ARTI T. PATEL M. Tech. FOOD BIOTECHNOLOGY 2009

CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM LACTIC ACID BACTERIAL ISOLATES

BY

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B. Sc. (Biotechnology)

DEPARTMENT OF DAIRY MICROBIOLOGY
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ANAND – 388 110, INDIA

2009

Registration No. 04-0482-2006

CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM LACTIC ACID BACTERIAL ISOLATES

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OF

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IN

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ABSTRACT

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Lactic Acid Bacteria (LAB) are widely used in the dairy and food industries from centuries. They play crucial role in food fermentation processes. Apart from production of lactic acid, flavouring compounds and bacteriocins; several strains of LAB secrete extracellular polysaccharides in favourable environments. In food industry, microbial EPS are used as thickeners or viscosifiers, stabilizing or emulsifying agents, texturizers, etc. EPS can find important place in health promotion too.

The present study was planned to find EPS producing lactic acid bacteria from foods and identify them. Subsequently, suitable isolates were checked for EPS production and finally the EPS was partially characterized. Thirty four samples of dahi, fruits, vegetables and other fermented foods were plated and incubated at 25 and 37°C for 24-48 h on Modified MRS agar. A total of 155 EPS^+ isolates were picked up and 65 were selected by morphological characterization. Further screening based on the production of highly mucoid colonies and ability to ferment skim milk left with 21 isolates. Finally, six isolates with rods and coccobacilli cell

morphology, catalase negative, skim milk fermenting and higher mucus producers were selected for further biochemical and molecular level identification.

The biochemical characterization of these six lactic acid bacterial isolates by API kits gave identification of two isolates as *Lactobacillus spp.*, one as *Leuconostoc spp.* and three as *Weissella spp.* The 16S rRNA gene amplification using universal primers was carried out to confirm species of the selected LAB by partial gene sequencing. The identification using BLAST tool identified one isolate as *Lactobacillus plantarum,* one as *Leuconostoc pseudomesenteroides* and four as *Weissella cibaria*.

Quantity of exopolysaccharide produced by the selected isolates ranged between 2.14 - 5.88 g/L. *L. plantarum* (86) produced highly significant amount of EPS (5.88 g/L) than other isolates. *W. cibaria* (138), *W. cibaria* (92), *W. cibaria* (85) and *W. cibaria* (145) produced 5.46, 4.88, 4.22 and 4.06 g/L of EPS, respectively. *Leu. pseudomesenteroides* (142) produced the lowest amount of EPS (2.14 g/L).

The monosaccharide compositional analysis of the EPS produced by selected LAB was analyzed by HPLC. The four major sugar standards run were glucose, fructose, galactose and rhamnose and surprisingly the EPS from all the isolates had very low amount of these sugar.

The selected LAB showed better tolerance to bile salt concentration (2.0%), pH (2.0) and phenol (0.5%) making them suitable candidate to be employed as probiotic.

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CERTIFICATE

This is to certify that the thesis entitled "**Characterization of Exopolysaccharides from Lactic Acid Bacterial Isolates**" submitted by Miss **Arti Thakarshibhai Patel (Registration No. 04-482-2006)** in partial fulfillment of the requirements for the degree of **Master of Technology** in **Food Biotechnology** of the Anand Agricultural University is a record of bonafide research work carried out by her under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Date: /10/2009 **(J. B. Prajapati)**

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CERTIFICATE-I

This is to certify that **Arti Thakarshibhai Patel** has successfully completed the comprehensive examination of Major and Minor subject held on **25/09/08** as required under the Regulation for **M. Tech.** degree in Dairy Science.

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Dated: 05 / 12 /2009

CERTIFICATE-II

This is to certify that the thesis entitled "**Characterization of exopolysaccharides from lactic acid bacterial isolates**" submitted for the degree of **Master of Technology** in the subject of **Food Biotechnology** embodies bonafide research work carried out by Ms. **Arti Thakarshibhai Patel (Regis. No. 04-0482-2006)** under my guidance and supervision and that no part of this thesis or research work has been submitted for any other degree. The assistance, guidance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by Advisory Committee on 04-12-2009.

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CERTIFICATE-III

This is to certify that the thesis entitled "**Characterization of exopolysaccharides from lactic acid bacterial isolates**" submitted by **Arti Thakarshibhai Patel** to the Anand Agricultural University, Anand in partial fulfillment of requirement for the degree of **Master of Technology** in Dairy Science in the subject of **Food Biotechnology** after suggestion and recommendation by external examiner was discussed and defended by the candidate before the following members of the Advisory Committee. The performance of the candidate in the oral examination on this thesis has been found satisfactory; we therefore, recommend that the thesis may be approved.

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CERTIFICATE - IV

This is to certify that **Ms. Arti Thakarshibhai Patel (Reg. No. 04-0482-2006)**, Department of Dairy Microbiology, Sheth M. C. College of Dairy Science**,** Anand has made all correction/modifications in the thesis entitled, "**Characterization of exopolysaccharides from lactic acid bacterial isolates**" which were suggested by the External Examiner and the Advisory Committee in the oral examination held on 04/12/2009. The final copies of the thesis duly bound and corrected were submitted on 05/ 12 /2009 are enclosed herewith for approval.

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APPROVED

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Place : Anand

Date : / /2009 [**Arti T. Patel**]

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LIST OF ABBREVIATIONS

%	Per cent
@	At the rate of
+ve	Positive
±	Plus or minus
≥	More than or equal to
° C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
AAU	Anand Agriculture University
AFLP	Amplified Fragment Length Polymorphism
AIDS	Acquired Immune Deficiency Syndrome
BLAST	Basic Local Alignment Search Tool
C.D.(0.05)	Critical difference at 5 per cent level
C.V.	Coefficient of variance
Ca	Calcium
Cal.	Calories
cfu	Colony forming unit
CHAPS	3-[(3-cholamidopropyl) dimethyl ammonio]-1-hydroxypropane-sulfonate
cm	Centimeter
CO_2	Carbon dioxide
CRD	Completely randomized design
DDBJ	DNA Data Bank of Japan
DM	Dry Matter
DNA	Deoxy Ribo Nucleic acid
DPW	Deproteinized whey
e.g.	Example
EDTA	Ethylene Diamine Tetra Acetate
EPS	Exopolysaccharide(s)
EPS^+	Exopolysaccharide producing
ESM	EPS Selection Medium
et al.,	Others
etc.	Extra
Fe^{+3}	Iron ion
g	Gram
g/L	Gram(s) per Liter
GC	Gas Chromatography
GCMS	Gas Chromatography/Mass Spectrometry

GIT	Gastrointestinal Tract
GLC	Gas Liquid Chromatography
GRAS	Generally Regarded As Safe
GYEA	Glucose Yeast Extract Acetate
H_2SO_4	Sulphuric Acid
HePS	Heteropolysaccharides
HoPS	Homopolysaccharides
HPLC	High Performance Liquid Chromatography
hrs	Hour(s)
i.e.	That is
ICAR	India Council of Agricultural Research
Kb	Kilo base
kg	Kilogram
KHz	Kilo hertz
L	Lactic culture, able to ferment lactose
LA	Lactic acid
LAB	Lactic Acid Bacteria(s)
lit.	Litre
M	Molar
m/s	Meter per second
max.	Maximum
Mg	Milli Gram
min	Minute(s)
ml	Milliliter
mm	Millimeter
MMRS	Modified deMan, Rogosa, Sharpe
Mn^{+2}	Manganese ion
Mr	Molecular mass
MRS	deMan, Rogosa, Sharpe
N	Normal
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
NDRI	National Dairy Research Institute
ng	nanogram
NH_4^+	ammonium
NL	Non lactic, not able to ferment lactose
No.	Number
Pa	Pascal
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
pH	Negative logarithm of hydrogen ion concentration

ppm	Parts per million
RAPD	Randomly Amplified Polymorphic DNA
REA	Restriction Endonuclease Analysis
RI	Refractive Index
rRNA	Ribosomal Ribo Nucleic Acid
S.Em	Standard Error of mean
SDS-PAGE	Sodium Dodesyl Sulphate- Polyacrylamide Gel Electrophoresis
Sec.	Second
TAE	Tris-acetate-EDTA
TCA	Tri-chloro Acetic acid
TLC	Thin Layer Chromatography
Triton X-100	Nonaethylene glycol octylphenol ether
Tween 40	Polyoxyethylene sorbitan monopalmitate
Tween 80	Polyoxyethylene sorbitan monooleate
USDA	United States Department of Agriculture
UV	Ultra violet
v/v	Volume by volume
-ve	Negative
viz.	Vide licet (namely)
w/v	Weight by Volume
w/w	Weight by Weight
Wt.	Weight
Zn^{+2}	Zinc ion

CHAPTER I

INTRODUCTION

Lactic Acid Bacteria (LAB) are widely used in the dairy and food industries from centuries. They play crucial role in food fermentation processes. Apart from production of lactic acid, flavouring compounds and bacteriocins; several strains of LAB secrete extracellular polysaccharides in favourable environments, such as milk (Cerning, 1990; Sikkema, Oba, 1998; Cerning, Marshall, 1999; De Vuyst, Degeest, 1999; Ricciardi, Clementi, 2000; Behare *et al*., 2008); vegetables and fruits (Son *et al*., 2008; Sarwat *et al.,* 2008;) and other fermented foods (Adebayo-tayo and Onilude, 2008; Behare *et al*., 2008; Cagno *et al*., 2006; Ludbrook *et al*., 1997; Prasher, 1996; Sanni *et al*., 2002; Savadogo *et al*., 2004; Smitinont *et al*. 1999; Van Geel Schutten *et al*., 1998; Vijayendra *et al*., 2008). The term exopolysaccharide (EPS) is used to describe both types of extracellular polysaccharides; i.e. either attached as capsule with bacterial cell wall or liberated in to the medium as ropy polysaccharide (Sutherland, 1972). These EPS play an important role in the improvement of physical properties of fermented milks, which act like a food stabilizer, viscosifier, emulsifier or gelling agent, providing a product with natural thickness (Sudherland, 1972). Some of the examples of EPS produced by lactic acid bacteria are dextran (*Leuconostoc mesenteroides)*, mutan (*Streptococcus mutans)* and fructan (*Streptococcus salivarius subsp. thermophilus)* (Montiville *et al*., 1978; Cerning, 1990). Gram negative bacteria *Xanthomonas campestris*, *Acetobacter xylinum* and *Sphingomonas paucimovilis* are also known to produce EPS xanthan, acetan and gellan respectively that are commercially available as food additives (Harvey and McNeil, 1998; Kumar *et al*., 2008). However, EPS extracted from Gram negative bacteria, although produced in larger

quantities, may not be preferred, as they are derived from non-food grade organisms and involve high cost of their recovery (De Vuyst *et al.,* 2001). Moreover, addition of purified EPS in to the food product may not have similar effects as EPS produced *in situ* by LAB during milk fermentation (Doleyres *et al.,* 2005). The properties of EPS in purified form differ considerably from the properties of EPS produced *in situ* (Duboc and Mollet, 2001), latter being more desirable approach.

The *in situ* EPS production may play useful role in the manufacture of a variety of cultured dairy products such as yoghurt, drinking yoghurt, cheese, cultured cream and milk-based dessert (Bauzar *et al*., 1997; Christiansen *et al.,* 1999). In addition, certain EPS produced by LAB are reported to exhibit beneficial effects on human health such as cholesterol-lowering ability, anticarcinogenic, immunomodulating and antitumoral activities; and prebiotic effects (Pigeon *et al.,* 1998; Chabot *et al.,* 2001; Dal bello *et al*., 2001; Korakli *et al*., 2002). It has been speculated that the increased viscosity of EPS containing foods may increase the residence time of ingested fermented milk in the gastrointestinal tract, which helps in transient colonization by probiotic bacteria (German *et al.,* 1999). For this reason, the use of EPS producing strains as natural source of food bio-thickeners with added health benefits has received much attention in recent years.

Some authors have reported direct correlation between the concentration of EPS and viscosity of fermented milk products, but no clear cut relation has been demonstrated (van Marly and Zoon, 1995; Dupont *et al.,* 2000) except that if a given strain produces more EPS the viscosity of fermented milk will increase (Sebastieni and Zelger, 1998; Ruas-Madiedo *et al.,* 2002; De Vuyst *et al.,* 2003). Type of EPS (Homo or Heteropolysaccharides), molecular size (chain length and molecular mass) and monosaccharide composition will certainly play a key role in improvement of functional properties of

fermented milk. So, understanding the nature and molecular characteristics of the polymer would help in determining its effect on physical properties.

Lot of work has been done on development of EPS producing cultures and some of the commercial companies have introduced EPS cultures for western products. However, in India, due to lack of supported research data and unavailability of EPS cultures of Indian origin, the commercial exploitation has not been possible. Moreover, the EPS producing strains used in western countries for preparation of fermented milks are specifically developed for particular product; their behavior may change from product to product. This suggests that there is a need to search new EPS producing strains of Indian origin that are made easily available for the commercial exploitation. The diverse microflora from indigenous fermented milks prepared in rural and urban areas using traditional methods may be a potential reservoir for isolation and exploitation of such EPS producing cultures.

Keeping this in view, the present work was planned with following objectives,

1. Isolation and identification of EPS producing lactic acid bacteria from various natural sources.
2. Phenotypic, Biochemical and Genetic characterization of selected isolates.
3. Extraction and purification of EPS from selected isolates.
4. Qualitative and quantitative analysis of EPS extracted from the selected isolates and its primary/ partial characterization.

CHAPTER - II

REVIEW OF LITERATURE

Before planning the present research work, the available literature related to lactic acid bacteria (LAB) and exopolysaccharides (EPS) was surfed. This chapter presents a review in relation to various aspects of lactic acid bacteria, their isolation and identification, production of exopolysaccharides, their characterization and their importance in health and food industries.

2.1. LACTIC ACID BACTERIA

The term LAB was coined in 1919 to denote bacteria that are able to ferment and coagulate milk. The bacteria in this group are those, which produce lactic acid mainly from lactose (Orla Jensen, 1919). A more relevant description of LAB which is that they are Gram positive, non-sporing, microaerphilic bacteria which produce lactate as their main fermentation product from fermentable carbohydrates (Kandler, 1983). They have high acid tolerance and survive pH lower than 5.0. This acid tolerance gives them a competitive advantage over other bacteria. Due to their lack of or limited ability to produce B-vitamins, nucleic acids and amino acids; the nutrient requirements of LAB are somewhat complex. Most of them are generally regarded as safe (GRAS), except for a few strains that are considered to be pathogenic. LAB commonly occurs in nutrient rich environments, such as plant surfaces in plant decaying material, foodstuffs, such as milk, meat, and fish (Viniegra-Gonzalez, 1984). They are also inhabitants of the the gastrointestinal tract (GIT) of man and animal (Kandler and Weiss, 1986). LABs are cocci, with the exceptions of *Lactobacillus* and *Carnobacterium* which are rods. Genera that resemble and which are phylogenetically related to LAB are: *Aerococcus*, *Bifidobacterium*, *Brochotrix*, *Listeria*, *Staphylococcus* and *Sporalactobacteria* (Stiles and Holzapfel, 1997). The optimum

temperature for the growth of LAB varies from 20-30°C (*Leuconostoc*, *Pediococcus*), 30-37°C (*Streptococcus* and *Enterococcus*) to 25-45°C for *Lactobacillus* (Dicks *et al*., 1995). Phylogenetically LAB can be devided into four super-clusters: (1) *Lactobacillus* (*Lb*) + *Pediococcus* (*Ped*); (2) *Leuconostoc* (*Leu*) + *Weisella* (*Wei*) + *Oenococcus* (*Oen*); (3) *Streptococcus* (*Str*) + *Lactococcus* (*Lc*) and (4) *Enterococcus* (*Ent*) + *Tetragenococcus* (T*etr*) + *Carnobacterium* (*Carn*) + *Vagococcus* (*Vag*) (Figure 2.1). (Martensson, 2002).

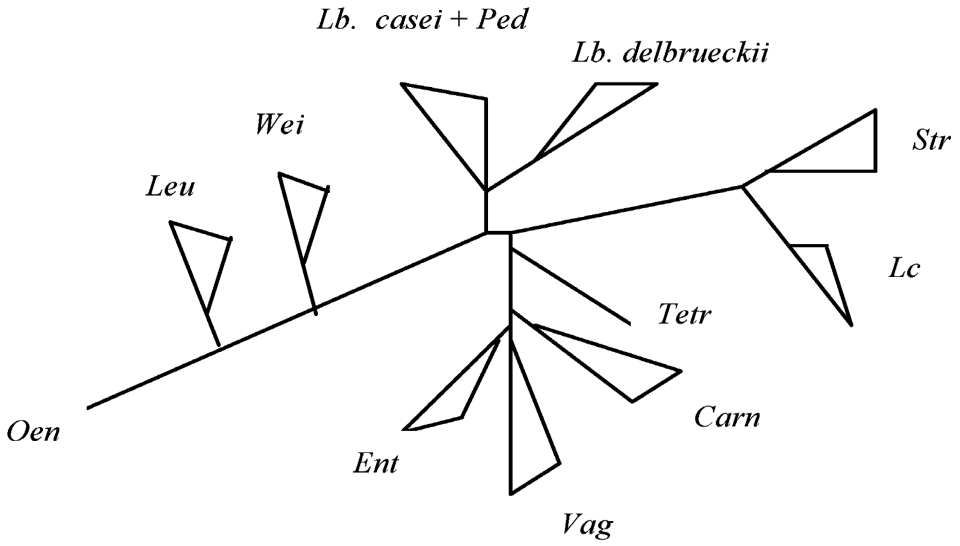


Figure 2.1 The phylogenetic tree of Lactic acid bacteria (adapted from Stiles and Holzapfel, 1997)

Today, these bacteria are indispensable in the manufacture of many products such as silage, dairy products, poultry products, vegetables and fruits. They are also exploited in the preparation of the plant seed products like fermented soy milk, peanut milk, sufu, shoyu, mahewn, ogi, fermented dough or sour dough bread (Smith and Palumbo, 1981). The lactic acid starters are commonly utilized in the manufacture of yoghurt, acidophilus milk, cultured buttermilk, sour cream, kefir, koumiss and varieties of cheeses.

Due to their long history of safe use in foods, most species of LAB are considered as commensal microorganisms with no pathogenic potential (Roberfroid, 2000a, b; Rastall and Gibson, 2004). The live cultures belonging to genus Lactobacillus along with Bifidobacterium species, is most widely encountered for providing numerous nutritional and health benefits including prevention of cancer, reduction in the level of serum cholesterol, management of normal gut flora and improvement in lactose utilization in lactose malabsorbers (Nelson and Gilliland, 1984; Jones *et al.,* 1985; Holzapfel and Schillinger, 2002; Wang, 2003; Saito, 2004; Mottet and Michetti, 2005; Senok *et al.,* 2005; Thomsen, 2006).

Metchnikoff (1908) hypothesized that lactobacilli, belonging to group lactic acid bacteria, in the gastrointestinal tract were important for the health and longevity of humans, since early 1900s (Gibson and Roberfroid, 1995). Primitive man was constantly exposed to various lactic acid bacteria from the moment of birth and these microorganisms established themselves to become important members of the indigenous flora. With the onset of technological revolution in twentieth century in the food industry, however, the type of organisms to which modern man is exposed to by eating has changed drastically. Nevertheless, the role of beneficial microbes is again being rediscovered since last 2-3 decades. The best means to check the effect of probiotics on human and animal health is to study *in vitro* and *in vivo* effect. *In vivo* experiments the beneficial effects of probiotics on gastrointestinal health (Khedekar *et al.,* 1990a; 1990b; Patel *et al.,* 1992), antibacterial activity against pathogenic and food poisoning bacteria (Khedekar *et al.,*1990c; 1994), cholesterol lowering effect (Ashar and Prajapati, 1998a) and beneficial effect on lipid profile (Ashar and Prajapati, 1998b; 2000; 2001), immunomodulatory effect (Patidar and Prajapati, 1999), anticarcinogenic activity have been proven by many researchers

with the pioneer contribution by Department of Dairy Microbiology, SMC college of Dairy Science, Anand since 1986.

2.2. EXOPOLYSACCHARIDES

Various Exopolysaccharides (EPSs) produced by bacteria have novel and unique physical characteristics and precise role according to the ecological niches of the microorganisms. EPSs are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. These sugar units are mainly glucose, galactose and rhamnose, in different ratios (De Vuyst, and Degeest, 1999; Welman and Maddox, 2003). The multifunctional bacterial biopolymers have emerged as new, industrially important polymeric materials, which are gradually proving economical at par with natural gums produced by marine algae and other plants (Welman and Maddox, 2003). Exopolysaccharides (EPS) have found extensive applications in food, pharmaceutical and other industries. Many species of gram-positive and gram-negative bacteria, fungi and also algae are known to produce EPS (Kumar *et al*., 2007). These long-chain polysaccharides that are secreted mainly into their surroundings during growth (Sutherland, 1972, 1977) and that are not permanently attached to the surface of the microbial cell and they are known as EPS where as the attached polysaccharide to the cell wall is known as CPS (capsular polysaccharides). The physical characteristics of EPSs are responsible for the slime-forming or mucoid trait of the microorganisms (Laws *et al*., 2001). Lactic acid bacteria (LAB) used generally as starter cultures may also produce EPS *in situ* during milk fermentation (e.g., yogurt and Scandinavian fermented milk *viili*). In addition, certain EPS produced by LAB are thought to have beneficial effects on human health such as cholesterol lowering ability (Pigeon *et al*., 2002), immunomodulating, and antitumoral activities (Kitazawa *et al*., 1998; Chabot *et al*., 2001), and prebiotic effects (Dal Bello *et al*., 2001; Korakli *et al*., 2002). For these reasons, the use of EPS-

producing strains as a natural source of food thickeners has received much attention in food industries recent years (Duboc and Mollet, 2001; Ruas-Madiedo and Reyes-Gavila´n, 2005; Behare *et al*., 2009a; 2009b).

2.3. EPS PRODUCING LACTIC ACID BACTERIA

A great variety of LAB, with some strains of Bifidobacteria (Abbad-Andaouse *et al*., 1995; Roberts *et al*., 1995; Hosono *et al*., 1997), are reported to produce exopolysaccharides. Most of them belong to the genera of *Leuconostoc, Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and some species of *Weissela*. Exopolysaccharides (EPSs) from lactic acid bacteria have found their most valuable application in the improvement of the rheology, texture and 'mouthfeel' of fermented milk products, such as yoghurt. There is a high consumer demand for smooth and creamy yoghurt products, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatin). Consumer demand for products with low fat or sugar content and low levels of additives, as well as cost factors, make EPSs a viable alternative (Welman and Maddox, 2003). The EPS producing LAB have been traditionally used in the Scandinavian fermented milk products to impart desirable texture and rheological properties (Macura and Townsley, 1984). The products made with ropy strains have smooth body, high viscosity and less syneresis than products made with non-ropy strains (Wacher-Rodarte *et al*., 1993). Often in the literature, to describe different EPS producing phenotypes, the term ropy, mucoid and slime have been interchangeably used. However, not all mucoid or slime producing cultures are ropy. The ropy colonies are able to form strand when touched with an inoculating loop, whereas, mucoid colonies have glistening and slimy appearance on agar plates and are not able to produce strands by this method (Vescovo *et al*., 1989; Dierksen *et al*., 1997). Nevertheless, some LAB (e.g. *Lactobacillus casei* CG11 and

Lactococcus lactis subsp. *cremoris* Ropy 352) can express both ropy and mucoid phenotypes depending on the culturing conditions (Cerning *et al*., 1994; Dierksen *et al*., 1997).

2.3.1. Isolation of EPS Producing Lactic Acid Bacteria

The EPS producing lactic bacteria are isolated from dairy and non-dairy environment using different media supplemented with one or more type of sugars (Table 2.2). The media used for isolation of EPS producing cultures are Liquid EPS Selection Medium (ESM) containing 90 g skim milk, 3.5 g yeast extract, 3.5 g peptone and 10 g/L glucose (van den Berg *et al*., 1993), Milk Indicator Agar and M17 Lactose Agar (Terzaghi and Sandine, 1975), MRS with high concentration of sugars (100 g / L) (van Geel Schutten *et al*., 1998) and Milk Agar (Mozzi *et al*., 2001) etc. Troili-Peterson (1899) was the first to isolate a slime producing bacterium *Bacterium lactis longi* from Swedish ropy milk “Langmjulk”, the bacterium renamed as *Lactococcus lactis* by Macy (1923). A systematic study on the microbiology of Finnish ropy milk has been completed by Sundaman (1953).

Ropy LAB formed 1-2 diameter Colonies on tomato agar medium after 3-4 days of incubation at 23°C. These strains when grown in sterile skim milk and in fresh milk gave a very viscous product. Majority of the strains belonged to the *Lactococcus* genus and *Lactococcus lactis* subsp. *cremoris* was the predominating species in this product. Extensive study on microflora of the Scandinavian ropy milk was made by Forsen (1966), who isolated slime producer *L. cremoris*, *L. lactis*, *L. diacetylactis* and *Leuconostoc spp*. from “Longmilk”. Bertelsen (1983) reported the ropy consistency of villi, a variety of Nordic ropy sour milk, was due to slime forming ability of *L. lactis* or *L. cremoris*. Toba *et al*. (1986) reported the isolation of the capsular bacteria from kefir grains on a new medium. Kefir grains were propagated at 18-22ºC in sterile skim milk and plated

on KPL agar and incubated at 30ºC for 7 days in anaerobic atmosphere. Isolates were gram positive rod shaped bacteria and identified as homofermentative lactobacilli. Latter, large numbers of ropy mesophilic lactococci were isolated from Swedish sour milk and finnish villi on M17 medium (Neve *et al*., 1988). Ropy colonies produced on media could be easily distinguished from non-ropy colonies by forming long, ropy filaments with an inoculation loop.

Table 2.1 Isolation of EPS producing LAB from dairy and non-dairy fermented foods

Fermented dairy and non-dairy food	Medium	EPS producing Isolates*	References
Cheeses and dairy product	Liquid ESM	30 (4.9%)	Van den Berg *et al*. (1993)
Soured milk, dahi, cream and ropy milk	MIA and M17 (L) Agar	44 (47.4%)	Prasher (1996)
Non dairy fermented foods	ESM-50 g/L glucose	11	Ludbrook *et al*. (1997)
Fermented foods	MRS with 100 g/L sucrose	60 (33%)	Van Geel-Schutten *et al.* (1998)
Thai fermented foods	MRS with 20 g/L sucrose	7 (6.7 %)	Smitinont *et al.* (1999)
Nigerian Fermented foods	Modified ESM	25 (16%)	Sanni *et al.* (2002)
Burkino faso fermented milk	MRS agar, Rogosa Agar, M17 Agar	13 (26 %)	Savadogo *et al*. (2004)
Italian sourdough	MRS-S (292 mM sucrose) agar	14	Cagno *et al*. (2006)
Indian Dahi	M-MRS with 5% sucrose, EPS medium	1	Vijayendra *et al*. (2008)
Belgien and Romanian dairy and cereal products	MRS agar, M-MRS agar with 5% sucrose	10 (5.74%)	Van der Meulen *et al.* (2007)
Vitis vinifera L. (Grape), Pakistan	Formulated medium with 5% sucrose	1	Sarwat *et al.* (2008)
Indian Dahi and raw milk	Deproteinized whey	47 (51.09 %)	Behare *et al*. (2008)
Korean traditional fermented vegetable (Dongchimi)	Defined medium containing 20% sucrose, 1.5% skim milk and 0.5% potato powder	1	Son *et al*. (2008)
Nigerian fermented foods	ESM (Exopolysaccharides selection medium)	103 (89.6%)	Adebayo-tayo and Onilude (2008)
Soil sample	Glucose Yeast Extract Acetate (GYEA) Broth	1	Kodali *et al*. (2009)

**Values in parentheses indicate percentage of EPS positive isolates from the total number of lactic cultures isolated.*

Gancel *et al*. (1989) developed ruthenium red indicator agar, in particular to differentiate EPS and non-EPS producing *Strep. thermophilus* strains. The EPS producing strains formed white colonies on agar medium due to the surrounding protective layer of EPS that prevents colorization of the cell, whereas, non-EPS producing strains formed pink colonies. The encapsulated strain of *Str. thermophilus* was isolated from the commercial yoghurt by Ariga *et al*. (1992). The ropy nature of the yoghurt could be attributed to ropy character of the strain which showed large capsule surrounding the cell stained with Indian ink. The liquid EPS selection medium was used by Van den Berg *et al*. (1993) for selection of EPS-producing LAB from sour dough, sausages, table olives, cheeses and other dairy products. After incubation at 30ºC for 24 h in Exopolysaccharides selection medium (ESM), the ropiness of the culture was determined by its resistance to flow through graduated pipettes. Results obtained showed that only 30 out of 607 LAB strains tested (4.9%) were EPS producers. Using a slightly modified ESM containing 50 g/ L-sucrose, Ludbrook *et al*. (1997) isolated 11 LAB strains from non dairy fermented foods which were able to produce EPS. Four EPS-positives LAB from 25 isolates were found in Nigerian fermented foods using the same modified ESM (Sanni *et al*., 2002).

Furthermore, the use of several carbohydrates (used separately) for the screening may improve the detection of EPS-producing strains. In that way, Van Geel Schutten *et al*. (1998) screened several Lactobacillus strains of different origins (fermented foods, gastrointestinal tract of animals, and human dental plaque) for EPS production in de Man Rogosa, and Sharpe medium supplemented with high concentrations of (100 g/L) of different sugars: glucose,

fructose, maltose, raffinose, sucrose, galactose or lactose. One hundred eighty-two strains were tested in these media and after 3 days of incubation at 37ºC, the cultures were precipitated with cold ethanol. Sixty strains produced EPS and 17 of them rendered more than 100 mg/ L, with the sucrose medium being the best for detecting the EPS phenotype. The authors attributed the higher percentage of positive isolates (33%) to the high content of sugar used in the media.

Different sugars were used for the screening of EPS-producing LAB from traditional Thai fermented foods by Smitinont *et al*. (1999). MRS agar plates containing 20 g/L of glucose, fructose, sucrose or lactose were streaked with the LAB isolates and incubated at 30ºC for 2-3 days. Seven out of 104 isolates produced slimy colonies on agar media containing sucrose. The EPS was confirmed in liquid medium by isolation of the EPS fraction. Two of the EPS-producing strains were identified as *Pediococcus pentosaceus* (AP-1 and AP-3), which produced 6.0 and 2.5 g / L of EPS, respectively. Micheli *et al*. (1999) isolated a capsular polysaccharide producing strain, LM-17, from kefir grains and identified as *Lactobacillus.* Mozzi *et al*. (2001) suggested a simplest milk agar medium to isolate EPS producing LAB that produce mucoid colonies on this medium. They screened two hundred one mesophilic and thermophilic LAB for ropiness in reconstituted skim milk (Mozzi *et al*., 2006). Thirteen EPS producing lactic isolates were reported from *Burkino faso* fermented milks (Savadogo *et al*., 2004). Targeting spacer region between 16S and 23S rRNA genes, the isolates were named as *Lact. delbrueckii*, *Lact. acidophilus*, *Lact. fermentum*, *Strep. thermophilus*, *Pediococcus* spp., *Leuc. mesenteroides* subsp. *mesenteroides*. Cagno *et al*. (2006) reported fourteen strains of LAB for EPS production from Italian sourdough. They reported seven isolates as *Weissella cibaria*, four as *Lactobacillus plantarum,* and three as *Pediococcus pentosaceus*. Two types of homopolysaccharides were synthesized:

glucans simultaneously with growth and fructans after 1 day of incubation. *W. cibaria* WC4 or *L. plantarum* LP9 synthesized ca. 2.5 g kg^{-1} EPS during sourdough fermentation with sucrose added. Compared to the sourdough started with an EPS-negative strain, the sourdough started with two of the isolated strains i.e. *W. cibaria* WC4 or *L. plantarum* LP9 were the best in increasing the viscosity, and the resulting bread had higher specific volume and lower firmness.

Exopolysaccharide producing non-ropy strain of *Leuconostoc sp*. CFR 2181 was isolated from dahi on M-MRS agar, containing sucrose (5%, w/v) as well as on a low cost EPS medium by Vijayendra *et al*. (2008). Behare *et al*. (2008) isolated 47 EPS producing mesophilic ropy strains of lactic acid bacteria from Indian dahi and raw milk using deproteinized whey based medium. Adebayo-tayo and Onilude (2008) studied 115 strains of LABs isolated and characterized from some fermented dairy (Nono, Fura, Yogurt, Wara) and non-dairy foods (Ogi and Fufu). They were identified as *L. fermentum, L. casei, L. plantarum, L. brevis, L. cellobiosus, L. delbrueckii, L. coryniformis, L. coprophilus, L. gensenii, L. lechmanii* and *Leu. paramesenteroides*. From the PCR and Ultrafiltration screened isolates, *Lactococcus lactis* (for homopolysaccharides) and *Lactobacillus curvatus* (for heteropolysaccharide) production reported for the first time by Van der Meulen *et al*. (2007). Sarwat *et al.* (2008) isolated mesophilic *L. mesenteroides* CMG713 strain from *Vitis vinifera L.* (Grape) using 15% added sucrose in the growth medium for the highest dextran production. On the basis of high enzyme activity, *L. mesenteroides* CNG713 showed maximum dextran production after 20h of incubation at 30°C with 15% sucrose at pH 7.0.

2.4. MOLECULAR TOOLS FOR CLASSIFICATION AND IDENTIFICATION OF LAB

The identification of lactobacilli and other lactic acid bacteria, using biochemical methods is difficult largely due to the need for plenty of biochemical tests along with the problems of highly resembling large number of species groups that are prone to transfer of plasmids among them. Hence, they alone are not sufficient for inter- and intra-species differentiation and need to be supplemented with sensitive molecular methods to obtain more reliable identification. Contrary to the phenotypic methods, molecular identification and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of phenotype **(**Singh *et al*., 2008). In fact, many *Lactobacillus* species have been reclassified on the basis of fresh information from advanced molecular techniques and their correct taxonomic status has been determined, such as *L. cellobiosus*, *L. pastorianus*, *L. arizonensis* have been reassigned to *L. fermentum* (Dellaglio, Torriani, & Felis, 2004), *L. paracollinoides* (Ehrmann & Vogel, 2005), and *L. plantarum* (Kostinek et al., 2005), respectively.

Many of the modern molecular tools are based on 16S ribosomal DNA sequences, complete or partial genomes or specific fluorescent probes that monitor the physiological activity of microbial cells. These high throughput approaches are increasingly applied to strains of lactic acid bacteria (LAB) (Kleerebezem *et al*., 2003) and bifidobacteria that provide health benefits and are marketed as probiotic bacteria. The primary purpose of these approaches is to provide proper strain identification as required for legal and good manufacturing practices. In addition, these identification tools can be used to trace and track LAB, including probiotics, in the

production phase and in food products as well as after consumption in the intestinal tract. Moreover, many of these identification tools can be instrumental in the selection of new strains or species of LAB or bifidobacteria as starters, for flavor developments, or to be developed into probiotics. Finally, a series of functional approaches are being developed and validated that can further be used in controlling quality (Amor *et al*., 2007). These are either based on generic microbial properties or specifically address the mechanisms by which LAB and probiotics may exert their functional or benefical effects. The generic systems are used to monitor the viability, vitality, or stress response of LAB during fermentation or in bioreactors or food products, whereas the specific ones have the potential to determine the performance of probiotic bacteria in all these systems as well as in the intestinal tract of the consumer (Amor *et al*., 2007)**.**

2.4.1. 16S rRNA PCR and Sequencing

Today, with the availability of rapid and automatic DNA sequencing technology, direct sequencing of the 16S rRNA gene has emerged as the most powerful and relatively easy one-step method for identification and classification of bacteria.

The determination of 16S rRNA sequences for the elucidation of the phylogeny of the LAB around 1990, initiated a rapid development of DNA probes for identification of these bacteria. Before that there had been some attempts to develop DNA probes based on selected fragments of a DNA library (Lars Axelsson, 2004). Identification of LAB with the use of 16S (or 23S rRNA)-targeted probes was developed and used for lactococci and enterococci (Klijn *et al*., 1991; Betzl *et al.,* 1990) lactobacilli from different niches,(Hertel *et al*, 1991; Hensiek *et al,* 1992; Vogel *et al*., 1994), carnobacteria from meat (Brooks *et al*., 1992), distinguishing vagococci from other LAB

(Williams *et al*., 1992), *S. thermophilus* (Ehrmann *et al,.* 1992) and even for distinguishing between the subspecies *lactis* and *cremoris* of *Lc. lactis* (Salama *et al*., 1991). It may also be interesting to determine the occurrence of specific groups of LAB. Genus- and group-specific probes have been developed for such purposes (Collins *et al*., 1993; Nissen *et al*., 1994; Williams *et al*., 1992).

The PCR technique is becoming more and more useful for identification and classification purposes. With this technique it is possible to amplify a gene or a part of a gene from a very limited number of cells for subsequent DNA sequencing. Conserved genes are analysed and compared by sequencing technique. This is a very powerful and accurate tool to characterize lactobacilli. Species-specific sequences are located in the first half of the 16S rRNA, but it is more accurate to sequence the whole gene, which has a length of about 1.5kb (Coeuret *et al.,* 2003). With automated sequencing systems and convenient direct PCR sequencing methods, it has become an easy task to determine the 16S rRNA sequence from any bacterium in a short time (Axelsson, 2004).

PCR can also be used in combination with other probing techniques (Klijn *et al*., 1991; Williams *et al*., 1992; Brooks *et al*., 1992) or oligonucleotide probes designed from 16S rRNA sequencing also can be used in PCR applications. A number of fingerprinting techniques based on PCR have also been developed. The most commonly known is randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al*., 1990) shown to be applicable for distinguishing strains of *Lb. acidophilus* group which has similar phenotype (Du Plessis and Dicks, 1995; Gancheva *et al*., 1999) although several methods can be used for this particular cluster of species. Another fingerprinting PCR-based method with similarities to RAPD is REP-PCR (with variants known as ERIC-PCR and BOX-PCR), and successful use for LAB has been reported (Ventura and Zink, 2002; Gevers *et al*., 2001; Sohier *et al*., 1999). Intergenic spacer sequence polymorphism in variants has also been used in PCR-

based methods (tDNA PCR, ISR-PCR) for species identification in LAB (Kabadjova *et al*., 2002; Baele *et al*., 2001, 2002; Nour, 1998). Other genotypic fingerprinting methods are based on restriction endonuclease cleaving of the chromosomal DNA with pulse field gel electrophoresis (PFGE). Therefore it is used as the principal method for creating unique "fingerprints" of defined commercial strains by vendors for starter cultures and/or probiotics. REA (Restriction Endonuclease Analysis), AFLP (Amplified fragment length polymorphism), a PCR-based REA and soluble protein patterns have also been used widely in identification and classification of LABs for its high discriminatory power.

In several studies the genetic methods described above have been compared in classifying LAB but each method has advantages and disadvantages and that one single method is not the solution for all applications, but rather that the methods complement each other when they are used at a time (Lars Axelsson, 2004).

To summarize, a number of alternatives to classical phenotypic/biochemical identification of LAB have emerged since 1990. Which one to use is often a matter of taste, probably depending on what methods a particular laboratory starts to use. For thorough identification/classification in bacterial systematics, it is still recommended to apply a polyphasic approach, (Vandamme *et al*., 1996, Lars Axelsson, 2004) i.e., using several phenotypic, chemotaxonomic, and genotypic methods.

2.5. TYPES OF EXOPOLYSACCHARIDES PRODUCED BY LACTIC ACID BACTERIA

Depending on their chemical composition, the EPS from LAB are classified as homopolysaccharides (**HoPS**), which contain a single

type of monosaccharide, and heteropolysaccharides (**HePS**), which comprise repeating units of different monosaccharides (De Vuyst *et al*., 2001; Ruas-Madiedo and Reyes-Gavila´n, 2005, Behare *et al*., 2008). The HoPS, composed of glucose are α- (dextran, mutan, and alternan) and *β*-glucans, whereas those containing fructose are fructans (levan and inulin-type) (Barker and Ajongwen, 1991; Monsan *et al*., 2001). The total yield of EPS produced by LAB can be influenced by composition of the medium and growth conditions (Degeest *et al.,* 2001b); HoPS are generally produced in larger quantities than HePS (Cerning, 1995; Van Geel-Schutten *et al*., 1999; Welman and Maddox, 2003).

2.5.1. Homopolysaccharides

Dextran, mutan and levan are the examples of homopolysaccharides produced by some *Lactobacillus*, *Leuconostocs* and *Streptococcus* species, of which, *Leuc. dextranicum* is a well-known dextran producer (Hamada and Slade, 1980; Montiville *et al*., 1978; Funane *et al*., 1995; Monchois *et al*., 1998; Monchois *et al*., 1999; van Geel Schutten *et al*., 1999). The homopolysaccharides are synthesized by anchored or secreted transglycosylases, which are able to catalyze the transfer of a corresponding glycosyl moity (Monsan *et al*., 2001).

Although each bacterial strain produces a unique glucan, a common structural feature of all dextrans is a high percentage (up to 95%) of α- 1, 6 linkages with a smaller proportion of α-1, 2, α-1, 3, or α-1, 4 linkages resulting in a highly branched molecule (Franz, 1986). Dextrans are synthesized outside the cell by dextransucrase, which catalyzes sucrose to produce D-fructose and D-glucose, and transfers the latter to an acceptor to form dextran.

Mutans are synthesized in a similar way by *S. mutans* and *S. sobrinus* (Montville *et al*., 1978). However, mutans differ from

dextrans in containing a high percentage of α-1, 3 linkages, which are attributed to the insoluble nature of this type of polymers (Hamada and Slade, 1980). Mutan polysaccharides are involved in the adhesion of oral flora microorganisms on the tooth surface to form the dental plaque (Hamada and Slade, 1980). No specific applications of mutan polymers have been developed up till recently (Monsan *et al*., 2001).

Some *S. salivarius* and *S. mutans* strains are able to produce fructans of the levan type with 2, 6-linked β-fructofuranoside residues (Cerning, 1990). The synthesis of this fructan has been studied in most detail in *B. subtilis* and *Zymomonas mobilis*, but no significant applications have as yet been developed involving this polysaccharide. An extracellular enzyme levansucrase is involved in hydrolyzing sucrose and transferring D-fructose to growing fructan chains to form levans.

2.5.2. Heteropolysaccharides

A wide range of LAB including mesophilic and thermophilic can produce heteropolysaccharides, which are composed of repeating units. The important genera from dairy point of view are *Lactococcus*, *Lactobacillus* and *Streptococcus*. The monosaccharide compositions of these EPS are mostly galactose and glucose, and also small amounts of rhamnose, fructose, mannose, and galactosamine (Cerning *et al*., 1990; van den Berg *et al*., 1995; Stingele *et al*., 1996; Grobben *et al*., 1997; Stingele *et al*., 1997; Welman and Maddox, 2003**;** Ruas-Madiedo and Reyes-Gavila´n, 2005). In comparison with the homopolysaccharides, the production of heteropolysaccharides by LAB is much lower i.e. 60 to 400 mg / L (Stingele *et al*., 1996). Generally, the heteropolysaccharides are synthesized intracellularly at the cytoplasmic membrane utilizing sugar nucleotides as precursors for the assembly of polysaccharide chains (Cerning, 1995).

Heteropolysaccharides are made by polymerization of repeating unit precursors formed in the cytoplasm (Cerning, 1990; De Vuyst and Degeest, 1999). Heteropolysaccharides contains backbone of repeated subunits that are branched (at positions C_2, C_3, C_4 or C_6) or unbranched and that consists of three to eight monosaccahrides (De Vuyst *et al*., 2001). The glycosyl transferases are the key enzymes for the biosynthesis of the EPS repeating unit, since they catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, thereby forming a glycosidic bond. After completion of a heteropolysaccharides repeating unit, it may be exported through the cell membrane, becoming polymerized in to a final heteropolysaccharides. Different types of heteropolysaccharides are secreted with respect to sugar composition and molecular mass, comparatively in larger than homopolysaccharides varying from 1.0×10^4 to 6.0×10^6 (Cerning, 1995; De Vuyst and Degeest, 1999; Behare *et al*., 2008).

2.6. BIOSYNTHESIS OF MICROBIAL EPSS

The application potential of EPSs for food is determined by their physical and rheological properties. Factors influencing these properties are the molecular mass, stiffness of the polymer, presence of sidechains, and presence of nonsaccharide components, such as organic (e.g. acetyl, pyruvyl, or succinyl groups) or inorganic (e.g. sulphate or phosphate groups) substituents. Genetic engineering may be applied as a tool to direct the EPS synthesis and introduce desired properties by altering the composition or chain length. This requires a proper understanding of the genetics and biochemistry of EPS biosynthesis.

2.7. FACTORS AFFECTING EPS PRODUCTION

Process for production of exopolysaccharide are characterized by the extreme rheology of the fermentation, product concentration, the diversity of subtle structural and conformational changes (which can occur throughout the entire process) and the discernable effect of these changes on the product's end application performance (Kumar *et al*., 2007).

There is no single set of culture conditions that guarantees high exopolysaccharide yields, since organisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH optima, which are the critical factors for maximum Exopolysaccharide production (Sutherland, 1972; Williams and Wimpenny, 1977). Physiological control is used to modulate the relative molecular mass (Mr), the pattern and number of residues and the degree of branching of the exopolysaccharide produced. The yield and quality of microbial exopolysaccharide are greatly affected by the nutritional and environmental conditions and an increase in polymer production is possible by manipulating the culture conditions (Kumar *et al*., 2007).

2.7.1. Carbon source

A wide variety of carbon sources, used to produce microbial exopolysaccharides, include sucrose, glucose, lactose, maltose, mannitol, sorbitol, whey, starch, sugar concentrates (Neosorb™, Cerelose™), methanol and C9 to C16 n-alkanes (Kumar *et al*., 2007). The type of carbon source influences the yield of exopolysaccharide (Morin, 1998). The size of the exopolysaccharide may also vary with the carbon source. For instance, alginate produced on fructose and glucose after 48 hours of growth had a maximum Mr of 500 kDa and 276 kDa, respectively (Conti *et al*., 1994). *Lactobacillus delbruckii* synthesizes different exopolysaccharides when grown on glucose or fructose (Grobben *et al*., 1996). When grown on fructose, the strain produced 25 mg/L exopolysaccharide composed of glucose and

galactose in the ratio 1:2.4. When the carbon source was switched to a mixture of fructose and glucose, the exopolysaccharide production increased to 80 mg/L while the sugar composition changed to glucose, galactose and rhamnose in a ratio of 1: 7: 0.8. However, Petry *et al*. (2000) reported contradictory observations wherein different carbon sources did not influence the component sugars of exopolysaccharide produced by *Lactobacillus delbruckii.* Degeest and De Vuyst (2000) and Escalante et al. (1998) made similar observations in case of *Streptococcus thermophilis*, where different carbohydrates did not result in variation in exopolysaccharide composition. They reported that carbon source invariably affects the total amount of polysaccharide produced. West and Strohfus (1998) have studied the effect of different carbon sources on gellan production by *Sphingomonas paucimobilis*. According to them, a number of carbon sources including glucose and corn syrup could support gellan production. However, the ability of carbon source to produce higher cell weight did not translate into increased gellan production.

2.7.2. Nitrogen source

The nitrogen sources currently being used for Exopolysaccharide production are ammonium sulfate, peptone, sodium nitrate, urea and yeast extract (Kumar *et al*., 2007). The use of organic nitrogen sources often results in a higher specific growth rate and exopolysaccharide production, which might be due to the addition of growth factors in trace amounts (Farres *et al.,* 1997). Further, some of the carbon found in the nitrogen source might serve as a substrate for exopolysaccharide production (De Souza and Sutherland, 1994). This contributes to increase in the C: N ratio, thus promoting the exopolysaccharide production (Morin. 1998). According to Vergas-Garcia *et al*. (2001), biomass levels were higher as the nitrogen concentration increased in the medium. However, under these conditions, exopolysaccharide synthesis showed an

opposite pattern to that observed for growth. Generally, exopolysaccharide production was higher at lower nitrogen concentration. Although it has been reported that supplementation with small amounts of combined nitrogen stimulates exopolysaccharide yield (Vermani *et al*., 1997). Gorret *et al*. (2001) demonstrated that addition of yeast extract to the medium improved both growth and exopolysaccharide production by *Propionibacterium acidi-propoinici*.

2.7.3. Source of Ions

Phosphorous content influences exopolysaccharide production (Kumar *et al*., 2007). In case of *Klebsiella spp*., maximum Exopolysaccharide could be produced in absence of phosphate ion (Farres *et al*., 1997). Omission of iron (Fe^{+3}), zinc (Zn^{+2}) and ammonium (NH_4^+) ions have no impact on the growth and exopolysaccharide production by *Lactobacillus bulgaricus*. Sugar composition of exopolysaccharide was also not affected by variation in the concentration of these ions (Grobben *et al*., 2000). Contradictory results have been reported for the iron requirement of lactic acid bacteria by Pandey *et al.* (1994). In case of *Lactobacillus casei*, addition of Mn^{+2}, alone or in combination with citrate, Ca^{+2} and SO_4^{-2}, are known to strongly stimulate exopolysaccharide production (Mozzi *et al*., 1995).

2.7.4. Oxygen and Aeration Rate

According to Lee *et al*. (2001), high aeration rate resulted in enhanced exopolysaccharide production and increased the viscosity of the culture broth in the case of marine bacterium *Hahella chejuensis*. Similar results were obtained by Yang and Liau (1998) where higher agitation and aeration appeared to be favorable for the formation of polysaccharide by *Ganoderma lucidum*. Transport of monosaccharides outside the cell involves coupling to C-55

Isoprenoid alcohol phosphate. Polymerization is performed by a polysaccharide polymerase outside the cell. Thus, molecular oxygen would be necessary for primary energy metabolism and also for the oxidation of sugar to the corresponding alcohol and for reoxidizing reduced pyridine nucleotides (Kumar *et al*., 2007). An increase in exopolysaccharide production may result as a consequence of better availability of oxygen and nutrients (Dassy *et al*., 1991; Bayer *et al*., 1990). The use of detergents may ameliorate oxygen concentration in the exopolysaccharide containing broth. In the presence of detergents, Xanthomonas cells are smaller than those seen in the absence of detergents. This may lead to higher oxygen uptake rate (OUR). On the other hand, an adverse effect of high oxygen-transfer rates was seen in the case of *Aureobasidium pullulans* (Weckner and Onken, 1991) and *Fusarium solani* (Rau *et al*., 1989).

2.7.5. Viscosity

Change in the rheology of a medium is a direct consequence of product formation or Exopolysaccharide secretion. During exopolysaccharide production, the broth develops non-Newtonian characteristics and may act as a pseudoplastic fluid where the measured viscosity decreases with increasing shear rate. This change in rheology can be caused by exopolysaccharide producing microorganisms, their behavior and product formation. This may also be due to lack of homogeneity in terms of mixing, mass, oxygen and heat transfer in the bioreactor (Kumar *et al*., 2007). Under such conditions, where the Exopolysaccharide producing microorganisms in non-Newtonian broth are exposed to gradients in a number of variables, the quality of the polysaccharide would be heterogeneous (i.e., changes in Mr, branching and rheological properties). Viscosity of the culture broth might result from polymer concentration with negligible contributions from the cells. Here, the

rheology can be used as a parameter to monitor exopolysaccharide production and quality (Morin, 1998).

2.7.6. Dilution rate

During fermentation, used for exopolysaccharide production, (in batch or fed-batch processes), the exponential growth phase and the exopolysaccharide synthesis do not occur simultaneously (Kumar *et al*., 2007). In batch cultures, polysaccharide synthesis takes place when the medium is depleted with one or more nutrients and it is often maximal in media with a high carbon/nitrogen ratio. Maximum levels of polysaccharides were observed at a low dilution rate of continuous cultures of *Pseudomonas, Alcaligenes* and *Klebsiella spp*. Lower dilution rate increases the residence time of microorganisms and promotes the utilization of excess carbon for the production of exopolysaccharide. At high dilution rates, the isoprenoid lipid could be insufficiently available for simultaneous synthesis of various surface polymers, including the exopolysaccharide (Sutherland, 1977).

2.7.7. Incubation temperature

An incubation temperature below the optimum growth temperature results in greater production of Exopolysaccharide (Cerning *et al*., 1992; Gancel and Novel, 1994). A low incubation temperature (32 °C) can cause reduction in growth rate and cell mass, which in turn resulted in long logarithmic phase of growth and higher viscosity as compared to high temperature (37 °C). The difference between optimal temperature for growth and exopolysaccharide production could also be a result of increased activities of enzymes involved in the synthesis of exopolysaccharide precursors (Kumar *et al*., 2007). For instance, production of GDP-mannuronic acid which is a precursor of alginic acid was enhanced at a sub-optimal growth temperature (Morin, 1998). Gorret *et al*. (2001) demonstrated

increased exopolysaccharide production at lower temperature in case of *Propionibacterium acidipropionici*. Such results could be explained by the mechanism proposed by Sutherland (1972), i.e. a decrease in temperature causes a decrease in growth rate and cell wall polymer biosynthesis, making more precursors available for exopolysaccharide synthesis. Paradoxically, Garcia-Garibay and Marshall (1991) have reported increase in production of Exopolysaccharide with increase in temperature in case of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Pediococci used as meat starters were found to produce exopolysaccharide when grown at 15–35 °C (Morin, 1998).

2.7.8. Incubation pH

Numerous microorganisms produce Exopolysaccharide in media buffered at neutral pH (Kumar *et al*., 2007). Many of the exopolysaccharide- producers require a constant pH for maximum production of exopolysaccharide (Morin, 1998). Others like *Neisseria meningitidis* produce more exopolysaccharide at acidic pH values (Morin, 1998). Exopolysaccharide produced by some meat starters were excreted when the starters were grown in culture media buffered between pH 5.2−6.5 and contained 2−4% sodium chloride (Van Beek, 1997). Poor bulk mixing may result in significant pH gradients in the bioreactor, which may generate problems in fermentation monitoring and control of pH (Morin, 1998). In case of *Propionibacterium acidi-propionici*, production of Exopolysaccharide was possible only between pH 5.3–6.5, suggesting that regulation of the biosynthetic pathway of exopolysaccharide production may be dependent on pH (Gorret *et al*., 2001).

2.7.9. Age of the exopolysaccharide producing cells and growth

Bergmaier *et al*. (2002) reported linear effect of age on lactic acid concentration in case of *Lactobacillus rhamnosus* RW-9595M. Studies on effect of physiological age and state on survival of *Pseudomonas aeruginosa* have been carried out by Skaliy and Eagon (1972), wherein they noticed the vital role of exopolysaccharide. Exopolysaccharide- producing microorganisms usually reach their optimal growth within the initial 24 h of incubation, whereas, maximal exopolysaccharide production occurs in the later stages of growth (e.g. during the stationary phase) (Morin, 1998). Other factors such as the presence of endogenous glycanolytic enzymes may affect the characteristics of the exopolysaccharide. Some polysaccharides are indeed subjected to enzyme

hydrolysis during the late phase of growth (Kumar *et al*., 2007). This degradation contributes to the reduction in viscosity of the culture medium. Such types of microbial enzymes (alginase) are reported in case of alginate production. Sutherland (1982) stated that there is a competition between EPS and cell-wall polymer (peptidoglycan, teichoic acids, lipopolysaccharides) biosynthesis for the isoprenoid glycosyl lipid carriers, and consequently EPS production is not growth-associated. Petry *et al*. (2000) reported majority of the EPS production during the stationary phase for two *Lactobacillus delbrueckii* subsp. *bulgaricus* strains. In case of a batch experiment with *Alteromonas macleodii* subsp. *fijiensis*, isolated from a deep sea vent, the production of EPS began at the end of the exponential phase and continued throughout the stationary phase, reaching a value of 6.0 g (dry weight) per liter at the end of 60 h (Raguenes *et al*., 1996). The size and form of the producing cells may vary during exopolysaccharide production (Kumar *et al*., 2007). *Sclerotium glucanicum* and *Sclerotium rolfsii* tend to grow as pellets surrounded with a layer of scleroglucan, which reduces production of this

polymer, possibly because of a reduced mass transfer rate to/from the cells.

2.7.10. Osmolarity

Osmolarity plays crucial role in both, growth and exopolysaccharide production (Kumar *et al*., 2007). Fast-growing *Rhizobium melilotii* produces two different water-soluble exopolysaccharides, namely succinoglycan and galactoglucan, the ratio of which is influenced by the osmolarity of the culture medium. In the absence of sodium chloride in the medium, the ratio of both Exopolysaccharides was 1:1. Upon increasing osmolarity of the culture medium up to 0.6 M NaCl, the proportion of sucinoglycan increased to 85% (Navrini *et al*., 1992).

2.7.11. Detergents

The presence of detergents influences the production of xanthan (Morin 1998). Effect of detergents such as Tween 40 (polyoxyethylene sorbitan monopalmitate) or Tween 80 (Polyoxyethylene sorbitan monooleate), CHAPS (3-[(3-cholamidopropyl) dimethyl ammonio]-1- hydroxypropane- sulfonate and Triton X-100 (nonaethylene glycol octylphenol ether) has been investigated by adding them after 24 h of growth of *Xanthomonas campestris*. An increase of 1.45 fold in xanthan production was observed at a low concentration (0.1 g/L) of Triton X-100. Here no severe foaming was observed because detergents were added after 24 h of growth when viscosity was already high. The addition of Triton X-100 improved xanthan production probably by altering the oxygen transfer rate and had no effect on the biomass production and broth viscosity. The addition of such detergents could affect factors controlling the rheological quality of xanthan such as its Mr (molecular weight). It has been suggested that, by interacting with

the *Xanthomonas campestris* membrane, detergents could enhance the polymerization process or the release of xanthan (Morin, 1998).

2.7.12. Carbon/nitrogen ratio (C/N or C : N ratio)

Carbon (C), nitrogen (N), phosphate (P) and oxygen (O) limitations are few of the factors that affect the conversion of the carbon source into polysaccharide(Kumar *et al*., 2007). For instance, N, C and O limitations affected the conversion of glucose into alginate and the proportion of mannuronate to glucuronate residues in *Pseudomonas mendocina*. Exopolysaccharide production is favored by a high carbon/nitrogen ratio, where 10:1 is considered to be the most favorable for maximal Exopolysaccharide production. Disappearance of nitrogen from the medium might also be a signal for exopolysaccharide synthesis, as observed for pullulan and scleroglucan (Morin, 1998).

2.8. ISOLATION AND PURIFICATION OF EPS

The cost of recovery of exopolysaccharide, including concentration, isolation and purification, is a significant part of the total production cost. Isolation of EPS generally presents few problems when it is secreted as an extracellular slime. The lack of physical attachment between polysaccharides and cell enables the use of differential centrifugation. Due to lower concentration of exopolysaccharide in the fermentation broth, the presence of contaminating solid (e.g. cells), solutes in the stream and the high viscosity of the fermentation liquid/broth creates separation problems. If the polysaccharide is in the capsule form it must be detached from the bacteria cell. Gentle stirring or mixing in a homogenizer may suffice, but sometimes more drastic procedures may have to be employed to remove. Finally, the EPS from the culture medium is precipitated by ethanol. Williamson (1959) reported the isolation of EPS produced by *Lactobacillus* species isolated from beer. The organism

was grown inside a sterile sac of dialysis tubing in a large volume of the medium. After growth, the concentrated culture was centrifuged and supernatant liquid dialyzed against running tap water for 3-4 days. The EPS was isolated by ethanol precipitation (2 Vol.) and the precipitated EPS was washed several times with 70% ethanol. Isolated EPS was then re-dissolved in distilled water and re-precipitated with ethanol (2 vol.) and freeze dried (Dunican and Seeley, 1965). Polysaccharides were precipitated from the cell free supernatant culture fluid (MRS broth) by two volumes of chilled 95% ethanol and kept overnight at 4ºC Two additional precipitations with ethanol and one with acetone (2 vol.) were sufficient to give relatively protein free samples of dextran.

Sharpe *et al*. (1972) reported the isolation and purification of slime produced by heterofermentative lactobacilli in MRS broth medium. EPS was isolated from cell free broth with three volume of ethanol and purified. Viscous matter was isolated from the culture medium (milk, whey, semi-synthetic medium) by precipitation with ethanol followed by the removal of proteins (Romanskaya and Dyment, 1982). Manca de Nadra *et al*. (1985) reported the isolation of EPS from the culture broth of *Lactobacillus bulgaricus* by ethanol precipitation.

Ariga *et al*. (1992) used the method of sonication (9 KHz, 200 W/10) for obtaining the polysaccharides of the encapsulated cell. The simplest procedure involves dialysis against water of the cultured medium (after cell removal by centrifugation) followed by lyophilization. This technique was used to isolate EPS from some *Lc. lactis* spp. *cremoris* strains grown in chemically defined media (Marshall *et al*., 1995 Van Kranenberg *et al*., 1997). Ethanol precipitation may be used to concentrate the EPS before dialysis for the isolation of EPS from thermophilic (yoghurt starter *Lb. delbrueckii* spp. *bulgaricus* and *Stre. thermophilus*) and mesophilic (lactococci and lactobacilli) LAB strains (Petry *et al*., 2000; Van Geel-

Schutten *et al*., 1999; Dal Bello *et al*., 2001; Degeest *et al*., 2001; Rimada and Abraham, 2001; Ricciardi *et al*., 2002). The most common procedure used for isolation from complex media involves TCA precipitation and protein removal by centrifugation, followed by concentration of the EPS by ethanol precipitation (Garcia-Garibay and Marshall, 1991; Cerning *et al*., 1994; Dupont *et al*., 2000; Frengova *et al*., 2000; Pham *et al*., 2000; Van Calsteren *et al*., 2002; Harding *et al*., 2003; Desai *et al*., 2006; Purwandari and Vasiljevic, 2009). The yields obtained by this method showed a maximum coefficient of variation between duplicates of 5 to 10%. Some workers have used enzyme pronase E (protease type XIV) from *Streptomyces griseus* to digest milk protein in culture medium. Pronase Enterococci has a wide range of substrate specificity and was used to isolate EPS produced by thermophilic yoghurt starters (Cerning *et al*., 1986, 1988; Mozzi *et al*., 1995; Bouzar *et al*., 1996, 1997) or mesophilic strains (Cerning *et al*., 1992; Mozzi *et al*., 1996; Torino *et al*., 2000, 2001). After heat inactivation of the pronase and concentration, the EPS fraction was precipitated with ethanol which showed a coefficient of variation of approximately 10%. Apart from protein removal and EPS precipitation, other procedures that have been used to purify the EPS fraction include membrane filtration techniques such as microfiltration, UF, and diafiltration (Tuinier *et al*., 1999b; Yanhg *et al*., 1999, 2000; Staaf *et al*., 2000; Levander *et al*., 2001).

The crude preparation of EPS obtained by ethanol precipitation (half volume) of cell free supernatant from a whey culture grown *Lactobacillus helveticus* var. *yoghurti* was purified by DEAE cellulose column chromatography with a 92.5% yield (Oda *et al*., 1983). Small amounts of proteins contaminating the polymer were removed by second DEAE step and CM cellulose column chromatography. Ethanol precipitated polysacchrides from milk culture containing *Streptococcus salivarius* subsp. *thermophilus* was purified by ion

exchange chromatography followed by gel filtration on Sephacryl S-1000 (Doco *et al*., 1990). Nakajima *et al*. (1990) reported that slime material obtained from culture supernatant in whey permeate medium contained 42% carbohydrate and 21% protein. With the insufficiency of separation of polysaccharides and protein, preparative SDS-PAGE purified polysaccharides with less than 0.5% protein. Doco *et al*. (1991) gave a rapid method for isolation and estimation of polysaccharides from skim milk fermented with *Streptococcus salivarius* subsp. *thermophilus* by coupled anion exchange and gel permeation high performance liquid chromatography. Polysaccharides produce by *Lactobacillus delbrueckii* subsp. *bulgaricus* in milk was isolated by treating it with 17 % (v/v) of 80 % TCA and centrifugation at 16000 rpm for 30 minutes (Garcia-Garibay and Marshall, 1991). Polymer was precipitated from the supernatant fluid by three volumes of ethanol and suspended in water (pH 4.0) and solution filtered through Whatman number 4. Similarly, Racine *et al*., 1991) reported that the polysaccharides produced by *Propionibacterium acidi-propionici* on whey based medium could be isolated by alcohol precipitation of the supernatant fluid.

Rimada and Abraham (2003) compared different EPS isolation procedures reported in the literature for recovery of EPS produced by *Lactobacillus kefir* in milk and deproteinized whey (DPW). For the isolation of kefir polysaccharide from milk and DPW, they reported higher EPS yield as compared to milk medium. Forty seven EPS producing mesophilic lactic acid bacteria were isolated from Dahi and raw milk by Behare *et al*. (2008) using DPW medium. Better yield of carbohydrate was obtained from *Lc. lactis* subsp*. lactis* B-6 and KT-24 by following ethanol precipitation. Purification by DEAE-cellulose ion exchange led to pure EPS with greater than 99.5% carbohydrate with negligible amount of protein content. Deproteinized whey based medium was used by Kodaikkal (2007)

for the isolation of EPS from the four strains of *Lactobacilli* from the culture collection of Dairy Microbiology Department, SMC college of Dairy Science, Anand Agricultural University, Anand. Using ethanol precipitation, the concentration of EPS released by individual strains of *L. acidophilus* ranged from 138.80 to 229.56 mg l^{-1}.

2.9. CHARACTERIZATION OF EPS

Detailed studies have been carried out by various workers to elucidate the structure and composition of slime. Williamson (1959) studied the chemical composition of slime produced by lactobacilli causing ropiness in beer and concluded that the extracellular slime of the strain usually consisted of dextran like polysaccharides (of mainly glucose units), nucleic acid being present only in certain cultures. Of the extracellular polysaccharides synthesized by the members of the genus *Lactobacillus*, homopolysaccharides glucan was synthesized using sucrose as the sole substrate (Dunican and Seeley, 1965). Purified glucan consisted of only one sugar glucose. Slimes from some heterofermentative species of the genus *Lactobacillus* have been studied by Sharpe *et al*. (1972). Chemical compositional studies indicated that glucose was the sole constituent sugar of all these polymers. Further treatment of these purified polysaccharides with dextranase indicated that the polysaccharide contained high proportion of α-1-6 glycosidic linkages and therefore were probably dextrans. Tamime and Robinson (1978) recovered polysaccharides from a yoghurt starter culture which was reported to be glucan as glucose was the only monomer detected by chromatography.

Characterization of the polysaccharides from *Lactobacillus brevis* revealed 98 to 99% carbohydrate and 0.045-0.065 % protein nitrogen. Acid hydrolysis and specific optical rotation values showed that these polymers are composed of α-D-glucose. Sabatie *et al*. (1988) stated that the comb like structure was too simple and

believed that there was a stronger evidence for a ramified structure. The chemical differences in various polysaccharides reflected the complexity of EPS synthesis from sucrose. Many factors including the type of growth media, incubation time, sucrose concentration and the presence of polysaccharides degrading enzymes probably influence the molecular weight and structure of these polymers.

Groux (1973) investigated the composition of slime secreted by *Lactobacillus bulgaricus* and reported that galactose was the most frequently encountered monomer within the polysaccharide. Arabinose, mannose and glucose were also present. Ranganathan *et al*. (1979) studied the composition of the slime produced by a strain of *Lactococcus lactis* and detected mannose, rhamnose and manuronic acid in the ropy material.

Romanskaya and Dyment (1982) showed that viscous extracellular polymer formed by *L lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L lactis* subsp. *diacetylactis* were carbohydrate-protein complexes and carbohydrate polymer included glucose, galactose and rhamnose. Oda *et al*. (1983) investigated the chemical composition of EPS produced by *Lactobacillus helveticus* var. j*oghurti* which was only composed of glucose and galactose monomers in the ratio of 1:2. The polymer had a high molecular weight and showed antitumor activity. Schellhaas (1984) characterized the extracellular slime produced by the bacterial starter and showed that exopolymers had a monosaccharide composition of galactose and glucose in 2:1 ratio as determined by GLC. Manca de Nadra *et al*. (1985) characterized extracellular polysaccharides of *Lactobacillus bulgaricus* strain isolated from yoghurt. It was constituted by glucose and fructose in 1:2 ratio. The predominant linkages were α-1, 4 and α-1,-6 glucosidic in a ratio of 1:1 and molecular size was 197,400. The polymer was composed of approximately 1000 hexose molecule. Cerning *et al*. (1986) isolated and characterized the EPS produced by *Lactobacillus bulgaricus* and

reported the water-soluble heteropolysaccahrides to be composed of galactose, glucose and rhamnose in an approximate molar ration of 4:1:1. The molecular weight of the polymer was about 500,000 daltons, and the intrinsic viscosity was 4.7 dl/g, indicating that the polymer had remarkable thickening properties.

Characteristics of polysaccharides produced from kefir grains were found to be similar to those of an encapsulated homofermentative *Lactobacillus* species, but different from that of *Lactobacillus kefir* (Toba *et al*., 1987). Polysaccharides from kefir grains and Lactobacillus were composed of D-glucose and D-galactose in a ratio of 1.0:0.96-1.09 whereas those from *Lactobacillus kefir* were in the ratio of 1.0: 0.74. It was concluded that an encapsulated homofermentative *Lactobacillus* is mainly responsible for polysaccharides production in the grains. The EPS produced by a *Streptococcus thermophilus* strain grown on skim milk was essentially composed of galactose and glucose as suggested by HPLC and GC studies (Cerning *et al*., 1988). Small amounts of xylose, arabinose, rhamnose and mannose were also identified. Doco *et al*. (1990) determined structural elements of an EPS produced in skim milk after only 3-4h of incubation. The polymer was composed of galactose, glucose and N- acetylgalactosamine in the ratio of 2:1:1. Its molecular weight was $1x10^6$ Daltons

Racine *et al*. (1991) characterized the polymer produced by *Propionibacterium acidi-propionici* on whey based media. The polysaccharides containing fractions were composed of glucose, galactose, mannose, rhamnose and fructose and had molecular weight of less than 5800 Dalton. Such a low molecular weight may explain the low viscosity value obtained as the length of the polysaccharides chain influences its rheological properties. Strains of *Streptococcus salivarius* subsp. *thermophilus* grown on partially deproteinized whey produced extracellular polysaccharides which contained L-rhamnose and D- galactose in the ratio of 1:1.47 (Ariga

et al., 1992). Methylation analysis showed the existence of 3 and 6 substituted D-galactose, 2-substituted L-rhamnose and non reducing terminal D-galactose. A Polysaccharide was also obtained from the cell surface after sonication. Molecular weights of both polysaccharides were determined to be a 9,000,000 and 1,100,000 Daltons by gel filtration method. Cerning *et al*., (1992) isolated and characterized the heteropolysaccharides produced by ropy mesophilic *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus casei* subsp. *casei* in milk and ultrafiltrate. The polysaccharides primarily consisted of galactose and glucose with the former predominating. However, small amounts of mannose, rhamnose and pentoses were also identified. Addition of either glucose or sucrose to both milk and ultrafiltrate modified the monosaccharide composition.

Marshall *et al*. (1995) recovered two types of EPS, charged and neutral from *Lactococcus lactis* subsp. *cremoris* LC 330. Gas chromatography of the o-methyloxime acetate derivatives showed that the charged polysaccharide was composed of glucose, rhamnose, galactose and glucosamine in an approximate ratio of 6:5:4:1. The neutral polysaccharide had a similar sugar content of glucose, galactose and glucosamine in an approximate ratio of 6:3:2. The carbohydrate content of the EPS isolated from *Lactococcus lactis* subsp. *lactis* PM-23 was 0.932 mg/mg of EPS which was composed of galactose and glucose (2:1) as determined by HPLC (Prashar, 1996). Faber *et al*. (2002) used GLC to determine monosaccharide composition of EPS produced by *S. thermophilus* 8S. The analysis revealed that the EPS contained galactose, glucose, ribose and N-acetyl-D-galactosamine in a molar ratio of 2:1:1:1. Savadogo *et al*. (2004) reported that glucose and galactose were the dominating sugars with small amounts of rhamnose, mannose, fructose, arabinose and xylose in EPS obtained from different LAB species.

All strategies for the structural determination of bacterial poysaccharides include preliminary depolymerization by total or partial acid hydrolysis with trifluoroacetic acid, HCL or H_2SO_4 at 100ºC to 120ºC for 2 to 8 h. These treatments yield mono or oligosaccharides that are frequently derivatized in to alditol acetates, trimethylsilylated (-)-2-butyl glycosides. The quantitative monosaccharide composition of an EPS has been analyzed in the past by TLC (Cerning *et al*., 1994; Lemoine *et al*., 1997). However this method has low discriminatory power and has been largely surpassed by more reliable liquid and gas chromatographic techniques. The qualitative and quantitative determination of EPS monosaccharides by HPLC involves the separation of monosaccharides by anion-exchange columns and detection by RI. Isocratic separations at 35 to 70ºC using 2.5 m*M* sulfuric acid as eluent were used to analyze the monomer composition of EPS produced by *Lactobacillus rhamnosus* (Van Calsteren *et al*., 2002) *Lb. delbruckii* spp. *bulgaricus* (Grobben *et al*., 1995) and *Str. thermophilus* strains (De Vuyst *et al*., 1998). Another technique used for the identification and quantification of mono- and oligosaccharides resulting from the partial hydrolysis of EPS is the high-performance anion-exchange chromatography pulse amperometric detection (HPAEC-PAD) (Gruter *et al*., 1992; Lemoine *et al*., 1997; Levander *et al*., 2001). Both HPLC-RI and HPAEC-PAD are liquid chromatographic methods that differ in sensitivity and accuracy (Cataldi *et al*., 2000). The most extensively used technology for the analysis of the monomer composition of EPS isolated from LAB is gas chromatography/mass spectrometry (GCMS). The monomeric composition of several EPS produced by different lactic acid bacteria strains such as *Lc. lactis* spp. *cremoris*, *S. thermophilus*, *Lb. delbruckii* spp. *bulgaricus*, *Lb. helveticus*, *Lb. sake*, *Lb. reuteri*, *Lb. rhamnosus* was determined using the GCMS

method (Marshall *et al*., 1995, 2001a; Roberts *et al*., 1995; Van den Berg *et al*., 1995; Bouzar *et al*., 1996, 1997; Van Geel-Schtten *et al*., 1999; Petry *et al*., 2000, 2003 Yang *et al*., 2000; Lipinski *et al*., 2003;).

2.10. FUNCTIONAL PROPERTIES OF EPS

Bacterial EPS influence the texture and rheology of fermented milk products at extremely low concentration so, understanding the nature and molecular characteristics of the polymer would help in determining its effect on physical properties (Tuinier *et al*., 1999a; Tuinier *et al*., 1999b; Kleerebezem *et al*., 1999). Most EPS are random coils with no fixed shape; they have randomly fluctuating tertiary structure. In most cases, EPS contributes to the thickening of a final product, which depends on the viscosifying ability of that polymer. The viscosifying ability of EPS in solution can be determined by some parameters like intrinsic viscosity, the specific volume occupied by the dispersed particle and the concentration of dispersed polymer. (Tuinier *et al*., 1999b). The specific volume of EPS in solution is determined by its molecular mass and its “radius of gyration” which is a measure of the size of the polymer in solution (Measure in nm). To obtain higher intrinsic viscosity, the molecular characteristics, either the molar mass (chain length) or the stiffness of the polysaccharide must increase (Laws and Marshall, 2001).

The lactic strains producing the exopolysaccharides with greater chain length may produce more viscous product. Two strains of *Strep. Thermophilus,* Sts and Rs, although produced similar amounts of EPS in milk: 135 mg l^{-1} for Rs and 127 mg l^{-1} for Sts , exhibit different viscosity values (measured using the Posthumus method, which measures flow rate) 39 and 126 respectively due to differences in molecular mass of the polymers (Faber *et al*., 1998).

The molecular mass of an exopolysaccharide of Rs strain was 2.6 × 10^6 and that of strain Sts was 3.7× 10^6 Da which produced more viscous product than former strain Rs. The *β* (1→4) linkages in the backbone of the polymer lead to stiffer chains whereas *β* (1→2) or *β* (1→3) linkages and α linkages lead to more flexible chains (Bianchi *et al*., 1986; Rees, 1977). The *L. lactis* subsp. *cremoris* B40 strain showed higher intrinsic viscosity and thickening efficiency because of the stiffer chains *β* (1→4) present in the backbone of EPS (Ruas-Madiedo *et al*., 2002). On the other hand the exopolysaccharide of *Strep. thermophilus* Sfi20 , which has two *β* sugars and one α sugar with 1→3 linkages in its backbone, suggested that the polymer was flexible (Navarini *et al*., 2001).

Ludbrook *et al*. (1997) isolated EPS from LAB of non dairy origin and assessed for their thickening properties by preparing 0.3% (w/v) EPS solution. The average intrinsic viscosity of the solution was found to be very low (ranged from 1 cps to 6 cps) and did not contribute to the thickening effect under specified conditions. Some authors has reported a direct correlation between the concentration of EPS and viscosity of stirred yoghurt, but no clear relation has been demonstrated (Van Marle and Zoon, 1995; Dupont *et al*., 2000) except that if a given strain produces more EPS the viscosity of fermented milk will increase (Sebastiani and Zelger, 1998; Ruas-Madiedo *et al*., 2002). The EPS with intrinsic viscosity below 1.5 M Kg^{-l} is not able to increase the viscosity of fermented milk (Ruas-Madiedo *et al*., 2002). De Vuyst *et al*., (2003) attempted to interpret the contribution of heteropolysaccharides to structure/function relationship of fermented milk. They observed no clear cut relationship between amount of EPS produced and viscosity of fermented milk. However, they stated that for high intrinsic viscosity, stiffer chains are required. In addition, the complexity of

the primary structure (size, monomer composition and side groups α- and *β*- linkages, branching) will influence the viscosifying effects of EPS solution (Yang *et al*., 2000).

2.11. APPLICATIONS OF EXOPOLYSACCHARIDES

EPS is produced by the majority of Gram-negative bacteria, some of which invest more than 70% of their energy in its production (Harder and Dijkhuizen., 1983). Many bacteria secrete polysaccharides that can have diverse functions, such as virulence factors, signaling molecules in bacterium–plant interactions, or protective agents. Some of these polymers have unique properties for food applications and are used as viscosifiers, stabilisers, emulsifiers or gelling agents (Table 2.2).

Table 2.2 Applications of bacterial polymers

Organism	Polysaccharide/biopolymer	Application
Leuconostoc mesenteriodes	Dextran	In veterinary medicine, in human medicine as blood plasma extender or blood flow improving agent and as cholesterol lowering agent, in separation technology, as molecular sieve and in aqueous two phase systems, as micro-carrier in tissue/cell culture (cross-linked dextran)
Pseudomonas aeruginosa and *Azotobacter vinelandii*	Alginate	As immobilization matrix for viable cells and enzymes, coating of roots of seedlings and plants to prevent desiccation, micro-encapsulation matrix for fertilizers, pesticides and nutrients, hypo-allergic wound-healing tissue
Acinetobacter calcoaceticus	Emulsan	-same as above-
Sphingomonas paucimobilis	Gellan	As stabilizer and suspending agent for foods. As gelling agent for solidifying culture media, especially for studying marine microorganisms
Streptococcus equii and *Streptococcus zooepidemicus*	Hyaluronic acid	As replacer of eye fluid in ophthalmic surgery, in artificial tear-liquid, synovial fluid replica, in wound healing, cosmetic industry (lotions, moisturizing agent)
Xanthomonas	Xanthan (E 415)	As viscosifier, stabilizer, emulsifier and suspending agent for food. In secondary and tertiary crude-oil recovery, in paints, pesticide and detergent formulations, pharmaceuticals, cosmetics, printing inks (to control viscosity, settling and gelation), in food as thickening and stabilizing agent, often used in combination with guar gum
Acetobacter spp.	Cellulose	In human medicine as temporary artificial skin to heal burns or surgical wounds, in nutrition as natural non-digestible fibers (which can be impregnated with amino acids, vitamins and minerals), as hollow fibers or membranes for specific separation technology, as acoustic membranes in audio-visual equipment
Rhizobium meliloti and *Agrobacterium radiobacter*	Curdlan	As a gelling agent, immobilization matrix, Curdlan along with zidovudine (AZT), displays promising high antiretroviral activity (anti AIDS-drug)
Alcaligenes faecalis var. *myxogenes*	Succinoglycan	-same as above-
Acetobacter	Acetan	As viscosifier and gelling agent for food.

xylinum		For production of sweet confectionary and vinegar.

(Kumar *et al*., 2007; Van Kranenburg *et al*., 1999)

2.11.1. *In vivo* applications

The precise role of the exopolysaccharide in exopolysaccharide producing bacteria, evident in different ecological niches, is dependent on the natural environment of the microorganism. Most of the functions ascribed to exopolysaccharide are of a protective nature. The ability of a microorganism to surround itself with a highly hydrated exopolysaccharide layer may provide it with protection against desiccation and predation by protozoans (Kumar *et al*. 2007). Also, the presence of a gelled polysaccharide layer around the cell may have paramount effects on the diffusion properties, both into and out of the cell (Dudman, 1977). As discussed in several reviews, many functions have been proposed for bacterial EPS (Table 2.3). They can be divided into four groups, functioning: a) as a physical protective barrier; b) as a response to environmental stress; c) in cell/cell recognition and interaction; or d) in biofilm formation/adhesion (Hall-Stoodley *et al*., 2004; Kodaikkal, 2007; Kumar and Anand, 1998; Weiner *et al.,* 1995).

Table 2.3 Exopolysaccharide and cell survival

Function	Survival advantage
Physical/protective barrier	Protection from desiccation, predation and the immune system. Resistance to toxins, antibiotics and poisons
Cell-cell recognition and interaction	Plant symbiosis, formation of nodules and microcolonies, invertebrate larvae settlement
Response to environmental stress	Sequestering and import of charged ions, production of excess reducing power
Adhesion and biofilm formation	Immobilization onto nutrient-rich surfaces, dissociation from nutrient-depleted surfaces, allows cells to survive over a wide range of

	different and extreme environmental conditions, e.g. at temperatures ranging from -5 to 120^{0}C, at pH values from 0 to 13 and at pressures as high as 100MPa.

(Weiner *et al.,* 1995; Denkhaus *et al*., 2007)

The production of exopolysaccharide in the form of capsules is eminent in pathogenic bacteria, wherein the pathogenicity of an organism depends on the rate of synthesis and the amount of Exopolysaccharide synthesized. Capsules enable evasion of phagocytosis. A noteworthy fact is that all capsular polysaccharides do not activate the immune system, which is due to the fact that their chemical structures may mimic the host cell surface components (Kumar *et al*. 2007). The lectins (polysaccharide binding proteins secreted by the plant, (e.g. Trifolin A) play a crucial role in the establishment of the symbiotic association between *Rhizobium* spp. and leguminous plants (Vanhooren and Vandamme 1998).

Exopolysaccharides play a principal role in the formation of cell aggregates, initiation of flocculation and similar processes (Kumar *et al*. 2007). This property is vital for wastewater treatment and soil aggregation (Sutherland, 2002). The presence of exopolysaccharide in adherent biofilms on inert and biological surfaces has been recognized for some time. However, the widespread incidence of these biofilms and their commercial implications in microbiological problems are as divergent as fouling of pipelines and the onset of dental caries (Vanhooren and Vandamme 1998).

Thus, EPS producing lactic acid bacteria are high in demand for the food industries in recent years because of their contribution to the rheology and texture properties of food products (Cerning & Marshall, 1999; Ruas-Madiedo & Reyes-Gavilan, 2005). Dahi is a popular fermented milk product of India, similar to yoghurt, consumed in almost every household (Prajapati & Nair, 2003) with

3.5-8% fat. Health awareness among consumers generated more demands for fat-free Dahi in the Indian dairy market. However, milk fat contributes to the flavour, body and texture development of the dairy products, removal leads to textural and functional defects in low fat yoghurt, cheeses, dahi (Behare *et al*., 2009). In case of low fat yoghurt and dahi, a lack of flavour, weak body and poor texture is the major problem (Haque & Ji, 2003; Guven *et al*. 2005). In this context, EPS producing LAB as 'biothickeners' can offer natural and more acceptable solution and can be the preferred approach to many additives (Chistiansen *et al*. 1999; De Vuyst *et al*. 2003). These cultures meet the consumer requirement for products with low levels of chemical additives (De Vuyst *et al*. 2001; Jolly *et al*. 2002), reduce the amount of total solids required without affecting the textural attributes (Wacher-Rodarte *et al*. 1993; De Vuyst *et al*. 2003) and improves sensory properties (Folkenberg *et al*. 2006). Low fat dahi made using different EPS producing cultures of *L. lactis* subsp. *lactis* PM23, *Strep. thermophilus* ST and *L. lactis* NCDC 191 found to be more acceptable in terms of body, texture and flavour as compared to dahi made with EPS negative culture NCDC 167. The microstructural studies showed that dahi made with EPS^{+} strains had more open structure and pores with discontinuous casein matrix than the controlled dahi, which had relatively compact linear structure (Praveen, 2000). To overcome the defects of low fat cheeses, manufacturers have used texture promoting or ropy cultures for many years particularly where addition of stabilizer is prohibited. These cultures may impart higher flavour intensity of the yoghurt due to the carbohydrate masking the flavour, mouth feel and other attributes may also be affected. To reduce the amount of added milk solids, to improve yoghurt viscosity, to enhance texture and mouthfeel and to avoid syneresis during fermentation or upon storage of the fermented milk products, EPS producing functional

starters are interesting. The apparent viscosity of skim milk gel made by both ropy cultures was increased as compared to that made by non-ropy cultures (Behare *et al*., 2009). Further they reported that combining two ropy cultures for yoghurt manufacture may not have always, additive effect.

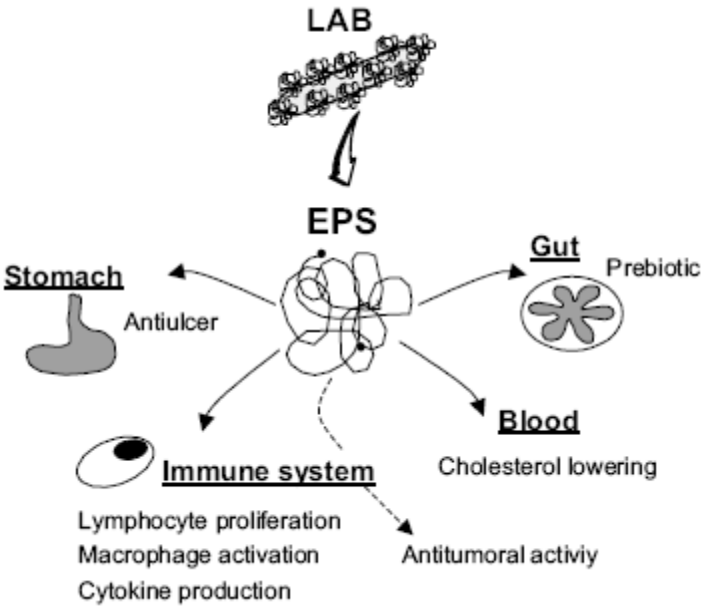
Besides yoghurt and cheeses, the other fermented milk products in which EPS cultures have been shown to affect product rheology are sour cream, kefir, dahi and European cultured dairy products. Use of slime producing *Strep. thermophilus*, strains greatly improved rheological properties of cream turo and number of other Hungarian cultured milk and cultured cream products (Obert, 1984). It is traditional self-carbonated slightly alcoholic fermented milk from Eastern Europe (Roginski, 1999; Tamime and Robinson, 1999). Kefir is prepared by kefir grains which consist of homofermentative and heterofermentative LAB, yeasts and acetic acid bacteria. These cells are embedded in kefiran, a slimy polysaccharide, which also found to affect texture of kefir (Micheli *et al*., 1999; Duboc and Mollet, 2001).

2.11.2. In vitro applications

The phenomenal demand for natural polymers for various industrial applications has led to a vibrant interest in exopolysaccharide production by microorganisms (Kumar *et al*. 2007). In recent years, there has been a substantial interest in the isolation and identification of new microbial polysaccharides that might have innovative uses as gelling agent, emulsifier, stabilizer or texture enhancing agent (Sutherland, 2001). Production of exopolysaccharide by a moderately halophilic bacterium has been reported by Iyer *et al*. (2001)**.**

2.11.2.1 Medicinal applications

Although having no taste of their own, the EPS from LAB increase the residence time, the milk products spend in the mouth, and hence impart an enhanced perception of taste (Duboc & Mollet, 2001). An additional hypothesized, physiological benefit is that EPS will remain longer in the gastro-intestinal tract, thus enhancing the colonization by probiotic bacteria (German *et al*., 1999).



Unlike the EPS produced by other microorganisms, many health benefits such as antiviral, antitumor (Kitazawa *et al*., 1998), cholesterol lowering ability (Pigeon, Cuesta, & Gilliland, 2002) and immunostimulatory activity (Chabot *et al*., 2001) have been attributed to EPS produced by LAB (Vijayendra *et al*., 2008). A low molecular weight heparin-like exopolysaccharide exhibiting anticoagulant property has been isolated from *Alteromonas infernus*, obtained from deep-sea hydrothermal vents (Colliec *et al*., 2001). Clavan, an L-frucose containing polysaccharide has a potential application in preventing tumor cell colonization of the lung, in controlling the formation of white blood cells, in the treatment of the rheumatoid arthritis, in the synthesis of antigens for antibody production and in cosmeceuticals as skin moisturizing agent (Vanhooren and Vandamme, 2000).

2.11.2.2 Gelling agent

Gelrite, obtained from *Pseudomonas spp*., is a new gelling polysaccharide with good thermal stability and clarity. It has been reported that gelrite is superior to agar (Lin and Casida, 1984). It forms a brittle, firm and optically clear gel upon deacetylation using mild alkali (Kang *et al*., 1982).

2.11.2.3 Emulsifiers

Surfactants and emulsifiers from bacterial sources have attracted attention because of their biodegradability and possible production from renewable resources. Emulsan produced by *Acinetobacter calcoaceticus* RAG-1 has been commercialized (Rosenberg *et al*., 1979). A viscous Exopolysaccharide has also been isolated from *Sphingomonas paucimobilis*. This polysaccharide stabilized emulsions more effectively than other commercial gums such as arabic, tragacanth, karaya and xanthan (Ashtaputre and Shah, 1995). Apart for Emulsan, an Exopolysaccharide produced by a marine bacteria, is reported to form stable emulsions with a number of hydrocarbons. This exopolysaccharide proved to be more efficient than the commercially available emulsifiers (Iyer *et al*., 2006).

2.11.2.4 Heavy metal removal

Contamination of the environment by heavy metals is of growing concern because of the health risks posed to humanity and animals. Cell bound polysaccharide produced by marine bacterium, *Zooglea sp*., has been reported to adsorb metal ions like chromium, lead and iron in solutions (Kong *et al,.* 1998). Biosorption of heavy metals by *Enterobacter cloaceae* is reported by Iyer *et al*. (2004, 2005a). However more work is required to confirm this property.

2.11.2.5 Enhanced oil recovery

The in situ production of xanthan- like polysaccharide in the oil-bearing strata has been suggested as a means of aiding tertiary oil recovery (Wells, 1977). *Volcaniella eurihalina* F2-7 is known to synthesize an exopolysaccharide, the rheological properties of which are stable to pH and inorganic salts, which makes it a suitable candidate for enhanced oil recovery (Calvo *et a.l*, 1995). Exopolysaccharide produced by *Enterobacter cloaceae* has been reported to have good viscosity even at high tem perature, which makes it a probable candidate for microbial enhanced oil recovery (Iyer *et al*., 2005b).

2.11.2.6 Source of monosaccharides

Certain bacterial extracellular homo- and hetero-polysaccharides are source materials to obtain unusual but valuable monosaccharide constituents e.g. L-fucose, L-rhamnose, L-altrose, D-mannose etc. which are otherwise difficult to obtain because chemical synthesis or extraction from plant or animal tissues is laborious, expensive and often in scant supply. *Clavibacter species* produce clavan, which is rich in D-fucose (Vanhooren and Vandamme, 2000). The Exopolysaccharide (containing 18.9% w/w L-fucose) produced by *Klebsiella pneumoniae* is a source of L-fucose (Vanhooren and Vandamme, 1998). Iyer *et al*. (2005c) reported the production of exopolysaccharide by *Enterobacter cloaceae* which is also rich in fructose.

Thus microbial EPS has multi-functional role in food and health.

CHAPTER – III

MATERIALS AND METHODS

The present study was planned to characterize new isolates of Lactic Acid Bacteria and their exopolysaccharides from various food sources. Materials and Methodologies used during the entire course of study are encompassed in this chapter.

3.1. ISOLATION OF EPS PRODUCING LACTIC ACID BACTERIA

3.1.1. Collection of samples

A total of 34 samples of different vegetables, cheese and fermented foods were collected from different sources such as household and local market. All the samples (Table 3.1) were collected in sterile glass bottles and stored under refrigeration (4°C) until they were analyzed.

Table 3.1 Collection of samples for isolation of EPS producing LAB from various sources

S.N.	City	Place	Type of sample	No. of samples
A.	Anand	Local Market	Cabbage	2
			Carrot	2
			Tomato	2
			Beet root	2
			Cucumber	2
			Fresh turmeric	2
			Orange	1
			Cheese	4
			Dahi	3
			Idli batter	1
		Laboratory made	Sauerkraut	1
			Fermented cucumber	1
			Fermented carrot	1
		House hold	Dahi	3
			Idli batter	1
B.	Ahmedabad	Local market	Cheese	2
			Idli batter	1
		House hold	Dahi	2
			Idli batter	1

Total	34

3.1.2. Culture media and reagents

The composition of media and reagents utilized during the course of experimentation is given in APPENDIX.

3.1.3. Plating of samples

Ten grams of each sample was weighed individually into Erlenmeyer flaks containing 90 ml of sterile saline (0.9% sodium chloride, Qualigens fine chemicals, Baroda). The peels of vegetables shaken thoroughly to dislodge the microorganisms and subjected to serial dilution and appropriate dilutions were plated onto modified de Man Rogosa Sharpe agar (M-MRS agar, Himedia, Banglore) containing sucrose at 5% w/v concentration (Sulab reagents, Baroda) by following the pour plate method and incubated at 30°C and 37°C for 24 to 48 hours anaerobically for mesophilic and thermophilic LABs (Smitinont *et al.*, 1999; Smitinont *et al.*, 2008).

3.1.4. Selection of colonies

After incubation period, the colonies produced by EPS^+ organisms showed mucoid appearance and/or formed long filaments on M-MRS agar when touched with the sterile wire loops. They were randomly picked up and transferred in to sterile M-MRS broth as well as skim milk tubes (10 ml). The EPS producing isolates were purified by streaking the cultures three times on agar medium. The lactic acid production was checked by fermentation of skim milk leading to coagulation.

3.1.5. Maintenance of EPS producing cultures

The isolates were sub cultured once in a week using M-MRS broth as well as 10% reconstituted skim milk and stored in a refrigerator at 4^0C. The promising cultures were also preserved on slants as well as

by freeze-drying (Genesis 25XL, The Vertis Company Ltd., NY) method.

3.2 CHARACTERIZATION OF EPS PRODUCING ISOLATES

The EPS^+ isolates were characterized for phenotypic and chemotaxonomic characteristics by morphological, cultural and various biochemical tests. It was identified up to species level according to Sharpe (1979), Holt *et al.*, (1994) and Vijayendra *et al*. (2008).

3.2.1. Morphological characterization

3.2.1.1. Gram's staining

Microscopic examination of the active cultures from M-MRS broth was done after Garm's staining. The Gram's reaction, shape and arrangements were recorded.

3.2.1.2. Capsule Staining

The formation of capsule surrounding the bacterial cell by selected isolates was examined by the method given by Anthonys (1931). The procedure involves preparation of bacterial smear from skim milk culture. The smear was allowed to completely air dry without heat fixing. Few drops of crystal violet were added and kept for two minutes. The smear was then rinsed by 20% copper sulfate and air dried smear was examined under oil immersion lens. In areas where the smear was thinner, cells and capsule was easily observed. The cells were stained dark while the capsule appeared as halos around the cells.

3.3. BIOCHEMICAL CHARACTERIZATION OF THE ISOLATES UP TO GENUS LEVEL

Isolates were subjected to various tests such as growth at different temperatures, salt tolerance, gas from glucose test, sugar fermentation and other biochemical tests for identification.

3.3.1. Catalase test

An aliquot of the 24h old culture grown in M17 broth or MRS broth was transferred on to a clean glass slide and a few drops of 3% hydrogen peroxide was poured over it and observed for reaction. Presence of effervescence indicates positive catalase test which is the indication of contamination of LAB with non lactic acid bacteria.

3.3.2. Growth at different temperatures

The test isolates were inoculated @2% in M-MRS broth tubes with 5%sucrose and incubated at 15, 30, 37 and 45^{0}C for 48 h. The growth was measured in terms of increase in turbidity and change in colour of the medium.

3.3.3. Salt tolerance

Salt tolerance of test isolates was tested by growing them in 10 ml aliquots with 2, 4, 6.5 % sodium chloride. Growth was assessed by increase in turbidity and change in colour of the medium after 48 h incubation at 30^{0}C.

3.3.4. Sugar fermentation test

Only promising (six) LAB isolates (isolate no. 85, 86, 92, 138, 142, 145) selected based on the production of EPS were subjected to species level identification by sugar fermentation test.

To classify strains biochemically, isolates were subjected to Hicarbohydrate ™ Kit (KB009, Himedia) to check sugar fermentation and the results were analysed manually.

The ability of the isolate to ferment sugar was tested with API 50CHL medium and API 50CH test plates (API systems, BioMerieux, France). Bacterial cells were inoculated according to the instruction provided by the manufacturer. The APIs were incubated at 30^{0}C and reactions were observed after 24 and 48h. The analysis of the results for the species level identification was done using API LABplus software with identification database version 5.1 (BioMerieux, France).

3.4. MOLECULAR CHARACTERIZATION OF SELECTED ISOLATES

The presumptive *Lactobacillus*, *Leuconostocs* and *Weissella* isolates identified on the basis of biochemical tests were confirmed by molecular methods like 16S rRNA based amplification and its partial sequencing.

3.4.1. Polymerase Chain Reaction

3.4.1.1. Preparation of templates for colony PCR

The selected lactic acid bacteria (Isolate no. 85, 86, 92, 138, 142, 145) were grown overnight on MRS (Merck, Germany) agar plates and single isolated colonies were suspended individually in 50 µl Tris EDTA buffer (10mM Tris-HCL and 1mM EDTA) and used as templates for PCR. The isolates no. 86 and 142 could not be amplified by this method and so they were subjected to genomic DNA extraction using DNA isolation kit.

3.4.1.2. Extraction of genomic DNA

The pure cultures of LAB (Isolate no. 86, 142) were inoculated in M-MRS broth and incubated at 30^0C. An aliquot of 2 ml cultures of log phase (after 7-8 hrs) was taken in sterile eppendorf tube and harvested by centrifugation in a micro-centrifuge (Biofuge 13, Heraeus Sepatech) at 12,000 rpm for 5 min at room temperature. The supernatant was discarded carefully. The pellets from respective cultures were washed with sterile phosphate buffer and then DNA was isolated using genomic DNA extraction kit (ZR Insect or Tissue DNA Kit-5, ZYMO Research Corporation). The concentration of the isolated DNA was checked Nanodrop (Nanodrop Technologies, INC. Wilmington, U.S.A, NanoDrop® ND-1000; full spectrum U.V-Vis spectrophotometer).

3.4.1.3. Oligonucleotide Primers

16S rRNA genes were amplified by PCR of the selected lactic acid bacteria using forward primer (F8) and reverse primer (R1492) from the published literature. This primer set is frequently used for molecular diversity studies because they result in a nearly full-length 16S rDNA product and are considered universal for the domain *Bacteria* (McCaig *et al*., 1999; Lu *et al*., 2003). Oligonucleotide primers were synthesized by Thermo, Finnzymes (Finland). The properties of these primers are shown below:

Primer name	**Primer sequence 5'-3'**	**Primer size**	**GC %**
Forward primer	AGA GTT TGA TCC TGG CTC AG	20 bp	50.0
Reverse primer	GGT TAC CTT GTT AGG ACT T	19 bp	42.1

3.4.1.4. PCR Reaction

Before setting up PCR reaction, all the reagents were thawed quickly except Taq DNA Polymerase. The PCR reaction mixture for the 16S rRNA gene containing 5X GC reaction buffers (Phusion™, Finnzyme, Finland), dNTPs and primers, was prepared and distributed equally in PCR reaction tubes. The final volume of the PCR mix was adjusted to 50 µl, as shown in Table 3.3.

Table 3.2 Details of master mix used for PCR

Reagents	**Concentration**	**Volume (µl)**
Sterile Milli Q water	-	35.5 µl
Primer (Forward)	10 µM	1 µl
Primer (Reverse)	10 µM	1 µl
Reaction Buffer (Finnzymes, Finland)	5X	10 µl
dNTPs (Biolab, U.K)	40 µM	1 µl

Taq DNA Polymerase (Finnzymes, Finland)	0.02U/ µl	0.5 µl
Template DNA	17-33 ng/50 µl	1 µl
Total volume		50 µl

3.4.1.5. PCR Cycling Steps

Initial DNA denaturation was performed by Lu *et al*. (2003) at 94°C for 2 min followed by 15 cycles of denaturation at 94°C for 1 min, annealing at 54°C; which was modified to improve amplification to initial denaturation at 98ºC for 5 min. The respective denaturation, annealing and extension temperatures modified for this study was 98°C for 10s, 50°C for 25s and 72°C for 1 min. This was repeated for 30 cycles in T-Professional 96 Thermocycler (Whatman Biometra, Germany). Finally, an additional extension was given at 72ºC for 5-10 min. After the run was completed, the amplified PCR products were held at 4ºC until further used.

3.4.1.6. Detection of PCR product

Agarose gel of 0.8% (w/v) concentration was prepared by dissolving the appropriate quantities of agarose (Saveen Werner, Sweden) in 1X TAE buffer (pH 8.0) for detection of PCR product. The DNA templates(5 µl) were mixed with 2 µl of 6X tracking dye (Fermentas) and 5 µl of 1X TAE buffer and loaded to the wells of the gel along with 10 µl of 1Kb DNA marker (O'gene ruler, Fermentas) to detect the band size. Electrophoresis was carried out at 100 volts (60 mA) current for 1 h in gel electrophoresis apparatus. After completion of electrophoresis, the gels were taken out, stained with ethidium bromide (Mercury, U.S.A). The bands were visualized by UV transilluminator (Gels were photographed using Kodak EDAS 290 digital camera).

3.4.1.7. Purification of PCR products

The PCR products were purified using QIAEX ® II Gel Extraction Kit (QIAGEN). This was followed by measuring the concentration of the purified DNA using the Nanodrop (Nanodrop Technologies, INC. Wilmington, U.S.A, NanoDrop® ND-1000; full spectrum U.V-Vis spectrophotometer). A concentration of 10-50ng/µl was prepared by diluting with autoclaved Milli-Q water as per the requirement of sequencing.

3.4.2. Sequencing

The purified PCR product was sent for sequencing to GATC Biotech, Germany (www.gatc-biotech.com). Sequencing was carried out with dideoxy method (Sanger *et al*., 1981). Sequencing was conducted in both the directions using 8F as forward and 1492 R reverse primers which were used in PCR.

3.4.2.1 Sequence analysis-identification

The 16S rDNA sequences were searched against GenBank (National Centre for Biotechnology Information; NCBI (www.ncbi.nlm.nih.gov) using the Advanced BLAST bioinformatics option to perform a quick search for highly similar sequences. It is accessible from the homepage of the NCBI.

3.5. PRODUCTION OF EPS BY SELECTED LAB SPECIES

The promising isolates were used for EPS production in M-MRS supplemented with sucrose (5% w/v). One milliliter of aliquots of actively grown cultures in M-MRS broth was transferred in a flask containing 100 ml of broth. The culture flasks were incubated at 30^0C for 10-12 hours. The fermented M-MRS flasks were kept at 4^0C till EPS isolation.

3.6. EXTRACTION OF EPS

The EPS produced by different LAB species in M-MRS broth containing 5% sucrose was isolated after removal of bacterial cells and protein by centrifugation. The fermented broth was distributed in 50 ml macro centrifuge tubes and centrifuged at 11000 g using

Refrigerated Centrifuge (Spinchron-R Centrifuge, Beckman). After, following the repetitive ethanol precipitation steps (Figure 3.1) the EPS was collected and dried in the same macrofuge tubes at 42^0C and analyzed.

3.6.1. Lyopholization of EPS

The tubes containing pre-dried and cooled EPS (at -20^0C overnight) was
then kept in a freeze drying unit (Labconco Lyph-Lock®, model 7750,

Ab Ninolab, Sweden) at least for 2 days. The contents were dried under vacuum. The crude freeze-dried EPS was collected in sample bottles, weighed and kept in refrigerator until further use.

Figure 3.1 Flow diagram for isolation of EPS from LAB isolates.

Inoculate an active culture in M-MRS broth (at the rate of 1%)
⇓
Incubate at appropriate temperature 10-12 h
⇓
Fermentation was stopped by adding 2.5% v/v of Trichloroacetic acid
(80% w/w)
⇓
Incubate overnight at 4°C
⇓
Centrifuge at 11000×g at 4°C/10 min
(Protein removal)
⇓
Supernatant + 2 volumes of cold ethanol (99.5%)
⇓
Keep at 4°C for 12 h
⇓
Centrifuge at 11,000 g/15 min at 4°C
⇓
Pellet
⇓
Re-suspend in 3 volumes of Milli-Q water
⇓
Refrigerated storage overnight
⇓
Add 2 volumes of cold ethanol (99.5%) kept at -20°C

⇓

Refrigerated storage for 12h

⇓

Centrifuge at 11000xg for 15 min at 4°C

⇓

EPS pellet

⇓

Re-suspend in water volumes of Milli-Q water and
Repeat the whole process once more

⇓

Drying (42°C)

⇓

Lyophilization
(Labconco Lyph-Lock®, model 7750, Ab Ninolab, Stockholm, Sweden)

(Modified from Purwandari, 2008)

3.7. QUANTIFICATION OF EPS

The quantification of EPS was done by measuring the dry weight of the freeze dried EPS after Ethanol-TCA precipitation method.

3.8. MONOSACCHARIDE COMPOSITION OF LACTIC ACID BACTERIAL EPS

Mixture of reference standards glucose, galactose, rhamnose and fructose were separated over high performance liquid chromatography (HPLC) under the standardized analytical conditions as follows:

Column and its Dimensions: Phenomenex Spherisorb-NH2 (25 cm x 4.6 mm)

Column Temperature : 90°C

Detector : Refractive Index Detector (RID)

Detector Temperature : 35°C

Mobile Phase : 90% aqueous acetonitrile

Injection Volume : 100 µl

Software Used : Waters Millenium 32 Software

Monosaccharide compositions of the LAB EPS were determined by hydrolyzing the material as per the method given by Smitinont *et al.* (1999). For the sugar analysis the 100 mg of dried EPS was dissolved in 10 mL of 1 N H_2SO_4 in a tightly sealed test tube. The solution was incubated at 100^0C in a water bath for 7 h. After being cooled and neutralized, the clear solution was collected and kept at 0-5^0C until analysis. Determination of monosaccharides was performed using HPLC (LC-3A, Shimadzu, Japan) with Phenomenex

Spherisorb-NH2 (25 cm x 4.6 mm) column and a refractive index detector using 90% aqueous acetonitrile (Merck, Germany) as mobile phase.

3.9. TESTS FOR CHECKING PROBIOTIC POTENTIAL OF SELECTED ISOLATES

The LAB isolates were subjected to various conditions to check probiotic potential of individual strain as described by Khedkar (1991). Various concentrations and gradients were used in triplicates in the study.

3.9.1. pH tolerance

To check pH tolerance by the selected isolates, pH of M-MRS broth was adjusted with 5N hydrochloric acid (Qualigens Fine Chemicals, Mumbai) using digital pH-meter (Ph Tester 30, EUTECH Instruments, OAKTON) at 4.0, 3.0 and 2.0. The LAB were inoculated in respective pH broth and incubated at 30°C for 72 h. Inoculated tubes that showed increased turbidity due to growth were noted as positive.

3.9.2. Phenol tolerance

The concentration of phenol (Merck, Mumbai) was adjusted at 0.3%, 0.4% and 0.5 percent (w/v) level in M-MRS broth. The LAB were inoculated in respective phenol broth and incubated at 30°C for 72 h. Inoculated tubes that showed increased turbidity due to growth were noted as positive.

3.9.3. Bile tolerance

Bile salt (Merck, Mumbai) was added in the M-MRS broth at 0.25%, 0.50%, 1% and 2 percent (w/v) and the tolerance tested by inoculation of the selected strains. The tubes were incubated at 30°C for 72 h.

3.10. STATISTICAL ANALYSIS

The data, for the quantity of EPS produced by selected isolates, taken from five replications were subjected to statistical analysis using Completely Randomized Design (CRD) as per the methods described in Steel and Torrie (1980). The significance was tested at 5 % level of significance using mean value, C.V. and C.D. were determined.

CHAPTER IV

RESULTS AND DISCUSSION

Many lactic acid bacteria, including mesophilic and thermophilic organisms, produces technologically important substances during fermentation of milk, that not only contribute to the improved physical properties of final product but also make the product more economical, nutritional and health beneficial. Of the many substances produced by different LAB, exopolysaccharides have received much attention during last decade. Use of EPS producing strains finds better alternatives to many commercially available additives such as stabilizers, viscosifiers, gelling agents, etc. The types of EPS produced by LAB differ from species to species and behave differently with physical properties of fermented milk products. Rational selection of LAB strains of different origin for EPS producing characteristics may help to find and exploit these strains commercially. The present study was taken up to isolate EPS producing LAB from dahi, vegetables and other fermented food products and then exploit them for improving rheological and sensory properties of food products and also find their use for various health benefits.

4.1. ISOLATION OF EPS$^+$ LACTIC ACID BACTERIA

With an objective to isolate EPS producing LAB, the total of 34 samples of dahi, fruits, vegetables and other fermented foods were plated on Modified MRS agar, and incubated at 25 and 37°C for 24-48 h. A total of 155 mucoid/ ropy colonies isolated from 34 these samples are shown in Table 4.1. All the colonies were picked up and transferred to modified MRS broth and incubated at either at 25 or 37° C for 24-48 h and observed for microscopic morphological features by Gram's staining.

Table 4.1 Number of EPS$^+$ isolates from various sources

Source	No. of Samples	No. of Isolates
Cabbage	2	10
Carrot	2	5
Tomato	2	16
Beet root	2	5
Cucumber	2	6
Fresh turmeric	2	16
Orange	1	1
Cheese	6	31
Dahi	8	41
Idli batter	4	18
Sauerkraut	1	6
Fermented cucumber	1	1
Fermented carrot	1	1
Total	34	155

Total of 65 isolates showing catalase negative test and with regular morphology of coccoidal short rod and rod shapes in pairs and chains were retained for further characterization. The broth cultures were purified by repetitive streaking on modified MRS agar till the purity of the isolates was ensured.

Literature shows a number of experiments dealing with isolation of slime producing cultures from natural food products from India and other countries.. Exopolysaccharide producing non-ropy strain of *Leuconostoc sp*. CFR 2181 was isolated from dahi on M-MRS agar, containing sucrose (5%, w/v) as well as on a low cost EPS medium by Vijayendra *et al*. (2008). Behare *et al*. (2008) isolated 47 EPS producing mesophilic ropy strains of lactic acid bacteria from Indian dahi and raw milk using deproteinized whey based medium.

Slime producing mesophilic LAB have been isolated from Swedish ropy milk (Nilsson, 1950; Macura and Townsley, 1984; Neve *et al*., 1988) and Finnish ropy milk (Sundman, 1953a; Forsen, 1966;

Bertelsen, 1983; Nakajima *et al*., 1990). The polysaccharides producing lactobacilli have been isolated from kefir grains on KPL agar by Toba *et al* (1986) and on Plate Count Agar by Pidoux *et al*. (1990). Typical 1-2mm mucoid colonies appeared after incubation at 30 and 42° C for 72h (Toba *et al*., 1986; Neve *et al*., 1988; Vescovo *et al*., 1989). EPS producing LAB isolates when touched with the inoculation loop exhibited increase in viscosity, consistency and formation of stringiness in skim milk whereas ropy colonies resulted in definite ropiness and a viscous product (Macy, 1923; Neve *et al*., 1988).

Four EPS positive LAB strains were isolated in Nigerian fermented foods using Modified ESM (Sanni *et al*., 2002). Savadogo *et al* (2004) isolated thirteen EPS^{+} lactic bacteria from Burkina faso fermented milks using MRS and M17 medium. One hundred and seventy four lactic acid bacteria strains isolated from dairy and cereal products were screened for the production of EPS. From the PCR and Ultrafiltration screened isolates, *Lactococcus lactis* (for homopolysaccharide) and *Lactobacillus curvatus* (for heteropolysaccharide) production reported for the first time by Van der Meulen *et al*. (2007). One hundred and three strains of LAB isolates were screened for their EPS producing activity from some traditional Nigerian fermented dairy (Nono, Fura, Yogurt, Wara) and non dairy food products (Ogi and Fufu) by Adebayo-tayo and Onilude (2008). Sarwat *et al.* (2008) isolated mesophilic *L. mesenteroides* CMG713 strain from *Vitis vinifera L.* (Grape) using 15% added sucrose in the defined growth medium for the highest dextran production. A novel *Leuconostoc citreum* S5 was isolated from Korean traditional fermented vegetable (Dongchimi) by Son *et al*. (2008). The strain isolated using 2% added sucrose with the MRS medium.

4.2. CHARACTERIZATION OF EPS PRODUCING LAB ISOLATES

4.2.1. Morphological Characterization

All the 155 EPS^+ isolates were examined microscopically for morphology (cocci and rods) and arrangements of cells by Gram staining. The proportion of cocci was higher as compared to coccidal (short rods) and rod shaped organisms. Most cells occurred as single, pair & long chains and in clusters for rod shaped and coccoidal organisms (Table 4.2).

Table 4.2 Morphological characterization of the isolates

Source	Isolate number	Gram's reaction	Morphology	Isolate number	Gram's reaction	Morphology
Tomato	1	+ve	Cocci	147	+ve	Coccobacilli
	2	+ve	Yeast	148	+ve	Cocci
	3	+ve	Cocci	149	+ve	Yeast
	4	+ve	Cocci	150	+ve	Rods
	5	+ve	Rods	151	+ve	Rods
	28	+ve	Cocci	152	+ve	Cocci
	34	+ve	Cocci	119	+ve	Cocci
	118	+ve	Cocci	122	+ve	Cocci
Dahi	9	+ve	Cocci	75	+ve	Cocci
	10	+ve	Cocci	110	+ve	Rods
	11	+ve	Cocci	123	+ve	Cocci
	12	+ve	Cocci	124	+ve	Cocci
	52	+ve	Cocci	129	+ve	Rods
	53	+ve	Cocci	132	+ve	Rods
	54	+ve	Rods	82	+ve	Yeast
	56	+ve	Rods	83	+ve	Yeast
	57	+ve	Rods	84	+ve	Cocci
	58	+ve	Rods	85	+ve	Coccobacilli
	59	+ve	Rods	86	+ve	Rods
	60	+ve	Cocci	87	+ve	Coccobacilli
	69	+ve	Cocci	88	+ve	Cocci
	70	+ve	Rods	89	+ve	Rods
	71	+ve	Rods	128	+ve	Cocci
	72	+ve	Cocci	130	+ve	Cocci
	73	+ve	Cocci	153	+ve	Cocci
	74	+ve	Rods	156	+ve	Rods
	90	+ve	Rods	157	+ve	Rods
	125	+ve	Cocci	163	+ve	Rods
	162	+ve	Rods			
Turmeric	17	+ve	Cocci	24	+ve	Cocci

Source	Isolate number	Gram's reaction	Morphology	Isolate number	Gram's reaction	Morphology
	20	+ve	Cocci	25	+ve	Cocci
	21	+ve	Cocci	98	+ve	Cocci
	22	-ve	Rods	99	+ve	Cocci
	23	Mix	Cocci	136	+ve	Rods
	106	+ve	Coccobacilli	137	+ve	Rods
	107	+ve	Cocci	138	+ve	Coccobacilli
	108	+ve	Cocci	109	+ve	Cocci
Idli batter	6	+ve	Cocci	77	+ve	Rods
	7	+ve	Cocci	78	+ve	Coccobacilli
	8	+ve	Yeast	79	+ve	Rods
	26	+ve	Cocci	80	+ve	Cocci
	27	+ve	Cocci	81	+ve	Cocci
	76	+ve	Rods	91	+ve	Cocci
	116	+ve	Cocci	92	+ve	Coccobacilli
	117	+ve	Cocci	160	+ve	Rods
	120	+ve	Cocci	161	+ve	Coccobacilli
Sauerkraut	29	+ve	Rods	113	+ve	Coccobacilli
	30	+ve	Cocci	114	+ve	Cocci
	31	+ve	Cocci	115	+ve	Cocci
Carrot	37	+ve	Cocci	96	+ve	Coccobacilli
	38	+ve	Cocci	97	+ve	Rods
	95	+ve	Coccobacilli			
Beet root	39	+ve	Cocci	93	+ve	Coccobacilli
	134	+ve	Cocci	94	+ve	Cocci
	127	+ve	Cocci			
Cheese	40	+ve	Rods	61	+ve	Rods
	41	+ve	Rods	62	+ve	Rods
	42	+ve	Coccobacilli	63	+ve	Rods
	43	+ve	Cocci	64	+ve	Cocobacilli
	44	+ve	Rods	65	+ve	Cocobacilli
	45	+ve	Cocci	66	+ve	Rods
	46	+ve	Rods	67	+ve	Cocobacilli
	47	+ve	Rods	68	+ve	Cocci
	48	+ve	Cocci	101	+ve	Cocci
	49	+ve	Rods	102	+ve	Cocci
	50	+ve	Cocci	104	+ve	Cocci
	51	+ve	Rods	105	+ve	Cocci
	126	+ve	Cocci	131	+ve	Cocci

Source	Isolate number	Gram's reaction	Morphology	Isolate number	Gram's reaction	Morphology
	135	+ve	Cocci	133	+ve	Cocci
	159	+ve	Cocci	154	+ve	Rods
	155	+ve	Rods			
Cabbage	13	+ve	Yeast	112	+ve	Cocci
	14	+ve	Cocci	144	+ve	Cocci
	35	+ve	Cocci	145	+ve	Coccobacilli
	36	+ve	Rods	146	+ve	Cocci
	32	+ve	Cocci	164	+ve	Cocci
Cucumber	139	+ve	Cocci	142	+ve	Coccobacilli
	140	+ve	Cocci	143	+ve	Coccobacilli
	141	+ve	Rods	165	+ve	Cocci
Fermented carrot	158	+ve	Rods			
Orange	121	+ve	Cocci			
Fermented cucumber	103	+ve	Cocci			

Key: +ve Positive; -ve Negative

Sixty five isolates, primarily showing rods and coccoidal type of morphology were selected based on microscopic examination for further study.

Lactic acid bacteria with different morphological features have been isolated from natural sources by various workers. Mavhungu (2006) reported twelve long rods in clusters, pairs or chain and six short rod types of LAB in clusters from 'Ting' (fermented maize and sorghum). Three rods, five cocobacilli, four spherical and one ovoid shaped EPS^{+} LAB isolates from Burkino Faso fermented milks were reported by Savadogo *et al*. (2004).

4.2.2. Preliminary Biochemical Characterization

Thirty one isolates as shown in Tale 4.3 were selected from among 65, based on colony characteristics in modified MRS agar. An

objective judgment was used to select highly mucoid colonies expecting them to produce more EPS. These 31 isolates were then tested for selected biochemical and physiological tests (Table 4.3) to identify their group.

Twenty seven out of 31 were screened as catalase negative cultures. All the catalase positive cultures did not ferment skim milk. Additionally five catalase negative isolates also could not ferment skim milk. Hence, we could get only 21 isolates which could coagulate milk and hence they were tentatively called as belonging to lactic acid bacteria. All the 31 isolates were cultured in MRS broth at different temperatures to know whether they are mesophilic or thermophilic types. Except isolates no. 74 and 85, all grew at 15C. At 45 C, total of 7 isolates could not multiply, showing them to be clearly mesophilic type. However, majority of the isolates grew at 15 as well as 45 C, indicating their broader temperature tolerance for growth and we could not place them in either mesophilic or thermophilic group.

Table 4.3 Preliminary biochemical tests for selected isolates

Isolate number	Catalase test	Skim milk fermentation	Growth at different temperatures			
			15ºC	25ºC	37ºC	45ºC
22	-ve	NL	+ve	+ve	+ve	+ve
46	-ve	L	+ve	+ve	+ve	+ve
47	-ve	L	+ve	+ve	+ve	+ve
49	-ve	L	+ve	+ve	+ve	+ve
56	-ve	L	+ve	+ve	-ve	-ve
61	-ve	L	+ve	+ve	-ve	-ve
66	-ve	L	+ve	+ve	+ve	+ve
71	-ve	L	+ve	+ve	+ve	+ve
74	-ve	L	-ve	+ve	+ve	+ve
78	+ve	NL	+ve	+ve	+ve	+ve
85	-ve	L	-ve	+ve	+ve	+ve
86	-ve	L	+ve	+ve	+ve	+ve
87	-ve	L	+ve	+ve	+ve	-ve
92	-ve	L	+ve	+ve	+ve	-ve
93	-ve	NL	+ve	+ve	+ve	+ve
97	+ve	NL	+ve	+ve	+ve	+ve

137	-ve	NL	+ve	+ve	+ve	+ve
138	-ve	NL	+ve	+ve	+ve	+ve
141	-ve	L	+ve	+ve	+ve	-ve
142	-ve	L	+ve	+ve	+ve	+ve
143	-ve	L	+ve	+ve	+ve	+ve
145	-ve	L	+ve	+ve	+ve	+ve
150	-ve	L	+ve	+ve	+ve	-ve
151	-ve	NL	+ve	+ve	+ve	-ve
152	+ve	NL	+ve	+ve	+ve	+ve
154	-ve	L	+ve	+ve	+ve	+ve
155	-ve	L	+ve	+ve	+ve	+ve
157	-ve	L	+ve	+ve	+ve	+ve
158	-ve	NL	+ve	+ve	+ve	+ve
160	+ve	NL	+ve	+ve	+ve	+ve
161	-ve	L	+ve	+ve	+ve	+ve

Key: +ve Positive; -ve Negative; L Lactic culture able to ferment lactose

NL Non lactic, not able to ferment lactose

Finally, six isolates with very good gum production and which are able to ferment milk properly were selected for further identification. Two additional tests, viz., gas production from glucose and growth in 6.5 % NaCl were carried out to confirm the genus level identification of the isolates. As shown in Table 4.5, the isolates No. 85, 86, 92, 138, 142 and 145 belonged to the groups of *Lactobacillus*, *Leuconostoc, Pediococcus and/or* W*eisella*. In the next phase, further biochemical identification with API test kits as well as Hi-Media test kits were carried out to characterize the isolates to species level.

Table 4.4 Preliminary characterization of selected isolates

CHARAC-TERISTICS	ISOLATES AND RESULTS					
	85	86	92	138	142	145
Cell form	Short rods	Long rods	Short rods with granular surface	Short rods	Elongated coccoides	Short rods
Cellular arrangement	Clusters	Pairs, chains	Forming Cluster	Chains, clusters	Single, Pairs	Clusters

Growth at:						
15°C	-	+	+	+	+	+
25°C	+	+	+	+	+	+
37°C	+	+	+	+	+	+
45°C	+	+	-	+	+	+
Gas from Glucose	-	-	-	-	-	-
Growth in:						
6.5% NaCl	+++	++	+	+	++	+
Catalase reaction	-	-	-	-	-	-
Probable Genus	*Lactococcus/ Leuconostoc/ Oenococcus*	*Lactobacillus*	*Weisella/ Leuconostoc/ Oenococcus*	*Lactobacillus/ Weisella/ Leuconostoc/ Oenococcus*	*Leuconostocs/ Weisella/ Oenococcus*	*Leuconostocs/ Weisella/ Oenococcus*

Key: +ve Positive; -ve Negative

The plate showing EPS producing colonies by isolate no 138 is shown.

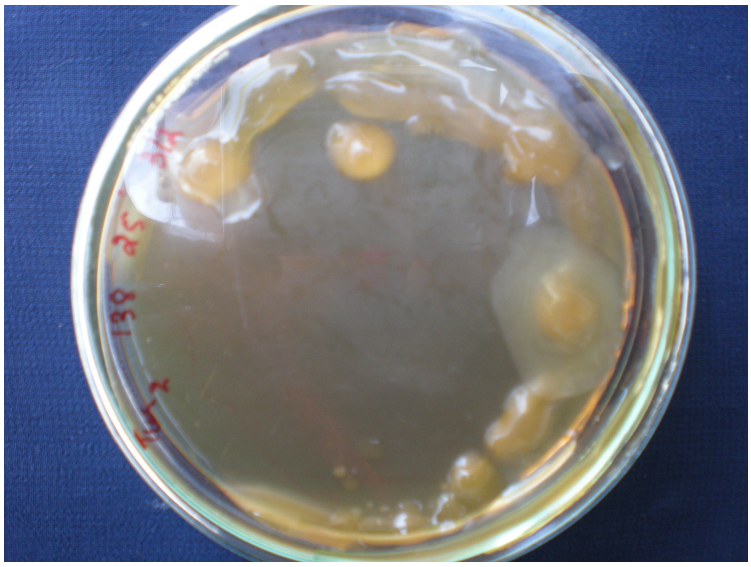
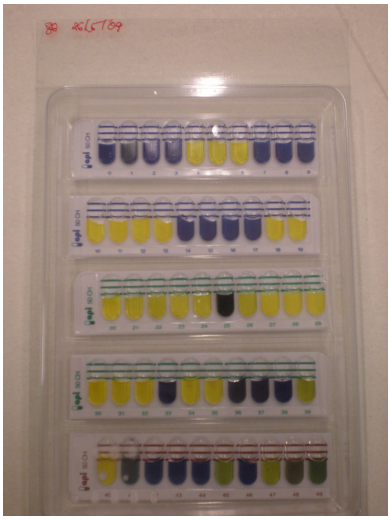


Figure 4.1 EPS production by isolate No 138 on modified MRS agar

4.2.3. Biochemical Identification by API Test Kit

The six selected isolates were subjected to species level biochemical identification by API test kit, (50CHL medium), Germany as well as by Hi-Carbo kit procured from M/s Himedia, Bangalore. The results obtained from Hi carbo kit were not clear and in several sugars it gave confusing status and hence, they are not reported in the present thesis.

Forty eight sugars used to check the characteristics of the isolates by API kit are described in Appendix I. The fermented sugars (+ve results) are indicated as colour change from violet to yellow, which can be seen from the plate photos for isolate no. 86, 142, 85, 92, 138 and 145 (Figure 4.2).



Isolate no 86

Isolate no 85

Isolate no 92

Isolate no 138

Isolate no 142

Isolate no 145

Figure 4.2 Plates showing API CHL50 test results of the six isolates

All the results from API test kit were interpreted by using the identification Biomerieux software version 5.1. The identification spectrum of the selected isolates is presented in Table 4.5.

Table 4.5 Identification of Isolates by API 50CHL

ISOLATE NO.	ISOLATED IDENTIFIED AS	LEVEL OF IDENTIFICA-TION (%)	REMARKS
85	*Weissella confusa*	99.5	Very good identification
	Lactobacillus brevis	0.4	Next choice
86	*Lactobacillus plantarum*	74.8	Acceptable identification to the genus
	Lactobacillus pentosus	25.1	
	Lactobacillus brevis	0.1	Next choice
92	*Weissella confusa*	74.3	Doubtful
	Lactobacillus brevis	23.9	
	Lactococcus lactis ssp. lactis	1.7	
	Lactobacillus plantarum	0.1	Next choice
138	*Lactobacillus brevis*	99.8	Very good identification
	Weissella confuse	0.1	Next choice
142	*Leuconostoc mesenteroides /dextranicum*	99.4	Very good identification
	Lactobacillus brevis	0.5	Next choice
145	*Weissella confusa*	99.6	Very good identification
	Lactobacillus brevis	0.3	Next choice

The identification spectrum of the isolates indicates that out of six isolates, three belong to genus Weissella, two to Lactobacillus and one to Leuconostoc.

Four isolates showed very good identification by software while isolate number 86 was acceptable up to genus level only and isolate 92 was doubtful. These results were compared with 16S rRNA sequencing.

API CHL tests kits have been used for identification of lactic acid bacteria earlier by several workers. Desai *et al*. (2006) found consistent classification results using API CHL medium for *Lactobacillus paracasei* and *L. rhamnosus* with the classification from the PCR assays with one exception for the *L. casei* and *L. zeae* as “unacceptable” profile. They indicated that this might be due to the limitation of biochemical profiling, when applied to closely related bacteria with intraspecific heterogeneity. Ten isolates of plaa-som samples including *Weissella cibaria* 110 (producing weissellicin 110), identified by API 50CHL kit by Srionnual *et al*. (2007). The utilization of carbon source was also determined indicating 91% homology with *Leuconostoc citrium* by Son *et al*. (2008).

The results obtained in our study may be interpreted with inherent limitations of the test kits. Hence, it is always advised to use other tools too for confirming the species. We used 16S rRNA sequencing to confirm the identification of the six isolates.

4.3. IDENTIFICATION OF SELECTED LABS BY MOLECULAR BIOLOGICAL TOOLS

4.3.1. 16S rRNA Polymerase Chain Reaction

After identification of species by biochemical tests, the species confirmation was carried out by PCR using universal primers for 16S ribosomal RNA gene as they are highly conserved genes of LAB.

Colony PCR of selected six isolates was performed using 16S rRNA gene universal primers (McCaig *et al*., 1999; Lu *et al*., 2003) but amplification was not obtained from isolate no 86 and 142 so they were subjected to DNA isolation by using a ZR Insect or Tissue DNA isolation kit and then amplified. The amplified PCR product of all the six isolates were run on 0.8% agarose gel, visualized by UV transilluminator and photographed using Kodak EDAS 290 digital camera. PCR product was purified using QIAEX ® II Gel Extraction Kit (QIAGEN) and the concentration was checked using Nanodrop (Nanodrop Technologies, INC. Wilmington, U.S.A, NanoDrop® ND-1000; full spectrum U.V-Vis spectrophotometer). The purified PCR product, for all six cultures, were ranged between 25 to 128 ng/µl and hence it was diluted with autoclaved Milli-Q water to bring it to working concentration of 10-50ng/µl for further discrimination by sequencing.

4.3.2. Sequence Analysis

The purified PCR product of the 16S rRNA gene from 6 isolates were sent to GATC Biotech, Germany for sequencing. Subsequently they were analysed by using BLAST tool of National Centre for Biotechnology Information (NCBI). The sequence similarity search for homology with the sequences available at Genebank, DDBJ and EMBL was carried out.

The sequence quality of isolates 86 and 142 was found ideal with 0% gaps and hence the identifications of 86 and 142 isolates were accurate. The 16S rRNA gene partial sequence of isolate number 86 which has been isolated from Indian *Dahi* sample, identified as *Lactobacillus plantarum* which showed 99% identity, with (0%) gaps, to NCBI homology search data of *Lactobacillus plantarum* strain KLDS 1.0610 [EU419598.1], an isolate of sour milk from china; *Lactobacillus plantarum* strain 37D2CCL02MX and 3DCCH01MX

([FJ538521] and [FJ538504]), isolated strains from sediments of cattle farm of Mexico; *Lactobacillus sp*. KLDS 1.0704 and KLDS 1.0706 ([EU600908.1] and [EU600910.1]), isolates from healthy human feces. The BLAST analysis of the 16S rRNA gene partial sequence of isolate no.142, was found 100% identical with *Leuconostoc pseudomesenteroides* strain L7 and SC8 ([DQ523483.1] and [AY929289.1], isolates from Ghanaian cocoa fermentations and traditional African food-Gari. Identification by phenotypic and biochemical tests also showed the same results as obtained by sequencing for these isolates.

The 16S rRNA partial sequences of four isolates (85, 92, 138, and 145) were identified as *Weissella cibaria* by the homologous sequencing data available at NCBI database. The isolate no. 85 isolated from *Dahi* and isolate no.92 from *Idli batter*, has shown good identities (97%), gaps (1%) with 16S rRNA, partial sequence of *Weissella cibaria* isolate R-32690 [AM491820.1], an isolate of Moroccan raw and traditional skimmed milk from Belgium; *Weissella sp*. KLDS 7.0701 [EU600924.1]; *Weissella cibaria* strain C2-32, C43-11c and C3-2 ([FJ429987.1], [FJ429984.1], [FJ429982.1]) isolated from semolina ecosystem of Italy. 16S rRNA partial gene sequence of cabbage isolate (145) and isolate of fresh turmeric (138) showed 96% identity with gaps 1% with *Weissella cibaria. Weissella cibaria* R-32690 [AM491820.1] an isolate of Moroccan raw milk, traditional skimmed milk of Belgium; and *Weissella cibaria* NRIC 0136 [AB362617.1].

The sequences of the isolates are shown as below:

Figure 4.3 16S rRNA gene sequences obtained by sequencing

For isolate No 85

TTTTGGGAGGGTCCCTTCTAATATGCGTGAGCCCATCCAAAAGTGGCGGAACGGGGGATAAAACGGGGGGAACTCC
TCCTTTTACCGGGGGGTACCTATTGAAAACAGTGTTTATACCCGGGATACCATAAGAAAACGCCAGGGTTGTTATT
AAAAGAAGGGTTCCGTAACCAATAAAGAAGGGTCCCCCGGGGCCATTAGTTAGTTGGGGAGGTAAGGGTTCACCAA
GACGGTTGATGCATAGCCGAGTTGAGAGACTGATCGGCCCCAATGGGACTGAGACACGGCCCATATTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGC

TCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGG
AACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCG
AGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTTTGGAAACTGGATGACTT
GAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAA
GGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCG
GGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCA
ACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCG
GAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCA
TGAGAGTTTGAACACCCAAAGCCGGTGGGGAACCTTCGGAGCCAGCCTCTAAGTGTCCGTGC

For isolate No 86

TGCAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGT
AACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACC
GCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGG
TAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAA
GAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTAT
TTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAA
CACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGA
TACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACG
CATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTA
GACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTA
CAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGC
CTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGAGAGTTTGAACACCCAAAGTCGGTGGGGAACCTTTAGGAACCAG

For isolate No 92

TTTGGAAGGGCTCTTTTGAATTTTGCGTGGCATTTTCTAAAAGGTGGGAACCGGTGGGTACACCGGGGGAAACTTC
CCCTTTAGCAGGGGATACCATTTGGAACCAGATGCTTAATCCCGTTAACCAATGCCAACCGCATGGTTGCTACTTA
AAGGATGGTTTTGCTTCCAATAAGGAATGGTCCCGCGGGGCCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGA
CGATGATGCATAGCCGAGTTGAGGGACTGATCGGCCACAATGGGACTGAGACACGGCCCATATTCTTACGGGAGGC
AGCAGTAGGGAATCTTCCCCAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCG
TAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAAC
GGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGC
GCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTTTGGAAACTGGATGACTTGAG
TGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGC
GGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCAC
ACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGG
ACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACC
CGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAA
TCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGA
GAGTTTTAACACCCAAAGCCGGTGGGGAACCTTCGGAGCCAGCCTCTAAGTGACGATGN

For isolate No 138

AAAGAGACGGTTGTTGTTCAAAATTTAAAAAGTTTTCCCAAAAAAGCGGTGGCTTTATCAAAGGTGGGGAAAGGGG
GGAGAACAGGTGGGAAACCCTCTTTTTGCGGGGGGATCCATTTGGGACCAGGTGCGTAAACCCGTTTAACAATAAC
AACCCGAAGGGTTGCTATTTAAAAAGTGGTTTTGGTATCAATTAAAGAATGGTCCCCCGGGGCCATTATTTAGTTG
GGGAAGTAAGGGTTCACCAAGACGGTGATGCATAGCCGAGTTGAGAGACTGATCGGCCCCAATGGGACTGAGACAC
GGCCCATATTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTG
TGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGA
CGGTATCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGC
TTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATG
GAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGG
ATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCAG
AGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
CGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGGCTGCA
ACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTA
CACACCGCCCGTCACACCATGAGAGTTTTAACACCCAAAGCCGGTGGGGAACCTTCGGAGCCAGCCTCTAAGTGAC
GATGC

For isolate No 142

TTAGACGGCTCCTTCCTAAAAGGTTAGGCCACCGGCTTTGGGCATTACAAACTCCCATGGTGTGACGGGCGGTGTG
TACAAGACCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGTCGAGT
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CACCGGCAGTCTCGCTAGAGTGCCCATCTGAATGCTGGCAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAA
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TCTAAAAGCTTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
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GCTTCGGCACTAAGAGGCGGAAACCTCCTAACACCTAGTGTTCATCGTTTACGGTGTGGACTACCAGGGTATCTAA
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TCCGGATAACGCTCGGGACATACGTATTACCGCGGCTGCTGGCACGTATTTAGCCGTCCCTTTCTGGTATGGTACC
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ACACGCGGCGTTGCTCCATCAGGCTTTCGCCCATTGTGGAAGATTCCCTACTGCAGCCTCCCGTAGGAGTTTGGGC
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CCAACTAACTAATGCACCGCGGATCCATCTCTAGGTGACGCCAAAGCGCCTTTTAACTTTGTGTCATGCGACACTG
AGTTTTATTCGGTATTAGCATCTGTTTCCAAATGTTATCCCCAGCCTTGAGGCAGGTTGTCCACGTGTTACTCACC
CGTTCGCCACTCACTTGAAAGGTGCAAGCACCTTTCGCTGTGC

For isolate No 145

TTGACAAAAAATAGAGAAGCTTTTCAATTTAGCGGTGCCCTCTAAAAGTGCGCGACGGGGTGACAAACGGGGGGAA
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GTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAA
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CGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTTTGGAAACTGGATGACTTGA
GTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGG
CGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCA
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AGAGTTTTAACACCCAAAGCCGGTGGGGAACCTTCGGAGCCAGCCTCTAAGTGACGATGC

The 16S rRNA partial sequences of four isolates (85, 92, 138, and 145) are less confident due to gaps (≥1%). The controversial identifications with the results from phenotypic and biochemical tests were observed by sequencing homology search data for 16S rRNA partial sequence for these isolates. By gene sequencing of 16S rRNA gene, they were identified as *Weissella cibaria*, which showed as ‘very good’ level of identification as *Weissella confusa* (in case of isolates 85, 138 and 145) and ‘doubtful’ profile in case of isolate no 92 with the API data base. The same controversial observations were made for two of the Lactobacillus isolates named *Lactobacillus casei* and *L. zeae* by Desai *et al*. (2006). Such observations illustrate the limitations of biochemical profiling when applied to closely related bacteria with intraspecific heterogeneity. Even though the sequences of four isolates (85, 92, 138 and 145) showed almost similar identities with *Weissella cibaria* strains with less percentage of identities, they are of more importance as they have not been reported by any workers from fermented food sources like idli batter and Dahi as EPS producing lactic acid bacteria.

4.4. SUMMARY OF IDENTIFICATION RESULTS

The identification results by morphological, biochemical and genetic characterization for the isolated LAB are shown in Table 4.6.

Table 4.6 Summary of results for identification of selected isolates

Name of Isolate	Source	Identification Results from Tests		
		Traditional test	API test	Sequencing analysis
85	Dahi	Weisella /Leuconostocs/ Oenococcus	*Weissella confusa*	*Weissella cibaria*
86	Dahi	Lactobacilli	*Lactobacillus plantarum/ Lactobacillus pentosus*	*Lactobacillus plantarum*
92	Idli Batter	Weisella/ Leuconostocs/ Oenococcus	*Weissella confusa/Lactobacillus brevis/ Lactococcus lactis ssp. lactis*	*Weissella cibaria*
138	Fresh Turmeric	Lactobacilli/ Weisella/ Leuconostocs/ Oenococcus	*Lactobacillus brevis*	*Weissella cibaria*
142	Cucumber	Leuconostocs/ Weisella/ Oenococcus	*Leuconostoc mesenteroides/dextranicum*	*Leuconostoc pseudomesenteroides*
145	Cabbage	Leuconostocs/ Weisella/ Oenococcus	*Weissella confusa*	*Weissella cibaria*

The identification shown by sequence analysis is more specific and reliable, while there is some scope of doubt in API kit results; hence the final identification of the six isolates is reported as follows:

Isolate no 86 as *Lactobacillus plantarum* by both, API biochemical test and 16S rRNA gene partial sequencing data.

Leuconostoc pseudomesenteroides in the case of isolate no 142, by 16S rRNA gene partial sequence data.

There was some doubt in sequences of 4 isolates identified as *Weissella cibaria* and hence repetition of the analysis and/or full sequence of the isolates or more sophisticated tools for species level differentiation may be useful to remove doubts.

4.5. PRODUCTION OF EPS BY SELECTED LAB

The production of EPS is gaining much importance in food and pharma industries because of its specific use. It is becoming very interesting field for research due to the suggested role of EPS in the mediation of adhesion promotion of the bacteria and its typical prebiotics effect.

Selected LAB isolates were grown in modified MRS broth at 30^0C for 24h and EPS was precipitated by TCA-Ethanol precipitation method. The quantity of EPS produced by particular strain was measured by the dry weight of the freeze dried EPS samples. The amount of EPS produced by different cultures was significantly ($P<0.05$) different (Table 4.7). The quantity of dried EPS of individual strains of LAB revealed that the values ranged from 2.14 to 5.88 g/L of medium.

Lactobacillus plantarum (86) produced the highest amount of EPS (5.88 g/L), which was at par with *Weissella cibaria* (138) (5.46 g/L). The EPS produced by *Weissella cibaria* (92) was 4.88 (g/L) which was comparable to *Weissella cibaria* (85), 4.22 g/L). *Weissella*

cibaria (145) was at par with isolate 85 with 4.06 (g/L) of EPS production. *Leuconostoc pseudomesenteroides* (142) produced the lowest amout of EPS (2.14 g/L) which was significantly lower ($P<0.05$) than all other isolates.

Table 4.7 Production of EPS by selected LAB strains

Strains	EPS production (Mean) (g/L)
Weissella cibaria (85)	4.22*
Lactobacillus plantarum (86)	5.88*
Weissella cibaria (92)	4.88*
Weissella cibaria (138)	5.46*
Leuconostoc pseudomesenteroides (142)	2.14*
Weissella cibaria (145)	4.82*
ANOVA TABLE	
S.Em ±	0.26
C.D. 0.05%	0.77
C.V%	12.82

*Significant at 0.05%

Four isolates of lactobacilli were tested for EPS production by Kodaikkal (2007). He found EPS concentration of 192.89 mg/L for *L. acidophilus* V3, 229.56 mg/L for *L. acidophilus* I4 mg/L , 213.52 mg/L *L. acidophilus* 22A for 138.80 mg/L for *L. acidophilus* LB1.

Exopolysaccharide (EPS) producing non-ropy mesophilic strain of lactic acid bacteria (*Leuconostoc sp*. CFR 2181) was isolated from dahi by Vijayendra *et al*. (2008). In shake flask fermentation for 72 h at 22°C, the quantity of EPS produced by the isolate was 13.8 g/L in modified MRS broth and 25.4 g/L in EPS medium (a newly formulated simplified synthetic medium).

Van der Meulen *et al*. (2007) isolated ten EPS producing Lactococci, Leuconostocs and Weisella strains which produced 0.02 to 17 g/L of EPS in M-MRS agar with 5% sucrose. Adebayo-tayo and Onilude (2008) isolated LABs using Exopolysaccharides selection medium (ESM) from Nigerian fermented foods. Their EPS ranged between 01.00-196.0 mg /L amongst the active producers of Exopolysaccharide i.e. *L. plantarum, L. fermentum, L. delbrueckii*, *Leu. mesenteroides ssp dextranicum*, *Leu. mesenteroides ssp mesenteroides, Leu. gelidium*, *L. casei, L. cellobiosus, Leu. amelbiosum, Lact*. *plantarum, Lact. piscium* strains. Among the 13 strains of *Leu. mesenteroides (*03.1-138.0 mg /L*),* the yoghurt isolate *Leu. mesenteroides ssp mesenteroides* (UMMY5) had highest production (118.2 mg/L). Among *L. plantarum* (0.10-185.2 mg/L*), strains* isolate *LPWO11* had the highest production.

Badel *et al*. (2008) reported *Leuconostoc sp*. able to produce more than 20 g/L of polysaccharide. Two strains from each *Leuconostoc mesenteroides and Rahnella aquatilis* had a tendency for higher EPS yield below 13°C (Tallgren *et al*., 1999). They observed almost 10 times higher amount of EPS (20-27g/L) than their own cell mass (2-3g/L), which showed the best EPS production at 30°C.

4.6. TESTS FOR CHECKING PROBIOTIC POTENTIAL OF SELECTED ISOLATES

The six isolates were tested for tolerance to bile salt, phenol and pH at different concentrations to check their probiotic potential in M-MRS broth medium at 30^0C. Table 4.8 shows the test results after 48h of incubation at 30^0C.

Table 4.8 Growth of selected isolates in presence of bile salt, phenol or at different pH.

Test condition	Test concentration	Extent of growth for Isolate No.					
		85	86	92	138	142	145
Bile	0.25%	+++	++	+++	+++	+++	+++
	0.50%	+++	++	++	+++	+++	+++
	1%	+++	++	+++	+++	+++	+++
	2%	+++	+	+++	+++	+++	+
pH	4.0	+++	++	+++	++	++	+++
	3.0	+	+	+	+	+	+
	2.0	+	+	++	+	++	+
Phenol	0.3%	+++	+++	++	+++	+++	+++
	0.4%	+++	+++	+++	++	++	+++
	0.5%	+	+	+	-	+	+

Key: - No growth, + slight growth, ++ moderate growth, +++ good growth

All the isolates were able to tolerate bile concentration up to 2% for 48 h and were able to grow up to pH 2.0. Phenol concentration up to 0.5% was tolerated by all five isolates, except *Weissella cibaria* (138), which was able to tolerate phenol concentration only up to 0.4%.

Hence, the newly isolated LAB strains have proven the preliminary fulfillment to be probiotic organism and further investigation is required for the rest probiotic characteristics. Together with the probiotic action, it can provide prebiotic potential as they are good producers of EPS.

Earlier Ashar and Prajapati (1998) had studied probiotic potential of lactobacilli by testing their tolerance to bile salt, bile deconjugation and cholesterol reducing properties. The intestinal isolate *Lactobacillus casei* I_4 showed highest tolerance to 0.3% sodium taurocholate followed by *L. acidophilus* V_3, C_2 and H_3 strains to MRS broth at 37^0C. Then the strains were tested for cholesterol lowering property by *in vivo* trials.

4.7 MONOSACCHARIDE COMPOSITION OF EPS

Model mixtures of glucose, galactose, rhamnose and fructose were separated over HPLC using RID. The retention times of these monosaccharides were 12.267, 10.338, 7.004 and 13.147 minutes respectively for glucose, rhamnose, galactose and fructose. Figure 4.5 show the monosaccharides compositions of EPS from isolate no. 85, 86, 92, 138 and 142 respectively of the same fraction. Figure 4.4 represents the chromatograph of blank experiment.

Purified EPS fractions of different LAB hydrolyzed with 1 N H_2SO_4, cooled, neutralized and then analyzed by High Performance Liquid Chromatography (HPLC) using Phenomenex Spherisorb-NH2 column and RI detector. The EPS of all the LAB isolates was found to be heteropolysaccharide composed of different sugars (Table 4.9).

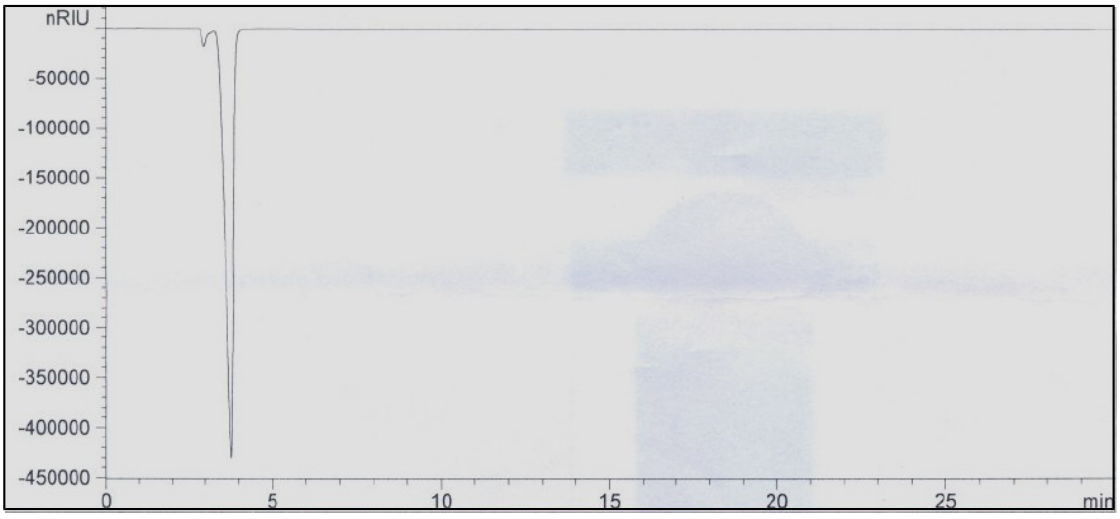

Figure 4.4. Chromatograph for Blank Experiment

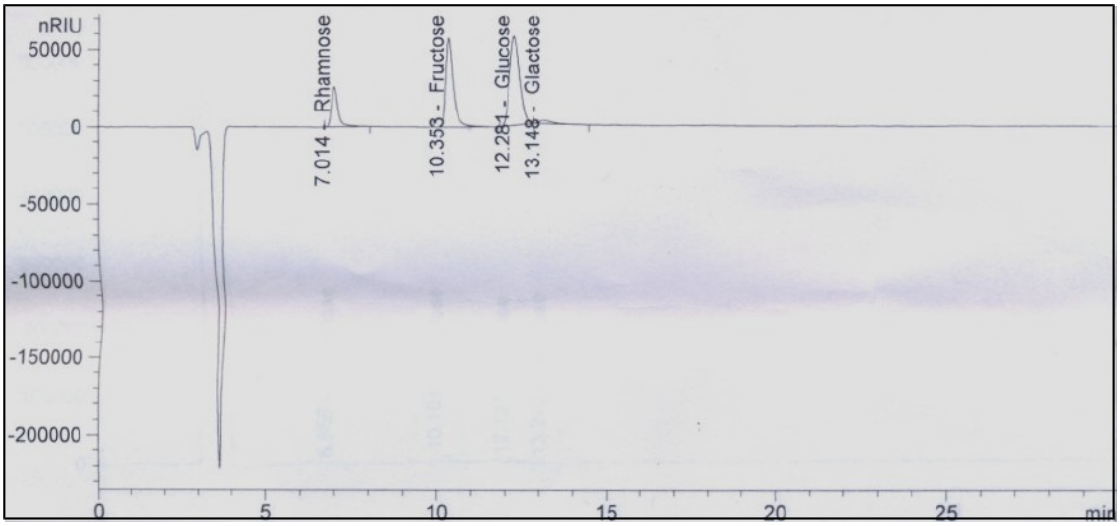

Figure 4.5. Chromatograph of Standard Sugars

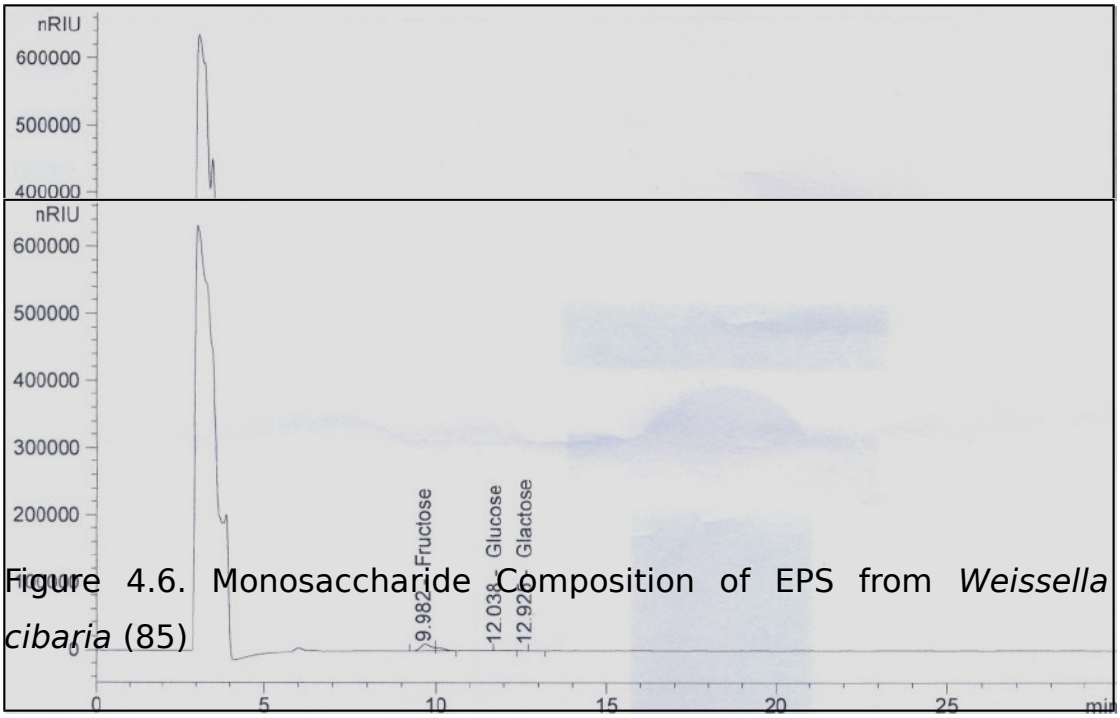

Figure 4.6. Monosaccharide Composition of EPS from *Weissella cibaria* (85)

Figure 4.7. Monosaccharide Composition of EPS from *Lactobacillus plantarum* (86)

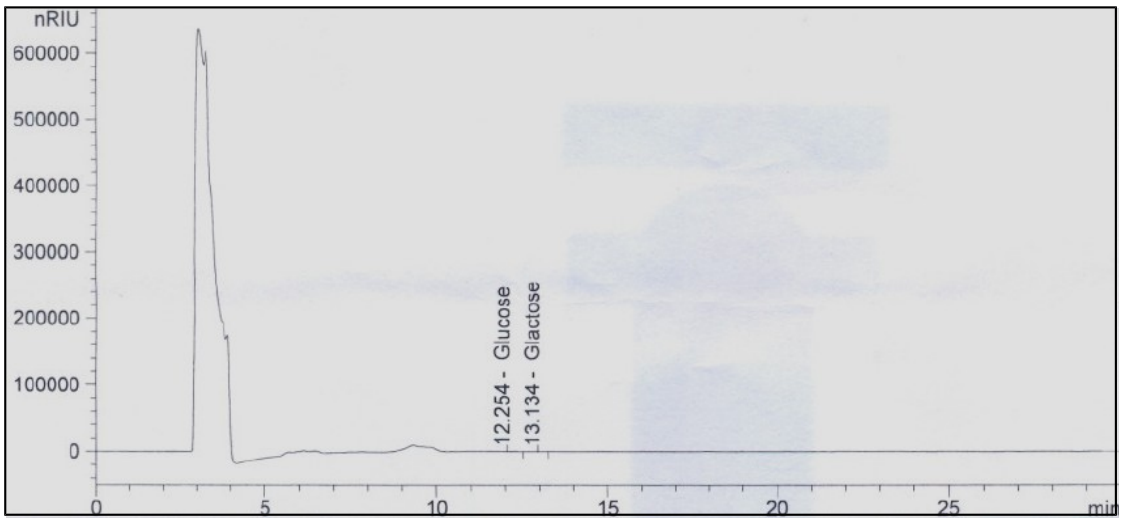

Figure 4.8. Monosaccharide Composition of EPS from *Weissella cibaria* (92)

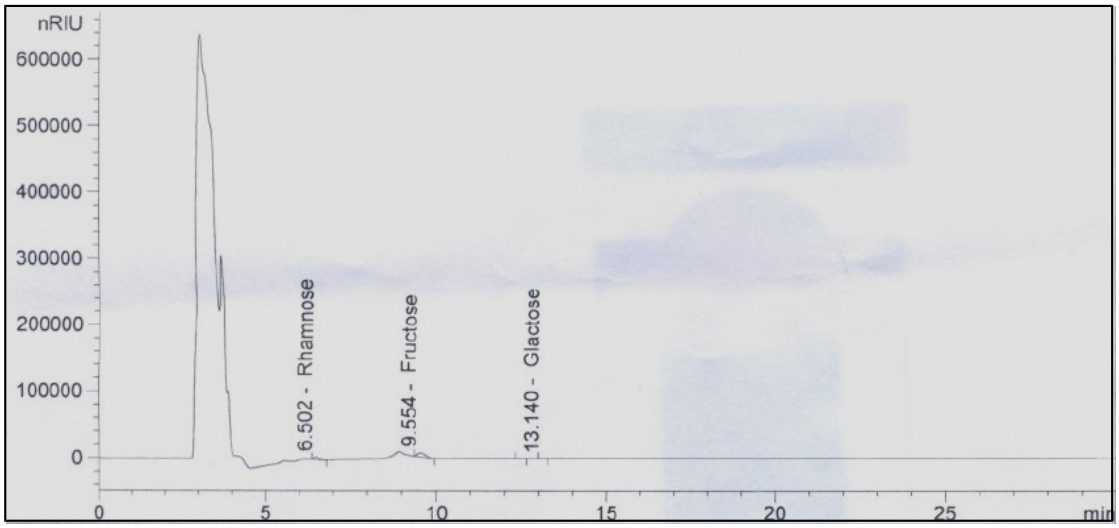

Figure 4.9. Monosaccharide Composition of EPS from *Weissella cibaria* (138)

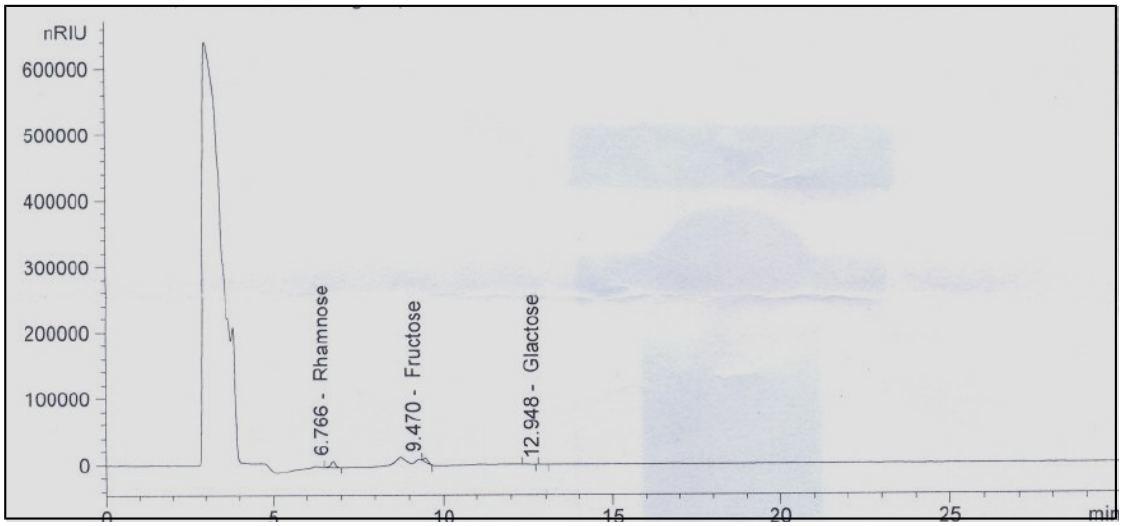

Figure 4.10 Monosaccharide Composition of EPS from *Leuconostoc pseudomesenteroides* (142)

The EPS of *Lactobacillus plantarum* (86) detected three sugars viz. glucose, fructose and galactose (Figure 4.6) in 0.07%, 1.29% and 0.82% respectively; *Weissella cibaria* (85) contained highest amount of glucose (0.92%) among all (Figure 4.7); *Weissella cibaria* (138) detected 0.02%, 1.33%, 0.03% and rhamnose 1.56% (Figure 4.9). Whereas, *Weissella cibaria* (92) had detected only two sugars viz. glucose (0.06%) and galactose (0.14%) (Figure 4.8); *Leuconostoc pseudomesenteroides* (142) has been found to be with highest rhamnose content (2.63%) among all the isolates (Figure 4.10). We could not run *Weissella cibaria (145)* due to some technical problems.

Table 4.9 Monomer composition of exopolysaccharides.

EPS producing strain	**Monomer composition**			
	Glucose	**Fructose**	**Galactose**	**Rhamnose**
Weissella cibaria (85)	0.92%	1.72%	0.44%	0.07%
Lactobacillus plantarum (86)	0.07%	1.29%	0.82%	ND
Weissella cibaria (92)	0.06%	ND	0.14%	ND
Weissella cibaria (138)	0.02%	1.33%	0.03%	1.56%
Leuconostoc pseudomesenteroides (142)	0.15%	0.66%	0.03%	2.63%

Key: ND= Not Detected

Smitinont et al. (1999) reported major monosaccharide, resulting from acid hydrolysis of EPSs, using the same method used in the present study. The strains *Pediococcus pentosaceus* AP-1 and AP-3 produced glucose, which accounted for 98.9% and 100% of total monosaccharides, respectively. Other minor peaks which appeared on the HPLC chromatogram of EPS from *Pediococcus pentosaceus* AP-1 are considered to be contaminants (hydrolysis side-products). Tallgren *et al*. (1999) reported homopolysaccharide producing t*wo strains of Leuconostoc mesenteroides* (dextran) and *Rahnella aquatilis* (levan), where as heteropolysaccharide (71% was glucose and 18% was fructose and 5% mannose and 5% galactose)

producing strain *Enterobacter amnigenus* from sugar beet. Van der Meulen *et al*. (2007) isolated nine homo polysaccharide (glucan) producing and one hetero polysaccharide (galactosamine: galactose: glucose for 2:3:1 ratio) producing strain of LABs from Belgian and Romanian dairy and cereal products.

Behare *et al*. (2008) found most LAB isolates, producing heteropolysaccharides in different ratios except Kt 24 EPS which was homopolysaccharide composed of only one type of sugar rhamnose. The EPS of *L. lactis* subsp. *lactis* B6 was found to be heteropolysaccharides composed of glucose and mannose in a molar ratio of 1:90.9; *S. thermophilus* IG16 in ratio of rhamnose to galactose was 5.3:1; *S. thermophilus* K2 contained glucose and mannose (1:7.4); *Lactobacillus fermentum* V10 in the ratio of 1:13:1.5 of glucose, rhamnose and galactose and *L. fermentum* NCDC containing rhamnose and galactose sugars in the ratio of 1:6.8.

In our study, the four major sugar standards were glucose, fructose, galactose and rhamnose and surprisingly the EPS from all the six isolates had very low amount of these sugar. This indicated that further identification with more sugar standards is necessary to reveal the exact monomer composition of EPS from these isolates. It also indicates that these EPS are heteropolysachharides.

CHAPTER - V

SUMMARY AND CONCLUSIONS

Lactic Acid Bacteria (LAB) are widely used in the dairy and food industries from centuries. They play crucial role in food fermentation processes. Apart from production of lactic acid, flavouring compounds and bacteriocins; several strains of LAB secrete extracellular polysaccharides in favourable environments. EPS in their natural environment are known to play a role in the protection of the microbial cell against phygocytosis, phage attack, antibiotics or toxic compounds, predation by protozoan, osmotic stress, adhesion to solid surfaces and in cellular recognition. In food industry, microbial EPS are used as thickeners or viscosifiers, stabilizing or emulsifying agents, texturizers as well as in gel-filtration to concentrate or recover proteins from liquid wastes such as whey and cereal waste systems and in other non-food purposes such as seed coating, etc.

Our results demonstrate the diversity of LAB in dairy and non-dairy fermented foods in India. The selected vegetables, fruits and fermented foods contain several species of LAB, which have potential to produce EPS were identified by phenotypic and genotypic characteristics. These strains can be used as starter culture with predictable characteristics and contribute to the development of small scale and commercial production of fermented food with consistent quality.

The present study was undertaken with an objective to isolate promising EPS^{+} lactic acid bacteria from fruits, vegetables and fermented foods of India and characterize EPS from selected LAB for monosaccharides composition.

Thirty four samples of dahi, fruits, vegetables and other fermented foods were tested and 155 EPS+ isolates were picked up by plating on modified MRS agar.

From among these, sixty five isolates, primarily showing rods and coccoidal type of morphology were selected for further study.

Based on objective judgment, 31 isolates producing highly mucoid colonies expecting them to produce more EPS, were tested to identify their group.

Twenty one isolates were identified as lactic acid bacteria, as they coagulated skimmilk within 48 h. Seven isolates did not grow at 45 ^{0}C and hence confirmed as mesophilic type. However, majority of the isolates grew at 15 as well as 45^{0}C, indicating their broader temperature tolerance for growth and we could not place them in either mesophilic or thermophilic group.

Finally, six catalase negative isolates (isolate no. 86 as long rods and isolate no 85,92,138,142 and 145 as short rods) with very good gum production and which are able to ferment milk properly were selected for two additional tests, viz., gas production from glucose and growth in 6.5 % NaCl. As per tests, the isolates were identified upto genus level as *Lactobacillus*, *Leuconostoc, Pediococcus and/or* W*eisella*.

The biochemical identification by API test software showed very good identification for four isolates. Isolate no 85 (99.5%), 138 (99.8%), and 145 (99.6%) were identified as *Weissella confusa* while 142 (99.4%) as *Leuconostoc mesenteroides /dextranicum.* Isolate number 86 was acceptable up to genus level only with 74.8% identity as *Lactobacillus plantarum* and isolate 92 had doubtful identification as *Weissella confusa*.

By 16S rRNA gene partial sequencing, isolate number 86 was identified as *Lactobacillus plantarum*.

The isolate no. 85, 92, 145 and 138 were identified as *Weissella cibaria.* The 16S rRNA partial sequences of four isolates (85, 92, 138, and 145) are less confident due to gaps (≥1%). Some controversial identifications with the results from phenotypic and biochemical tests were observed by sequencing homology search data for 16S rRNA partial sequence for these isolates but they have not been reported by any workers from fermented food sources like idli batter and Dahi as EPS producing lactic acid bacteria.

The extraction and precipitation of the EPS from 24h/30^{0}C incubated, modified MRS broth medium was done by TCA-Ethanol precipitation method. The quantity of EPS produced by particular strain was measured by the dry weight of the freeze dried EPS samples. The quantity of dried EPS of individual strains of LAB revealed that the values ranged from 2.14 to 5.88 g/L of medium.

The amount of EPS produced by different cultures was significantly ($P<0.05$) different. *Lactobacillus plantarum* (86) produced the highest amount of EPS (5.88 g/L), which was at par with *Weissella cibaria* (138) (5.46 g/L). The EPS produced by *Weissella cibaria* (92) was 4.88 (g/L) which was comparable to *Weissella cibaria* (85) (4.22 g/L). *Weissella cibaria* (145) was at par with isolate 85 with 4.06 (g/L) of EPS production. *Leuconostoc pseudomesenteroides* (142) produced the lowest amount of EPS (2.14 g/L) which was significantly lower ($P<0.05$) than all other isolates.

The monosaccharide compositional analysis of the EPS produced by selected LAB was analyzed by HPLC. All the isolates produced heteropolysaccharide. The four major sugar standards run were glucose, fructose, galactose and rhamnose and surprisingly the EPS from all the isolates had very low amount of these sugar. The

heteropolysaccharide of *Weissella cibaria* (85), *Weissella cibaria* (138) and *Leuconostoc pseudomesenteroides* (142) detected all the four sugars (glucose 0.92%, 0.02% ,0.15%; fructose 1.72%, 1.33%, 0.66%; galactose 0.44%, 0.03%, 0.03% and rhamnose 0.07%, 1.56%, 2.63%, respectively). The EPS of *Lactobacillus plantarum* (86) was found with three sugars viz. glucose (0.07%), fructose (1.29%) and galactose (0.82%) and whereas *Weissella cibaria* (92) had detected only two sugars viz. glucose (0.06%) and galactose (0.14%).

The six isolates were tested for tolerance to bile salt, phenol and pH at different concentrations to check their probiotic potential in M-MRS broth medium at 30^0C. All the isolates were able to tolerate bile concentration up to 2% for 48 h and were able to grow up to pH 2.0. Phenol concentration up to 0.5% was tolerated by all five isolates, except *Weissella cibaria* (138), which was able to tolerate phenol concentration only up to 0.4%. Hence, the newly isolated LAB strains have proven the preliminary fulfillment to be probiotic organism and further investigation is required for the rest probiotic characteristics. Together with the probiotic action, it can provide prebiotic potential as they are good producers of EPS.

Based on the results, the following inferences were made:

- The identification shown by sequence analysis is more specific and reliable, while there is some scope of doubt in API kit results; hence the final identification of the six isolates is reported as follows:
  - Isolate no 86 as *Lactobacillus plantarum* by both, API biochemical test and 16S rRNA gene partial sequencing data.

  - *Leuconostoc pseudomesenteroides* in the case of isolate no 142, by 16S rRNA gene partial sequence data.
  - There was some doubt in sequences of 4 isolates identified as *Weissella cibaria* and hence repetition of the analysis and/or full sequence of the isolates or more sophisticated tools for species level differentiation may be useful to remove doubts.
- *Lactobacillus plantarum* (86) produced the highest amount of EPS (5.88 g/L).
- The monosaccharide compositional analysis of the EPS produced by selected LAB was analyzed by HPLC. All the isolates produced heteropolysaccharide. The four major sugar standards run were glucose, fructose, galactose and rhamnose and surprisingly the EPS from all the isolates had very low amount of these sugar.
- *Lactobacillus plantarum* (86) able to tolerate 2%bile concentration, pH 2.0 and phenol concentration up to 0.5% for 48 h.

Possible future work:

1. To remove confusions in identification and to check whether the species are novel or not by full genome sequencing.
2. To check characteristic biochemical properties of EPS such as linkages, viscosity, compositional analysis for moisture, carbohydrate and protein.
3. To define standard condition for highest EPS production by the new strains.
4. *In vitro* and *in vivo* testing for the strains as probiotics producing higher amount of EPS.

5. *In vivo* testing for the potential of EPS as prebiotic.
6. To test rheological and sensory quality of food with new LAB strains.
7. Food application of EPS to check its efficiency as emulsifier, viscosifier, thickener.

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APPENDIX

COMPOSITION AND METHODS FOR PREPARATION OF CULTURE MEDIA AND REAGENTS USED IN THE STUDY

1. Sterilized Skim Milk

Skim milk powder was reconstituted and then distributed in test tubes and flasks in required quantities. It was then autoclaved to a temperature of 121°C (15 psi) and holding at this temperature for 20 min.

2. Saline solution and dilutions

Sodium chloride (Qualigens fine chemicals, Baroda) dissolved at 0.9 percent in distilled water. It was distributed in test tubes and flasks in required quantity and then autoclaved to a temperature of 121°C (15 psi) and holding at this temperature for 20 min.

2. Phosphate Buffer for Preparing Dilutions (IS: 1479, part-III, 1962)

Stock solution of phosphate buffer was prepared by dissolving 34.0 g of KH_2PO_4 in 500 ml distilled water. pH was adjusted to 7.2 with 0.1 N NaOH solution and the total volume was made up to 1 liter with distilled water.

For use as dilution water, 1.25 ml of the stock phosphate buffer solution was diluted to 1 liter with distilled water. It was then filled in 9 ml aliquots in tubes and sterilized by autoclaving at 121°C for 20 min.

4. Modified MRS Agar Medium (De Man *et al*., 1960)

The suggested quantity (67.15 g) of dehydrated powder of MRS agar (Himedia Laboratories Pvt. Ltd., Mumbai, India) was suspended in 1000 ml distilled water with 5% added sucrose. The contents were then heated to boiling to dissolve the medium completely. The

medium was cooled and the pH of the agar medium was adjusted to 6.5 ± 0.2. The medium was distributed in flask (100 ml quantity) of 250 ml capacity, and was sterilized at 121°C (15 p.s.i.) for 15 min.

Composition of MRS agar

Ingredients	**g / liter**
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Agar	12.00

5. Biochemical test by API 50CHL medium

API 50 CH is a standardized system, associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. API 50 CH is used in conjunction with API 50 CHL Medium for the identification of *Lactobacillus* and related genera and with API 50. The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates.

COMPOSITION OF THE STRIP

The composition of the API 50 CH strip is given below in the list of tests :

Strip 0 - 9

Tube	Test Active ingredients	QTY (mg/cup.)
0	CONTROL	–
1 GLY	GLYcerol	1.64
2 ERY	ERYthritol	1.44
3 DARA	D-ARAbinose	1.4
4 LARA	L-ARAbinose	1.4
5 RIB	D-RIBose	1.4
6 DXYL	D-XYLose	1.4
7 LXYL	L-XYLose	1.4
8 ADO	D-ADOnitol	1.36
9 MDX	Methyl-β-D-Xylopyranoside	1.28

Strip 10 – 19

Tube	Test Active ingredients	QTY (mg/cup.)
10 GAL	D-GALactose	1.4
11 GLU	D-GLUcose	1.56
12 FRU	D-FRUctose	1.4
13 MNE	D-MaNnosE	1.4
14 SBE	L-SorBosE	1.4
15 RHA	L-RHAmnose	1.36
16 DUL	DULcitol	1.36
17 INO	INOsitol	1.4
18 MAN	D-MANnitol	1.36
19 SOR	D-SORbitol	1.36

Strip 20 - 29

Tube	Test Active ingredients	QTY (mg/cup.)
20 MDM	Methyl-αD-Mannopyranoside	1.28
21 MDG	Methyl-αD-Glucopyranoside	1.28
22 NAG	N-AcetylGlucosamine	1.28
23 AMY	AMYgdalin	1.08
24 ARB	ARButin	1.08
25 ESC	ESCulin	1.16
26 SAL	SALicin	1.04
27 CEL	D-CELlobiose	1.32
28 MAL	D-MALtose	1.4
29 LAC	D-LACtose (bovine origin)	1.4

Strip 30 - 39

Tube	Test Active ingredients	QTY (mg/cup.)
30 MEL	D-MELibiose	1.32
31 SAC	D-SACcharose (sucrose)	1.32
32 TRE	D-TREhalose	1.32
33 INU	INUlin	1.28
34 MLZ	D-MeLeZitose	1.32
35 RAF	D-RAFfinose	1.56
36 AMD	AmiDon (starch)	1.28
37 GLYG	GLYcoGen	1.28
38 XLT	XyLiTol	1.4
39 GEN	GENtiobiose	0.5

Strip 40 - 49

Tube	Test Active	QTY

	ingredients	**(mg/cup.)**
40 TUR	D-TURanose	1.32
41 LYX	D-LYXose	1.4
42 TAG	D-TAGatose	1.4
43 DFUC	D-FUCose	1.28
44 LFUC	L-FUCose	1.28
45 DARL	D-ARabitoL	1.4
46 LARL	L-ARabitoL	1.4
47 GNT	potassium GlucoNaTe	1.84
48 2KG	potassium 2-KetoGluconate	2.12
49 5KG	potassium 5-KetoGluconate	1.8

The quantities indicated may be adjusted depending on the titer of the raw materials used.

6. Trichloro Acetic acid

Trichloro acetic acid (Merck, Germany) was taken at 4:1 quantity with milli Q water and 80% w/w solution was prepared in required quantity.

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Dated: 05 / 12 /2009

DECLARATION

This is to certify that the whole of the research work reported in the thesis in partial fulfillment of the requirement for the award of the degree of **Master of Technology** in Dairy Science in the subject of **Food Biotechnology** is the result of investigations done by undersigned under the direct guidance and supervision of **Dr. J. B. Prajapati,** Professor, Dairy Microbiology Department, Sheth M. C. College of Dairy Science, Anand Agricultural University, Anand and no part of the research work has been submitted for any other degree so far.

Place: Anand (**Arti Thakarshibhai Patel**)
Date: 05 /12/2009

Countersigned by

(Major Advisor)
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