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Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis

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L. DE VUYST, F. VANDERVEKEN, S. VAN DE VEN AND B. DEGEEST. 1998. Four *Streptococcus thermophilus* strains (*Strep. thermophilus* BTC, *Strep. thermophilus* LY03, *Strep. thermophilus* 480 and *Strep. thermophilus* Sfi20) have been examined for their exopolysaccharide production capacity. All strains produced a polymer composed of the neutral sugars glucose and galactose, but in different ratios. It was clearly shown that the biosynthesis of exopolysaccharides from *Strep. thermophilus* LY03 is growth-associated and hence displays primary metabolite kinetics. The monomer ratio of the exopolysaccharide synthesized did not vary throughout the fermentation cycle. The production kinetics and exopolysaccharide yields were strongly dependent on the fermentation conditions. Physical factors such as temperature, pH and oxygen tension as well as chemical factors (medium composition, initial lactose concentration, carbon/nitrogen levels) were of utmost importance.

INTRODUCTION

In the food industry, many polysaccharides are used as thickeners or viscosifiers, stabilizing or emulsifying agents, and as gelling and water-binding agents or texturizers (Sutherland 1986). The majority of these additives are of plant origin, for instance starch, locust bean gum, guar gum and alginate. Xanthan is the only approved microbial polysaccharide; it is produced by *Xanthomonas campestris*, a phytopathogenic, non-GRAS (Generally Recognized As Safe) bacterium. In addition, most of the plant carbohydrates used are chemically modified to improve their rheological properties (Roller and Dea 1992). Their use is hence strongly restricted, especially in the European Union (E numbers).

Also, many 'food grade' micro-organisms produce extracellular polysaccharides, in particular lactic acid bacteria (Cerning 1990, 1995), propionibacteria (Cerning 1995) and bifidobacteria (Andaloussi *et al.* 1995; Roberts *et al.* 1995). They are not yet, however, exploited by food manufacturers (Roller and Dea 1992). On the other hand, their use could result in a safe, natural end-product that may have an important impact on the development of novel products, especially food products with enhanced, rheological properties and improved stability.

Exopolysaccharides from lactic acid bacteria can be subdivided into three groups: (i) α-glucans, mainly composed of α-1,6- and α-1,3-linked glucose residues, namely dextrans (*Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum*) and mutans (*Streptococcus mutans* and *Strep. sobrinus*); (ii) fructans, mainly composed of β-2,6-linked fructose molecules, such as levan (*Strep. salivarius*); (iii) heteropolysaccharides produced by mesophilic (*Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) and thermophilic (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lact. helveticus* and *Strep. thermophilus*) lactic acid bacteria (Cerning 1990). The latter group of exopolysaccharides has not yet been studied in much detail (Cerning 1995). However, they play an important role in the rheology, texture and mouth feel of fermented milk drinks (Cerning 1990). For instance, the creamy, smooth texture is one aspect of the quality of yoghurt which seems to be determined by the ability of the yoghurt bacteria to produce exopolysaccharides. In addition, some polysaccharides may contribute to human health, either as a non-digestible food fraction (Gibson and

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Roberfroid 1995), or because of their anti-tumoral activity (Oda *et al.* 1983).

Until now, only a few heteropolysaccharides from *Streptococcus thermophilus* have been isolated and so far, the structures of only two have been elucidated (Doco *et al.* 1990; Stingele *et al.* 1996; Bubb *et al.* 1997). Information on biosynthesis and culture conditions (Cerning *et al.* 1988, 1990; Petit *et al.* 1991; Ariga *et al.* 1992; Gancel and Novel 1994a,b; Mozzi *et al.* 1995) and on the molecular organization (Stingele and Mollet 1995; Stingele *et al.* 1996) of exopolysaccharides from thermophilic lactic acid bacteria is also very scarce and the production kinetics of exopolysaccharide formation are poorly described. In addition, exopolysaccharide quantification is based mainly on indirect methods, either colorimetric techniques resulting in exopolysaccharide yields expressed as glucose equivalents (Petit *et al.* 1991; Cerning *et al.* 1994), or dextran equivalents (Garcia-Garibay and Marshall 1991; Gancel and Novel 1994a), or viscosity measurements which are not necessarily related to exopolysaccharide yields (Cerning *et al.* 1986, 1988).

In this paper, the capacity of four *Streptococcus thermophilus* strains to produce exopolysaccharides was investigated. For quantification of exopolysaccharides, an improved and easy method was used which enabled direct isolation of the secreted polymers. The production kinetics of exopolysaccharide biosynthesis by *Strep. thermophilus* LY03 under different environmental conditions are described, and it is shown that exopolysaccharide biosynthesis is mainly growth-associated.

MATERIALS AND METHODS

Bacterial strains and media

Four *Streptococcus thermophilus* strains were used for exopolysaccharide production: one strain was isolated from Greek yoghurt (*Strep. thermophilus* 480, provided by Prof. G. Kalantzopoulos, Agricultural University of Athens, Athens, Greece) and three strains were isolated from industrial yoghurt starter cultures (*Strep. thermophilus* BTC, *Strep. thermophilus* LY03 and *Strep. thermophilus* Sfi 20, kindly provided by Dr J.-P. Grill, Université de Nancy, Nancy, France, Prof. V. Marshall, The University of Huddersfield, Huddersfield, UK, and Dr B. Mollet, Nestec Ltd, Lausanne, Switzerland, respectively). The strains were stored at −80 °C in de Man Rogosa Sharpe (MRS) broth (de Man *et al.* 1960) plus 25% (v/v) glycerol. Before experimental use the cultures were propagated twice in MRS (Oxoid, Basingstoke, UK) at 42 °C; the transfer inoculum was 1% (v/v) of a 12 h culture grown in fresh medium.

Several media (500 ml) were tested in 1 litre bottles for their capacity to support *Strep. thermophilus* growth and/or exopolysaccharide production. These were de Man-Rogosa-Sharpe or MRS medium, M17 medium of Terzaghi and Sandine (1975), a modified semi-defined medium (SDM medium; van den Berg *et al.* 1995) containing 20 g l^{-1} lactalbumin hydrolysate (Oxoid) (instead of NZ-case Plus and yeast nitrogen base), and skimmed milk medium (further referred to as milk medium or SM medium) composed of (g l^{-1}) skimmed milk powder (SMP) (Dairy Industry INCO Kallo, Belgium), 100, peptone (Oxoid), 10, and yeast extract (Merck, Darmstadt, Germany), 5. In MRS, M17 and SDM media, several carbon sources were tested, namely glucose, galactose and lactose. Milk medium was also used for exopolysaccharide production (unless otherwise indicated). A solid tryptone medium consisting of (g l^{-1}) trypton (Oxoid), 10, yeast extract (Merck), 10, lactose, 5, sucrose, 5, calcium carbonate, 3, and dipotassiumhydrogenphosphate, 0·5, was used to determine cell counts. Solid media were prepared by the addition of 15 g l^{-1} granulated agar (Oxoid) to the broth.

Isolation of exopolysaccharides

Isolation of exopolysaccharides was carried out in flasks containing 500 ml of (milk) medium and performed in four steps. First, (milk) proteins were removed from the fermentation liquor by precipitation with one volume of 20% trichloroacetic acid, followed by centrifugation of cells and proteins (25 000 *g*, 20 min, 4 °C). Second, exopolysaccharides were precipitated overnight with an iso-volume of acetone, followed by centrifugation of the precipitate (25 000 *g*, 30 min, 4 °C), after which the pellet was redissolved in ultra-pure water. Third, residual proteinaceous material was precipitated with trichloroacetic acid and removed by centrifugation as described above. Fourth, the exopolysaccharides were isolated by acetone precipitation (one volume) and harvested by spinning for further analysis or quantification. To optimize this method, several organic solvents were compared: ethanol, isopropanol and acetone. After each step, the content of residual proteins (BioRad Protein Assay Kit, serial number 500–0116; BioRad Laboratories, CA, USA) and monosaccharides (Anthron method; Scott and Melvin 1953) was determined.

Monomer analysis

The exopolysaccharides harvested as described above were washed via a supplementary acetone precipitation, and lyophilized. A 1 ml aliquot of a 15 mg ml^{-1} lyophilisate solution was hydrolysed with either 6 N trifluoroacetic acid during 3 h at 100 °C, or with 0·6 N hydrochloric acid during 4 h at 100 °C, in a sealed recipient. The monosaccharide composition of the hydrolysate of four independent trials was determined by HPLC (see below) and the relative proportion of the peak areas was calculated to estimate the monomer composition.

Fermentation experiments

A 2 l Biostat® B and 15 l Biostat® C fermenter (B. Braun Biotech International, Melsungen, Germany) with a working volume of 1·5 l and 12 l, respectively, were used to study the kinetics of exopolysaccharide production. The Biostat® C fermenter was sterilized *in situ* at 121 °C for 30 min. The skimmed milk was sterilized separately (30 min at 121 °C) and aseptically pumped into the fermenter. The fermenter was inoculated with approximately 1% (v/v) of an exponentially growing culture of the exopolysaccharide-producing strain. The inoculum was obtained by propagating a fresh culture twice at 42 °C for 12 h. The fermenter was operated at 42 °C (or the temperature indicated). Slow agitation (100 rev min^{-1} seemed to be the most appropriate agitation rate; unpublished results) was maintained to keep the fermentation broth homogenous. The pH was controlled on line at 5·5 (unless otherwise indicated) by automatic addition of 10 N NaOH. Aeration was provided only when studying the influence of the oxygen tension. A constant oxygen tension of 5 and 10% could be maintained by adjusting the aeration rate to 1 and 2 l min^{-1}, respectively.

Sampling

Over a period of 24 h or more, samples were aseptically withdrawn from the fermentation vessel to determine exopolysaccharide yield (EPS), lactic acid (LA) concentration, and residual lactose (S), glucose (S_{glu}) and galactose (S_{gal}) concentrations. Agitation rate, pH, oxygen tension and base supply were monitored on line (MicroMFCS for Windows™NT software). Samples were immediately cooled on ice. Biokinetic parameters such as the maximal specific growth rate (μ_{max}) and the exopolysaccharide yield coefficient ($Y_{EPS/S}$) were calculated. Determination of the former parameter was based on NaOH consumption (see below).

Growth determination

Growth was followed by on-line registration of the amount of NaOH required to neutralize the lactic acid accumulated and hence, the amount of glucose (equimolar to lactose) consumed. Maximal specific growth rates (μ_{max}) were estimated by linear regression (indicated by the correlation coefficient r^2) from the plots of ln *B vs t*, where *B* is the amount of base used in ml l^{-1} and *t* the time in h. Cell numbers (cfu ml^{-1}) were estimated by plate counts on solid tryptone medium.

Quantitative determination of exopolysaccharide yield

To quantify exopolysaccharide production, 0·5 l samples were aseptically withdrawn. High molecular mass exopolysaccharides were isolated according to the procedure described above, washed with acetone, dried during 48 h at 42 °C, and weighed. The amount of exopolysaccharide was expressed as polymer dry mass per litre (mg PDM l^{-1}). The standard deviation was calculated as 20%.

Residual sugars and lactic acid determination

Residual sugar levels and lactic acid concentration were determined by high pressure liquid chromatography (HPLC) using a Waters chromatograph (Waters Corp., Milford, MA, USA) equipped with a Waters 410 differential refractometer, a Waters column oven, a Waters 717 plus autosampler and Millenium® software (version 2.10). Cells and solid particles were removed from 1·5 ml samples (appropriately diluted with ultrapure water) by micro-centrifugation (13 000 *g*, 20 min). Proteins were removed by the addition of an isovolume of 20% trichloroacetic acid, centrifuged (13 000 *g*, 20 min) and filtered through a nylon syringe filter (Euro-Scientific, Lint, Belgium). A 30 µl portion was injected into a Polyspher® OA KC column (Merck, Darmstadt, Germany) held at 35 °C. A 0·005 N H_2SO_4 solution was used as the mobile phase at a fixed flow rate of 0·4 ml min^{-1}. The confidentiality intervals for lactose, glucose, galactose and lactic acid were determined as $\pm 0{\cdot}040$ g l^{-1}, $\pm 0{\cdot}035$ g l^{-1}, $\pm 0{\cdot}006$ g l^{-1} and $\pm 0{\cdot}025$ g l^{-1}, respectively.

RESULTS

Exopolysaccharide production capacity of *Streptococcus thermophilus*

Four *Strep. thermophilus* strains (*Strep. thermophilus* LY03, *Strep. thermophilus* Sfi20, *Strep. thermophilus* 480 and *Strep. thermophilus* BTC) have been examined for their exopolysaccharide production capacity. Fermentation characteristics of the four strains are listed in Table 1. Growth of the *Strep. thermophilus* strains tested only occurred in MRS and M17 medium with glucose or lactose as energy source. Galactose could not be used as energy source. In SDM medium, no growth of the tested strains could be observed. However, milk coagulation and hence, growth of all tested strains, took place in SM medium. Although SM medium also seemed to be an excellent medium for supporting exopolysaccharide production by *Strep. thermophilus* strains, no exopolysaccharides could be isolated from the complex MRS and M17 medium or the semi-synthetic SDM medium fermented with *Strep. thermophilus*. Visual observation of slime production was very difficult. This may be due to the very low amounts of exopolysaccharides produced by *Strep. thermophilus* (Cerning *et al.* 1988, 1990) in contrast with, for instance, *Xanthomonas* (De Vuyst and Vermeire 1994). In addition, strong coagulation of the milk took place because of

Table 1 Important fermentation characteristics of the isolated, exopolysaccharide-producing *Streptococcus thermophilus* strains and monomer composition of their exopolysaccharides

Exopolysaccharide-producing strain	Cell number (cfu ml^{-1})	Final pH	EPS (mg PDM l^{-1})*§		Monomer composition¶
			SDM	SM	
Streptococcus thermophilus 480	$2{\cdot}9 \times 10^8$†	4·4†	ng	24	gal:glu, 3:1
Streptococcus thermophilus LY03	$6{\cdot}3 \times 10^8$†	4·1†	ng	32	gal:glu, 4:1
Streptococcus thermophilus BTC	$5{\cdot}3 \times 10^8$†	4·3†	ng	26	gal:glu, 3:1
Streptococcus thermophilus Sfi20	$5{\cdot}7 \times 10^8$†	4·2†	ng	20	gal:glu, 3:1
Lactobacillus sake 0-1	$8{\cdot}9 \times 10^8$‡	4·6‡	1560	0	rha:glu, 2:3

* SDM, modified semi-defined medium: SM, milk medium (see Materials and Methods).
† Determined in SM medium.
‡ Determined in SDM medium.
§ 0·5 l sample analysed after 18 h; ng = no growth.
¶ gal, galactose; glu, glucose; rha, rhamnose (determined using two different methods, see Materials and Methods).

the pH-uncontrolled experimental conditions due to acid production and proteolytic activity. The monosaccharide composition of the isolated exopolysaccharides is also represented in Table 1. All *Strep. thermophilus* exopolysaccharides were composed of the neutral sugars glucose and galactose. The galactose/glucose ratio varied from strain to strain, ranging from 3:1 to 4:1. Solutions of these exopolysaccharides (10 g l^{-1}) displayed a drop in viscosity with increasing shear rate as well as hysteresis (results not shown).

Kinetics of exopolysaccharide production

Figure 1 represents a typical fermentation profile of an exopolysaccharide-producing *Strep. thermophilus* strain (in this case, *Strep. thermophilus* LY03) grown in milk medium. Exponential growth took place during approximately 5 h. The stationary phase began after about 10 h of fermentation. During growth, lactose was hydrolysed intracellularly to glucose and galactose. Glucose was completely converted into lactic acid (by glycolytic degradation) and galactose was excreted into the fermentation liquor (due to a lactose/galactose antiport transport mechanism). However, part of the galactose—probably after conversion to glucose—must be incorporated in the synthesized exopolysaccharide. This may explain the small differences in lactic acid (derived from glucose) and galactose levels—which are supposed to be equimolar stoichiometrically—throughout the course of fermentation. The exopolysaccharide was produced mainly during the exponential growth phase. The production started after about 4 h of fermentation and reached a maximum of 247 mg PDM l^{-1} after 7 h of fermentation. This indicates primary metabolite kinetics. After having reached a local minimum (94 mg PDM l^{-1}) during the deceleration phase (after about 9 h of fermentation), an increase in exopolysaccharide production could again be observed at the beginning of the stationary phase with a maximum of 352 mg PDM

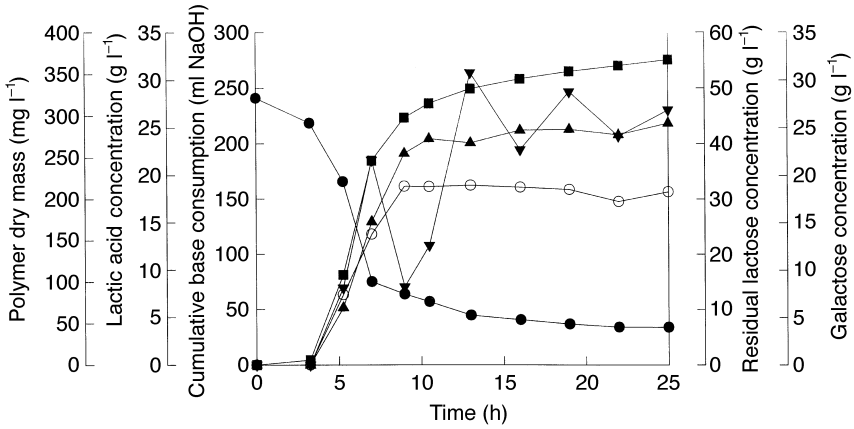

Fig 1 Batch fermentation profile of *Streptococcus thermophilus* LY03 growth and exopolysaccharide production at 42 °C and constant pH 5·5. Cells were grown in a Biostat® C fermenter containing 12 l SM medium. (▼), polymer dry mass; (▲), lactic acid; (■), cumulative base consumption; (●), residual lactose concentration; (○), galactose concentration

l^{-1} after 13 h of fermentation. The same fermentation profile was observed in all but one of the fermentations carried out. To verify the monomeric composition of the second exopolysaccharide peak, a composition analysis was done on all samples taken throughout the whole fermentation cycle. It was found that the same galactose/glucose ratio of 4:1 could be determined at any time during fermentation, possibly indicating that only one exopolysaccharide was produced. These data further indicate that exopolysaccharide production, as lactic acid accumulation, also occurs in the stationary phase. Finally, after 12 h of fermentation, the exopolysaccharide yield decreased, possibly due to enzymatic degradation.

Influence of medium composition on *Streptococcus thermophilus* growth and exopolysaccharide production

Streptococcus thermophilus LY03 fermentations were carried out in milk media with varying concentrations of the complex nitrogen source (peptone and/or yeast extract). All fermentations were performed at 42 °C and constant pH 5·5. The results are shown in Table 2. It is clear that both yeast extract and peptone are indispensable, not only for a high exopolysaccharide yield (166 mg PDM l^{-1} in the presence of both complex nitrogen sources compared to only 13 mg PDM l^{-1} without peptone or yeast extract), but also to support good growth. Indeed, most *Strep. thermophilus* strains do not grow well in the absence of a peptide or amino acid source because they lack an efficient proteinase capable of proteolytically degrading the caseins (Zourari *et al.* 1992). When one or both nitrogen sources were absent, it took much longer to reach the stationary phase. In the latter case, the exponential growth phase was followed by a linear growth phase because of nitrogen starvation.

Influence of fermentation temperature on *Streptococcus thermophilus* growth and exopolysaccharide production

Streptococcus thermophilus LY03 fermentations were carried out in milk medium at 25, 30, 37, 42, 50 and 55 °C. All fermentations were performed at constant pH 5·5. An overview of the most important fermentation parameters is given in Table 3. No growth was observed at 25 °C and 55 °C. Hence, *Strep. thermophilus* LY03 shows good growth within the temperature range 30 to 50 °C, with 42 °C the optimal growth temperature. In contrast, exopolysaccharide production took place only between 30 and 42 °C; at 50 °C, no exopolymer could be isolated. Within the temperature range of growth, the specific growth rate varied from 0·71 h^{-1} to 1·60 h^{-1}, and increased from 0·85 h^{-1} to 1·60 h^{-1} when the temperature increased from 30 to 42 °C. At non-optimal growth temperatures (both 30 and 50 °C), the specific growth rate decreased to about one half the maximal value under ideal growth conditions (42 °C). The energy of activation E_a for growth could be calculated as 41 kJ mol^{-1} (Arrhenius-plot not shown). It was also noticeable that a linear, prolonged active growth phase occurred at non-optimal growth temperatures. During all fermentations, exopolysaccharide production displayed the profile described above. The exopolysaccharide yield increased from low (30 °C) to ideal (42 °C) growth temperature and decreased to zero at higher temperatures. The best exopolysaccharide yield was obtained at 42 °C, namely 352 mg PDM l^{-1}. The product yield coefficient was also highest at this temperature (about 0·01 g PDM (g lactose)$^{-1}$). Exopolysaccharide degradation was less pronounced at higher fermentation temperatures. Finally, it has to be mentioned that for all fermentations, rather high levels of lactose remained unconsumed (Fig. 1), indicating non-optimal fermentation conditions such as pH, mixing, substrate supply, etc.

Influence of a constant fermentation liquor pH on *Streptococcus thermophilus* growth and exopolysaccharide production

Without pH control, low levels of exopolysaccharide were isolated from the fermented medium (Table 1). Therefore, several *Strep. thermophilus* LY03 fermentations were carried out at different constant pH values (4·9, 5·5, 6·2 and 6·9). The fermentations were carried out in milk medium and at

Table 2 Influence of the milk medium composition on *Streptococcus thermophilus* LY03 growth and exopolysaccharide production. The fermentations were carried out in a Biostat® B fermenter at 42 °C and constant pH 5·5. When the stationary phase was reached, a 0·5 l sample was asceptically withdrawn for further analysis

Medium composition*	EPS_{max} (mg PDM l^{-1})	μ_{max} (h^{-1})	Time of sampling (h)
10% SMP	13	0·71 ($r^2 = 0·996$)	27
10% SMP+0·5% YE	54	1·06 ($r^2 = 0·994$)	29
10% SMP+1·0% P	56	0·42 ($r^2 = 0·994$)	24
10% SMP+1·0% P+0·5% YE (= SM)	166	1·50 ($r^2 = 0·994$)	6

* SMP, skimmed milk powder (contains 56% lactose); P, peptone; YE, yeast extract.

Table 3 Important parameters of *Streptococcus thermophilus* LY03 fermentations carried out in milk medium (SM) at different temperatures. All fermentations were performed in a Biostat® C fermenter

Temperature (°C)	EPS_{max} (mg PDM l^{-1})	μ_{max} (h^{-1})	$(Y_{EPS/S})_{max}$ [g PDM (g lactose)$^{-1}$]	Time of $(EPS)_{max}$ (h)
30	202	0·85 ($r^2 = 0·998$)	0·006	16
37	325	1·13 ($r^2 = 0·996$)	0·007	11
42	352	1·60 ($r^2 = 0·999$)	0·008	13
50	0	0·71 ($r^2 = 0·996$)	0·000	–

42 °C, the optimal temperature for both growth and exopolysaccharide production. It is clear that growth was best at pH $\geqslant$ 5·5. The lag phase did not differ significantly (data not shown). The exponential growth phase was most remarkable at pH 6·2 and less characteristic at pH 6·9. All lactose was consumed at both pH 6·2 and 6·9. The course of the exopolysaccharide levels again displayed the typical profile described above. However, the two exopolysaccharide production phases were much more pronounced at pH 6·9, while exopolymer degradation was less pronounced at pH 5·5 but pronounced drastically at pH 4·9 (data not shown). The specific growth rate, exopolysaccharide yield and specific slime production were maximal at pH 6·2 and averaged 1·72 h^{-1}, 491 mg PDM l^{-1} and 0·01 g PDM (g lactose)$^{-1}$, respectively. At pH values above 6·2 and under 5·5, the exopolysaccharide yield decreased significantly (Table 4).

Influence of fermentation liquor constant oxygen tension on growth and exopolysaccharide production of *Streptococcus thermophilus*

Streptococcus thermophilus LY03 fermentations were carried out in an enriched milk medium (containing 10% skimmed milk powder, 4·4% extra lactose, 2·0% peptone and 1·0% yeast extract) at 42 °C and controlled pH 6·2, with continuous aeration at 1 and 2 l min^{-1}, and without aeration (aeration rate of 0 l min^{-1}). With an increasing aeration rate from 0 and 1 to 2 l min^{-1}, the specific growth rate decreased (from 2·36 h^{-1} and 2·15 h^{-1} to 1·82 h^{-1}, respectively) as well as the exopolysaccharide yield (from 550 mg PDM l^{-1} and 360 mg PDM l^{-1} to 260 mg PDM l^{-1}, respectively).

Influence of carbon/nitrogen levels on *Streptococcus thermophilus* growth and exopolysaccharide production

To determine the effect of carbon and nitrogen levels on both growth and exopolysaccharide production of *Strep. thermophilus* LY03, three additional fermentations were carried out (Table 5). Doubling the initial SMP concentration (and hence, lactose concentration) affected the exopolysaccharide yield significantly, increasing it from 278 mg PDM l^{-1} to 352 mg PDM l^{-1}. The initial lactose concentration did not influence the specific growth rate; in both cases it averaged 1·70 h^{-1}. However, since SMP contains additional nutritive constituents (e.g. amino acids, vitamins, nucleic acid precursors, etc.) the increase of EPS production cannot be directly correlated with the increase in lactose. To determine whether nitrogen was a limiting factor, exopolysaccharide production was studied with varying carbon/nitrogen levels in the fermentation broth at both pH 5·5 and 6·2 (Table 5). It is clear that an improved exopolysaccharide yield was achieved when

Table 4 Important parameters of *Streptococcus thermophilus* LY03 fermentations carried out in milk medium (SM) at 42 °C and at different, constant pH values. All fermentations were carried out in a Biostat® C fermenter

Constant pH	EPS_{max} (mg PDM l^{-1})	μ_{max} (h^{-1})	$(Y_{EPS/S})_{max}$ [g PDM (g lactose)$^{-1}$]	Time of $(EPS)_{max}$ (h)
4·9	73	0·97 ($r^2 = 0·998$)	0·002	15
5·5	352	1·60 ($r^2 = 0·999$)	0·008	13
6·2	491	1·72 ($r^2 = 0·999$)	0·010	12
6·9	106	1·66 ($r^2 = 0·994$)	0·002	12

Table 5 Influence of the carbon/nitrogen ratio on *Streptococcus thermophilus* LY03 growth and exopolysaccharide production. The fermentations were carried out in a Biostat® C fermenter at 42 °C and constant pH

Medium composition*	pH	EPS_{max} (mg PDM l^{-1})	μ_{max} (h^{-1})	Time of $(EPS)_{max}$ (h)
5·0% SMP+1.0% P+0·5% YE	5·5	278	1·83 ($r^2 = 0·999$)	8·5
10·0% SMP+1·0% P+0·5% YE (= SM)	5·5	352	1·60 ($r^2 = 0·999$)	13
10·0% SMP+4·4% L+1·0% P+0·5% YE	5·5	246	1·84 ($r^2 = 0·999$)	6
10% SMP+1·0% P+0·5% YE	6·2	491	1·72 ($r^2 = 0·999$)	12
10% SMP+4·4% L+2·0% P+1·0% YE	6·2	546	2·36 ($r^2 = 0·999$)	6

* SMP, skimmed milk powder (contains 56% lactose); L, lactose; P, peptone; YE, yeast extract.

both the carbon and nitrogen concentration were increased under optimal physical conditions.

DISCUSSION

In this paper, exopolysaccharides from four different *Streptococcus thermophilus* strains were isolated using a four-step protocol, including two protein and two exopolysaccharide precipitation steps; the exopolysaccharides were analysed and quantified, and the production kinetics of one were studied.

All *Strep. thermophilus* exopolysaccharides isolated during this study were composed of the neutral sugars galactose and glucose in a ratio varying from 3 : 1 to 4 : 1. Variations in the monosaccharide composition of the exopolysaccharides isolated from different *Strep. thermophilus* strains may indicate structural differences among the different polymers or, depending on the culture conditions, production of several polymers by the same strain, as has been suggested by Ariga *et al.* (1992), Cerning *et al.* (1994), Gancel and Novel (1994a,b) and Marshall *et al.* (1995), or variation of the monomer ratio during fermentation as postulated by Cerning *et al.* (1994) and Grobben *et al.* (1995). However, we could show that the galactose/glucose ratio of *Strep. thermophilus* LY03 exopolysaccharide does not vary throughout the batch cycle, at least under the same substrate and environmental conditions. On the other hand, structural analysis combined with rheological studies on exopolysaccharides from several lactic acid bacteria revealed that there is indeed considerable variation among the different exopolysaccharides. However, differences in monomer composition among related strains due to different culture conditions or isolation and purification procedures may not be excluded (Cerning 1990, 1995). The monosaccharides occurring most frequently in the various exopolysaccharides from lactic acid bacteria are glucose and galactose, of which galactose is the most important (Cerning 1990, 1995), but rhamnose (Cerning *et al.* 1986, 1988; Nakajima *et al.* 1990, 1992; Gruter *et al.* 1993; Grobben *et al.* 1995), mannose (Petit *et al.* 1991), fructose (Manca de Nadra *et al.* 1985), arabinose and xylose (Cerning *et al.* 1988, 1992), or sugar derivatives such as *N*-acetylgalactosamine (Doco *et al.* 1990; Petit *et al.* 1991) and *N*-acetylglucosamine (Cerning *et al.* 1994), are also found. Ariga *et al.* (1992) isolated a completely different exopolysaccharide produced by *Strep. thermophilus* OR 901, consisting of L-rhamnose and D-galactose in a 1·00 : 1·47 ratio; *Lact. casei* CG11 produces a heteropolysaccharide mainly composed of glucose (approximately 75%) and rhamnose (approximately 15%) (Kojic *et al.* 1992) and small amounts of galactose and mannose (Cerning *et al.* 1994). It still has to be determined whether the monomer composition can influence the rheology of solutions and fermented milk drinks prepared with these exopolysaccharides. Until now, it has been postulated that the functional properties of exopolysaccharides are influenced by their primary structure (Sutherland 1994) and it has been shown that the viscosity of pure exopolysaccharide solutions is dependent on the average molecular mass distribution (van den Berg *et al.* 1995). In addition, shear-thinning properties are important for food applications when considering processing costs (manufacturer) and mouth feel (consumer) of food products.

The production of intracellularly synthesized exopolysaccharides by different lactic acid bacteria varies widely from 57 to 424 mg l^{-1} with *Lact. delbrueckii* ssp. *bulgaricus* (Cerning *et al.* 1986, 1990), 53 to 337 mg l^{-1} with *Strep. thermophilus* (Cerning *et al.* 1988, 1990), 30–85 mg l^{-1}, 100–600 mg l^{-1} and 105–150 mg l^{-1} with *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Lact. casei* subsp. *casei*, respectively (Cerning *et al.* 1992) and 105–168 mg l^{-1} with bifidobacteria (Andaloussi *et al.* 1995; Roberts *et al.* 1995). *Lactobacillus sake* 0–1 produces from 1·3 g l^{-1} (van den Berg *et al.* 1995) to more than 1·5 g.l^{-1} (Janssens, B. and De Vuyst, L., unpublished

results). Our results indicate that milk is the best medium for exopolysaccharide production from *Strep. thermophilus* with a yield varying from 13 (pH free) to 546 mg l^{-1} (constant pH of 6·2) for *Strep. thermophilus* LY03. No slime production could take place in media such as MRS and M17, classical culture media for lactic acid bacteria. However, Petit *et al.* (1991), and Kojic *et al.* (1992) and Mozzi *et al.* (1995), found exopolysaccharide biosynthesis in MRS (pH-controlled) and M17 (free pH) medium, respectively. They quantified the exopolysaccharide yield colorimetrically.

Detailed fermentation profiles of exopolysaccharide-producing lactic acid bacteria cannot be found in the literature. The limited quantitative data available are based either on indirect quantitative techniques or on a limited number of samples. Our results clearly show that exopolysaccharide biosynthesis is growth-associated. The growth-associated biosynthesis of exopolysaccharides from *Strep. thermophilus* LY03 is supported by the need for an equilibrated carbon/nitrogen ratio, and a direct relationship between optimal growth conditons (temperature, pH and oxygen tension) and exopolysaccharide yields. Glucose is completely converted into lactic acid to yield the necessary energy; enough nitrogen is necessary for the synthesis of essential cell components, while the cells actively produce exopolysaccharides in the presence of an appropriate carbon source. In contrast with mesophilic lactic acid bacteria (Kontusaari and Forsén 1988; Cerning *et al.* 1992; van den Berg *et al.* 1995), maximal exopolysaccharide production with *Strep. thermophilus* LY03 was stimulated at temperatures optimal for growth. However, an increased exopolysaccharide production at 32 or 37 °C compared to a higher fermentation temperature of 42 °C was observed for some *Strep. thermophilus* and *Lact. delbrueckii* subsp. *bulgaricus* strains (Kojic *et al.* 1992; Gancel and Novel 1994a; Mozzi *et al.* 1995). On the other hand, some authors clearly showed enhanced exopolymer levels with increased temperatures (Garcia-Garibay and Marshall 1991; Grobben *et al.* 1995). Finally, a coupled effect between *Strep. thermophilus* LY03 growth and exopolysaccharide production could be observed with varying pH. At low, non-optimal pH, both growth rate and exopolysaccharide production were low. At a well defined, ideal pH (6·2), the conversion of sugar in polymer took place efficiently, while sugar was preferentially converted into energy for both growth and maintenance at higher pH values. Similar observations were made for *Lact. sake* 0–1 fermentations (van den Berg *et al.* 1995). Also, with some *Strep. thermophilus*, *Lact. delbrueckii* subsp. *bulgaricus* and *L. lactis* strains, exopolysaccharide biosynthesis improved considerably if the pH was kept constant at about 6·0 (Cerning 1995; Mozzi *et al.* 1996). In contrast with the postulations of Cerning (1990), exopolysaccharide production increased with decreasing constant oxygen tension, in turn resulting in better growth. The growth-associated biosynthesis reported here is in strong contrast to the hypothesis of Sutherland (1972), which stated that exopolysaccharide production occurs at low growth rates, explained by the fact that slower growth goes hand-in-hand with reduced biosynthesis of cell wall polymers, hence making more precursor molecules available for exopolysaccharide biosynthesis. Sutherland's conclusions are, however, based on the analysis of the production kinetics of exopolymers from Gram-negative bacteria such as *Xanthomonas campestris*, *Rhizobium* spp., *Klebsiella* spp., *Pseudomonas* spp., *Acetobacter* spp. and *Escherichia coli*. However, secondary metabolite kinetics have also been proposed for lactic acid bacteria (Manca de Nadra *et al.* 1985; Kojic *et al.* 1992; Gancel and Novel 1994a). On the other hand, growth-associated exopolymer production (primary metabolite kinetics) has been observed for several lactic acid bacterial strains (Petit *et al.* 1991; Dueñas *et al.* 1995; Grobben *et al.* 1995; van den Berg *et al.* 1995). The production of exopolysaccharides during the stationary growth phase could not be completely excluded, as shown by our results. Both growth-associated and non-associated production kinetics were also observed by Manca de Nadra *et al.* (1985) and Kojic *et al.* (1992). These dual production kinetics may be explained in several ways, including: a temporary limited slime production; production of low molecular mass and high molecular mass exopolysaccharide material; production of an insoluble and a soluble slime fraction; an environmental signal, for instance, a modified carbon/nitrogen ratio induces biosynthesis after growth resulting in a second production phase. Although some authors are convinced that the nature of the substrate cannot influence the composition of the exopolysaccharides produced (Manca de Nadra *et al.* 1985; van den Berg *et al.* 1995), others found that the chemical composition of the environment causes variation in the exopolymer composition (Petit *et al.* 1991; Kojic *et al.* 1992; Cerning *et al.* 1994; Grobben *et al.* 1996). Also, the carbon/nitrogen ratio would play an important role in the production of exopolysaccharides, as was shown before by De Vuyst *et al.* (1987) and De Vuyst and Vermeire (1994). It has further been shown that exopolysaccharide biosynthesis and degradation could be induced by several environmental factors (Gancel and Novel 1994a,b; Dierksen *et al.* 1995). A decreased exopolysaccharide level upon prolonged fermentation may be the result of a physiologically changing cell environment (Gancel and Novel 1994a,b), degradation by glycohydrolytic activity (Cerning *et al.* 1988, 1990, 1992; Gancel and Novel 1994b), or reversible DNA rearrangements resulting in different cell types with different exopolymer production capacities (Gancel and Novel 1994b). It could be shown by our results that fermentation temperature and pH influence exopolysaccharide degradation.

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