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Kinetic analysis of growth and sugar consumption by *Lactobacillus fermentum* IMDO 130101 reveals adaptation to the acidic sourdough ecosystem

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ABSTRACT

The effect of pH on growth and carbohydrate metabolism of *L. fermentum* IMDO 130101 was investigated. Pronounced acid tolerance occurred together with marked responses in sugar metabolism due to acid stress. In accordance with the environment from which this strain was isolated, glucose and fructose metabolism remained active at low pH. Fructose was quantitatively converted into mannitol under all conditions tested, yielding an energetic advantage to the strain. Modelling of growth, sugar consumption, lactic and acetic acid production, and mannitol production of *L. fermentum* IMDO 130101 allowed the estimation of its basic biokinetic parameters when growing under simulated sourdough conditions. The obtained kinetic data underline the competitiveness of the strain in an acidic environment.

1. Introduction

Sourdough has long been recognised as having important technological properties, such as the improvement of bread texture and flavour and the delay of staling (Hansen, 2004; De Vuyst and Neysens, 2005). However, the microbiology of this highly competitive and stressful environment and the physiological adaptations of the competing microbiota to this ecosystem are just beginning to be understood (Spicher and Stephan, 1999; Salim ur et al., 2006). Lactic acid bacteria (LAB) and yeasts play a key role in the sourdough fermentation process (Salovaara, 1998; De Vuyst and Vancanneyt, 2007). For reasons that are as yet poorly understood, sourdoughs are stable ecosystems. Typically, one or several yeast strains and LAB strains form a stable association for very long periods of time. These stable associations arise because of an array of influencing factors in different sourdoughs. The dominance of *Lactobacillus sanfranciscensis* strains in type I sourdoughs has been ascribed to the sourdough fermentation technology applied, while the production of an antibiotic (reutericyclin) produced by strains of *Lactobacillus reuteri* that inhibits most of the sourdough LAB is responsible for the stability of German sourdoughs with *L. reuteri* as dominating species (Gänzle et al., 2000; Messens and De Vuyst, 2002; De Vuyst and Neysens, 2005).

LAB are adapted to live in microaerophilic environments that are rich in nutrients, including the presence of sugars, and peptides or amino acids (Makarova et al., 2006). Glucose is fermented either via the homofermentative or heterofermentative pathway. Homofermentatively, one mole of glucose is converted into two moles of lactate via glycolysis (Embden–Meyerhof–Parnas pathway). In the heterofermentative or Warburg–Dickens pathway, one mole of glucose is converted into one mole of lactate and one mole of ethanol or acetic acid, depending on the availability of external electron acceptors (Zaunmüller et al., 2006). Maltose, the main fermentable sugar in sourdough enters the cell through a maltose/H^+ symporter (Neubauer et al., 1994). Recently, evidence has been presented for the possibility of a maltose phosphotransferase system in LAB (Le Breton et al., 2005). Maltose can be split by a maltose phosphorylase (Egloff et al., 2001) or an α-D-glucosidase (Antuna and Martinezanaya, 1993; Decort et al., 1994) into two moles of glucose. The use of a maltose phosphorylase provides sourdough LAB with an energetic advantage (Stolz et al., 1993; Ehrmann and Vogel, 1998; Aisaka et al., 2000; Gänzle et al., 2007).

An important functionality of sourdough LAB is the capacity to reduce fructose to mannitol, which confers an extra energetic advantage on the strain (Stolz et al., 1995a,b; Hammes et al., 1996; Fernandez and Zuñiga, 2006). Two pathways have been identified that lead to production of mannitol in LAB. In homofermentative LAB, intracellular fructose-1-phosphate is reduced to mannitol-1-phosphate when using this hexose as electron acceptor instead of pyruvate (Wisselink et al., 2005). A phosphatase releases free mannitol, which is then excreted. Heterofermentative LAB reduce extracellular fructose (as alternative electron acceptor) to mannitol through the enzyme mannitol dehydrogenase, thereby regenerating NAD^+. Concomitantly, heterofermentative LAB avoid the need to reduce acetyl phosphate to ethanol for NAD^+ regeneration. Instead, acetyl phosphate is converted

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into acetate with extra generation of ATP, which increases the energy yield significantly (Gobbetti et al., 1995; Saha, 2006).

In the course of previous investigations, several interesting *Lactobacillus* strains have been isolated from laboratory sourdough fermentations. From sourdough fermentation with rye flour, the *Lactobacillus fermentum* strain IMDO 130101 has been isolated, which demonstrated the capacity to reduce fructose to mannitol (Van der Meulen et al., 2007; De Vuyst and Vancanneyt, 2007; De Vuyst and Neysens, 2005). *L. fermentum* was first isolated in 1901 by Beijerinck and has been detected in a wide variety of environments, including food products and the human body (Beijerinck, 1901; Fons et al., 1997; Silvester and Dicks, 2003; Strahinic et al., 2007). Concerning fermented foods, *L. fermentum* has repeatedly been reported as one of the dominant microorganisms in sourdough fermentations and in the preparation of traditional African fermented foods (Kazanskaya et al., 1983; Spicher and Lönner, 1985; Hounhouigan et al., 1993; Halm et al., 1996; Hamad et al., 1997; Hayford et al., 1999; De Vuyst and Vancanneyt, 2007). Certain strains have been recognized as good producers of mannitol (von Weymarn , 2002a,b; Aarnikunnas et al., 2003). In the last decade, most research interest has been directed at efficient industrial processes for mannitol production, avoiding the catalytic hydrogenation of fructose that operates with low efficiency, yet little attention has been paid to characterizing the ecological importance of mannitol production (von Weymarn et al., 2002a,b).

The aim of the present study was to quantitatively characterize growth, sugar consumption, lactic and acetic acid production, and mannitol production of *L. fermentum* IMDO 130101, and to characterize the physiological adaptation of this strain to the sourdough ecosystem. More particularly, the goal was a thorough knowledge of the metabolic adaptation to the sugars present in sourdough through kinetic analysis and mathematical modelling. Although modelling is usually applied to study pathogenic and spoilage microorganisms, the present study contributes to the development of the area of 'positive' predictive microbiology, dealing with the kinetics of growth and metabolite production of beneficial microorganisms, such as LAB (Leroy et al., 2002). So far, primary modelling of the bacterial sugar metabolism in sourdough has been restricted to *L. amylovorus* DCE 471 (Leroy et al., 2006).

2. Materials and methods

2.1. Strain, media, and growth conditions

L. fermentum IMDO 130101 was the sourdough LAB strain used throughout this study. The strain was stored at −80 °C in wheat sourdough simulation medium (W-SSM), supplemented with 25% (vol/vol) glycerol as cryoprotectant. W-SSM was also used as the medium to perform simulated wheat sourdough fermentations, and had the following composition (per L): carbohydrates, see below; wheat peptone, 12 g; granulated yeast extract, 12 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; KH_2PO_4, 4 g; K_2HPO_4, 4 g; Tween 80, 1 mL; and vitamin solution, 1 mL. The latter had the following composition (per L): cobalamine, 0.2 g; folic acid, 0.2 g; nicotinamide, 0.2 g; pantothenic acid, 0.2 g; pyridoxal-phosphate, 0.2 g; and thiamine, 0.2 g. The carbohydrate concentrations, applied as single sugars (10 g per L) or as sugar mixtures, were (per L): glucose, 0.5 g; fructose, 0.5 g; maltose, 10.0 g; and sucrose, 2.0 g. All chemicals were obtained from VWR International (Darmstadt, Germany). The pH of the medium was adjusted to the desired value prior to sterilisation (121 °C, 20 min). If necessary, final pH corrections were done in the fermentor prior to inoculation. Solid medium was prepared by adding 1.5% (wt/vol) agar (Oxoid Ltd., Basingstoke, UK) to the broth.

2.2. Fermentation experiments

Fermentations were carried out in 15-L Biostat® C fermentors (Sartorius/B. Braun Biotech International, Melsungen, Germany). The fermentor was sterilized *in situ* at 121 °C for 20 min. The energy source was sterilized separately and added aseptically to the fermentor. The fermentation temperature was kept at 30 °C. The pH of the medium was kept constant through automatic addition of a 10 N NaOH solution to the fermentation broth. The stirring speed was fixed at 100 rpm to keep the medium homogeneous. Sterile air was continuously blown through the head space of the fermentor at a rate of 1 L min^{-1}. Temperature, pH, agitation, and airflow were controlled on line (Micro MFCS for Windows NT software, B. Braun Biotech International). The inoculum was prepared through three subcultures of 12 h in W-SSM. The first two subcultures were carried out in 10 mL of medium, the third subculture in 100 mL.

The influence of the energy source on growth and sugar consumption by *L. fermentum* IMDO 130101 was studied by applying single carbohydrates and mixtures of carbohydrates in W-SSM at constant pH 5.5. To investigate the influence of pH on growth and sugar consumption, fermentations were carried out at constant pH in W-SSM medium at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. All fermentations were carried out in duplicate during 24 h. The minimum and maximum pH for growth were confirmed by small-scale fermentations in glass bottles (100 mL W-SSM during 72 h).

2.3. Analyses

During fermentation, samples were regularly withdrawn from the fermentor for determination of cell counts (colony forming units, cfu). Cell counts were obtained by plating tenfold serial dilutions in saline (0.85% NaCl solution, wt/vol) on W-SSM agar. Every measurement was performed on three independent samples.

The concentration of lactic acid was determined by high performance liquid chromatography (HPLC) as described by Makras Van Acker and De Vuyst (2005). A Waters chromatograph (Waters Corp., Milford, MA, USA) was used, which was equipped with a 2414 differential refractometer, a 600 S controller, a column oven, and a 717plus autosampler. An ICSep ICE ORH-801 column (Interchim, Montluçon, France) was used with 10 mN H_2SO_4 as mobile phase at a flow rate of 0.4 mL min^{-1}. The column temperature was kept at 35 °C. To remove proteins from the samples, 350 µl of Carrez A reagent [3.6% (wt/vol) of $K_4(Fe(CN)_6).3H_2O$] and 350 µl of Carrez B reagent [7.2% (wt/vol) of $ZnSO_4.7H_2O$] were added to 700 µl of cell-free culture supernatant. After centrifugation (16,060 ×*g* for 15 min) the supernatant was filtered (0.2-µm filters; Minisart high-flow, Sartorius), and injected (30 µL). Calibration was performed with external standards. Samples were analyzed in triplicate and the results are represented as the average of these three independent measurements. The error on the measurements is represented as the standard deviation.

The concentrations of glucose, fructose, sucrose, maltose, and mannitol were determined by high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) as described by Van der Meulen et al. (2007) but using a standard addition protocol. Briefly, an ICS 3000 chromatograph with a CarboPac™ PA10 column (Dionex, Sunnyvale, CA, USA) was used. The mobile phase, at a flow rate of 1.0 mL min^{-1}, consisted of ultra-pure water (0.015 µS cm^{-1}; eluent A) and 250 mM NaOH (eluent B), with the following gradient: 0.0 min: 87% A, 13% B; 20.0 min: 87% A, 13% B; 40.0 min: 15% A, 85% B; 41.0 min: 100% B; 49.0 min: 100% B; 50.0 min: 87% A, 13% B; and 65.0 min: 87% A, 13% B. Due to matrix interference, quantification was carried out with standard addition. Four standard solutions with the following composition were prepared (g per L): ultra-pure water (solution A); 5.00 maltose, 1.00 sucrose, 0.25 glucose, 0.25 fructose, and 0.25 mannitol (solution B); 10.00 maltose, 2.00 sucrose, 0.50 glucose, 0.50 fructose, and 0.50 mannitol (solution C); 15 maltose, 3.00 sucrose, 0.75 glucose, 0.75 fructose, and 0.75 mannitol (solution D). For each time point, 300 µL of