CHAPTER 3

ISOLATION OF LACTIC ACID BACTERIA AND PROBIOTIC CHARACTERIZATION
3.1. Introduction

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

Probiotics are live microorganisms thought to be beneficial to the host organism. According to the definition by FAO/WHO, probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". The probiotic concept is open to a large number of applications in various fields relevant to human and animal health. Developing probiotic food and feed is a key research and development area for future functional food markets. Probiotic products include exopolysaccharides (EPS), vitamins, enzymes, capsules or tablets, and some fermented foods containing microorganisms. The mechanisms of probiotic action include the production of inhibitory substances, blocking of adhesion sites, competition for nutrients, stimulation of immunity and degradation of toxin receptor (Çakır, 2003). A successful probiotic strain is expected to be of a) human origin for human usage, this criteria is important for species dependent health effects, b) acid and bile tolerant, for survival
through intestine (Holzapfel et al., 2001), c) adherent to mucosal surface, to improve immune system, d) safe for food and clinical use, e) clinically validated and documented for health effects and f) possessing good technological properties (Çakır, 2003). They create a healthy equilibrium between beneficial and potentially harmful microorganisms in the gut, by competitive exclusion and by the production of organic acids, enzymes, vitamins, antioxidants, exopolysaccharides and antimicrobial compounds preventing intestinal infection (Salminen et al., 1996). The employment of antibiotics in animal feeding can be substituted with the development of products with probiotic characteristics.

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

3.2. Materials and Methods

3.2.1. Bacterial Strains and Growth Conditions

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

3.2.2. Isolation of Lactic Acid Bacteria

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

3.2.3. Production of Lactic Acid and Reducing Sugar Consumption

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

3.2.4. Probiotic Characterization Studies

3.2.4.1. Tolerance to Inhibitory Substances

Probiotic properties of the isolates were tested by checking their tolerance to acid, salt, phenol and bile salts. Tolerance to above mentioned inhibitory substances was studied in MRS broth by inoculating a culture of cell density $10^9$ CFU. Varying pH, 2.5, 3, 4 and 5 were studied. Sodium chloride: 4, 5, 8 and 12 % (w/v); 0.2-0.5 g phenol/100 ml and 0.3, 0.5 and 0.8 g sodium taurocholate /100ml were the concentration of other
inhibitory substances chosen for the study. The cultures were incubated at 37 °C and the optical density was noted at 620 nm after 24 h.

3.2.4.2. Hydrophobicity of Strains

The degree of hydrophobicity of the strains was determined by employing the method described by Thapa et al (2004). This method was based on adhesion of cells to hexadecane droplets. Cultures were grown in 10 ml MRS broth at 37 °C for 24 h and centrifuged at 6000 x g for 5 min to collect the cell pellet which was washed and re-suspended in 10 ml of Ringer solution (6 % NaCl, 0.0075 % KCl, 0.01 % CaCl₂ and 0.01 % NaHCO₃). The absorbance at 600 nm was measured. Cell suspension was then mixed with equal volume of n-hexadecane and mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min and absorbance at 600 nm of the lower phase was recorded. The percentage hydrophobicity of strain adhering to hexadecane was calculated using the equation:

\[
\text{Hydrophobicity (\%)} = \frac{\text{OD}_{600} \text{ (initial)} - \text{OD}_{600} \text{ (with hexadecane)}}{\text{OD}_{600} \text{ (initial)}} \times 100
\]

3.2.4.3. Mucin Adhesion Assay

The isolates were grown at 37 °C in Lactobacillus MRS broth supplemented with 0.1 % mucin (Sigma, USA) for 24 h to induce binding (Jonsson et al., 2001). Microtitre plate wells were coated with mucin (100 µg/µl mucin in 50 mM Na₂CO₃ buffer, pH 9.7, per well) and incubated overnight at 4 °C with slow rotation. The reaction was then blocked with PBS with 1 % Tween 20 for 1 h and washed with PBST (PBS supplemented
with 0.05 % Tween 20, pH 7.3) (Roos & Jonsson, 2002). The bacterial strains were grown as described above, washed once in PBST and diluted (A$_{595}$ = 0.5 ± 0.02) in to the same buffer. Bacterial suspension (100 µl) was added to each well and incubated for 1 h at 30 ºC. The wells were washed with PBST and absorbance taken at 405 nm.

3.2.4.4. Antimicrobial Activity

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

3.2.4.5. Antibiotic Resistance Study

All the tests were performed in Mueller-Hinton agar (Oxoid). Mueller-Hinton agar was inoculated with 50 µl of the isolates of prior adjusted OD 0.6. Inoculum was spread evenly over the entire surface of the plate by swabbing in three directions.
Antibiotic discs of vancomycin (10 µg), a glycopeptide inhibitor of cell wall and erythromycin (15 µg), an inhibitor of protein synthesis were firmly applied to the surface of the agar plates dried previously. The plates were incubated at 37 °C overnight and diameters of the zone of inhibition measured.

3.2.5. Screening for Exopolysaccharide Production

3.2.5.1. Quantitative Estimation of Exopolysaccharide Production

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

3.2.5.2. Exopolysaccharide Producing Phenotype Identification by Staining Techniques

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

3.2.5.2.1. Alcian Blue Staining

Alcian blue staining is a method which can be adopted to stain exopolysaccharides or capsular polysaccharides produced by bacterial cells. A bacterial
smear was prepared on a glass slide and stained using alcian blue (1% in 3% glacial acetic acid, whose pH adjusted to 2.5 with acetic acid) for 5 min and washed thoroughly in water. The slide was viewed under phase-contrast microscope.

3.2.5.2.2. Ruthenium Red Agar Method

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

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

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

For the molecular identification method, the bacterial strain was grown in MRS broth and the genomic DNA isolated as described in Chapter 7 (section 7.2.2). Multiplex Polymerase Chain Reaction (PCR) was performed in mixtures (25 μl) containing 50 ng of DNA, 1.5mM MgCl₂, the four deoxynucleoside triphosphates at 150 μM each, each primer (27F 5’ AGAGTTTGATCCTGGCTCAG 3’,1492R 5’ TACGGTTACCTTGGTAC ACTT 3’, L. plantarum specific primer 5’ TGAACAGTTACTTCAGATA 3’ (Uchida et al., 2004)) at 10 pM in Taq buffer, and 0.5 U of Taq polymerase. The PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) by following the programs as described. The amplification profile consisted of one cycle at 95 °C for 3 min, then 30 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min and finally, one
cycle at 72 °C for 8 min. Amplicons were analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate-EDTA buffer. A 1 kb DNA ladder (Fermentas) was used to identify the molecular sizes of the bands.

3.2.7. Experimental Statistics

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

3.3. Results and Discussion

3.3.1. Isolation and Characterization of Lactic Acid Bacteria

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

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

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

![Graph of Lactic Acid Production and Reducing Sugar Consumption](image_url)

**Fig. 3.1. Lactic acid production (bar) and reducing sugar (line) consumption**
3.3.3. Probiotic Characterization Studies

3.3.3.1. Tolerance to Inhibitory Substances

The main functional aspect concerning a probiotic is its survival in the gastrointestinal tract. The survival of bacteria in gastric juice depends on their ability to tolerate low pH and high bile concentration, as the survival of the probiotic strain may be influenced by acidic stress caused by accumulation of metabolic end products of starters or by strain itself and the amount of bile produced in the tract daily. From the study it was visible that most of the isolates obtained grew at pH 5 and the isolates from cabbage, goat excreta, SG1, SG2, W3, MC1, WJ1, and MCJ1 showed growth even in pH 2.5 (Table 3.1) establishing their presence in an acidic condition even after 24 h. The growth of LAB lowers the pH due to lactic acid production and it is this acidification process which is the most desirable side-effects of their growth. More than 2 litres of gastric juice is secreted each day into the stomach (Morelli, 2000). This gastric juice renders the stomach pH to approximately 2.0 (Murthy et al., 2000). The presence of food raises the pH value to the level of 3 (Erkkilä & Petäjä, 2000) low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolonged shelf life. Acidity has a negative impact on bacterial physiology by altering enzymatic activities leading to dissipation of the proton motive force and expression of acid response proteins (Champomier-Vergès et al., 2002). The optimum pH for most bacteria is near the neutral point (pH 7.0). Certain bacteria are acid tolerant and will survive at reduced pH levels. Notable acid-tolerant bacteria include the Lactobacillus and Streptococcus species which play a role in the fermentation of dairy and vegetable products.
The concentration of bile to be used in the selection of probiotic species for human beings must be 0.3 % (w/v). This is so because the isolated microorganism may show tolerance to high concentrations of bile. Almost all the isolates could tolerate up to 0.5 % oxgall, where as W1, W2 from whey and WJ1 from human baby excreta showed less tolerance to 0.8 % oxgall (Table 3.1). According to Pancheniak and Soccol (2005), isolates which showed 0.3 % tolerance to bile could be used as probiotic for swine. The daily average of biliary flow is very high in swine, around two litres for each 40 kg of swine (Pancheniak & Soccol, 2005) as compared to that of an adult human (70 kg) that produces 400 to 800 ml of bile daily.

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

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

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

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

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

3.3.3.3. *Mucin Adhesion Assay*

Mucus provides protective functions in the gastrointestinal tract and plays an important role in the adhesion of microorganisms to host surfaces. The potentially important property of probiotic microbes is their ability to interact with epithelial cells in
Table 3.1 Tolerance of isolated LAB towards varying growth inhibitory substances

<table>
<thead>
<tr>
<th>Isolates</th>
<th>NaCl</th>
<th>pH</th>
<th>Phenol</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 %</td>
<td>8 %</td>
<td>12 %</td>
<td>5</td>
</tr>
<tr>
<td>CB1</td>
<td>0.11</td>
<td>0.033</td>
<td>0.56</td>
<td>1.749</td>
</tr>
<tr>
<td>CB2</td>
<td>0.24</td>
<td>0.04</td>
<td>0.065</td>
<td>0.781</td>
</tr>
<tr>
<td>CB3</td>
<td>0.98</td>
<td>0.04</td>
<td>0.065</td>
<td>1.754</td>
</tr>
<tr>
<td>CB4</td>
<td>0.8</td>
<td>0.8</td>
<td>0.051</td>
<td>1.709</td>
</tr>
<tr>
<td>CB5</td>
<td>1.147</td>
<td>1.497</td>
<td>0.041</td>
<td>1.773</td>
</tr>
<tr>
<td>G1</td>
<td>1.84</td>
<td>0.07</td>
<td>0.058</td>
<td>1.516</td>
</tr>
<tr>
<td>G2</td>
<td>1.614</td>
<td>0.044</td>
<td>0.045</td>
<td>1.492</td>
</tr>
<tr>
<td>G3</td>
<td>1.819</td>
<td>0.077</td>
<td>0.064</td>
<td>1.571</td>
</tr>
<tr>
<td>SD1</td>
<td>0.035</td>
<td>0.049</td>
<td>0.039</td>
<td>0.758</td>
</tr>
<tr>
<td>SD2</td>
<td>0.045</td>
<td>0.04</td>
<td>0.046</td>
<td>0.823</td>
</tr>
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<td>SD3</td>
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<td>0.032</td>
<td>0.039</td>
<td>0.413</td>
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<tr>
<td>SD4</td>
<td>0.033</td>
<td>0.039</td>
<td>0.055</td>
<td>0.746</td>
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<td>W1</td>
<td>0.538</td>
<td>0.026</td>
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<tr>
<td>W2</td>
<td>0.036</td>
<td>0.039</td>
<td>0.055</td>
<td>0.537</td>
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<tr>
<td>W3</td>
<td>1.359</td>
<td>0.035</td>
<td>0.036</td>
<td>1.48</td>
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<tr>
<td>SG1</td>
<td>2.158</td>
<td>0.806</td>
<td>0.061</td>
<td>1.631</td>
</tr>
<tr>
<td>SG2</td>
<td>2.114</td>
<td>0.927</td>
<td>0.056</td>
<td>1.696</td>
</tr>
<tr>
<td>MC1</td>
<td>1.012</td>
<td>0.09</td>
<td>0.076</td>
<td>1.62</td>
</tr>
<tr>
<td>WJ1</td>
<td>1.1</td>
<td>0.118</td>
<td>0.11</td>
<td>1.55</td>
</tr>
<tr>
<td>MCJ1</td>
<td>1.12</td>
<td>0.092</td>
<td>0.08</td>
<td>1.521</td>
</tr>
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</table>

Standard deviation was found to be ± 0.05.
the intestinal tract, which may promote retention and host-bacterial communication. This study was another approach of hydrophobicity determining the interaction of microbes with epithelium. Mucus consists of a complex mixture of highly glycosylated proteins (mucins) and glycolipids which covers the epithelial cells of the intestine (Dekker et al., 2002). The adhesion of the microorganisms to mucins has been reported to be mediated by proteins in most cases (Roos & Jonsson, 2002). In the human intestinal tract, the layer of mucus may vary in thickness from about 30 to 300 μm, generally increasing in thickness from the small intestine to the rectum. The results were found to be coinciding with the previous experiment with all the isolates showing a mucin binding ability of ± 2 % of hydrophobicity. The isolate MC1 was showing 25 % mucin binding property (Table 3.2).

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

3.3.3.4. Antimicrobial Activity

Lactic acid bacteria have been useful at promoting bacterial interference by the production of inhibitory substances like organic acids, free fatty acids, ammonia, diacetyl, hydrogen peroxide and bacteriocins. Hydrogen peroxide is produced by many of the lactics. The antimicrobial effect is based on the oxidative properties that
Table 3.2 Surface hydrophobicity and mucin adhesion of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Hydrophobicity (%)</th>
<th>Mucin Adhesion Assay (%)</th>
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<tbody>
<tr>
<td>CB1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>CB2</td>
<td>11</td>
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<td>CB3</td>
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<td>G1</td>
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<tr>
<td>WJ1</td>
<td>9.7</td>
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</tr>
<tr>
<td>MCJ1</td>
<td>9.8</td>
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Standard deviation was found to be ± 0.05.
results in irreversible changes in the microbial cell membrane. Isolates from snake
gourd, MC1, W3, G2, G3, and CB4 showed antimicrobial activity against potential
human pathogens such as *Shigella sonnei*, *Shigella flexneri*, *Staphylococcus aureus*
and *E. coli*. The zone of inhibition is tabulated in Table 3.3.

Table 3.3 Antimicrobial activity of LAB isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Zone of inhibition (mm)</th>
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<td></td>
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<td>S. aureus</td>
<td>E. coli</td>
<td><em>Shigella sonnei</em></td>
<td><em>Shigella flexneri</em></td>
</tr>
<tr>
<td>CB1</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MC1</td>
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<td>WJ1</td>
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<td>MCJ1</td>
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</table>
3.3.3.5. Antibiotic Resistance

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

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

3.3.4. Screening for Exopolysaccharide Production

3.3.4.1. Quantitative Estimation of Exopolysaccharide Production

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

### Table 3.4 Performance of LAB isolates against Gram positive spectrum antibiotics

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Diameter of zone of inhibition (cm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Erythromycin (15 µg)</td>
<td>Vancomycin (10 µg)</td>
</tr>
<tr>
<td>CB1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB2</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>CB3</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>CB4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB5</td>
<td>-</td>
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</tr>
<tr>
<td>G1</td>
<td>1.0</td>
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<td>0.9</td>
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<tr>
<td>SD1</td>
<td>-</td>
<td>2.5</td>
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<tr>
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<tr>
<td>SD3</td>
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<td>1.5</td>
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<td>SD4</td>
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<td>SG2</td>
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<td>1.0</td>
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<tr>
<td>MC1</td>
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<td>WJ1</td>
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<td>1.3</td>
</tr>
<tr>
<td>MCJ1</td>
<td>1.1</td>
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</tbody>
</table>

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

![Exopolysaccharide production profile of LAB isolates](image)

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

3.3.4.2. **Exopolysaccharide Producing Phenotype Identification by Staining Techniques**

Exopolysaccharide producing phenotypes were confirmed by the below mentioned techniques.
3.3.4.2.1. Alcian Blue Staining

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

3.3.4.2.2. Ruthenium Red Agar Method

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

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

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

The molecular identification method by 16S rRNA sequencing by means of a multiplex PCR also coincided with the results obtained by conventional method of characterization. Two bands were obtained with the multiplex PCR reaction (Fig. 3.4), performed with the specific primers. A band of size ~1508 bp specific for eubacterial
16SrRNA and a second band of ~491 bp size specific for the *plantarum* species were obtained by PCR. The 16SrRNA sequence (Fig. 3.5) was aligned and compared with other 16SrRNA gene sequences in the GenBank by using the NCBI Basic Local alignment search tools BLAST n program. The blast results of the PCR amplicons showed 100 % identity with *Lactobacillus plantarum* (Fig. 3.6). The gene sequence was submitted in GenBank and assigned with a GenBank accession number JQ809467.

**Fig. 3.3. Phenotypic characterization by a) Phase-contrast microscopy of alcian blue staining, (b) Ruthenium red agar method**
Fig. 3.4. Multiplex PCR of 16S rRNA of MC1: Lane 1: 1 kb DNA ladder; Lane 2: Eubacterial 16S rRNA (~1508 bp) and *Lactobacillus plantarum* specific 16S rRNA (~491 bp)

Fig. 3.5. 16S rRNA sequence of MC1 identified as *Lactobacillus plantarum* MTCC 9510
Chapter 3: Isolation & Probiotic characterization

**Fig. 3.6.** Sequence identity of 16SrRNA of MC1 with *Lactobacillus plantarum*
3.4. Conclusion

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