Process Characteristics of Exopolysaccharide Production by *Streptococcus thermophilus*

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SUMMARY: The kinetics of growth and exopolysaccharide (EPS) production from *Streptococcus thermophilus* were studied and optimized for different physical (temperature, pH, aeration rate) and chemical (carbon/nitrogen ratio) factors in milk medium. From these experiments, it was clear that EPS production displays primary metabolite kinetics. Using the optimized conditions, EPS production could also be established in de Man Rogosa Sharpe medium. Growth-associated EPS production, bacterial growth and other fermentation data were translated into a mathematical model.

Introduction

Exopolysaccharides (EPS) produced by mesophilic or thermophilic lactic acid bacteria (LAB) are of commercial interest for their potential application as 'natural' texturizers, viscosifiers and syneresis-lowering agents[1,2]. The amounts of EPS produced by LAB are however very low and their production is unstable. Therefore, production kinetics, molecular organisation and functional properties have to be studied and optimized[1,3].

Streptococcus thermophilus is able to produce EPS of the heteropolysaccharide type, but until now, only four EPS structures have been elucidated[3,4,5,6]. Furthermore, little information on the production kinetics of EPS by *S. thermophilus* is available and is sometimes

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contradictory[1,7,8,9,10,11]. Therefore, we studied and optimized the technological properties of EPS production through detailed fermentation analysis.

Experimental

Bacterial strain. The *Streptococcus thermophilus* LY03 strain used in this study was isolated from an industrial starter culture for yoghurt production and kindly provided by The University of Huddersfield, Huddersfield, England.

Media. Both de Man Rogosa Sharpe (MRS) medium[12] with lactose as a carbon source, and a milk medium consisting of skimmed milk powder (SMP) enriched with peptone and yeast extract and/or lactose (experiment dependent concentrations), were used for fermentation studies.

Isolation of exopolysaccharides. Isolation of EPS was performed in four steps when using milk medium[13]. First, proteins were removed from the fermentation liquor by precipitation with one volume of 20 % trichloroacetic acid (TCA), followed by centrifugation of cells and proteins. Second, EPS were precipitated overnight with an isovolume of acetone, followed by centrifugation. The EPS material was redissolved in ultrapure water. Third, residual proteinaceous material was precipitated with TCA and removed by centrifugation as described above. Fourth, the EPS were finally isolated by precipitation with acetone (one volume) and harvested by spinning on a rod. The second TCA and acetone precipitation steps of this procedure were omitted when using MRS medium.

For quantitative sampling during fermentation experiments, EPS were isolated as described above, dried and weighed thereafter giving the amount of EPS in g polysaccharide dry mass (PDM)/L.

Fermentation experiments. All fermentations were carried out in an *in situ* sterilizable 15 L Biostat® C fermentor (B. Braun Biotech International, Melsungen, Germany) with a working volume of 12 L, operating at 42° C (unless otherwise indicated). The pH was controlled at 6.2 (or the pH indicated) on-line by automatic addition of 10 N NaOH. When studying the influence of the oxygen tension, aeration with sterile air (aerobic conditions) or nitrogen (anaerobic conditions) was provided; otherwise microaerophilic conditions (no aeration) were applied.

Inocula for all experiments were prepared in two steps. First, 9.5 mL MRS broth was inoculated with 500 µL of a freshly prepared *S. thermophilus* LY03 culture. After 12 h of

incubation (tube) at 42° C, this culture was used to inoculate an Erlenmeyer flask containing 100 mL modified MRS broth (identical to the medium used in the fermentation experiment afterwards). After 12 h of growth at 42° C, this second preculture was used to inoculate the fermenter (12 L).

Samples were aseptically withdrawn from the fermentation vessel to measure growth (as cell dry mass (CDM) determinations), exopolysaccharide yield (determined on 0.5 L samples) and residual lactose (S), residual galactose (Gal) and lactic acid (LA) concentrations. Agitation rate, pH, oxygen tension and base supply were monitored on-line (MicroMFCS for Windows™NT software, B. Braun Biotech International, Melsungen, Germany). The maximal specific growth rate (μ_{max}, 1/h) was calculated from the maximal slope when plotting logarithmic cell dry mass values in function of time. Residual sugar levels and lactic acid were determined by high pressure liquid chromatography (HPLC) using a Waters chromatograph (Waters Corp., Milford, MA, USA) equipped with a Polyspher® OA KC column (Merck, Darmstadt, Germany) kept at 35°C. As a mobile phase, 0.005 N H_2SO_4 was used at a fixed flow rate of 0.4 mL/min.

Modelling. All model simulations were performed using Euler integration on an IBM compatible Pentium PC. All modelled parameters were calculated using the 'least squares' method, for which the difference between modelled and experimental values is reduced to a minimum.

Results and Discussion

Optimisation of physical process conditions for EPS production with *Streptococcus thermophilus* LY03

The physical process conditions for EPS production with *S. thermophilus* LY03 were optimised in milk medium enriched with peptone and yeast extract.

Temperature. Fermentations were carried out at different temperatures, ranging from 25 to 55°C (Fig. 1). It is clearly shown that no growth took place at 25 and 55°C and that maximal growth as well as maximal EPS production was observed at 42°C. The correlation between maximal growth and maximal EPS production is a first indication of the growth-associated kinetics of EPS production.

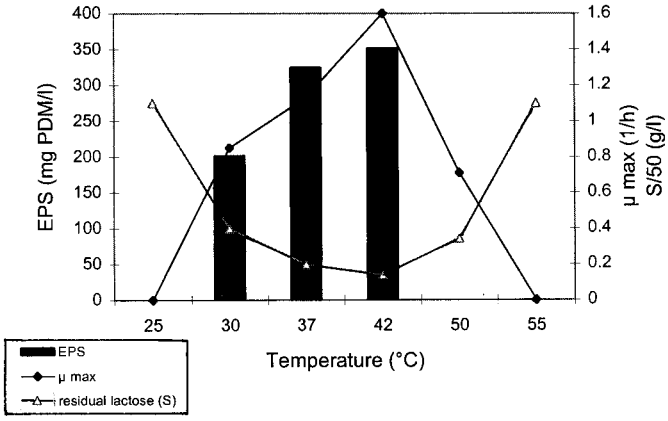

Fig. 1: Influence of the temperature on *Streptococcus thermophilus* LY03 growth and exopolysaccharide (EPS) production; pH 5.5; 10 % SMP + 1 % peptone + 0.5 % yeast extract; PDM: polysaccharide dry mass; μ_{max}: maximal specific growth rate.

pH. It was further shown that EPS production without pH control is very low[13]; therefore a comparison of growth and EPS production of *S. thermophilus* LY03 during fermentations with different constant pH values was made (Fig. 2). Again, best growth conditions - the ideal pH was 6.2 - correlated with highest EPS production.

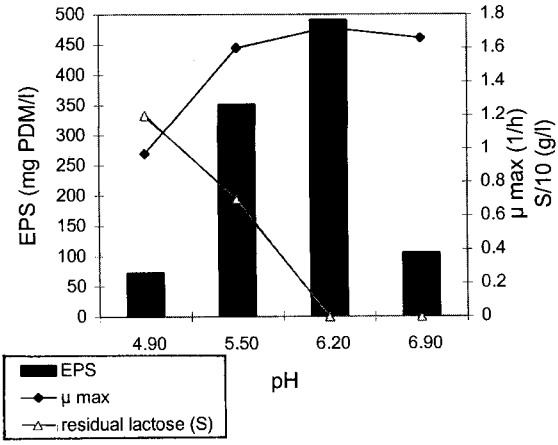

Fig. 2: Influence of a constant pH on *Streptococcus thermophilus* LY03 growth and EPS production; T = 42°C; 10 % SMP + 1 % peptone + 0.5 % yeast extract. Legend as in Fig. 1.

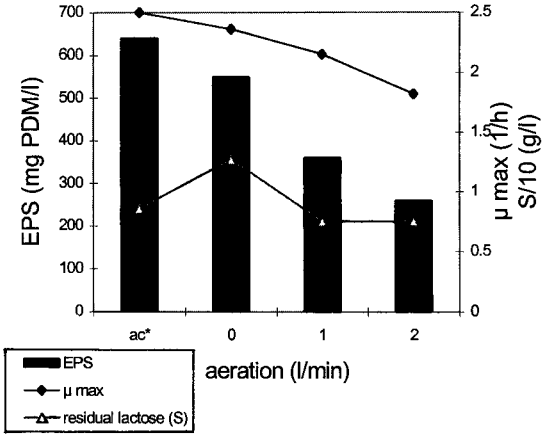

Fig. 3: Influence of oxygen tension on *Streptococcus thermophilus* LY03 growth and EPS production (ac* anaerobic conditions); T = 42°C; pH = 6.2; 10 % SMP + 2 % peptone + 1 % yeast extract + 4.4 % extra lactose. Legend as in Fig. 1.

Oxygen tension. Application of increasing aeration rates confirmed the above described findings. Biopolymer production and growth decreased with increasing aeration rates (Fig. 3). This can be easily explained for growth, since *S. thermophilus* is a microaerophilic microorganism. EPS production, however, contrasts with literature data[1], where higher yields are predicted in 'stress' conditions.

Primary metabolite kinetics. The above described results can be summarized by stating that EPS production by *S. thermophilus* LY03 is growth-associated (primary metabolite kinetics, cf. Fig. 6), a property that seems characteristic for thermophilic lactic acid bacteria. This behavior does not accord with some literature data[10]. However, similar findings were reported by others[14,15]. By contrast, mesophilic lactic acid bacteria seem to produce higher amounts of EPS at rather low temperatures[10,16].

Optimisation of chemical process conditions for EPS production with *Streptococcus thermophilus* LY03

C/N ratio. Using the above described optimized physical process conditions, fermentations were carried out in milk medium, applying different C/N ratios by varying the concentration

of lactose (C) and complex nitrogen (N) (Fig. 4). One optimal C/N ratio seemed to be most efficient for both growth and EPS production. Both carbon and nitrogen source needed to be increased until a certain level, in view of obtaining higher EPS production levels. Increasing only the lactose or only the nitrogen content was not sufficient to obtain higher amounts of polysaccharide. To obtain maximal EPS production one has to take into account a well defined C/N ratio and a maximal C/N content.

Using these optimised process conditions, a 10-fold increase of EPS yield was obtained as compared with the production under non-optimised process conditions[13].

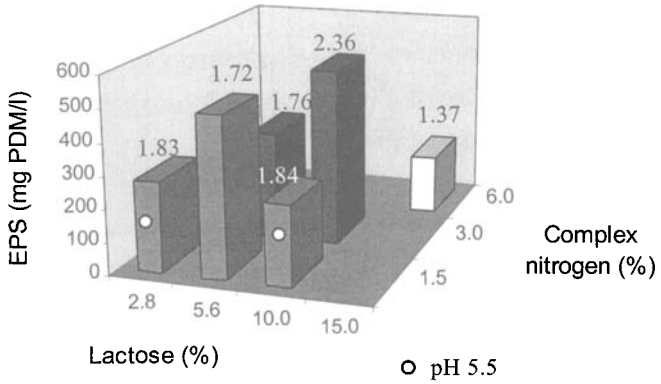

Fig. 4: Influence of carbon and nitrogen source levels on *Streptococcus thermophilus* LY03 fermentations in milk medium containing varying concentrations of lactose and complex nitrogen (T = 42°C; pH = 6.2); complex nitrogen always consisted of a 2:1 ratio of peptone:yeast extract; μ_{max} is indicated above each bar. EPS: exopolysaccharide, PDM: polysaccharide dry mass.

EPS production in MRS. MRS medium (in which no EPS production took place under non-optimised conditions[13]) was used for different fermentations to demonstrate again that C/N ratio plays an important role, and to show that less-complex media can be used for EPS production when using the right process conditions (Fig. 5). Again, maximal EPS production occurred at one optimal C/N ratio.

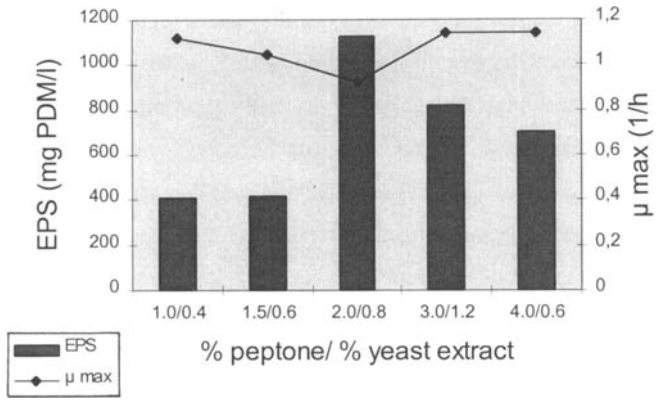

Fig. 5: Influence of nitrogen source levels on *S. thermophilus* LY03 growth and EPS production in MRS medium (T = 42°C, pH = 6.2, initial lactose concentration = 7.5 %). Legend as in Fig. 1.

Modelling

All data mentioned above were used to set up a basic model for describing the growth and EPS production kinetics of *S. thermophilus* LY03. The model is based on the following equations:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \qquad (1)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - m_S X \qquad (2)$$

$$\frac{dGal}{dt} = -\frac{1}{Y_{S/Gal}}\frac{dS}{dt} \qquad (3)$$

$$\frac{dLA}{dt} = -\frac{1}{Y_{S/LA}}\frac{dS}{dt} \qquad (4)$$

$$\frac{dEPS}{dt} = k_f\frac{dX}{dt} \qquad \text{if } X < X' \qquad (5)$$

$$\frac{dEPS}{dt} = -k_d(EPS - EPS_r) \qquad \text{if } X > X' \text{ or } S = 0 \qquad (6)$$

where equation (1) is the dynamic growth equation, which upon integration yields the logistic curve (X is the cell dry mass concentration, g/l; μ_{max} is the maximal specific growth rate, 1/h; X_{max} is the maximal attainable cell dry mass concentration, g/l). Equation (2) expresses lactose consumption by the maintenance energy model (S is the lactose concentration, g/l; $Y_{X/S}$ is the cell yield coefficient, g cell dry mass/(g lactose); m_s is the maintenance coefficient, g lactose/(g cell dry mass)/h). Equations (3) and (4) describe galactose (Gal) excretion into the medium and lactic acid (LA) formation, respectively (both yield coefficients in g/g). Equation (5) is the mathematical translation of the growth-associated EPS production with a specific EPS formation constant k_f (g/g) and equation (6) represents the constant degradation of EPS after the cell dry mass reached a certain value X'. The specific degradation rate is given by k_d (1/h); EPS_r is the fraction of EPS which remains unaffected in the medium.

This model is able to describe the growth and EPS production quite well (Fig. 6). Biokinetic parameters can be calculated from it: $\mu_{max} = 1.60\ h^{-1}$, $Y_{X/S} = 0.095$ g CDM.(g S)$^{-1}$, $m_s = 0.16$ g S. (g CDM)$^{-1}h^{-1}$, $k_f = 195$ mg PDM.(g CDM)$^{-1}$, $k_d = 0.60\ h^{-1}$, $X_{max} = 4.90$ g CDM.l^{-1}, X' = 4.65 g CDM.l^{-1}, $EPS_{max} = 905$ mg PDM.l^{-1}, $EPS_r = 700$ mg PDM.l^{-1}. Furthermore, it will be possible by extending this model to predict bacterial growth and EPS production behaviour in different media, which is useful for further optimization of the production through better feeding strategies (e.g. fed-batch).

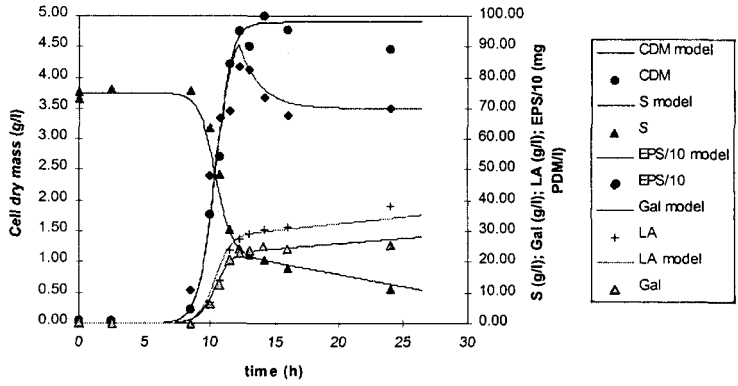

Fig. 6: Comparison of experimental data and modelled values from a *S. thermophilus* LY03 fermentation carried out in MRS medium (4 % peptone; 1.6 % yeast extract; 7.5 % lactose) at pH 6.2 and T = 42°C. S: substrate concentration (lactose), Gal: galactose, LA: lactic acid, EPS: exopolysaccharide, PDM: polymer dry mass.

Conclusions

Process characteristics for growth and EPS production of *Streptococcus thermophilus* LY03 were studied and optimized. Physical factors (temperature, pH, oxygen tension) did influence the specific growth rate and the EPS production significantly. Bacterial growth and EPS production seemed to be correlated, and yielded primary metabolite kinetics. Nutritional factors were studied and from these fermentations, it was concluded that, to obtain higher EPS levels, both carbon and nitrogen concentrations must be increased simultaneously, relative to a well defined C/N ratio. Using these optimized process conditions, EPS production also took place in MRS medium. Finally, these results were mathematically 'translated' into a basic model, which fits the experimental data.

Aknowledgements

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