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Effect of medium composition and temperature and pH changes on exopolysaccharide yields and stability during *Streptococcus thermophilus* LY03 fermentations

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Abstract

To increase the exopolysaccharide (EPS) yields from *Streptococcus thermophilus* LY03 and to unravel the nature of the EPS degradation process, fermentation experiments were carried out with this strain in a customized MRS medium, using different additional carbohydrates or amino acids possibly related to growth and EPS production. No significant increase of the EPS yields or activities of the enzymes α-phosphoglucomutase, UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase that are correlated with EPS production, or of the activity of dTDP-glucose pyrophosphorylase involved in the rhamnose synthetic branch of EPS biosynthesis, was observed. The EPS monomer composition remained unchanged for all experiments. Fermentations with a sudden temperature increase or lowered pH were carried out as well to try to avoid EPS degradation upon prolonged fermentation. It was demonstrated that EPS degradation took place enzymatically. Incubations of purified high-molecular-mass EPS with cell-free culture supernatant or cell extracts showed its degradation by enzymes with an endo-activity. This glycohydrolytic activity probably encompasses several enzymes having a molecular mass lower than 50,000 and 10,000 Da, and seems to be rather stable at high temperature and low pH. These results contribute to a better understanding of the physiological and chemical factors influencing EPS production and degradation.

Keywords: Exopolysaccharide; *S. thermophilus*; EPS biosynthesis; EPS degradation

1. Introduction

The exopolysaccharide (EPS)-producing properties of lactic acid bacteria (LAB) have been extensively studied during the last few years (Cerning and Marshall, 1999; De Vuyst and Degeest, 1999; Ricciardi and Clementi, 2000; De Vuyst et al., 2001). The main advantage of LAB EPS is that they are produced by food-grade microorganisms and can contribute to the proper consistency and texture of fermented foods. However, low yields and high degradation rates of in situ produced EPS are important bottlenecks for the industrial processor (De Vuyst et al., 2001). A number of studies on physical and chemical cultivation conditions of *Streptococcus thermophilus* to obtain higher

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EPS yields have been performed (Degeest et al., 2001b). The physical factors of utmost importance are incubation temperature, pH, oxygen tension, agitation speed, and incubation time (Gancel and Novel, 1994a,b; Degeest and De Vuyst, 1998; De Vuyst et al., 1998). Chemical factors determining the EPS yield of *S. thermophilus* are the carbohydrate source, the nitrogen source, the carbon/nitrogen ratio, and the presence or absence of other medium components, e.g. salts and vitamins (Gancel and Novel, 1994a,b; Degeest and De Vuyst, 1998, 1999, 2000; De Vuyst et al., 1998). For *S. thermopilus*, also the genetic organisation of *eps* gene clusters, and the enzymes involved in the biosynthesis and interconversion of sugar nucleotides (EPS precursors) or in the linkage of the different sugar molecules (glycosyltransferases) receive much attention (Griffin et al., 1996; Stingele et al., 1996, 1999a,b; Escalante et al., 1998; Low et al., 1998; Bourgoin et al., 1999; Almirón-Roig et al., 2000; Degeest and De Vuyst, 2000; Ramos et al., 2001). Details about the metabolic pathways involved are known now (Fig. 1).

Many studies showed decreased EPS amounts after prolonged incubation of *S. thermophilus* (Macura and Townsley, 1984; Cerning et al., 1988, 1990; Gancel and Novel, 1994b; De Vuyst et al., 1998; Degeest and

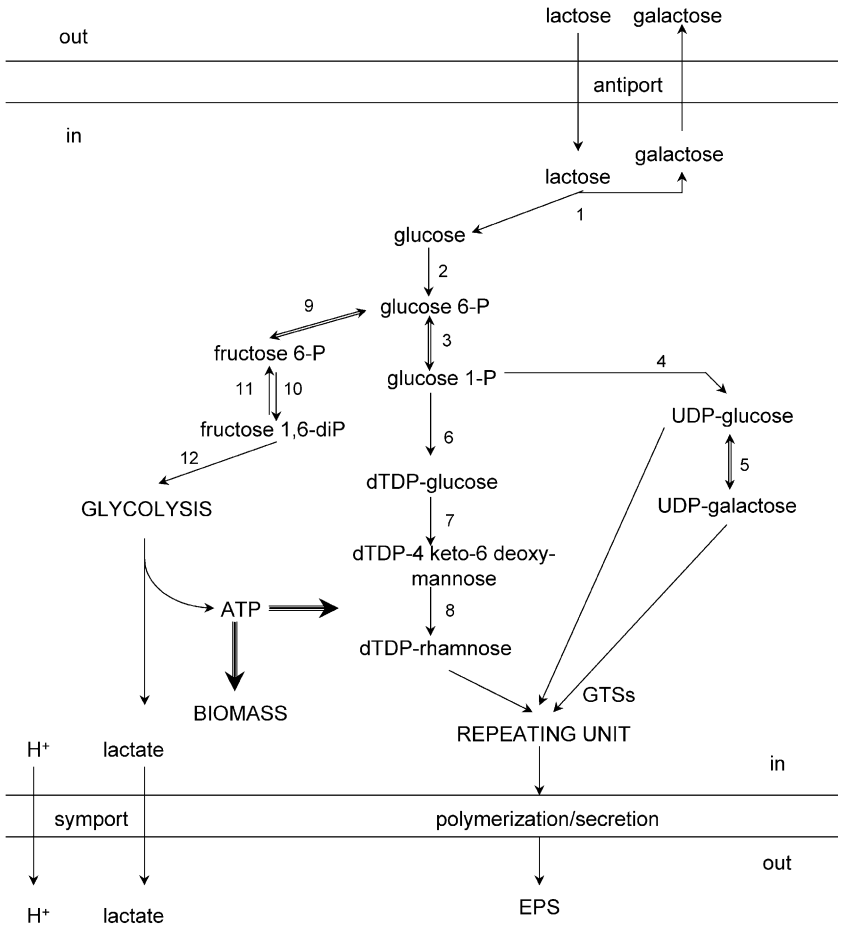

Fig. 1. Diagram of the metabolic pathways involved in lactose catabolism and exopolysaccharide biosynthesis by *S. thermophilus*. The numbers refer to the enzymes involved: (1) β-galactosidase, (2) glucokinase, (3) α-phosphoglucomutase, (4) UDP-glucose pyrophosphorylase, (5) UDP-galactose 4-epimerase, (6) dTDP-glucose pyrophosphorylase, (7) dehydratase, (8) epimerase reductase, (9) phosphoglucose isomerase, (10) 6-phosphofructokinase, (11) fructose-1,6-bisphosphatase, (12) fructose-1,6-diphosphate aldolase. GTS = glycosyl transferase.

De Vuyst, 1999; Pham et al., 2000). Suggestions have been made that this degradation during fermentation is due to a change in the physical or chemical factors linked or not to the activity of glycohydrolases. For instance, EPS degradation was less pronounced at higher fermentation temperatures and was drastically favoured at pH 4.9 for *S. thermophilus* LY03 fermentations (De Vuyst et al., 1998). Pham et al. (2000) found a more pronounced reduction of EPS yields in the case of lactose-grown *Lactobacillus rhamnosus* R cells than in glucose-grown cells. Cerning et al. (1988) suggested that this EPS degradation is due to an enzyme, possibly a glucohydrolase, which progressively destroys the polymer. The slow rate of reduction in viscosity of EPS-containing media suggests that the mode of enzymatic action involves exotype mechanisms, successively splitting glycosidic linkages which results in polymers with lower molecular mass (Pham et al., 2000). Although this link between EPS degradation and glycohydrolase activity has been put forward several times, clear evidence of the existence and effect of glycohydrolases, degrading heteropolysaccharides from LAB, has only been demonstrated for the *Lb. rhamnosus* R strain (Pham et al., 2000). It is further well known that homopolysaccharide-producing LAB strains synthesize EPS hydrolases besides glycosyltransferases, enabling some of them to utilize the resultant oligosaccharides for intracellular metabolism (Colby and Russell, 1997). Different glycohydrolases, both hydrolytic and eliminase enzymes, for other microbial EPS were described by Sutherland (1999).

S. thermophilus LY03 produces both high-molecular-mass and low-molecular-mass heteropolysaccharides composed of galactose/glucose/(*N*-acetyl) galactosamine in an averaged ratio of 2:1:1 (Degeest and De Vuyst, 1999; Degeest et al., 2001a). Whereas in milk EPS levels of less than 600 mg l^{-1} have been obtained (De Vuyst et al., 1998), fermentations in MRS media yielded up to 1500 mg l^{-1}, depending on the carbon/nitrogen ratio (Degeest and De Vuyst, 1999) and the nature of the carbohydrate source used (Degeest and De Vuyst, 2000). EPS production displayed primary metabolite kinetics, followed by degradation upon prolonged fermentation (De Vuyst et al., 1998; Degeest and De Vuyst, 1999).

In this study, we tried to increase EPS yields by stimulating bacterial growth and EPS production through additions to the fermentation medium of specific carbohydrates or amino acids, possibly related to EPS biosynthesis (Keevil et al., 1984; Christensen et al., 1999; De Vuyst et al., 2001). Concomitantly, the correlation of EPS yields with enzyme activities involved in sugar nucleotide biosynthesis was examined. Further, we tried to reduce EPS degradation by changing physical factors during the fermentation process. Finally, incubations of EPS with cell extracts or cell-free culture supernatant, fractionated through ultrafiltration, were studied, to unravel the nature of the degradation process.

2. Materials and methods

2.1. Bacterial strains, growth conditions and media

S. thermophilus LY03 (kindly provided by Prof. Dr. V.M. Marshall, University of Huddersfield, Huddersfield, United Kingdom) 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 25% (v/v) glycerol (de Man et al., 1960). To obtain fresh cultures, the bacteria were propagated twice (12 h at 42 °C) in the medium identical to the one used for the fermentations later on. The fermentor inoculum was always prepared in two steps. First, 10 ml of medium was inoculated with 100 μl of a freshly prepared culture. This culture was further transferred twice (1.0%, v/v) to finally use the second preculture as fermentor inoculum.

A customized MRS medium was used as basic EPS production medium; it contained (g l^{-1}): peptone (Oxoid), 30; yeast extract (Merck, Darmstadt, Germany), 12; Lab Lemco (Oxoid), 8; K_2HPO_4, 2; sodium acetate, 5; triammonium citrate, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.038; and Tween 80 (1 ml l^{-1}) (Degeest and De Vuyst, 1999). Lactose (0.22 M) was used as basic carbohydrate source. Modified compositions consisted of either 0.03 M ribose or 0.04 M α-D-glucose 1-phosphate as additional carbohydrate source, or either 14.2 mM arginine or 23.3 mM aspartic acid (doubled concentration as compared to the customized MRS medium) as supplementary amino acids.

2.2. Fermentation experiments and sampling

All fermentations were carried out in customized MRS medium (unless otherwise specified) in 5 l Biostat©© CT or 10 l Biostat© C (B. Braun Biotech International, Melsungen, Germany) fermentors using a 1.0% (v/v) inoculum. Two types of fermentations were carried out, namely production fermentations to isolate EPS at the end of the exponential growth phase (after approximately 8 h of incubation), and fermentations during which samples were taken aseptically at regular time intervals. These samples were used to determine one or more of the following fermentation parameters: biomass concentration (cell dry mass, CDM), residual sugar concentration, EPS yield (polymer dry mass, PDM), EPS monomer composition, and enzyme activities. All fermentations were carried out at a constant temperature of 42.0±0.1 °C (unless otherwise specified), and at a constant pH 6.2±0.1 (unless otherwise specified) by on line, automatic addition of 10 N NaOH. An agitation rate of 100 rpm was programmed to keep the fermentation broth homogeneous.

The biomass concentration, the amount of EPS produced (both high-molecular-mass EPS [HMM-EPS] and low-molecular-mass EPS [LMM-EPS]), the EPS monomer composition analyses, the residual sugar concentration determinations, the maximum specific growth rate (μ_{max}, h^{-1}) and the maximum carbohydrate consumption rate (r_{max}, h^{-1}) were determined or calculated as described before (Degeest and De Vuyst, 1999). For the standard deviations of the EPS isolations, EPS monomer composition analysis, and residual sugar concentrations, see also Degeest and De Vuyst (1999). Cell extracts were prepared, and activities of the enzymes α-phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase and dTDP-glucose pyrophosphorylase were determined in duplicate, as described by Degeest and De Vuyst (2000).

2.3. Determination of exopolysaccharide degradation

To determine whether EPS were degraded due to enzymes present in the fermentation broth after prolonged incubation, different experiments were performed.

In a first stage, fermentations were carried out with either an increased temperature step or a decreased pH step at the end of the exponential growth phase. For the latter one, the pH was dropped from 6.2±0.1 to 3.0±0.1, kept for 3 h at pH 3.0±0.1, and restored to pH 6.2±0.1. For the fermentation with an increased temperature step, the temperature was increased from 42±0.1 to 90±0.1 °C at the end of the exponential growth phase, and then restored to 42±0.1 °C after 3 h. The amount of HMM-EPS and LMM-EPS was determined at different time points: just before the temperature and pH step, just after the temperature and pH step to see if there was any influence on EPS levels, and twice during the stationary phase to see the effect of temperature and pH.

In a second stage, incubations of purified HMM-EPS with either cell-free culture supernatant (possible source of extracellular EPS-degrading enzymes) or cell extracts (possible source of intracellular EPS-degrading enzymes) were performed. HMM-EPS were produced through fermentation. Before use, HMM-EPS were purified by extensive dialysis against ultrapure water, using Spectra/Por membranes (Merck) with a molecular mass cut off (MMCO) of 3500, and subsequently freeze-dried. Cells were isolated from fermented medium by centrifugation (20 min, 11,000 rpm, 4 °C), and used to prepare cell extracts as described by Degeest and De Vuyst (2000), except that a sodium acetate buffer (pH 5.0) was used to suspend the cells. The protein content of the cell extracts was determined with a BioRad Protein Assay Kit (serial number 500-0116; BioRad Laboratories, Hercules, CA, USA). Fractionation of both cell extracts and cell-free culture supernatant was performed through ultrafiltration, using tangential flow modules (Vivaflow, Vivascience, Lincoln, United Kingdom), equipped with membranes with a MMCO of 10,000, 50,000, and 100,000. As a negative control, cell extracts and cell-free culture supernatant were heated during 5 min at 100 °C to inactivate the enzymes. Non-fractionated cell extracts and cell-free culture supernatant without heat treatment were used as a positive control. Incubations of 100 mg of purified freeze-dried HMM-EPS were performed at 42 °C with 250 ml of culture supernatant or 50 ml of sodium acetate buffer (pH 5.0) containing cell extract (3.7 mg/ml). To all incubation mixtures 0.02% NaN_3 was added to prevent bacterial growth. As an additional control, all samples were also incubated without addition of purified HMM-EPS. Before incubation,

and after 12 h and 24 h of incubation, all samples were assayed in three ways. First, HMM-EPS and LMM-EPS were isolated as described above, freeze-dried, and weighed. Second, to identify exo-acting enzymes, liberation of reducing sugars and/or monomers present in the sample were measured by HPLC (De Vuyst et al., 1998) and by the Somogyi–Nelson technique (Nelson, 1944; Somogyi, 1945). Third, the total sugar content (derived from EPS, monomers present in the sample, and liberated reducing sugars) was determined by the Anthron method (Scott and Melvin, 1953). All assays were performed in triplicate. The standard deviation was 20.0% for the EPS isolation method, 0.5 g l^{-1} for the HPLC analyses, 0.005 g l^{-1} for the Somogyi–Nelson method, and 0.005 g l^{-1} for the Anthron method. Finally, the molecular mass of the purified HMM-EPS, and all isolated EPS after 24 h of incubation, was determined using gel filtration chromatography applying a Sephacryl S-400 gel (Pharmacia, Uppsala, Sweden) with 50 mM phosphate/NaOH buffer (pH 6.8) and 0.15 M NaCl as eluent. A dextran standard series was used to estimate the molecular mass (Degeest and De Vuyst, 1999).

3. Results

3.1. Effect of the addition of carbohydrates or amino acids on bacterial growth and exopolysaccharide production of S. thermophilus LY03

First, 0.03 M ribose was added as an additional carbohydrate source. Ribose is the main component of the sugar nucleotides, the precursor molecules in EPS biosynthesis. As seen from Table 1 bacterial growth, EPS production and EPS monomer composition were identical to the control fermentation (0.22 M lactose as the sole carbohydrate source). This was not surprising because ribose was not taken up since the ribose concentration in the medium remained unchanged. The maximum carbohydrate consumption

Table 1
Effect of the addition of carbohydrates or amino acids to the fermentation medium on both bacterial growth and exopolysaccharide production during *S. thermophilus* LY03 fermentations[a]

Carbohydrate source(s) or amino acids added	μ_{max} (h^{-1}) (r^2)	r_{max} (h^{-1}) (r^2)	$Biomass_{max}$ (g CDM l^{-1}) (h)	$(HMM\text{-}EPS)_{max}$ (mg PDM l^{-1}) (h) Gal:Glu	$(LMM\text{-}EPS)_{max}$ (mg PDM l^{-1}) (h) Gal:Glu	$(Total\ EPS)_{max}$ (mg PDM l^{-1}) (h) Gal:Glu HMM Gal:Glu LMM
0.22 M lactose (control)	1.1 (0.997)	0.3 (0.999)	4.5 (10.00)	821 (8.50) 4.14:1.00	766 (10.00) 4.19:1.00	1142 (10.00) 3.91:1.00 4.02:1.00
0.22 M lactose+0.03 M ribose	1.0 (0.976)	0.3 (0.998)	4.5 (16.50)	949 (15.75) 3.18:1.00	362 (12.50) 4.10:1.00	1117 (15.75) 3.98:1.00 3.89:1.00
0.22 M lactose+0.04 M α-D-glucose 1-phosphate	1.1 (0.982)	0.3 (0.989)	5.3 (13.00)	837 (12.00) 3.79:1.00	429 (10.00) 3.89:1.00	979 (12.00) 3.85:1.00 3.79:1.00
0.22 M lactose, 14.2 mM added arginine	1.1 (0.985)	0.3 (0.977)	3.9 (14.50)	987 (12.75) 3.80:1.00	410 (24.25) 4.20:1.00	1158 (12.75) 4.00:1.00 3.78:1.00
0.22 M lactose, 23.3 mM added aspartic acid	1.0 (0.973)	0.3 (0.965)	3.9 (14.25)	812 (13.25) 3.88:1.00	290 (25.75) 4.50:1.00	967 (13.25) 3.65:1.00 3.60:1.00

[a] Fermentations were performed in customized MRS broth at 42 °C and at a controlled pH 6.2. The biokinetic parameters μ_{max} and r_{max} were estimated through modelling of the experimental data (the correlation coefficient r^2 is given in parentheses).

rate of lactose was identical to the one of the control fermentation. Second, α-D-glucose 1-phosphate as a key intermediate in the EPS biosynthesis pathway was added (0.04 M) to the fermentation medium. Besides the fact that a somewhat increased maximum biomass concentration (5.3 g CDM l^{-1}) was observed after 13 h of fermentation, no other effects were seen as compared to the control fermentation. As was seen for ribose, also α-D-glucose 1-phosphate remained unaffected in the fermentation medium, and the maximum carbohydrate consumption rate of lactose remained unchanged. In another attempt to influence bacterial growth and EPS production, the concentrations of the amino acids arginine or aspartic acid were doubled as compared to the control fermentation. Arginine is believed to increase the conversion of α-D-glucose 6-phosphate into α-D-glucose 1-phosphate in oral streptococci (Keevil et al., 1984). Aspartic acid is believed to serve as a precursor for other amino acids and for nucleotides in LAB (Christensen et al., 1999). Both amino acids can further generate metabolic energy, based on production of ATP via substrate level phosphorylation or decarboxylation and electrogenic transport, respectively (Konings et al., 1995, 1997; Abe et al., 1996). Energy is indispensable for both cell growth and growth-associated EPS production. Again, no changes were observed in the fermentation profile and fermentation parameters, except for a lower maximum biomass concentration of 3.9 g CDM l^{-1} in both cases.

3.2. Effect of the addition of carbohydrates or amino acids on activities of key enzymes

The activities of four key enzymes involved in sugar nucleotide biosynthesis, as compared to EPS yields, were determined at three different time points for all fermentations described above. The results are displayed in Table 2. For all enzymes, a similar trend was seen. In general, the observed activities were highest at the end of the exponential growth phase, the point at which the total EPS yield was maximum (Fig. 2). For the fermentation where α-D-glucose 1-phosphate was added as an additional carbohydrate source, a slight increase of the activity of the enzyme α-phosphoglucomutase was observed. The activity of this enzyme was comparable for all other fermentations (including the control fermentation). For the activities of the two enzymes UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase, no significant differences were observed for any of the samples. Only for the fermentation where α-D-glucose 1-phosphate was added, somewhat lower activities were seen. Finally, no activity at all was observed for dTDP-glucose pyrophosphorylase.

3.3. Influence of temperature and pH changes on exopolysaccharide degradation

To study the influence of physical factors on EPS degradation upon prolonged fermentation, two additional fermentations were carried out with either a sudden temperature increase at the end of the exponential growth phase or an abrupt pH drop, after which the initial settings of temperature or pH were restored. All results, compared with a control fermentation and carried out at a constant temperature of 42 °C and at a constant pH of 6.2, are displayed in Fig. 3.

At sampling points 1 and 2 at the end of the exponential growth phase (just before and after the temperature or the pH shift), a comparable EPS yield was observed for all experiments. At sampling point 3 (during the stationary phase), clear differences were observed in the control fermentation as compared to the experiments with a temperature or pH shift. The amount of HMM-EPS was lower for the control experiment, while the amount of LMM-EPS was higher, indicating EPS degradation. For the experiments carried out with a temperature or pH shift, almost no difference was observed for the EPS yields between sampling points 1, 2, and 3, indicating no EPS degradation. After restoring the initial fermentation temperature or pH, however, the amounts of HMM-EPS and LMM-EPS were again comparable with the control fermentation, indicating EPS degradation. For all fermentations, a lower total EPS yield was observed at the final sampling point 4.

3.4. Indication that exopolysaccharide degradation takes place enzymatically

Both cell extracts and cell-free culture supernatant, fractionated by ultrafiltration or not, were tested for their ability to degrade EPS. For the HMM-EPS incubated with cell-free culture supernatant, average values of 20 g l^{-1} of lactose and of 19 g l^{-1} of

Table 2
Amounts of high-molecular-mass exopolysaccharide (HMM-EPS), low-molecular-mass exopolysaccharide (LMM-EPS), total exopolysaccharide (total EPS), and activities of four key enzymes involved in sugar nucleotide biosynthesis in cell extracts of *S. thermophilus* LY03[a]

Carbohydrate source or amino acid added	0.22 M lactose (control)			0.22 M lactose+0.03 M ribose			0.22 M lactose+0.04 M α-D-glucose 1-P			0.22 M lactose, 14.2 mM arginine added			0.22 M lactose, 23.3 mM aspartic acid added		
Time point	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Amount of exopolysaccharide produced in mg PDM l^{-1}															
HMM-EPS	0	821	495	0	949	103	0	837	212	33	987	379	33	812	522
LMM-EPS	603	207	0	362	168	197	429	142	389	210	171	338	123	155	210
Total EPS	603	1028	495	362	1117	300	429	979	601	243	1130	717	156	967	732
Enzyme activities in nmol (mg cell protein)$^{-1}$ min^{-1}															
α-Phospho-glucomutase	581±44	829±7	715±18	602±33	615±12	599±46	820±22	845±44	799±32	610±45	790±67	802±13	610±32	799±12	655±34
UDP-glucose pyrophosphorylase	137±11	199±2	175±5	111±13	134±16	122±9	97±6	100±10	102±12	150±14	210±15	220±14	155±12	200±9	201±22
UDP-galactose 4-epimerase	281±60	396±38	351±44	190±41	285±12	257±13	181±22	182±33	170±39	190±22	201±12	179±63	200±22	352±41	320±13
dTDP-glucose pyrophosphorylase	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0

[a] *S. thermophilus* LY03 was grown in batch cultures in customized MRS medium with lactose or combinations of lactose and an additional carbohydrate or amino acid at 42 °C and at a constant pH 6.2. Samples were taken at three time points of the fermentation course, representing the exponential growth phase (point 1), the end of the exponential growth phase (point 2), and the stationary phase (point 3). Each value is always the average of two measurements.

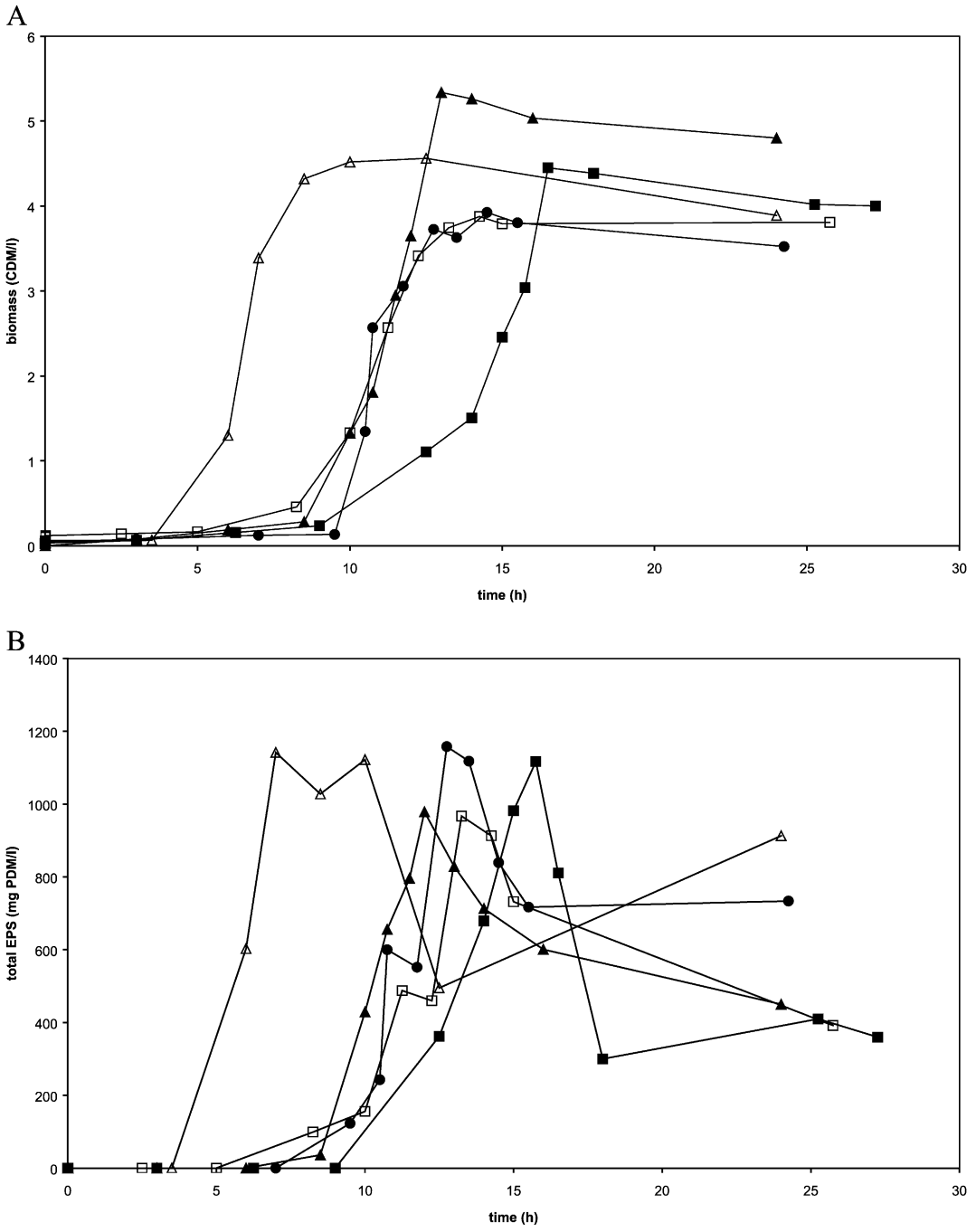

Fig. 2. Fermentation profiles for the addition of ribose (0.03 M, ■), α-D-glucose 1-phosphate (0.04 M, ▲), arginine (14.2 mM, ●), and aspartic acid (23.3 mM, □) to MRS with 0.22 M of lactose at 42 °C and a controlled pH of 6.2 (control fermentation, without any addition, △): growth (A) and exopolysaccharide production (B).

galactose were observed, as determined by HPLC. These values remained unchanged for all samples and for the controls before incubation, and after 12 and 24 h of incubation, indicating that no extra monomers were released during incubation. For the HMM-EPS incubated with cell extracts, no monomers were detected by HPLC for any of the samples (controls included), neither before incubation nor after incubation for 12 and 24 h. Using the Somogyi–Nelson technique, an average value of 35.00 g l^{-1} of glucose

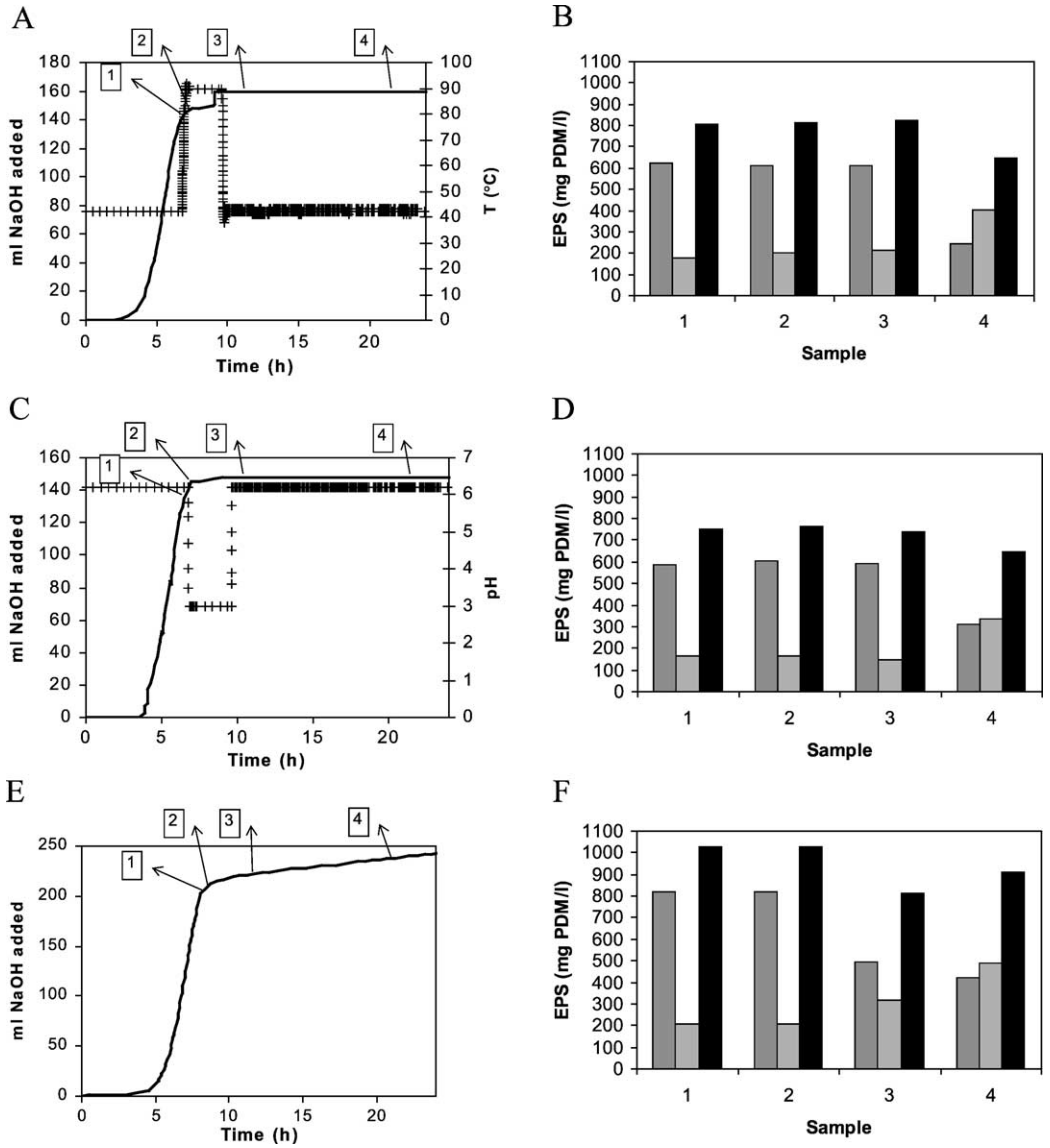

Fig. 3. Fermentations carried out in customized MRS medium with *S. thermophilus* LY03 applying a temperature (A and B) or pH shift (C and D). Graphs (E) and (F) represent the control fermentation at 42 °C and pH 6.2. In graphs A, C, and E, the cumulative base consumption is represented by a full line. The temperature (graph A) and pH shift (graph C) is represented by dashed lines. The concomitant amounts of high-molecular-mass ■, low-molecular-mass ■, and total ■ exopolysaccharides are represented in graphs (B), (D), and (F), respectively. The four sampling points are displayed on the graphs.

equivalents was observed for all HMM-EPS samples prior to incubation and after incubation with cell-free culture supernatant for 12 and 24 h. These observations show again that no extra monomers were released during incubation, indicating that EPS-degrading enzymes possibly present in cell-free culture supernatant are acting as endo-enzymes. However, for the samples incubated with cell extracts, a

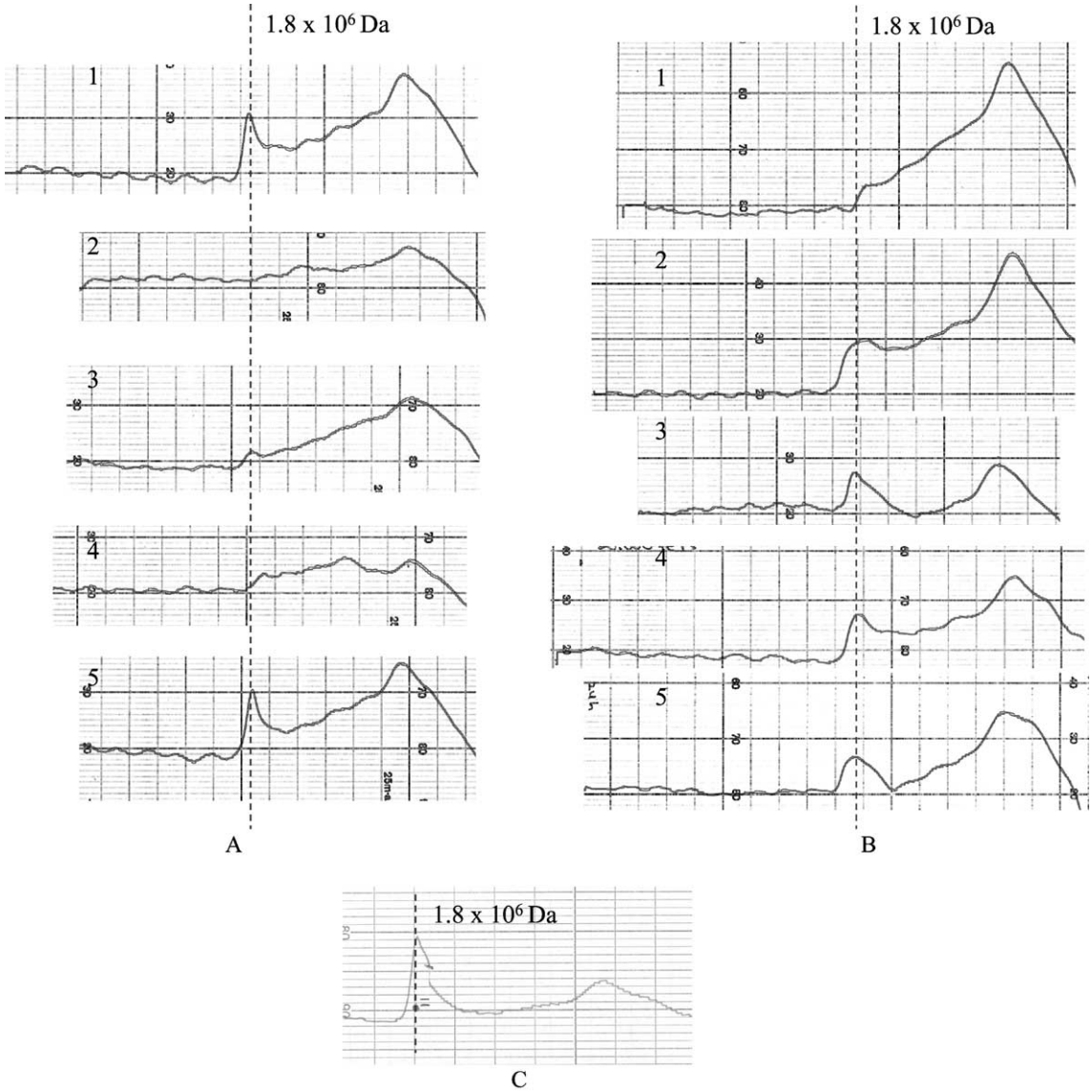

Fig. 4. Gel filtration chromatograms of purified, high-molecular-mass exopolysaccharides produced by *S. thermophilus* LY03, after incubation with (A) cell extracts, and (B) cell-free culture supernatant. Chromatogram (C) represents the purified high-molecular-mass EPS, added to all samples, before incubation. The numbers displayed on the chromatograms represent the treatment of the fractions: 1, without treatment (positive control); 2, heat treatment of 5 min at 100 °C (negative control); 3, filtrate obtained after ultrafiltration with a MMCO of 10,000 Da; 4, filtrate obtained after ultrafiltration with a MMCO of 50,000 Da; 5, filtrate obtained after ultrafiltration with a MMCO of 100,000 Da. The vertical dotted line indicates a molecular mass of 1.8×10^6 Da.

slight liberation of monomers was seen after 24 h of incubation, because the monomer concentration was 0.01 g l^{-1} higher after 24 h of incubation as compared to the start of the experiment, indicating exo-activity of enzymes present. The total amount of reducing sugars, determined by the Anthron method, was higher for HMM-EPS incubated with culture supernatant as compared to HMM-EPS incubated with cell extracts. This was ascribed to the residual sugars in the supernatant (as was shown by the HPLC analyses). However, due to fluctuations of the values observed for all samples measured, no conclusions could be drawn regarding endo- or exo-activity of the enzymes involved, based on this technique. Apparently, the Anthron method interfered with other components in the samples. After isolation of EPS from all samples, the added EPS could always be recovered. All EPS were, however, recovered as a pelleted fraction, representing only LMM-EPS. This indicates that the HMM-EPS concentration was too low for its isolation in suspension as a floating fraction. Therefore, gel filtration was performed on the EPS isolated from all samples incubated during 24 h, including purified HMM-EPS initially added to the samples. These results are displayed in Fig. 4. The purified HMM-EPS displayed two peaks, one peak at a molecular mass of 1.8×10^6, and a broad peak around 70,000 Da (Fig. 4C). The latter peak is caused by a contamination with glucomannans derived from yeast extract present in the EPS production medium as has been shown by gel filtration and high performance liquid chromatography with amperometric detection of yeast extract and purified EPS only (data not shown). In the culture supernatant used, LMM-EPS was also present, explaining the higher LMM peaks (around 70,000 Da) for all samples where HMM-EPS was incubated with culture supernatant (Fig. 4B). For the HMM-EPS, incubated for 24 h with cell-free culture supernatant, degradation was observed in all samples, including the heat-treated control (Fig. 4B). However, this degradation was less pronounced for the filtrates obtained after ultrafiltration through membranes with a MMCO of 10,000, indicating that the EPS-degrading enzymes display a molecular mass of above 10,000 (Fig. 4B3). For the HMM-EPS, incubated for 24 h with cell extracts, degradation was observed in all samples (including the heat-treated control), but was most pronounced for the filtrates obtained by ultrafiltration through membranes with a MMCO of 10,000 and 50,000 Da (Fig. 4A). This indicates that the EPS-degrading enzymes display a molecular mass of less than 50,000 and 10,000 Da. Also the heat-treated controls displayed EPS-degrading activity (Fig. 4A2 and B2), indicating incomplete inactivation or heat stability of the enzymes involved. The fact that the molecular mass of the HMM-EPS decreased gradually might indicate that the enzymes display both exo- and endo-activity.

4. Discussion

S. thermophilus LY03 produces a high-molecular-mass and a low-molecular-mass EPS (Degeest and De Vuyst, 1999). It has been shown before that EPS production is maximum in complex media (De Vuyst et al., 1998; Degeest and De Vuyst, 1999). Complex nutrients like yeast extract and peptone are necessary for both cell growth and EPS formation indicating the role of vitamins, purines and pyrimidines, and minerals in these processes. It is, however, difficult to find out what the actual components in favour of EPS biosynthesis are. Whereas the carbohydrate source essentially provides the necessary energy for both cell growth and EPS production, a fraction of the carbohydrate source is used for the biosynthesis of biomass and EPS precursors (Degeest and De Vuyst, 2000). Amino acids are not directly involved in EPS production but serve as carbon and nitrogen sources that are essential for growth. Because EPS production is growth-associated in *S. thermophilus* (De Vuyst et al., 1998) and because EPS production is an energy-demanding process (De Vuyst et al., 2001), either increased carbohydrate levels or increased amino acids uptake might favour EPS production. However, neither carbohydrates like α-D-glucose 1-phosphate or ribose, or amino acids like arginine or aspartic acid affect bacterial growth, EPS production, EPS monomer composition, or the activity of key enzymes (α-phosphoglucomutase, UDP-glucose-pyrophosphorylase, UDP-galactose 4-epimerase, and dTDP-glucose pyrophosphorylase) involved in sugar nucleotide biosynthesis (Fig. 1). If one speculates that a very low amount of α-D-glucose 1-phosphate was used, the decreased activities of the enzymes UDP-glucose-pyrophosphorylase and dTDP-glucose pyrophosphor-

ylase, and the slight increase of α-phosphoglucomutase activity may be explained as follows. α-Phosphoglucomutase, responsible for the interconversion of α-D-glucose 6-phosphate into α-D-glucose 1-phosphate and vice versa, might be more active to compensate for the increased level of α-D-glucose 1-phosphate by changing the equilibrium towards α-D-glucose-6-phosphate. This in turn might explain the slightly lowered activities of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase, acting in the direction of the formation of UDP-glucose and UDP-galactose from glucose 1-phosphate, respectively. The fact that no enzyme activity was observed for dTDP-glucose pyrophosphorylase in any of the samples is due to the lack of rhamnose in the EPS repeating unit (Grobben et al., 1996; Breedveld et al., 1998; Degeest and De Vuyst, 2000; Degeest et al., 2001a).

Another way of obtaining increased EPS yields, or of better maintaining high EPS yields, during the fermentation process is inhibiting EPS degradation. This is very important from an industrial point of view. Both temperature and pH shifts towards values that inactivate enzymes, at the harvesting time of the fermentation, are promising. This temperature and pH influence indicates that EPS degradation takes place enzymatically (De Vuyst et al., 1998; Pham et al., 2000). Remarkably, however, was the delayed degradation of the HMM-EPS at the end of the fermentation process when both temperature and pH values were restored. Besides a possible heat and pH stability of the enzymes involved, the late release of intracellular enzymes upon cell lysis could be responsible for this. As demonstrated in this study, enzymes of the glycohydrolase-type acting as endo-enzymes are present, in both cell extracts and cell-free culture supernatant.

The small differences observed for HMM-EPS concentrations during incubation with cell-free culture supernatant or cell extracts may be explained by stating that different glycohydrolases are present. Pham et al. (2000) observed activities of the enzymes α-glucosidase, β-glucosidase, α-D-galactosidase, β-D-galactosidase, β-glucuronidase, and α-L-rhamnosidase. They found the highest specific activity in cell-bound fractions. Furthermore, the purified glycohydrolases α-glucosidase and β-glucuronidase were shown to be rather stable in a wide temperature and pH range and possessed molecular masses of about 40,000. This can explain on one hand the degradation we observed in the samples with heat-treated cell-free culture supernatant and cell extracts (negative control), and on the other phenomenon of further degradation upon prolonged fermentation when the pH drop and temperature increase are restored.

To conclude, increasing EPS yields or changing EPS monomer composition by addition of specific carbohydrates or amino acids, possibly related to cell growth and/or EPS production, was not pronounced. It is, however, interesting that EPS production can be stabilised by applying a heating or acidification step. This indicates that EPS breakdown is caused by glycohydrolase-type enzymes. These enzymes probably display endo-activity, and are present in both cell extracts and cell-free culture supernatant. They posses a molecular mass lower than 50,000 and 10,000, and seem to be rather stable at high temperature and low pH. A further characterization of these glycohydrolases is necessary to develop methods to prevent EPS breakdown in industrial exploitation.

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