test variables sodium alginate, calcium chloride and curing time. The probability value 0.0023, 0.014 and 0.04 respectively for sodium alginate, calcium chloride and curing time ensures the factors to be significant in the encapsulation of cells and in the release of EPS. The quadratic effect of all the factors and the interaction effect of sodium alginate and curing time was found to be significant, indicating that the interaction between sodium alginate and curing time is necessary for the encapsulation condition and in the fnal release of EPS.

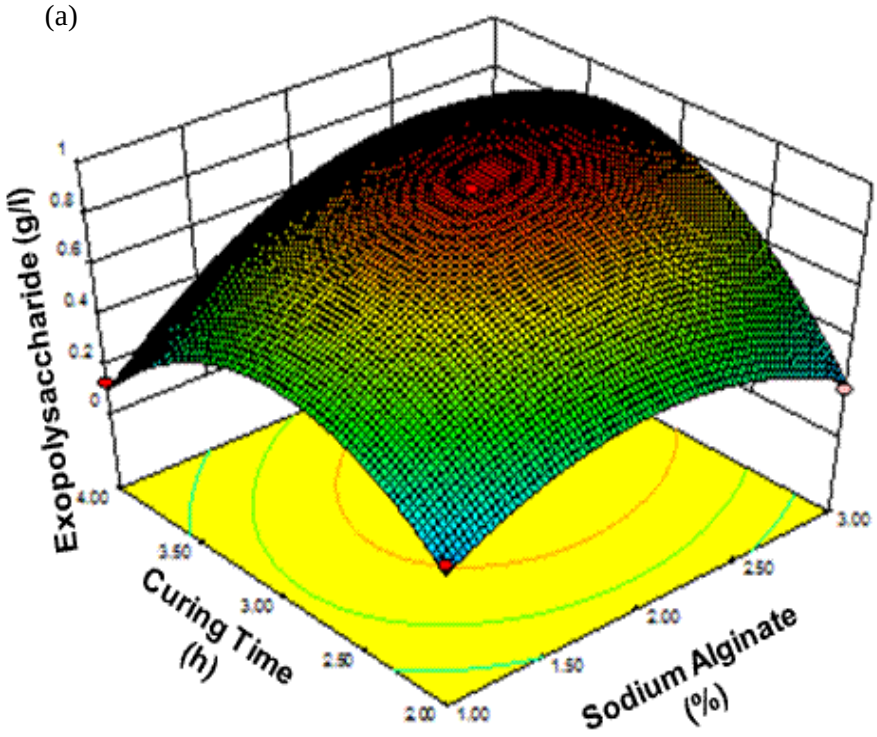
The response of the RSM was shown as 3D response surface graphs and contour plots, which gives infinite number of combinations of the two factors selcted keeping the other constant. The maximum amount of EPS (0.9 ± 0.09g/l) was released by the encapsulated cells in a combination of sodium alginate 2 %, calcium chloride 0.5 M and curing time 3 h with minimum cell leakage. It was noted that the amount of EPS released was more in this combination (Table 6.1) and also the cell leakage at regular intervals was less compared to the rest of the compilations (Table 6.3).

Table 6.3 Cell leakage observed at different time intervals for various runs

Run	*Sodium alginate (%)*	*Calcium chloride (M)*	*Curing time (h)*	*Cell Leakage (24 h)*	*Cell Leakage (48 h)*	*Cell Leakage (72h)*
1	3	0.55	2	0.02	0.03	0.06
2	1	1	3	0.02	0.05	0.08
3	3	0.55	4	0.02	0.03	0.06
4	3	0.1	3	0.004	0.02	0.03
5	2	0.55	3	0.01	0.03	0.07
6	2	0.55	3	0.01	0.03	0.08
7	2	0.55	3	0.02	0.03	0.07
8	2	1	2	0.02	0.05	0.12
9	2	1	4	0.03	0.06	0.12
10	2	0.1	4	0.01	0.03	0.06
11	1	0.1	3	0.33	0.98	1.33
12	1	0.55	4	0.01	0.03	0.07
13	2	0.55	3	0.02	0.03	0.06
4	2	0.1	2	0.01	0.02	0.07
15	3	1	3	0.10	0.12	0.17
16	2	0.55	3	0.02	0.03	0.07
17	1	0.55	2	0.01	0.03	0.09

The ANOVA showed the interaction effect of sodium alginate and curing time (Fig. 6.2) to be significant in the encapsulation of cells and in the relaese of EPS through the matrix. The interaction effects of sodium alginate and calcium chloride (Fig. 6.3), calcium chloride and

(a)



(b)

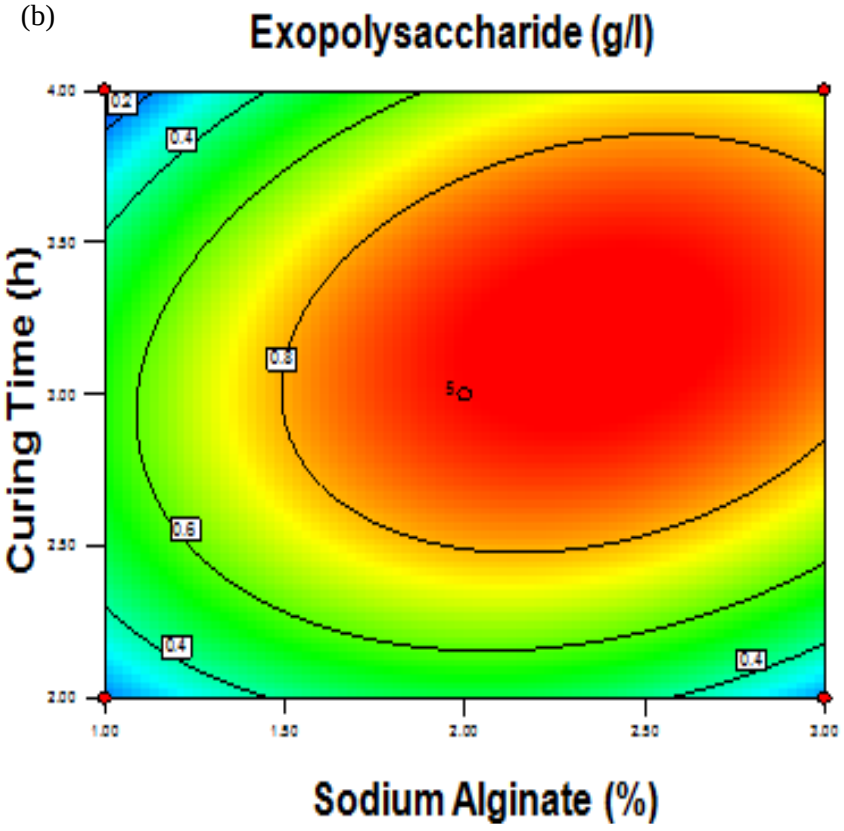


Fig. 6. 2. Interaction effect of sodium alginate and curing time on encapsulation and release of exopolysaccharide (a) RSM graph (b) contour plot

curing time were not found to be significant (Fig. 6.4). The appropriate concentration of sodium alginate was found to be 2 %. The higher the concentration of alginate, the lower will be the death rate of cells in beads and also there will be lower diffusion rate of sugar into the beads, hence the concentration of alginate should be medium, supporting the result obtained.

The lower diffusion rate of glucose in more concentrated alginate gels is due to a decrease in the number and length of pores rather than a decrease in the pore diameter (Hannoun *et al.*, 1986). Increasing curing time will result in harder beads with less EPS release, hence the curing time should not be lower or higher. In the experiment curing time of 3 h was found to be optimum in the encapsulation of cells and EPS release.

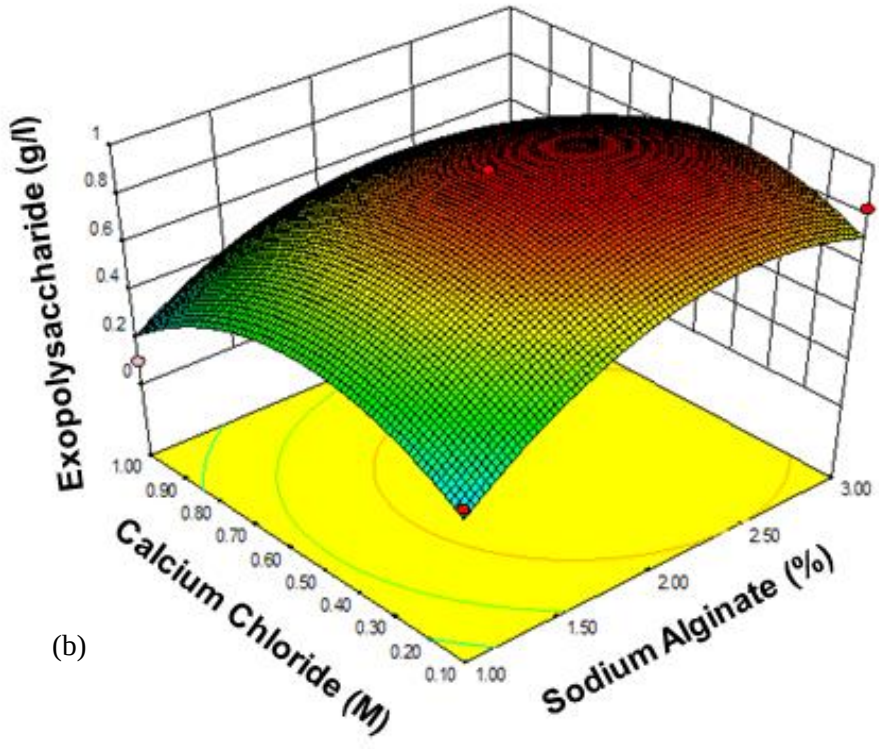
6.3.2. Validation of the Model

The model was validated in shake flask level by the conditions predicted by the software. The experiments showed actual values nearer to the predicted values supporting the data and the model as valid (Fig. 6.5).

There was not much deviations in the observed as well as predicted values showing the significance and validity of the Box-behnken design for encapsulation. The maximal production observed during validation was 0.9 g/l as in the initial experiment against the predicted value of 0.94 g/l.

The organism used in the present study for encapsulation is *L. plantarum*. Based on the organism and the product of interest, the alginate concentration required for immobilization may differ (Lu *et al.*, 1988; Nasin *et al.*, 1989; Nampoothiri *et al.*, 1998) and accordingly it has to be optimized. Concentration and type of alginate used for immobilization determines the properties of beads, as alginate may contain different

(a)



(b)

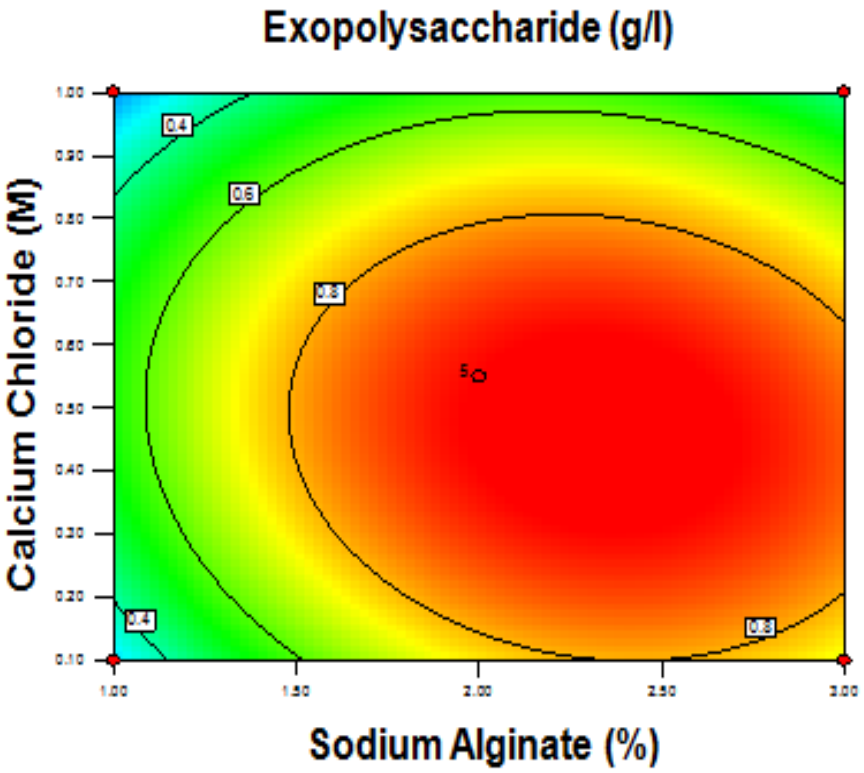
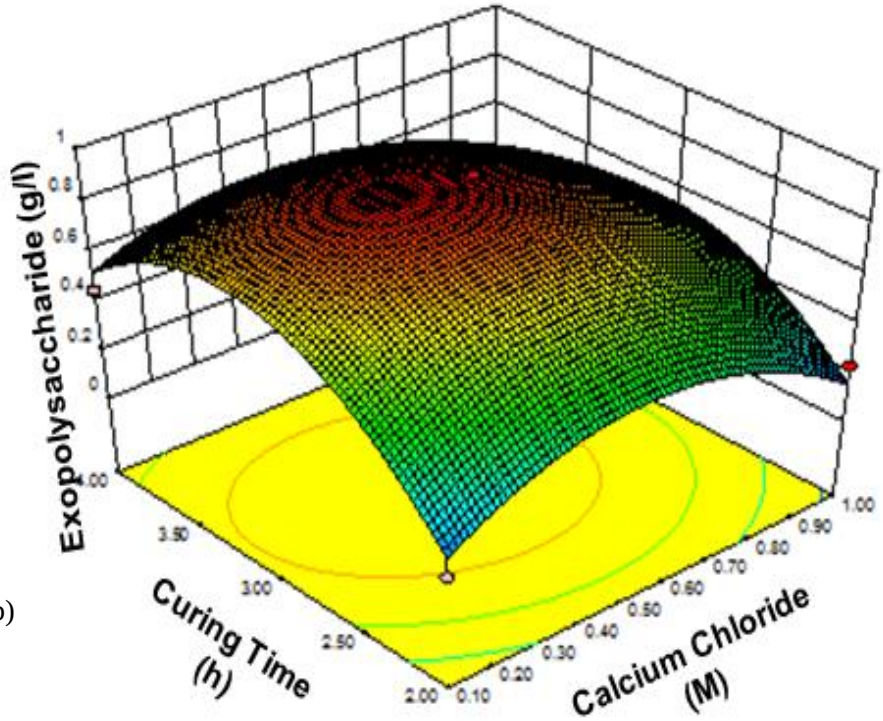


Fig. 6. 3. Interaction effect of sodium alginate and calcium chloride on encapsulation and release of exopolysaccharide (a) RSM graph (b) contour plot

(a)



(b)

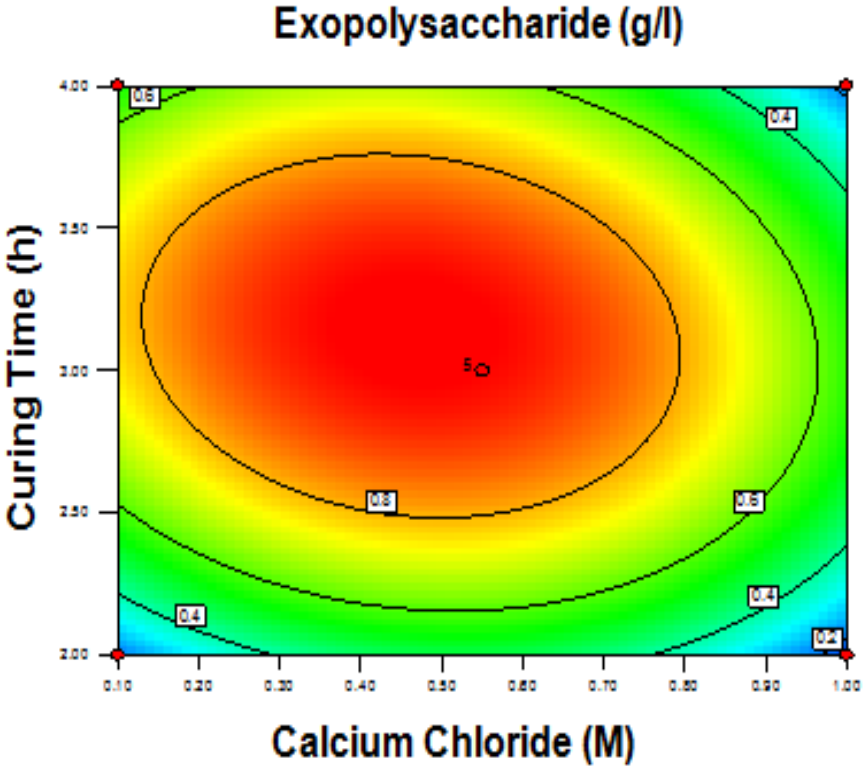


Fig. 6. 4. Interaction effect of calcium chloride and curing time on encapsulation and release of exopolysaccharide (a) RSM graph (b) contour plot

proportions of mannuronic acid and guluronic acid (Zhang *et al.*, 2000). From the experiments it was found that the appropriate amount of alginate required for the proper encapsulation of *L. plantarum* cells was 2 %.

Alginate forms a gel in the presence of divalent cations, such as Ca^{2+}, which link specifically to the G-Blocks (guluronic acid block) by binding to the free carboxyl groups. The mechanical stability of the alginate bead is critical in the physiological environment where divalent ions, which are involved in the network formation, are exchanged with other ions in the environment. This results in a loosening of the G-G bonds, with a subsequent increase in porosity, swelling of the capsule and release of the biomaterial or cell (Thu *et al.*, 1996). Alginate beads are unstable in the presence of other metal ions like Mg^{2+} or K^{+}.

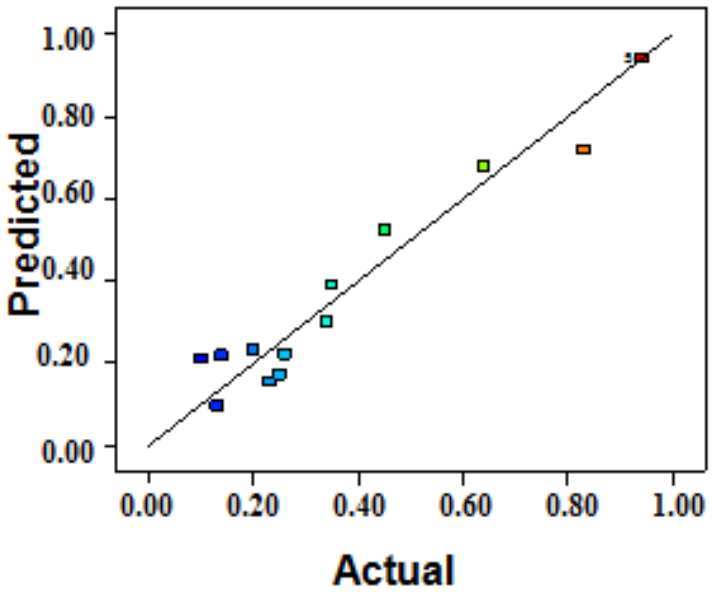


Fig. 6. 5. Predicted Vs Actual values of EPS production

Hence, attention was taken to modify the production medium in such a way that it is devoid of potassium and magnesium ions. Optimum conditions of the selected variables were defined on the basis of the maximum amount of EPS released and with minimum cell leakage. The behaviour of entrapped cells depends on the nature of biological material in beads (Garbayo *et al.*, 2004).

6.3.3. Comparison of Free and Encapsulated Lactobacillus plantarum

A comparison of EPS production was done between free as well as encapsulated cells to see their influence on production. On comparison it was found that the EPS production was three fold at an inoculum volume of 25 x 10^9 CFU than 1 x 10^9 CFU, when encapsulated. The reason could be more amount of viable inoculum than the second case. One of the advantages of encapsulation technology is the possibility of supplying fermentation reactions with more viable inoculum (biomass). It was clear from the experiment with 25 x 10^9 CFU and 1 x 10^9 CFU inoculum volumes that in both cases the encapsulated cells gave more production (Fig. 6.6) than the free cells of same inoculum volumes in batch fermentation.

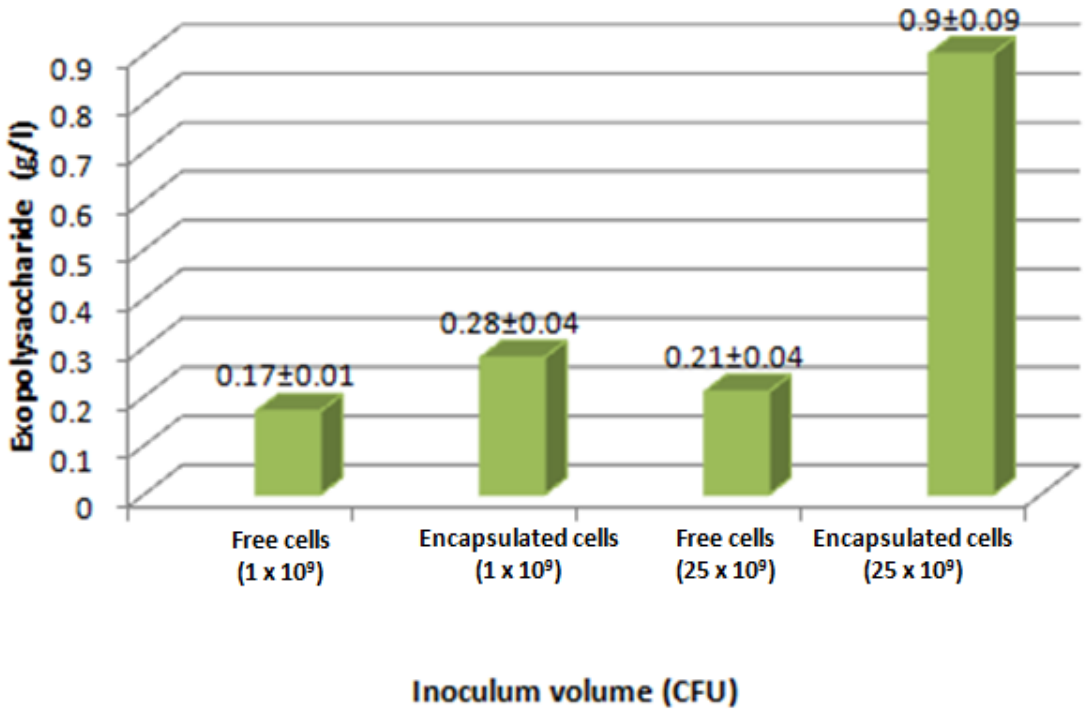


Fig. 6.6. Exopolysaccharide production by free and encapsulated *L. plantarum*

This could be because the competition between cells is less when encapsulated than in the case of free cells. It was concluded from the study that more amount of inoculum in the encapsulated condition can give rise to more production.

6.3.4. Reusability of Beads

A repeated batch study with the encapsulated *L. plantarum* showed production at the same level even in the second run. The inoculum volume 25 x 10^9 CFU was selected for the study as the production was higher at the particular inoculum volume.

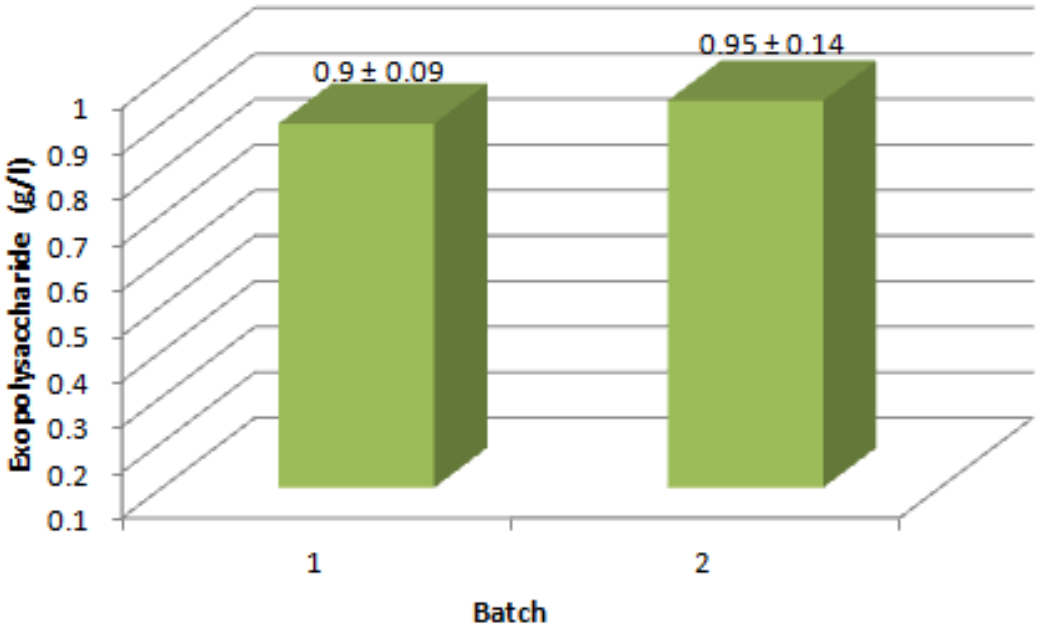


Fig. 6. 7. Repeated use of encapsulated *Lactobacillus plantarum* for exopolysaccharide production

The estimation of EPS production after each batch of 72 h showed that the encapsulated cells could give a production at the same level even in the subsequent batch. The stability in the production confirmed the viability and efficiency of cells in the subsequent batch pointing to the performance of encapsulated cells.

Only very few research has taken place in EPS production with immobilized cells. The mucoid properties of *Lb. rhamnosus* RW 9595M was exploited by Bergmaier et al (2003) for cell immobilization by adsorption on solid porous supports (ImmobaSil®). They reported a high concentration of EPS (1.7 g/l) during repeated immobilized cell cultures after

four cycles of 7h incubation period in supplemented whey permeate. The volumetric productivity of EPS was seen to be increased in immobilized cell culture system than the free cell culture system in batch fermentation. Boza et al (2004) reported that the process of encapsulating *Beijerinckia sp.* isolated from sugar cane roots mainly influenced cell growth and EPS production, due to physiological alterations resulting from the encapsulation process using a spray dryer. They could conclude that the encapsulation in malt dextrin was a viable technique to obtain inoculum of *Beijerinckia* for use in fermentations.

Microencapsulation of probiotic cells has been suggested to enhance cell resistance to freeze and freeze-drying. Shah and Ravula (2000) reported a higher stability for *L. acidophilus* and *B. longum* immobilized in alginate beads compared with free cells during storage of frozen dairy desserts and for *B. longum* in ice cream (Sheu *et al.*, 1993). The addition of cryoprotective agents in the alginate solution increased the protective effects of immobilization and gave survival rates as high as 90 % compared against a 40 % survival rate for free cells. Similarly, Lee and Heo (2000) reported that there was a decrease in the death rate of *B. longum* cells encapsulated in calcium alginate beads when exposed to simulated gastric juice and bile salt solution. Microencapsulation enhanced the survival of probiotic cultures compared to free cells in acidic conditions (Ding & Shah, 2009). The survival of probiotic cultures in yogurts was enhanced by microencapsulation on storage for over 7 weeks. The textural property (smoothness) of yoghurts was altered by the addition of probiotic cultures. The EPS production by the probiotic cultures, incorporation of the encapsulant (sodium alginate) and the filler material (starch) might have influenced the textural attributes (Kailasapathy, 2006) of yoghurts.

Encapsulation helps to protect the core material from its environment until it is released. This strategy was made use of in the case of encapsulation of the strains for probiotic use, as encapsulated bacteria will be protected from the harsh conditions until it

reach the gastrointestinal region where it has to be released. Considering probiotic approach, the efficiency of added probiotic bacteria depends on the dose level and their viability must be maintained throughout storage, products shelf-life and they must survive the gut environment (Kailasapathy & Chin, 2000). Hence viability of probiotic bacteria is of paramount importance in the market ability of probiotic-based food products.

EPS producing lactic acid bacteria strains are used in yoghurt, sour cream and whipped toppings to improve their rheological properties, viscosity, to prevent syneresis and to replace stabilizers (Shihata & Shah, 2002). EPS produced by these strains being not sufficient to encapsulate themselves fully, the problem of clogging of the bead by the product of interest is out of question. It can co-encapsulate both EPS (prebiotic ingredient) and probiotic bacteria within the same capsule enhancing growth and multiplication of these bacteria through symbiotic effects when they are released in the gastro-intestinal tract.

6.4. Conclusion

The microencapsulation technology was found to be an effective method for retaining the efficiency of cells even in repeated batches. In a broad sense, microencapsulation can be used for many applications in the food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavours, colours or odours, extending the shelf life and protecting components against nutritional loss. It is clear from the experiment that the used matrix, calcium alginate was proper for the encapsulation of *L. plantarum* as there was minimum leakage of cells and maximum release of EPS without interfering in its production and release. In fact, either the EPS producing encapsulated probiotic strain *Lactobacillus plantarum* or the extracted EPS can act as an ingredient in the products that can be used for consumption or it can serve as a synbiotic (probiotic/prebiotic combination).

CHAPTER 7

MOLECULAR IDENTIFICATION OF EPS BIOSYNTHETIC GENES

CHAPTER 7

MOLECULAR IDENTIFICATION OF EPS BIOSYNTHETIC GENES

7.1. Introduction

Lactic acid bacteria (LAB) are adjunct of an important group of probiotic bacteria and are already used in many probiotic dairy products (Lourens-Hattingh & Viljoen, 2001). The three major developments that have brought the research on these powerhouses of the probiotic and dairy industry at the present level are the genomic revolution that has been quickly implemented in LAB with the sequence analysis of plasmids, bacteriophages, and now genomes or collections of genomes, the high throughput experimentation that became available and shown to be of particular use for LAB and the development of systems and synthetic biology approaches. These systems solutions integrate all aspects of metabolism, genetics and application of LAB. Many mobile elements that also include conjugative transposons were found to encode important industrial characteristics such as lactose and citrate metabolism, proteinase, bacteriocin and exopolysaccharide (EPS) production, as well as bacteriophage insensitivity.

A variety of tools has been developed to generate genetically modified LAB, such as cloning, chromosome modification and expression systems. The most popular transformation system for generating directed genetic alterations in LAB is electroporation with self-replicating vectors. Among the expression systems, the Nisin **c**ontrolled gene **e**xpression system (NICE®), developed at NIZO Food Research, Netherlands, has gained interest in this area. It has the advantage of over-expression of homologous and heterologous genes for functional studies and to obtain large quantities of specific gene products (www.mobitec.com). Tightly controlled gene expression can be obtained with the help of

this. For exploitation of the auto-induction mechanism of nisin for gene expression, the genes for the signal transduction system nisK and nisR were isolated from the nisin gene cluster and inserted into the chromosome of *L. lactis* subsp. *cremoris* MG1363 (nisin-negative) and created the strain NZ9000 (Kunji *et al.*, 2003). When a gene of interest is subsequently placed behind the inducible promoter PnisA on a plasmid (pNZ8148, food grade vector) or on the chromosome, expression of that gene can be induced by the addition of sub-inhibitory amounts of nisin (0.1–5 ng/ml) to the culture medium. However, this expression becomes impossible in the case of other lactococcus strains and strains of other LAB genera not engineered with the signal transduction system, necessitating the presence of a second plasmid (pNZ9530) harbouring the regulatory genes (www.mobitec.com).

Today, there is a growing need for new strains of LAB that carry the probiotic traits and having favourable health effects on human and animals. Hence focus was given on the development of LAB strains with probiotic characteristics and increased production of nutraceutical like exopolysaccharides. EPS producing lactic acid bacteria (LAB) are used to improve the texture of fermented dairy products in dairy industry. The genetics of bacterial EPS production is shown to be linked to *eps* gene clusters, which could be of chromosomal or plasmid origin. If it is plasmid encoded, conjugal transfer of this EPS plasmid can be applied to obtain new EPS-producing strains, as has been demonstrated for the 40 kb EPS plasmid pNZ4000 in *Lactococcus lactis* (VanKranenburg *et al.*, 1997), containing 12 kb *eps* operon (*eps*RXABCDEFGHIJKL). This operon is driven by a promoter upstream of *eps* R which is involved in regulation of the operon (Fig. 1.8, chapter 1). Polymerization and chain length are driven by *eps* A and B along with *eps* I and *eps* K involved in polymerization and export respectively. The proteins expressed by the other genes *eps* DEFGH function as glycosyl transferases (EC 2.4.1.x.) (Jolly & Stingele, 2001).

Glycosyl transferases (GTFs) are key carbohydrate-interacting enzymes involved in the synthesis of complex carbohydrate structures, which offer the advantages of high regio- and stereo specificities compared to a chemical approach, as well as the potential availability of many different glycosidic linkages.

Since the similarity between *eps* clusters from lactic acid bacteria being striking, identification of new wild type LAB strains producing unique EPS is a considerable challenge that could benefit from screening methods for the genetic elements involved. Sequence information about the EPS gene clusters in LAB is important to identify strains producing novel EPS with potent functional activities. For the evaluation of genetic structure of EPS biosynthesis, the genomic DNA of the isolate was explored. The genomic DNA was mainly explored for the presence of priming glycosyl transferase, for the successful application of genetic modification techniques for the improvement of this organism and its use in food industry. This becomes essential to see the homology of the predicted gene products being expressed.

The present chapter deals with the amplification of EPS biosynthetic genes such as *epsA* from plasmid DNA of *L. lactis* NIZO B40 and priming glycosyl transferase from genomic DNA of the isolate, *L. plantarum* (MC1) as well as from plasmid DNA of *L. lactis* NIZO B40, their cloning into pTZ57R/T vector and sequencing.

7.2. Materials and Methods

7.2.1. Microorganism and maintenance

Lactobacillus plantarum was sub-cultured in MRS agar at 37 ºC. *E. coli* DH5α was subcultured in Luria Bertani agar (Hi-media, Mumbai, India) slants at 37 ºC. These strains were sub-cultured twice in a month and glycerol stocks were prepared for long term storage at -80 ºC.

7.2.2. Genomic DNA Preparation of Lactobacillus plantarum

Total DNA was extracted from culture (1.5 ml) harvested in the mid-log phase (OD_{600} of 0.5-1) employing the method of Savadogo et al (2004). Cells were collected by centrifugation (3000xg, 10 min, 4 °C) and frozen for at least 1 h at -20 °C. The thawed pellet was washed in 1 ml TES buffer (6.7 % sucrose, 50 mM Tris-HCl, 1mM EDTA, pH 8.0) and resuspended in 300 µl STET buffer (8 % sucrose, 5 % Triton X-100, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0). Seventy-five microlitres of lysis buffer (TES containing 1330 U/ml mutanolysine and 40 mg/ml lysosyme) was added and the suspension was incubated at 37°C for 1 h. After addition of 40 µl preheated (37°C) 20 % SDS in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. One hundred microlitres of TE buffer was added and the lysate was extracted with 1 volume phenol/chloroform/isoamyl alcohol (49:49:1). Phases were separated by centrifugation (18000 x g, 5 min) using phase lock gel tubes (Eppendorf). The aqueous phase was carefully mixed with 70 µl 5M NaCl, 1 ml isopropanol and DNA precipitated on ice for 15 min. The DNA was collected by centrifugation (20000 x g, 30 min, 4°C) and the pellet washed in ice-cold 70 % ethanol. Finally, DNA was dried and resuspended in 100 µl TE. Genomic DNA was analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer.

7.2.3. Plasmid DNA Preparation of Lactococcus lactis NIZO B40

High molecular weight plasmid DNA from *L. lactis* NIZO B40 was isolated using the protocol mentioned by Anderson and McKay (1983). *L. lactis* NIZO B40 cells were grown overnight in M17 broth at 32 ºC. 2 % (v/v) of inoculum from this was inoculated into modified Elliker Broth medium designated as lysis broth (0.5 % tryptone, 0.25 % yeast extract, 0.25 % gelatin, 0.4 % sodium chloride, 0.15 % sodium acetate, 1 % glucose and 20 mM DL-threonine). Culture was propagated in lysis broth for 4 h at 32 ºC and cells were

harvested by centrifugation. The pelleted cells were resuspended in 379 µl of solution I (6.7 % sucrose, 1 mM EDTA (pH 8.0), 50 mM Tris) and incubated at 37 ºC for 15 min. 96.5 µl of solution II (lysozyme 10 mg/ml in 25 mM Tris (pH 8.0)) was added and incubated for 30 min at 37 ºC. To the reaction mixture, added 48.2 µl solution III (0.25 M EDTA (pH 8.0), 50 mM Tris). 27.6 µl of SDS (20 % w/v in 50 mM Tris-20 mM EDTA, pH 8.0) was added and mixed immediately. Hereafter, the mixture was incubated for 20-30 min at 37 ºC and vortexed at highest setting for 30 S for complete lysis. 27.6 µl of fresh 3.0 N NaOH was added and mixed gently by intermittent inversion or swirling for 10 min. 49.6 µl of 2 M Tris HCl (pH 7.0) was added and continued gentle mixing for 3 min. Then 71.7 µl of 5.0 M NaCl was added. 700 µl of phenol-saturated with 3 % NaCl was added and mixed thoroughly and centrifuged for 5 min and the aqueous phase was separated and extracted with 700 µl chloroform-isoamyl alcohol (24:1). The aqueous phase was removed and precipitated with 1 volume of isopropanol and incubated at 0 ºC for 30 min and centrifuged for 5 min. The excess isopropanol was removed and resuspended in 20 µl 10 mM Tris-1 mM EDTA (pH 7.5). Plasmid DNA was analyzed by electrophoresis in 0.7 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer.

7.2.4. Gradient Polymerase Chain Reaction (PCR) Conditions and Gene Amplification

The presence of priming glycosyl transferase gene in the genomic DNA of *L. plantarum* isolate was verified by using primers (Integrated DNA Technologies, USA) designed from the priming glycosyl transferase gene sequence of *Lactobacillus rhamnosus* ATCC 9595 (Fig. 7.1). The primers used were rham F GGTACC ATGGAGACTGCATCTAAGCAC (with Kpn I site underlined) and rham R1 AAGCTT TTAATAGGCTCCAGTTGGATG (with Hind III site underlined).

The PCR mixtures (25 µl) contained 50 ng of DNA, 1.5mM $MgCl_2$, four deoxynucleoside triphosphates at 150 µM each, forward and reverse primers at 10 pM in *Taq* buffer, and 0.5 U of *Taq* polymerase. The PCR reactions were performed on an Eppendorf

>gb|AY659976.1|:15178-15846 *Lactobacillus rhamnosus* strain ATCC 9595 Wzd (wzd), Wze (wze), Wzx (wzx), WelF (welF), WelG (welG), WelH (welH), WelI (welI), Wzy (wzy), WelJ (welJ), Wzm (wzm), RmlA (rmlA), RmlC (rmlC), RmlB (rmlB), RmlD (rmlD), WelE (welE), Wzr (wzr), Wzb (wzb), ClpL (clpL), and Nrp (nrp) genes, complete cds

ATGGAGACTGCATCTAAGCACAGACAAACTACCGTTGAACAAGTTGATCTTGGGGGG
TTAACACCGACATATCTAGTAACTAAACGTTGTTTCGATTTCTTAGCCAGTTTTGTGGT
CTTGTTCTGCTTAGTGGTGTTTTTTTAATTTTGTCCATTCTGATCAAAATAGATGTCGC
ACGGGAAAATTTTTTATTCGCAGACAAGAATGGGAAAAGATGGTAGGACGTTTAAAT
GTGGAAGTTTCGATCCATGGTAACTGGAGCGGACAAGATGGTCGACAAATTACTTAA
AAAGAATGACGTTGAGGGGGCAATGTTTAAAATTAAGCATGATCCGCGAATTACGCG
TGTTGGACGAGTCATTCGAAAATACAGCCTTGATGAACTGCCACAATTATATAATGTT
CTTCGTGGTGATATGAGTTTGGTTGGACCAAGACCTCCGTTGCCACGTGAAGTCGTCA
AGTATACAGATTATGATCGTCAACGGTTAGCAGTTGTTCCTGGTGTTACGGGATTGTG
GCAGGTTTCGGGACGAAACGAATTAAGTTTTGATGAAATGGTCAGGTTGGACATTCA
GTACATCAACAATGCGTGTGTTACTGAAGATCTTCGAATTTTATTCAAAACTGTTCTTG
TTGTCGTTCATCCAACTGGAGCCTATTAA

Fig. 7. 1. Priming glycosyl transferase gene sequence of *Lactobacillus rhamnosus* ATCC 9595

Mastercycler (Eppendorf, Hamburg, Germany) by following the programs as described. A gradient PCR (Eppendorf, India) was performed for the amplification of the gene. The amplification program consisted of one cycle at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 40-55 °C for 30 s and 72 °C for 1 min and finally, one cycle at 72 °C for 8 min.

Simialry, PCR was executed to amplify EPS biosynthetic genes, *epsA* and *priming glycosyl transferase* from *L. lactis* NIZO B40 plasmid using the specific primers (Meulen *et al.*, 2007) as listed in Table 7.1.

The program for the amplification of genes consisted of one cycle at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 40-55 °C for 30 s and 72 °C for 1 min and finally, one cycle

at 72 °C for 8 min. Amplicons were analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer. A 1 kb DNA ladder (Fermentas) was used to identify the molecular sizes of the bands. PCR products were purified using QIAquick gel extraction kits were obtained from Qiagen (Germany).

Table 7. 1 Specific primers for amplification of EPS biosynthetic genes from plasmid DNA of *L. lactis* NIZO B40

Gene targeted	*Oligonucleotide primers*
epsA	epsA F:TAGTGACAACGGTTGTACTG
	epsA R: GATCATTATGGACTGTCAC
Priming glycosyl transferase	Pg F:TCATTTTATTCGTAAAACCTCAATTGAYGARYTNCC
	pg R:AATATTATTACGACCTSWNAYYTGCCA

7.2.5. Gene Amplification and Cloning into pTZ57R/T Vector

Genes amplified from the genomic DNA of *L. plantarum* and the plasmid DNA of *L. lactis* NIZO B40 using the program, one cycle at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 46.3 °C for 30 s and 72 °C for 1 min and finally, one cycle at 72 °C for 8 min were eluted and purified using QIAquick gel extraction kit (Qiagen, Germany). The amplicons were ligated into pTZ57R/T vector (Fig. 7.2) and transformed into *E. coli* DH5α cells as described below. The pTZ57R/T cloning vector is a linearized and ddT tailed vector for direct use in cloning of PCR products, generated with *Taq, Tth, Tfl* or other DNA polymerases or polymerase mixtures, which add extra adenines to the ends of PCR products.

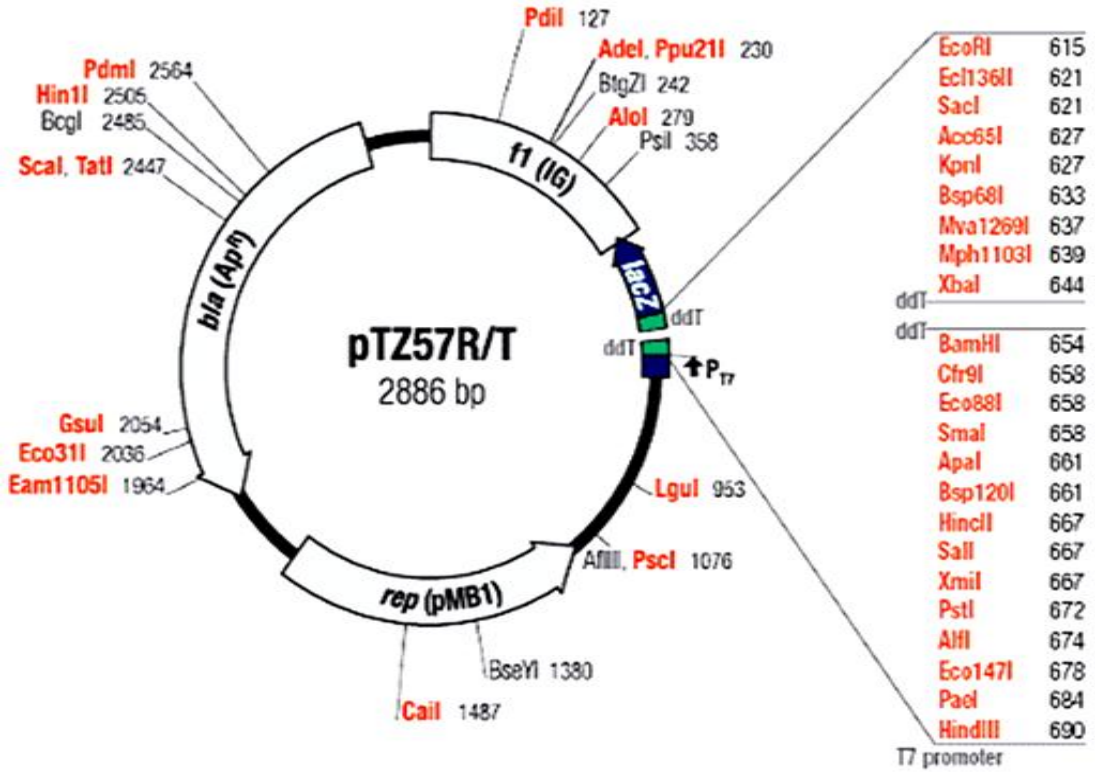


Fig. 7. 2. Vector map of pTZ57R/T

The PCR product was ligated in to the pTZ57R/T vector based on the reaction protocol as mentined in the kit (InsTAclone™ PCR Cloning Kit, Fermentas, USA). The 10 µl ligation mixture, with 1 µl pTZ57R/T vector, 3 µl insert, 2 µl ligation buffer and 1 µl ligase, was set at 16 °C for 9 h and finally ligase was inactivated at 65 °C for 10 min. The ligated products were transformed into *E. coli* DH5α cells.

7.2.5.1. Preparation of Competent E. coli Cells and Transformation

Overnight culture (1 %) of *E. coli* DH5α was inoculated into 50 ml LB broth until the optical density (O.D) was 0.6. The appropriately grown culture was centrifuged at 3000 rpm for 10 min and the pellet resuspended into 10 ml $MgCl_2$ (100 mM) and centrifuged at 3000 rpm for 10 min. The pellet obtained was washed with 10 ml 100 mM $CaCl_2$ and kept in ice for 40 min and centrifuged at 3000 rpm for 10 min. Pellet was again resuspended in 1 ml FTB

buffer (20 ml FTB : 17 ml 100 mM $CaCl_2$ + 3 ml 100 % glycerol) and transferred into eppendorfs as 150 µl aliquots.

10 µl of ligated products were added into 150 µl of *E. coli* DH5α competent cells. It was kept in ice for 40 min (Undisturbed). Heat shock was applied for 90s at 42 °C and kept in ice for 2 min. 900 µl of LB was added and incubated in shaking condition at 37 °C for 1 h. 100 µl of culture was plated onto LB-ampicillin plate and incubated overnight at 37 °C.

7.2.5.2. Plasmid DNA Preparation from Transformed E. coli cells

Single colonies were inoculated in to LB-ampicillin broth and incubated overnight at 37 °C. Plasmid DNA was isolated using kit obtained from Fermentas (USA) as explained. 1.5 ml *E. coli* cell culture in the log phase was centrifuged at 12, 500 x g for 5 min and the pellet was suspended in 250 µl of resuspension solution (with RNase A) and vortexed. 250 µl of lysis solution was added and the tubes were inverted 4-6 times. Then 350 µl of neutralization solution was added and inverted as before. It was followed by a centrifugation at 12, 500 x g for 5 min and the supernatant loaded on to GeneJET™ spin column provided with the kit. Again centrifuged at 12, 500 g for 1min and added 500 µl of Wash Solution and centrifuged at 12, 500 x g for 30-60s. The flow-through was discarded and the empty column was centrifuged for 1min. Finally, the column was kept in a fresh eppendorf and plasmid eluted by adding 50 µl of elution buffer to the column. The column was incubated for 2min and centrifuged at 12, 500 x g for 2 min to obtain the plasmid. Plasmid was loaded on to 1 % agarose gel and stained with ethidum bromide for visualization.

7.3. Results and Discussion

7.3.1. Genomic DNA Preparation of Lactobacillus plantarum

Genomic DNA was extracted from the isolate *L. plantarum* (Fig.7.3). The main problem that encountered with genomic DNA extraction was incomplete lysis of the cell wall. The main reasons behind the problem could be the presence of teichoic acids in the cell

wall. Some particular teichoic acids, lipoteichoic acids, have a lipid component and can assist in anchoring peptidoglycan, as the lipid component is embedded in the membrane (Delcour *et al.*, 1999). In Gram-positive bacteria, S-layer is attached to the peptidoglycan layer. This was overcome by facilitating lysozyme degradation of the cell wall with 40 mg/ml lysozyme and subsequent sodium dodecyl sulfate (SDS)-mediated lysis of the cell membrane. Mixing of the

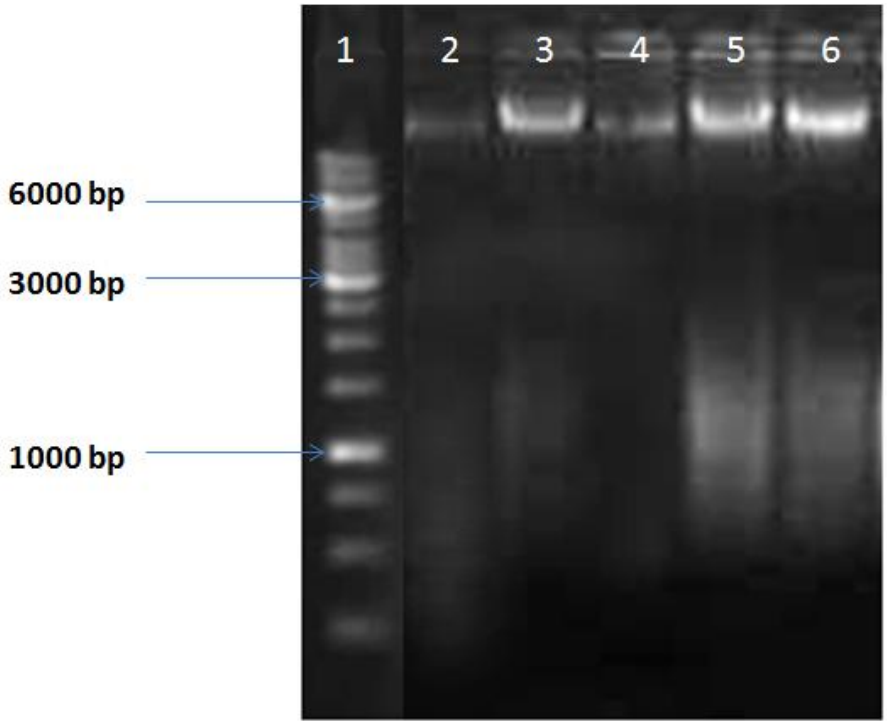


Fig. 7. 3. Genomic DNA isolation from *Lactobacillus plantarum*: Lane 1: 1 kb DNA ladder; Lane 2-6: Genomic DNA

DNA solution immediately after the addition of SDS enabled a more thorough distribution of the detergent and facilitated more efficient lysis of the cell population. The method was simple and quick and it produced sufficient amounts of genomic DNA, suitable for cloning procedures without further purification, from small volumes of cultures.

7.3.2. Plasmid DNA Preparation of Lactococcus lactis NIZO B40

High molecular weight plasmid around 43 kb was extracted (Fig. 7.4) easily, employing the above described protocol. This plasmid isolation method was based on alkaline-mediated denaturation of chromosomal DNA and extraction of protein with salt saturated phenol and chloroform-isoamyl alcohol. Problems with incomplete lysis encountered when other protocols were tried. This was overcome by growing cells in the

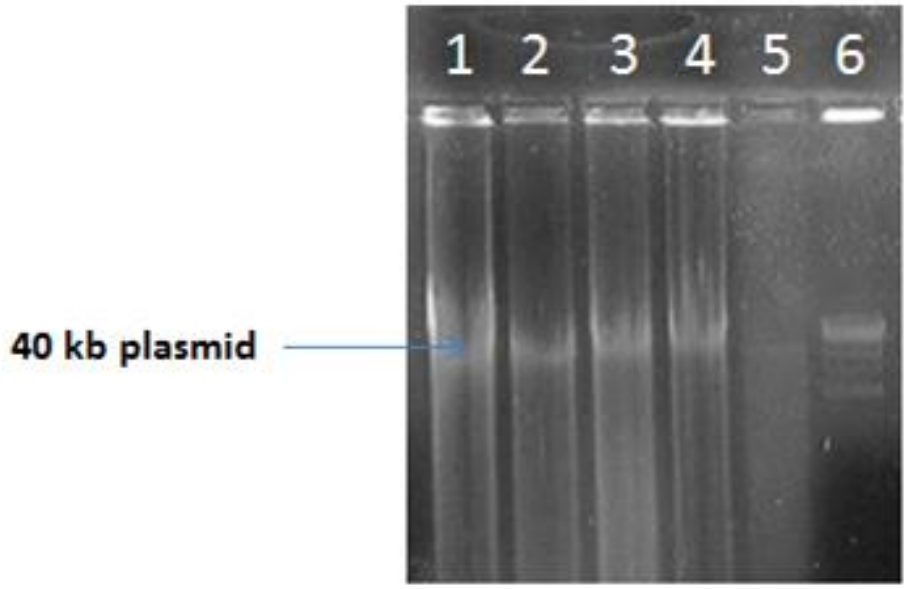


Fig. 7. 4. *Lactococcus lactis* NIZOB40 plasmid DNA: Lane 1-5 Plasmid DNA; Lane 6: 48 kb lambda mix DNA marker

presence of 20 mM DL-threonine for 4 h to weaken the cell wall, facilitating lysozyme degradation of the wall and subsequent sodium dodecyl sulfate (SDS)-mediated lysis of the cell membrane. Mixing of the DNA solution immediately after the addition of SDS enabled a more thorough distribution of the detergent and facilitated more efficient lysis of the cell population. The method was simple and quick and it produced sufficient amounts of plasmid

DNA, suitable for cloning procedures without further purification, from small volumes of cultures.

7.3.3. Gradient Polymerase Chain Reaction (PCR) Conditions and Gene Amplification

The genetics of bacterial EPS production has been described for a few lactic acid bacteria strains (Germond *et al.*, 2001) and shown to be linked to *eps* gene clusters encoding putative gylcosyl transferases (Jolly & Stingele, 2001). A search for *priming glycosyl transferase* gene in the genomic DNA of *L. plantarum* was performed by gradient PCR. But more than one amplicon of varying sizes was obtained in a cycle reaction. Presence of unspecific secondary bands in PCR reaction may hinder or even prevent further analyses or an unequivocal assessment of the PCR result. This could be overcome by optimizing PCR conditions such as titrating the magnesium, template, primer, dNTP and Taq polymerase concentration, adding detergents or by Hot Start PCR, Touch-down PCR, reducing the PCR cycles or gradient PCR. The selection of the annealing temperature is the most critical component for optimizing the specificity of a PCR reaction. In most cases, this temperature must be empirically tested. The PCR is normally started at 5°C below the calculated temperature of the primer melting point (T_m). However, the possible formation of unspecific secondary bands shows that the optimum temperature is often much higher than the calculated temperature (>12°C). Further PCR reactions with gradually increasing temperatures are required until the most stringent conditions have been found. In such cases, the gradient PCR enables rapid testing of the optimum temperature conditions on one block and in one experiment. During the PCR, a temperature gradient, which can be programmed between 1°C and 20 °C, is built up across the thermo block. This allows the most stringent parameters for every primer set to be calculated with the aid of only one single PCR reaction.

In the present experiment, a gradient PCR programmed between 40-55 °C at 2 °C difference was executed for the search of priming glycosyl transferase gene. The result showed bands at two different temperatures, 46.3 and 52.1 °C (Fig. 7.5). The bands were more visible at 46.3 °C, which was selected as the optimum annealing temperature for the primers rham F and rham R1. There were two additional bands of ~1300 bp and 1000 bp along with the expected 680 bp fragment.

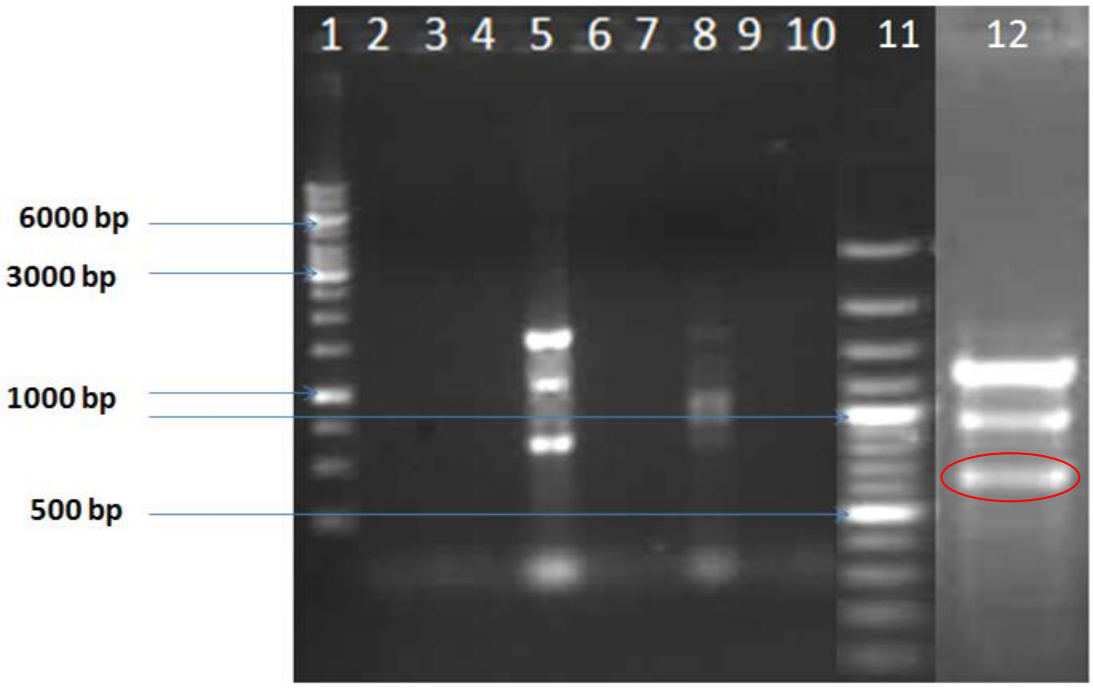


Fig. 7. 5. Gradient PCR (40-55 °C) of *Lactobacillus plantarum* genomic DNA: Lane 1: 1 kb DNA ladder; Lane 5: PCR amplicon at 46.3 °C; Lane 8: PCR amplicon at 52.1 °C; Lane 11: 100 bp DNA ladder; Lane 12: PCR amplicon obtained for priming glycosyl transferase at 46.3 °C (encircled: expected band)

The exploration of the high molecular weight plasmid DNA of *L. lactis* NIZO B40 with the gradient program between 40-55 °C at 2 °C difference for amplification of *epsA* gene resulted in a band of ~780 bp in all temperatures except 40 and 44 °C.

Eventhough, the amplification of plasmid DNA for *priming glycosyl transferase* gene resulted in more than one band in almost all the temperatures, the band of expected size (~481 bp) was observed only at 46. 3 °C (Fig. 7.6). In short, PCR reactions were performed for the *priming glycosyl transferase* gene of *L. plantarum* (isolate) and priming glycosyl transferase and *epsA* genes of *L. lactis* NIZO B40. PCR reactions generated gene fragments of three different base pairs (~680 bp, ~1000bp and ~1300 bp) from the genomic DNA of

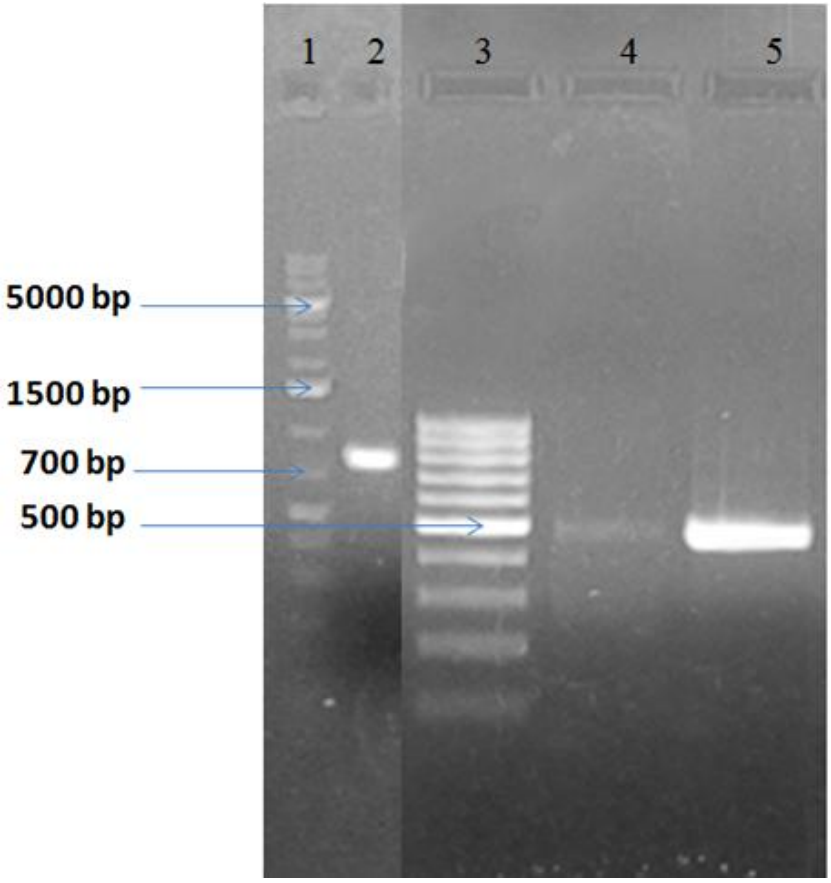


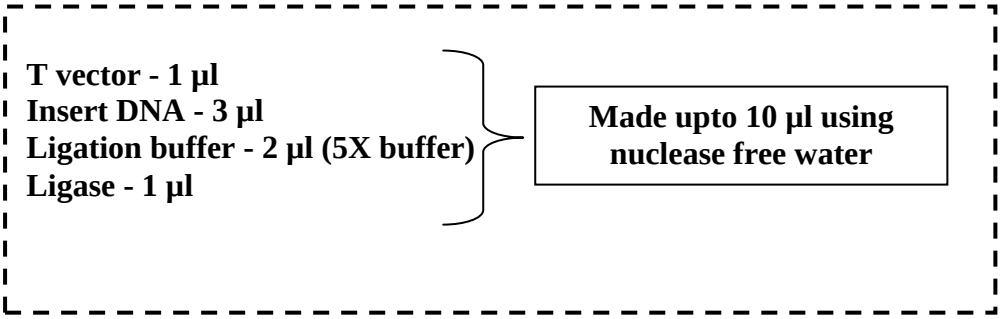
Fig. 7. 6. PCR amplification of *Lactococcus lactis* plasmid DNA: Lane 1: 1 kb DNA ladder; Lane 2: Amplicon obtained for *epsA* (~780 bp); Lane 3: 100 bp DNA ladder; Lane 5: Amplicon obtained for priming glycosyl transferase (~ 481 bp)

L.plantarum. Gene fragments of ~780 bp and ~481 bp corresponding to the *epsA* and *priming glycosyl transferase* genes were obtained from the plasmid DNA of *L. lactis* NIZO B40. The

size of priming glycosyl transferase reported from lactic acid bacteria genomic DNA is of 680 bp size and that from plasmid DNA is of 481 bp. Similarly, epsA reported from plasmid DNA of lactic acid bacteria is of 780 bp size.

7.3.4. Cloning of amplified genes into pTZ57R/T Vector

All the above amplicons generated using the primers were ligated into pTZ57R/T vector. The ligation reaction was performed as follows:



The reaction was set at 16 °C for 9 h and finally ligase was inactivated at 65 °C for 10 min. The ligated products were transformed into *E. coli* DH5α cells. The confirmation of clone of *L. plantarum* amplicons in pTZ57R/T vector was performed by PCR reaction of the cloning vector with insert using the primers rham F and rham R1 (Fig. 7.7). The cloned fragments were sequenced.

Comparative alignment of the query sequences was done using BLAST program. The sequencing result (Table 7.2) showed that the query sequences of the PCR amplicons obtained from the genomic DNA of *L. plantarum* (~680 bp, ~1000bp and ~1300 bp), analysed for priminig glycosyl transferase gene, were not showing similarity to priming glycosyl transferase of *Lactobacillus* or *Lactococcus* species, instead it showed similarity to a transposase of *L. plantarum*, cloning vector pJAP8 and hypothetical protein or fumarate reductase genes of *L. plantarum* respectively.

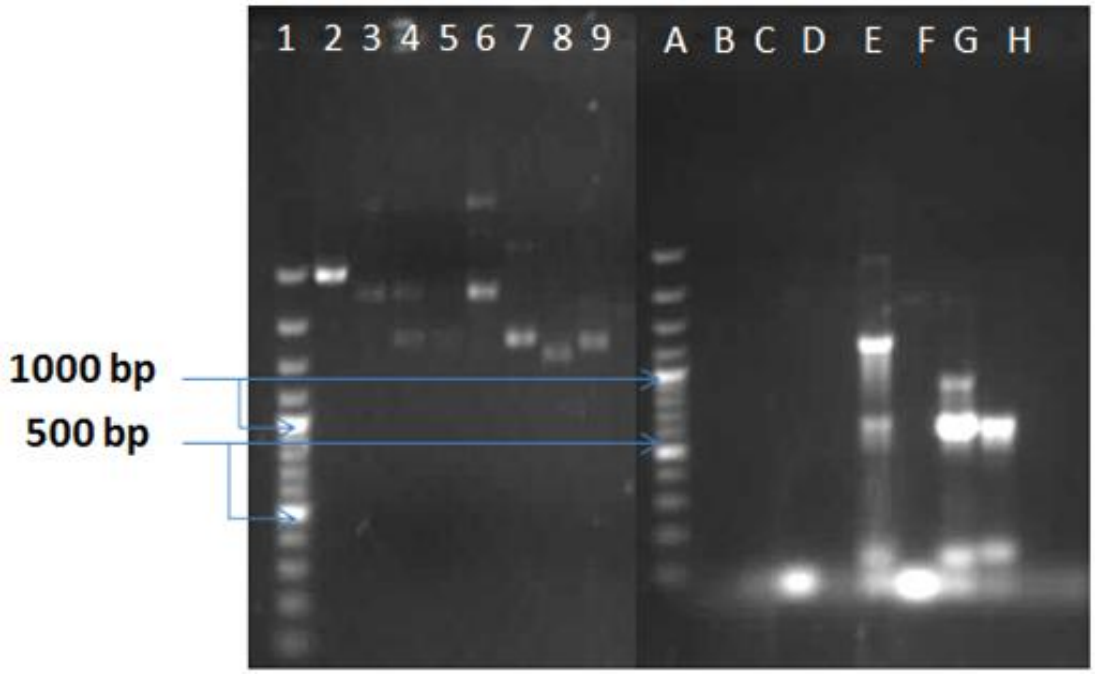


Fig. 7. 7. Cloning of PCR amplicons of *L. plantarum* into pTZ57R/T vector and confirmation of clone: Lane 1& A: 100 bp DNA ladder; Lane 2-9: T vector harbouring PCR amplicons; Lane E, G & H: PCR amplicon (T vector with ~1300 bp fragment, ~1000 bp fragment, ~680 bp fragment)

As per the blast result, the maximum identity of the 680 bp fragment was to *Lactobacillus plantarum* insertion sequence ISLpl2 OrfA gene, partial cds; OrfB, complete cds; insertion sequence ISLpl1 transposase TraISLpl1 (traISLpl1), and P87 genes, complete cds. It is interesting to note that transposable elements were identified in the vicinity or even within EPS gene clusters (Jolly *et al.*, 2002; Péant *et al.*, 2005; Dan *et al.*, 2009) and this points to the fact that the transposase gene amplified from the genomic DNA of *L. plantarum* strain could be inside the EPS gene cluster or in the near vicinity of the cluster.

According to the report by Dan et al (2009), there is a possibility that the transposase gene might be inserted between putative glycosyl transferase genes as in *L. fermentum*. This

particular insertion in no way affected the expression of mRNA in the EPS gene cluster of *L. fermentum*.

The hypothetical protein gene amplified (~1300 bp), while analysing for the priming glycosyl transferase gene, is located in the MFS (Major Facilitator Super family) region of the genomic DNA as per the BLAST result. The MFS is one of the two largest families of membrane transporters, the other being the ATP-binding cassette (ABC) superfamily. It includes a large and diverse group of secondary transporters of uniporters, symporters and antiporters. There are about 17 distinct families within the MFS according to phylogenetic analysis. MFS proteins facilitate the transport across cytoplasmic or internal membranes of a variety of substrates including simple sugars, oligosaccharides, inositols, drugs, neurotransmitters, aminoacids, nocleosides, organophosphate sters, Krebs cycle metabolites and ions (Pao *et al.*, 1998). They do so using the electrochemical potential of the transported substrates. MFS proteins are typically 400 to 600 amino acids in length, and the majority contain 12 transmembrane alpha helices (TMs) connected by hydrophilic loops. The N- and C-terminal halves of these proteins display weak similarity and may be the result of a gene duplication/fusion event. Based on kinetic studies and the structures of a few bacterial superfamily members, GlpT (glycerol-3-phosphate transporter), LacY (lactose permease), and EmrD (multidrug transporter), MFS proteins are thought to function through a single substrate binding site, alternating-access mechanism involving a rocker-switch type of movement. Bacterial members function primarily for nutrient uptake and as drug-efflux pumps to confer antibiotic resistance. Some MFS proteins have medical significance in humans such as the glucose transporter Glut4, which is impaired in type II diabetes and glucose-6-phosphate transporter (G6PT), which causes glycogen storage disease when mutated. These data imposes the coincidence of this hypothetical protein identified from *L. plantarum* being involved in sugar transport helping in the EPS biosynthesis.

The result shows that the priming glycosyl transferase of LAB varies among species. Specific primers designed from known sequence of *L. rhamnosus* ATCC 9595 were not enough to amplify the respective portion from *L. plantarum* pointing to the importance of degenerate primers in amplifying similar gene from a different species and the homology of the particular sequences within the genus. This homology difference in the priming glycosyl transferase unveils that the EPS produced by *L. plantarum* will be structurally different from the EPS of other *Lactobacillus* spp.

Table 7.2 Sequencing results of gene amplicons from genomic DNA of *L. plantarum*

PCR amplicons cloned into pTZ57R/T vector	***Sequencing result***
Amplicon (~680 bp) for *Priming glycosyl transferase*	*Lactobacillus plantarum* insertion sequence ISLpl2 OrfA gene, partial cds; OrfB, complete cds; insertion sequence ISLpl1 transposase TraISLpl1 (traISLpl1), and P87 genes, complete cds
Amplicon (~1000 bp) for *Priming glycosyl transferase*	Cloning vector pJAP8, complete sequence
Amplicon (~1300 bp) for *Priming glycosyl transferase*	*Lactobacillus plantarum* JDM1, complete genome, hypothetical protein or fumarate reductase/succinate dehydrogenase flavoprotein

Similarly, the confirmation of clones obtained from the gene fragments of *L. lactis* in pTZ57R/T vector (Fig. 7.8) was performed by PCR reaction of the cloning vector with insert using the primers eps A F, epsA R, pg F and pg R. The clones in pTZ57R/T vector were sequenced and the similarity based on the BLAST program is displayed in Table 7.3.

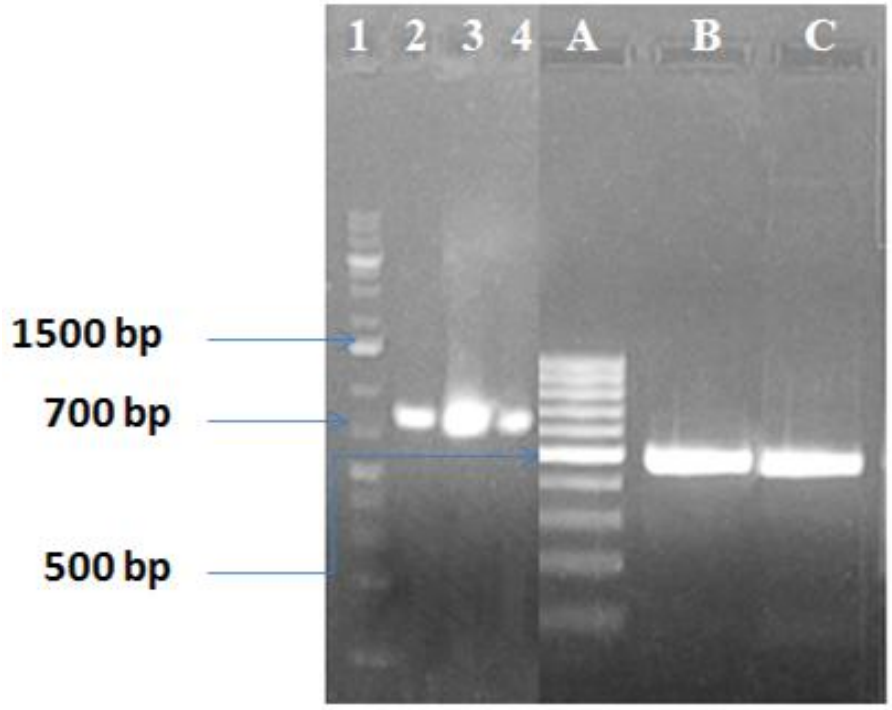


Fig. 7. 8. Confirmation of clone of PCR amplicons of *L. lactis* in pTZ57R/T vector: Lane 1& A: 1kb DNA ladder; Lane 2-4: eps A PCR amplicons from T vector (~780 bp); Lane B& C: priming glycosyl transferase PCR amplicon from T vector (~481 bp)

The BLAST result showed similarity of epsA amplicon (~780 bp) with EPS synthesis gene cluster of *S. thermophilus*. The similarity was obtained with partial sequence of EPS cluster. This reveals the presence of EPS gene cluster involved in EPS biosynthesis, in the plasmid DNA of *L. lactis*. The amplicon obtained (~481 bp), while analysing for priming glycosyl transferase, from *L. lactis* showed coincidence towards hydrolase of HAD super family from *S. thermophilus*. The glycosidic bond(s) between two or more carbohydrates or the bond between a carbohydrate moiety and a non-carbohydrate moiety is cleaved by glycosyl hydrolases (EC 3.2.1 to 3.2.3). The similarity of the gene sequence was particular to phosphatase of the hydrolases, which are involved in the carbohydrate uptake of cells.

Table 7.3 Sequencing results of gene amplicons from plasmid DNA of *L. lactis*

PCR amplicons cloned into pTZ57R/T vector	***Sequencing result***
Amplicon (~780 bp) for *epsA*	*Streptococcus thermophilus strain MTC310 exopolysaccharide synthesis gene cluster, partial sequence*
Amplicon (~481 bp) for *Priming glycosyl transferase*	*Streptococcus thermophilus* LMD-9, complete genome, hydrolase of HAD super family

7.4. Conclusion

The search for EPS biosynthesis gene such as priming glycosyl transferase (links first sugar of the repeating unit to a lipid carrier) from *L. plantarum* (MC1, NIIST isolate) and priming glycosyl transferase and *epsA* (involved in the polymerisation and chain length of EPS) from *L. lactis* (NIZO B40) revealed the homology and differences in the particular gene sequences of lactic acid bacteria. The amplification for priming glycosyl transferase gene from the genomic DNA of *L. plantarum* resulted in more than one amplicon, one of which showing homology to a transposase and the other to a hypothetical protein sequence of *L. plantarum*. As transposase genes have been identified inside EPS gene cluster and in the vicinity of the gene cluster of many lactic acid bacteria, the possibility of this identified gene being inside an EPS gene cluster or in the near vicinity of the cluster cannot be ruled out. The hypothetical gene amplified may be involved in sugar transport promoting EPS biosynthesis. The results point to the fact that the priming glycosyl transferase gene in different species of *Lactobacillus* showed differences which require degenerate primers to get amplified and the

homology difference can result in the production of structurally different EPS of specific physico-chemical properties. At the same time, the analysis of *L. lactis* plasmid for epsA and priming glycosyl transferase genes resulted in amplicons showing homology to EPS synthesis gene cluster of *S. thermophilus* and hydrolase of HAD super family of *S. thermophilus* respectively. The functionality of these genes has to be proved further.

CHAPTER 8

EXPLORATION OF THE POSSIBLE APPLICATIONS OF EPS IN THERAPEUTICS

CHAPTER 8

EXPLORATION OF THE POSSIBLE APPLICATIONS OF EPS IN THERAPEUTICS

8.1. Introduction

Exopolysaccharides have found multiple applications in pharmaceutical, food and various other industries. Exopolysaccharides possess the potential to contribute to human health as pre-biotics or due to its anti-tumour, anti-ulcer, immunomodulating or cholesterol-lowering activities (Ruas-Madiedo *et al.*, 2002). Natural or bio-polysaccharides derived from microbial and plant sources were reported to have antioxidant activities (Kong *et al.*, 2010) and anti-tumour activity (Leung *et al.*, 2006). The major biotechnological advantages of microbial polysaccharides are short fermentation process and easily formed and stable emulsions. These polysaccharides usually possess low cytotoxicity and side-effects which make them good candidates for immunotherapy against cancer and as anti-oxidants.

Reactive Oxygen Species (ROS) are found to be involved in various biological processes resulting in the development or progression of several diseases. Exopolysaccharides are found to participate in the removal of free radicals, there by functioning as potent anti-oxidants. Mechanism of antitumour action of polysaccharides consists in the stimulation of certain components of the immune system, mainly T and B-lymphocytes, macrophages and induction of interleukin release by NK cells. Polysaccharides are most often administered parenterally, sometimes orally, when the presence of peptide fragment allows for such route. Method of administration of these compounds, resulting mostly from their chemical structure is not burdensome to patients, which is the undoubted advantage of these compounds.

An important feature concerned with the bioactivity of immunomodulatory polysaccharides is the importance of its structure-function relationship. Molecular weight, tertiary structure or conformation and composition can affect the biological activity of polysaccharides. In general, polysaccharides in a configuration with β (1–3), (1–4) or (1–6) branch chains are necessary for activity and complex branch-chained polysaccharides with anionic structures and higher molecular weights have greater immunostimulating activities (Cleary *et al.*, 1999). Differences in bioactivity may be due to differences in receptor affinity or receptor-ligand interaction on the cell surface (Mueller *et al.*, 2000). There have been numerous studies on the chemical properties and bioactivities of intracellular polysaccharide isolated from plants and fungi. However, the properties of extracellular EPS produced in liquid or submerged fermentation are not well characterised and documented.

The present chapter deals with the anti-oxidant and anti-tumour activity of the EPS produced by *L. Plantarum* MTCC 9510. It unveils the therapeutic role of the EPS and its relation to the elucidated structure.

8.2. Material and Methods

8.2.1. Cell lines and Maintenance

Cell lines that have been made use for the experiments are discussed in detail in chapter 2 (section 2.1.3). The cell lines in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Grand Island, NY, USA) supplemented with10 % fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin, were maintained at 37 ºC in a 5 % CO_2 humidified incubator.

8.2.2. In vitro Assay for Anti-oxidant Activity

Anti-oxidant activity of crude and pure EPS was investigated by reducing power assay (Oyaizu, 1986). The concentration of crude and pure EPS selected for the studies were

in the range of 0.05-1 mg/ml. Reaction was carried out in a mixture containing 2.5 ml of sample (0.05–1 mg/ml), 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1 %, w/v) by incubating at 50 °C for 20 min. Trichloroacetic acid (2.5 ml 10 %, w/v) was added to the reaction mixture and was centrifuged at 5000 rpm for 10 min. The upper layer (5 ml) was mixed with 0.5 ml of fresh $FeCl_3$ (0.1 %, w/v), and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. De-ionized water and ascorbic acid were used as blank and control respectively.

8.2.3. In vitro Assay for Cytotoxicity

Cytotoxicity of *L. plantarum* EPS in normal cells was checked using L929 fibroblast cell line. Approximately 5 x 10^3 cells in 100 µL of DMEM (10 % FBS) were seeded per well in 96 well plates. The cells were incubated overnight at 37°C in a humidified incubator of 5 % CO_2. The cells were then treated with EPS of different concentrations $1x10^{-6}$ - 50 mg/ml. The cells were incubated for 24, 48 and 72 h at 37°C under the same conditions. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed (Loosdrecht *et al.*, 1994) to study the inhibitory effect of the polysaccharide in normal cells.

Growth Inhibition Rate (GIR) = 100 - Proliferation Rate

$$\textbf{Proliferation Rate (PR)} = \frac{\textbf{A}_{570}\ \textbf{of treated cells}}{\textbf{A}_{570}\ \textbf{of control}} \times 100$$

8.2.4. Anti-tumour Activity of L. plantarum Exopolysaccharide

Anti-tumour activity of *L. plantarum* EPS was observed in human breast adenocarcinoma cells (MCF-7), by morphological apoptosis method and *in vitro* assay method.

8.2.4.1. Morphological Apoptosis Determination

Human breast adenocarcinoma cells (MCF-7) treated with *L. plantarum* EPS ($1x10^{-6}$ - 25 mg/ml) were observed morphologically by fluorescent microscopy for apoptosis. Two different morphological staining techniques employing fluorescent DNA binding dyes, Hoechst and Ethidium Bromide, Acridine Orange (EB/AO) were made use for the purpose. For Hoechst staining, approximately 10^6 cells were placed in a test tube and centrifuged at 300 x g for 5 min. Supernatant was removed and 500 µl of DMEM was added and pre-warmed to 37 °C. The mixture was mixed gently and 5 µl of Hoechst 33342 stock solution (1 mg/ml) was added and mixed again. The solution was incubated at 37°C for 45 min. After treatment, the cells were washed three times with 1X PBS and viewed under microscope. EB/AO staining was performed by adding 25 µL of 1X EB/AO to the treated cells and viewed under microscope (Ribble *et al.*, 2005).

8.2.4.2. In vitro Assay for Anti-tumour Activity

The human breast adenocarcinoma cells (MCF-7), were seeded in 96 well plates at a concentration of approximately 5 x 10^3 cells per well in 100 µL of DMEM (10 % FBS). The cells were incubated overnight at 37°C in a humidified incubator of 5 % CO_2. The cells were then treated with EPS of varying concentrations: $1x10^{-6}$ - 25 mg/ml. The cells were incubated for 24, 48 and 72 h at 37°C in a humidified incubator of 5 % CO_2. The anti-tumour property of the polysaccharide was studied by cellular viability assessment employing MTT assay (Loosdrecht *et al.*, 1994). The anti-tumour effect of the compound was compared with a commercially available anti-cancer drug doxorubicin in the same concentrations as the polysaccharide.

8.2.5. In vitro Assay for Lymphocyte Proliferation

Lymphocytes were isolated from heparinised blood of a healthy, adult male volunteer by Ficoll- Hypaque™ centrifugation method of Boyum (1968). Cells were washed twice in

0.5 ml RPMI 1640 with 10 % fetal bovine serum (FBS) by centrifugation at 3500 rpm for 2 min. The final pellet was suspended in 10 ml PBS. Approximately 2 x 10^4 lymphocytes in RPMI 1640 (10 % FBS) medium were seeded per well in 96-well plates and incubated with *L. plantarum* EPS of various concentrations: $1x10^{-6}$ - 25 mg/ml. Cells were incubated at 37°C for 72 h in a humidified incubator which maintained a constant atmosphere of 5 % CO_2. Finally, the lymphocyte proliferation was checked by MTT assay. The absorbance A_{570} nm of the cells was measured in an ELISA reader (BIO-RAD 550, American).

8.2.6. Experimental Statistics

All treatments and assays have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

8.3. Results and Discussion

8.3.1. In vitro Assay for Anti-oxidant Activity

Reducing power of a compound can serve as a significant indicator of anti-oxidant activity. Antioxidants are able to reduce Fe^{3+}/ferricyanide complex to its ferrous form. This Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al.*, 2002), which gives the measure of reducing power of the compound. Experiment with crude and pure EPS showed that the EPS possessed anti-oxidant activity in a relatively lower level. On comparison to ascorbic acid, the anti-oxidant activity of both crude and pure EPS was observed to be around 3 %. Fig. 8.1 displays the reducing power of crude and pure EPS. The antioxidant activity of crude and pure EPS was found to be increasing with increasing concentration. Crude EPS exhibited a little bit higher anti-oxidant activity than the pure EPS. This could be mainly due to the presence of smaller concentration of proteins in the crude EPS. The particular chemical nature of the EPS could be the reason for its relatively low level of anti-oxidant property. Chemical modification of the polysaccharide such as alkylation,

acylation, reduction, oxidation, sulphonation etc could improve the property. It has been reported by Du et al (2010) that the sulphated derivative of a polysaccharide from *Tremella aurantialba* fruit bodies presented an intense increase in the biological activity on comparison to the native polysaccharide .

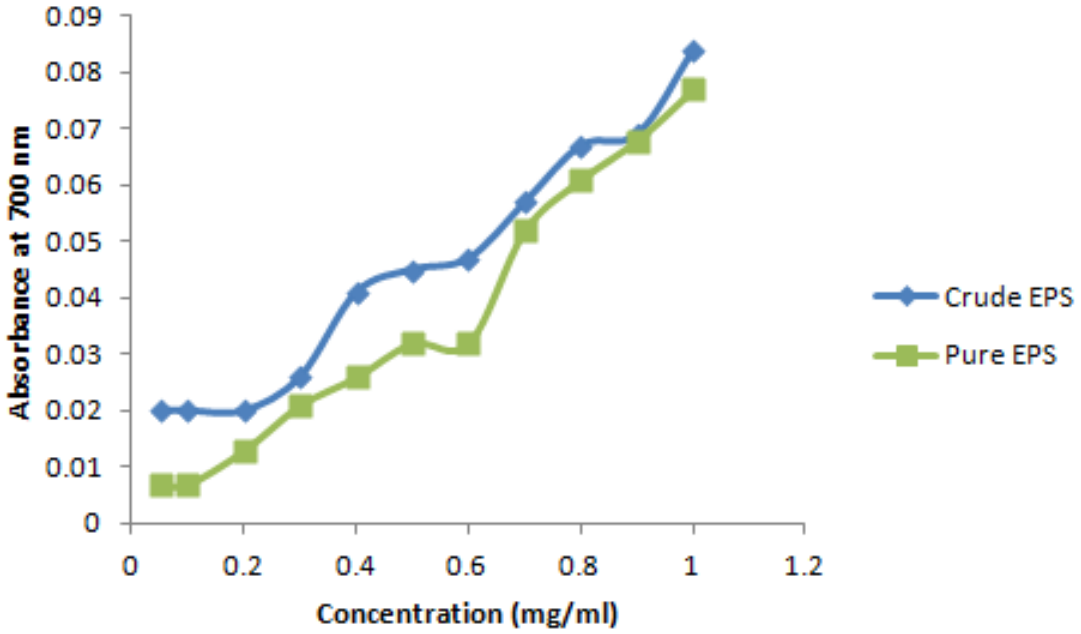


Fig. 8. 1. Reducing power of crude and pure *L. plantarum* EPS

8.3.2. In vitro Assay for Cytotoxicity

The cytotoxicity of EPS in normal fibroblast cells (L929) was measured in terms of cellular viability by MTT assay. MTT assay is a sensitive colorimetric substitute for radioisotopes in cell proliferation and cytotoxicity studies. Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells (Fig. 8. 2).

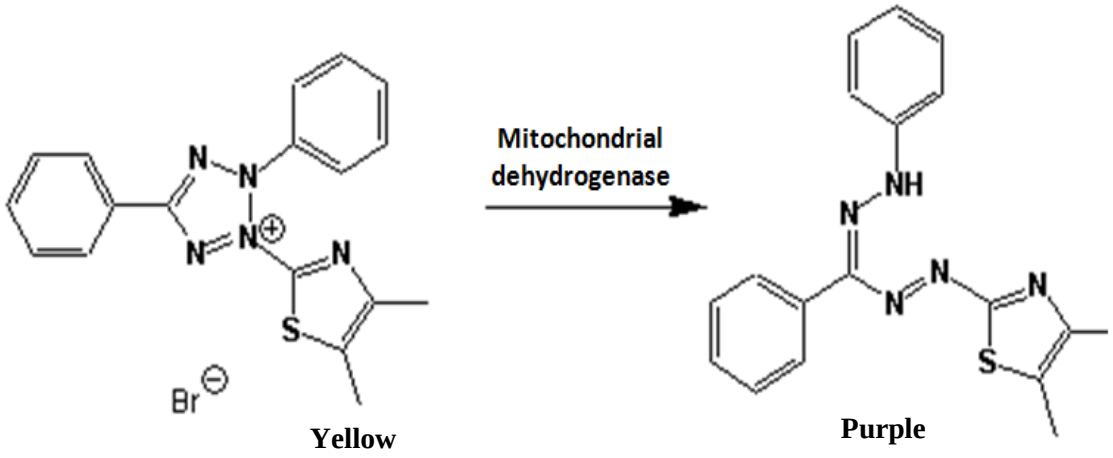


Fig. 8. 2. Reduction of MTT by mitochondrial dehydrogenase

This reduction takes place only when mitochondrial dehydrogenase enzymes are active and therefore conversion can be directly related to the number of viable (living) cells.

The *L. plantarum* EPS exhibited no inhibitory effect on normal cells up to 25 mg/ml concentration. The result obtained was same for all incubation periods, 24, 48 and 72 h (Fig. 8. 3). Cytotoxicity was observed in normal cells only at higher concentration 50 mg/ml in all incubation periods.

Since the polysaccharide does not possess any inhibitory effect up to 25 mg/ml on normal cells, the compound could be considered for anti-tumour studies. It becomes necessary to study the toxicity of anti-cancer compounds in normal cells as the compound should not compromise normal cells. The experiment proves that the compound will be selective if chosen as an anti-cancer agent. The solubility of the compound in water is another selective factor for its anti-cancer studies as most of the anti-cancer drugs are not water soluble (Gollahon *et al.*, 2011) and are administered with vehicles which bring out serious side effects.

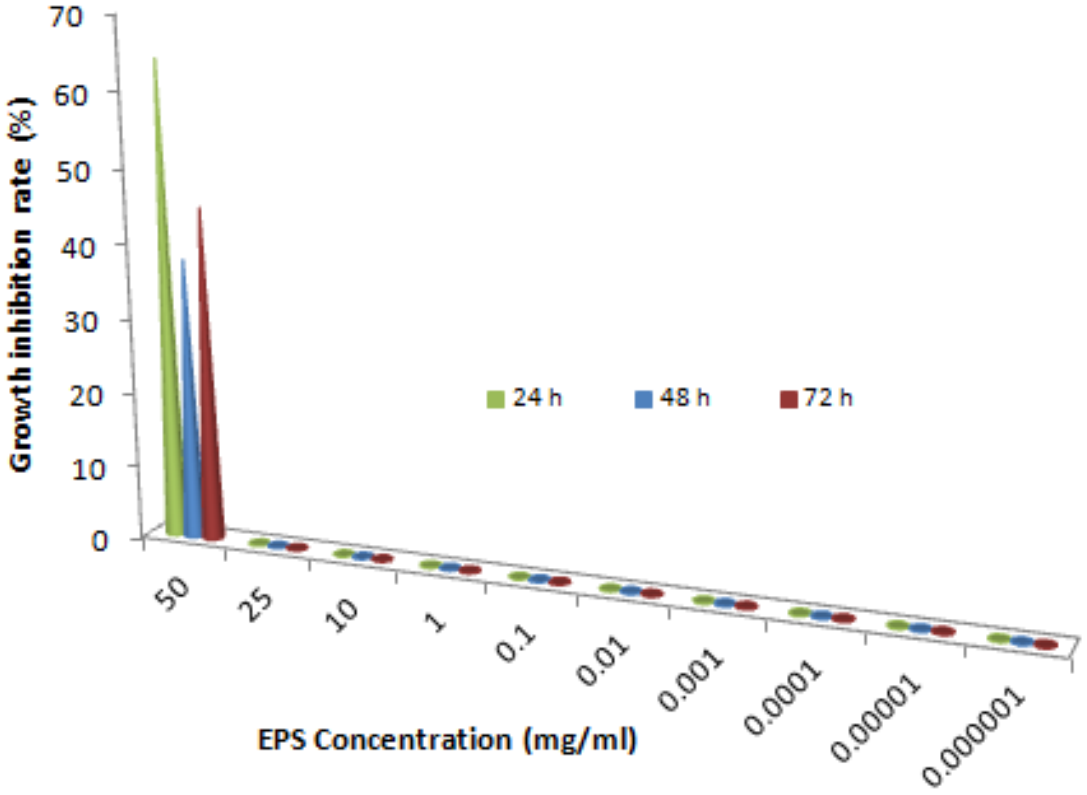


Fig. 8. 3. Cytotoxicity of *L. plantarum* EPS in L929 cells

8.3.3. Anti-tumour Activity of L. plantarum Exopolysaccharide

8.3.3.1. Morphological Apoptosis Determination

The MCF7 breast cancer cell line is among the most used and well characterized. These cells are ER^+, PR^+ and $her2/neu^+$ (Creighton *et al.*, 2008). Highly condensed chromatin bodies are the specific features of apoptotic cells. Each apoptotic body has a fragmented piece of nucleus surrounded by a viable cell membrane. These apoptotic cells can be easily differentiated from a normal cell by fluorescent microscopy. The present experiment employed Hoechst and EB/AO stains. Hoechst 33342 is a permeable DNA dye that binds preferentially to A-T base-pairs. In Hoechst staining, the nucleus of live and dead cells is

stained blue. The difference is in the intensity of the colour which will be more in dead cells due to chromatin condensation. Fig. 8. 4. shows the control cells and treated cells stained with Hoechst and EB/AO. It was observed during the experiment that the *L. plantarum* EPS was showing apoptotic activity in breast adenocarcinoma cells (MCF-7).

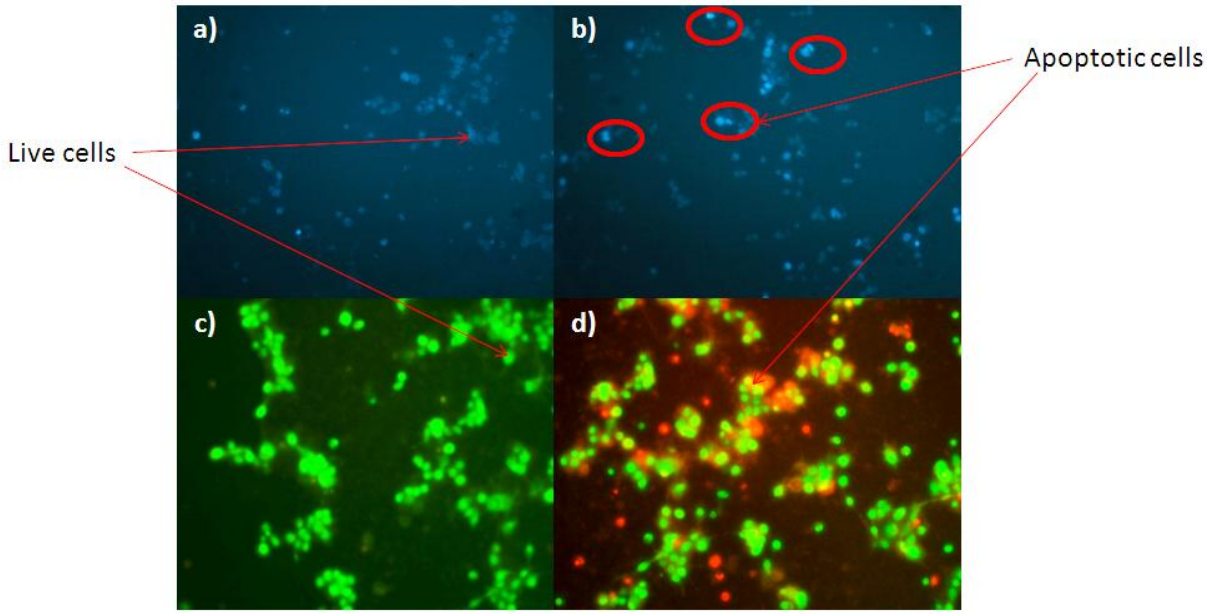


Fig. 8. 4. Hoechst staining a) MCF-7 control cells without treatment b) MCF-7 cells treated with EPS; EB/AO staining c) MCF-7 control cells without treatment d) MCF-7 cells treated with EPS

Hoechst stained apoptotic cells were blue in colour with condensed chromatin. This was confirmed by the EB/AO staining which could distinguish orange apoptotic cells from green live cells. The two fluorescent dyes ethidium bromide and acridine orange, allow rapid and easy recognition of live and dead cells when visualized by fluorescence microscopy, which is the advantage of EB/AO staining over Hoechst staining. Acridine orange stains live cells green while ethidium bromide stains dead cells red-to-orange, depending on the filter

employed in the microscope. The excitation and emission maxima for acridine orange are 500 and 530 nm while for ethidium bromide 510 and 595 nm respectively. Early-stage apoptotic cells are stained green as they take up acridine orange but not ethidium bromide, whereas nonviable cells take up both dyes and are stained orange. The microscopic images showed a higher percentage of apoptotic cells in the EPS treated cells.

8.3.3.2. In vitro Assay for Anti-tumour Activity

Anti-tumour activity of *L. plantarum* EPS in MCF-7 cells was quantitatively assessed by MTT assay in MCF-7 adenocarcinoma cells. Since the EPS exhibited cytotoxicity at a concentration of 50 mg/ml, the particular concentration was avoided from anti-tumour studies in MCF-7 cells. The result (Fig. 8. 5) shows that the EPS could inhibit the growth of tumour cells even at lower concentration of 1 ng/ml when incubated for 72 h. Growth inhibition was observed from 48 h onwards for 10 ng/ml concentration and from 24 h onwards for 100 ng/ml concentration. The percentage of growth inhibition in these concentrations was in the range of 1- 4 %. The inhibition percentage was observed to be 9 – 22 % for 1- 100 µg/ml concentrations. With 1 mg/ml concentration, an inhibition of 28 % was obtained in 72 h. Even though the IC_{50} for the compound was attained at 10 mg/ml on comparison to the control, doxorubicin of IC_{50} 0.1 mg/ml, the absence of any side effects in normal cells under *in vitro* conditions could make the polysaccharide a good candidate of study in anti-tumour aspect.

The particular anti-tumour property of the polysaccharide could be mainly attributed by the (1, 3) linkages present in the structure. It has already been reported that exopolysaccharides with β (1, 3) linkages exhibit anti-tumour properties. This property is initiated by the binding of glucans to β-glucan receptor, such as dectin-1 (Brown & Gordon, 2001; Taylor *et al.*, 2002), immune cells. Dectin-1 cooperates with Toll-like receptors (TLRs), and many other surface receptors for the recognition of different microbial products

such as fungal cell wall, lipopolysaccaharide, lipoprotein, flagellin and bacterial DNA (Underhill, 2003). The positive advantages of the compound are masked by the IC_{50} (10 mg/ml). This problem could be overcome by modifying the chemical structure of the polysaccharide.

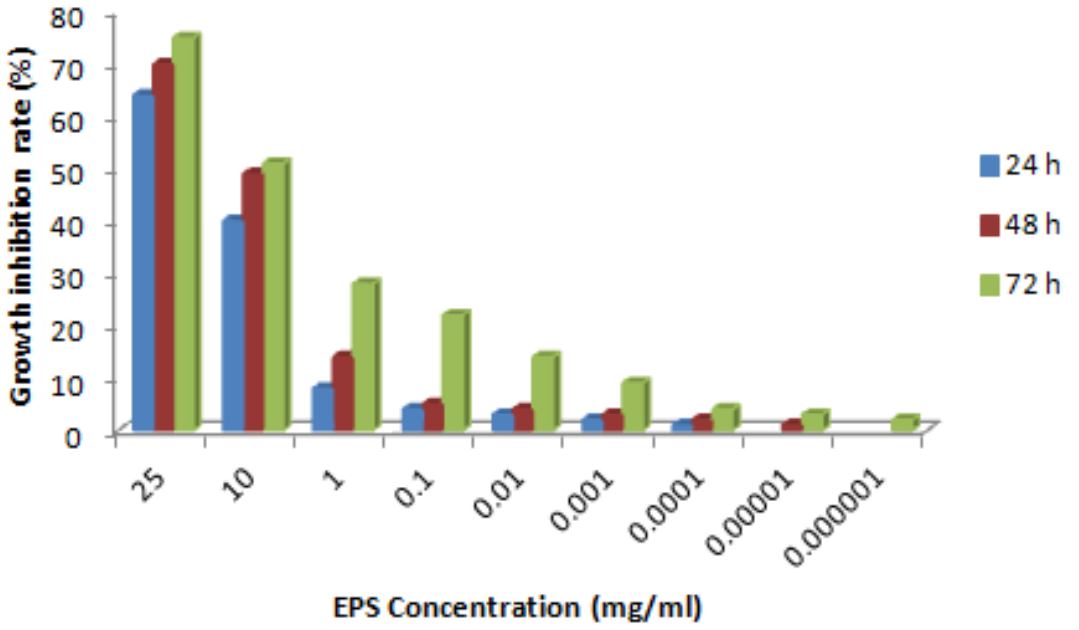


Fig. 8. 5. Anti-tumour activity of *L. plantarum* EPS by MTT assay

It is of importance that chemical modifications of the polysaccharide by oxidation, reduction, transglycosylation or sulphonation can decrease the IC_{50} and improve the anti-tumour properties. The carboxymethylated linear (1, 3)-α-glucans from *Amanita muscaria* and *Agrocybe cylindracea* shows high potent antitumor activity against Sarcoma 180 and immunomodulating activity in mice (Yoshida *et al.*, 1996) compared to its native form. These studies show that the chemical modification of polysaccharides might be an effective approach of improving the biological activities of polysaccharides. There are possibilities that the compound could exhibit a better anti-tumour property in a different cell line and under

in vivo conditions. The mode of action of the EPS is through apoptosis but the detailed mechanism behind the action requires further *in vitro* and *in vivo* studies.

8.3.4. In vitro Assay for Lymphocyte Proliferation

Lymphocytes consist of various subpopulations with distinctive functions, which play important roles in immune responses. Activation and proliferation of these subpopulations can be achieved by treating them with mitogens. Measurement of proliferative responses of lymphocytes is a fundamental technique for the assessment of their biological responses to various stimuli. The immunologic action of polysaccharides may begin with activating effector cells such as lymphocytes, macrophages, natural killer (NK) cells. These exopolysaccharides are generally considered as a kind of biological response modifiers which are able to restore or enhance various immune responses *in vivo* and *in vitro*. This means that it causes no harm and places no additional stress on the body and instead helps the body to adapt in various environmental and biological stresses, supporting some of the major systems as nervous, hormonal and immune systems, as well as regulatory functions.

In the experiment, *L. plantarum* EPS exhibited lymphocyte proliferation property in the range 1 x 10^{-5} – 10 mg/ml concentration (Fig. 8.6). The other two concentrations (1 x10^{-6} and 25 mg/ml) were found to be inhibitory. The concentration of 0.1 mg/ml was found as the best in lymphocyte proliferation with approximately 20 % proliferation rate.

These results show that EPS from *L. plantarum* is possessing immunomodulatory effect which explains its role as an anti-cancer compound. The proliferation is a complex process involving delivery of single signal or group of signals to the cell membrane and activation of intracellular enzyme pathways. The stimulated immune pathway of exopolysaccharides is different from that of lipopolysaccharides. The ability to induce or enhance cytokine production could be a major mechanism by which EPS exerts its immunomodulatory property.

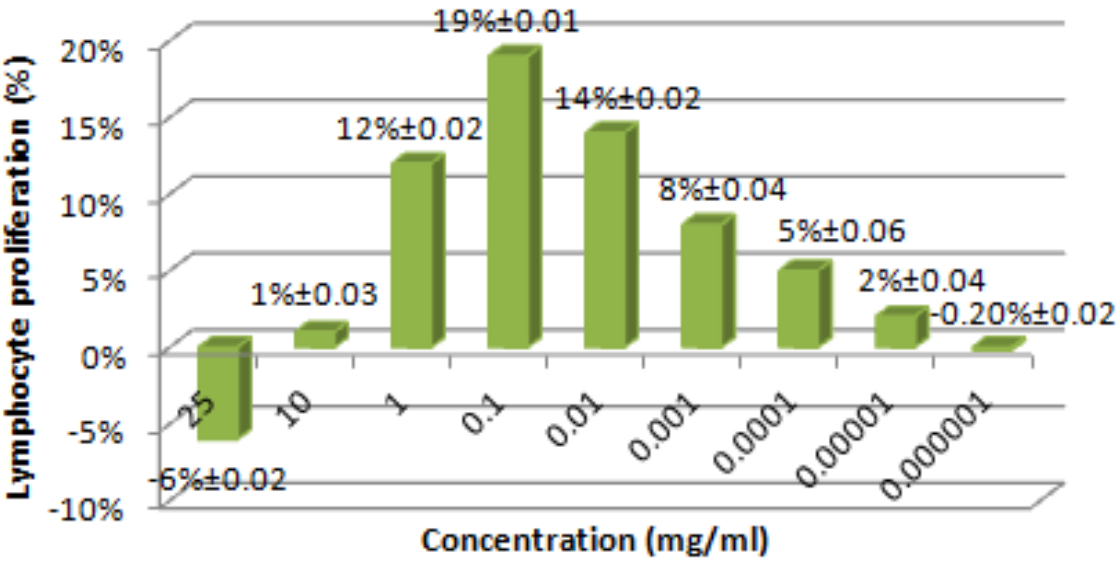


Fig. 8. 6. Lymphocyte proliferation of *L. plantarum* EPS

Mainly, mannose-rich polysaccharides collaborate with Toll like Receptors (TLRs) for the activation of immunity. So there is a possibility of the particular polysaccharide to act with TLRs as it comprises glucose and mannose as its subunits. The understanding of this complex process requires further *in vitro* and *in vivo* experiments. Kim et al (2007) reported that macrophage activation by lactic acid bacteria EPS resulted in the release of TNF-α. The experiment by Liu et al (2011) proved that the EPS isolated from lactic acid bacteria can promote macrophage growth and induce production of pro-inflammatory responses in murine macrophage cell lines.

8.4. Conclusion

The great majority of chemical compounds identified as anti-cancerous are also toxic to normal cells. Hence, the discovery and identification of new safe drugs has become an important goal of research in the biomedical sciences. This chapter has discussed about the anti-oxidant, anti-tumour and immunomodulating properties of EPS purified from

Lactobacillus plantarum. The findings show that the exopolysaccharide could be used, as an anti-cancer agent taking into consideration its non-toxicity towards normal cells, upon suitable chemical modifications. The main advantage of the compound is the absence of cytotoxicity in normal cells and its water solubility. The study implies that the EPS can destroy tumour cells and can activate immune system (lymphocyte proliferation). The particular properties could be attributed to the particular structure composition of the EPS comprising glucose and mannose linked by α and β (1, 3) linkages and its molecular weight. The future scope of this study would be the chemical modification of the polysaccharide for the development of a potent anti-cancer drug with low IC_{50}.

CHAPTER 9

EXOPOLYSACCHARIDE IN FOOD INDUSTRY: PREVENTION OF SYNERESIS

CHAPTER 9

EXOPOLYSACCHARIDE IN FOOD INDUSTRY: PREVENTION OF SYNERESIS

9.1. Introduction

Starch is one of the most abundant and widely distributed components in foodstuffs such as bread and noodles. Commercial starches are obtained from seeds, particularly corn, waxy corn, high amylose corn, wheat and various rices and also from tuber or roots of potato, sweet potato and cassava. Its gelatinization is commonly achieved by cooking in the presence of water. On cooling, starch granules recrystallize to form a solid gel, a process known as retrogradation. A decrease in temperature causes a reduction in the kinetic energy that facilitates the amylose molecules to associate and form a three-dimensional network. As a consequence, water is squeezed out of the gel, a process generally referred as syneresis (Fig. 9.1), while intermolecular interaction between amylose molecules becomes stronger and gel shrinks.

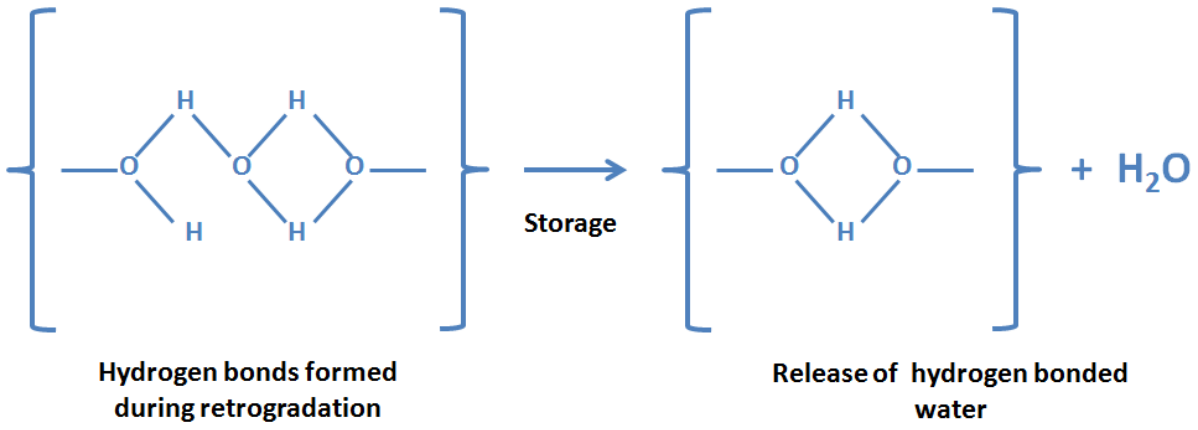


Fig. 9. 1. Syneresis of starch gel, showing release of water from amylose gel

Syneresis negatively affects the functional and sensory properties of foods (Zheng *et al.*, 1998; Sikora *et al.*, 2003). It should be minimized without interfering with the native properties of food products. This could be controlled by chemical modifications, but blending starch or milk products with polysaccharide hydrocolloids can function as an alternative to the expensive chemical modification (Appelqvist & Debet, 1997). Most polysaccharides used by the food industry as bio-thickeners are derived from plants such as starch, pectin, guar and seaweed (carrageenan, alginate). These are not always readily available and their rheological properties often do not match those required. Hence, most polysaccharides of plant origin require chemical modifications to improve their structure and rheological properties. The consequence of these chemical modifications is that the polysaccharides carry heavy restrictions over their usage in food products.

Interest in using microbial exopolysaccharides in food processing has been increased in recent years because of several reasons like their nature of polydispersity, water binding and low solution viscosity, their ease of production by fermentation and their ease of manipulation by recombinant DNA technology (Johns & Noor, 1991; Morris, 1995). Starch/hydrocolloid mixtures are widely used to modify and control the texture of foodstuffs. The addition of a hydrocolloid strongly influences the gelatinization and retro-gradation of starch. Specifically, food hydrocolloids are used to thicken gel, control syneresis, stabilize an emulsion or suspension, function as a coating and bind water. It has been demonstrated that the structure of the hydrocolloid, including the type and number of monosaccharide backbone as well as the type, number and distribution of side units, determines its characteristics and behaviour in solutions. Understanding such properties will lead to improvements in the formulation of starch-based foods. All these aspects had thrown light into the study of exopolysaccharides from *Lactobacillus plantarum* on prevention of syneresis in starch and their rheological characterization. Understanding this aspect emphasizes the efficiency of

Lactobacillus plantarum exopolysaccharide in syneresis reduction and it was proved a potential candidate replacing carboxy-methyl cellulose, to improve the texture of starch-containing foods by increasing the viscosity of the final product and also by holding the water released.

9.2. Materials and Methods

9.2.1. Raw Materials

The raw materials, wheat and cassava starches used for syneresis studies were obtained from Sd- fine chemicals, Mumbai and Central Tuber Crops Research Institute (CTCRI), Trivandrum.

9.2.2. Preparation of starch-exopolysaccharide dispersion

Starch powder was dispersed in distilled water and cooked for 15 min in a boiling water bath with gentle mixing until thickening of paste. The mixture was allowed to reach room temperature before being aliquoted. Polysaccharide dispersion was made separately. EPS was dispersed in distilled water and then stirred in a magnetic stirrer until complete dissolution. Starch suspension and EPS dispersion were mixed before being aliquoted. Sodium benzoate or sodium meta bisulphite was added to the mixture to prevent microbial activity (1 g/l).

9.2.3. Estimation of Syneresis

The extent of syneresis in the formulations was estimated according to the protocol mentioned by Viñarta et al (2006). The liquid phase length (Δh) separated above the sedimented phase was measured in two day interval time throughout the storage at 4 °C for 20 days. The degree of syneresis was represented by $\Delta h/h_0$, where h_0 stands for the initial height (in cm) of the sample dispersion.

9.2.4. Selection of Starch

Cassava starch and wheat starch were chosen at 2 % (w/v) concentration for the initial studies. Concentrations of the wheat starch experimented were 2, 3, 4, 5 & 6 % (w/v) and for the subsequent studies, 6 % (w/v) wheat starch was used since the degree of syneresis was higher in 6 % wheat starch and it was stable throughout the storage.

9.2.5. Comparison of EPS Efficacy with Carboxy Methyl Cellulose

The efficacy of EPS in prevention of syneresis was compared with a control, carboxy methyl cellulose (CMC, Sigma Aldrich, USA), a viscosifier currently in use in food industry. The concentrations of wheat starch chosen for the experiment were 2, 4 and 6 % (w/v) and the concentration of CMC and *L. plantarum* EPS chosen for the comparison studies was 0.2 % (w/v).

9.2.6. Prevention of Syneresis in Starch by exopolysaccharide

The ability of exopolysaccharides (EPS) produced by *Lactobacillus plantarum*, to minimize the liquid separation (syneresis) experienced by cooked starch pastes during refrigeration was evaluated. The starch samples or mixture (starch and polysaccharide) were poured into screw-cap plastic tubes to reach a final volume of 15 ml. Tubes were held vertically at 4 °C for 20 days, unless otherwise stated and the extent of syneresis was estimated as mentioned above (section 9.2.3).

9.2.7. Rheological characterization of wheat starch-exopolysaccharide hydrocolloid

The rheological characterization of wheat starch, wheat starch-carboxy methyl cellulose and wheat starch-exopolysaccharide was studied using Paar Physica Moderate Compact Rheometer MCR 15 (Pongsawatmanit & Srijunthongsiri, 2008). Test samples were subjected to shear rate 0-100 s^{-1} at 25 ºC. The apparent viscosity of the samples was

calculated at each point and the relationship between shear rate and shear stress was evaluated. The apparent viscosity was expressed in mPa s. The behaviour of wheat starch-exopolysaccharide at low and high concentrations of EPS was evaluated.

9.3. Results and Discussion

9.3.1. Selection of Starch

Starches of two different natures were selected for the study. Cassava starch is a tuber starch while wheat starch is a cereal grain type. Cassava starch is oval truncate in shape with an average size of 25 µm while the other is round lenticular of the same size. The tuber and root starches have a lower fat content than the grain starch. Out of the two different starches (2 % w/v) explored, the percentage of syneresis observed was more in wheat starch compared to cassava starch (Fig. 9.2).

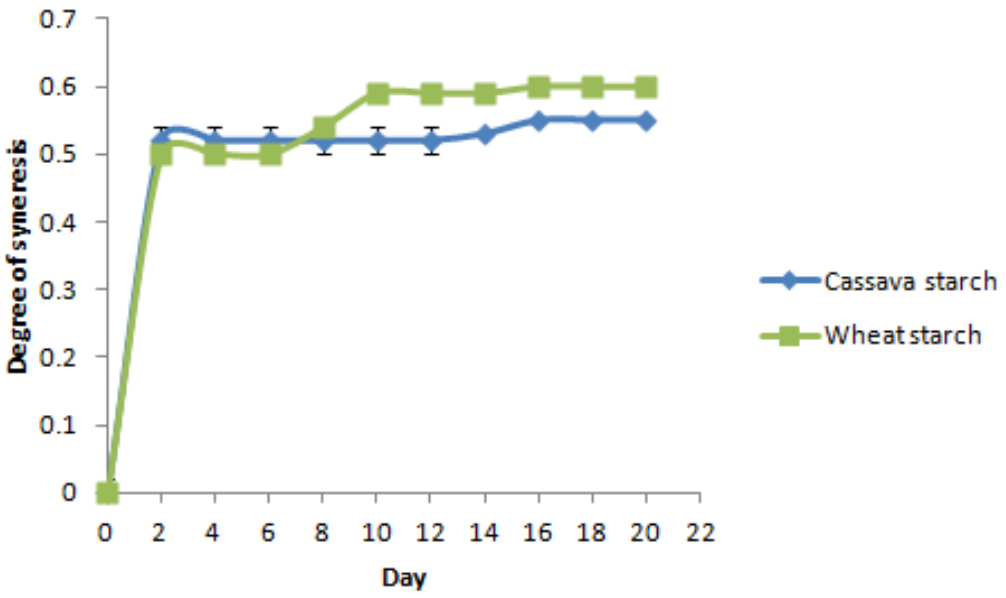


Fig. 9. 2. Syneresis in wheat and cassava (2 % w/v) starches

It has been discussed by Zheng et al (1998) that root and tuber starches exhibit high stability to cold storage. The result obtained indicated that the percentage of syneresis in wheat starch

has to be dealt with rather than the cassava starch. So wheat starch was selected for the syneresis studies using exopolysaccharide from *L. plantarum*.

9.3.2. Comparison of EPS Efficacy with Carboxy Methyl Cellulose

Carboxy Methyl cellulose (CMC) or cellulose gum is used in food science as a viscosity modifier or thickener and to stabilize emulsions in various products including ice-cream. Taking into consideration this aspect, low viscosity CMC was used as a control to compare with EPS from *L. plantarum* which is of low viscous nature at a concentration of 0.2 %. This particular concentration of the hydrocolloids was chosen for comparison, as a minimum concentration. Usually, polymers exhibit significant influence in their flow behaviour even from 0.2 % and hence are used at lower concentrations in food preparations in the range of 0.2-1 %. Positive effects may be attained at levels as low as 0.1–1 % (Sikora *et al.*, 2003; Gimeno *et al.*, 2004; Sadar, 2004). On comparison, the EPS from *L. plantarum* was found to have a better efficacy than CMC in prevention of syneresis in cooked starch pastes. The performance of *L. plantarum* EPS in all the three starch concentrations (2, 4 and 6 %) was impressive than CMC.

The percentage of syneresis inhibition in 6 % (w/v) starch with 0.2 % (w/v) *L. plantarum* EPS was higher (30 %) than the other two on second day and observed a drop on the fourth day onwards and reached 8 % on the 20^{th} day on storage at 4 °C (Fig. 9.3). Interestingly, in a similar formulation (6 % (w/v) starch with CMC, there was no syneresis inhibition at all. But in the other two formulations, 2 and 4 %, the percentage of inhibition increased up to 17 and 25 % with 0.2 % (w/v) *L. plantarum* EPS respectively and it was almost stable throughout the storage. The percentage inhibition difference of CMC and *L. plantarum* EPS in these two formulations was nearly 2-8 %. In other words, the EPS from *L. plantarum* exhibited a 2-8 % higher efficiency than the commercially used viscosifier, CMC.

The percentage inhibition of syneresis with *L. plantarum* was promising in all the formulations, but of varying degree. But the inhibition was not stable throughout storage at 4°C in 2 % and 6 % wheat starch formulations. The effect of both the hydrocolloids in 6 % wheat starch was not satisfactory. Taking into consideration this aspect, 6 % wheat starch was considered for the subsequent studies with *L. plantarum* EPS as CMC had no effective inhibition at the particular lower concentration.

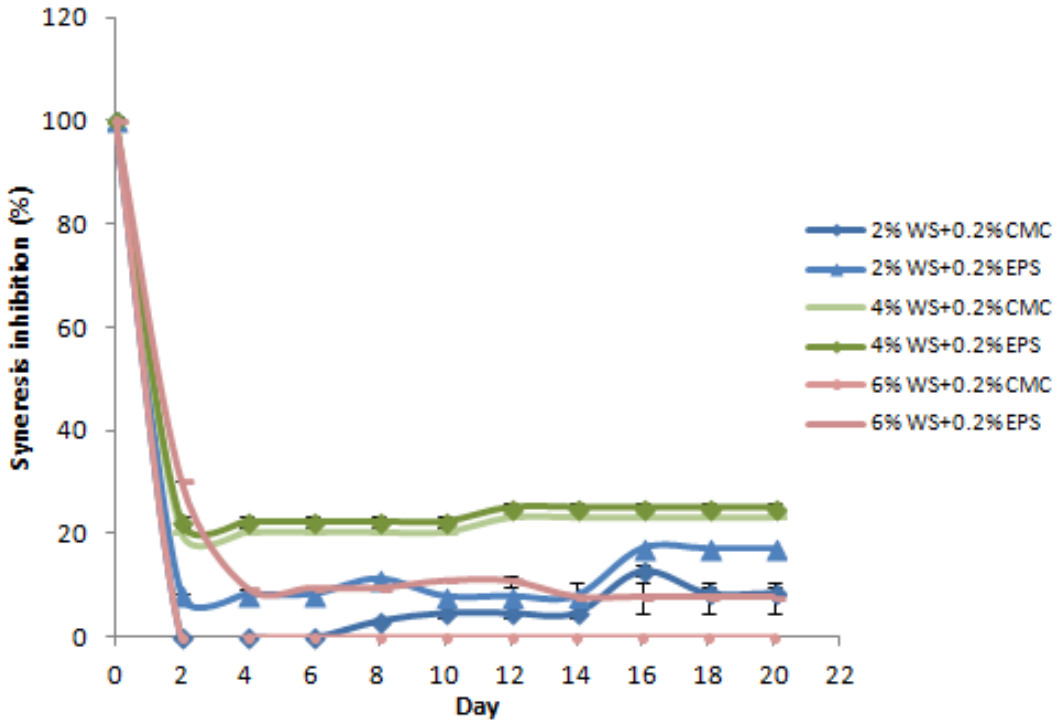


Fig. 9. 3. Comparison of syneresis inhibition of CMC and *L. plantarum* EPS in different starch concentrations

This experiment focuses the importance of *L. plantarum* EPS as a substitute of CMC in food preparations. CMC derived from bulky components, or pulp cellulose, of plant material, is chemically derivatized to make it water soluble while the EPS from *L. plantarum* is readily soluble in water. *L. plantarum* EPS performs effectively in the prevention of syneresis in addition to the role of a viscosifier.

9.3.3. Prevention of Syneresis in Starch by exopolysaccharide

As mentioned in the above section, prevention of syneresis was observed in all starch concentrations (2, 4 and 6 % w/v) with 0.2 % (w/v) *L. plantarum* EPS. Varying concentrations (0.2-1 % (w/v)) of *L. plantarum* EPS in 6 % (w/v) starch were found to be effective. Even though, inhibition in syneresis was observed with all concentrations, the rate of inhibition was around 50 % with 1 % EPS while it was 8 % with 0.2 % EPS (Fig. 9.4). All other concentrations displayed a percentage of inhibition between these values. With 0.4 %, EPS an inhibition of 9 % was obtained. The inhibition that could be attained with 0.6 and 0.8 % of EPS was almost same, 42 %.

EPS have the ability to bind water, which promotes the syneresis prevention on addition to cooked starch pastes. This was proved with the experiment that the incorporation of EPS in starch containing foods can reduce syneresis. Phase separation in aqueous solutions was observed at higher concentrations of *L. plantarum* EPS (Fig. 9.5), thereby bringing out an enhancement in the viscosity of the mixed system. EPS prevent syneresis and improve product stability firstly by increasing the viscosity and elasticity of the final product and secondly by binding hydration water. The importance of hydrocolloids in food industry has been discussed by many researchers as it is used to improve mouth-feel, texture, visual and taste perception, storage stability, mechanical protection and prevention of syneresis in the final food products (Broadbent *et al.*, 2003; Hassan *et al.*, 2003; Sikora *et al.*, 2003).

9.3.4. Rheological characterization of wheat starch-exopolysaccharide hydrocolloid

The rheological behaviour of wheat starch, wheat starch-carboxy methyl cellulose and wheat starch-exopolysaccharide hydrocolloid was investigated by shear stress controlled rheometry. It is speculated that the increased viscosity of EPS-containing foods may increase the residence time of ingested fermented product in the gastrointestinal tract and therefore be

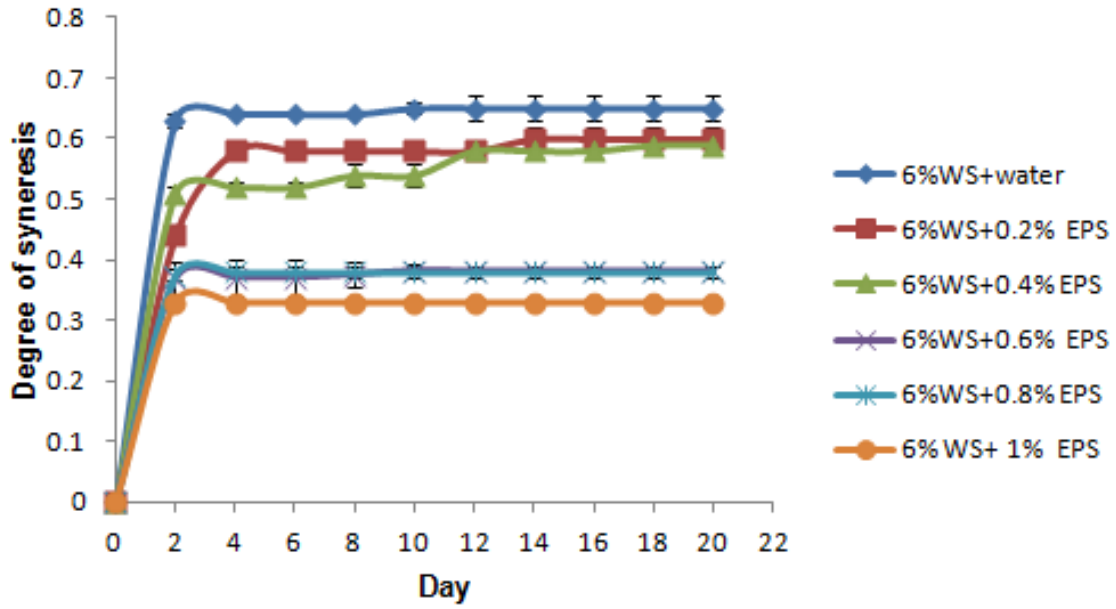


Fig. 9. 4. Prevention of syneresis in wheat starch (WS, 6 % (w/v)) by *Lactobacillus plantarum* exopolysaccharide (EPS) at different concentrations

beneficial to a transient colonisation by probiotic bacteria. Fig. 9.6 displays the changes in the apparent viscosities of wheat starch-water, wheat starch-carboxy methyl

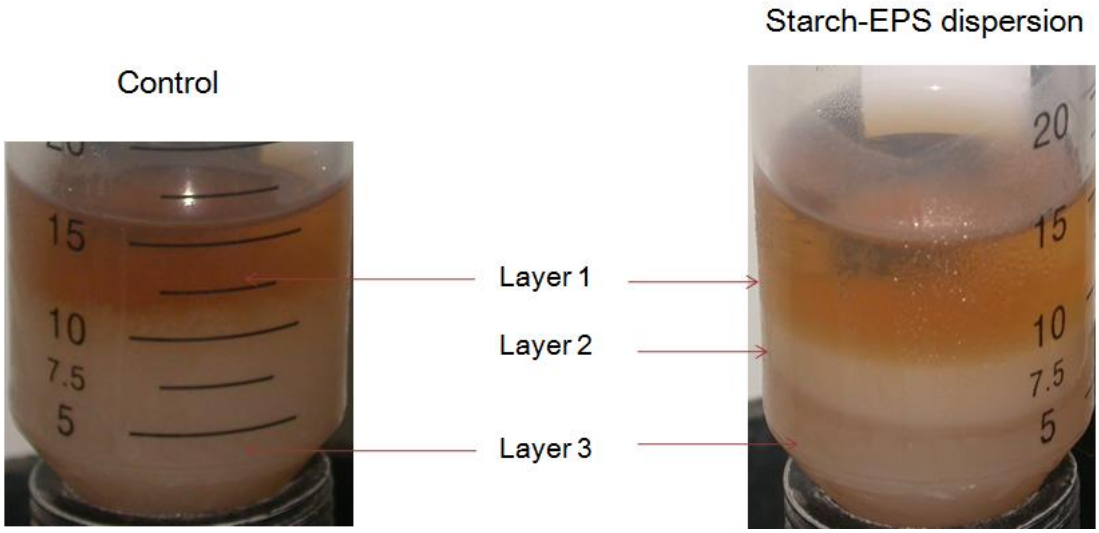


Fig. 9. 5. Phase separation observed in wheat starch- EPS dispersion at higher concentrations

cellulose and wheat starch-exopolysaccharide (wheat starch-EPS) dispersion. It was found that the starch-EPS hydrocolloid displayed higher apparent viscosity than the one without EPS. The viscosity of the starch-EPS hydrocolloid was higher than the control (starch-carboxy methyl cellulose hydrocolloid) in same concentration. Increasing the concentration of EPS in the starch suspension increased the apparent viscosity of the mixture. The incorporation of the *L. plantarum* EPS hydrocolloid at a concentration of 0.2 % could increase the viscosity by 14 %. The viscosity of the starch-EPS hydrocolloid was higher than

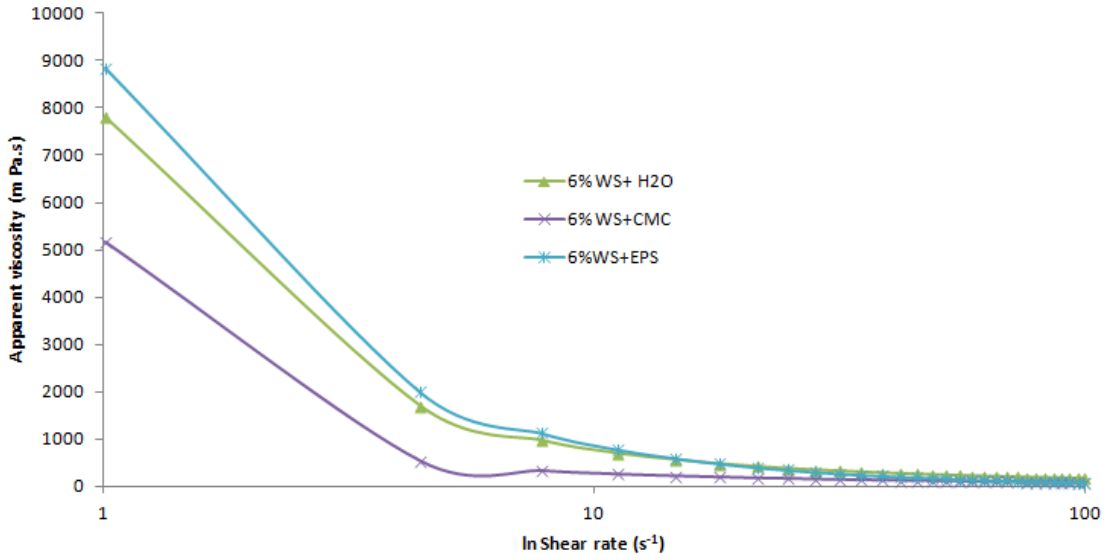


Fig. 9. 6. Shear-thinning behaviour of wheat starch (WS-water) suspension, wheat starch-carboxy methyl cellulose (WS-CMC), wheat starch-exopolysaccharide (WS-EPS) hydrocolloids

the control, starch-carboxy methyl cellulose hydrocolloid at same concentration. Increasing the concentration of EPS in the starch suspension increased the apparent viscosity of the mixture. The apparent viscosity of the mixture had an increase of 28 % when the concentration of EPS was increased to 1 % (Fig. 9.7).

In studies undertaken on starch/hydrocolloid blends, synergistic effects were observed that resulted in an increase in the viscosity of the mixtures compared with starch or

hydrocolloid alone (Sandstedt & Abbott, 1964). The synergistic increase in paste viscosity might be considered to be the result of at least two effects. First, there may be interaction between exudate from the granule (solubilized amylose and low-molecular-weight amylopectins) and the hydrocolloid (EPS). Second, the addition of EPS would mean that the forces being exerted on the granules in the shear field are much larger than those encountered in starch- water suspensions of equal starch concentration. These increased forces should significantly affect granule breakdown and the amount of material exuded into the medium.

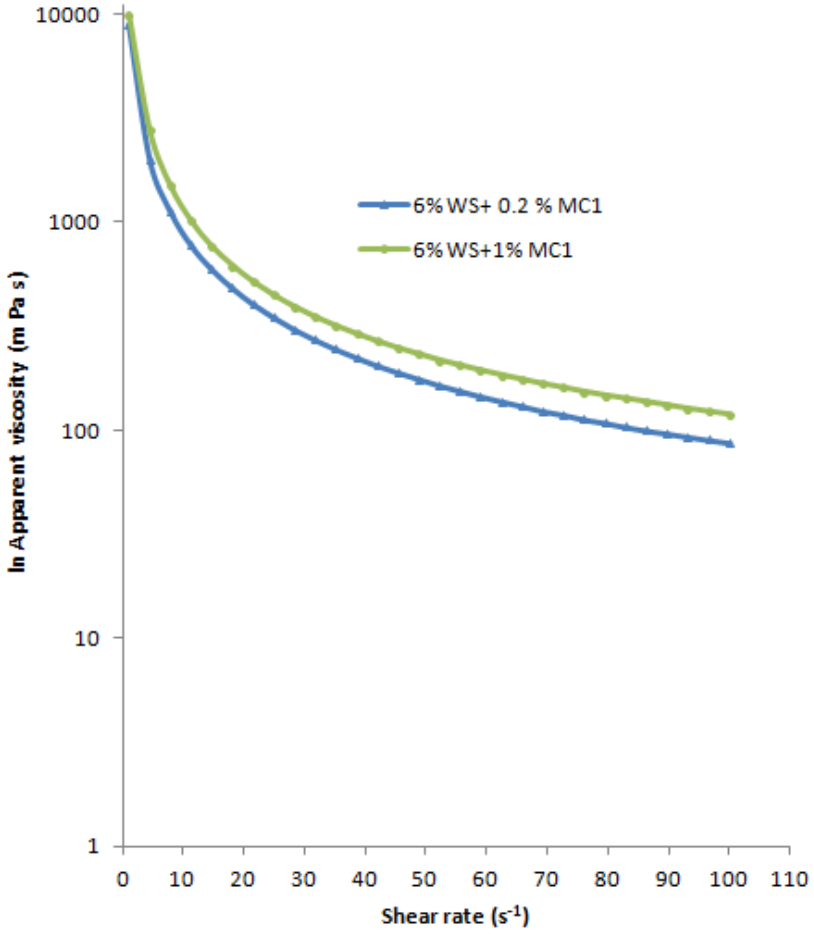


Fig. 9. 7. Apparent viscosity changes observed in wheat starch-EPS (0.2 %) and wheat starch-EPS (1 %)

The dispersions exhibited a non-Newtonian and pseudo-plastic behaviour. The relation between shear stress and shear rate was not linear, which is the characteristic of a non-Newtonian fluid. The dispersions exhibited a shear-thinning behaviour as the apparent viscosity showed an increase at lower shear rate and decrease at higher shear rate. The shear-thinning behaviour of the dispersions is more evident when both apparent viscosity and shear rate are plotted in logarithmic scale (Fig. 9.8). From the figure, it is evident that the dispersions obey the power-law. Power-law region, *i.e.*, the straight line region, is the portion where the apparent viscosity is not at all constant. In this region, we can approximate the behaviour by:

$$\ln\eta = a + b \ln\theta \qquad (1)$$

Finally by using the connection between apparent viscosity, shear stress and shear rate we write the equation as:

$$\tau = K\theta^{n} \qquad (2)$$

where τ is the shear stress, K, the consistency coefficient, θ, shear rate and n, power-law index. The power-law index for the dispersions was observed in the range 0.6-0.9 *i.e.*, <1.

Similar behaviour was observed for the wheat starch-exopolysaccharide dispersions in all the concentrations of exopolysaccharide. The application of exopolysaccharides in varying industrial areas is mainly due to their rheological properties that allow the formation of viscous solutions at low concentrations (0.05–1.0 %) and stability over wide temperature, pH and ionic strength ranges (Kumar & Mody, 2009). Incorporation of hydrocolloids in dairy foods or beverages is a well-known strategy to provide viscosity, stability and water-holding

capacity. This strategy of incorporation of hydrocolloids to provide the above characteristics has been made use of in the present experiment. The success of EPS application mainly depends on its ability to bind to the water and its efficacy to increase the viscosity of the final product and these were successfully attained in this particular case. This thermal stable EPS from *Lactobacillus plantarum* isolate along with its ability to bind water and increase viscosity, find its role in food industry as it can withstand heat processing and can protect foodstuffs during storage and transportation.

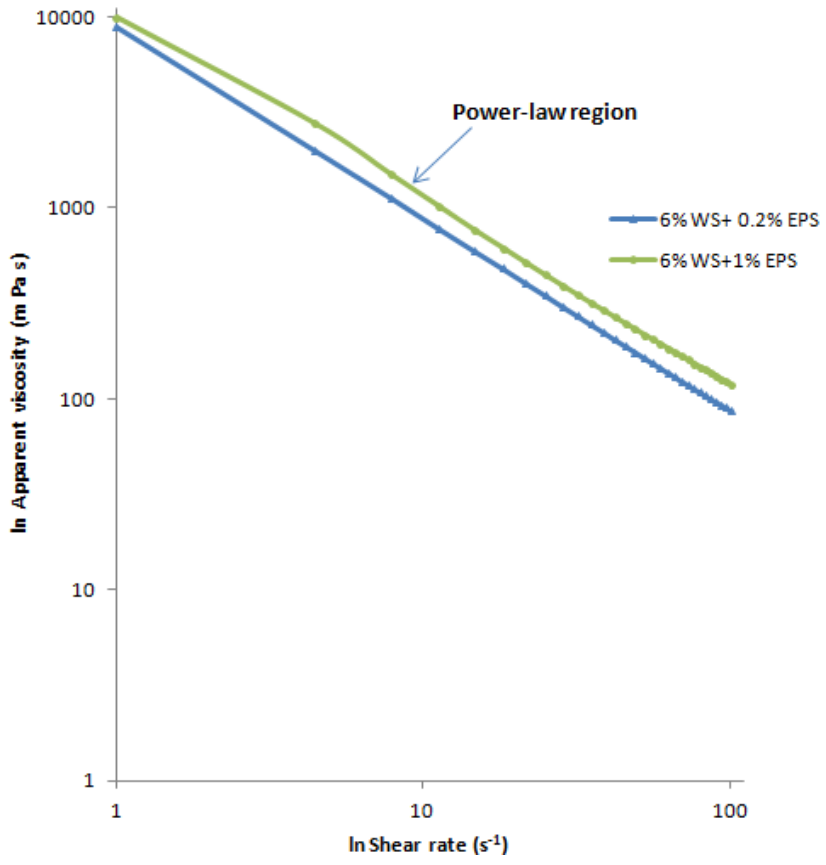


Fig. 9. 8. Pseudoplastic wheat starch-EPS dispersions obeying power-law

9.4. Conclusion

The study revealed that the incorporation of exopolysaccharides in starchy foods can increase the viscosity of the food and thereby improve the appearance and texture properties

of the food. The dispersions exhibited a non-Newtonian and pseudo-plastic behaviour. Phase separation was observed in the dispersions when higher concentrations of *L. plantarum* EPS was used and a shear-thinning behaviour was eminent as the apparent viscosity showed an increase at lower shear rate and decrease at higher shear rate. The impact of exopolysaccharide in the prevention of syneresis in starch throws light into its use as an ingredient in starch-containing foods for better texture and mouth-feel. The improvement of viscosity of the final product and prevention of syneresis makes the *Lactobacillus plantarum* EPS an effective substitute of CMC as viscosifier in foods.

CHAPTER 10
SUMMARY AND CONCLUSION

CHAPTER 10

SUMMARY AND CONCLUSION

The main reason behind the limited use of microbial exopolysaccharides in industry is associated with its economically feasible production. Demand for natural polymers for various industrial applications has led to a vibrant interest in exopolysaccharide (EPS) production by microorganisms. Bacterial exopolysaccharides cover a broad range of complex chemical structures and consequently different properties. The modulation of biochemical properties of exopolysaccharide require a thorough understanding of its biosynthetic pathway and the relation between the structure of EPS and the functional effect provided by them on incorporation into the food matrix. In particular LAB have QPS (qualified presumption of safety) status and the EPS produced by these bacteria can be considered as food grade-additives. The thesis mainly discussed the isolation and identification of a probiotic *Lactobacillus plantarum*, fermentative production of exopolysaccharide by the strain, its purification, structural characterisation and possible applications in food industry and therapeutics.

The studies on the probiotic characterization explored the tolerance of the isolated LAB cultures to acid, bile, phenol, salt and mucin binding. These are some of the key factors that could satisfy the criteria for probiotic strains. LAB exhibited Gram positive spectrum antibiotic resistance or sensitivity and produced antimicrobial substances which inhibited the growth of potential human pathogens. Based on the overall characteristics of the cultures, one of the isolates, MC1 identified to be *Lactobacillus plantarum* MTCC 9510 with promising EPS production and prominent probiotic features was selected for the present studies. Even though a lower hydrophobic interaction was expressed by the strain with mucin, it could serve as a better probiotic due to its EPS production as hydrophobic interaction is not that necessary for probiosis.

The important factors required for a high EPS production in submerged fermentation was investigated with a collection of statistical and mathematical approach. A highly significant quadratic polynomial obtained by the Box-Behnken model gave infinite number of combinations of the two factors, selected based on the single parameter optimization, keeping the other factor constant. A production titre of 1.21 g/l was attained in a medium combination of yeast extract 40 g/l, lactose 40 g/l and ammonium sulphate 5.5 g/l. From the experiments, the model was found to be significant with yeast extract and lactose as the most significant factors influencing EPS production. The production attained by *L. plantarum* is very promising on comparison to other strains mentioned in literature with an additional impact of the strain being a probiotic. This point focuses the importance that the explored lactic acid bacteria can favour the industry with a high EPS production and as a starter culture.

Polysaccharides possess the greatest potential for structural variability. A deeper understanding of the diversity of carbohydrates can help in the understanding of the applications of the compound. Chapter 5 of the thesis explains the structural elucidation of EPS employing spectroscopic and chromatographic techniques. The studies helped in the exploration of the hetero-polysaccharide sequence from *L. plantarum* MTCC 9510. The sequence of the EPS produced is comprised of - α-D-glucose- α-D-mannose- β-D-glucose- trisaccharide repeats. The molecular weight studies showed that the EPS is of high molecular weight. Techniques like Thermo Gravimetric Analysis (TGA), Fourier Transform Infra-red Spectroscopy (FT-IR), Gas Chromatography (GC), mass spectrometry, one dimensional NMR and two-dimensional NMR techniques like Correlation Spectroscopy (COSY), Nuclear Over-hauser Effect Spectroscopy (NOESY), Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Quantum Coherence (HMQC) were employed in the structure prediction.

As encapsulation technology can be used for many applications in the food industry (including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release of products), masking flavours, colours or odours, extending the shelf life and protecting components against nutritional loss) the method was exploited in the submerged fermentation studies. Calcium alginate, the matrix used for encapsulation was found to be effective with minimum leakage of cells and maximum release of EPS without interfering in its production and release. This enlightens the synbiotic (probiotic/probiotic combination) aspect of the encapsulation technology. A production titre of EPS (0.9 ± 0.09g/l) was attained by the encapsulated cells in a combination sodium alginate 2 %, calcium chloride 0.5 M and curing time 3 h with minimum cell leakage. The comparison between free and encapsulated cells under the same conditions bestowed upon the encapsulation technique with encapsulated cells performance. The reusability of the encapsulated cells was alsofound to be satisfactory.

The exploration of the genomic DNA of *L. plantarum* for EPS biosynthesis gene such as priming glycosyl transferase and plasmid DNA of *L. lactis* for priming glycosyl transferase and epsA revealed the homology and difference in the particular gene sequences of lactic acid bacteria. The studies unveiled a biosynthetic gene and hydrolase from the plasmid DNA and a transposase and hypothetical protein from the genomic DNA. The exposition of transposase could be useful as transposase genes have been identified inside EPS gene cluster and in the vicinity of the gene cluster of many lactic acid bacteria. The hypothetical gene in the MFS region could also play an efficient role in the EPS biosynthesis. The functionality of these genes has to be confirmed further before going in to the expression of the particular gene in *L. plantarum*. At the same time the amplification of genes other than priming glycosyl transferase with specific primers designed from already reported priming glycosyl transferases point to the homology difference in the genes within the same genus. The

homology difference can aid in the production of structurally different EPS of specific physico-chemical properties.

The thesis also explored the bioactivities of EPS from *L. plantarum*. As majority of chemical compounds identified as anti-cancerous are toxic to normal cells, the discovery and identification of new safe drugs has become an important goal of research in the biomedical sciences. The thesis has explored the anti-oxidant, anti-tumour and immunomodulating properties of EPS purified from *Lactobacillus plantarum*. The presence of (1, 3) linkages and its molecular weight presented the EPS with anti-oxidant, anti-tumour and immunomodulating properties under *in vitro* conditions. The immunostimulation property of the compound in human lymphocytes was seen as satisfactory at a concentration of 0.1 mg/ml with 20 % proliferation rate. The anti-tumour studies on breast adenocarcinoma cell line (MCF-7) exhibited a higher IC_{50} for the compound. The anti-tumour properties were studied by morphological apoptosis determination and MTT assay. The relatively higher IC_{50} of the compound could be explored in future as the chemical modification of the polysaccharide could bring down the IC_{50} for the development of a potent anti-cancer drug which shows non- cytotoxicity and the most preferred water soluble property.

The functional aspect of EPS from *L. plantarum* in food industry was studied in detail by investigating its role in the prevention of syneresis in starchy food stored in cold temperature. The experiments showed that the incorporation of exopolysaccharides at a particular concentration ranging from 0.1–1 % in starchy foods can increase the viscosity of the food and hence improving the appearance and texture properties of the food. The incorporation of EPS at a concentration of 1 % could reduce syneresis by 50 % and increase viscosity by 28 %. The performance was compared with CMC, a viscosifier currently in use in food industry. The impact of exopolysaccharide in the prevention of syneresis in starch throws light into its use as an ingredient in starch-containing foods for better texture and

mouth-feel. The improvement of viscosity of the final product and prevention of syneresis makes the *Lactobacillus plantarum* EPS an effective substitute of CMC as viscosifier in foods.

The successful probiotic features of the culture *Lactobacillus plantarum* MTCC 9510 with its promising role in the prevention of syneresis and improvement of viscosity along with anti-cancer property makes the strain a very efficient candidate for the probiotic industry. Even though relatively low hydrophobic interaction with the host epithelial cells was shown by the strain, the beneficial effects can be exerted on the host by the metabolite, exopolysaccharide thereby rendering the direct role of the bacteria not that necessary for probiosis. The encapsulation technology could intensify the probiotic performance of the strain with increased viability and release of exopolysaccharide, presenting the emergence of a potential symbiotic. The exopolysaccharide titre obtained from the bioprocess using the particular strain showed favourable experimental conditions. The physical and chemical characterization of EPS was enabled with the data obtained from various spectroscopic and chromatographic techniques. For molecular weight determination size exclusion chromatography was suitable as EPS possess zero charge. Due to the particular polydisperse nature of EPS an apparent average molecular weight was determined. NMR spectroscopy, an established technique for structure elucidation, was preferred as it unveils the details of glycosidic linkages and monomers of the EPS. At the same time, the particular structure of the exopolysaccharide, with α (1, 3) and β (1, 3) linkages, produced by *L. plantarum* doesn't eliminate its role as a prebiotic in the probiotic industry. It could be a promising food ingredient in dairy products and the suitable chemical modifications can furnish the exopolysaccharide with enhanced anti-cancer property.

BIBLIOGRAPHY

Adapa S & Schmidt K (1998) Physical properties of low-fat sour cream containing exopolysaccharide-producing lactic acid bacteria. *Journal of Food Science* **63**, 901–903.

Adnan AFM & Tan IKP (2007) Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. *Bioresource Technology* **98**, 1380–1385.

Ahmad N & Muhammadi (2007) Characterization of exopolysaccharide produced by *Bacillus* strain CMG1447. *Journal of chemical society of pakistan* **29**, 346-351.

Ahmed N, Soda ME, Hassan A & Frank J (2005) Improving the textural properties of an acid-coagulated Karish cheese using exopolysaccharide producing cultures. Lebensmittel- Wissenschaft und- Technologie *Food Science and Technology* **38,** 843-847.

Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T & Callanan M (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacilus acidophilus* NCFM. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 3906–3912.

Anderson D & McKay L (1983) Simple and rapid method for isolating large plasmid DNA from lactic Streptococci. *Applied and Environmental Microbiology* **46**, 549-552.

Andersson H, Asp NG, Bruce A, Roos S, Wadstrom T & Wold AE (2001) Health effects of probiotics and prebiotics. A literature review on human studies. *Scandinavian Journal of Nutrition* **45**, 58 – 75.

Angyal SJ (1992) The composition of reducing sugars in solution: Current aspects. *Advances in Carbohydrate Chemistry and Biochemistry* **49**, 19–35.

Annuk H, Hynes SO, Hirmo S, Mikelsaar M & Wadstrom T (2001) Characterization and Differentiation of lactobacilli by lectin typing *Journal of Medical Microbiology* **50**, 1069-1074.

Appelqvist IAM & Debet MRM (1997) Starch-biopolymer interactions- A review. *Food Review International* **13**, 163-224.

Aslim B, Ksekdag ZNY, Beyatli Y & Mercan N (2005) Exopolysaccharide production by *Lactobacillus delbruckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains under different growth conditions. *World Journal of Microbiology and Biotechnology* **21**, 673–677.

Barker SA, Bourne EJ, Stacey M & Whiffen DH (1954) Infrared spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society* **75**, 171–176.

Barker SB & Summerson WH (1941) The colorimetric determination of lactic acid in biological material. *Journal of Biological Chemistry* **138**, 535–554.

Batchelor RA, Araguchi GE, Hull RA & Hull S (1991) Regulation by a novel protein of the bimodal distribution of lipopolysaccharide in the outer membrane of *E. coIi*. *Journal of Bacteriology* **173**, 5699-5704.

Bergmaier D, Lacroix C & Champagne CP (2003) Exopolysaccharide production during batch cultures with free and immobilized *Lactobacillus rhamnosus* RW-9595M. *Journal of Applied Microbiology* **95**, 1049-1057.

Bidlack W (1998) New technologies for healthy foods and nutraceuticals. *Journal of the American College of Nutrition* **17**, 296–297.

Bodera P & Chcialowski A (2009) Immunomodulatory effect of probiotic bacteria. *Recent Patents on Inflammation and Allergy Drug Discovery* **3**, 58-64.

Bouzar F, Cerning J & Desmazeaud M (1996) Exopolysaccharide production in milk by *Lactobacillus delbrueckii* ssp *bulgaricus* CNRZ 1187 and by two colonial variants. *Journal of Dairy Science* **79**, 205-211.

Boyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation. Supplement* **21**, 77-89.

Boza Y, Neto LP, Costa FAA & Scamparini ARP (2004) Exopolysaccharide production by encapsulated *Beijerinckia* cultures. *Process Biochemistry* **39**, 1201-1209.

Braissant O, Decho AW, Przekop KM, Gallagher KL, Glunk C, Dupraz C & Visscher PT (2009) Characteristics and turnover of exopolymeric substances in a hypersaline microbial mat. *FEMS Microbiology Ecology* **67**, 293–307.

Briczinski EP & Roberts RF (2002) Production of an exopolysaccharide-containing whey protein concentrate by fermentation of whey. *Journal of Dairy Science* **85**, 3189–3197.

Broadbent JR, McMahon DJ, Welker DL, Oberg CJ & Moineau S (2003) Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: A review. *Journal of Dairy Science* **86**, 407-423.

Brown GD & Gordon S (2001) Immune recognition. A new receptor for beta-glucans. *Nature* **413**, 36-37.

Bubb WA (2003) NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts in Magnetic Resonance Part A* **19A**, 1-19.

Çakır İ (2003) Determination of some probiotic properties on Lactobacilli and Bifidobacteria. *Ankara University, Doctoral Thesis*.

Canquil N, Villarroel M, Bravo S, Rubilar M & Shene C (2007) Behavior of the rheological parameters of exopolysaccharides synthesized by three lactic acid bacteria. *Carbohydrate Polymers* **68**, 270–279.

Capela P, CHay TK & P SN (2006) Effect of cryoproyectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-driedyoghurt. *Food Research International* **39**, 203-211.

Černá M, Barros AS, Nunes A, Rocha SM, Delgadillo I, Čopíková J & Coimbra MA (2003) Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. *Carbohydate Polymers* **51**, 383–389.

Cerning J & Marshall VM (1999) Exopolysaccharides produced by the dairy lactic acid bacteria. *Recent Research developments in Microbiology* **3**, 195-209.

Cerning J, Renard C, Thibault JF, Bouillanne C, Landon M, Desmazeaud M & Topisirovic L (1994) Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure-analysis of the polymer. *Applied and Environmental Microbiology* **60**, 3914-3919.

Chabot S, Yu H-L, Léséleuc LD, Cloutier D, Clasteren M-RV, Lessard M, Roy D, Lacroix M & Oth D (2001) Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6, IL-12 in human and mouse cultured immunocomponent cells, and IFN-γ in mouse splenocytes. *Le Lait* **81**, 683-697.

Champagne CP, Gardner NJ, Lacroix C & (2007) Fermentation technologies for the production of exopolysaccharide synthesizing *Lactobacillus rhamnosus* concentrated cultures. *Electronic Journal of Biotechnology* **10** ISSN: 0717-3458

Champomier-Vergès M-C, Manguin E, Mistou M-I, Anglade P & Chich J-F (2002) Lactic acid bacteria and proteomics: current knowledge and perspectives. *Journal of Chromatography B* **771**, 329-342.

Chaubey M & Kapoor VP (2001) Structure of a galactomannan from the seeds of Cassia angustifolia Vahl. *Carbohydrate Research* **332**, 439–444.

Chi Z, Su CD & Lu WD (2007) A new exopolysaccharide produced by marine Cyanothece sp. 113. *Bioresource Technology* **98**, 1329–1332.

Chung YC, Chang CT, Chao WW, Lin CF & Chou ST (2002) Antioxidative activity and safety of the 50 % ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *Journal of Agricultural and Food Chemistry* **50**, 2454-2458.

Claesson M, Li Y, Leahy S, Pijkeren JCC, Cerdeño-Tárraga A, Parkhill J, Flynn S, O'Sullivan G, Collins JK, Higgins D, Shanahan F, Fitzgerald G, Sinderen Dv & Toole P (2006) Multireplicon genome architecture of *Lactobacillus salivarius*. *Proceedings of the National Academy of Sciences* **10317**, 6718-6723.

Cleary A, Kelly G & Husband A (1999) The effect of molecular weight and beta 1,6 linkages on priming of macrophage function in mice by (1,3)- beta-D-glucan. *Immunology and Cell Biology* **77**, 395-403

Corcoran BM, Stanton C, Fitzgerald GF & Ross RP (2005) Survival of probiotic Lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology* **71**, 3060-3067.

Creighton CJ, Massarweh S, Huang S, ATsimelzon, Hilsenbeck SG, Osborne CK, Shou J, Malorni L & Schiff R (2008) Development of resistance to targeted therapies transforms the clinically associated molecular profile subtype of breast tumor xenografts. *Cancer Research* **68**, 7493-7501.

Dabour N & LaPointe G (2005) Identification and molecular characterization of the chromosomal exopolysaccharide biosynthesis gene cluster from *Lactococcus lactis* subsp. *cremoris* SMQ-461. *Applied and Environmental Microbiology* **71**, 7414–7425.

Dag S (2005) Structural studies of some bacterial lipopolysaccharides and extracellular polysaccharides using nmr spectroscopy and mass spectrometry. *Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala*.

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 and Biochemistry* **73**, 2656-2664.

DeFelice SL (1992) The nutraceutical initiative: a recommendation for U.S. Economic and regulatory reforms. *Genetic Engineering News* **12**, 13-15.

Degeest B, Vaningelgem F & Vuyst LD (2001) Microbial physiology, fermentation kinetics, and process engineering of heteropolysaccharide production by lactic acid bacteria. *International Dairy Journal* **11**, 747-757.

Dekker J, Rossen JW, Buller HA & Einerhand AW (2002) The MUC family: an obituary. *Trends in Biochemical Sciences* **27**, 126–131.

Delcour J, Ferain T, Deghorain M, Palumbo E & Hols P (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria *Antonie van Leeuwenhoek* **76**, 159-184.

de Roos NM & Katan MB (2000) Effects of probiotic bacteria on diarrhoea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *American Journal of Clinical Nutrition* **71**, 405–411.

De Smet I, Hoorde LV, Saeyer ND, Woestyne MV & Verstraete W (1994) In vitro study of bile salt hydrolase BSH activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. *Microbial Ecology in Health and Disease* **7**, 315-329.

De Vuyst L, DeVin F, Vaningelgem F & Degeest B (2001) Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *International Dairy Journal* **11**, 687-707.

Ding WK & Shah NP (2009) Effect of various encapsulating materials on the stability of probiotic bacteria. *Journal of Food Science* **74**, M100–M107.

Du X-j, Zhang J-s, Yang Y, Tang Q-j, Jia W & Pan Y-j (2010) Purification, chemical modification and immunostimulating activity of polysaccharides from Tremella aurantialba fruit bodies. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)* **11**, 437-442.

Duboc P & Mollet B (2001) Applications of exopolysaccharides in the dairy industry. *International Dairy Journal* **11**, 759-768.

Dubois M, Gilles K, Hamilton J, Rebers P & Smith F (1956) Colorimetric methods for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F & Collins JK (2001) In-vitro selection criteria for probiotic bacteria of human origin: correlation with in-vivo findings. *American Journal of clinical nutrition* **73**, 386S-392S.

Dupont I, Roy D & Lapointe G (2000) Comparison of exopolysaccharide production by strains of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* grown in chemically defined medium and milk. *Journal of Industrial Microbiology and Biotechnology* **24**, 251–255.

Durlu-Özkaya F, Aslim B & Ozkaya M (2007) Effect of exopolysacaccharides EPSs produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* strains to bacteriophage and nisin sensitivity of the bacteria. *LWT* **40**, 564–568.

Edina CN (2007) Investigation of the efficiency and mode of action of lactic acid bacteria suitable for probiotic food production. *Doctoral Thesis*.

Erkkilä S & Petäjä E (2000) Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Science* **55**, 297–300.

Faber EJ, Kamerling JP & Vliegenthart JFG (2001) Structure of the extracellular polysaccharide produced by *Lactobacillus delbrueckii* subsp *bulgaricus* 291. *Carbohydrate Research* **331**, 183-194.

Filippov MP (1992) Practical infrared spectroscopy of pectic substances. *Food Hydrocolloids* **6**, 115–142.

Fogli-Cawley J, Dwyer J, Saltzman E, McCullough M, Troy L, Meigs J & Jacques P (2007) The 2005 dietary guidelines for Americans and risk of the metabolic syndrome. *Journal of Clinical Nutrition* **86**, 1193–1201.

Fuller R (1989) A review: Probiotics in man and animals. *Journal of Applied Bacteriology* **66**, 365–378.

Garai-Ibabe G, Aeizaga J, Aznar R, Elizaquivel P, Prieto A, Irastorza A & Dueñas MT (2010) Screening and selection of 2-branched (1,3)-β-D-glucan producing lactic acid bacteria and exopolysaccharide characterization. *Journal of Agricultural and Food Chemistry* **58**, 6149-6156.

Garbayo I, Vilchez C, Vega JM, Nava-Saucedo JE & Barbotin JN (2004) Influence of immobilization parameters on growth and lactic acid production by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* co-immobilized in calcium alginate gel beads. *Biotechnology Letters* **26**, 1825–1827.

Germond J-E, Delley M, Dámico N & Vincent S (2001) Heterologuous expression and characterization of the exopolysaccharide from *Streptococcus thermophilus* sfi39. *European Journal of Biochemistry* **268**, 5149-5156.

Gilliland S, Nelson C & Maxwell C (1985) Assimilation of cholesterol by *Lacobacillus acidophilus*. *Applied and Environmental Microbiology* **492**, 377–381.

Gilliland SE & Walker DK (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science* **73**, 905–911.

Gimeno E, Moraru CI, Kokini JL & (2004) Effect of xanthan gum and CMC on the structure and texture of corn flour pellets expanded by microwave heating. *Cereal Chemistry* **81**, 100–107.

Giraud E, Brauman A, Keleke S, Leong B & Raimbault M (1991) Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology* **36**, 379–383.

Goh K (2004) Isolation and characterisation of bacterial exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 and *Sphingomonas elodea* ATCC 31461. *Doctoral Thesis*.

Goktepe I, Juneja VK & Ahmedna M (2006) Probiotics in food safety and human health. *Boca Raton, FL: Taylor and Francis group*.

Gollahon L, Jeong Y, Finckbone V, Lee K & Park J-S (2011) The natural product NI-07, is effective against breast cancer cells while showing no cytotoxicity to normal cells. *The Open Breast Cancer Journal* **3** 31-44.

Gorin P (1981) Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. In: R. Tipson, & D. Horton (Eds). *Advance in carbohydrate chemistry and biochemistry, New York: Academic Press*, 13–104.

Gorret N, Maubois JL, Engasser JM, Ghoul M & (2001) Study of the effects of temperature, pH and yeast extract on growth and exopolysaccharide production by

Propionibacterium acidi-propionici on milk microfiltrate using a response surface methodology. *Journal of Applied Microbiology* **90**, 788–796.

Hallack LF, Passos DS, Mattos KA, Agrellos OA, Jones C, Mendonça-Previato L, Previato JO & Todeschini AR (2010) Structural elucidation of the repeat unit in highly branched acidic exopolysaccharides produced by nitrogen fixing Burkholderia. *Glycobiology Advance* **20,** 338-347

Hannoun B, Stephanopoulos G & (1986) Diffusion coefficients of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes. *Biotechnology and Bioengineering* **28**, 829–835.

Hassan AN, Ipsen R, TJanzen & BQvist K (2003) Microstructure and rheology of yogurt made with cultures differing only in their ability to produce exopolysaccharides. *Journal of Dairy Science* **86**, 1632–1638.

Holt JH, Krieg NR, Sneath PHA, Staley JT & Williams ST (1994) Group 17: gram-positive cocci. *In W. R. Hensyl (Ed.) Bergey's Manual of Determinative Bacteriology (9th ed.). Baltimore, MD, USA: Williams & Wilkins.,* 527-558.

Holzapfel WH & Schillinger U (2002) Introduction to pre- and probiotics *Food Research International* **35**, 109 -116.

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J & Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of clinical nutrition* **73**, 365S–373S.

Hou RC, Lin MY, MWang M & Tzen JT (2003) Increase of viability of entrapped cells of *Lactobacillus delbrueckii* spp. *Bulgaricus* in artificial sesame oil emulsions. *Journal of Dairy Science* **86**, 424-428.

Huang J & Adams MC (2004) In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology* **91**, 253-260.

Ismail B & Nampoothiri KM (2010) Production, purification and structural characterization of an exopolysaccharide produced by a probiotic *Lactobacillus plantarum* MTCC 9510. *Archives of Microbiology* **192**, 1049–1057.

Johns MR & Noor E (1991) Recovery and purification of polysaccharides from broth. *Australian Journal of Biotechnology* **5**, 73–77.

Jolly L & Stingele F (2001) Molecular organization and functionality of exopolysaccharide gene clusters from lactic acid bacteria. *International Dairy Journal* **11**, 733-745.

Jolly L, Newell J, Porcelli I, Vincent S & Stingele F (2002) *Lactobacillus helveticus* glycosyl trasnferases: from genes to carbohydrate synthesis. *Glycobiology* **125**, 319-327.

Jonsson H, Strom F & Roos S (2001) Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* in vitro. *FEMS Microbiology Letters* **204**, 19–22.

Kailasapathy K & Chin JC (2000) Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacerium* spp. *Immunology and Cell Biology* **78**, 80–88.

Kailasapathy K (2006) Survival of free and encapsulated probioticbacteria and their effect on the sensoryproperties of yoghurt. *LWT - Food Science and Technology* **39**, 1221-1227.

Kato I, Endo K & Yokokura T (1994) Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice. *International Journal of Immunopharmacology* **16**, 29–36.

Kenji F, Tala S, Kentaro N, Fiame L, Tadashi N, Kumi Y, Akitsugu S, Hidemasa M & Tadasu U (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.

Khurana HK (2006) Development of technology for extended shelf life fruit lassi. *Ph.D. Dissertation; NDRI Deemed University, Karnal Haryana – India.*

Kiely LJ & Olson NF (2000) The physicochemical surface characteristics of *Lactobacillus casei*. *Food Microbiology* **17**, 277–291.

Kim DW, Cho SB, Lee HJ, Chung WT, Kim KH & Hwangbo J (2007) Comparison of cytokine and nitric oxide induction in murine macrophages between whole cell and enzymatically digested Bifidobacterium sp. obtained frommonogastric animals. *Journal of Microbiology* **45**, 305–310.

Kim JU, Kim Y, Han KS, Oh S, Whang KY, Kim JN & Kim SH (2006) Function of cell-bound and released exopolysaccharides produced by *Lactobacillus rhamnosus* ATCC 9595. *Journal of Microbiology and Biotechnology* **16**, 939-945.

Kim Y, Oh S, Yun HS, Oh S & Kim S (2010) Cell-bound exopolysaccharide from probiotic bacteria induces autophagic cell death of tumour cells. *Letters in Applied Microbiology* **51** 123-130.

Kinsella J (1988) Food lipids and fatty acids: importance in food quality, nutrition and health. *Food Technology* **42**, 124-142.

Kitazawa H, Itoh T, Tomioka Y, Mizugaki M & Yamaguchi T (1996) Induction of IFNγ and IL-1α production in macrophages stimulated with phosphopolysaccharide produced by *Lactococcus lactis* ssp. *cremoris*. *International Journal of Food Microbiology* **31**, 99-106.

Kitazawa H, Yamaguchi T, Fujimoto Y & Itoh T (1993) An analysis of mitogenic response of phosphopolysaccharide, a B-cell mitogen produced by *Lactococcus lactis* ssp. *cremoris* to spleen cells. *Animal Science and Technology* **64**, 807-812.

Klein G, Hallmann C, Casas LA, Abad J, Louwers J & Reuter G (2000) Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. *Journal of Applied Microbiology* **89**, 815–824.

Klein G, Pack A, Bonaparte C & Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* **41**, 103– 105.

Kong FL, Zhang MW, Kuang RB, Yu SJ, Chi JW & Wei ZC (2010) Antioxidant activities of different fractions of polysaccharide purified from pulp tissue of litchi (Litchi chinensis Sonn.). *Carbohydrate Polymers* **81**, 612-616.

Kotilainen L, Rajalahti R, Ragasa C & Pehu E (2006) Health enhancing foods: opportunities for strengthening the sector in developing countries. *Agriculture and Rural Development Discussion Paper 30*.

Kreisman LSC, Friedman JH, Neaga A & Cobb BA (2007) Structure and function relations with a T-cell-activating polysaccharide antigen using circular dichroism. *Glycobiology* **17**, 46–55

Kumar AS & Mody K (2009) Microbial exopolysaccharides: Variety and potential applications. *In Microbial production of biopolymers and polymer precursors – Applications and perspectives. Caister Academic Press*.

Kunji ERS, Slotboom D-J & Poolman B (2003) *Lactococcus lactis* as host for overproduction of functional membrane proteins. *Biochimica et Biophysica Acta* **1610**, 97- 108.

Lambo-Fodge AM, Öste R & Nyman MEGL (2006) Short chain fatty acid formation in the hindgut of rats fed native and fermented oat fibre concentrates. *Brazilian Journal of Nutrition* **96**, 47-55.

Lamothe GT, Jolly L, Mollet B & Stingele F (2002) Genetic and biochemical characterization of exopolysaccharide biosynthesis by *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Archives of Microbiology* **178**, 218-228.

Landersjö C, Yang Z, Huttunen E & Widmalm G (2002) Structural studies of the exopolysaccharide produced by *Lactobacillus rhamnosus* strain GG (ATCC 53103). *Biomacromolecules* **3**, 880-884.

Lee JW, Shin JG, Kim EH, Kang HE, Yim IB, Kim JY, Joo HG & Woo HJ (2004) Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of *Lactobacillus casei* and *Bifidobacterium longum*. *Journal of Veterinary Science* **5**, 41–48.

Lee K-M & Gilmore DF (2005) Formulation and process modeling of biopolymer polyhydroxyalkanoates, PHAs production from industrial wastes by novel crossed experimental design. *Process Biochemistry* **40**, 229-246.

Lee K-Y & Heo T-R (2000) Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. *Applied and Environmental Microbiology* **66**, 869-873.

Leung MYK, Liu C, Koon JCM & Fung KP (2006) Polysaccharide biological response modifiers. *Immunology Letters* **105**, 101–114.

Lin E-S & Chen Y-H (2007) Factors affecting mycelial biomass and exopolysaccharide production in submerged cultivation of *Antrodia cinnamomea* using complex media. *Bioresource Technology* **98**, 2511–2517.

Lin TY & Chien MFC (2007) Exopolysaccharide production as affected by lactic acid bacteria and fermentation time. *Food Chemistry* **100**, 1419-1423.

Liu C-F, Tseng K-C, Chiang S-S, Lee B-H, Hsu W-H & Pan T-M (2011) Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *Journal of the Science Food and Agriculture* **91**, 2284-2291

Looijesteijn PJ & Hugenholtz J (1999) Uncoupling of growth and exopolysaccharide production by *Lactobacillus lactis* subsp. *cremoris* NIZO B40 and optimization of its synthesis. *Journal of Bioscience and Bioengineering* **88**, 78–182.

Looijesteijn PJ, Trapet L, Vries Ed, Abee T & Hugenholtz J (2001) Physiological function of exopolysaccharides produced by *Lactococcus lactis*. *International Journal of Food Microbiology* **64**, 71-80.

Loosdrecht AAvd, Beelen RH, Ossenkoppele GJ, Broekhoven MG & Langenhuijsen MM (1994) Aetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of Immunological Methods* **14**, 311-320.

Lourens-Hattingh A & Viljoen BC (2001) Yogurt as probiotics carrier food. *International Dairy Journal* **11**, 1–17.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* **193**, 265–275.

Lu WM, Chen WC & (1988) Production of L-glutamate using entrapped living cells of Brevibacterium ammoniagenes with calcium alginate gels. *Proceedings of the National Science Council, Taipei, Taiwan* **6**, 400 – 406.

Macedo MG, Lacroix C, Gardner NJ & PChampagne C (2002) Effect of medium supplementation on exopolysaccharide production by *Lactobacillus rhamnosus* RW-9595 M in whey permeate. *International Dairy Journal* **12**, 419–426.

Maeda H, Zhu X, Omura K, Suzuki S & Kitamura S (2004a) Effects of an exopolysaccharide kefiran on lipids, blood pressure, blood glucose, and constipation. *Biofactors* **22**, 197-200.

Maeda H, Zhu X, Suzuki S, Suzuki K, Kitamura S & (2004b) Structural characterization and biological activities of an exopolysaccharide kefiran produced by *Lactobacillus kefiranofaciens* WT-2BT. *Journal of Agriculture and Food Chemistry* **52**, 5533-5538.

Majumdar I, D'Souza F, Bhosle NB & (1999) Microbial exopolysaccharides, effect on corrosion and partial chemical characterization. *Journal of Indian Institute of Sciences* **79**, 539-550.

Mathlouthi M, Koenig J & (1986) Vibrational spectra of carbohydrate. *Advance in Carbohydrate Chemistry and Biochemistry* **44**, 7–89.

Meulen RVd, Grosu-Tudor S, Mozzi F, Vaningelgem F, Zamfir M, Valdez GFd & Vuyst LD (2007) Screening of lactic acid bacteria isolates from dairy and cereal products for exopolysaccharide production and genes involved. *International Journal of Food Microbiology* **118,** 250–258.

Miller G (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**, 426–429

Morelli L (2000) In vitro selection of probiotic Lactobacilli: a critical appraisal. *Current issues in intestinal microbiology* **1**, 59-67.

Morris VJ (1995) Bacterial polysaccharides, in: A. L. Stephens (Ed.). *Food Polysaccharides and their Application, Marcel Dekker, New York*, 341–371.

Mozzi F, Vaningelgem F, Hébert EM, Meulen RVd, Moreno MRF, Valdez GFd & Vuyst LD (2006) Diversity of heteropolysaccharides-producing lactic acid bacterium strains and their polymers. *Applied and Environmental Microbiology* **72**, 4431-4435.

Mueller A, Raptis J & Rice P (2000) The influence of glucan polymer structure and solution conformation on binding to (1->3)-beta-D-glucan receptors in a human monocyte-like cell line. *Glycobiology* **10**, 339-346.

Murthy M, Kumar V, R Nagendra & Khem S (2000) Delineation of beneficial characteristics of effective probiotics. *The Journal of the American Nutraceutical Association* **3**, ISSN 1521-4524.

Muthukumarasamy P & Holley RA (2007) Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing micro-encapsulated probiotic lactic acid bacteria. *Food Microbiology* **24**, 82–88.

Nakajima H, Suzuki Y & Hirota T (1992) Cholesterol lowering activity of ropy fermented Milk. *Journal of Food Science* **576**, 1327-1329.

Nampoothiri KM, Pandey A & (1998) Immobilization of *Brevibacterium* cells for the production of L-glutamic acid. *Bioresource Technology* **63**, 101–106.

Nasin M, Dhouib A, Zorguani F, Kriaa H & Ellouz R (1989) Production of Lysine by using immobilized living *Corynebacterium* spp. cells. *Biotechnology Letters* **11**, 865–870.

Niness KR (1999) Nutritional and health benefits of inulin and oligofructose. *Journal of Nutrition* **129**, 1402S-1406S.

Otero MC, Ocana VS & Macias ENM (2004) Bacterial surface characteristics applied to selection of probiotic microorganisms. *Methods in Molecular Biology* **268**, 435–440.

Ouwehand A, Salminen S & Isolauri E (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek* **82**, 279-289.

Oyaizu M (1986) Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition* **44**, 307-315.

Özer B, Kirmaci HA, Senel E, Atamer M & Hayaloglu A (2009) Improving the viability of Bifidobacterium bifidum BB-12 and *Lactobacillus acidophilus* LA-5 in white brined cheese by microencapsulation. *International Dairy Journal* **19**, 22-29

Pancheniak ER & Soccol CR (2005) Biochemical characterization and identification of probiotic Lactobacillus for swine. *B.CEPPA Curitiba* **23**, 299–310.

Pao SS, Paulsen IT & Saier MH (1998) Major Facilitator Superfamily. *Microbiology and Molecular Biology Reviews* **62**, 1–34

Parvez S, Malik KA, Kang SA & Kim HY (2006) Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology* **100**, 1171-1185.

Péant B, LaPointe G, Gilbert C, Atlan D, Ward P & Roy D (2005) Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. *Microbiology* **151**, 1839-1851.

Pereira DIA & Gibson GR (2002) Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Critical Reviews in Biochemistry and Molecular Biology* **374**, 259-281.

Pham PL, 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. *Applied and Environmental Microbiology* **66**, 2302-2310.

Pongsawatmanit R & Srijunthongsiri S (2008) Influence of xanthan gum on rheological properties and freeze-thaw stability of tapioca starch. *Journal of Food Engineering* **88,** 137-143.

Prevost H & Divies C (1988) Continuous pre-fermentation of milk by entrapped yoghurt bacteria. I. Development of the process. *Milchwissenschaft* **43**, 621–625.

Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet A-C, Zwahlen M-C, Rouvet M, Altermann E & Barrangou R (2004) The genome sequence of the probiotic

intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2512-2517.

Purama RK & Goyal A (2005) Dextransucrase production by *Leuconostoc mesenteroides*. *Indian Journal of Microbiology* **2**, 89–101.

Rafter J (2004) The effects of probiotics on colon cancer development. *Nutrition Research Reviews* **17**, 277-284.

Ranganathan N, Friedman EA, Tam P, Rao V, Ranganathan P & Dheer R (2009) Probiotic dietary supplementation in patients with stage 3 and 4 chronic kidney disease: a 6-month pilot scale trial in Canada. *Current Medical Research and Opinion* **25**, 1919-1930.

Rayes N, Seehofer D, Theruvath T, Schiller RA, Langrehr JM, Jona S, Bengmark S & Neuhaus P (2005) Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation-a randomized, double-blind trial. *American J Transplantation* **5**, 125-130.

Reale M, Boscolo P, Bellante V, Tarantelli C, Nicola MD, Forcella L, Li Q, Morimoto K & Muraro R (2011) Daily intake of *Lactobacillus casei Shirota* increases natural killer cell activity in smokers. *British Journal of Nutrition*, doi: 10.1017/S0007114511005630.

Reddy GV, Shahani KM, Friend BA & Chandan RC (1983) Natural antibiotic activity of *L. acidophilus* and *bulgaricus*. III. production and partial purification of bulgarican from *Lactobacillus bulgaricus*. *Cultured Dairy Products Journal* **18**, 15-19.

Reid AA, Champagne CP, Gardner N, Fustier P & Vuillemard JC (2007) Survival in food systems of *Lactobacillus rhamnosus* R0011 microentrapped in whey protein gel particles. *Journal of Food Science* **72**, M31–M37.

Ribble D, Goldstein NB, Norris DA & Shellman YG (2005) A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnology* **5**, 12.

Rijnaarts HHM, Norde W, Bouwer EJ, Lyklema J & Zehnder AJB (1993) Bacterial adhesion under static and dynamic conditions. *Applied and Environmental Microbiology* **59**, 3255–3265.

Rokka S & Rantamäki P (2010) Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. *European Food Research and Technology* **231**, 1-12.

Roos S & Jonsson H (2002) A high-molecular-mass cellsurface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* **148**, 433–442.

Ruas-Madiedo P, Gueimonde M, Margolles A, Reyes-Gavilán CGDL & 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, Hugenholtz J & Zoon P (2002) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Journal* **12**, 163-171.

Sa´nchez J, Martı´nez B, Guille´n R, Jime´nez-Dı´az R & Rodrı´guez A (2006) Culture conditions determine the balance between two different exopolysaccharides produced by Lactobacillus pentosus LPS26. *Applied and Environmental Microbiology* **72**, 7495-7502.

Saarela M, Mogensen G, Fonden R, Matto J & Mattila-Sandholm T (2000) Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology* **84**, 197–215.

Sadar LN (2004) Rheological and textural characteristics of copolymerized hydrocolloidal solutions containing curdlan gum. *MSc Thesis; Faculty of the Graduate School of the University of Maryland, College Park, MD, USA*.

Salminen S (2001) Human studies on probiotics: aspects of scientific documentation. *Scandinavian Journal of Nutrition* **45**, 8-12.

Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Frank A, Gibson GR, Isolauri E, Moreau M-C, Roberfroid M & Rowland I (1998) Functional food science and gastrointestinal physiology and function. *Brazilian Journal of Nutrition* **80**, S147-S171.

Salminen S, Laine M, Wright A, Voupiovarikila JTV, Mattila-sandholm T & (1996) Delevelopment of selection criteria for probiotic strain to assess their potential in functional food. a nordic and european approach. *Bioscience and Microflora* **15**, 61–67.

Salminen S, Ouwehand A, Benno Y & Lee YK (1999) Probiotics: how should they be defined? *Trends in Food Science and Technology* **10**, 107-110.

Sambrook J, Fritsch F & Maniatis T (1989) *Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.*

Sánchez J, Martínez B, Guilleń R, Jimeńez-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.

Sánchez-Medina I, Gerwig GJ, Urshev ZL & Kamerlinga JP (2007) Structural determination of a neutral exopolysaccharide produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* LBB.B332. *Carbohydrate Research* **342**, 2735–2744.

Sandstedt RM & Abbott RC (1964) A comparison of methods for studying the course of starch gelatinization. *Cereal Science Today* **9**, 13-26.

Savadogo A, C A T Ouattara1, Savadogo PW, Barro N, Ouattara AS & Traoré AS (2004) Identification of exopolysaccharides-producing lactic acid bacteria from Burkina Faso fermented milk samples. *African Journal of Biotechnology* **3,** 189-194.

Säwén E, Huttunen E, Zhang X, Yang Z & Widmalm G (2010) Structural analysis of the exopolysaccharide produced by *Streptococcus thermophilus* ST1 solely by NMR spectroscopy. *Journal of Biomolecular NMR* **47**, 125-134.

Shah NP & Ravula RR (2000) Microencapsulation of probiotic bacteria and their survival in frozen fermented dairy desserts. *Australian Journal of Dairy Technology* **55**, 139-144.

Sharpe ME (1979) In F. A. Skinner, & D. W. Lovelock (Eds.) Identification methods for microbiologists *London: Academic*, 233–259.

Shene C & Bravon S (2007) Whey fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* for exopolysaccharide production in continuous culture. *Enzyme and Microbial Technology* **40**, 1578–1584.

Sheu TY, Marshall RT & Heymann H (1993) Improving survival of culture bacteria in frozen desserts by microentrapment. *Journal of Dairy Science* **76**, 1902-1907.

Shihata A & Shah NP (2002) Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria. *International Dairy Journal* **12**, 765–772.

Sikora M, Juszczak L, Sady M & Krawontka J (2003) Use of starch/xanthan gum combinations as thickeners of cocoa syrups. *Nahrung/Food* **47**, 106-113.

Stadlbauer V, Mookerjee RP, Hodges S, Wright GA, Davies NA & Jalan R (2008) Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis. *Journal of Hepatology* **48**, 945-951.

Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch PB & Ross RP (2001) Market potential for probiotics. *American Journal of clinical nutrition* **73**, 476S-483S.

Stingele F, Neeser J-R & Mollet BI (1996) Identification and characterization of the eps exopolysaccharide gene cluster from *Streptococcus thermophilus* Sfi6. *Journal of Bacteriology* **178**, 1680-1690.

Sun Z, Chen X, Wang J, Zhao W, Shao Y, Wu L, Zhou Z, Sun T, Wang L, Meng H, Zhang H & Chen W (2011) Complete genome sequence of *Streptococcus thermophilus* strain ND03. *Journal of Bacteriology* **193**, 793-794.

Surrenjav U, Zhang L, X J Xu , Zhang XF, Zeng FB & (2006) Effects of molecular structure on antitumor activities of (1 →3)- beta-D-glucans from different *Lentinus Edodes*. *Carbohydater Polymers* **63**, 97–104.

Sutherland IW (2002) A sticky business. Microbial exopolysaccharides: current products and future trends. *Microbiology Today* **29**, 70-71.

Tao YZ, Zhang LN, Cheung PCK & (2006) Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. *Carbohydrate Research* **341**, 2261–2269

Taylor PR, Brown GD, Reid DM, Willment JA, Martinez-Pomares L, Gordon S & Wong SY (2002) The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *Journal of Immunology* **169**, 3876-3882.

Thapa N, J Pal & Tamang JP (2004) Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India. *World Journal of Microbiology & Biotechnology* **20**, 599- 607.

Thu B, Bruheim P, Espevik T, Smidsrod O, Soon-Shiong P & Braek GS- (1996) Alginate polycation microcapsules. II. Some functional properties. *Biomaterials* **17**, 1069-1079.

Tieking M & Ganzle M (2005) Exopolysaccharides from cereal associated lactobacilli. *Trends in Food Science and Technology* **16,** 79–84.

Timmerman HM, Koning CJM, Mulder L, Rombouts FM & Beynen AC (2004) Monostrain, multistrain and multispecies probiotics – a comparison of functionality and efficacy. *International Journal of Food Microbiololgy* **96,** 219–233.

Tok E & Aslim B (2010) Cholesterol removal by some lactic acid bacteria that can be used as probiotic. *Microbiology and Immunology* **54**, 257-264.

Tsuda H & Miyamoto T (2010) Production of exopolysaccharide by *Lactobacillus plantarum* and the prebiotic activity of the exopolysaccharide. *Food Science and Technology Research* **16**, 87 – 92

Tsujisaka Y & Mitsuhashi M (1993) Pullulan in industrial gums: polysaccharides and their derivatives. *Whistler, R., BeMiller, J. N., Eds.; Academic: San Diego*, 447-460.

Tunier R (1999) An exocellular polysaccharide and its interactions with proteins. *Ph.D. Dissertation. Wageningen University, The Netherlands.*

Uchida M, Amakasu H, Satoh Y & Murata M (2004) Combinations of lactic acid bacteria and yeast suitablefor preparation of marine silage. *Fisheries Science* **70** 507–517.

Underhill DM (2003) Toll-like receptors: networking for success. *European Journal of Immunology* **33**, 1767-1775.

Usui T, Yamaoka N, Mastuda K, Tuzimura K, Sugiyama H, Seto S & (1973) 13C nuclear magnetic resonance spectra of glucobiose, glucotriose, and glucans. *Journal of the Chemical Society, Perkin Transactions* **1**, 2425–2432.

Vanhaverbeke C, Bosso C, Colin-Morel P, Gey C, Gamar-Nourani L, Blondeau K, Simonet J-M, Heyraud A & (1998) Structure of an extracellular polysaccharide produced by *Lactobacillus rhamnosus* strain C83. *Carbohydrate Research* **314**, 211-220.

VanKranenburg R, Marugg JD, Swam IIV, Willem NJ & Vos WMd (1997) Molecular characterisation of the plasmid-encoded eps gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. *Molecular Microbiology* **24**, 387–397.

Verma S (2009) An empirical analysis of consumer's attitude towards OTC health supplements in India. *International Journal of Indian Culture and Business Management* **21**, 1-10.

Vescovo M, Scolari GL & Bottazi V (1989) Plasmid-encoded ropiness production in *Lactobacillus casei* ssp *casei*. *Biotechnology Letters* **11**, 709-712.

Vicente-García V, Ríos-Leal E, Calderón-Domínguez G, Cañizares-Villanueva RO & Olvera-Ramírez R (2004) Detection, isolation, and characterization of exopolysaccharide produced by a strain of *Phormidium* 94a isolated from an arid zone of Mexico. *Biotechnology and Bioengineering* **85**, 306-310.

Vijayendra SVN, Palanivel G, Mahadevamma S, Tharanathan RN & (2009) Physico-chemical characterization of a new heteropolysaccharide produced by a native isolate of heterofermentative Lactobacillus sp. CFR-2182. *Arch Microbiol* **191**, 303-310.

Viñarta SC, Molina OE, Figueroa LIC & Fariña JI (2006) A further insight into the practical applications of exopolysaccharides from *Sclerotium rolfsii*. *Food Hydrocolloids* **20**, 619–629.

Vincent SJ, Faber EJ, Neeser JR, Stingele F, Kamerling JP & (2001) Structure and properties of the exopolysaccharide produced by *Streptococcus macedonicus* Sc136. *Glycobiology* **11**, 131–139.

Wang Y-X & Lu Z-X (2005) Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by Boletus spp. ACCC 50328. *Process Biochemistry* **40**, 1043-1051.

Welman AD & Maddox IS (2003) Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends in Biotechnology* **21**, 269-274.

Wood BJB & Holzapfel WH (1995) The genera of lactic acid bacteria. *1st ed. Blackie Academic and Professional, Glasgow, United Kingdom*.

Wood BJB & Warner PJ (2003) Genetics of lactic acid bacteria. *Kluwer Academic/Plenum Publishers, New York, NY*.

www.glyco.ac.ru.

www.mobitec.com.

Yang J, Zhang W, Shi P, Chen J, Han X & Wang Y (2005) Effects of exopolysaccharide fraction (EPSF) from a cultivated *Cordyceps sinensis* fungus on c-Myc, c-Fos, and VEGF expression in B16 melanoma-bearing mice. *Pathology- Research and Practice* **201**, 745-750.

Yoshida I, Kiho T, Usui S, Sakushima M & Ukai S (1996) Polysaccharides in fungi .37. Immunomodulating activities of carboxymethylated derivatives of linear (1-]3)-alpha-d-glucans extracted from the fruiting bodies of *Agrocybe cylindracea* and *Amanita muscaria*. *Biological and Pharmaceutical Bulletin* **19**, 114-121.

Zhang W, Kim JH, Franco CMM, Middelberg APJ & (2000) Characterisation of shrinkage of calcium alginate gel membrane with immobilized *Lactobacillus rhamnosus*. *Applied Microbiology and Biotechnology* **54**, 28–32.

Zhang Y, Li S, Zhang C, Luo Y, Zhang H & Yang Z (2011) Growth and exopolysaccharide production by *Lactobacillus fermentum* F6 in skim milk. *African Journal of Biotechnology* **10**, 2080-2091.

Zheng GH, Sosulski FW & (1998) Determination of water separation from cooked starch and flour pastes after refrigeration and freeze-thaw. *Journal of Food Science* **63**, 134–139.

ANNEXURES

TABLE AI : MAJOR INSTRUMENTS USED

EQUIPMENT	MODEL
Autoclave	Tomy, Japan
Referigerated Centrifuge	Remi instruments, India; Eppendorf, Germany; Hitachi, Japan
Deep Freezer (-80 ºC)	Sanyo, Japan
Electron Microscope	JEOL JSM 5600LV, 115 Japan
Electrophoresis Unit	Bangalore Genei Pvt. Ltd, India; Biorad, USA
Fourier Transform-Infrared Spectrometer	Shimadzu, IR Prestige-21, Japan
Gas Chromatograph	Hewlett-Packard 4890A, Agilent Technologies, Palo Alto, CA, USA
Gel Documentation	Lark Biosciences, Chennai
Hot Air Oven	Kemi Instruments, India
Incubating Water Bath	Julabo, Germany
Incubator	Sanyo, Japan; Innova4230, New Brunswick Scientific, USA
Ion Exchange and Gel Filtration Column	Amersham Biosciences, UK
Laminar Air Flow Chamber	Clean Air System, India
Lyophilizer	Operon, Korea; SCANVAC, Labogene, Denmark
MALDI Spectrometer	Axima CFR^{+}, Applied Biosystems, Boston, MA
Nanodrop	Thermo Scientific, USA
NMR Spectrometer	Bruker Avance II-500, Bruker Co., USA
PCR Machine	Eppendorf, India
Peristaltic Pump	Gilson, USA
pH Meter	Systronics, India
Phase Contrast Microscope	Leica DMLS, Leica Microsystems, Germany
TGA apparatus	Shimadzu H-50, Japan
UV-VIS Spectrophotometer	UV-1601, Shimadzu, Japan
Weighing Balance	Mettler Toledo, Mumbai, India

TABLE AII : EPS PRODUCTION MEDIUM

Constituents	Concentration (g/l)
Lactose	40
Yeast Extract	40
Ammonium Sulphate	5.5
Di-Potassium Hydrogen Phosphate	2
Sodium Acetate	5
Magnesium Sulphate	0.1
Manganese Sulphate	0.05
Tween 80	1

TABLE AIII : MRS MEDIUM

Constituents	Concentration (g/l)
Glucose	20
Yeast Extract	15
Beef Extract	5
Peptone	5
Ammonium Sulphate	5.5
Di-Potassium Hydrogen Phosphate	2
Ammonium Citrate	2
Sodium Acetate	5
Magnesium Sulphate	0.1
Manganese Sulphate	0.05
Tween 80	1

TABLE AIV : LURIA BERTANI (LB) MEDIUM

Constituents	Concentration (g/l)
Beef Extract	10
Peptone	10
Sodium Chloride	5
Agar	15

RESEARCH OUTPUT

I PUBLICATIONS

1. **Ismail Bindhumol** and Nampoothiri K M**,** (2010), Production, purification and structural characterization of an exopolysaccharide produced by a probiotic *Lactobacillus plantarum* MTCC 9510, Archives of Microbiology 192:1049–1057
2. **Ismail Bindhumol** and Nampoothiri K M, (2010), Exopolysaccharide production and prevention of syneresis in starch using encapsulated probiotic *Lactobacillus plantarum*, Food Technology and Biotechnology, *48* (4) 484–489
3. Aswathy, R.G., **Ismail, Bindhumol**, John, R.P., & Nampoothiri, K. M. (2008). Evaluation of the probiotic characteristics of newly isolated Lactic acid bacteria. Applied Biochemistry and Biotechnology, 151, 244–255
4. **Ismail Bindhumol** and Nampoothiri K M , Two dimensional NMR spectroscopic analysis of an exopolysaccharide from probiotic bacteria unveiling its role in starchy food, (Communicated)
5. **Ismail Bindhumol** and Nampoothiri K M**,** Molecular genetics, production and structural variations and potential markets of exopolysaccharides (EPS) from probiotic Lactic acid bacteria, (Communicated)
6. **Ismail Bindhumol** and Nampoothiri K M**,** Exposition of anti-tumour activity of exopolysaccharides (EPS) from probiotic Lactic acid bacteria, (Communicated)

II INTERNATIONAL/NATIONAL CONFERENCE PAPERS

1. **Ismail Bindhumol** and Nampoothiri K M (2011), Exopolysaccharide of - α-D-glucose, α-D-mannose and β-D-glucose - sequence purified from probiotic Lactic acid bacteria, International Conference on New Horizons in Biotechnology, November 26-29, Trivandrum, India, P 224-225.
2. **Ismail Bindhumol** and Nampoothiri K M (2010), Rheological characterization of an exopolysaccharide from probiotic *Lactobacillus plantarum* and its application

in prevention of syneresis in starch, International Conference on Genomic Sciences, November 12-14, at Madurai Kamraj University, Tamil Nadu, India, P 69.

3. **Ismail Bindhumol**, Divya Jayakumar Beena , Dhanya Gangadharan, Ashok Pandey and Nampoothiri K M (2010), Isolation of genes involved in the biosynthesis of exopolysaccharides (EPS) and folic acid from probiotic lactic acid bacteria, International Congress on Bioprocess in Food Industries, Curitiba, Brazil.
4. **Ismail Bindhumol** and Nampoothiri K M (2009), Exopolysaccharide production using free and encapsulated *Lactobacillus plantarum*, Emerging Trends in Biotechnology, December 4-6, at Banaras Hindu University, Varanasi, India, P 187-188.
5. **Ismail Bindhumol,** RG Aswathy, and Nampoothiri K M (2008). Optimization of production medium for exopolysaccharides using a newly isolated *Lactobacillus plantarum.* International Congress on Bioprocesses in Food Industries, November 6-8, at Osmania University, Hyderabad, India. P 119-120.
6. **Ismail Bindhumol**, RG Aswathy, Nampoothiri K M and Ashok Pandey (2007). Nutraceuticals from lactic acid bacteria having probiotic qualities, International Conference on New Horizons in Biotechnology, November 26-29, Trivandrum, India, P 250-251.
7. RG Aswathy, **Ismail Bindhumol**, Rojan P John, Nampoothiri K M and Ashok Pandey (2006). Screening of newly isolated lactic acid bacteria for probiotic application, Proceedings of the 3 rd Convention of BRSI and International Conference on "Exploring Horizons in Biotechnology: A Global Venture"

November 2-4, at Sardar Patel University and Charutar Vidyamandal, Vallabh Vidyanagar –388 120, Gujarat, India, P 250.

III NUCLEOTIDE GENEBANK SUBMISSION

Ismail B and K M Nampoothiri (2012) 16S rRNA of exopolysaccharide producing probiotic *Lactobacillus plantarum* MTCC 9510; GenBank Accession Number: JQ 809467

IV AWARDS & HONOURS

Japanese Monbukagakusho Fellowship 2009

CSIR SRF Fellowship 2009