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Production of Exopolysaccharides by Strains of *Streptococcus thermophilus*

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Two strains of *Streptococcus thermophilus* producing capsular and capsular-ropy exopolysaccharides (EPS) were examined for their growth and EPS production in M17 medium supplemented with glucose, galactose or lactose and incubated at 30, 37 or 42°C for 24 hours. Growth parameters (viable cells, OD, lactate production, pH) and EPS production were determined. Flow behavior of the EPS dispersions was assessed as a function of concentration and temperature. Culture growth during incubation was affected by types of sugar, temperature and time. Growth was enhanced by glucose, lactose and higher incubation temperature. EPS concentration in the medium was greater in the presence of glucose and galactose. Despite the restricted growth conditions, the capsular strain produced comparable levels of EPS to the capsular-ropy strain even under sub-optimal incubation temperature

Key words: *Streptococcus thermophilus*, exopolysaccharides, growth conditions

Considerable attention has been given to bacterial exopolysaccharides (EPS) in foods arising their ability to provide potential health benefits to consumers (Looijesteijn *et al*. 2001) and their application as thickening agents in processed products (Cerning 1990). Although certain bacterial species can produce considerable amounts of EPS, yoghurts prepared with strains *Streptococcus thermophilus* resulted in less than 0.1% EPS in the final product (Cerning 1990). Nevertheless, EPS play an important role in the development of yoghurt texture, with the type of EPS exerting a greater effect than their concentration (Vaningelgem *et al*. 2004). In general, two strain-dependent types of EPS (Mozzi *et al*. 2006) have been frequently assessed for their effects on yoghurt texture, namely 'ropy' and 'capsular'. Certain bacterial strains may even produce a mix of these two types in various proportions (Zisu and Shah 2003; Zisu and Shah 2005). Furthermore, combinations of the two types of EPS producing cultures improved not only the total EPS production but also yoghurt texture (Marshall and Rawson 1999).

Numerous studies have been conducted in order to enhance our understanding of the factors governing EPS production and the mechanisms by which they affect the yoghurt texture. Most of the EPS produced during yoghurt fermentation are heteropolysaccharides, and their production may be unpredictable due to a plasmid-related instability (Boels *et al*. 2001). EPS production, thickening properties, molecular mass and structural conformation are greatly affected by environmental factors (Ruas-Madiedo *et al.* 2005). In some species, these factors only affected EPS yield, because their monosaccharide composition remained unchanged (Looijesteijn and Hugenholtz 1999). Strain selection, temperature, pH and growth stage (Aslim *et al*. 2005), nitrogen source (Degeest *et al.* 2002), and metabolizable sugars (Mozzi *et al*. 2003) are among the factors influencing EPS production. EPS production by some *S. thermophilus* strains was coupled to growth (Vin *et al*. 2005). However, in some mesophilic bacterial strains, EPS were mainly produced at sub-optimal growth conditions (Cerning 1990). Depending on the strain and growth conditions, maximum yield of EPS may be achieved in the exponential (Duenas *et al*. 2003) or stationary growth phase (Gancel and Novel 1994). At the end of growth phase, there are some indications that EPS undergoes undesirable enzymatic degradation (Pham *et al*. 2000; Degeest *et al*. 2002). The type of carbon source apparently governs the total amount of EPS produced with a clear strain-dependence (Mozzi *et al*. 2001). For example, *S. thermophilus* LY03 produced more EPS with lactose than with glucose (Degeest and de Vuyst 2000a). Nitrogen compounds in the growth medium are necessary during formation of sugar nucleotides, essential precursors for EPS assembly. However, the type of nitrogen source appears to be less important in EPS production than is the carbon source (Duenas *et al*. 2003).

Although several types of EPS have been reported to influence yoghurt texture, and some mechanisms on their interactions with milk proteins has been proposed, there is a lack of information on the rheological properties of EPS produced by yoghurt cultures. Such information can be useful for predicting the possible interaction of EPS with milk component(s) to influence the texture of yoghurt. Therefore, this study aimed to examine EPS production by two strains of *S. thermophilus* which produced capsular-ropy and capsular EPS. We reported the effect of fermentation conditions including temperature, time, and carbon source on its production.

MATERIALS AND METHODS.

Bacterial Strains. The two strains of *S. thermophilus* examined in the study were kindly provided by Australian Starter Culture Research Centre (Werribee, Australia). *S. thermophilus* ST 1275 produces mainly ropy with a smaller portion of capsular EPS, while ST 285 produces only

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capsular EPS (Zisu and Shah 2003; 2005). The frozen (-80°C) cultures in 300 mL L^{-1} glycerol were activated by growing them twice in M17 medium (Amyl Media, Merck Pty Ltd., Kilsyth, Victoria, Australia) at 37°C for at least 24 hours.

Medium. The medium used in these experiments was basal M17 medium supplemented with galactose, glucose or lactose at 20 g L^{-1}. The cultures were inoculated into 30 mL of the sterile medium in 50 mL Falcon tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA) and incubated at 30, 37 or 42°C for 24 hours, during which samples were periodically collected for analysis of all growth parameters and determination of EPS concentration.

Bacterial Counts. Determination of viable cell counts was carried out by serial dilutions in sterile 1 g L^{-1} peptone water, and incubation at the corresponding temperature for 24 hours to obtain sufficient cell growth. Cell concentration was additionally determined based on optical density at 650 nm.

EPS Determination. EPS assessed in the rheological studies were extracted from the culture grown in M17 medium by precipitation with ethanol (van Geel-Schutten *et al*. 1998). Initially, fermentation was stopped by adding 20 g L^{-1} TCA and keeping in a cool room (4°C) overnight. This was followed by protein removal by centrifugation at 11 000 × g and 4°C for 10 min (Model J2-HS, Beckman, Fullerton, California, USA). The supernatant was collected and mixed with two volumes of ethanol. The mixture was allowed to stand for twelve hours for complete precipitation of EPS. This procedure was repeated twice, after which the EPS was freeze-dried (Dynavac freeze drier, Dynavac Eng. Pty. Ltd., Melbourne, Australia). The concentration of EPS was assessed using the phenolsulphuric acid method (Dubois *et al*. 1956). Lactic acid concentration was determined by an HPLC method (Donkor *et al*. 2005). The method uses dilute 0.01 mol/L H_2SO_4 as a mobile phase with flow rate of 0.6 mL min^{-1}, and 300 x 7.8 mm ion-exchange Aminex HPX-87H column (Bio-Rad Laboratories, Australia) coupled to a UV detector (Varian Analytical Instruments, Walnut Creek, CA, USA) at 220 nm. The column temperature was maintained at 65°C.

Statistical Analysis. A randomized split plot in time block design was applied to the design of the fermentation experiments. This was set up with four main effects: strain (two levels), sugar types (three levels), temperatures (three levels), and fermentation time (six levels: 0, 3, 6, 9, 12, 24 hours). This design was replicated twice with at least two sub samplings. Results were analyzed using a General Linear Model procedure. The level of significance was set at P=0.05. The best fit correlational analysis for rheological data was carried out using the Rheoplus software (v2.81, Anton Paar).

RESULTS

Bacterial Counts. Viable cell counts were significantly (P<0.05) affected by all factors: strain, temperature, type of sugar, fermentation time and their interactions. The capsular strain consistently showed slower growth compared to the capsular-ropy culture, regardless of the growth conditions. The maximum cell numbers of the capsular strain were $2.2x10^8$ CFU mL^{-1}, achieved in glucose-M17 medium at 42°C. In contrast, the capsular-ropy culture grew to $1.6x10^{10}$ CFU mL^{-1} in lactose-M17 medium at 37°C (Fig 1a, b). Higher temperatures (37 and 42 °C) resulted in greater cell growth than at 30°C for both strains and all types of sugar.

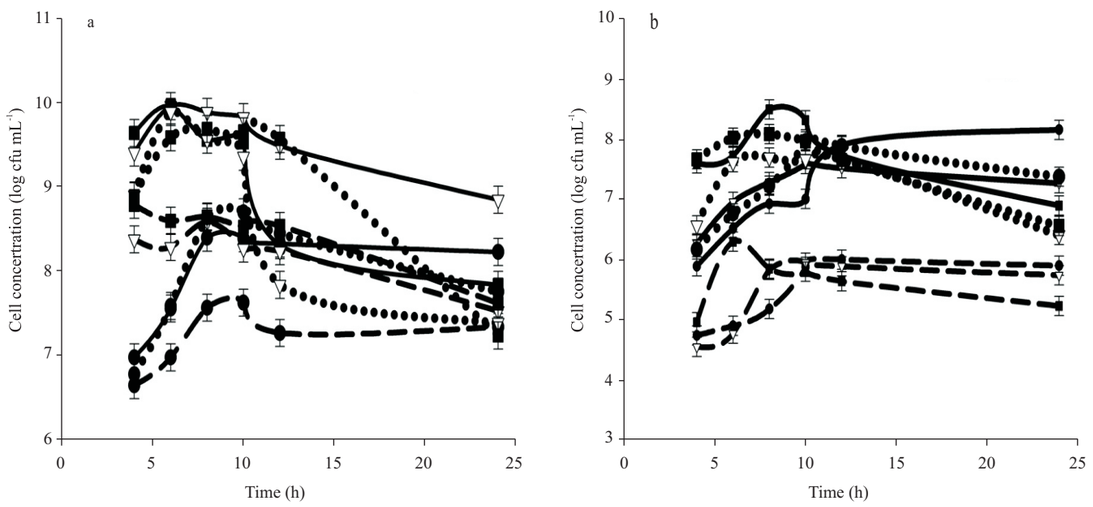

Fig 1 Viable cell counts of capsular-ropy (a) and capsular (b) strain of *Streptococcus thermophilus* cultivated in a M17 medium supplemented with glucose (solid line), galactose (dashed line) or lactose (dotted line) at 30°C (□), 37°C (▯) or 42°C (▽) for 24 h.

Table 1 Cell growth parameters of the capsular-ropy and capsular strain of *Streptococcus thermophilus* as affected by temperature and sugar source during growth phase

Strain	Temperature, (°C)	Time, (hour)	Type of sugar	pH	Lactic acid concentration, (mg mL^{-1})	OD*
Capsular-ropy	30	8	galactose	6.9^{cB}	0.53^{aA}	0.07^{cA}
			lactose	5.9^{aB}	0.67^{bA}	0.86^{bA}
			glucose	6.3^{bB}	0.87^{cA}	0.72^{bA}
		24	galactose	6.9^{cA}	0.54^{aA}	0.09^{aA}
			lactose	5.9^{bB}	0.66^{bB}	0.95^{bA}
			glucose	5.5^{aB}	0.86^{cA}	1.73^{cA}
	37	8	galactose	6.8^{cAB}	0.55^{aAB}	0.16^{aA}
			lactose	5.8^{bB}	0.68^{bA}	1.23^{bB}
			glucose	5.1^{aA}	0.88^{cA}	2.52^{cB}
		24	galactose	6.7^{cA}	0.68^{bC}	0.20^{aA}
			lactose	5.8^{bB}	0.57^{aA}	1.08^{bA}
			glucose	5.0^{aA}	0.89^{cA}	2.08^{cB}
	42	8	galactose	6.6^{bB}	0.58^{aB}	0.29^{aA}
			lactose	5.3^{aA}	0.77^{bB}	1.75^{bC}
			glucose	5.1^{aA}	0.89^{cA}	2.53^{cB}
		24	galactose	6.5^{bA}	0.60^{aB}	0.28^{aA}
			lactose	5.3^{aA}	0.74^{bC}	1.67^{bB}
			glucose	5.1^{aA}	0.89^{cA}	2.78^{cB}
Capsular	30	8	galactose	6.9^{cA}	0.55^{aA}	0.24^{cC}
			Lactose	5.9^{bA}	0.65^{bA}	0.94^{aB}
			glucose	5.3^{aA}	0.84^{cB}	2.12^{bB}
		24	Galactose	6.8^{cA}	0.56^{aA}	0.27^{cB}
			lactose	5.9^{bA}	0.66^{bA}	1.01^{aB}
			glucose	5.3^{aA}	0.83^{cB}	2.11^{cB}
	37	8	galactose	6.8^{cA}	0.54^{aA}	0.20^{aA}
			lactose	5.9^{bA}	0.64^{bA}	0.57^{bA}
			glucose	5.4^{aA}	0.84^{cB}	1.69^{cB}
		24	galactose	6.6^{cA}	0.57^{aA}	0.14^{aA}
			lactose	5.9^{bA}	0.66^{bA}	0.66^{bB}
			glucose	5.4^{aA}	0.84^{cB}	1.51^{cA}
	42	8	galactose	6.9^{cA}	0.59^{aB}	0.20^{aA}
			lactose	5.9^{bA}	0.66^{bA}	0.57^{bA}
			glucose	5.4^{aA}	0.76^{cA}	1.59^{cA}
		24	galactose	6.9^{cA}	0.57^{aA}	0.16^{aA}
			lactose	6.1^{bB}	0.62^{bA}	0.44^{bA}
			glucose	5.4^{aA}	0.78^{cA}	1.60^{cA}
SEM**				0.101	0.020	0.108

*Different small caps in a column denote significant (P<0.05) difference among means of the same culture, temperature and time but different sugar types. The different capital letters depict significant (P<0.05) difference among means of the same culture, sugar type and time but different temperature. **SEM, standard error of means, with P=0.05.

M17 medium containing glucose or lactose was more growth supportive than that with galactose for both strains and in all temperatures.

Positive effects of higher fermentation temperature, glucose or lactose on cell growth was also confirmed by other growth parameters such as optical density, pH and lactic acid production (Table 1). Statistically, OD readings were only influenced by the type of sugar and its interactions with other factors (P<0.05). Fermentation time and the type of strain individually had no effect on cell growth as measured by OD, but their interactions did (P<0.05). Sugar type and its interaction with other factors significantly (P<0.05) affected the final pH of the medium. Lactic acid concentration was similarly influenced by the factors examined. The types of sugar substantially (P<0.05) influenced lactate concentration, where glucose produced most lactic acid (~0.80-0.87±0.020 mg mL^{-1}), whereas galactose produced the lowest levels ~0.55-0.62±0.02 mg mL^{-1}. In most cases, from the 8th hour onwards, lactate concentration was either increased or remained unchanged.

EPS Concentration. The EPS production was only affected by the type of sugar and the fermentation time, and their interactions with other factors (P<0.05). The amount of EPS produced by the two strains was not significantly different (P>0.05). Maximum EPS concentration produced by the capsular-ropy strain was 441.0±36.2 mg L^{-1} in galactose-M17 at 42°C after 12 hours. Similarly, the capsular strain produced 563.0±36.2 mg L^{-1} with glucose at 30°C after 12 hours (Fig 2a, b). EPS production of the capsular strain at all temperatures was relatively high and the difference among them was negligible. Notably in our study, EPS production was significantly (P<0.05) affected by incubation time though not statistically significant (P>0.05), higher temperature tended to enhance EPS production by the capsular-ropy strain in all types of sugar (Fig 2a,b). On the other hand, the capsular strain appeared to prefer lower

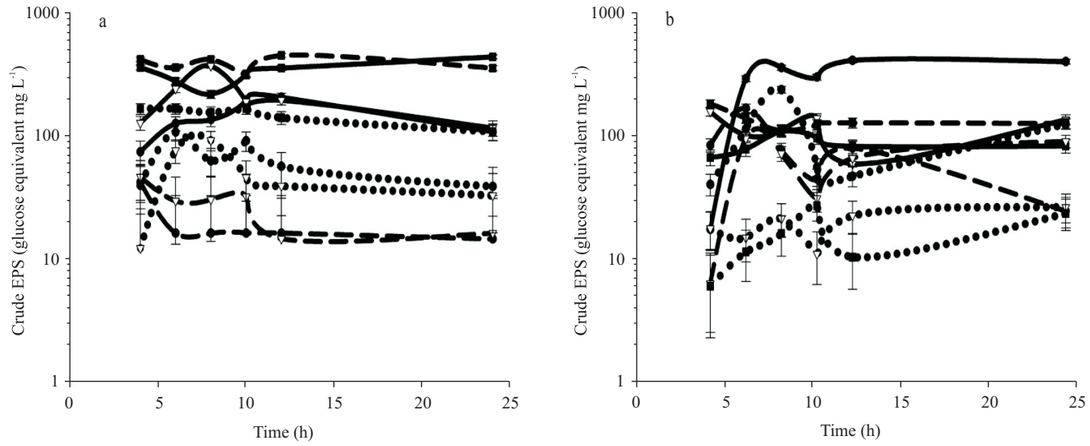

Fig 2 Exopolysaccharides (EPS) concentration, expressed as glucose equivalent, produced by the capsular-ropy (a) and the capsular (b) strain of *Streptococcus thermophilus* grown in a M17 medium supplemented with glucose (solid line), galactose (dashed line) or lactose (dotted line), at 30°C (□), 37°C (▯) or 42°C (▽) for 24 h.

temperature (30°C) and produced more EPS when grown on glucose or lactose (Fig 2a, b). M17 medium containing galactose poorly supported cell growth, however, the EPS concentration in this medium was relatively high. In medium with lactose at all temperatures, and glucose or galactose at low temperature (30°C), maximum EPS concentration was reached after 8 hours. Afterwards, the EPS concentration was often slightly reduced towards the end of fermentation. The pattern of EPS production over time by the capsular strain was somewhat similar to that of the capsular-ropy counterpart, except that EPS concentration in galactose medium at low temperature (30°C) remained stationary after 4 hours. Moreover, EPS concentrations in lactose medium at low temperatures (30 and 37°C) were low, and did not show any considerable change until the end of fermentation.

DISCUSSION

Bacterial Counts. Our results are in agreement with previous work showing comparable cell numbers of *S. thermophilus* grown in lactose- and glucose-containing medium (Degeest and de Vuyst 2000b). In contrast to these findings, van den Bogaard *et al*. (2000) found that *S. thermophilus* grew poorly on glucose (Poolman 2002). Growth of the *S. thermophilus* strain in the glucose based medium requires the activity of phosphoglucomutase whose activity may be supressed in the presence of lactose (Levander and Radstrom 2001). Degeest *et al*. (2000b) also showed that galactose did not support the growth of a *S. thermophilus* strain.

EPS Concentration. Our experiment showed that both strains produced considerable amounts of EPS in M17 medium, even higher than as reported by others (Aslim *et al.* 2006). As in this experiment, no difference in EPS production of both ropy and non-ropy strains was found by Mozzi *et al*. (2006). Most of *S. thermophilus* strains posses a Leloir system for EPS synthesis (Mora *et al*. 2002). The three sugars examined in our study supported EPS production, as has reported elsewhere (Chervaux *et al*. 2000), with galactose frequently related to EPS production (Mozzi *et al*. 2001). Our result was in contrast to previous report that glucose poorly supported EPS production compared to lactose (Degeest *et al*. 2000a), but similar to a more recent work (Shene *et al*. 2008) that EPS production of *S. thermophilus* strain was higher on glucose-containing medium. The growth-associated nature of EPS production of *S. thermophilus* strains has been reported previously, with the EPS produced mainly during logarithmic phase (Ruas-Madiedo *et al*. 2005; Shene *et al*. 2008). The maximum production of EPS seemingly varies with strains and is not always linear with temperature of fermentation, as also shown by previous report (Shene *et al*. 2008).

Galactose was a predominant (Goh *et al*. 2005) and a consistent (Goh *et al.* 2005; Mozzi *et al*. 2006) primary unit in the structure of the EPS backbone in several *S. thermophilus* strains, although most of them did not utilize galactose (Mora *et al*. 2002) or metabolized galactose only at the end of growth phase (de Vin *et al*. 2005). However, several reports found discrepancies between theoretical values and actual galactose concentration in the medium, suggesting a flux to other metabolites including lactic acid or even assimilation of galactose into EPS in the Gal$^-$ strains of *S. thermophilus* (de Vin *et al.* 2005). Galactose appeared to contribute to EPS anabolism rather than the cell growth (Mozzi *et al.* 2001). The availability of galactose might be initiated by the activation of galactose symporter in the absence of lactose (de Vin *et al*. 2005) or low lactose/galactose ratio (Poolman 2002). Moreover, galactose catabolism is also possible by the activation of some enzymes in Leloir pathway such as galactokinase in a lactose-depleted environment (de Vin *et al.* 2005). The EPS concentration in the medium was reduced at the end of the growth phase. This could be related to shortage of ATP required for EPS polymerization (Welman *et al.* 2006). Moreover, cell lysis (Mozzi *et al.* 2003) could lead to enzymatic degradation of EPS (Pham *et al.* 2000) towards the late stages of the growth.

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