Influence of ions on growth and production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772

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Several lactic acid bacteria produce exopolysaccharides (EPS), either attached to the cell wall or excreted into the environment as slime material. EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) and *Streptococcus thermophilus* play an important role in improving the texture and stability of yogurt and preventing syneresis (Cerning, 1990; Nakajima *et al.* 1990). The amount and composition of the EPS produced by lactic acid bacteria are dependent on a number of factors, such as temperature, initial pH, carbon source and the availability of minerals, vitamins and other medium components.

In previous work it was shown that the production and sugar composition of the EPS from *Lb. bulgaricus* NCFB 2772 are affected by the carbohydrate source (Grobben *et al.* 1995, 1996). In a simplified defined medium, from which several vitamins and trace elements were omitted, EPS production by *Lb. bulgaricus* significantly increased, although growth of the strain was reduced (Grobben *et al.* 1998).

EPS may contribute to the uptake of nutrients and metal ions when these are present in very low concentrations (Cerning, 1990), and it has been suggested that limiting amounts of nutrients in the growth medium in combination with a carbon excess may stimulate the formation of EPS (Sutherland, 1972). On the other hand, some ions may be essential to the functions of enzymes involved in EPS production. However, most investigations on the role of ions in the production of EPS have revealed that increasing ion concentrations stimulate the formation of EPS. Mozzi *et al.* (1995*a*) found that growth and EPS production by *Lb. casei* grown in APTgl broth was stimulated by the addition of $MnSO_4$ or triammonium citrate, but the addition of Mg^{2+} or K^+ only stimulated growth and not the specific production of EPS. With *Lactobacillus* sp. KPB-167B, the addition of 5 mM-$CaCl_2$ to MRSL medium resulted in an enhancement of the polysaccharide kefiran (Yokoi & Watanabe, 1992). Racine *et al.* (1991) found that increasing phosphate levels stimulated EPS production by *Propionibacterium acidi-propionici*. In the present study, we have investigated the

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influence of omitting or adding low concentrations of a number of ions on the growth and EPS production by *Lb. bulgaricus* NCFB 2772 grown in batch culture on defined medium.

materials and methods

Microorganism and growth experiments

Lb. bulgaricus NCFB 2772 was obtained from the National Collections of Industrial and Marine Bacteria (Institute of Food Research, Reading RG6 6BZ, UK). The strain was stored at −80 °C in MRS broth medium containing 150 ml glycerol/l and reactivated in MRS broth medium (De Man *et al.* 1960) at 37 °C for 16 h.

Growth experiments were conducted at 37 °C and initial pH 6·0 with 83 mM-glucose as the carbohydrate source in static, nitrogen-flushed, sealed 115 ml glass bottles containing 50 ml defined medium whose composition was given by Grobben *et al.* (1995, 1998). The medium was sterilized by passing it through a 0·2 μm sterile filter. Growth was monitored after 48 h incubation by measuring the absorption at 600 nm or as cell dry weight from a standard curve of A_{600} *v.* cell dry weight (Grobben *et al.* 1995). Sugars and lactic acid were determined by HPLC as described by Grobben *et al.* (1995). To evaluate the requirements for nutrients, the single omission technique was used, and growth experiments were performed as described previously (Grobben *et al.* 1998). All experiments were performed at least in duplicate.

Batch culture experiments at controlled pH were performed in a glass fermenter (Applikon, NL-3100 AC Schiedam, The Netherlands) with a working volume of 600 ml. The pH of the cultures was maintained at 6·0 ± 0·1 by titration with NaOH, the growth temperature was 40 °C and the agitation rate 100 rev./min. The cultures were kept under anaerobic conditions by flushing the fermenter with nitrogen gas. Glucose was added as the carbohydrate source at 83 mM.

Isolation and sugar composition of the exopolysaccharides

Trichloroacetic acid was added to cultures to a final concentration of 136 g/l and the mixtures were centrifuged (27000 ***g***, 4 °C, 20 min). The EPS were isolated by precipitation with three volumes of cold ethanol (Grobben *et al.* 1995). The total carbohydrate content of the EPS was determined using the phenol–sulphuric acid procedure of Dubois *et al.* (1956). To determine the monomeric sugar composition, the EPS were hydrolysed at 121 °C for 1 h in 1 M-trifluoroacetic acid, dried under a stream of N_2 gas at 45 °C and resuspended in distilled water. The solution containing the hydrolysates was analysed by HPLC (Grobben *et al.* 1995).

results and discussion

The effect of decreased Mn^{2+} concentrations on growth and EPS production by *Lb. bulgaricus* indicated that Mn^{2+} was essential for growth of the strain. Table 1 shows that at very low Mn^{2+} concentrations there was a lower specific EPS production. At Mn^{2+} concentrations $\geqslant$ 2·5 μM, the specific EPS production did not differ from the value found under standard conditions (252 μM-$MnCl_2$), and further increases in Mn^{2+} concentration (up to 500 μM) had no effect. Compared with the EPS produced under standard conditions, only that produced at 2·5 μM-Mn^{2+} contained a lower amount of rhamnose monomer. The results were not in agreement with those obtained with *Lb. casei* CRL 87, in which the addition of Mn^{2+}, alone or in combination with citrate, Ca^{2+} or SO_4^{2-}, strongly stimulated EPS production (Mozzi

Table 1. *Effect of initial $MnCl_2$ concentration on growth and exopolysaccharide production by* Lactobacillus delbrueckii *subsp.* bulgaricus†

(Values are means for three independent experiments)

$MnCl_2$ concn, μM	A_{600}	Exopolysaccharide concn, mg/l	Specific exopolysaccharide production, mg/mg cell dry weight‡	Exopolysaccharide composition Glc:Gal:Rha§
0·5	0·1±0·0	ND	ND	ND
1·5	0·5±0·0	5·8±0·3	15·5±0·6	ND
2·5	1·2±0·1	24·2±2·3	26·9±0·9	1·0:5·4±0·1:0·3±0·0
5·0	1·3±0·1	25·0±2·3	25·6±1·5	1·0:5·9±0·3:0·6±0·0
10·0	1·2±0·0	28·2±1·7	31·3±1·2	1·0:5·9±0·4:0·6±0·1
30·0	1·5±0·0	30·5±3·0	27·1±1·9	1·0:6·3±0·2:0·7±0·1
40·0	1·4±0·1	31·0±3·1	29·5±2·0	1·0:6·2±0·4:0·6±0·0
90·0	1·7±0·2	34·8±2·8	27·3±1·0	1·0:6·8±0·6:0·7±0·0
252·0	1·9±0·2	42·3±3·3	29·7±1·2	1·0:6·2±0·3:0·7±0·1

ND, not determined.

† *Lb. bulgaricus* NCFB 2772 was grown in batch cultures in a defined medium at 37 °C and initial pH 6·0.

‡ Specific exopolysaccharide production was calculated using a standard curve of A_{600} against cell dry weight (Grobben *et al.* 1995).

§ Amounts of galactose and rhamnose were related to the amount of glucose. Glc, glucose; Gal, galactose; Rha, rhamnose.

et al. 1995*a*, *b*). *Rhizobium meliloti* JJ-1 produced four times as much EPS in media containing 500 μM-Mn^{2+} compared with media without added Mn^{2+} (Appanna, 1988).

The influence of decreased concentrations of phosphate on the growth and EPS production of *Lb. bulgaricus* was investigated in pH-controlled batch cultures. Since the reduction in phosphate concentration might have caused a rapid fall in pH under acidifying culture conditions, we chose to perform this experiment under controlled pH. PO_4^{3-} ions were replaced by Cl^- ions in order to balance Na^+ and K^+ concentrations. The addition of Cl^- ions up to 100 mM had no effect on the growth and EPS production by the strain (results not shown). It was found that at concentrations $< 2{\cdot}0$ mM phosphate became growth limiting (Table 2). The specific EPS production was lower at these phosphate concentrations. With increasing phosphate concentrations, growth and specific EPS production were comparable to those under standard growth conditions (21·5 mM-phosphate) for the strain. The EPS produced at low phosphate concentrations had a lower amount of rhamnose (Table 2). It is already known that growth of lactic acid bacteria is possible in the presence of phosphate (MacLeod & Snell, 1947). With *Propionibacterium acidi-propionici*, EPS production increased in the presence of phosphate (Racine *et al.* 1991). *Pseudomonas* sp. NCIB 11264 produced less EPS in media containing small amounts of phosphate, but this was mainly caused by the low buffering capacity of the growth medium. In a buffered medium, the amount of EPS is only slightly affected by the phosphate concentration (Williams & Wimpenny, 1977). Previously, no effect of phosphate on EPS production by lactic acid bacteria has been reported.

We found that the omission of Fe^{2+}, Zn^{2+} or NH_4^+ ions had no effect on the growth of *Lb. bulgaricus*, and EPS production and sugar composition were not affected by variations in the concentrations of these components. Conflicting results have been reported for the iron requirements of lactic acid bacteria. Pandey *et al.* (1994) investigated 23 strains of lactic acid bacteria, including several lactobacilli, and stated that none of these strains needs iron for growth. In addition, *Lb. plantarum* grows well in the absence of iron (Archibald, 1983). On the other hand, the *Lactobacillus* strains investigated by Ledesma *et al.* (1977) all require Fe^{2+}.

Table 2. *Effect of initial phosphate concentration on growth and exopolysaccharide production by* Lactobacillus delbrueckii *subsp.* bulgaricus†

(Values are means for three independent experiments)

Phosphate concn, mM	A_{600}	Exopolysaccharide concn, mg/l	Specific exopolysaccharide production, mg/mg cell dry weight‡	Exopolysaccharide composition Glc:Gal:Rha§
0·0	0·04±0·0	ND	ND	ND
1·0	0·7±0·1	19·2±2·1	36·6±0·3	1·0:5·9±0·2:0·3±0·0
2·0	1·8±0·1	78·8±3·3	58·3±6·1	1·0:6·8±0·4:0·5±0·0
5·0	2·4±0·2	109·7±4·0	60·9±6·9	1·0:6·4±0·4:0·7±0·1
10·0	2·2±0·1	98·9±4·7	59·9±1·9	1·0:6·6±0·4:0·6±0·0
21·5	2·5±0·2	110·2±3·3	58·7±2·9	1·0:6·8±0·2:0·7±0·1

ND, not determined.

† *Lb. bulgaricus* NCFB 2772 was grown in batch cultures in a defined medium at 37 °C and initial pH 6·0.

‡ Specific exopolysaccharide production was calculated using a standard curve of A_{600} against cell dry weight (Grobben *et al.* 1995).

§ Amounts of galactose and rhamnose were related to the amount of glucose. Glc, glucose; Gal, galactose; Rha, rhamnose.

Furthermore, we showed that an increased citrate concentration inhibited growth of *Lb. bulgaricus*. When 100 mM-citrate was present, the final A_{600} of the culture was only 0·9. Neither specific EPS production nor sugar composition of the EPS was affected by a high citrate concentration. In previous work, we found that completely omitting citrate from the growth medium resulted in lower growth and EPS production (Grobben *et al.* 1998). The inhibitory effect of high concentrations of citrate on the growth of lactic acid bacteria was explained by the fact that citrate ions form complexes with bivalent metallic ions such as Mg^{2+} and Mn^{2+}, and these complexes have been shown to be unavailable for growth. The addition of higher concentrations of Mg^{2+} and Mn^{2+} can overcome the toxic effects of citrate (MacLeod & Snell, 1947). In contrast to the case with *Lb. casei*, Mozzi *et al.* (1995*a*) found no stimulatory effect of citrate addition on EPS production.

The addition of nutrients such as phosphate or manganese in growth-limiting amounts influenced the production of EPS only at very low concentrations. At these concentrations, specific EPS production decreased and the EPS contained lower amounts of rhamnose. In previous work it was found that at low cell densities, for instance in the early logarithmic growth phase, the EPS contained a lower amount of rhamnose relative to the amount of glucose monomers (Grobben *et al.* 1995). Further research on the amounts and compositions of the EPS produced by *Lb. bulgaricus* grown at low cell densities will be needed to clarify these findings.

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