RESEARCH PAPER
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Studies on the effect of carbon source on adhesion properties of lactic acid bacterial strains

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The influence of the carbon sources present in the reconstituted MRS medium on adhesion properties of lactic acid bacterial strains *Enterococcus durans* Afm50, *Lactococcus lactis* Gfm34 and *Lactococcus lactis* Brd10 was examined. The results demonstrated that variation in the carbon sources in the reconstituted MRS medium had a significant influence on the cell surface hydrophilicity/hydrophobicity of all strains but remained the Lewis- acid/base characteristics in the outer cell surface of LAB strains. The weakly hydrophilic strains *E.durans* Afm50 and *L.lactis* Gfm34 displayed high electronegative charge whereas strong hydrophilic strain *L.lactis* Brd10 had less electronegative charge measured in terms of zeta potential as a function of pH. Model surface of polystyrene microtiter plate on adhesive ability of LAB strains revealed that the strains adhered with varying abilities on the carbon sources. Knowledge of the bacterial adhesion on various carbon sources can have advantages in food biotechnological processes.

Key words : Lactic acid bacteria, Carbon source, Hydrophobicity/Hydrophilicity, Surface charge, Adhesion

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INTRODUCTION

Lactic acid bacteria (LAB) are ubiquitous in nature and widespread in dairy, meat products, cereals, millets and fermented foods (Hutkins, 2006). LAB strains are widely used in food industry for the production of not only in fermented milk, vegetables, sausages but also in fruit- based, vegetable-based (Karovicova *et al*., 2002) and cereal-based products (Angelov *et al*., 2006; Lamsal and Faubion, 2009). The surface properties of LAB are of major importance in fermentation technology (Boonaert and Rouxhet, 2000). In food industry, bacterial adhesion is the initial step of colonization on a solid surface subsequent biofilm formation. The adhesion of bacteria to food matrix is required for efficient utilization of substrate (Reeves *et al*., 1997; Imam and Harry- O' Kuru, 1999). Immobilization of LAB was effective in continuous inoculation of yogurt and cheese making (Champagne *et al*., 1994). LAB adhesion to the components of colloidal food matrices was also found to contribute to the stability of food emulsions (Ly *et al*, 2006a, b).

Fermentable substrates are complex in nature with the presence of various components such as proteins, lipids and carbohydrates which are involved in physico-chemical interactions. When bacteria are to be used, they can interact physico-chemically with these components. These interactions are governed by van der Waals, electrostatic interactions and cell surface hydrophobicity (Briandet *et al*., 2001; Liu *et al*., 2004). The surface properties of bacteria are dependent on the composition of fermentation medium. Consequently, the interactions of bacteria with surface exposed during bioprocessing may be strongly influenced by the composition of the fermentation medium (Schar-Zammaretti *et al*., 2005). Studies have been done on variations in microbial surface properties using various established media (Dufrene and Rouxhet, 1996), using slight variation in the formulation of commercial media (Millsap *et al*., 1997), using various carbon sources (Neufeld *et al*., 1980) or using variations in the concentration of a simple carbohydrate in complex medium (Bowen and Cooke, 1989). A better knowledge of these aspects could help us to understand that different cell surface constituents have influence in bacterial behavior. This is important since adhesion properties of LAB are of considerable technological importance in selection of food matrices and for development of food formulations.

RESEARCH METHODOLOGY

Bacterial strains and growth conditions :

The LAB strains *Enterococcus durans* Afm50, *Lactococcus lactis* Gfm34 and *Lactococcus lactis* Brd10 developed for use in food processing with characteristics *viz*., amylolysis, glycansucrase activity and broad spectrum of antibacterial activity, respectively were used in this study. LAB cultures were kept at -20°C in de Man Rogosa Sharpe (MRS) broth containing 25 per cent (v/v) glycerol. Bacteria were sub cultured twice in MRS broth at 37°C for 48 h. The amylolytic strain *E. durans* Afm50 was grown in modified MRS medium containing 20 g l^{-1} of starch instead of glucose.

Studies on hydrophobicity/hydrophilicity using MATS method:

The hydrophobic/hydrophilic character of the LAB strains and their Lewis acid/base characteristics were evaluated by the MATS (Microbial adhesion to solvents) test (Bellon-Fontaine *et al*., 1996). Three different solvents were used in this study: hexadecane, (apolar solvent); chloroform (a mono polar and acidic solvent) and ethyl acetate (a mono polar and basic solvent). Microbial adhesion to hexadecane reflects cell surface hydrophobicity or hydrophilicity whereas chloroform and ethyl acetate were regarded as a measure of electron donor/ basic and electron acceptor/acidic characteristics of bacterial cell surface, respectively.

The effect of growth in different carbon sources on adhesion properties of LAB strains was studied by pre-culturing in MRS medium and then transferred to MRS media modified with sucrose, maltose and starch. Stationary phase cells of approximately 10^8 cfu ml^{-1} were used in this study. The absorbance of the cell suspension was measured at 600 nm (OD_0). After mixing the solvents with bacterial suspension, the absorbance was measured (OD_1). The microbial adhesion to each solvent was calculated using the formula :

$$\text{Percentage of adhesion} = 1-(OD_0 / OD_1) \times 100$$

Estimation of surface charge of bacterial cells :

Electrophoretic mobility (EM) was measured to determine the cell surface net charge of the bacteria (Giaouris *et al*., 2009). Prior to the measurements, bacterial cells in the early stationary phase were harvested by centrifugation (7000 × g, 20 mins, 4°C), washed twice with 1.5 mM NaCl and resuspended in the same solution to get a final concentration of approximately 10^7 cfu ml^{-1}. The EM as a function of pH was determined in 1.5 mM NaCl. The pH of the bacterial suspension was adjusted to pH values of 3.5, 4.5, 5.5, 6.5, 7.0 and 8.0 by adding either nitric acid (HNO_3, Sigma) or potassium hydroxide (KOH, Sigma). The EM in the terms of zeta potential was measured with a zeta potential analyzer at 150 V (Zeta sizer Nano zs90; Malvern Instruments Ltd, Malvern, UK). Before injection of each bacterial suspension in the measurement chamber, electrodes were rinsed with Milli-Q™ water followed by the bacterial suspension. The bacterial suspension was used to fill clear disposable zeta cells (ATA scientific, Australia) immediately prior to zeta potential measurements. The zeta potential of bacterial cell surface was expressed as mV.

Bacterial adhesion to polystyrene microtiter plates :

The ability of the LAB strains to adhere to polystyrene micro-titer plates was evaluated using the method described by Van Merode *et al*. (2006), with some adaptations. Bacterial strains were cultivated in the MRS broth containing with different carbon sources (sucrose, starch and maltose). Bacteria from cultures in stationary phase were suspended in either 150mM NaCl, to provide an optical density at 600 nm of 0.1. A portion (200 µl) of this bacterial suspension was transferred to a well of a sterile 96-well polystyrene micro titer plate. This was allowed under static conditions for 3 h at 25^0 C. After the wells were washed twice with 150mM NaCl to remove the unbound cells, the wells were stained with 1 per cent (w/v) crystal violet (Sigma) for 30 min to quantify the amount of adhering bacteria. After staining being washed twice with 200 µl of deionized water, the crystal violet was solubilized in 200 ìl of an ethanol: acetone mixture (80:20, v/v). Dye absorbance at 630 nm (A_{630} nm) was measured using a microtiter plate reader. As a control, wells were filled with 150 mM NaCl solution without bacteria.

Statistical analysis :

All the experiments were carried out in triplicate. All the data were subjected to statistical analysis using analysis of variance (ANOVA) (Gomez and Gomez, 1984) at $p<0.05$ levels which considered as statistically significant.

RESEARCH FINDINGS AND ANALYSIS

It is known that any food component contains carbohydrate such as simple sugars (glucose, maltose) as well as polymers (starch, cellulose). The cell wall of bacteria represents a significant structural component and differences in their outer layer reflect the adaptations of the organism to specific environmental conditions (Beveridge and Graham, 1991). In this study, changes in different aspects of bacterial cell adhesion properties due to the variation in carbohydrate source in the growth medium were demonstrated. Variations in the carbohydrate source had a significant influence on the growth/cell density of the strains and reflect the physiological changes in the strains (Begovic *et al*., 2010). In the present study, when the affinity of cultures was tested to hexadecane by reconstituting the carbon sources in the MRS medium from glucose to sucrose, maltose or starch, a change in cell surface hydrophilicity/ hydrophobicity of all strains was exhibited (Fig.1). The weakly phenotypic *E.durans* Afm50

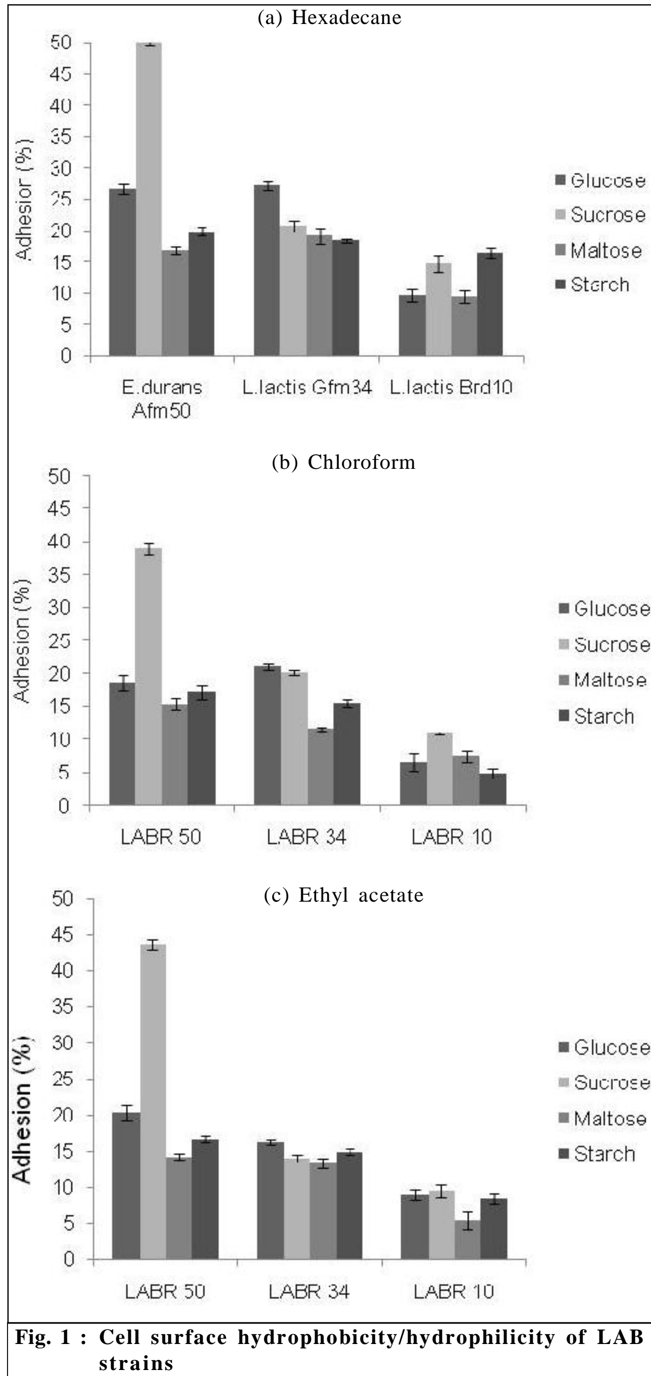

Fig. 1 : Cell surface hydrophobicity/hydrophilicity of LAB strains

when grown in sucrose medium, the cell surface changed from hydrophilicity to hydrophobicity as adhesion percentage recorded was 50 per cent. Shifting the culture from glucose to maltose and starch containing growth medium reduced the affinity percentage to hexadecane to nearly 10 per cent. The strains *L.lactis* Gfm34 and *L.lactis* Brd10 showed varied affinity to different carbon sources. The variation of strains in the hydrophilic /hydrophobic surface to different carbon sources might be related to differences in concentration of nitrogen or carbon in carbohydrate form. The hydrophilic nature of LAB strains might be due to the presence of polysaccharides in their surface (Dufrene *et al*., 1997; Pelletier *et al*., 1997) and cell surface hydrophobicity was also correlated with the concentration of nitrogen or carbon and inversely correlated with the concentration of oxygen of outer cell wall (Mozes *et al*., 1988; Boonaert and Rouxhet, 2000). The influence of composition of fermentation medium on surface properties was investigated by Schar-Zammaretti *et al*. (2005) on *Lactobacillus acidophilus* and Begovic *et al*. (2010) on *L. rhamnosus* and changes in hydrophilicity of the strains with even small changes in MRS composition was demonstrated. Regardless of the carbon sources used, the affinity values for chloroform and ethyl acetate remained lower than the values for hexadecane for all the three strains. The Lewis- acid/base characteristics were not changed for the LAB strains when the carbohydrate source was changed in the medium as the strains retained both electron accepting and weakly electron donating nature.

The surface electrical properties of the three LAB strains were measured in terms of zeta potential as a function of pH. The bacterial cell charge was attributed to cell wall constituents of phosphate and carboxyl groups, proteins, etc (Boonaert and Rouxhet, 2000). Electrostatic charges have been found to influence the adhesion of bacteria to solids (Martinez – Martinez *et al*., 1991). For relatively hydrophilic organisms, electro-kinetic potential is the main mode of adhesion (Stevik *et al*., 2004). In the present study, the weakly hydrophilic strains *E. durans* Afm50 and *L.lactis* Gfm34 exhibited high electronegative charge at whole pH values ranged from 3.5 to 8.0 while low electronegative charge was observed in the strong hydrophilic surface strain *L.lactis* Brd10. The strain *L.lactis* Brd10 reached isoelectric point at pH 4.5 (Fig. 2). The electrostatic charge on microbial surfaces is caused by dissociation of various inorganic groups like carboxyl and amino group, located on the outer surface, and situated in deeper layers of the cell wall (vas Loosdrecht *et al*., 1987). High negative charge at low pH for the strain *E. durans* Afm50 and *L.lactis* Gfm34 inform the absence of surface S-layer in the strains but the presence of strong polysaccharide coating (Schar-Zammaretti and Ubbink, 2003). The decrease in zeta potential between pH 3.5 and 6.5 is likely be caused by the increase in the dissociation of weak acidic groups of polysaccharides constituents of the cell outer surface (Poortinga *et al*., 2001).

A model polystyrene surface was used to understand the adhesion of bacteria to a solid surface. The strains used here adhered with varying abilities on the carbon sources glucose, sucrose, maltose and starch at high ionic strength of

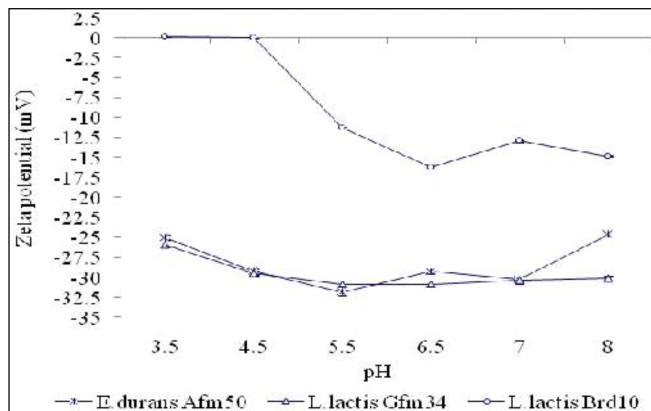

Fig. 2 : Cell surface electrical charge of LAB strains

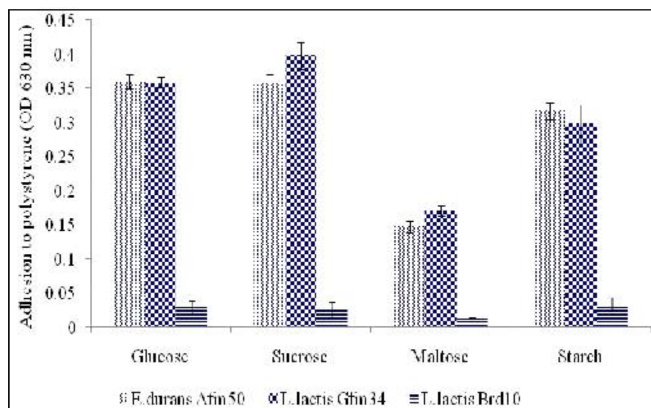

Fig. 3 : Influence of carbon sources on adhesion of LAB strains to polystyrene plates

150mM NaCl. The weakly hydrophilic strain of *E.durans* Afm50 and *L.lactis* Gfm34 displayed higher adhesion than the strong hydrophilic strain *L.lactis* Brd10 for all carbon sources. Adhesion was low in maltose for all the strains. The glycansucrase producing *L.lactis* Gfm34 showed high adhesion on sucrose. The amylolytic strain *E. durans* Afm50 adhered better on glucose and sucrose; however, on starch the strain demonstrated high adhesion on comparison with other two strains (Fig.3). Slight preference of carbon source to adhesion in relation to their functional enzyme activity was observed in *E.durans* Afm50 and *L.lactis* Gfm34. Adhesion may be increased or decreased by changes in the bacterial surface if adhesive interactions are increased or reduced depending on the type (e.g. hydrogen bonding, charge) and number of physico-chemical interactions between the solid surface and a bacterial surface (Mceldowney and Fletcher, 1986).

Conclusion :

In this study, there was a change in cell surface hydrophilicity when carbon source was varied but remained unchanging of electron donor/electron acceptor nature in the outer surface of the LAB strains. Knowledge of bacterial adhesion of these starter cultures on various carbon sources can have advantages in food biotechnological processes. Furthermore, understanding the process whereby bacterial adhesion to food substrate may lead to advances in the technology of whole cell-immobilization on food matrices, a process with many food industrial applications.

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