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Full Length Research Paper

Growth and exopolysaccharide production by *Lactobacillus fermentum* F6 in skim milk

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Growth and exopolysaccharide (EPS) production by *Lactobacillus fermentum* F6 isolated from traditional dairy products in Inner Mongolia of China were studied when the strain was grown in 10% (w/v) reconstituted skim milk under different culture conditions. The results showed that culturing of *L. fermentum* F6 at a temperature (37°C) and pH (6.5) optimal for growth was also favorable for EPS production. Supplementation in 10% (w/v) skim milk with different carbon sources (glucose, lactose, galactose and fructose) increased EPS production; glucose being more effective than other sugars. Supplementation with whey protein concentrate (WPC) at 0.5% (w/v) resulted in about two-fold increase in EPS production. A maximum of 44.49 mg/l of EPS was produced by *L. fermentum* F6 in the skim milk medium supplemented with 2% (w/v) glucose and 0.5% (w/v) WPC at 37°C and at initial pH 6.5. Monosaccharide analysis showed that the EPS of *L. fermentum* F6 was composed of glucose and galactose in a molar ratio of 4:3. The molecular mass of the EPS was determined to be 3.54×10^6 Da. A 1% (w/v) aqueous solution of the EPS showed relatively high viscosity, indicating the potential of this EPS to be used as a viscosifying agent in food products.

Key words: Lactic acid bacteria, *Lactobacillus fermentum*, exopolysaccharides.

INTRODUCTION

There have been many studies on exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) during the last decade (Nichols et al., 2005; Vaningelgem et al., 2004; Ruas-Madiedo et al., 2005; Celik et al., 2008). EPSs produced by LAB have received increasing attention mainly because these biopolymers play an important role in the improvement of physical properties of fermented food products. It has been shown that EPSs of LAB contribute to the texture, mouth-feel, taste perception and stability of fermented products (Hernández et al., 2009; Girard and Schaffer-Lequart, 2007; Ahamed et al., 2005). Since LAB strains have a GRAS (generally regarded as safe) status, the use of EPS-producing LAB could result in safe and natural products with reduced use of stabilizers (Duboc and Mollet, 2001). Furthermore, it has been reported that EPSs of LAB have anti-tumoral (Oda et al., 1983), anti-ulcer (Nagaoka et al., 1994), immunomodulating (Chabot et al., 2001; Makino et al., 2006) or cholesterol-lowering activity (Kitazawa et al., 1998; Korakli et al., 2002).

Although many LAB strains have been reported to produce EPSs, the yield of EPS is generally low (Ruas-Madiedo et al., 2002), and applications of these polymers in the improvement of physical properties of food

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Abbreviations: EPS, Exopolysaccharide; **WPC,** whey protein concentrate; **LAB,** lactic acid bacteria; **GRAS,** generally regarded as safe; **MRS,** Man Rogosa Sharpe; **°T ,** titratable acidity; **HPLC,** high-performance liquid chromatography; **DEAE,** diethylaminoethyl.

products are limited (Degeest and De Vuyst, 1999). Currently, dextran produced by *Leuconostoc mesenteroides* is the only commercial EPS product used in the food industry. Previous studies have shown that EPS production by LAB can be improved by manipulating medium composition (Degeest et al., 2002; Duenas et al., 2003; Lee et al., 2007), such as carbon source (Looijesteijn et al., 1999; Cerning et al., 1994), nitrogen source (Degeest and De Vuyst, 1999; Zisu and Shah, 2003), and environmental conditions, that is, temperature (Nichols et al., 2005; Vaningelgem et al.,2004), pH (Kimmel et al., 1998; Degeest et al., 2002), oxygen tension (Gamar-Nourani et al., 1998) and incubation time (Pham et al., 2000; Lin and Chang Chien, 2007). For some EPS-producing LAB strains, such as those from *Streptococcus* (Escalaute et al., 1998; Urshev et al., 2006; Faber et al., 2001), *Lactococcus* (Ramos et al., 2001; Dabour et al., 2005) and *Lactobacillus* (Desai et al., 2006; Rodríguez-Carvajal et al., 2008; Briczinski and Roberts, 2002; Torino et al., 2005), manipulating their physiological conditions could result in increased EPS biosynthesis.

In the search of EPS-producing LAB strains for potential industrial applications, we found that a *Lactobacillus fermentum* strain F6 isolated from traditional dairy products in Inner Mongolia of China produced a viscous EPS when grown in skim milk. In order to further understand the EPS biosynthesis properties of *L. fermentum* F6, growth and EPS production by this strain in skim milk were investigated under different culture conditions. The EPS was isolated, purified and characterized with respect to monosaccharide composition, molecular mass and viscosity properties of the polymer.

MATERIALS AND METHODS

Bacterial strain

L. fermentum F6 was obtained from LAB Collection of Inner Mongolia Agricultural University, Hohhot, China. It was used as the EPS-producing strain throughout this study. The strain was stored at-80°C in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom), containing 20% (v/v) glycerol (de Man et al., 1960). Before experimental use, the bacteria were propagated three times consecutively in MRS broth at 37°C for 16 h.

Fermentation experiments

To investigate the effect of the initial pH of the medium and temperature on the growth and EPS production by *L. fermentum* F6, the organism was grown at various initial pHs (5.0, 5.5, 6.0, 6.5 and 7.0) at 37°C, or at different temperatures (25, 30, 37 and 42°C) at pH 6.5 in 10% (w/v) skim milk (Fonterra Cooperative Group Limited, New Zealand). The effect of carbon source on EPS production was studied by supplementation in the skim milk with 2%(w/v) of different sugars (glucose, lactose, galactose and fructose).

The effect of nitrogen source on EPS production was studied by supplementation in the skim milk with 0.5 or 1.0% (w/v) whey protein concentrate (WPC, Fonterra Cooperative Group Limited, New Zealand). During the fermentation, samples were taken at 8 h intervals for the determination of EPS production, viable cell counts and titratable acidity (°T). The total EPS concentration was determined by the phenol-sulfuric method (Dubois et al., 1956), using glucose as a standard. Cell numbers (CFU per milliliter) were estimated by plating MRS agar for incubation at 37°C for 48 h.

Isolation and characterization of EPS

EPS was isolated from the fermented sample using a modified procedure previously described by Yang et al. (1999). Briefly, the sample culture was heated at 100°C for 15 min to inactivate enzymes potentially capable of polymer degradation. Then, it was cooled and added with trichloroacetic acid to a final concentration of 4% (w/v). After centrifugation (12500 ×g for 30 min at 4°C) to remove the precipitated proteins and bacteria, the supernatant was mixed with a double volume of cold ethanol and then stored at 4°C for 24 h. The precipitated EPS was collected by centrifugation (12500 ×g for 30 min at 4°C), dissolved in deionized water for dialysis against deionized water at 4°C for 24 h and then lyophilized. Further purification of the EPS was performed by anion exchange chromatography on the DEAE-Cellulose column (2.6 × 30 cm), eluted with distilled water at a flow rate of 1 ml/min. Peak fractions containing polysaccharides (50 ml) were pooled, dialyzed and lyophilized. The lyophilized sample (160 mg) was further separated by gel filtration using a Sepharose CL-6B column (2.6 × 100 cm) (Amersham Pharmacia Biotech, Sweden) eluted with 0.9% (w/v) NaCl at a flow rate of 0.5 ml/min. Fractions (5.0 ml) were collected and monitored for carbohydrates (phenol/sulfuric acid test: absorbance at 490 nm). The eluted fractions containing carbohydrates were pooled, dialyzed with water and freeze-dried.

The monosaccharide composition of EPS was determined by a method described by Honda et al. (1989) and Yang et al. (2005). Briefly, the purified polysaccharide sample (1 mg) was hydrolyzed with 1 ml of 2 M trifluoroacetic acid at 120°C for 2 h, derivatized with 1-phenyl-3-methyl-5-pyrazolone and subsequently analyzed by high-performance liquid chromatography (HPLC) with a four-unit pump (Agilent Technologies, Wilmington, USA) and a Shim-pak VP-ODS column (4.6 × 150 mm) with detection by absorbance monitoring at 245 nm. The mobile phase consisted of 82% sodium phosphate (50 mM, pH 7.0) and 18% acetonitrile (v/v), and the sample was eluted at a flow rate of 1.0 ml/min.

The viscosity of the aqueous solution of the purified EPS was determined using an AR-500 dynamic rheometer (TA Instruments, USA) by a method described by Yang et al. (1999). A 1% (w/v) of the EPS solution was prepared by dissolving the freeze-dried polysaccharide material in deionized water. The viscometry measurements were performed at 20°C with increasing shear rates of up to 300 s^{-1}.

Statistical analysis

All fermentations were carried out in duplicate independent experiments. For determination of EPS quantification, bacterial counts and titratable acidity, samples were withdrawn in duplicate, and the results are presented as a mean ± standard error.

RESULTS

Effect of initial pH

The effect of the initial pH of medium on the bacterial growth and EPS production by *L. fermentum* F6 was studied when the strain was grown at 37°C in skim milk

adjusted to different pH. *L. fermentum* F6 was found to grow better and produce more EPS at higher pH (6.0, 6.5 and 7.0), with the most EPS production (14.61 mg/l at 32 h) at pH 6.5 (Figures 1a and b). The maximal amounts of EPS produced at pH 5.0, 5.5, 6.0 and 7.0 were 5.86 mg/l (40 h), 7.86 mg/l (40 h), 10.11 mg/l (32 h) and 8.32 mg/l (32 h), respectively. Both the bacterial growth and EPS production by *L. fermentum* F6 seemed to be strongly affected by the acidic condition of the medium. As shown in Figure 1b, the viable counts increased slowly during the initial stage of growth at lower pH (5.0 and 5.5), reaching the maximum of 1.90×10^7 and 2.24×10^7 cfu/ml at 24 h, respectively. Due to the poor growth at the initial pH 5.0, there was no significant change in the titratable acidity of the culture, being around 45 °T during the 48 h of growth, much lower than that (108.15 °T at 48h) obtained with the initial pH 6.5 (Figure 1c). Therefore, the initial pH of 6.5 was most suitable for the growth and EPS production by *L. fermentum* F6, and it was used for the following experiments.

Effect of temperature on EPS production

Figure 2 shows the influence of temperature on the bacterial growth and EPS production by *L. fermentum* F6 grown in the skim milk at the initial pH of 6.5 and at 25, 30, 37 and 42°C. The strain was found to produce more EPS with better acidifying capability at higher temperatures (37 and 42°C) (Figures 2a and c). However, less biomass was produced during the log and stationary phases when the strain was grown at 42°C than at other temperatures tested (Figure 2b). At 37°C, *L. fermentum* F6 showed the optimal growth (7.76×10^7 cfu/ml at 24 h) with best acidifying capability (107.25 °T at 48 h) and EPS production (14.75 mg/l at 32 h), when compared with the maximal amounts of EPS, 8.18, 8.86, 14.75 and 12.90 mg/l produced at 32 h, at 25, 30, 37 and 42°C, respectively.

Effect of carbon source on EPS production

The effect of supplementation in the skim milk with different carbon sources on the growth and EPS production by *L. fermentum* F6 was investigated when the strain was cultivated at 37°C and at the initial pH of 6.5 for 48 h. Addition of 2% (w/v) of glucose, fructose, galactose or lactose to the skim milk resulted in different extent of increase in the bacterial growth and EPS production, with glucose giving the most biomass (2.69×10^8 cfu/ml at 24 h) and most EPS production (Figures 3a and b). The effectiveness of these sugars on the EPS production was in the order of glucose (33.05 mg/l at 32 h), fructose (16.40 mg/l at 40 h), lactose (13.94 mg/l at 48 h) and galactose (12.50 mg/l at 32 h). In addition, supplementation with glucose increased significantly the acidification capacity of *L. fermentum* F6 with the titratable acidity of 87.5 °T when compared with the control of 47.5 °T at the end of fermentation. In contrast, no significant increase in the acidification capacity of *L. fermentum* F6 was observed with supplementation of the other sugars tested (Figure 3c).

Effect of WPC on EPS production

The influence of supplementation in the skim milk with WPC on the growth and EPS production by *L. fermentum* F6 was investigated when the strain was grown at 37°C and at the initial pH of 6.5 for 48 h. Addition of 0.5 or 1% (w/v) of WPC to the skim milk increased clearly the bacterial growth, acidification capacity and EPS production by *L. fermentum* F6 (Figure 4). The maximal yields of EPS were obtained after 32 h of growth, being 30.38, 33 and 14.98 mg/l, for the supplementation of 0.5 and 1% (w/v) WPC, and the control (without supplementation), respectively (Figure 4a). Figure 4b also shows that during the late stationary phase *L. fermentum* F6 continued to grow well when added with WPC in the skim milk. However, for the control, the growth declined after 24 h from 4.57×10^7 to 1.66×10^6 cfu/ml at 48 h, though the capability of acidification of the strain increased throughout the fermentation (Figures 4b and c).

EPS production under optimized culture conditions

There was a marked increase in the growth and EPS production by *L. fermentum* F6 when it was grown under the optimized culture conditions determined above, that is, at the initial pH 6.5, 37°C in 10% (w/v) skim milk supplemented with 2% (w/v) glucose and 0.5% (w/v) WPC (Figure 5a). After 24 h, *L. fermentum* F6 sustained growth with cell counts around 1.05×10^8 cfu/ml and with increasing acidity up to 90 °T at 48 h (Figure 5b). A maximal EPS yield of 44.49 mg/l was obtained at 32 h with the control being 15.11 mg/l.

Isolation and chemical analysis of EPS

The culture of *L. fermentum* F6 grown under the optimized culture conditions described above was used for isolation of EPS by ethanol precipitation. The EPS was purified to homogeneity by anion exchange chromatography on diethylaminoethyl (DEAE)-cellulose and size-exclusion chromatography on Sepharose CL-6B, showing a single peak for the polysaccharide (Figure 6). The EPS was shown to be a neutral polysaccharide since it was not adsorbed onto the DEAE-cellulose anion exchange column eluted with water. Monosaccharide analysis of the purified EPS sample by HPLC showed two distinct peaks, corresponding to glucose and galactose in a molar ratio

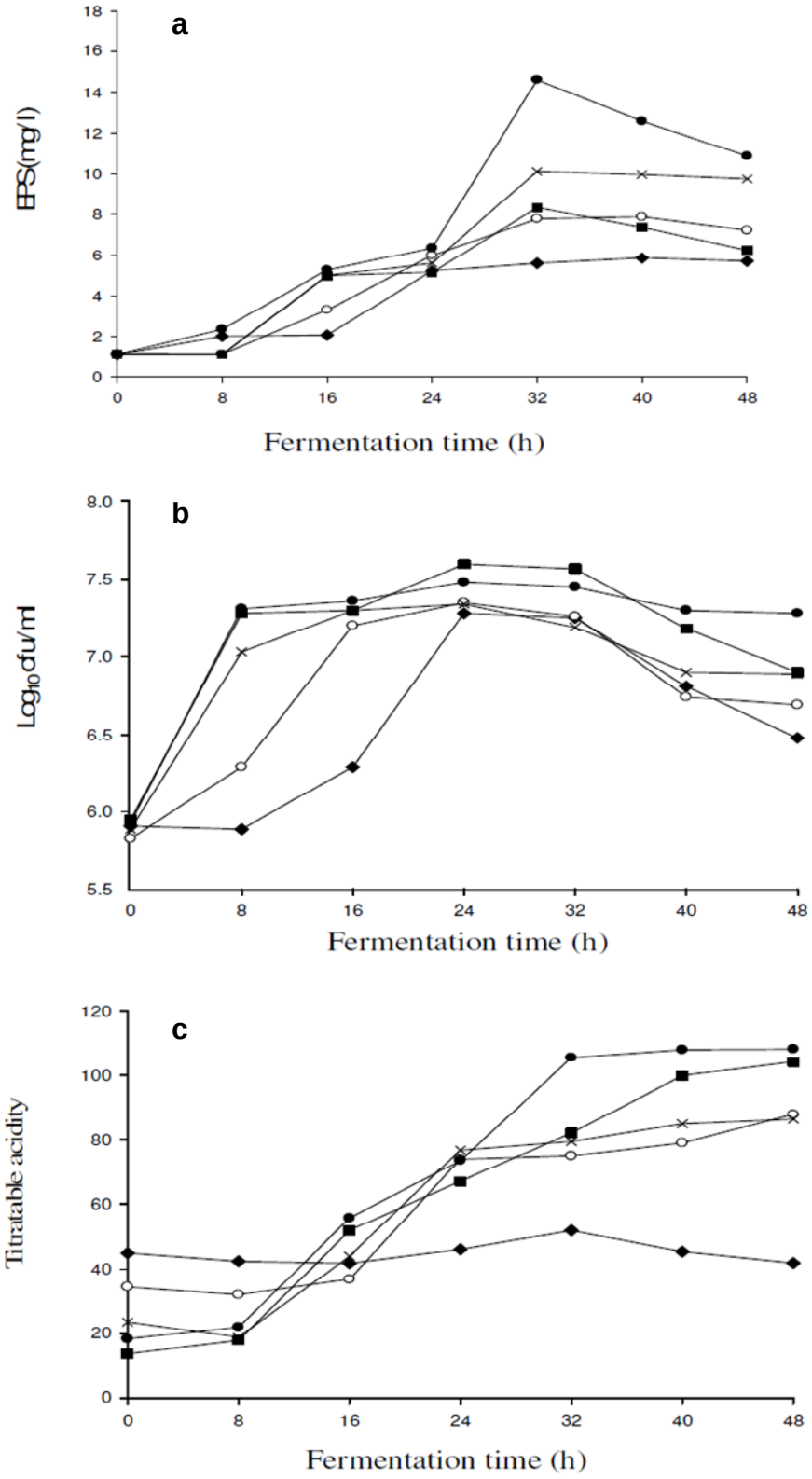

Figure 1. Influence of different initial pH of growth medium. pH 5.0 (◆), 5.5 (○), 6.0 (×), 6.5 (●) and 7.0 (■), on the yield of EPS (a), bacterial counts (b) and titratable acidity (c) during fermentation by *L. fermentum* F6 in skim milk at 37°C.

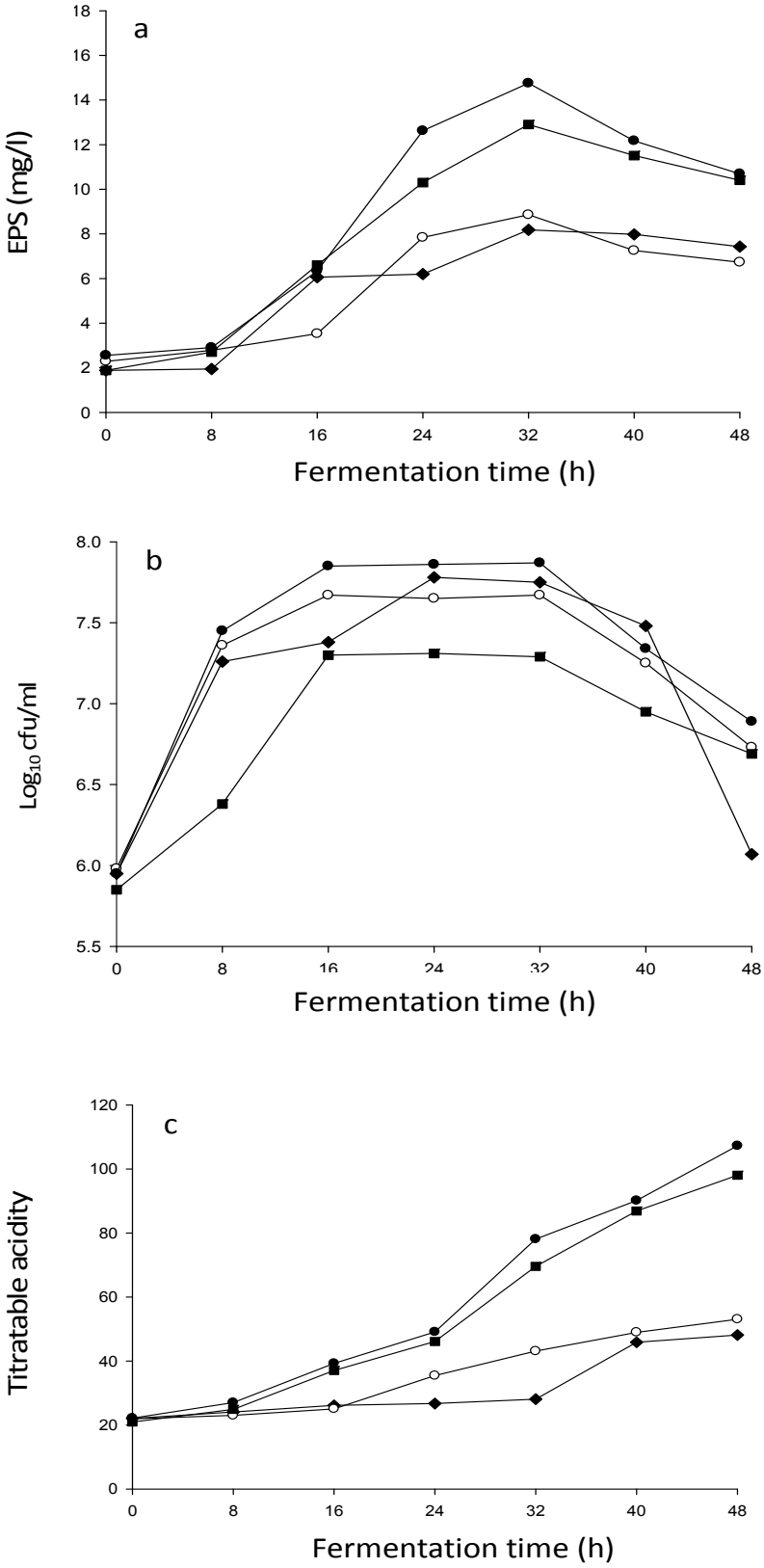

Figure 2. Influence of different temperature of 25 (◆), 30 (○), 37 (●) and 42°C (■) on the yield of EPS (a), bacterial counts (b) and titratable acidity (c) during fermentation by *L. fermentum* F6 in skim milk at initial pH 6.5.

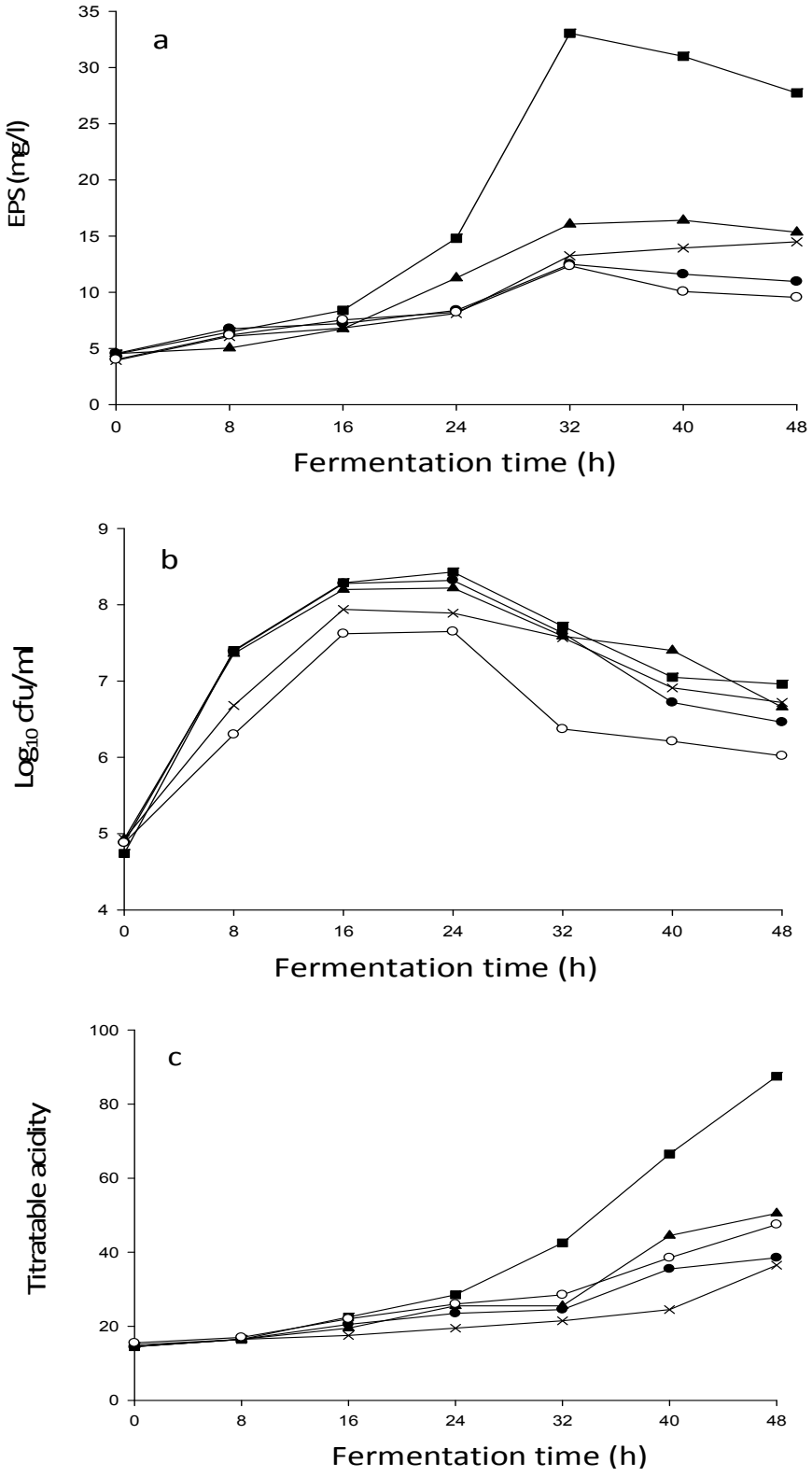

Figure 3. Effect of supplementation with different carbon sources of glucose (■), lactose (×), galactose (●) and fructose (▲) in comparison with the control (○) without supplementation, on the yield of EPS (a), bacterial counts (b) and titratable acidity (c) during fermentation by *L. fermentum* F6 in skim milk at 37°C and at initial pH 6.5.

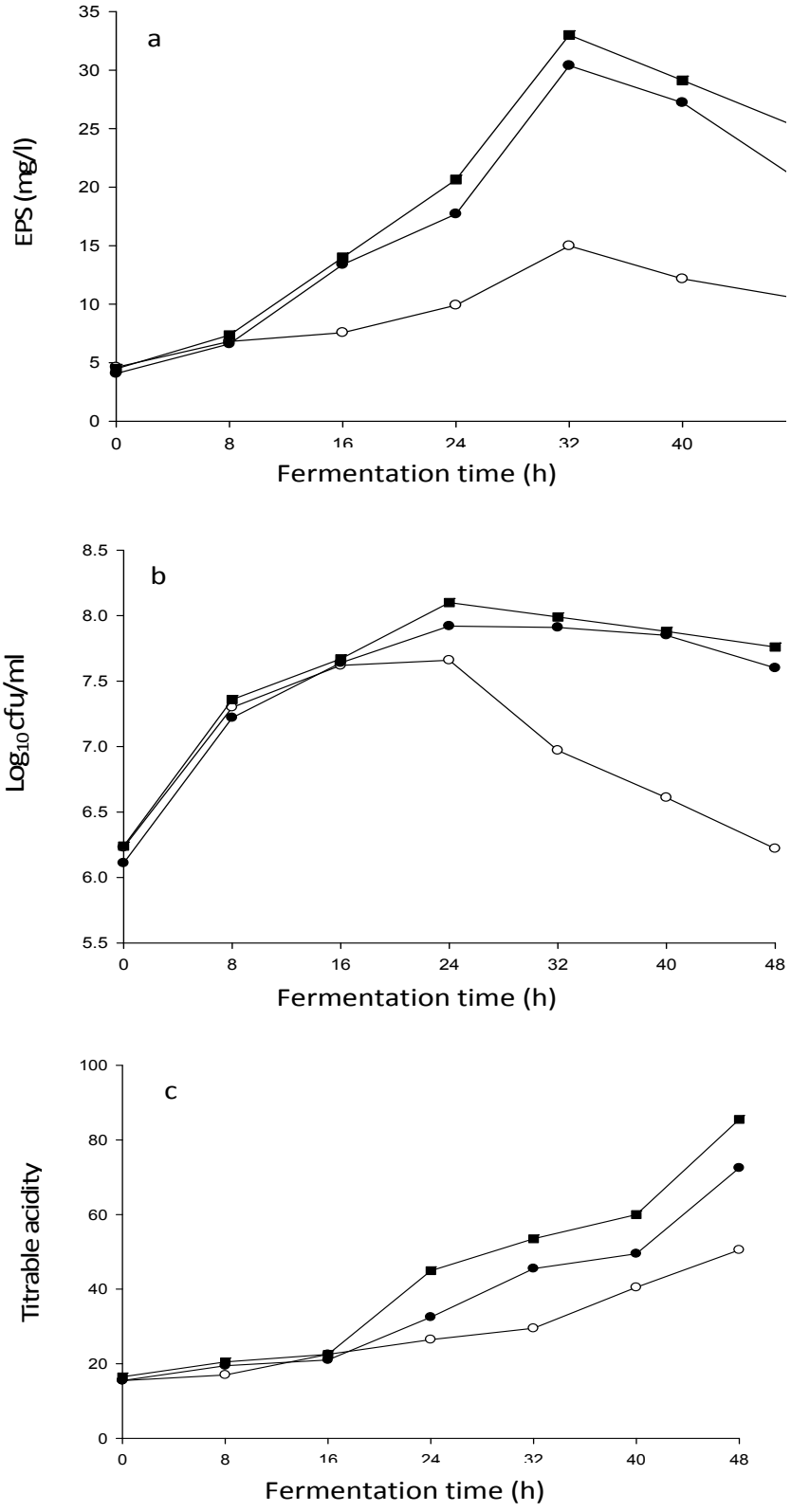

Figure 4. Effect of supplementation with whey protein concentrate at 0.5 (w/v) (●) and 1.0% (w/v) (■), when compared with the control (○) without supplementation, on the yield of EPS (a), bacterial counts (b) and titratable acidity (c) during fermentation by *L. fermentum* F6 in skim milk.

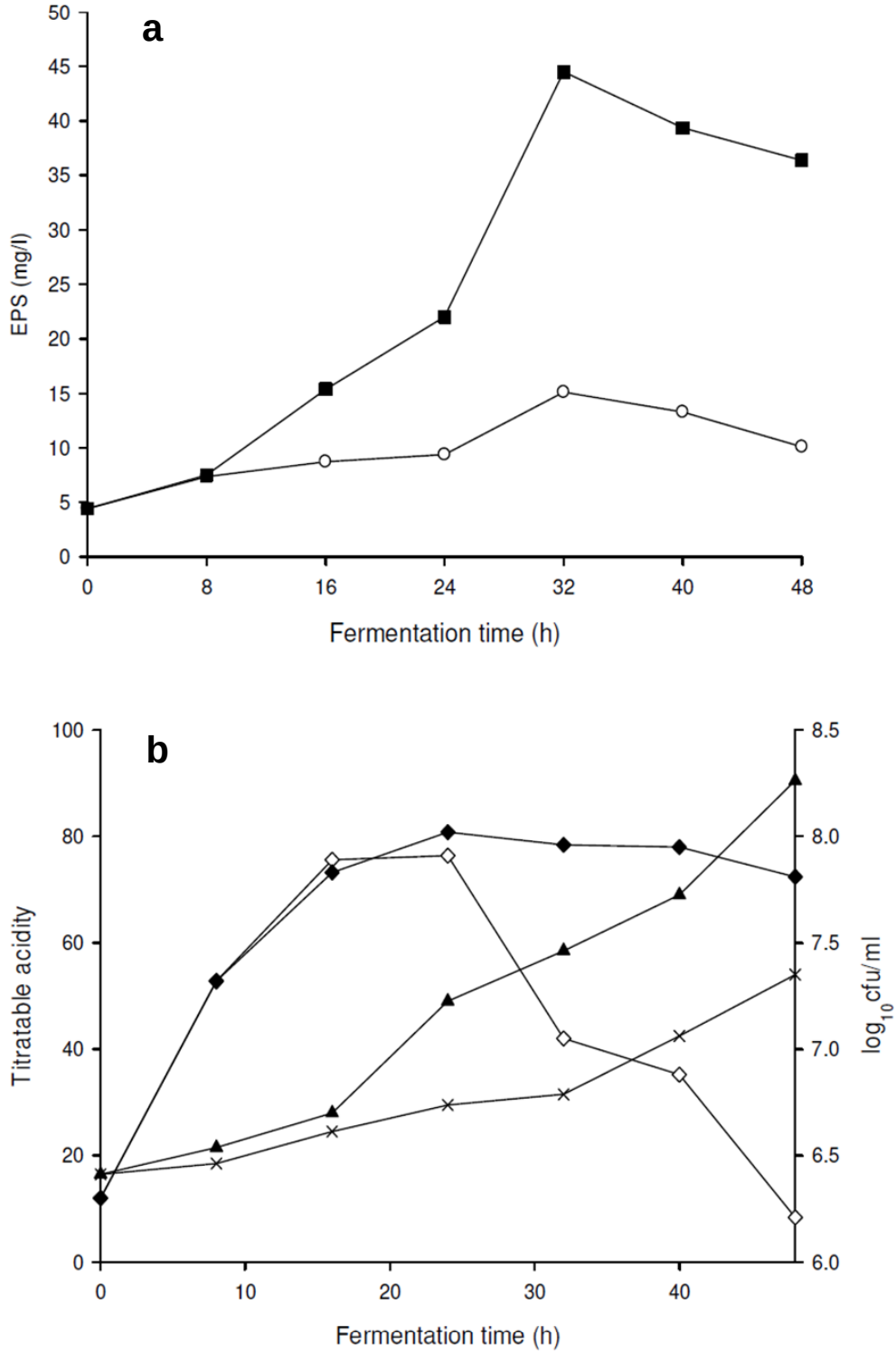

Figure 5. (a) EPS production of *L. fermentum* F6 in skim milk supplemented with 2% (w/v) glucose and 0.5% (w/v) whey protein concentrate (■) as compared with the control (○) without these supplementations; (b) profiles for bacterial counts (◆) and titratable acidity (▲) during fermentation by *L. fermentum* F6 in skim milk supplemented with 2% (w/v) glucose and 0.5% (w/v) whey protein concentrate, when compared with the profiles for bacterial counts (◇) and titratable acidity (×) of the control without these supplementations. All the experiments were performed at 37°C and at initial pH 6.5.

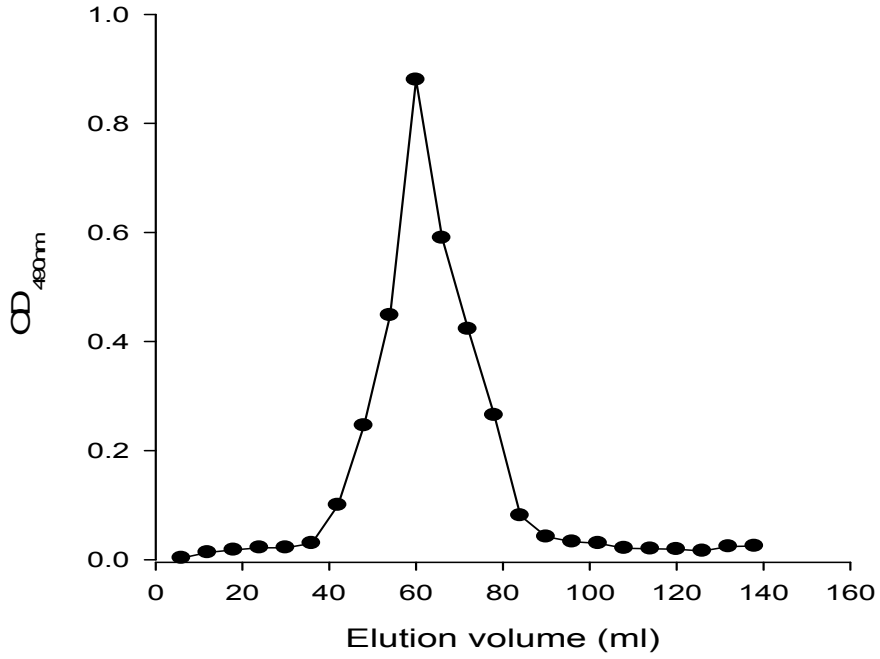

Figure 6. Purification of the EPS produced by *L. fermentum* F6 by gel filtration chromatography on Sepharose CL-6B, showing a single peak of the polysaccharide.

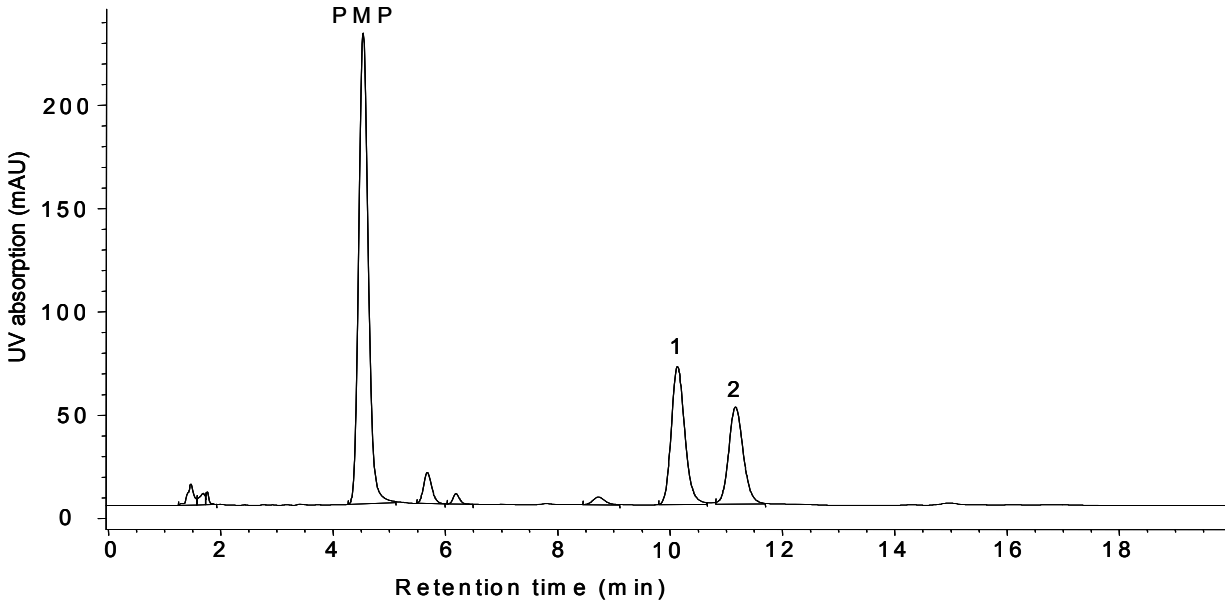

Figure 7. Monosaccharide analysis of the purified EPS sample by HPLC of the PMP derivatives of the acid hydrolysate of the EPS produced by *L. fermentum* F6, showing two peaks for glucose (peak 1) and galactose (peak 2). PMP, 1-phenyl-3-methyl-5-pyrazolone.

of 4:3 (Figure 7). The molecular mass of the EPS was determined to be 3.54×10^6 Da.

The aqueous solution of the purified EPS (1%, w/v) of *L. fermentum* F6 gave relatively high viscosity, that is, 226 mPa.s at a shear rate of 11.7/s. The drastic decrease in viscosity of the EPS solution from 226 to 94 mPa.s with increasing shear rate from 11.7 to 299.9/s clearly showed the non-Newtonian behavior (shear-thinning) of the EPS

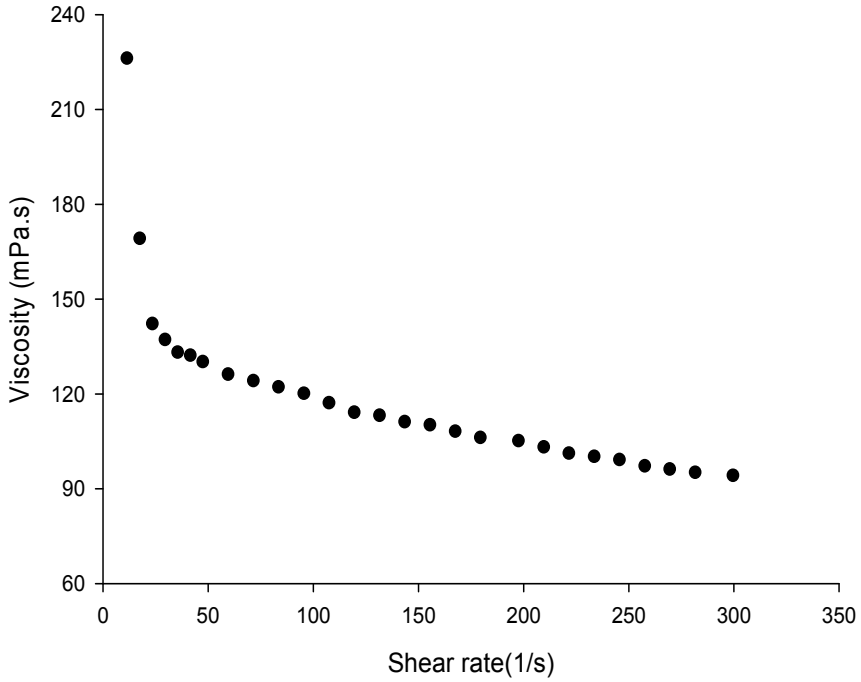

Figure 8. Viscosity-shear rate profile of 1% (w/v) aqueous solution of the EPS produced by *L. fermentum* F6 at 20°C.

solution (Figure 8).

DISCUSSION

Previously, *L. fermentum* TDS030603 was reported to produce a viscous EPS composed of a tetrasaccharide repeating unit with galactose and glucose in the molar ratio of 2:5 (Leo et al., 2007). In the present study, we showed that another *L. fermentum* strain F6 isolated from traditional dairy products in Inner Mongolia of China also produced a viscous EPS consisting of galactose and glucose, but with different molar ratio of the monosaccharides (gal: glu =4:3). EPS production by *L. fermentum* F6 was affected by environmental factors such as growth temperature and pH and composition of growth medium, and it was shown to be growth-linked, like other EPS-producing LAB strains reported earlier (De Vuyst and Degeest, 1999; Kimmel et al., 1998; Knoshaug et al., 2000).

Under the growth conditions used in this study, EPS production by *L. fermentum* F6 decreased to different extents during the late stationary phase of growth (after 32 h). This was probably because of the action of glycohydrolases possibly produced in the culture that catalyzed the degradation of polysaccharides, resulting in decreased EPS yields (Pham et al., 2000; Cerning et al., 1992). EPS degradation upon prolonged incubation was also reported with EPSs produced by other LAB strains (Mozzi et al., 2001; Degeest et al., 2001; De Vuyst et al., 1998), and less degradation of polysaccharide was observed when the strains were grown at lower temperatures and pH than those for the optimal growth (Degeest et al., 2002; Lin and Chang Chien, 2007). However, for some EPS-producing LAB strains, e.g. *Streptococcus thermophilus* ST111, EPS produced during the fermentation was not degraded since maximum EPS yield was obtained at the end of fermentation (Vaningelgem et al., 2004).

L. fermentum F6 was shown to produce more EPS when the strain was grown at optimal temperature (37°C) and pH (6.5) (Figures 1 and 2). Similarly, *L. fermentum* TDS030603 was found to produce more EPS at 37 than at 40°C (Leo et al., 2007). Mozzi et al. (1996) found a correlation between the optimum growth temperature (37 - 42 °C) and maximum EPS production by *Lactobacillus casei* strain CRL 870. However, the optimal conditions for EPS production by *Propionibacterium acidipropionici* DSM 4900 were different from those for optimal growth (Gorret et al., 2001). Low temperatures enhanced the EPS production by *Lactobacillus sake* 0-1 (Degeest et al., 2001; Dick et al., 1995). A shift from the optimum growth temperature (37°C) to the optimum EPS secretion temperature (25°C) of *L. rhamnosus* strain C83 led to a 19% increase in EPS concentration (Gamar-Nourani et al., 1998). Although the optimal pH for EPS production varied in different strains of LAB, it was often found to be close to pH 6.0 (De Vuyst et al., 1998; Dick et al., 1995). Maximum growth and EPS production by *S. thermophilus* ST111 were obtained at a constant pH 6.2 in milk medium

(Vaningelgem et al., 2004).

Figure 3 indicates that glucose was more effective than fructose, lactose or galactose as a carbon source for the growth and EPS biosynthesis of *L. fermentum* F6. This was probably due to more efficient use of glucose than other sugars as energy source or precursor for the EPS synthesis of this strain (Looisjesteijn et al., 1999). Other researchers also found that glucose was an efficient carbon source for EPS production by many bacteria (Cerning et al., 1994; West, 2003). *Lactococcus delbrueckii* subsp. *bulgaricus* NCFB 2772 produced three times more EPS with glucose than with fructose as a sugar source (Grobben et al., 1996). *Lactococcus lactis* subsp. *cremoris* NIZO B40 produced about nine times more EPS with glucose than with fructose as a sugar source under acidifying conditions (Looisjesteijn et al., 1999; Looijesteijn and Hugenholtz, 1999). In addition, Sanchez et al. (2006) found that the yields of EPS produced by *L. pentosus LPS26* are significantly influenced by the carbon source such as glucose. Gancel and Novel (1994) found a correlation between the optimum carbon source (glucose or fructose) and more EPS production by an industrial strain *S. thermophilus* S22.

Figure 4a shows that supplementation of additional nitrogen source such as WPC (0.5% or 1%, w/v) in the skim milk increased markedly the EPS production of *L. fermentum* F6. However, no significant increase in EPS production was observed when the concentration of WPC was increased from 0.5 (w/v) to 1% (w/v), probably due to the fact that supplementation of 0.5% (w/v) WPC provided enough nitrogen source for the growth and EPS production by *L. fermentum* F6. Zisu and Shah (2003) found that the EPS yield of *S. thermophilus* 1275 increased with supplementation of 0.5% (w/v) WPC in the skim milk, and the appearance of the EPS increased at higher WPC concentrations. A fivefold increase of EPS yield was obtained for *S. thermophilus* ST 111 when it was grown in milk medium supplemented with whey protein hydrolysate (Vaningelgem et al., 2004).

This study showed that EPS production by *L. fermentum* F6 could be improved by optimization of environmental conditions such as initial pH of growth medium, temperature and composition of the medium. A significant increase in EPS production of *L. fermentum* F6 with a maximum EPS yield of 44.49 mg/l was obtained when the strain was grown at 37°C and at initial pH 6.5 in the skim milk supplemented with 2% (w/v) glucose and 0.5% (w/v) WPC. A 1% (w/v) of the aqueous solution of the EPS showed relatively high viscosity, indicating the potential of this EPS for applications as a viscosifying agent to improve the physical properties of food products.

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