Biochemistry and physiology of sourdough lactic acid bacteria

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Lactic acid bacteria (LAB) are the dominant microorganisms in sourdoughs, and the rheology, flavour and nutritional properties of sourdough-based baked products greatly rely on the activity of LAB. The newer developments on the biochemistry and physiology of this group of bacteria are considered here, with particular emphasis on carbohydrate and nitrogen metabolism, responses to environmental stresses, production of anti-microbial compounds and nutritional implications.

Introduction

The biochemistry and physiology of sourdough lactic acid bacteria (LAB) have received extensive attention during the last decade for giving an explanation of the microbial colonisation of the natural sourdough environment, which affects the rheology, flavour and nutritional properties of baked goods. Carbohydrate and nitrogen

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metabolisms deserved the major interest but other biochemical mechanisms have also been considered. A striking property of many sourdough LAB is their enormous flexibility and potential with respect not only to catabolic substrates and anabolic products but also with respect to the continuous changes in the surrounding environment.

Sourdoughs are very complex biological ecosystems since the microbial composition and the interactive effects among bread making processes and ingredients (Gobbetti, 1998). Basically, three standard protocols of sourdough fermentations are distinguished (namely, type I, II, and III), but artisanal and industrial technologies also largely use other traditional protocols. As a general rule, LAB are the dominant organisms in sourdoughs and in many cases they co-exist with yeasts which are also present in elevated numbers (Vogel et al., 1999). As shown for several European sourdoughs that had been propagated for a long time, selection leads to the predominance of unique LAB communities with large genotypic and phenotypic variability (Corsetti et al., 2003; De Vuyst et al., 2002; Gobbetti, 1998).

This review focuses on the more recent knowledge of the metabolisms of carbohydrates and nitrogen compounds, environmental adaptation, anti-microbial activity and effects on the nutritional properties of baked goods which are strictly related to the biochemistry and physiology of sourdough LAB.

Metabolism of carbohydrates

Facultative (e.g. *Lactobacillus plantarum* and *Lactobacillus alimentarius*) and obligately heterofermentative (e.g. *Lactobacillus sanfranciscensis* and *Lactobacillus pontis*) LAB which use, respectively, the Embden–Meyerhof–Parnas (EMP) and phoshogluconate pathways for hexose fermentation, are commonly found in sourdoughs. Behind these main energy routes, the phenotypic responses to low and variable nutrient conditions involve the use of external acceptors of electrons, the hierarchical and/or simultaneous use of various energy sources, often coupled with inducible uptake systems, and/or the interactions with endogenous and exogenous enzymes.

Use of external acceptors of electrons

Overall, the competitiveness of obligatory heterofermentative lactobacilli in sourdoughs is explained by their combined use of maltose and external electron acceptors (Vogel et al., 1999). During the phosphogluconate pathway,
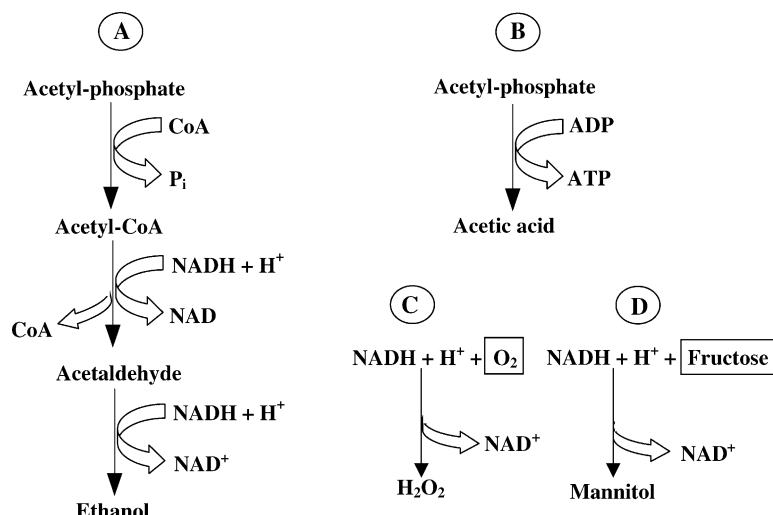
Fig. 1. Use of fructose and oxygen as external electron acceptors by *Lb. sanfranciscensis*. (A) Two-carbon branch of the phosphogluconate pathway; (B) acetate kinase reaction; (C) use of oxygen; and (D) fructose as external electron acceptors.

additional energy may be generated by the activity of acetate kinase which, in the presence of electron acceptors, allows the recycling of NAD^+ without the need of ethanol formation. External electron acceptors used by *Lb. sanfranciscensis* mainly include fructose, which is reduced to mannitol (Korakli & Vogel, 2003), and oxygen (Fig. 1). The mannitol dehydrogenase of *Lb. sanfranciscensis* has an apparent molecular mass of 44 kDa and catalyses both the reduction of fructose to mannitol and the oxidation of mannitol to fructose. The optimal temperature and pH for these activities were 35 °C and 5.8–8.0, respectively (Korakli & Voge, 2003). The use of fructose as an external electron acceptor was also shown in *Leuconostoc mesenteroides* (Erten, 1998). A few *Lb. sanfranciscensis* produce mannitol although fructose is not fermented (De Vuyst et al., 2002). When synthesized, mannitol could be used by *Lb. plantarum* strains as an energy substrate. Its anaerobic consumption was shown in the presence of electron acceptors such as ketoacids (e.g. pyruvate) and yields further to lactate (Liu, 2003). Fructose may have also another effect in sourdough fermentation, especially when maltose-positive and -negative LAB are associated (Gobbetti, 1998). In most of the cases, *Lb. sanfranciscensis* hydrolyses maltose and accumulates available glucose in the medium in a molar ratio of *ca.* 1:1 (Gobbetti, Corsetti, & Rossi, 1994; Stolz, Böcker, Vogel, & Hammes, 1993). Nevertheless, for some strains, glucose accumulation and maltose phosphorylase activity were not observed when cells were grown with maltose plus fructose (De Vuyst et al., 2002). It is also believed that hexokinase activity is induced in the presence of glucose or fructose (Stolz, Hammes, & Vogel, 1996). Hence, the non-phosphorylated glucose formed upon the cleavage of maltose by maltose phosphorylase was possibly immediately converted by hexokinase activity, which in turn was induced by fructose in the medium. Thus, in the presence of both maltose and fructose in the medium, induction of hexokinase activity

does occur, explaining why no glucose accumulation was found (De Vuyst et al., 2002).

The lag phase, growth rate and cell yield of *Lb. sanfranciscensis* were positively affected by oxygen and 225 μM Mn^{2+} (De Angelis & Gobbetti, 1999). Oxygen, used as an external acceptor of electrons, activated the acetate kinase reaction through its reduction to H_2O_2 and aerobiosis induced the expression of a *ca.* 12.5 kDa true superoxide dismutase, probably Mn-dependent. LAB have a number of enzymes which are involved with oxygen or its toxic derivatives. NADH:H_2O_2 oxidase, NADH:peroxidase and L-cysteine uptake system were specifically used for the detoxification of H_2O_2 in sourdough lactobacilli (De Angelis & Gobbetti, 2004). Recently, it has been shown that *Lactococcus lactis*, a species isolated from sourdough also, could grow via a respiratory metabolism when oxygen and a heme source are present. Strains, isolated from plant environments may benefit from a respiratory metabolism in term of the long survival due to the fact that these niches may contain oxygen and may provide sources of iron-charged porphyrin (Gaudu et al., 2002).

Overall, the practical relevance for using external acceptors of electrons is the change of the fermentation quotient (lactate/acetate molar ratio) (Spicher, 1983) which positively affects the baking, sensorial and shelf-life properties of sourdough bread (Gobbetti, 1998).

Hierarchical and/or simultaneous use of various energy sources

Bacteria use carbon sources in a strictly controlled hierarchical manner for which they have developed global control mechanisms (Titgemeyer & Hillen, 2002). This is achieved via carbon catabolite repression (CCR), which is, in Gram-positive bacteria, the result of global transcriptional control (activation and repression) and inducer exclusion through the seryl-phosporylated form of the phosphotransferase HPr (HPr-Ser-P) in concert with the pleiotropic

regulator CcpA (catabolite control protein). Based on these mechanisms, when bacteria are exposed to a mixture of carbon sources, they choose the substrate that yields the maximum profit for growth or obligatory use non-conventional substrates.

Lactobacillus alimentarius 15F, *Lactobacillus brevis* 10A, *Lactobacillus fermentum* 1F, and *Lb. plantarum* 20B showed higher growth rate, cell yield, acidification rate and acetic acid production when xylose, ribose or arabinose, instead of maltose, were added to the culture medium (Gobbetti, Lavermicocca, Minervini, De Angelis, & Corsetti, 2000). Sourdough fermentation by *Lb. plantarum* 20B with addition of pentosan extract and α-L-arabinofuranosidase from *Aspergillus niger* (which produced arabinose from pentosans) increased the acidification rate, titratable acidity and acetic acid concentration, without a variation in the synthesis of lactic acid, compared to a traditional sourdough (Fig. 2). In facultatively heterofermentative LAB such as *Lb. plantarum*, the presence of pentoses may induce phosphoketolase and, therefore, the phosphogluconate pathway. The existence of specific pentose permeases has been detected in cell-free extracts of several facultatively heterofermentative LAB. Arabinose is fermented through the lower half of the 6-phosphogluconate pathway and, in this case, only 1 mole of $NADH + H^+$ has to be oxidised, which is done in the 3-carbon branch of the heterofermentative pathway. There is, therefore, no need to form acetaldehyde and ethanol; instead, an extra mole of ATP and acetate is formed from acetyl-phosphate.

Co-fermentations are metabolic alternatives which enable sourdough LAB to use non-fermentable substrates, thus increasing their adaptability. A co-metabolism of citrate and maltose or glucose was observed in *Lb. sanfranciscensis* (Gobbetti & Corsetti, 1996). After a large screening on 170 strains of sourdough LAB, *Lb. alimentarius* M106 and M137, *Lactobacillus hilgardii* S32, *Lb. brevis* AM11, *Lb. fermentum* I4 and *Lb. brevis* AM8 had a higher cell yield, growth rate

and acetic acid production on a mix of arabinose, xylose or ribose plus maltose than on maltose alone as carbon sources. In the co-fermentation process, pentoses were preferentially consumed instead of maltose (Gobbetti *et al.*, 1999).

The formation of pyruvate and lactate may also derive from the obligatory use of a range of non-conventional substrates such as amino acids. Serine is deaminated to ammonia and pyruvate, which is reduced to lactate. Pyruvate is produced directly (e.g. alanine) or indirectly (aspartate) from amino acids by transamination (Liu, 2003). Evidences are also available that some sourdough LAB may degrade lactate to acetate. *Lactobacillus buchneri* catabolises lactate to acetate via pyruvate, through a NAD^+-independent lactate dehydrogenase (LDH), during sugar–glycerol co-fermentation (McFeeters & Chen, 1986). Lactate degradation during sourdough fermentation may have an impact on sourdough flavour and texture due to the formation of acetate and CO_2 (Liu, 2003).

Interactions with enzymes

Endogenous and added (exogenous) enzymes have important effects to achieve improved and uniform product quality of cereal foods. The actual trend is to use a complex mixture of exogenous microbial enzymes which act in a synergistic way (Si, 1996). Enzymes may interact with the sourdough LAB thus promoting effects on the microbial metabolism. Selecting the most useful combination of sourdough LAB and exogenous enzymes could be of great importance in modern biotechnology of baked goods. Eleven LAB species were used alone or in association with microbial glucose-oxidase, lipase, endoxylanase, α-amylase or protease to produce sourdough (Di Cagno *et al.*, 2003). Only *Leuconostoc citreum* 23B, *Lc. lactis* subsp. *lactis* 11M and *Lb. hilgardii* 51B were positively influenced by added enzymes. Lactic acid fermentation was increased and markedly accelerated. All the enzymes affected the acidification activity of *Lb. hilgardii* 51B, including their addition in mixture, leading to a marked decrease of the time of fermentation. The addition of lipase, endoxylanase and α-amylase increased the production of acetic acid by *Lb. hilgardii* 51B. The molar ratio between acetic acid and ethanol increased from *ca.* 0.38 to *ca.* 0.78–1.1.

Metabolism of nitrogen compounds

It has been well established that almost all LAB have multiple (4 up to 14 amino acids) auxotrophy (Calderon, Loiseau, & Guyot 2003). LAB, therefore, depend on proteolytic systems that allows degradation of proteins. The proteolytic system consists of an extracellularly located serine-proteinase, transport systems specific for di/tri-peptides and oligopeptides (>3 amino acid residues) and a multitude of intracellular peptidases. Compared to dairy LAB, the proteolytic system of sourdough LAB has been poorly characterised and the effect on baked goods properties has been shown only in part. The most recent studies have dealt with the general characterisation of
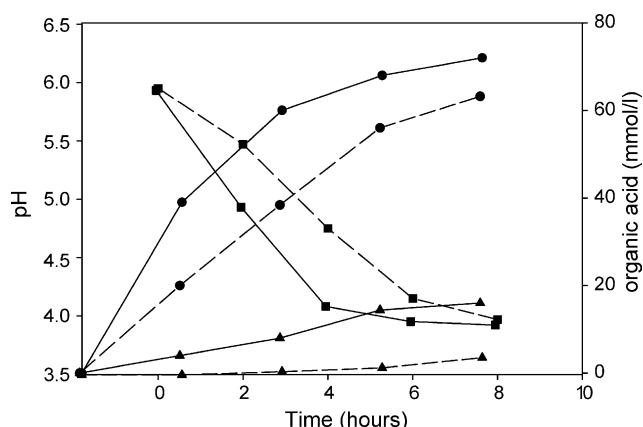

Fig. 2. Kinetics of pH (■) and lactic (●) and acetic (▼) acids production (mmol/l) during sourdough fermentation by *Lactobacillus plantarum* 20B with pentosans and α-L-arabinofuranosidase from *Aspergillus niger* (—) and without the addition of improvers (– – –). Adapted from Gobbetti *et al.* (2000).

proteolysis during sourdough fermentation and with the catabolism of peptides and amino acids.

Proteolysis during sourdough fermentation

Overall, sourdough fermentation with LAB resulted in an increase of amino acid concentrations during fermentation, whereas dough fermentation with yeasts alone reduced the concentration of free amino acids. This enhanced proteolysis is attributed either to the proteolytic activity of sourdough LAB and/or to an activation of proteolysis by cereal enzymes under the acidic conditions of sourdough fermentation (Gobbetti, 1998; Gobbetti *et al*., 1994; Thiele, Gänzle, & Vogel, 2002).

Based on their proteolysis profile patterns towards albumins and globulins, *Lb. alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A and *Lb. hilgardii* 51B were selected and used in sourdough fermentation (Di Cagno *et al*., 2002). Compared to a chemically acidified dough, 37–42 polypeptides, distributed over a wide range of pIs and molecular masses, were hydrolysed by selected LAB as shown by two-dimensional electrophoresis (2-DE). Albumins, globulins and gliadins were shown to be hydrolysed, while glutenins were not degraded. Interestingly, proteolysis among the four strains was highly different even though no difference was found in acidification properties. Similar results were found for proteolysis during sourdough fermentation for pizza production (Pepe, Villani, Oliviero, Greco, & Coppola, 2003).

Whole and pooled cells of selected *Lb. alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A and *Lb. hilgardii* 51B, at a concentration of 10^9 CFU/ml, were active towards Pro- and Gly-Pro-*p*-NA, Z-Gly-Pro-NH-trifluoromethylcoumarin, Val-Pro, Pro-Gly and Gly-Pro-Ala, which are substrates relatively specific for iminopeptidase, dipeptidyl-peptidase, prolyl-endopeptidase, prolidase, prolinase and aminopeptidase activities, respectively (Di Cagno *et al*., 2004) (Table 1). Practically, the pool of these sourdough lactobacilli seemed to have a pattern of specialized peptidases capable of hydrolysing all the different peptide bonds which potentially include the imino acid proline. Proline is unique among the 20 amino acids due to its cyclic structure which imposes many restrictions on the structural aspects of peptides and proteins and confers particular biological properties (Cunningham & O'Connor, 1997). When assayed *in vitro*, the selected LAB showed a complete hydrolysis of the fragments 31–43 (Di Cagno *et al*., 2002) and 62–75 of the A-gliadin and of the epitope 33-mer (Di Cagno *et al*., 2004), which were shown to be responsible for celiac sprue (CS) (Shan *et al*., 2002). These hydrolysing activities are not widespread in dairy LAB (Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Stepaniak, 1999). Under the above experimental conditions (Di Cagno *et al*., 2004), selected sourdough lactobacilli (cells and cytoplasmic extracts) were used.

The effect of acidification and of endogenous wheat proteinases (Thiele *et al*., 2002), which have an optimum pH

Table 1. Enzyme activity[a] of selected sourdough LAB strains of *Lb. alimentarius* (15M), *Lb. brevis* (14G), *Lb. sanfranciscensis* (7A) and *Lb. hilgardii* (51B) on various substrates containing proline residues

Substrate	Substrate concentration (mM)	Unit of activity (U)
Pro-*p*-NA	2.0	0.3 ± 0.01[b]
Gly-Pro-*p*-NA	2.0	5.2 ± 0.03
Z-Gly-Pro-NH-trifluoromethyl-coumarin	2.0	12.3 ± 0.4[c]
Val-Pro	2.3	2.1 ± 0.03[d]
Pro-Gly	2.3	1.9 ± 0.04
Gly-Pro-Ala	2.0	2.2 ± 0.02
Bradykinin	0.3	11.1 ± 0.3
Fragments 62–75 A-gliadin	0.45	15.0 ± 0.5
33-Mer	0.2	0.2 ± 0.01

[a] Each value is the average of three enzyme assays, and standard deviations were calculated.
[b] A unit of enzyme activity (U) on *p*-NA substrates was defined as the amount of enzyme, which produced an increase in absorbance at 410 nm of 0.01/min.
[c] A unit on Z-Gly-Pro-NH-trifluoromethylcoumarin was the amount of enzyme which produced an increase in fluorescence of 0.1/min.
[d] A unit on di-, tri- and polypeptides was the amount of enzyme which liberates 1 μmol of substrate/min.

at 3.0–4.0 (Bleukx & Delcour, 2000; Kawamura & Yonezawa, 1982), must be considered important for proteolysis in the dough, especially for long time sourdough fermentations. By using fluorescence labelled wheat proteins it was shown that proteolytic breakdown of proteins was enhanced at low pH (Thiele, Gänzle, & Vogel, 2003). Similarly, the reduction of inter-protein disulfide bonds during dough mixing enhances the solubility of gliadins and glutenins, and lactic and acetic acids further swell gluten proteins (Thiele *et al*., 2002).

Catabolism of amino acids

The catabolism of amino acids by LAB has implications with regard to the sensory properties of baked goods and has also an important role for obtaining energy in nutrient-limited conditions (Christensen, Dudley, Pederson, & Steele, 1999). Catabolic reactions such as deamination, decarboxylation, transamination and side chain modification may yield ketoacids, ammonia, amines, aldehydes, acids and alcohols, which are essential for taste and aroma of baked goods (Kieronczyk, Skeie, Olsen, & Langsrud, 2001). Together with transaminases and methionine γ-lyase, cystathionine lyases are key enzymes in sulphur metabolism. A comparison between dairy and sourdough LAB showed that cystathionine lyase activities were markedly higher in the former strains but strains which also populate both the food ecosystems, e.g. *Lactobacillus reuteri* and *Lc. lactis* subsp. *lactis*, had a considerable activity (Curtin *et al*., 2001). A homo-tetrameric *ca*. 160 kDa cystathionine γ-lyase was purified to homogeneity from *Lb. reuteri* DSM 20016 (De Angelis, Curtin, McSweeney, Faccia, & Gobbetti, 2002).

The enzyme was active towards a range of amino acids and amino acid derivatives, including methionine. The N-terminal sequence was MKFNTQLIHGGNSED, which had 100% homology with cystathionine β-lyase of *Lb. reuteri* 104R (Accession number: CAC05298).

Arginine deiminase (ADI) pathway

The ADI pathway is comprised of three enzymes: arginine deiminase (ADI, EC 3.5.3.6) which degrades arginine into citrulline and ammonia; ornithine transcarbamoylase (OTC, EC 2.1.3.3), which cleaves citrulline into carbamoyl phosphate and ornithine; and carbamate kinase (CK, EC 2.7.2.2), which produces ATP, ammonia and carbon dioxide through dephosphorylation of carbamoyl phosphate. A fourth membrane transport protein, which catalyses an electroneutral exchange between arginine and ornithine, is also fundamental (Christensen *et al.*, 1999; Liu & Pilone, 1998; Tonon, Bourdineaud, & Lonvaud-Funel, 2001). The expression of the ADI pathway in industrial microorganisms, such as sourdough LAB, could be of great practical significance. The cytoplasmic extracts of 70 strains of the most frequently isolated sourdough LAB were screened initially for ADI, OTC, and CK activities (De Angelis, Mariotti, *et al.*, 2002). Only obligately heterofermentative strains such as *Lb. sanfranciscensis* CB1, *Lb. brevis* AM1, AM8, and 10A, *Lb. hilgardii* 51B and *Lactobacillus fructivorans* DD3 and DA106 showed all the three enzyme activities. Of the 24 strains of *Lb. sanfranciscensis* tested, only CB1 had ADI pathway enzyme activities. *Lb. sanfranciscensis* CB1 showed the highest activities and the three enzymes were purified from this microorganism to homogeneity (Table 2). ADI, COTC, and CK had apparent molecular masses of *ca*. 46, 39, and 37 kDa, respectively, and the pIs were in the range of 5.07–5.2. The COTC, CK and especially ADI enzyme activities were well adapted to pH (acidic, pH 3.5–4.5) and temperature (30–37 °C) conditions which are usually found during sourdough fermentation. Internal peptide sequences of the three enzymes had the highest level of homology with ADI, COTC and CK of *Lactobacillus sakei*. *Lb. sanfranciscensis* CB1 expressed the ADI pathway during sourdough fermentation with 1–43 mM added arginine. Arginine catabolism in *Lb. sanfranciscensis* CB1 depended on the presence of a carbon source and arginine; glucose at up to *ca*. 54 mM did not exert an inhibitory effect, and the pH was not relevant for induction. The pH of sourdoughs fermented by *Lb. sanfranciscensis*

CB1 was dependent on the amount of arginine added to the dough. A low supply of arginine (6 mM) during sourdough fermentation by *Lb. sanfranciscensis* CB1 favours the expression of the ADI pathway which promotes: (i) enhanced cell growth and survival which positively interfere with the constant microbial composition; (ii) enhanced tolerance to acid environmental stress; and (iii) greater production of ornithine, which improve the organoleptic characteristics of the sourdough through the synthesis of 2-acetyl pyrroline, responsible for the roasty note of the wheat bread crust.

Environmental stress responses

Being unicellular, with a high volume/surface ratio, bacteria are exposed intensively to physical and chemical variations in their surroundings. Therefore, it is not surprising to find that highly dynamic interactions between the cell and its environment have evolved as these are a prerequisite for survival (Teixeira de Mattos & Neijssel, 1997). The field of environmental stress responses is vast and related proteomics of LAB have considered *Lc. lactis* (Casadei, Ingram, Hitchings, Archer, & Gaze, 2001; Sanders, Venema, & Kok, 1999) as a model organism but a few studies have dealt with sourdough LAB also. Knowledge about proteomics of sourdough LAB may permit: (i) tools to be developed for screening for tolerant or sensitive strains; (ii) enhanced use through the optimisation for growth, acidification, proteolysis and synthesis of volatile compounds; and (iii) enhanced growth and/or survival by appropriate preservation methods. Some of the findings related to oxygen adaptation (see section 'Use of external acceptors of electrons') and ADI pathway (see section 'Arginine deiminase (ADI) pathway') were previously described, the others which have been studied for sourdough LAB will be considered in this section.

Heat stress

The greatest effect induced by high temperature is protein denaturation but membranes and nucleic acids have also been identified as cellular sites of heat injury (Teixeira, Castro, Mohacsi-Farkas, & Kirby, 1997). Heat stress also disturbs the trans-membrane proton gradient, leading to a decrease of the intracellular pH (Piper, 1993). When cells are exposed to heat shock, responses occur via increased synthesis of a group of evolutionary-conserved heat shock proteins (HSPs) (De Angelis & Gobbetti, 2004), which promote the correct folding of nascent polypeptides, assembly of protein

Table 2. Characterization of the arginine deiminase (ADI), ornithine transcarbamoylase (cOTC) and carbamate kinase (CK) purified from *Lb. sanfranciscensis* CB1

Enzyme	N-terminal sequence	Optimum T (°C)	pI	Optimum pH	Sensibility to cations and inhibitors	MM (kDa)
ADI	RDNGNLLKRPGK	30	5.18	5.0	Hg^{2+}, Zn^{2+}, Cu^{2+}, PMSF	46
cOTC	GLAEKSAFTTA	30	5.2	5.2	PMSF, Fe^{2+}, Ni^{2+}	39
CK	SATAQTHGNGPQV	40	5.07	5.07	*N*-Ethylmaleimide, iodacetamide, EDTA, DL-propargylglycine, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}	37

Adapted from De Angelis *et al.*, (2002).

complexes, degradation and translocation of proteins. Recently, the heat shock response was studied by comparing *Lb. plantarum* strains isolated from cheeses and sourdoughs (De Angelis, *et al*., 2004). A rather similar response was found. When mid-exponential phase cells of *Lb. plantarum* were adapted to 42 °C for 1 h, the heat resistance to 72 °C for 90 s increased *ca*. 3 log cycles. Two-DE analysis of protein expression by control and heat-adapted cells showed changes in the level of expression of 31 and 18 proteins in mid-exponential and stationary phase cells, respectively. Nine proteins which were commonly or differently induced in the adapted mid-exponential and stationary phase cells were subjected to N-terminal sequencing. All the sequences showed 100% of identity with the deduced amino acid sequences from the complete genome sequence of *Lb. plantarum* WCFS1 (Kleerebezem *et al*., 2003). Proteins were identified as DnaK, GroEL, trigger factor, ribosomal proteins L1, L11, L31, and S6, DNA-binding protein II HlbA, and CspC, by comparison to known proteins of various species (Table 3).

Cold stress

When exposed to abrupt temperature downshifts, bacteria undergo severe physiological disturbance such as reduction in membrane fluidity, changes in the level of DNA supercoiling, and the formation of stable secondary structures in DNA and RNA that impair replication, transcription and protein synthesis (Graumann & Marahiel, 1998). To overcome these deleterious effects and to ensure that cellular activity will be resumed or maintained at low temperature, bacteria have to develop a transient adaptive cold-shock response. While sourdough LAB may naturally adapt to temperature downshifts, they continue to grow at a reduced rate after a temperature decrease of *ca*. 20 °C below their optimum (De Angelis *et al*., 2005). *Lb. sanfranciscensis*, *Lb. plantarum*, *Lb. brevis*, *Lb. hilgardii*, *Lb. alimentarius* and *Lb. fructivorans* grew in wheat flour hydrolysate (WFH) at 15 °C by increasing the lag phase (from *ca*. 2–5 h) and the generation time (from *ca*. 10–18 h). The survival after freezing of *Lb. plantarum* DB200, *Lb. brevis* H12, *Lb. plantarum* 20B and *Lb. sanfranciscensis* CB1 was only 1.0, 0.25, 0.12, and 0.04%, respectively. When the cells cultivated at 30 °C were cold-adapted at 15 °C for 2 h before freezing, cell recovery increased: *ca*. 10-, 25- and 100-fold for *Lb. sanfranciscensis* CB1, *Lb. plantarum* DB200 and *Lb. brevis* H12, and *Lb. plantarum* 20B, respectively. Upon cold-adaptation, 2-DE showed the over expression of 14–18 proteins depending on the strains. The universal primers, CSPU5 and CSPU3 were used to amplify DNA sequences of *Lb. plantarum* 20B and DB200, *Lb. brevis* H12 and *Lb.*

Table 3. Proteins identified by N-terminal sequencing[***]			
Homologous protein[a]	**N-terminal sequences**	**Identities (%)[b]**	**A.N.[c]**
DnaK	ASNKIIGIDLGTTNS	93% *Lactobacillus sanfranciscensis*	Q8KML6
	::::.::::::::::		
	ASNKVIGIDLGTTNS		
GroEL	AKELKFSEDARSAML	93% *Enterococcus durans*	Q8GBC4
	:::::::::::.:::		
	AKELKFSEDARAAML		
Trigger factor	AKELKFSEDARSAML	75% *Staphylococcus epidermidis* ATCC 12228	Q8CNY4
	:::::::::::.:::		
	AKELKFSEDARAAML		
Ribosomal protein L1	AAKWEKKEGNQGELT	66% *Bacillus cereus* ATCC 14579	NC_004722.1
	:. :::::::: ::		
	AT-WEKKEGNEGVLT		
50S ribosomal L11 protein	AKKSKQYQDAAKLVD	86% *Streptococcus mutans* UA159	Q8DSX9
	::..:.. .::::::		
	AKRGKKYVEAAKLVD		
DNA-binding protein II, HlbA	AKKVANVVKLQIPAG	83% *Lactobacillus delbrueckii* subsp. *bulgaricus*	Q8KQE1
	:::: :.::::::::		
	AKKVENIVKLQIPAG		
CspC	-ANKAELVNNVAAATN	100% *Lactobacillus plantarum*	Q9FCV6
	::::::::..::: :.		
	MANKAELVSEVAAKTK		
Ribosomal protein L31	MEHGTVKWFNADKGF	80% *Corynebacterium glutamicum* ATCC 13032	Q8NS12
	::::::::::::::		
	MEHGTVKWFNADKGF		
30S ribosomal protein S6	MQKGIHPDYHLVVFQ	81% *Oceanobacillus iheyensis* HTE831	Q8EKV4
	:.: ::::::: ::::		
	MKKDIHPDYHAVVFQ		

***Refers to the proteins identified in the study of De Angelis, *et al*. (2004).

[a] Similarity of the amino acid sequence to a sequence found in the database. The similarity searches were done by using the BLAST at NCBI non-redundant databases and SWALL database.

[b] Based on the number of amino acids matching in the test sequence.

[c] Accession number in SwissProt and Trembl databases.

sanfranciscensis CB1. The deduced amino acid sequences from all the sourdough lactobacilli displayed high sequence similarity with cold-shock proteins (CSPs), which represent one of the five subfamilies belonging to a superfamily of proteins containing the cold-shock domain (CSD), which consists of *ca*. 70 amino acids and harbours the nucleic acid binding motifs RNP-1 and RNP-2 (Weber, Fricke, Doll, & Marahiel, 2002). The RNP-1 of *Lb. sanfranciscensis* CB1, *Lb. plantarum* DB200 and *Lb. brevis* H12 showed the sequence KGYGFI (De Angelis *et al*., 2005), which was identified in several CSPs such as CspL of *Lb. plantarum* (Mayo *et al*., 1997) and CspA of *Lactobacillus casei* (Francis & Stewart, 1997).

Acid stress

Acid is an important environmental stress which occurs in LAB during sourdough fermentation and storage. The pK_a of lactic acid is 3.86 and in the non-dissociated form it enters the cells by a carrier-mediated electroneutral process. At cytoplasmic pH, lactic acid dissociates, determining the stationary phase of growth, even if nutrients are still available (Piard & Desmazeaud, 1991). The same mechanism is generally promoted by acetic acid. Several mechanisms regulate the homeostasis of intracellular pH (pHi) and the proton-translocating ATPase is the most important for fermentative bacteria (Hutkins & Nannen, 1993). Survival under acidic conditions is positively affected by an adaptation to low pH, a mechanism known as acid-tolerance response (ATR) (Foster & Hall, 1991). After growth at a constant pH of 6.4, the survival of mid-exponential phase cells of *Lb. sanfranciscensis* CB1 decreased dramatically when suddenly subjected to pH 3.2–3.4, as set by lactic acid or by a mixture of lactic and acetic acids (molar ratio of *ca*. 2.3) (De Angelis, Bini, Pallini, Cocconcelli, & Gobbetti, 2001). The tolerance factor induced by adaptation to pH 5.0 for 1 h was *ca*. 4×10^3 after 10 h of exposure to pH 3.2–3.4. Two constitutively acid-tolerant mutants of *Lb. sanfranciscensis* CB1 were isolated following a prolonged exposure (24 h) to the challenge pH 3.2 (Fig. 3). Studies on the acid adaptive responses by 2-DE showed the induction of 15 acid shock proteins (ASPs) in *Lb. sanfranciscensis* CB1. The mutant strains of *Lb. sanfranciscensis* CB1 showed other different phenotypic features compared to the parental strain: (i) increased aminopeptidase activity under acid conditions; (ii) higher growth at 10 °C and in the presence of 5% NaCl; and (iii) increased acidification rate during sourdough fermentation under acidic conditions (De Angelis *et al*., 2001).

High hydrostatic pressure stress

High hydrostatic pressures (100–1000 MPa) are used in food preservation as an alternative to heat treatments (Palou, Lopez-Malo, Barbosa-Canovas, & Swanson, 1999). Microbial barotolerance during commercial pressurization may represent a serious threat to the safety and stability of pressure-processed foods. The effect of high pressures on the
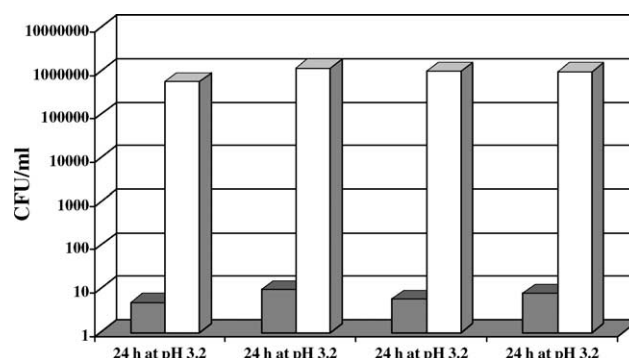
Fig. 3. Survival of *Lb. sanfranciscensis* CB1 to repeated lactic acid stress at pH 3.2. Stress resistance of control cells (■) and mutant cells (□). The four bars indicate different cycles of stress treatment. Adapted from De Angelis *et al*. (2001).

protein expression of *Lb. sanfranciscensis* DSM 20451 was investigated by 2-DE (Drews, Weiss, Reil, Parlar, Wait, & Görg, 2002). The level of expression of 12 proteins within the pH range of 3.5–9.0 was increased or decreased more than 2-fold. Two pressure dependent proteins showing the same molecular mass (*ca*. 15 kDa) and different pI (*ca*. 4.0 and 4.2) were identified as putative homologs/paralogs to CSPs of *Lc. lactis* subsp. *lactis*. Other pressure dependent proteins of *Lb. sanfranciscensis* DSM 20451 had homology with the ribokinase RbsK of *Lb. casei*, GMP synthetase of *Lc. lactis* subsp. *lactis* and elongation factor Tu (Drews *et al*., 2002).

Anti-bacterial activity

Bread and other leavened baked products can become contaminated with spoilage bacteria or moulds. LAB have been shown to possess both anti-bacterial and anti-fungal properties and sourdough addition is an effective procedure to preserve bread from spoilage since it complies with the consumer request for additive-free products (Messens & De Vuyst, 2002).

Anti-bacterial activity

Besides various compounds (e g. organic acids, hydrogen peroxide, diacetyl), sourdough LAB can inhibit the growth of other, usually related microorganisms, by producing bacteriocins or other substances, such as the low-molecular-mass antibiotic reutericyclin produced by *Lb. reuteri* LTH2584 (Höltzel, Gänzle, Nicholson, Hammes, & Jung, 2000). Anti-bacterial compounds may also regulate microbial interactions in complex food systems. Till today only a few bacteriocins or bacteriocin-like inhibitory substances (BLIS) produced by sourdough LAB have been characterized. They include bavaricin A by *Lactobacillus bavaricus* MI401 (Larsen, Vogensen, & Josephsen, 1993), plantaricin ST31 by *Lb. plantarum* ST31 (Todorov *et al*., 1999) and BLIS C57 by *Lb. sanfranciscensis* C57 (Corsetti, Gobbetti, & Smacchi, 1996). More recently, after a screening of 437 *Lactobacillus* strains isolated from 70 sourdoughs, BLIS production was shown by five other lactobacilli (*Lactobacillus pentosus* 2MF8 and 8CF, *Lb. plantarum* 4DE and 3DM and

Lactobacillus sp. CS1) (Corsetti, Settanni, & Van Sinderen, 2004). Moreover, other LAB isolated from food systems other than sourdoughs, such as *Lactobacillus amylovorus* DCE 471 (Messens, Neysens, Vansieleghem, Vanderhoeven, & De Vuyst, 2002) and *Lc. lactis* M30 (Corsetti *et al.*, 2004) have been showed to produce bacteriocins or BLIS of interest for sourdough fermentation. All the above substances are resistant to heat and acidity and some of them are active against *Bacillus*, *Staphylococcus* and *Listera* spp. (Corsetti *et al.*, 2004; Messens & De Vuyst, 2002). The activity of reutericyclin (Gänzle & Vogel, 2003) and BLIS synthesized by *Lb. pentosus* 2MF8 and *Lc. lactis* M30 (Corsetti *et al.*, 2004; Hartnett, Vaughan, & van Sinderen, 2002) has also been shown *in situ*. Reutericyclin formation contributed to the stable persistence of *Lb. reuteri* in sourdough and was active against *Lb. sanfranciscensis* (Gänzle & Vogel, 2003). The BLIS of *Lc. lactis* M30 was effective in reducing the growth of some LAB frequently prevailing during sourdough propagation and may influence the complex sourdough microflora by supporting the implantation and stability of insensitive bacteria such as *Lb. sanfranciscensis* (Corsetti *et al.*, 2004).

Anti-moulds activity

Till today, the anti-fungal activities of LAB have received limited attention but the interest is rapidly increasing to respond to consumer demands for minimally processed foods. A mixture of acetic, caproic, formic, propionic, butyric and *n*-valeric acids, acting in a synergistic way, in which caproic acid played a key role, was responsible for the *in vitro* inhibitory activity of *Lb. sanfranciscensis* CB1 against moulds responsible for bread spoilage such as *Fusarium*, *Penicillium*, *Aspergillus* and *Monilia* (Corsetti, Gobbetti, Rossi, & Damiani, 1998). Phenyllactic and 4-hydroxy-phenyllactic acids have been discovered in culture filtrate of two sourdough-isolated *Lb. plantarum* strains (21B and 20B) showing inhibitory activity against *Aspergillus*, *Penicillium*, *Eurotium*, *Endomyces* and *Monilia* (Lavermicocca *et al.*, 2000). Phenyllactic acid (PLA) was contained at the highest concentration in the bacterial culture filtrate and showed the highest activity. The anti-fungal activity of *Lb. plantarum* 21B was also found in sourdough bread. Compared to breads started with *Saccharomyces cerevisiae* 141 alone, the sourdough bread produced with the association of *S. cerevisiae* 141 and *Lb. plantarum* 21B delayed fungal contamination until 7 days of storage at room temperature (Lavermicocca *et al.*, 2000). Recently the minimal fungicidal or inhibitory concentration of PLA has been evaluated against 23 fungal strains belonging to 14 species of *Aspergillus*, *Penicillium* and *Fusarium* isolated from cereals and bakery products (Lavermicocca, Valerio, & Visconti, 2003). Less than 7.5 mg/ml of PLA were required to obtain 90% growth inhibition for all the strains, while at a concentration of ≤ 10 mg/ml of PLA, 19 strains out of 23 were killed.

Nutritional implications

It is well documented that the use of sourdough has positive nutritional implications too. Some of these nutritional properties are directly related to the biochemical features of sourdough LAB. A few examples of the main and more recent findings will be considered below.

Celiac Sprue (CS)

CS, also known as celiac disease or gluten-sensitive enteropathy, is one of the most common food intolerances, occurring in 1 out of every 130–300 persons of the European (Sollid, 2002) and United States (Fasano *et al.*, 2003) populations. Upon ingestion of gluten, CS patients suffer of self-perpetuating mucosal inflammation characterized by a progressive loss of absorbtive villi and hyperplasia of the crypts. Prolamins of wheat (α-, β-, γ- and ω-gliadin subgroups), rye (e.g. secalin) and barley (e.g. hordein) are hydrolysed, during endoluminal proteolytic digestion, and release a family of closely related Pro- and Gln-rich polypeptides, which are responsible for an inappropriate T-cell mediated immune response (Silano & De Vincenzi, 1999). A 33-mer peptide was shown to be a potent inducer of gut-derived human T-cell lines in 14 CS patients (Shan *et al.*, 2002). The large proportion and location of proline residues in the amino acid sequences of these toxic peptides makes them extremely resistant to further proteolysis (Hausch, Shan, Santiago, Gray, & Khosla, 2003). Although peptidases capable of hydrolysing Pro- and Gln-rich peptides are located in the intestinal brush-border (Andria, Cucchiara, De Vizia, Mazzacca, & Auricchio, 1980), these epitopes withstand enzymatic processing for more than 24 h (Shan *et al.*, 2002). The prolyl-endopeptidase of *Flavobacterium meningosepticum*, which is not related to bread biotechnology, was the only enzyme proposed as detoxifying agent for 33-mer, suggesting a strategy for an oral peptidase supplement therapy for CS patients. A cocktail of selected sourdough lactobacilli, with high potentialities to degrade Pro- and Gln-rich gliadin oligopeptides, including the 33-mer (see section 'Proteolysis during sourdough fermentation'), was used to produce a sourdough bread made of wheat and non-toxic flours according to an ancient bread technology (Di Cagno *et al.*, 2004). Under these conditions, a fermented wheat sourdough (24 h) was mixed subsequently with non-toxic flours (oat, milled and buckwheat) and allowed to ferment for 2 h before baking. This type of bread was technologically suitable: the volume was *ca.* one half of that started with baker's yeast and the texture was comparable with that of wheat sourdough breads. This bread, containing 30% of wheat, was compared with a bread made of the same ingredients but fermented with baker's yeast alone. After 24 h of fermentation, wheat gliadins and low-molecular-mass alcohol-soluble polypeptides were hydrolyzed almost totally (Fig. 4). An acute *in vivo* challenge, based on the intestinal permeability tests (Greco *et al.*, 1991), was carried out with both the types of bread on 17 CS patients recruited voluntarily after at least 2 years on a gluten-free diet

(Di Cagno *et al.*, 2004). Thirteen of the 17 patients showed a marked alteration of the intestinal permeability after the ingestion of the bread started with baker's yeast. When the same 13 CS patients ingested the same dose of gluten (*ca.*

2 g) in bread started with lactobacilli, they showed values of intestinal absorption, which did not differ significantly from the baseline value. Four of the 17 patients did not respond to gluten after ingesting the baker's yeast or sourdough breads.
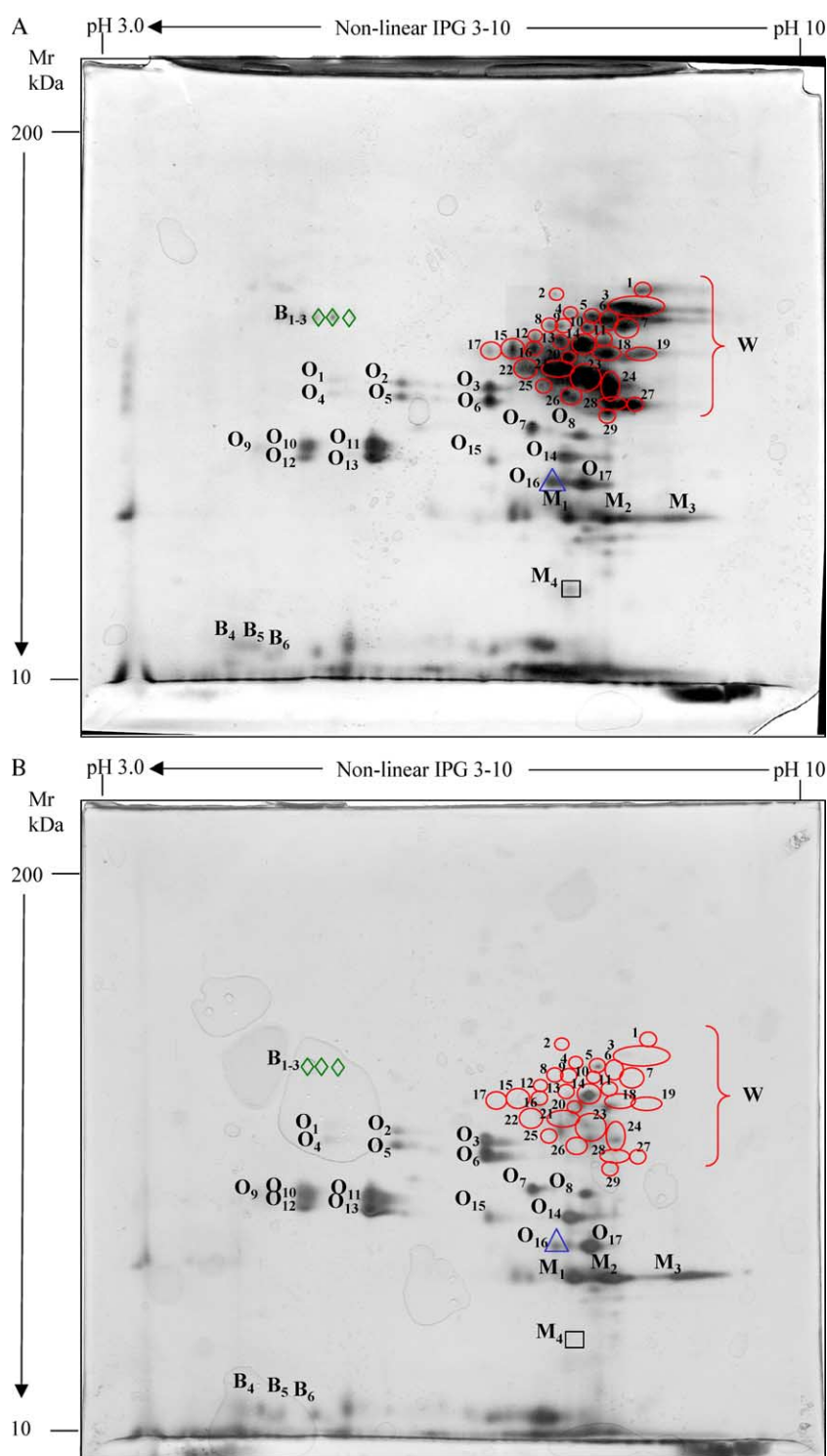

Fig. 4. 2-DE electrophoresis analysis of the prolamin protein fractions of different doughs made of a mixture of wheat (30%), oat (10%), millet (40%), and buckwheat (20%) flours. (A) Chemically acidified dough incubated at 37 °C for 24 h, and (B) sourdough started with selected LAB at 37 °C for 24 h. Prolamin polypeptides from wheat [spot W (1–29)], oat [spot O (1–17)], millet [spot M (1–4)], and buckwheat flours [spot B (1–6)] are shown. The numbered ovals, triangles, squares and diamonds refer to hydrolysed prolamins from wheat, oat, millet, and buckwheat, respectively. Adapted from Di Cagno *et al.* (2004).

Phytate degradation

Phytic acid [myo-inositol hexakis (dihydrogen-phosphate)] constitutes 1–4% by weight of cereal grains, legumes and oilseeds, being a source of myo-inositol and the major storage form of phosphorous (Pandey, Szakacs, Soccol, Rodriguez-Leon, & Soccol, 2001). This molecule is highly charged with six phosphate groups extending from the central myo-inositol ring. For this property, phytic acid is considered to be an anti-nutritional factor for humans and animals as it acts as an excellent chelator of cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} and as it complexes the basic amino acid group of proteins, thus decreasing the dietary bio-availability of these nutrients (Dvorakova, 1998; Wodzinski & Ullah, 1996). Phytic acid has also some positive effects on nutrition for prevention of colonic cancer (Graf & Eaton, 1985) and lowering of cholesterol (Jariwalla *et al.*, 1990). Phytase [myo-inositol hexakis (dihydrogenphosphate) phosphohydrolase, EC 3.1.3.8] catalyses the hydrolysis of phytic acid into myo-inositol and phosphoric acid via penta- to mono-phosphate thus decreasing or eliminating the anti-nutritional effect of phytic acid. Endogenous phytase activity may be contained in the wheat and rye flours but its level greatly varies with the variety and crop year, and, generally, is considered to be insufficient to significantly decrease the amount of phytic acid (Cossa, Oloffs, Kluge, Drauschke, & Jeroch, 2000). It was shown by Fretzdorff and Brümmer (1992) for wheat and rye flours that in chemically acidified doughs the endogenous phytase activity was highest in the pH range of 4.3–4.6. This range is lower than the optimum pH range obtained in aqueous solutions (pH 5.0–5.5) (Lásztity & Lásztity, 1990). The phytase activity was studied in *Lb. plantarum*, *Lactobacillus acidophilus* and *Leuc. mesenteroides* subsp. *mesenteroides* isolated from sourdough and cultivated in a whole-wheat flour medium (Lopez *et al.*, 2000). The sourdough fermentation was shown to be more efficient than yeast fermentation in reducing the phytate concentration in whole wheat bread (Lopez *et al.*, 2001, 2003). The phytase activity of 12 species of sourdough LAB was screened. It was intracellular only, largely distributed among the species, and strains of *Lb. sanfranciscensis* possessed the highest levels of activity (De Angelis *et al.*, 2003). A monomeric *ca.* 50 kDa phytase was purified to homogeneity from *Lb. sanfranciscensis* CB1, which exhibited the highest hydrolysing activity on Na-phytate after reaching the stationary phase of growth. The phytase was optimally active at pH 4.0 and 45 °C. The substrate specificity was dependent on the type of phosphate ester and the highest hydrolysis was found towards adenosine-5'-tri-, di- and mono-phosphate. Compared to these substrates, the activity on Na-phytate was also relevant. The enzyme was thermo-stable after exposure to 70 °C for 30 min. *Lb. sanfranciscensis* CB1 cells or the correspondent cytoplasmic extract were used to ferment a sourdough at 37 °C for 8 h; a marked decrease (64–74%) of the Na-phytate concentration was found compared with the unstarted sourdough. The sourdough started with *Lb. sanfranciscensis* CB1 cells was re-used for several times and phytase activity was maintained to a considerable level (De Angelis *et al.*, 2003).

Concluding remarks

Several years ago the scientific community defined the sourdough as "a traditional product with a great future". Since this time a large number of studies dealt with the physiology and biochemistry of sourdough LAB. They greatly improved the understanding of the carbohydrate and nitrogen metabolisms with repercussions on the manipulation, propagation and storage of this natural starter. Proteomics and nutritional studies on sourdough LAB have started. The trends for the next years could be to enlarge the use of the sourdough and to industrially show that it represents "a unique traditional product to get an otherwise un-reproducible quality of baked goods".

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