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SHORT CONTRIBUTION

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Effect of temperature and pH on growth and product formation of *Lactococcus lactis* ssp. *lactis* ATCC 19435 growing on maltose

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Abstract *Lactococcus lactis* ssp. *lactis* ATCC 19435 is known to produce mixed acids when grown on maltose. A change in fermentation conditions only, elevated temperatures (up to 37 °C) and reduced pH values (down to 5.0) resulted in a shift towards homolactic product formation. This was accompanied by decreased growth rate and cell yield. The results are discussed in terms of redox balance and maintenance, and the regulation of lactate dehydrogenase and pyruvate formate-lyase.

Introduction

When lactic acid was produced from wheat flour hydrolysate by *Lactococcus lactis* ssp. *lactis* ATCC 19435 (*L. lactis* 19435), the product formation rate and the product pattern were found to change with temperature and pH (Åkerberg et al. 1998). Maximum lactic acid formation has been observed at 37 °C for *Lactobacillus casei* (Hujanen and Linko 1996) and at 33.5 °C for *L. lactis* 19435 (Åkerberg et al. 1998). The optimal pH for growth and product formation has been established to be around 6 for both lactococci and lactobacilli (Parente et al. 1994; Wijtzes et al. 1995; Yoo et al. 1996; Åkerberg et al. 1998). By-product formation decreases with decreasing pH (de Ley 1962; Snoep et al. 1990; Nannen and Hutkins 1991; Yoo et al. 1996) and was lowest at pH 5 and 30 °C for *L. lactis* 19435 (Åkerberg et al. 1998).

L. lactis 19435 is known to form a mixed-acid product on maltose (Lohmeier-Vogel et al. 1986; Qian et al. 1994; Sjöberg et al. 1995; Åkerberg et al. 1998). In the present study, anaerobic fermentations in a semi-defined medium at elevated temperatures (30–37 °C) and reduced pH values (5.0–6.5) showed that the formation of mixed acids was redirected towards homolactic fermentation.

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Materials and methods

Microorganism and inoculum preparation

Lactococcus lactis ssp. *lactis* ATCC 19435 (American Type Culture Collection, Rockville, Md., USA), stored at −80 °C, was plated on M17 agar and incubated for 24 h at 30 °C. A single colony was transferred to a new plate and incubated in the same way. Colonies from this plate were used to inoculate a 5-ml pre-inoculum, which was incubated in an orbital incubator (Gallenkamp INR-200, Leicester, UK) for 4–10 h at 140 rpm, 30 °C. The cells were subsequently harvested by centrifugation (8000 *g*, 2 min, 4 °C), resuspended in 1.5 ml NaCl 9 g/l, transferred to a 30-ml inoculum and incubated in the same way for another 11–18 h. The bacteria were harvested by centrifugation (6400 *g*, 10 min, 4 °C; Beckman J2-21, Beckman Instruments Inc., Fullerton, Calif., USA) and resuspended in 22 ml NaCl 9 g/l. This suspension was used to inoculate the fermentor.

Media composition

For the propagation of the pre-inocula, a rich medium was used that contained, (l^{-1}) 5 g yeast extract (Merck, Darmstadt, Germany), 1 g casamino acids (Difco, Detroit, Midn., USA), 2.5 g K_2HPO_4, 2.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 10 g maltose.

The inocula and cultures were grown on a semi-defined medium containing (l^{-1}): casamino acids 10 g, K_2HPO_4 2.5 g, KH_2PO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, yeast nitrogen base (without amino acids, Difco) 5 g, asparagine 0.4 g, reduced glutathione 10 mg, uracil 60 mg, adenine 30 mg, guanine 30 mg, vitamin solution 10 ml, trace element solution 1 ml and maltose 5 g. The vitamin solution consisted of the following components (mg/l): D-biotin 10, pyridoxal-HCl 206, folic acid 100, riboflavin 100, niacinamide 100, thiamine-HCl 100, Calcium D-panthotenate 95, *p*-aminobenzoic acid 10. The trace element solution contained (g/l): Ca_2EDTA 15, $ZnSO_4 \cdot 7H_2O$ 4.5, $MnCl_2 \cdot 2H_2O$ 1, $CoCl_2 \cdot 6H_2O$ 0.3, $CuSO_4 \cdot 5H_2O$ 0.3, $Na_2MoO_4 \cdot H_2O$ 0.4, $CaCl_2 \cdot 2H_2O$ 4.5, $FeSO_4 \cdot 7H_2O$ 3, H_3BO_3 1 and KI 0.1. The solutions of vitamins, trace elements, nucleic acid bases, yeast nitrogen base, asparagine and reduced glutathione were filter-sterilised (0.2 μm) and added to the media aseptically. The other components were autoclaved separately.

Fermentor set-up

Batch cultures were carried out in a computer-controlled 0.6-l glass vessel (SARA, Belach Bioteknik AB, Stockholm, Sweden) (Hofvendahl et al. 1996). The working volume was 0.5 l; temperatures, 30, 35 and 37 °C; pH values, 5.0, 5.75 and 6.5 controlled with 250 g/l NaOH; agitation, 120 rpm; and N_2 flow over the culture, 0.1 l/min. The gas flow rate was controlled by a mass flow controller (Bronkhorst Hi-Tec, Ruurlo, The Netherlands) and the exhaust gas was passed through a condenser cooled by tap water. The initial pH was adjusted with 20% H_2SO_4.

Sampling and analysis

Samples were withdrawn every 30 min and the concentrations of maltose, glucose, lactic acid, acetic acid, formic acid and ethanol in undiluted samples were measured by HPLC, as described previously (Åkerberg et al. 1998). Growth was followed by measuring the absorbance at 620 nm in a Schimadzu UV-120-02 spectrophotometer (Kyoto, Japan). The concentration of cell mass (c), expressed as dry weight per litre, was determined in selected samples (Åkerberg et al. 1998). The relations between c (g/l) and absorbance (at $A > 0.5$) was determined to be: $c = 0.47 \times A - 0.2$. Most fermentation experiments were carried out in duplicate, and the mean values are reported. The error was less than 10% for growth, sugar consumption and product formation.

Results and discussion

Lactococcus lactis ssp. *lactis* ATCC 19435 was grown anaerobically in batch culture on maltose and the temperature and pH were varied one at a time. Under standard conditions (pH 6.5 and 30 °C) all the maltose was consumed within 8 h and converted to lactic acid, formic acid, acetic acid and ethanol (Fig. 1A). When the temperature was increased to 37 °C (Fig. 1B) or the pH reduced to 5.0 (data not shown) the fermentation pattern was similar, but resulted in a slightly lower cell mass and less by-product formation.

The maximum specific growth rate, the ratio of lactic acid to the total amount of products and the cell yield per mole of substrate consumed were calculated when all the maltose had been consumed at various temperatures and pH values (Table 1).

The flux to lactic acid increased at elevated temperatures and reduced pH values at the expense of cell mass and by-product formation. When the temperature was increased from 30 °C to 37 °C at pH 6.5, the ratio of lactic acid to the total amount of products increased by 23%. Simultaneously, the cell yield decreased by 34%. The growth rate was almost constant, with a slight maximum (10% higher) at 35 °C. A decrease in pH from 6.5 to 5.0 at 30 °C resulted in an increase in the ratio of lactic acid over the total amount of products by 65% and a decrease in the cell yield by 56%. The highest value of the growth rate was at pH 5.75. Growth and product formation in glucose-grown cells followed the same pattern at reduced pH and increased temperature as did maltose-grown cells (Hofvendahl 1998). However, the amount of the by-products formic acid, acetic acid and ethanol was smaller during fermentation of glucose.

All the parameters studied: product formation, cell yield and growth rate, showed greater variation with pH than with temperature. The growth-inhibiting effect seen at low pH was coupled to the production of lactic acid.

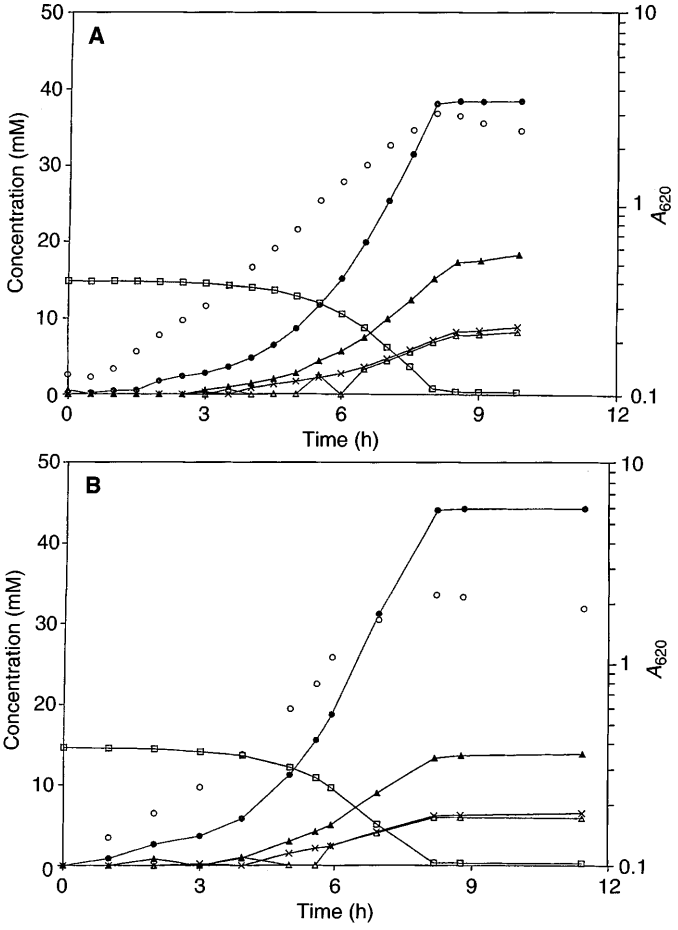

Fig. 1A, B *Lactococcus lactis* 19435 fermenting maltose at 30 °C (**A**) and 37 °C (**B**), pH 6.5. A_{620} (O) and concentrations of maltose (□), lactic acid (●), formic acid (▲), acetic acid (×) and ethanol (△)

Table 1 Growth rate, product formation and cell yield when all maltose was consumed at various temperatures and pH values

Temperature °C	pH	Maximum specific growth rate (h^{-1})	Lactic acid/total amount of products (mol/mol)	Cell yield (g/mole maltose)
30	5.0	0.35	0.86	39
30	5.75	0.62	0.68	57
30	**6.5**	**0.50**	**0.52**	**88**
35	6.5	0.56	0.55	65
37	6.5	0.51	0.64	58

Lactococci have been found to withstand extracellular pH values down to 5.7 (Kashket 1987) or 5.0 (Nannen and Hutkins 1991). Under these conditions, both the intracellular accumulation of the lactate anion (Russel 1992) and the uncoupling of ATP synthesis (Kashket 1987) have been claimed to inhibit growth.

The molar ratio of formic acid produced to acetic acid plus ethanol was 1.0–1.1 in all fermentations (data not shown), indicating that pyruvate formate-lyase converts pyruvate not converted by lactate dehydrogenase (Thomas et al. 1979; Snoep et al. 1990). It has been reported that the by-product formation is pH-dependent such that more by-products, i.e. more acid equivalents, are produced at higher pH (de Ley 1962; Nannen and Hutkins 1991; Yoo et al. 1996). Pyruvate formate-lyase is inactivated at low pH, resulting in reduced formic acid and acetyl-CoA and increased lactic acid production (Takahashi et al. 1982). This has been suggested to be due to the sensitivity of the enzyme to high redox potentials (Lindmark et al. 1969), observed both at decreased pH and at increased temperatures (Jacob 1970).

Also lactate dehydrogenase is pH-sensitive, so that the activation mediated by fructose 1,6-bisphosphate is reduced at increased pH (Wittenberger and Angelo 1970), with less lactic acid being formed. The NADH/NAD^+ ratio has also been reported to influence the lactate dehydrogenase activity, so that an increased ratio inhibits the enzyme (Garrigues et al. 1997). At low pH this ratio decreases (Snoep et al. 1991), which is in concordance with the observations in the present study.

The shift towards a more homolactic product pattern was accompanied by a reduction in growth rate and cell yield. These effects could be related, since the production of acetate in the mixed-acid product results in a higher ATP yield than the homolactic one. The increased amount of ATP could increase the cell yield. On the other hand, the effects might also be results of separate events. A decreased cell yield at increased temperatures and reduced pH has been ascribed to an increased maintenance requirement (Harrison and Loveless 1971; Topiwala and Sinclair 1971; Mainzer and Hempfling 1976; Wijtzes et al. 1995). Examples of maintenance processes are turnover of cell material, maintenance of concentration and electrochemical gradients, motility and futile cycles (Pirt 1975). The maintenance coefficient was elucidated (data not shown) and indicated a non-constant value, i.e. that the maintenance increased at elevated temperatures and reduced pH values.

The change of the flux from cell mass and by-product formation to lactic acid and maintenance observed at elevated temperatures and reduced pH values could be used to increase the yield in an industrial process. The only drawback is then the reduced growth rate, which translates into reduced volumetric productivity.

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