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Effect of pH on *Lactobacillus fermentum* growth, raffinose removal, α-galactosidase activity and fermentation products

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Abstract In this study, the behaviour of *Lactobacillus fermentum* CRL 722 and CRL 251 were evaluated under different pH conditions (pH 6.0, 5.5, 5.0, 4.5) and without pH control. Growth was similar under all conditions assayed except at pH 4.5. These microorganisms were able to eliminate raffinose, a nondigestible α-oligosaccharide (NDO) found in soy products, showing a consumption rate of 0.25 g l^{-1} h^{-1} (pH 6.0–5.0). The removal of raffinose was due to the high α-galactosidase (α-gal) activities of these lactic acid bacteria, which was highest at pH 5.5 (5.0 U/ml). The yield of organic acids produced during raffinose consumption was also highest at this pH. The results of this study will allow selection of the optimum growth conditions of *L. fermentum* with elevated levels of α-gal to be used in the reduction of NDO in soy products when used as starter cultures.

Introduction

Soy products have an excellent reputation for high protein content and high amino acid quality. However, human consumption of soy products has been hampered by the presence of nondigestible oligosaccharides (NDO) (i.e. α-galactosides such as raffinose and stachyose) in soybeans, which are not eliminated by processing (Leske et al. 1993). NDO are best defined as "carbohydrates with a degree of polymerisation of two or more, which are soluble in 80% ethanol and are not susceptible to digestion by pancreatic and brush border enzymes" (Quigley et al. 1999). Hydrolytic digestion of α-galactosides in the small intestine is relatively weak since mammals do not possess the α-galactosidase (α-gal) necessary to hydrolyse their α-1,6 linkages in the gut (Slominski 1994). In humans, these NDO are metabolised by microorganisms in the large intestine, liberating huge amounts of gas, which can cause gastrointestinal disorders to sensitive individuals. They represent approximately 4–6% of the dry mass of soybean meal and have been reported to increase the incidence of diaorrhea in rats (Kuriyama and Mendel 1917).

The use of microbial α-gal is a promising solution for the degradation of these NDO in soy products. Lactic acid bacteria (LAB) have been consumed in fermented foods by humans for centuries without any obvious adverse effects (Fuller 1992). LAB such as *Lactobacillus plantarum*, *L. fermentum*, *L. brevis*, *L. buchneri* and *L. reuteri* are able to hydrolyse α-galactosides into digestible carbohydrates during vegetable fermentations. In lactobacilli, many α-gals have been characterised at the biochemical and physiological levels. In our laboratory, α-gal from bifidobacteria and lactobacilli have been characterised (Garro et al. 1993, 1994) and purified from lactobacilli such as *L. fermentum* (Garro et al. 1996). The molecular characterisation of an α-gal from *L. plantarum* has also recently been published (Silvestroni et al. 2002).

The object of this study was to determine the optimal growth parameters and α-gal production of *L. fermentum* in response to changes in pH conditions and the use of *L. fermentum* for the removal of raffinose. *L. fermentum* CRL 722 and CRL 251 are able to eliminate raffinose during fermentation under a variety of pH conditions due to their high α-gal activities. These LAB could thus be used during soy product fermentation in order to eliminate NDO.

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Materials and methods

Micro-organisms and growth conditions

The strains *L. fermentum* CRL 251 and CRL 722 used in this study were obtained from the Culture Collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). Before experimental use, cultures were propagated (2%, v/v) twice in sterile MRS medium containing raffinose instead of glucose (MRS+Raf) and incubated at 37°C for 16 h. Raffinose was sterilised separately (0.22 μm filtration) and added to the MRS broth without glucose to give a final concentration of 1% (w/v).

Fermentations were performed in a 1.0-l fermentor (New Brunswick Scientific, Edison, N.J.) filled with MRS+Raf with an agitation rate of 100 rpm. The temperature was maintained at 37°C and automatically buffered to desired pH values of 6.0, 5.5, 5.0 or 4.5·by addition of sterile 1 M NH_4OH or HCl.

Sampling

Fermentations were allowed to proceed for 24 h; samples were aseptically withdrawn at 0, 2, 4, 6, 8, 10, 12 and 24 h from the fermentation vessel and immediately cooled on ice to determine optical density, cell viability, residual sugars, organic acids, α-gal activity and pH.

The optical density (OD) of samples was measured at 560 nm in a Bausch and Lomb Spectronic 20 spectrophotometer (Rochester, Minn.).

Cell viability was determined in sterile MRS agar. Serial dilutions of each sample were pour plated in triplicate and incubated 37°C for 48 h. Results were expressed as colony forming units per millilitre (cfu/ml). The specific growth rate (μ_{max}) was calculated from the slope of a semi-logarithmic plot of cfu/ml as a function of time.

For evaluation of residual sugars and organic acids, samples were centrifuged at 10,000 *g* for 10 min at 4°C and supernatants were stored at −20°C until analysis.

Lactic and acetic acids were determined by HPLC (Isco model 2360, Lincoln, Neb.) using an Aminex HPX87H column (Bio-Rad, Richmond, Calif.) with a flow rate of 0.6 ml/min 5 mM sulfuric acid at 210 nm.

Raffinose, saccharose, melibiose, glucose, galactose and fructose were quantified by HPLC coupled to a differential refractometer (LKB, model 2142, Bromma, Sweden) using a REZEX RSO oligosaccharides column (200×10 mm, Phenomenex, Torrance, Calif.) at a column temperature of 70°C using HPLC grade water as the eluant at a flow rate of 0.3 ml/min. Samples were deproteinised before sugar determination as previously described (Mital et al. 1973).

α-Galactosidase activity determination

Samples (5 ml) were centrifuged at 10,000 *g* for 10 min at 4°C and the cell pellet was washed twice with 5 mM McIlvaine buffer (Na_2HPO_4-citric acid, pH 5.8; McIlvaine, 1921) and resuspended in 0.5 ml of the same buffer (final $OD_{560\ nm}$=20). Cells were disrupted with 500 mg glass beads (0.10–0.11 mm, Sigma) and shaking at maximum speed on a vortex mixer for five cycles of 1 min each with 5 min pauses on ice in between. Cellular debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C and the supernatant was kept on ice until analysis. α-Gal activity was determined using a modified version of the method of Church et al. (1980), where 1 U α-gal is defined as the amount of enzyme that releases 1.0 μmol *p*-nitrophenol (pNP)/min. Briefly, to a 45 μl sample, 15 μl 10 mM *p*-nitrophenyl-α-D-galactopyranoside (pNPG) was added and incubated at 37°C for 15 min; 900 μl 0.25 M Na_2CO_3 was added to stop the reaction. Absorbance at 405 nm was measured using a VersaMax tuneable microplate reader (Molecular Devices, Sunnyvale, Calif.). Protein concentration was determined using the Bio-Rad protein assay based on the method of Bradford (1976) using bovine serum albumin as a standard.

Reproducibility

All results presented in this paper are the average of three assays. The variation among results was less than 10%. Results were expressed as mean ± standard deviation, and their significance was analysed using Student's *t* test.

Results

This study evaluated the optimal growth parameters of two strains of *L. fermentum*, CRL 251 and CRL 722, under different pH conditions. The following results refer to strain CRL 722; differences between the strains will be discussed later.

Effect of pH on microbial growth

The microbial growth of *L. fermentum* CRL 722 (Fig. 1, Table 1) was noticeably slower at pH 4.5 (μ_{max}=0.78 h^{-1}) than at all other pH values assayed (μ_{max}=1.15–1.25 h^{-1}). However, the OD_{560nm} reached similar levels (3.3–3.6) at the end of the fermentation (24 h) regardless of the pH. After 6 h of fermentation, the microorganism reached a stationary phase of growth at pH 6.0, 5.5, 5.0 and without pH control, whereas at pH 4.5 this growth phase was reached after 8 h. Biomass production was slightly higher at pH 5.5 and pH 5.0 (7.2 mg) than at pH 6.0 (6.9 mg) and without pH control (6.7 mg), and significantly higher than at pH 4.5 (4.5 mg). Similar results can be observed in cell viability, which is highest at pH 5.5 (9.35 log cfu/ml) and lowest at pH 4.5 (8.84 log cfu/ml). Values for the

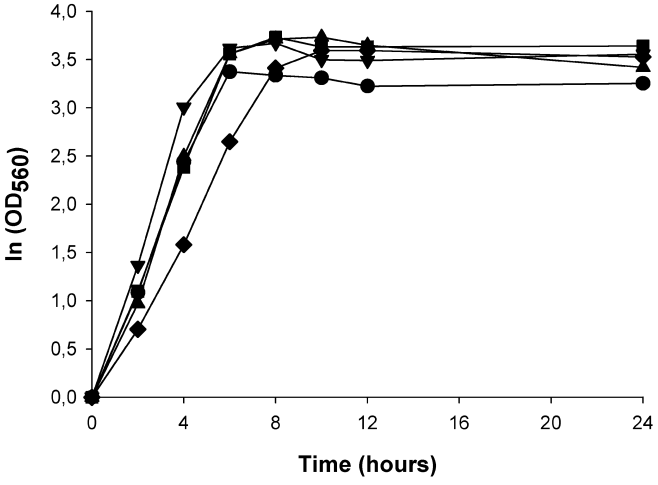

Fig. 1 Growth of *Lactobacillus fermentum* CRL 722 on MRS +raffinose (Raf) at pH 6.0 (*circles*), 5.5 (*inverted triangles*), 5.0 (*squares*), 4.5 (*diamonds*) and without pH control (*triangles*). Results are expressed as ln (OD_{560}) vs time, where OD_{560} is the OD at 560 nm at a given time/OD at the start time

fermentation without pH control followed the behaviour observed at pH 5.0.

Effect of pH on raffinose removal

Raffinose, which is the principal energy source in MRS medium, was completely utilised by *L. fermentum* CRL 722 at all pH values tested (Fig. 2). The only difference observed due to pH was the rate of consumption of this NDO. At pH 4.5, raffinose was eliminated after 10 h of fermentation, whereas at all other pH values, this sugar diminished to undetectable levels after only 6 h growth showing that, at pH 4.5, the consumption rate (0.10 g l^{-1} h^{-1}) is nearly five times lower that at the other pH conditions assayed (0.50 g l^{-1} h^{-1}, see Table 1). Small amounts of glucose were detected at pH 4.5 after 10 h growth (0.4 g/l), whereas at the other pH values tested, the same concentration was observed 4 h earlier (Fig. 2). Only trace amounts of saccharose, melibiose, galactose and fructose were detected in this study.

Effect of pH on α-gal activity

The maximum specific α-gal activity was reached earlier at pH 5.5 and pH 5.0 and without pH control (2 h) than at pH 6.0 and pH 5.0 (6 h and 10 h, respectively, see Fig. 3, Table 1). Once the maximum specific α-gal activity was reached at each pH, the levels remained constant until the end of the fermentation. After 24 h fermentation, the specific activity was significantly lower (1.0 U/mg) at pH 4.5 than at pH 6.0, 5.5 and 5.0 and without pH control (1.8, 1.9, 1.7 and 1.8 U/mg, respectively) (Fig. 3). As shown in Table 1, during the exponential growth phase, α-gal production was higher at pH 5.5 (5.0 U/ml) than at pH 5.0 (4.4 U/ml), without pH control (4.1 U/ml) and pH 6.0 (3.5 U/ml), and lowest at pH 4.5 (3.2 U/ml). After 24 h fermentation, α-gal production was higher at pH 5.5, 5.0 and without pH control (4.2, 3.7 and 3.0 U/ml respectively) than at pH 4.5 and 6.0 (2.4 and 2.3 U/ml, respectively).

Table 1 Metabolism and α-galactosidase activity of *Lactobacillus fermentum* CRL 722 during exponential growth phase at pH 6.0, 5.5, 5.0, 4.5 and without pH control (*n/c*)

	pH				
	6.00	5.50	5.00	4.50	n/c
Growth					
μmax (h^{-1})	1.15	1.25	1.20	0.78	1.22
Cell viability log (cfu ml^{-1})	9.12	9.35	9.03	8.84	9.30
Biomass (mg)	6.90	7.20	7.20	4.50	6.70
α-Galactosidase					
Production (U ml^{-1})	3.50	5.00	4.40	3.20	4.10
Specific activity (U mg^{-1})	1.90	1.90	1.50	1.50	1.60
Rate consumption (g l^{-1} h^{-1})					
Raffinose	0.25	0.25	0.25	0.10	0.25
Rate production (g l^{-1} h^{-1})					
Lactic acid	0.71	0.80	0.59	0.38	0.86
Acetic acid	0.26	0.32	0.40	0.13	0.35
$Yield_{p/s}$[a]	3.94	4.50	3.82	3.33	4.84

[a]Millimoles lactic and acetic acid produced per millimole raffinose consumed

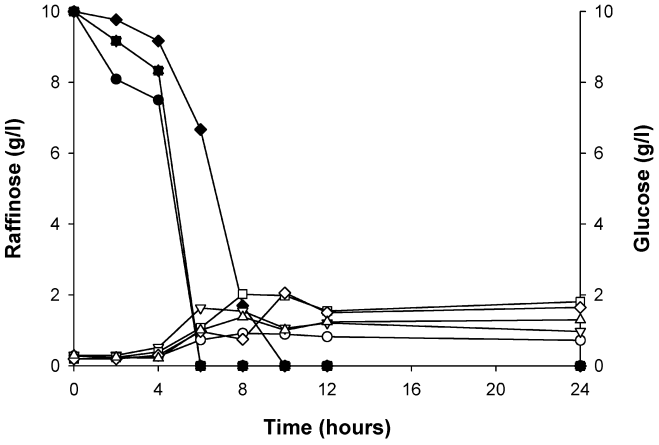

Fig. 2 Residual Raf (*filled symbols*) and glucose (*open symbols*) during growth of *L. fermentum* CRL 722 at pH 6.0 (*circles*), 5.5 (*inverted triangles*), 5.0 (*squares*), 4.5 (*diamonds*) and without pH control (*triangles*)

Effect of pH on the production of organic acids

Acetic and lactic acid production followed the same patterns as growth (Figs. 1, 4). There was a lag phase at pH 4.5, where the maximum organic acid production was reached only after 10 h growth whereas at the other pH values assayed, this maximum was obtained after only 6 h growth (Fig. 4). At the end of the fermentations, lactic acid was present at similar concentrations regardless of pH (between 3.9 and 4.4 g/l for lactic acid). However, mid-

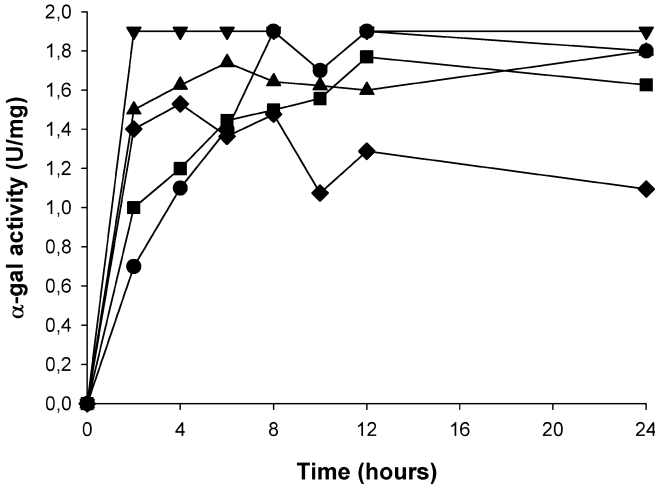

Fig. 3 Specific α-galactosidase (α-gal) activity of *L. fermentum* CRL 722 grown at pH 6.0 (*circles*), 5.5 (*inverted triangles*), 5.0 (squares), 4.5 (*diamonds*) and without pH control (*triangles*)

range pH (5.0 and 5.5) showed a slightly higher production of acetic acid (2.3 and 2.9 g/l, respectively) than pH 6.0, 4.5 and without pH control (1.7, 1.6 and 1.3 g/l respectively). Acetic acid production was fastest at pH 5.0, while at pH 5.5 and without pH control this production was similar but slightly slower than at pH 5.0 (Table 1). The molar ratios of acetic/lactic acids at the point of maximum production (6 h) for pH 5.0, 5.5 and 6.0 were 2.0, 3.4, 3.7, respectively, while at pH 4.5 the maximum production was at 10 h with a molar ratio of 3.2. The molar product yield at the same times at pH 5.0, 5.5, 6.0 and 4.5 was 3.8, 4.5, 3.9 and 3.3, respectively.

Differences between *L. fermentum* CRL 722 and CRL 251

Growth, cell viability, sugar consumption and organic acid production were similar for both strains of *L. fermentum* studied. However, α-gal activity of *L. fermentum* CRL 722 is 1.5 times higher than the CRL 251 strain under the conditions evaluated in this study.

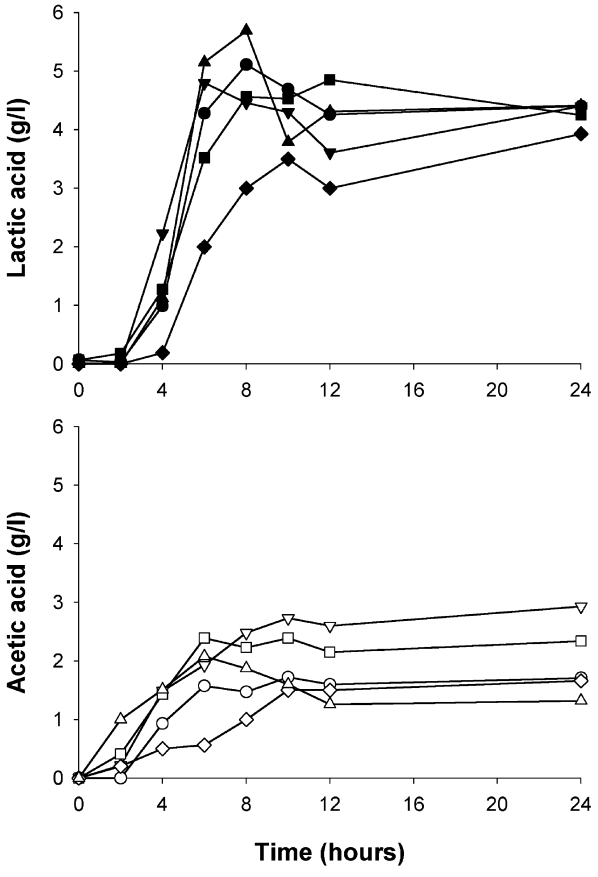

Fig. 4 Acetic (*empty symbols*) and lactic (*filled symbols*) acid production by *L. fermentum* CRL 722 at pH 6.0 (*circles*), 5.5 (*inverted triangles*), 5.0 (*squares*), 4.5 (*diamonds*) and without pH control (*triangles*). Results are expressed as (X–X_0) where X is the concentration at a given time (T) and X_0 at the start time (T_0)

Discussion

L. fermentum is a LAB that has been isolated in a variety of milk and vegetable derived products (Stiles and Holzapfel 1997; Gardiner et al. 2002). These lactobacilli, especially *L. fermentum* CRL 722 and CRL 251, are currently being studied in our laboratory due to their high α-gal activity and possible use in fermented soy products (Garro et al. 1998).

The results obtained (Fig. 1, Table 1) indicate that bacterial growth of *L. fermentum* CRL 722 and CRL 251 was not noticeably affected by the external pH of the fermentation medium in this study except at pH 4.5. This allows us to conclude that these microorganisms are able to adapt to different ambient pH. One important observation was that, even though the growth of these bacteria is independent of the pH, the final concentration is always higher at pH 5.5.

Due to the relative importance of NDO removal in soy products, raffinose consumption of *L. fermentum* CRL 251 and CRL 722 was studied. In our trials, raffinose, which is the principal energy source in MRS fermentation medium, was completely eliminated by both strains studied at all pH values tested (Fig. 2). However, these microorganisms depleted raffinose from the fermentation medium much more slowly at pH 4.5 than at the other pH values tested , probably due to the longer lag phase in growth at the lower pH. The appearance of glucose could be explained by the high α-gal activity of this strain, since this enzyme favours hydrolysis of raffinose, releasing galactose and sucrose, and an invertase (data not shown) will hydrolyse the β-(1,2)-fructofuranoside bond of this disaccharide, releasing fructose and glucose. Similar results were obtained in our laboratory with other microorganisms (Garro et al. 1998, 1999). These findings are in agreement with those reported by Mital and Steinkrauss (1975), where raffinose and stachyose were completely utilised after 12 and 25 h, respectively, by *L. fermenti* in soymilk.

α-Gal production was higher at pH 5.5, 5.0, and without pH control than at pH 4.5 and 6.0 (Fig. 3). This result was once again proportional to final bacterial concentrations after fermentation, since more bacteria are found at pH 5.5 and 5.0 than at the other pH values. Specific α-gal activity was not influenced by pH except at pH 4.5 (Fig. 3). This result suggests that α-gal production is not dependent on growth, since the level of α-gal remained the same during all growth phases after the initial lag. In soymilk, *L. fermentum* displayed highest α-gal activity after 6 h growth, but diminished quickly afterwards (Garro et al. 2003); pH control could be used to conserve this important activity.

By definition, *L. fermentum* is considered a heterofermentative bacterium, capable of fermenting glucose into equimolecular amounts of lactate, carbon dioxide, and acetate or ethanol (Kandler 1983). However, certain modifications to the culture conditions may change the prevalence of these products. In our study, *L. fermentum* was able to produce lactic acid (between 3.9 and 4.4 g/l), with the final concentration not being influenced by pH

(Fig. 4). Production of acetic acid was most affected at pH 5.0 and 5.5 than at the other pH values assayed (Fig. 4). This difference is directly proportional to the total amount of bacteria at each pH. The final concentration of lactic acid was 2–4 times higher than that of acetic acid. Its production was probably limited by medium composition and not by bacterial growth (Montelongo et al. 1993). Acetic and lactic acid are generally recognised as food preservatives since they inhibit the growth of bacteria and fungi (Adams and Hall 1988). Recently, Zaika (2002) published a study showing that organic acids such as lactic and acetic acids can impair the growth of *Shigella*, which is the leading cause of gastrointestinal illness throughout the world. Cabo et al. (2002) showed that acetic acid produced by LAB or present in the culture medium (MRS) can inhibit the growth of four species of fungi of relevance to the cheese industry.

Finally, since all of the parameters studied showed that both strains of *L. fermentum* tested (CRL 722 and CRL 251) were similar except for the levels of α-gal activity, it is safe to assume that these microorganisms are closely related. Since *L. fermentum* CRL 722 has a slightly higher α-gal activity than CRL 251 and all other strains tested in our laboratory (research in progress), the former is an ideal candidate for raffinose removal.

L. fermentum CRL 722 and CRL 251 were able to eliminate raffinose during fermentation in broth cultures under a variety of pH conditions. Since *L. fermentum* is able to grow in soymilk, these LAB could thus be used during soy product fermentation in order to eliminate NDO previous to their consumption (Garro et al. 2003). The results of this study will allow the selection of the optimum growth conditions of *L. fermentum* with elevated and maintainable levels of α-gal to be used in the reduction of NDO in soy products.

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