

GROWTH AND EXOPOLYSACCHARIDE PRODUCTION BY *STREPTOCOCCUS THERMOPHILUS* ST1 IN SKIM MILK

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ABSTRACT

To analyze the exopolysaccharide (EPS) production by *Streptococcus thermophilus* ST1, cultures were cultivated in 10% (w/v) reconstituted skim milk under different growth conditions including various temperatures and pHs of growth medium, supplementation of the medium with various carbon sources (glucose, lactose, sucrose, galactose and fructose) and nitrogen source (whey protein concentrate, or WPC). The results showed that most EPS production by strain ST1 was obtained at a temperature (42°C) and pH (6.5) optimal for its growth. Supplementation of the skim milk medium with either carbohydrates or WPC increased both growth and polymer formation by different extents, with sucrose being most effective among the carbon sources tested. Under the optimal cultural conditions, i.e. pH 6.5, 42°C with 2% (w/v) sucrose and 0.5% (w/v) WPC, 135.80 mg l⁻¹ of EPS was produced by strain ST1. The monosaccharide composition of the EPS was determined to be glucose and galactose (2:1), and the molecular mass of the EPS was 3.97 × 10⁶ Da. The aqueous solution of the EPS at 1% (w/v) showed relatively high viscosity, indicating the potential of this EPS-producing *S. thermophilus* strain for applications in the improvement of physical properties of fermented milk products.

Key words: Exopolysaccharide, *Streptococcus thermophilus* ST1, Skim milk

INTRODUCTION

There has been an increasing interest in the exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) during the last decade. These biopolymers may function as viscosifiers, texturizers, or emulsifying agents to improve

the texture and consistency of fermented milk (35). EPSs produced by LAB have been shown to play an important role in the prevention of syneresis (whey separation), a common problem in yogurt manufacturing (15). *In situ* production of EPSs by yogurt bacteria has been suggested to be used as an alternative to the addition of stabilizers, e.g. animal

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hydrocolloids (gelatin and casein) or chemically modified plant carbohydrates (starch, pectin and guar gum) (36). Since LAB strains are generally food-grade microorganisms with a GRAS (Generally Recognised As Safe) status, the use of EPS-producing LAB in food fermentation could result in safe and natural products with improved stability (9). All-natural products have become increasingly popular during the recent years (31).

The characteristics of EPS production by LAB vary considerably with the strains with respect to EPS yield, rheological properties, monosaccharide composition and structure of EPS (28,38). Physiologically, EPS synthesis by LAB is influenced by many factors, e.g. temperature, pH, and components of growth medium and fermentation time, among others (9,27). *Streptococcus thermophilus* strains have been reported to produce EPSs from 50 to 350 mg l⁻¹ (3). *S. thermophilus* LY03 was shown to produce the highest amount of EPS at 42°C and at pH 6.2 (10), and it produced more EPS with lactose than glucose as the carbon source (7). Looijesteijn and Hugenholtz (21) found a higher production of EPS by *Lactococcus lactis* spp. *cremoris* B40 with glucose than when fructose was used as substrate. For some *S. thermophilus* strains used in a proto-cooperative yoghurt culture, the presence of *L. delbrueckii* subsp. *bulgaricus* was necessary to ensure their proper growth and EPS production (35,25). Supplementation of the skim milk medium with whey protein concentrate (WPC) or whey protein hydrolysates increased EPS production by *S. thermophilus* ST111 (32,35). The use of WPC increased buffering capacity of the medium, thus decreasing the acidic effects on EPS production during fermentation (9).

During our studies on the EPS production by LAB, we found in the LAB collection of our laboratory an EPS-producing strain, *S. thermophilus* ST1 that produced a viscous culture when grown in skim milk. Since *S. thermophilus* is commonly used as one of the starter strains for yoghurt manufacturing, it would be interesting to study the

characteristics of EPS production in view of improving the physical properties of yoghurt by using EPS-producing strains. Therefore, the present work was carried out to study the effect of different cultural conditions on the EPS production by *S. thermophilus* ST1, aiming to improve the EPS yield of this strain for possible dairy applications. In addition, the EPS was isolated, and the monosaccharide composition, molecular mass and viscosity properties of the EPS were studied.

MATERIALS AND METHODS

Bacterial strain

S. thermophilus ST1 obtained from the Culture Collection of NARCC (Northeast Agricultural Research Center of China) was used throughout this study. The strain was stored at -80°C in reconstituted skim milk powder at 10% (w/v, Fonterra, New Zealand) containing 30% (v/v) glycerol. *S. thermophilus* ST1 was propagated three times consecutively using a 1% (v/v) inoculum in 10% (w/v) skim milk at 42°C for 18 h before use.

Growth experiments

Batch cultures of *S. thermophilus* ST1 were performed using 10% (w/v) skim milk in 1500 ml Erlenmeyer flasks for 40 h, to study the influence of different factors on bacterial growth, acidification and EPS production. The effect of the initial pH of the medium was studied at 37°C with pH 5.0, 6.0, 6.5, and 7.0, the pH being adjusted with 0.1 M NaOH and 0.1 M HCl. The effect of temperature was studied at 30, 37 and 42°C. The influence of supplementation with 2% (w/v) glucose, lactose, sucrose, galactose, fructose or 0.5% (w/v) WPC was studied at 42°C.

For each experiment, a 1% inoculum of 18 h grown culture was added to 1000 ml of the medium, and the pH was allowed to drop freely during bacterial growth. Samples (50 ml) were aseptically withdrawn at 0 h and every 8 h thereafter to determine the amount of EPS and pH. A fresh sample (1 ml) was also taken for immediate enumeration of the bacterial

counts by serial decimal dilutions. A 1 ml aliquot of inoculated skim milk was sampled and plated using the pour plating technique. Solidified agar plates were incubated aerobically at 42°C for 48 h. A digital display pH meter (PB-10, Sartorius, Germany) was used to determine pH.

Isolation and analyses of EPS

After incubation at 42°C for 32 h in 10% (w/v) skim milk, cultures (1000 ml) were heated at 100°C for 15 min to inactivate the enzymes potentially capable of polymer degradation, and cells were removed by centrifugation (10,000 × g, 10 min, 4°C). The supernatant was precipitated with two volumes of chilled absolute ethanol. After standing overnight at 4°C, the resultant precipitate was collected by centrifugation (12,000 × g, 20 min, 4°C), dissolved in distilled water, dialyzed against distilled water at 4°C for 24 h, and lyophilized. The lyophilized powder (100 mg) was dissolved in water, loaded onto a DEAE-Cellulose column (2.6 × 30 cm), and eluted with distilled water at a flow rate of 1 ml min⁻¹. Fractions were collected every 5 min and peak fractions containing polysaccharides (50 ml) were pooled, dialyzed and lyophilized. The lyophilized sample (30 mg) was further separated by gel filtration on Sepharose CL-6B column (2.6 × 100 cm) (Amersham Pharmacia Biotech, Sweden) eluted with 0.9% (w/v) NaCl at a flow rate of 0.4 ml min⁻¹. Fractions were collected every 20 min and the peak fractions were pooled, dialyzed with water and lyophilized. Fractions were monitored for sugars by a method described by Dubois *et al.* (12).

The monosaccharide composition of EPS was determined by a method described by Honda *et al.* (17) and Yang *et al.* (37). 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 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⁻¹.

The molecular mass of EPS was determined by gel filtration chromatography of the isolated EPS on a Sepharose CL-6B column (1.6 × 100 cm), calibrated with dextran standards (Mw 2000, 500, 150, 70 and 40 kDa; Fluka Chemie GmbH, Buchs, Switzerland), and eluted with 0.9% NaCl at a flow rate of 0.16 ml min⁻¹. The carbohydrate content of each fraction (3.2 ml) was determined by the phenol-sulfuric acid assay (12) using glucose as a standard.

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.* (38). A 1% (w/v) solution of EPS was prepared by dissolving the freeze-dried polysaccharide material in deionized water. The viscometry measurements were performed at 20°C with increasing shear rates up to 300 s⁻¹.

Statistical analysis

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

RESULTS AND DISCUSSION

Effects of initial pH on EPS production

As shown in Figure 1b, *S. thermophilus* ST1 showed a similar growth pattern at all pH levels tested, growing fast during the first 8 h and then entering stationary phase. However, at pH 5.0, the bacterial growth was poor, showing low bacterial counts with only slight decrease in pH during the growth.

Figure 1a shows that EPS production by *S. thermophilus* ST1 was clearly affected by the initial pH of the medium. At

pH 6.5, the EPS production increased rapidly during the first 16 h of growth, and reached a maximal EPS yield of 45.10 mg l⁻¹ at 24 h. Comparatively, the strain produced less EPS at pH 5.0, 6.0 and 7.0 with a maximal yield at 40 h, 11.47 mg l⁻¹, 35.58 mg l⁻¹ and 18.53 mg l⁻¹, respectively. The decreased EPS production, e.g. at pH 5.0 by *S. thermophilus* ST1 could be explained by the high acidity of the medium that might cause acid stress to the cells (35). EPS-producing bacteria

could protect the cells from environmental stress by controlling the release of the produced EPS (1), and in some cases by forming a capsule around the cells (29). Similar findings were also reported for the LAB strains from kefir grains with pH-dependent production of an extracellular Kefiran; acidic stress (pH 4.5-4.9) caused a drastic decrease in the Kefiran production (4).

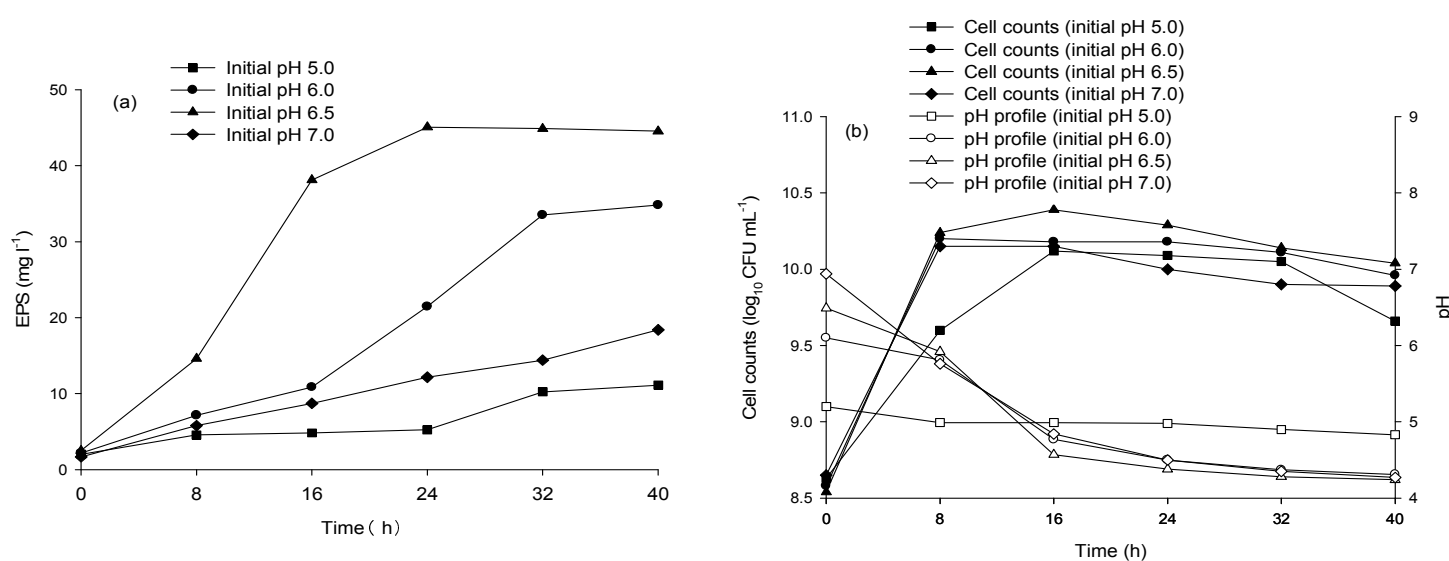


Figure 1. Effect of the initial pH of medium on growth and EPS production by *S. thermophilus* ST1. Cultivation of strain ST1 in skim milk at 37°C and at different initial pH was monitored by the yield of EPS (a), the cell counts and the pH profile of the culture (b).

Effect of temperature on EPS production

The effect of temperature on EPS production by *S. thermophilus* ST1 was investigated with skim milk at 30, 37 and 42°C (Figure 2a and 2b). The results showed that at the optimal temperature (42°C) for growth, this strain produced the highest amount of EPS with the maximal yield of 59.06 mg l⁻¹ at 32 h. The strain produced much less EPS at 30 and 37°C with the maximal yields of 21.47 mg l⁻¹ at 24 h and 34.35 mg l⁻¹ at 16 h, respectively.

Studies on the kinetics of EPS synthesis by *S. thermophilus*

ST1 at different temperatures (Figure 2) and at different pHs (Figure 1) indicated that EPS production by *S. thermophilus* ST1 was growth-linked. The optimal conditions such as temperature (42°C) and pH (6.5) for the growth of *S. thermophilus* ST1 were favorable for its EPS production. However, there were also reports that optimal conditions for EPS production by some LAB strains were different from those for their optimal growth (13). A number of LAB strains were shown to produce more EPS at a lower temperature for their optimal growth (19). Acidic stress that inhibited bacterial growth could stimulate EPS production by

some LAB strains (39). EPS production has been considered a mechanism of bacterial self-protection against unfavorable

environmental factors, such as dehydration, high acidity and phage attack, among others (30).

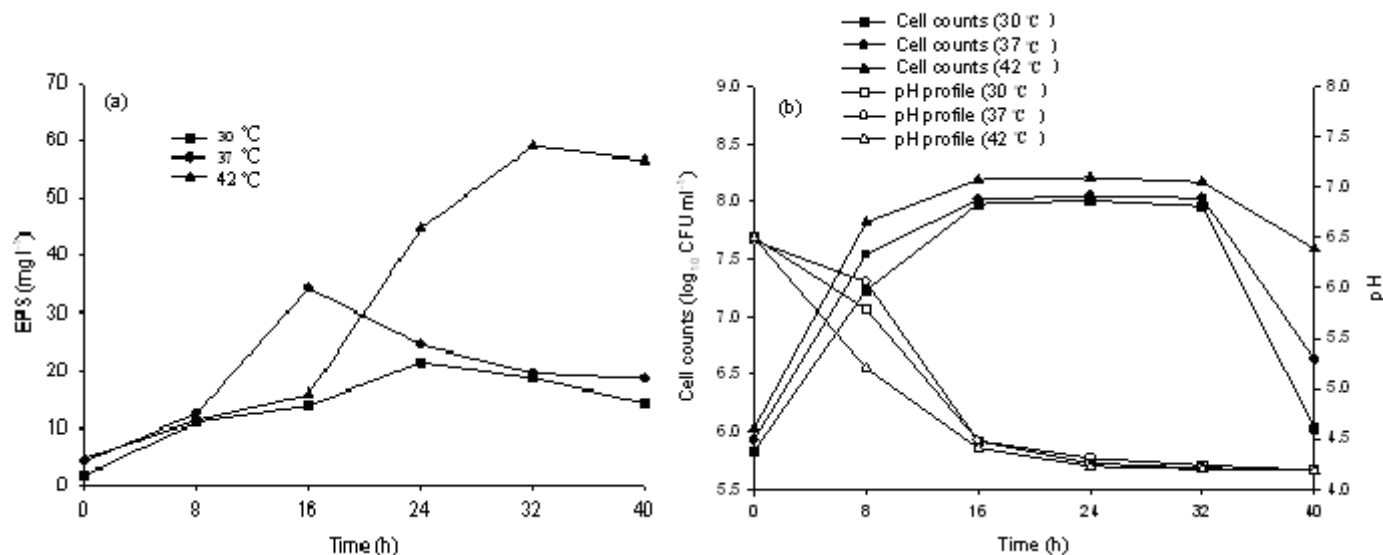


Figure 2. Effect of temperature on growth and EPS production by *S. thermophilus* ST1. Cultivation of strain ST1 in skim milk at different temperature was monitored by the yield of EPS (a), the cell counts and the pH profile of the culture (b).

Effect of supplementation with carbohydrates on EPS production

The effect of supplementation with different carbohydrates (glucose, lactose, sucrose, galactose, fructose) on EPS formation by *S. thermophilus* ST1 grown in skim milk for 32 h at 42 °C is shown in Table 1. There was an increased bacterial growth, achieving populations of around log 8.2 CFU ml⁻¹ at 32 h, when 2% (w/v) of fructose, lactose, glucose or sucrose were added to the skim milk, whereas the control reached log 8.01 CFU ml⁻¹ at 32 h. The addition of any of the sugars to the culture resulted in a significant increase in EPS yield, showing values of 64.52 mg l⁻¹, 66.39 mg l⁻¹, 69.35 mg l⁻¹, and 73.28 mg l⁻¹, respectively, with the addition of fructose, lactose, glucose, and sucrose, whereas the EPS yield obtained for the control was of 45.63 mg l⁻¹ at 32 h. The EPS yield was also higher with galactose (60.93 mg l⁻¹), when compared to

that of the control, although no obvious increased bacterial growth (log 7.98 CFU ml⁻¹) was observed with this sugar.

The effect of carbon source on growth and EPS production by LAB was reported to be dependent on the specific strain, the type of sugar, and the properties of the cell carbohydrate metabolism (24). Previously, glucose, lactose, and galactose were found to be suitable carbon sources for EPS synthesis by *L. helveticus* ATCC 15807 in a chemically defined medium. Nevertheless, this strain displayed much less growth with galactose than with glucose or lactose (34). *L. casei* CG11 was grown well with galactose, glucose or sucrose in a basal minimum medium, but it produced much less EPS with galactose than with the other sugars (2). Inhibition of the activity of some key enzymes for EPS synthesis might decrease EPS synthesis, as shown for *L. sakei* 0-1 when this strain was grown on galactose, giving a low EPS yield (8). In the present study, supplementation with galactose stimulated EPS

formation, but not the growth of *S. thermophilus* ST1, suggesting that most of the sugar is employed for EPS biosynthesis and little or none as an energy source for growth

(6). Further studies are needed to elucidate the mechanism involved in the different effect of carbohydrates on EPS synthesis by *S. thermophilus* ST1.

Table 1. Effect of supplementation in the skim milk medium with different carbon sources on the growth and EPS production by *S. thermophilus* ST1 grown at 42°C for 32 h^a

Medium	pH	EPS (mg l ⁻¹)	Cell counts (log ₁₀ CFU ml ⁻¹)
Skim milk	4.20±0.03	45.63±0.53	8.03±0.02
Skim milk+2% galactose	4.45±0.06	60.93±0.38	8.03±0.05
Skim milk+2% fructose	4.24±0.01	64.52±0.67	8.25±0.08
Skim milk+2% lactose	4.30±0.01	66.39±0.29	8.18±0.07
Skim milk+2% glucose	4.29±0.05	69.35±0.78	8.20±0.09
Skim milk+2% sucrose	4.32±0.07	73.28±0.46	8.23±0.11

^a All the values are expressed as the means ± standard deviations of duplicate measurements using the cultures at 32 h of growth.

Effect of supplementation with WPC on EPS production

Figure 3 shows that supplementation of the skim milk with 0.5% (w/v) WPC increased both the bacterial growth and the EPS production by *S. thermophilus* ST1. Throughout fermentation, both the bacterial counts and the EPS yields maintained at higher levels with the supplementation of WPC than those of the control. At 24 h, the maximal amount of EPS (82.70 mg l⁻¹) was produced with the supplementation of WPC, compared to 41.10 mg l⁻¹ observed for the control. At the end of fermentation, the EPS yield with the supplementation of WPC was 52.74% higher than that obtained without supplementation.

Milk was reported not to be a suitable medium for EPS synthesis by *S. thermophilus*, due to the absence of certain vitamins, peptides, and amino acids, essential for bacterial growth and EPS synthesis (5,10,26,33). In addition, limited proteolytic activity and inefficient peptide transport mechanism of these strains also limit their growth rate (20). However, for some *S. thermophilus* strains that had the capability of catabolizing larger proteins, addition of yeast extract, peptone (14) or WPC (39) to milk was found to increase the growth and EPS synthesis by providing necessary peptides and amino acids for the cells (16). The results obtained in this study, regarding the increased growth and EPS production of *S. thermophilus* ST1 with the supplementation of WPC in skim milk were in agreement with

these previous findings.

Figure 3 also shows that addition of 0.5% (w/v) WPC to skim milk caused a delay on pH decrease during the initial 24 h fermentation, probably due to the buffering effect of WPC. Previous studies showed that partial replacement of skim milk with WPC could enhance the buffering capacity of yogurt, which caused a delay on pH decrease during yogurt production, resulting in an increased bacterial growth and EPS production (18, 32).

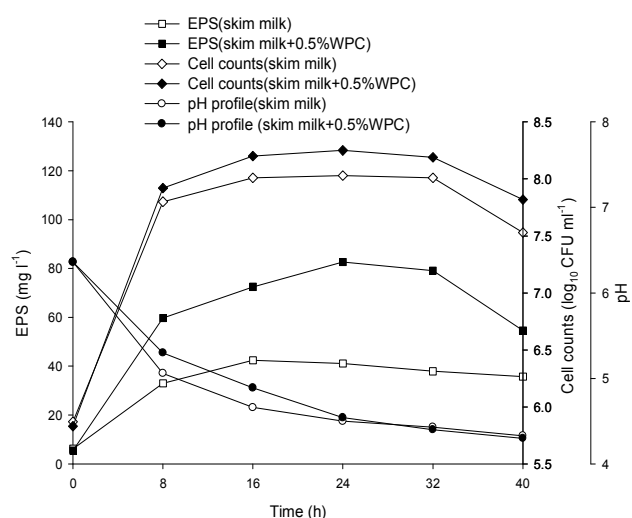


Figure 3. EPS production, cell counts and pH profile of the culture of *S. thermophilus* ST1 grown at 42°C in skim milk supplemented with WPC at 0.5% (w/v).

EPS isolation and characterization

S. thermophilus ST1 produced a maximal amount of 135.80 mg l⁻¹ of EPS, when this strain was grown under the optimal conditions determined above, i.e. 42°C in skim milk supplemented with 2% (w/v) sucrose and 0.5% (w/v) WPC. The EPS isolated from the above cultures was purified to homogeneity by anion exchange chromatography on DEAE-Cellulose and gel filtration chromatography on Sepharose CL-6B, showing a single peak for the polysaccharide (Figure 4). The EPS of *S. thermophilus* ST1 was shown to be a neutral polysaccharide, since it was not adsorbed onto the DEAE-Cellulose anion exchange column eluted with water. Monosaccharide analysis by HPLC of the purified EPS produced by *S. thermophilus* ST1 showed two distinct peaks, corresponding to glucose and galactose in a molar ratio of 2:1 (Figure 5). Previously, several *S. thermophilus* strains, such as SFi39 (22), EU20 (23), STD, CH101 (11), and THS (25) were also found to produce EPSs containing glucose and galactose, but in different molar ratios of the monosaccharides. Additional sugar components, such as rhamnose, ribose, fucose or *N*-acetyl-D-galactosamine were also found in the EPSs produced by *S. thermophilus* strains (1,30). The molecular mass of the EPS produced by *S. thermophilus* ST1 was determined to be 3.97×10⁶ Da. The molecular mass of heteropolysaccharides produced by LAB usually ranges from 4.0×10⁴ Da to 6.0×10⁶ Da (30).

The aqueous solution of the purified EPS (1%, w/v) of *S. thermophilus* ST1 gave relatively high viscosity, i.e. 406.6 mPa.s at a shear rate of 4.46 s⁻¹. The drastic decrease in viscosity of the EPS solution from 406.6 to 24.16 mPa.s, with increasing shear rate from 4.46 to 279.3 s⁻¹ clearly showed the non-Newtonian behavior (shear-thinning) of the EPS solution (Figure 6).

In summary, EPS production by *S. thermophilus* ST1 was dependent on cultural conditions, such as growth temperature, the initial pH of growth medium, and composition of the medium, such as carbon and nitrogen sources. Conditions suitable for growth of *S. thermophilus* ST1 were found to be favorable for its EPS production. Optimization of cultural conditions for *S. thermophilus* ST1 resulted in a significant increase in EPS production by 130%. The viscous nature of the EPS of *S. thermophilus* ST1 as shown in this study indicated the possible

application of this EPS-producing strain in the improvement of physical properties of fermented milk products.

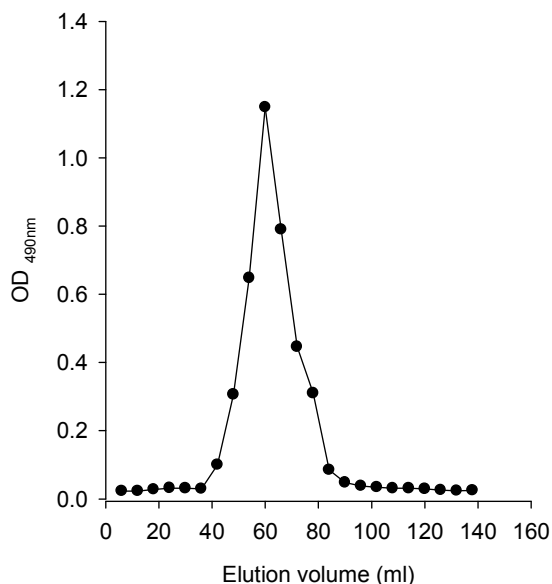


Figure 4. Purification of the EPS produced by *S. thermophilus* ST1 by gel filtration chromatography on Sepharose CL-6B, showing a single peak of the polysaccharide.

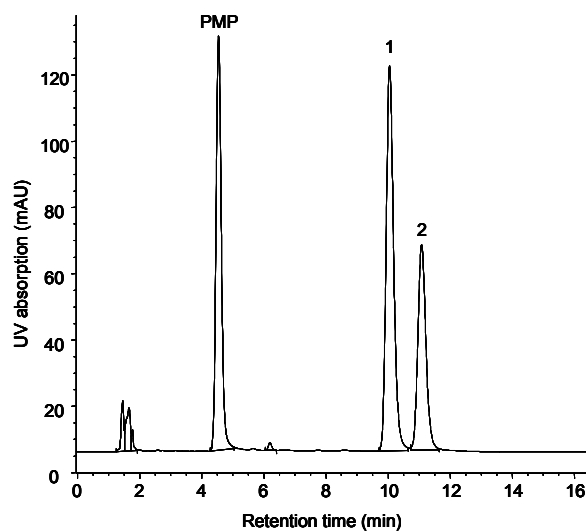


Figure 5. Monosaccharide analysis of the purified EPS sample by HPLC of the PMP derivatives of the acid hydrolysate of EPS, showing two peaks for glucose (peak 1) and galactose (peak 2). PMP, 1-phenyl-3-methyl-5-pyrazolone.

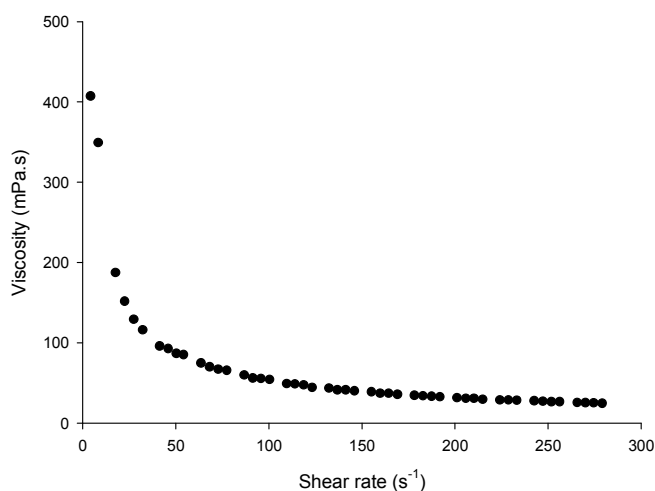


Figure 6. Viscosity-shear rate profile of 1% (w/v) aqueous solution of the EPS produced by *S. thermophilus* ST1 at 20°C.

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