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Fermentation Characteristics of Exopolysaccharide-Producing Lactic Acid Bacteria from Sourdough and Assessment of the Isolates for Industrial Potential

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Lactic acid bacteria (LAB) with antimicrobial activity and high exopolysaccharide (EPS) production ability isolated from sourdough were studied for their fermentation characteristics as potential new starter cultures. The values of pH, titratable acidity, and viable cell counts were 4.06±0.009–4.50± 0.015, 0.787±0.020%–1.172±0.018%, and 8.78±0.08–8.98± 0.06 log CFU/ml, respectively. In order to select probiotics with a high survival rate in the gut, isolates were tested to assess resistance against the artificial gastric acid and bile juice. Viable LAB counts were significantly (p<0.05) affected by the acidity. At pH 2.0, the total declines in the initial bacterial counts were 4.52±0.07 log for *S. thermophilus* St-Body-1, >7.98±0.03 log for *E. flavescens* DU-10, >7.95±0.05 log for *E. faecium* DU-12, and 3.15± 0.06 log for *L. amylovorus* DU-21. Among the strains, *L. amylovorus* DU-21 was the only strain that had bile tolerance under simulated gastrointestinal conditions. In order to improve EPS production by *L. amylovorus* DU-21, the influence of carbon source was studied. When glucose was used as a carbon source, EPS production dramatically increased to 17.19±0.28 g/l (p<0.05). The maximum cell growth (10.012±0.012 log CFU/ml) and EPS production (18.71±0.19 g/l) were achieved when 15 g/l of glucose was employed as the carbon source.

Keywords: Exopolysaccharides, *Lactobacillus amylovorus*, probiotic, gastrointestinal stress tolerance, starter culture

In 1989, Fuller defined "probiotic" as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. This has become the most widely accepted definition [13]. There is some debate as to whether the concept of probiotic should include dead microorganisms, or even bacterial fragments [49]. Naidu *et al.* [31] introduced the concept of "Probiotic Active Substance", as a cellular complex of lactic acid bacteria (LAB) that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independently of the viability of lactic acid bacteria. Nowadays, most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* [35]. However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces* [40, 41], and *Propionibacterium* [14] are also considered as probiotic microorganisms. It is clear that the selection of new strains provides an exciting challenge both to science and industry. However, despite that probiotic microorganisms are claimed to promote health, the mechanisms involved have not been fully elucidated yet. Approaches for selection of an "ideal" strain are therefore still difficult and indeed require considerable resources. Desirable technical features, and factors related to health promotion or health sustainment, serve as important criteria for strain selection [20]. It is generally considered now that three major categories should be taken into account as key criteria for selection of an appropriate strain: general aspects, including origin, identity, safety, and resistance (*e.g.*, to mutations and environmental stress, and to the antimicrobial factors prevailing in the upper gastrointestinal tract; technical aspects (growth properties *in vitro* and during processing, survival and viability during transport and storage); and functional aspects, as well as beneficial features. An important criterion when selecting a probiotic strain is its ability to survive the acidic conditions of the gastric tract. Acid and bile tolerances are important in potential probiotics, as bile tolerance is required for bacterial growth and survival in the small intestine, and acid tolerance is required for the bacteria to survive passage through the stomach, as well as to survive in food [19, 28]. The primary barrier to the survival of probiotic microorganisms in the stomach is mainly the low pH, which is related to the high hydrochloric acid concentration of the secreted gastric acid [21]. Acid- and bile-tolerant strains have been found, and some of them are on the market. However, less than

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10% of the strains tested can grow in gastrointestinal juice [7], and the survival of the LAB depends on the strain and the individual who has ingested the bacteria [8].

Certain strains of LAB are able to synthesize exopolysaccharides (EPS) that are secreted into their environment such as milk [3, 5, 36, 44]. In particular, for the production of yoghurt, drinking yoghurt, cheese, fermented cream, and milk-based desserts, EPS-producing LAB are very important [2, 4, 6, 9]. EPS contribute to the texture, mouth-feel, taste perception, and stability of the final product. In the yoghurt business, there is increasing interest by the consumer for stirred yoghurt products with a smooth and creamy texture. During yoghurt fermentation, lactose present in the milk is converted to lactic acid, which decreases the pH of the product. As a consequence, casein micelles are destabilized when the pH reaches about 5.3 and irreversibly coagulate to form a gel [34]. Stirred yoghurts are obtained by mild homogenization of the milk coagulum after fermentation. However, the mechanical treatment strongly affects the rheology of the coagulum and favors syneresis (serum separation) since the network formed by the gel is broken. Several solutions were proposed to improve the texture of fermented milk products and reduce syneresis. Texture and stability are affected by the culture conditions [37, 42] and the manufacturing procedure. Well-known technological approaches to improve the quality of the product comprise an increase in milk solids such as fat, proteins [38], or sugars (sucrose, fructose), and addition of stabilizers such as pectin, starch, alginate, or gelatin, when permitted by national legislation. However, these approaches do not address an increasing consumer demand for products with low (or reduced) fat, low sugar, low cost, and with as few food additives as possible [22]. An answer to this challenge is to incubate the starter cultures at suboptimal growth temperature and/or to take advantage of the EPS produced naturally by LAB used as the starter culture in the fermentation. The growth temperature regulates different metabolic pathways. The cell growth was either inhibited or limited by a compound other than glucose. When glucose was depleted, the EPS concentration often started to decline. This is observed frequently in fermentations of other EPS-producing LAB and is possibly due to a combination of glucose limitation and activation of glycohydrolases [36].

Results obtained from our previous study [23] suggest the possibility and potential of developing the lactic acid bacteria-containing probiotic yoghurt. The aim of this work was to determine and compare some probiotic characteristics and resistance to biological barriers of LAB isolated from sourdough. In addition, we tested various carbon sources that might influence the production of EPS from the culture fluid of LAB, and determined the optimal concentration for cell growth and EPS production.

Materials and Methods

Microorganisms

Enterococcus flavescens DU-10, *E. faecium* DU-12, and *Lactobacillus amylovorus* DU-21, producer of the exopolysaccharides, were used throughout this study. All strains were isolated from traditional sourdough (type I sourdough) and stored at −25°C in de Man, Rogosa, and Sharpe (MRS) medium (Difco, USA) containing 15% (v/v) glycerol (Sigma Chemical Co., U.S.A.) as a cryoprotectant. *Streptococcus thermophilus* St-body-1 (Chr. Hansen, Denmark) was used as an indicator strain for the estimation of industrial potential. The strains were propagated twice in MRS broth at 37°C for 18 h before experimental use and inoculated at 5 log CFU/ml into MRS broth and incubated at 37°C for 24 h.

Fermentation Experiments

Cell growth was determined by measuring the optical density of the culture fluid at 600 nm (OD_{600}; UV-1201, Shimadzu, Japan) and by determination of the number of colony forming units (CFU) by plating serial dilutions on BCP agar (Eiken Chemical Co., Japan) after an incubation at 37°C for 24 h. The pH was measured with a pH meter (model 420A; Orion, U.S.A.) for 10 min at room temperature. The titratable acidity was assessed by following the AOAC titration method using 0.1 N NaOH [1]. Approximately 10 g of the culture fluid was diluted with approximately 20 ml of distilled water before titration. Titratable acidity was expressed as percentage of lactic acid, determined using the following equation:

$$\text{Lactic acid (\%)} = \frac{0.1\text{ N NaOH required} \times 0.1\text{ N NaOH factor} \times 0.009}{\text{Weight of sample}} \times 100$$

Determination of Acid and Bile Tolerances

The artificial gastric juice was prepared by supplementing MRS broth with pepsin (Junsei, Japan). The MRS broth was prepared by adjusting to pH 2.0, 2.5, 3.2, and 7.2 (control) with 1 N HCl (OCI, Korea), respectively. The pepsin solution was filter sterilized using a 0.45-μm-pore-size cellulose nitrate filter (Toyo Roshi Co., Ltd., Japan) and was then added to MRS broth (pH 2.5) to a final concentration of 1,000 units/ml. The LAB were incubated at 37°C for 24 h and then centrifuged (6,000 rpm for 10 min, 4°C). The collected cells were then resuspended in sterile saline (0.85% NaCl). The cells were inoculated at 8 log 10 CFU/ml into artificial gastric juice and incubated at 37°C for 3 h. The bacterial counts were determined with BCP agar plates.

Bile tolerance was determined in MRS broth containing 1.0% bile acids (10% oxgall; Difco, U.S.A.). Before testing for bile tolerance, all strains were incubated at 37°C for 3 h in artificial gastric juice (pH 2.0) and then inoculated at 1% (v/v) into MRS broth containing 1.0% bile acids. Cultures were incubated at 37°C for 24 h. The bacteria were plated and enumerated after 24 and 48 h of incubation.

Determination of Suitable Carbon Source for EPS Production

For the production of EPS, the existing MRS medium was modified. To examine the effect of carbon source on cell growth and EPS production, different carbon sources (fructose, galactose, glucose, lactose) were provided at a concentration of 20.0 g/l. To find the optimal concentration of carbon source for cell growth and EPS production, LAB were cultivated under different carbon concentrations ranging from 0 to

40 g/l. All LAB were grown in modified MRS broth (Difco, U.S.A.) at 37°C for 48 h.

Isolation and Quantification of Exopolysaccharides (EPS)

EPS were isolated from the fermented sample according to a modified method of Yang *et al.* [47]. After incubation, cultures were heated at 100°C for 20 min to inactivate potential EPS hydrolases and improve detachment of EPS from the microbial cell walls. Trichloroacetic acid (Junsei, Japan) solution was added to the fermented sample to give a final concentration of 4% (w/v), and the precipitated protein and bacteria were removed by centrifugation (12,000 rpm for 40 min at 4°C). The supernatant was then mixed with an equal volume of ethanol, stored at 4°C for 24 h, and centrifuged, as described above, to collect the precipitated EPS. The precipitate was resuspended in 25 ml of distilled water. EPS solutions were dialyzed (molecular weight cut-off 12,000) at 4°C for 24 h against distilled water. After 24 h of freezing at -80°C, followed by 24 h of freezeddrying by lyophilization at -18°C, the dry weight of the precipitated EPS was determined.

Statistical Analysis

The mean and standard deviation of data were calculated using the SAS Statistical Analysis for Windows v.9.1 (SAS Institute Inc., Cary, N.C., U.S.A.). Significance of differences were determined by ANOVA and Duncan's multiple range tests at the level of $p<0.05$.

Results

Fermentation Characteristics of LAB Isolated from Sourdough

Fig. 1 shows the growth patterns and viable cell counts that occurred when the four LAB (*S. thermophilus* St-Body-1, *E. flavescens* DU-10, *E. faecium* DU-12, and *L. amylovorus* DU-21) were inoculated into MRS broth medium. These four LAB used in this study grew well in MRS broth and their populations stabilized after 21 h, 15 h, 15 h, and 18 h incubation, respectively. After 24 h, the maximum viable cell concentration was found in *L. amylovorus* DU-21 with 8.98±0.06 log CFU/ml, followed by *E. faecium* DU-12 with 8.95±0.05 CFU/ml. Fermented dairy products require probiotic bacteria at 7 log CFU/ml in order to give health effects in the gastrointestinal tract when consumed [43]. In fermented milk, LAB are present in important concentrations (more than 9 log CFU/ml at the end of fermentation, an amount that is maintained over 8 log CFU/ml for some probiotic products to which many biologically inactive cells can be added) [46]. The viable cell counts ranged from 8.78±0.08 log CFU/ml to 8.98± 0.06 log CFU/ml. These results were within the range obtained by Shah [43] and Yoon *et al.* [48].

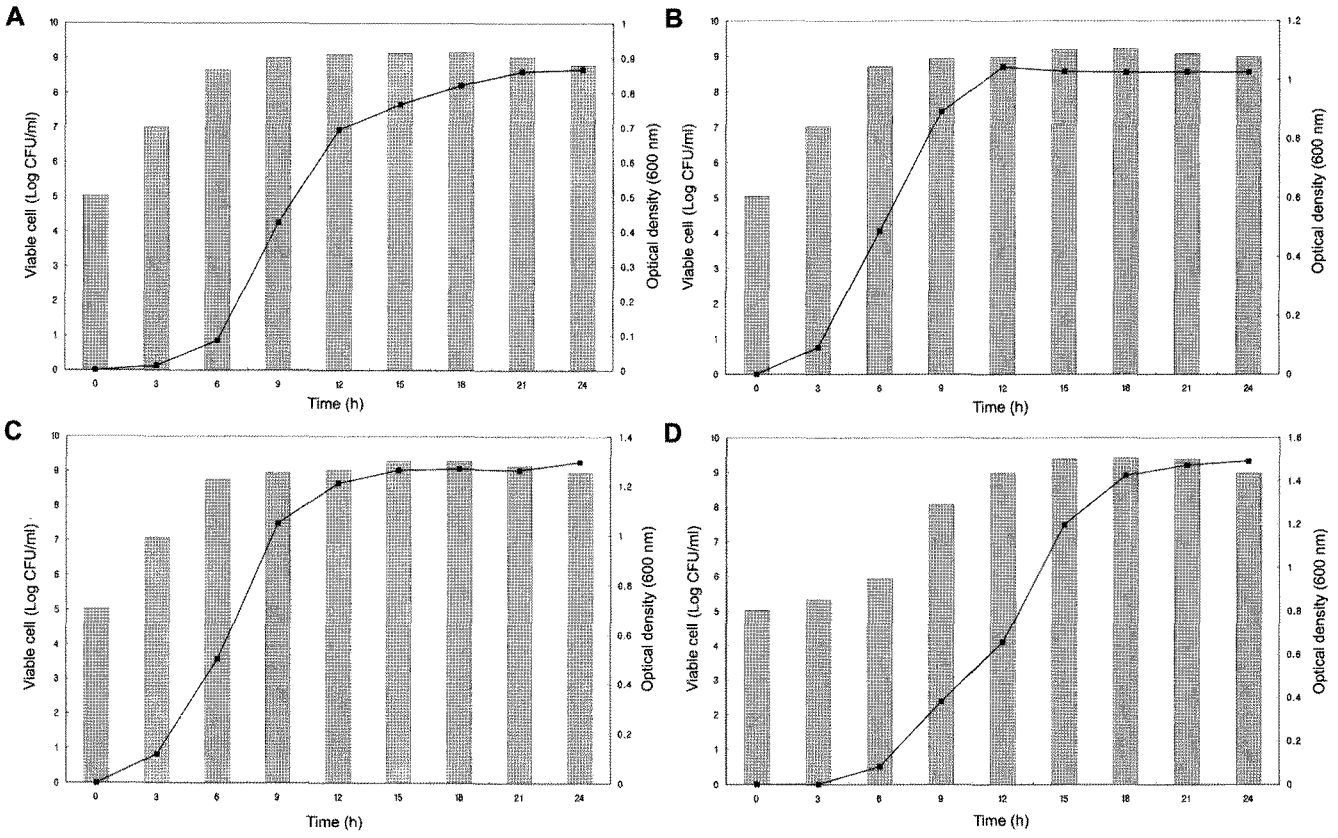

Fig. 1. Profile of viable cell count and optical density by lactic acid bacteria in MRS broth at 37°C.
▒: Viable cell count; ■–■: Optical density. **A.** *Streptococcus thermophilus* St-Body-1; **B.** *Enterococcus flavescens* DU-10; **C.** *Enterococcus faecium* DU-12; **D.** *Lactobacillus amylovorus* DU-21.

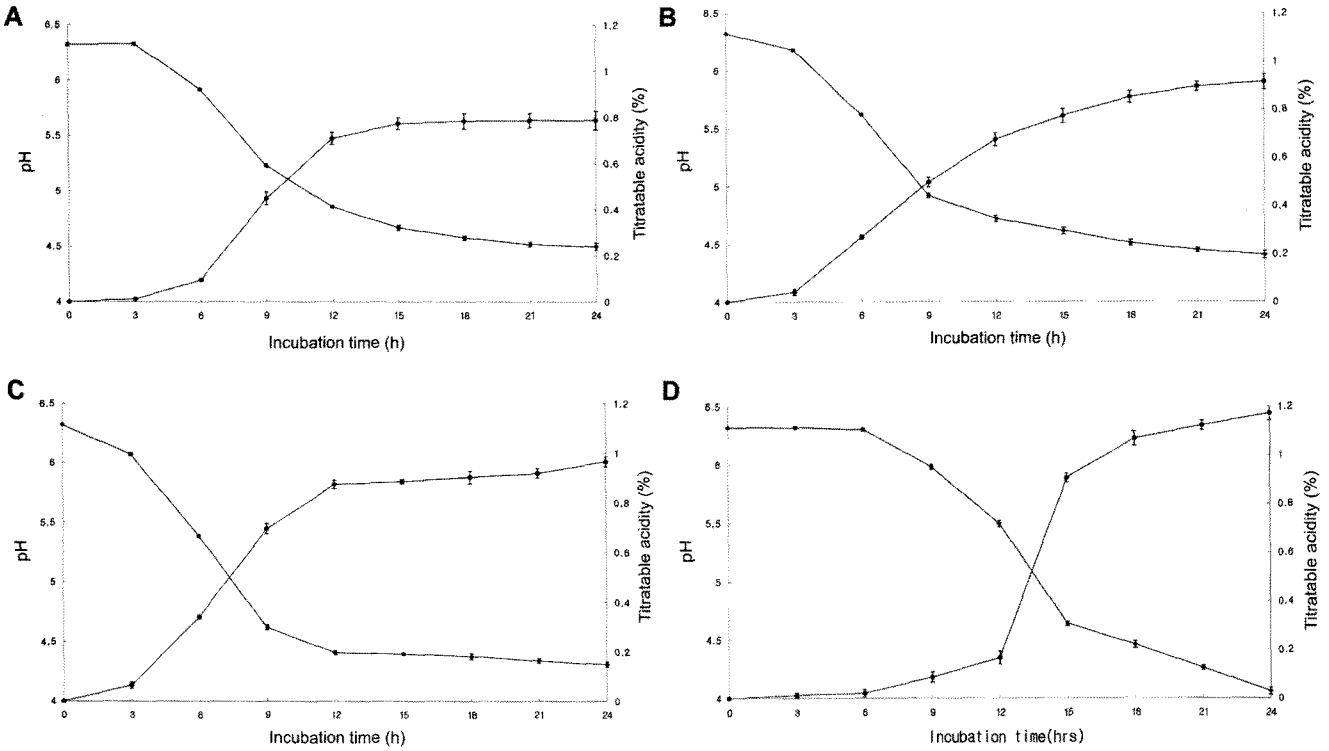

Fig. 2. Changes in pH and titratable acidity for lactic acid bacteria grown in MRS broth at 37°C.
◆–◆: pH; ●–●: titratable acidity. **A.** *Streptococcus thermophilus* St-Body-1; **B.** *Enterococcus flavescens* DU-10; **C.** *Enterococcus faecium* DU-12; **D.** *Lactobacillus amylovorus* DU-21.

The pH changes and titratable acidity of the four LAB are shown in Fig. 2. The results showed that pH ranged from 4.06±0.009 to 4.50±0.015, and titratable acidity from 0.787±0.020% to 1.172±0.018%. The final pH values of MRS broth inoculated with the LAB isolated from sourdough were lower than that of the fermented MRS broth inoculated with *S. thermophilus* St-Body-1. The formation of the yoghurt gel is related to pH decrease and culture behavior during fermentation. According Lee and Lucey [27], the development of the elastic gel structure with a solid-like behavior starts at a pH of around 5.6, causing changes in the micelle structure due to solubilization of colloidal calcium phosphate, and was terminated at a pH of around 4.6. pH changes of the samples were lower than those reported by Lee and Lucey [27]. Significant acid production and associated drop in pH values by most of the four LAB were observed during the incubation period. The highest titratable acidity, expressed as the amount of lactic acid, was detected from *L. amylovorus* DU-21 (1.172±0.018%) and the minimum production was detected from *S. thermophilus* St-Body-1 (0.787± 0.020%). The lactic acid production results were similar to those reported by Damiani *et al.* [10], and Şimşek *et al.* [45].

Table 1. Survival of probiotic lactic acid bacteria isolated from sourdough in artificial gastric acid and artificial bile juice.

Strains[a]	0 h (pH 7.2) (log CFU/ml)	Artificial gastric juice			Artificial bile juice	
		3 h (pH 2.0) (log CFU/ml)	3 h (pH 2.5) (log CFU/ml)	3 h (pH 3.2) (log CFU/ml)	Without oxgall (log CFU/ml)	With 10% oxgall (log CFU/ml)
ST	8.780±0.08	4.26±0.05	6.88±0.06*	4.26±0.05	8.39±0.04	6.49±0.03**
EFDU-10	8.982±0.03	<1.0*	4.46±0.06*	5.73±0.09*	-	-
EFDU-12	8.953±0.05	<1.0*	4.30±0.03*	5.38±0.05*	-	-
LADU-21	8.985±0.02	5.83±0.06*	7.95±0.05*	8.05±0.01*	8.93±0.04	8.68±0.05**

[a]Lactic acid bacteria isolated form sourdough. ST: *Streptococcus thermophilus* St-Body-1; EFDU-10: *Enterococcus flavescens* DU-10; EFDU-12: *Enterococcus faecium* DU-12; LADU-21: *Lactobacillus amylovorus* DU-21.
*Means that the viable cell count shown is significantly different ($p<0.05$) from that before acid treatment (pH 7.2, 0 h) shown in the same row.
**Means that the viable cell counts after bile treatment is significantly different ($p<0.05$) from that without bile treatment shown in the same row.

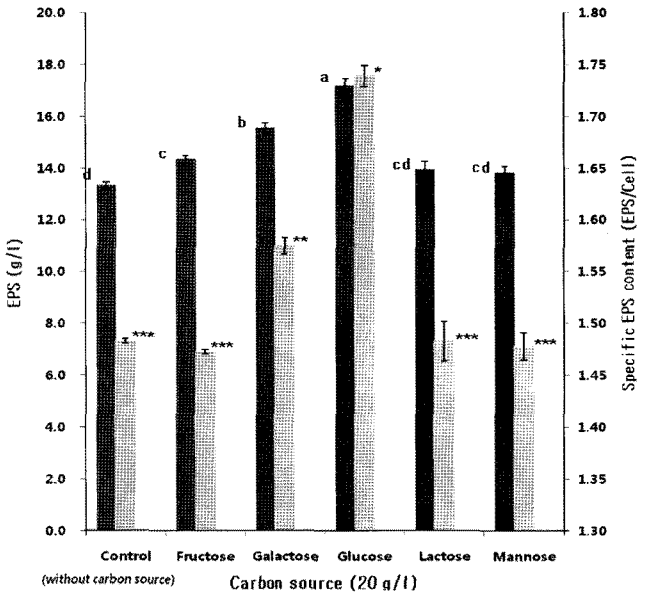

Fig. 3. Effect of carbon source on EPS production by *Lactobacillus amylovorus* DU-21 at 37°C.
■: EPS; ▒: Specific EPS content.[a-d] *-***, Mean with the same letter are not significantly different, α=0.05.

Survival Rates Under Simulated Gastrointestinal Conditions

The gastrointestinal tract is the major location to affect the viability of LAB cells. According to Havennar and Husis [18], the stabilities of LAB cells obtained from either *in vivo* or *in vitro* study are similar. In this study, the viable LAB counts were determined after 3 h incubation in the artificial gastric juice. Results from Table 1 show that viable LAB counts were significantly ($p<0.05$) affected by the acidity. Although *L. amylovorus* DU-21 showed the highest level of survival during 3 h incubation in artificial gastric juice, all LAB showed a significant losts ($p<0.05$) in viability after their incubation for 3 h with the artificial gastric juice. At pH 2.0, the total declines in the initial bacterial counts were 4.52±0.07 log (*S. thermophilus* St-Body-1), >7.98±0.03 log (*E. flavescens* DU-10), >7.95±0.05 log (*E. faecium* DU-12), and 3.15±0.06 log (*L. amylovorus* DU-21).

The bile in animal intestine is also an important factor that affects the LAB viability [15]. In this study, LAB strains survived from acid (pH 2.0) treatment were cultured in MRS broth with 10% oxgall so that its tolerance to bile could be evaluated. After 24 h cultivation, effects of bile salt on the growth of the LAB were observed (Table 1). Among the strains, *L. amylovorus* DU-21 was the only strain that had bile tolerance under simulated gastrointestinal conditions. In a previous study, Gilliland *et al.* [16] showed that bile resistance could differ among strains of one single species of enteric lactobacilli and that this difference could account for differences in the ability to colonize the intestinal tract of calves.

Effect of the Carbon Source on EPS Production of *L. amylvorus* DU-21

In order to improve EPS production by *L. amylovorus* DU-21, the influence of carbon source was studied. Five carbon sources were tested. Results of the EPS production are shown in Fig. 3. Maximum EPS production was affected by the carbon source. Fructose, galactose, glucose, lactose, and mannose showed a maximum EPS production of 14.34±0.15 g/l, 15.55±0.22 g/l, 17.19±0.28 g/l, 13.94±0.32, and 13.82±0.25, respectively ($p<0.05$). EPS production without carbon source by *L. amylovorus* DU-21 in MRS broth at 37°C for 48 h was 13.32±0.094 g/l. The specific EPS contents ranged from 1.473±0.002 EPS/cell to 1.793±0.010 EPS/cell. When glucose was used as a sole carbon source, EPS production and specific EPS content dramatically increased to 17.19±0.28 g/l and 1.793±0.010 EPS/cell, respectively. Lactose and mannose were less favorable carbon sources for EPS production of *L. amylovorus* DU-21. This indicated that glucose might be easier to use than any other carbon sources for biosynthesis of EPS. Similar results for EPS production were reported from *L. acidophilus* CH-2 and *L. delbrueckii* ssp. *bulgaricus* LB12 [24].

To determine the optimal concentration of glucose for cell growth and EPS production, *L. amylovorus* DU-21 was cultivated under different carbon concentrations ranging

Table 2. Effect of glucose on cell growth and EPS production by *Lactobacillus amylovorus* DU-21.

Glucose (%)	Final pH[b]	Titratable acidity (%)	Viability(log CFU/ml)	EPS production (g/l)	Specific EPS content (EPS/cell)
0	4.06±0.01[a]	1.17±0.02[c]	8.985±0.094[e]	13.32±0.09[c]	1.483±0.005[de]
0.5	3.74±0.05[a]	1.91±0.06[a]	9.823±0.015[d]	14.47±0.15[bc]	1.473±0.013[e]
1.0	3.81±0.01[a]	1.82±0.04[ab]	9.862±0.006[cd]	15.53±0.06[bc]	1.575±0.005[c]
1.5	3.66±0.02[a]	2.21±0.07[a]	10.012±0.012[a]	18.71±0.19[a]	1.869±0.017[a]
2.0	3.75±0.10[a]	1.91±0.12[a]	9.884±0.105[bc]	17.19±0.28[ab]	1.739±0.010[b]
3.0	3.79±0.12[a]	1.87±0.15[a]	9.922±0.021[b]	15.07±0.21[bc]	1.519±0.018[d]
4.0	3.87±0.09[a]	1.61±0.10[bc]	9.888±0.265[bc]	14.89±0.10[bc]	1.480±0.025[de]

[a-e]Mean with the same letter in the same column are not significantly different, α=0.05.
* Initial pH: 6.32.

from 0 to 40 g/l. As shown in Table 2, the concentration of glucose affected cell growth, EPS production, and specific EPS content ($p<0.05$). The maximum cell growth (10.012±0.012 log CFU/ml), EPS production (18.71±0.19 g/l), and specific EPS content (1.869±0.017 EPS/cell) were achieved when 15 g/l of glucose was employed as the carbon source.

DISCUSSION

In this study, some important characteristics of LAB isolated from sourdough were evaluated before using them for probiotic use. Since these LAB are nonpathogenic and nontoxic bacteria, the first trial was focused on the determination of their resistances to simulated gastrointestinal transit conditions.

A major problem for the deliberate use of LAB isolated from sourdough is their limited resistance to stress conditions occurring upon preparations; e.g., drying, heat, and acid [46]. Data reported here showed that LAB isolated from sourdough could be used for yoghurt fermentation. Characterization of *L. amylovorus* DU-21 showed that it was bile- and acid-tolerant. The results of this study indicate that *L. amylovorus* DU-21 is a safe probiotic with the potential to reduce serum cholesterol. Cholesterol-lowering effects may be due in part to the deconjugation of bile salts by strains of bacteria that produce the enzyme bile salt hydrolase (BSH) [33].

Studies of the application of EPS produced by sourdough LAB have primarily focused on heteropolysaccharides (HePS) from lactobacilli in dairy fermentations. Growth conditions (pH, temperature, and incubation time) and medium composition (carbon, nitrogen sources, and other nutrients) can affect the EPS yield and the sugar composition. HePS are produced in small amounts, usually below 0.5 g/l [26]. Homopolysaccharides (HoPS) are synthesized by extracellular glucan- and fructosyltransferases using sucrose as the glycosyl donor [30]. Sucrose is rarely fermented as such by sourdough LAB, although some strains display sucrose hydrolysis through levansucrase activity [17]. De Vuyst *et al.* [11] reported that *L. amylovorus* strains displayed very little growth in the presence of sucrose as the sole energy source. Furthermore, sucrose uptake was not stimulated if other sugars (glucose, maltose, and/or fructose) were added and neither did sucrose interfere with the consumption of these sugars. Leroy *et al.* [29] have also demonstrated that glucose is of primary importance for the cell growth and EPS production of *L. amylovorus* strains. When glucose was used as a carbon source in this study, EPS production dramatically increased to 17.19±0.28 g/l. The maximum cell growth and EPS production were achieved when 15 g/l of glucose was employed as the carbon source. These results were in accordance with De Vuyst *et al.* [11] and Leroy *et al.* [29].

EPS represent only a small fraction of the current biopolymer market. Factors limiting the use of microbial EPS are their economical production, which requires a thorough knowledge of their biosynthesis and an adapted bioprocess technology, the high costs of their recovery, and the non-food bacterial origin of most of them [12]. Strains of generally recognized as safe (GRAS) microorganisms, and in particular, LAB that are able to produce EPS in large enough quantities are an interesting alternative for food uses of EPS [3, 5, 40]. Moreover, LAB can be used for the production of EPS, particularly for the production of yoghurt, drinking yoghurt, cheese, fermented cream, and milk-based desserts, to improve their rheology and mouth-feel [12]. Finally, it has been suggested that EPS produced by food-grade bacteria may contribute health benefits [22, 25, 32, 39]. Therefore, EPS from LAB have potential for development and exploitation as food additives or functional food ingredients with both health and economic benefits. Hence, novel microbial biopolymers may fill gaps in the market-available polymers or may replace traditional food products in terms of improved rheological and stability characteristics.

According to the results of this study, *L. amylovorus* DU-21 is a potentially important starter culture organism because it produces lactic acid, EPS, and has bile and acid tolerances. Further studies will be required to determine the mechanism underlying the cholesterol-lowering effect. It will also be necessary to test more animals, using varying doses of bacteria over longer times, to assess the long-term probiotic potential of *L. amylovorus* DU-21. We must also study the potential properties of adhesion that could result from the presence of the EPS.

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