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Mixed-acid fermentation and polysaccharide production by *Lactobacillus helveticus* in milk cultures

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Abstract

Lactobacillus helveticus grown in milk with pH control at 6.2 had a slower growth rate ($\mu = 0.27\ h^{-1}$) and produced less exopolysaccharide (49 mg l^{-1}) but increased lactic acid production (425 mM) compared to cultures without pH control ($\mu = 0.5\ h^{-1}$, 380 mg exopolysaccharide l^{-1}, and 210 mM lactate), respectively. Both cultures displayed a mixed-acid fermentation with formation of acetate, which is linked not only to citrate metabolism, but also to alternative pathways from pyruvate.

Introduction

Lactobacillus helveticus, an obligated homofermentative lactic acid bacteria (LAB), plays an important role in the dairy industry as starter culture for the manufacture of acid milks, hard-cheeses and Mozzarella-like cheeses. The incorporation of exopolysaccharide (EPS)-producing strains into the starter culture would avoid the use of additives of plant or animal origin which are not allowed in most European Union countries (Gibson & Roberfroid 1995), resulting in the development of novel products with enhanced rheological properties.

The metabolism of citrate in milk to produce acetate and/or diacetyl is also important for the flavour development of many fermented dairy products. However, there are few reports concerning the metabolism of citrate by thermophilic lactobacilli (Hickey *et al.* 1983).

This paper deals with the fermentation pattern of and the EPS production by *L. helveticus* growing in milk as free and pH-controlled fermentation runs.

Materials and methods

Microorganism and growth conditions

Lactobacillus helveticus ATCC 15807 was grown in sterile (115 °C, 20 min), 10% (w/v) reconstituted non-fat skim milk, in a fermenter, working volume of 2000 ml, at 37 °C for 60 h. Batch cultures were carried out without aeration, as free- and controlled-pH (adjusted at pH 6.2 with sterile 1 M NH_4OH) fermentation runs. Temperature (37 °C), agitation (100 rev min^{-1}) and pH were controlled automatically.

Fermentations were allowed to proceed for 60 h; samples were aseptically withdrawn at different intervals from the fermentation vessel and immediately cooled on ice to determine EPS yield, cell viability, end-products, citrate consumption, residual lactose and its hydrolysis products (glucose and galactose). The specific growth rate (μ_{max}) was calculated from the slope of a semi logarithmic plot of c.f.u. ml^{-1} vs. time. Cell viability was determined by plating in mass appropriated dilutions in MRS agar (De Man *et al.*

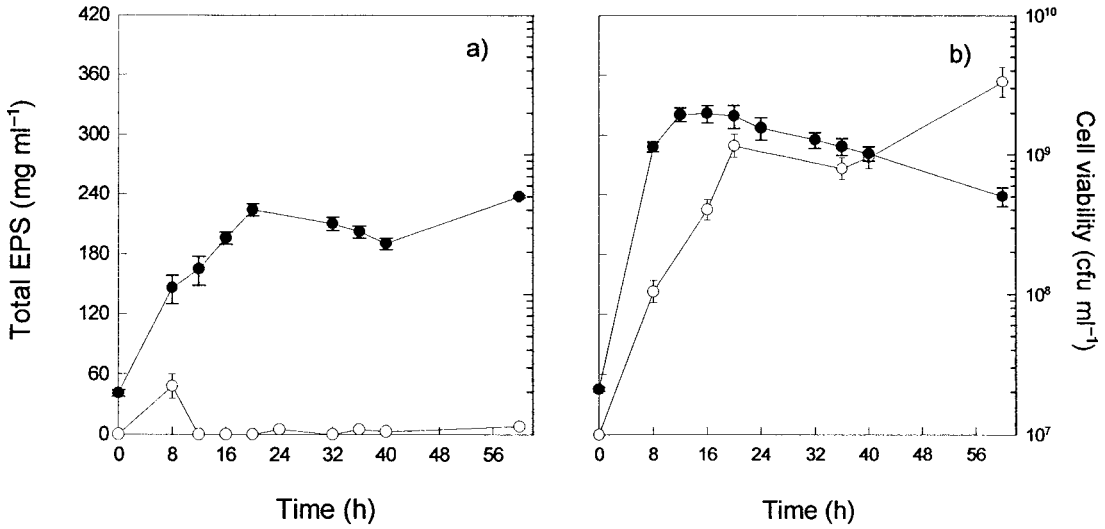

Fig. 1. Cell viability (–●–) and EPS production (–○–) by *Lactobacillus helveticus* in milk batch cultures; (a) pH 6.2, (b) free-pH.

1960). The plates were incubated at 37 °C for 48 h and the results expressed as c.f.u. ml^{-1}.

Quantification of exopolysaccharide and monomer analysis

Isolation of EPS was carried out in 100 ml samples treated with Pronase E Type XIV (Sigma) to hydrolyse milk proteins. The EPS were isolated by centrifugation, precipitated with ethanol (Mozzi *et al.* 1996), freeze-dried in 2 ml distilled water, and applied on a Sepharose 4B (Sigma) column to determine its molecular weight (MW). The EPS was eluted with 0.05 M Tris/HCl buffer (pH 7), and the column calibrated with a mixture of dextrans (Sigma) (MW 39 100; 73 000; 515 000 and 2 000 000) at 0.25 mg ml^{-1} each. The MW of the EPS was determined by using a graphic plot of the MW log of dextrans against the elution volume. The monosaccharide composition of the purified and hydrolysed (3 M trifluoroacetic acid, at 100 °C for 6 h) EPS was determined by HPLC on a Rezex ROA-Organic Acid column (Phenomenex) at 55 °C using water as mobile phase at 0.6 ml min^{-1}. The relative proportion of the peak areas was calculated to estimate the monomer composition. Total EPS (expressed as mg l^{-1}) was estimated by the phenol sulphuric method (Dubois *et al.* 1956) using glucose as standard.

Other determinations

The residual lactose, glucose and galactose as well as lactate and acetate were determined by HPLC as described above; citrate was determined by enzymatic methods (Boehringer Mannheim GmbH, Germany). For HPLC analysis, samples were previously clarified with Carrez solution (Boehringer). Results were expressed in mM.

Reproducibility

All results presented in this paper are the average of three assays. The variations among results were less than 10%.

Results and discussion

The growth kinetics of and the lactic acid and EPS production by *L. helveticus* ATCC 15807 in milk batch cultures with and without pH control was quite different (Figures 1–3). At pH 6.2 (Figure 1a), cultures displayed a low specific growth rate ($\mu = 0.27$ h^{-1}), cell viability (3×10^8 c.f.u. ml^{-1}) and EPS formation (49 mg l^{-1}) but a high lactic acid production (425 mM) (Figure 2a); this fact would indicate that the sugar metabolism was mainly diverted to lactate rather than to the synthesis of EPS. The lactic acid accumulated in the medium at pH 6.2 seemed to be the key factor for the early beginning of the stationary phase and the low cell viability (Figure 1a). In contrast, cultures grown without pH control grew at a higher rate ($\mu = 0.5$ h^{-1}) and produced 83% more EPS (360 mg l^{-1}) (Figure 1b) but 49% less lactate (210 mM) (Figure 2b).

The isolated EPS were polymers of high MW (8.2×10^5 to 2×10^6) containing high amounts of

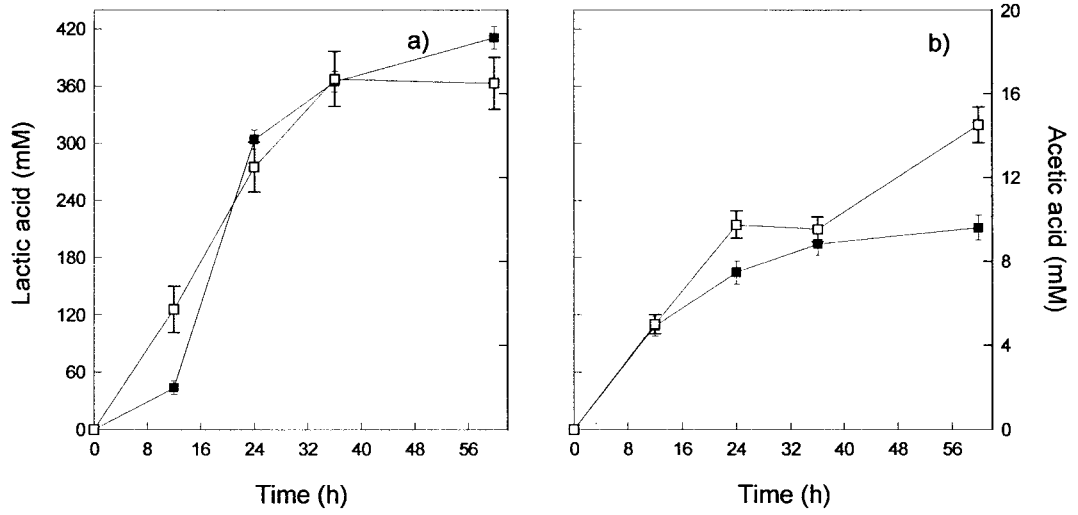

Fig. 2. Production of lactic acid (–■–) and acetic acid (–□–) by *Lactobacillus helveticus* in milk batch cultures; (a) pH 6.2, (b) free-pH.

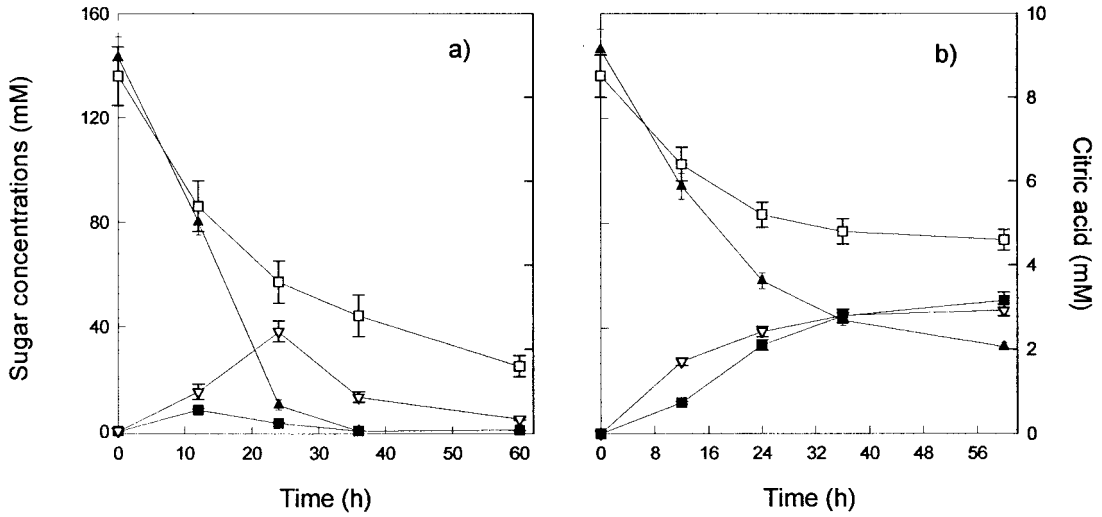

Fig. 3. Residual lactose (–▲–), galactose (–▽–), glucose (–■–), and citric acid (–□–) in milk batch cultures of *Lactobacillus helveticus*; (a) pH 6.2, (b) free-pH.

phosphate (81% in average), glucose and galactose in ratios of 1:1 (pH 6.2) and 2:1 (free pH), and traces of rhamnose (6.1% of the total sugar content). The purified EPS showed no differences in their chemical composition. However, cultures were non-ropy at pH 6.2 possibly due to the amount of lactic acid produced and/or to the presence of hydrolytic enzymes (Cerning *et al*. 1988).

Lactose concentration was almost zero after 24 h of fermentation at pH 6.2 remaining ca. 40 mM sugar in free-pH cultures (Figures 3a and 3b). *L. helveticus* ATCC 15807 released glucose and galactose moieties during growth in milk, the fate of each being dependent on the incubation time and the culture conditions. At pH 6.2 (Figure 3a), the microorganism completely consumed both sugars, which remained at a high concentration (42–46 mM) in free-pH cultures after 60 h of fermentation (Figure 2b). The steady formation of lactate after lactose depletion at the former pH (Figure 2b) might be related to the consumption of the glucose and galactose moieties.

L. helveticus ATCC 15807 in free- and controlled-pH fermentations displayed a mixed-acid fermentation with formation of lactate as the main end-product

(210–425 mM, respectively) and acetate, which was linked to the citrate metabolism during the exponential phase (up to 12 h) (Figures 2a and 2b). The citrate present in milk was almost totally metabolised at pH 6.2 remaining ca. 4.6 mM in cultures without pH control. The total amount of acetate produced after 60 h of fermentation by free- (13.4 mM) and controlled-pH (16.5 mM) cultures clearly indicates that it was synthesised through alternative pathways from pyruvate.

Following is uptake, citrate is cleaved by citrate lyase producing acetate (without ATP formation) and oxalacetate in equimolecular concentrations. The oxalacetate can be subsequently decarboxylated to pyruvate, which in turn would be split into acetate – via the pyruvate dehydrogenase and acetate kinase pathway – resulting in ATP production (Marty-Teysset *et al.* 1996, Garrigues *et al.* 1997). The decarboxylation of oxalacetate would play a role in acidic environments. According to Magni *et al.* (1999), the uptake of citrate, coupled to the efflux of lactate produced during glycolysis, is a regulatory mechanism for maintaining the pH homeostasis through the consumption of protons in the decarboxylation of oxalacetate. Besides, the production of acetate, a more antimicrobial compound than lactate, can aid homofermentative lactic acid bacteria in competition with other less acid-tolerant microbes both in natural habitats and in envisaged applications.

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