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# *Lactobacillus reuteri* CRL 1100 as starter culture for wheat dough fermentation

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**Abstract**

The effect of sucrose on the fermentation balance of *Lactobacillus reuteri* CRL 1100 and the invertase activity of this strain in wheat dough and culture medium (MRSs) was evaluated. The enzyme activity was dependent on the environmental pH releasing glucose and fructose from sucrose hydrolysis. Glucose was used as carbon source, while fructose was mainly used as electron acceptor to produce mannitol up to 10 h of fermentation. Thereafter, fructose seemed to be metabolized by the heterofermentative pathway, which determined an increase in the concentration of acetate ($6\,mmol\,l^{-1}$), lactate ($2\,mmol\,l^{-1}$) and ethanol ($1\,mmol\,l^{-1}$) and the lack of mannitol formation after glucose depletion. The fermentation balance of *Lb. reuteri* CRL 1100 during the dough fermentation resulted in lower (63%) ethanol, higher (75%) acetate production and soluble carbohydrates concentrations, like MRSs cultures. This fermentation profile would be important to obtain an optimal growth of yeast and the optimal bread flavor and taste.


*Keywords:* *Lactobacillus reuteri*; Sucrose fermentation; Wheat dough

## 1. Introduction

Lactic acid bacteria (LAB) and yeasts have been used for years in bread making to improve the dough properties (Collar, 1996), organoleptic characteristics (Hammes et al., 1996), nutritional value (Lopez et al., 2001) and the shelf life of bread (Lavermicocca et al., 2000). These benefits result from an appropriate balance between the metabolism of yeasts and LAB strains, for which knowledge of the biochemical characteristics and the technological properties of the microorganisms is required (De Vuyst and Neysens, 2005).

The performance of LAB strains in the food matrix has mainly been studied by characterization of the acidification parameters, such as pH, total titratable acidity, and lactic and acetic acids production during sourdough fermentation (Hammes and Gänzle, 1998). Furthermore, the acetate production by heterofermentative metabolism is of primary importance for the development of flavor and for a proper fermentation quotient (FQ = lactate/acetate molar ratio) (Rosenquist and Hansen, 1998). Production of suitable end products during dough fermentation depends on the availability of soluble carbohydrates (glucose, fructose, maltose, and sucrose), which are in low concentrations ($2–5\,mmol\,100\,g^{-1}$) in wheat flour. The addition of sucrose to wheat dough is a common practice in bakery to improve the yeasts growth (Gobbetti, 1998). Besides, the organic acids production by LAB would contribute to the production of aroma compounds (Meignen et al., 2001).

In sourdoughs, the sugar utilization by LAB strains depends on the microorganism, the type of sugar, the presence of yeasts, and the manufacture conditions (Hammes and Gänzle, 1998; Martinez-Anaya, 2003). On the whole, *Lactobacillus* (*Lb.*) *reuteri* strains isolated from homemade doughs ferment different sugars, e.g., sucrose, melibiose, rafinose, fructose, or glucose (unpublished), while most *Lactobacillus sanfranciscensis* strains isolated from traditional sourdoughs only ferment glucose and maltose (Corsetti et al., 2001). The stability of specific

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microbial associations, e.g., LAB–yeasts in doughs fermentations, mainly depends on noncompetitive conditions for the main carbon source. In this regard, the invertase activity (β-fructofuranosidase enzyme) of the strain *Lb. reuteri* CRL 1100 (Cuezzo de Ginés et al., 2000), an infrequent enzyme in LAB that hydrolyzes sucrose into the moieties fructose and glucose, would be of ecological importance in the sourdough ecosystem.

The present study was undertaken to evaluate the effect of sucrose on the fermentation of wheat dough by *Lb. reuteri* CRL 1100. Due to the complexity of the dough ecosystem, sucrose fermentation was also determined in culture medium.

## 2. Materials and methods

### 2.1. Microorganism and growth conditions

*Lb. reuteri* CRL 1100, previously isolated from homemade wheat dough, belongs to the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA), Tucumán, Argentina. The strain was grown in MRS broth (De Man et al., 1960) at 37 °C for 16 h at least twice prior to experimental use. The cells were harvested by centrifugation at $8000 \times g$ for 10 min at 4 °C (IEC model B-22M, International Equipment Company, USA), washed twice with 10 mmol $l^{-1}$ potassium phosphate buffer (pH 7.0) and suspended in sterile distilled water to give 9 log cfu $ml^{-1}$. The cell suspension was inoculated (2%) in MRS broth in which glucose (2%) was replaced by 1% of glucose (MRSg), fructose (MRSf) or sucrose (MRSs). At 2-h intervals, samples were taken and the cell counts were determined by plate dilution method (proper dilution inoculated in mass) using MRS plus 1.5% w/v agar. The plates were incubated at 37 °C for 48 h and the results were expressed as log cfu $ml^{-1}$. The specific growth rate ($\mu$) was calculated from the slope of a semi-logarithmic plot of cell viability versus time. End products (organic acids and alcohols) and sugar concentrations were determined by enzymatic methods and HPLC.

Cells from cultures in MRSs, MRSf, and MRSg were harvested by centrifugation ($8000 \times g$ for 15 min at 4 °C), washed twice with 10 mmol $l^{-1}$ potassium phosphate buffer (pH 7.0) and suspended (30%, w/v) in 200 mmol $l^{-1}$ acetate buffer (pH 5.0). The cells were disrupted (French Pressure Cells Thermo Spectronic, USA) and centrifuged ($10{,}000 \times g$ for 15 min at 4 °C); the supernatant (CE, cell-free extract) obtained was used for the enzyme determination.

### 2.2. Invertase activity

The enzymatic activity was determined with enzymatic kits (Boehringer-Manheim, Germany) by measuring the glucose released. The assay mixture contained 20 µl of 1000 mmol $l^{-1}$ sucrose in 0.2 mmol $l^{-1}$ acetate buffer (pH 5.0), and 50 µl of CE to a final volume of 0.2 ml. The reaction was carried out at 37 °C for 15 min and stopped by heating the mixture in boiling water for 10 min. One unit invertase activity (U) was defined as the amount of enzyme required to produce 1 µmol glucose per ml per min. Specific activity was expressed as U per mg of protein (U $mg^{-1}$ protein).

### 2.3. Protein determination

The protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

### 2.4. Carbohydrates, organic acids, and ethanol determinations

Glucose, sucrose, maltose, and acetic acid were determined by enzymatic methods (Boehringer-Manheim). Mannitol, fructose, ethanol, and lactic acid were determined by HPLC (ISCO 2350 model) using an ion-exclusion Aminex 87H column (300 mm × 78 mm, Bio Rad) under the following conditions: mobile phase (2.5 mmol $l^{-1}$) $H_2SO_4$, flow rate 0.6 ml $min^{-1}$, column temperature 45 °C. A refractive index (Knauer K-2301) and UV (210 nm) (ISCO V 4 model) detectors were used for determining fructose, mannitol, and ethanol, and for detecting lactic acid, respectively, connected to a software (Peak Simple II) for data analyses.

### 2.5. Dough fermentation

Commercial wheat flour (000 type) containing (%, w/w dry basis) 71.1 carbohydrates, 2.8 fiber, 10 protein, and 1.3 fat was used to prepare the doughs by mixing flour (100 g), 45 ml water, and 15 ml *Lb. reuteri* CRL 1100 cell suspension (9 log cfu $ml^{-1}$) ($D_{1100}$). When needed, sucrose was added to a final concentration of 1.97 mmol 100 $g^{-1}$ dough ($DS_{1100}$). A dough (Dc) without *Lb. reuteri* prepared under the same conditions as $D_{1100}$ and $DS_{1100}$ was used as control. Cycloheximide (0.01 mg $g^{-1}$), a yeast inhibitor, was added to doughs to avoid the growth of indigenous yeasts.

The dough fermentations were carried out in flasks (400 $cm^3$) at 30 °C and samples were drawn aseptically at 0, 6 and 24 h of fermentation. Cell counts were determined by plate dilution method in MRS agar and the plates were incubated at 37 °C for 48 h. The fermented dough samples (10 g) were homogenized with distilled water (90 ml) in a homogenizer (The Virtis Company, Gardiner, NY) and centrifuged at $8000 \times g$ for 10 min. The pH value, total titratable acidity, soluble carbohydrates, organic acids, and alcohols (ethanol and mannitol) concentrations were determined in the dough aqueous extracts supernatants.

### 2.6. Statistical analysis

The results of three independent assays performed with three replicates each were expressed as mean ± S.D. Data

were compared by the one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. The statistical significance ($P<0.05$) was determined with the Minitab-12 software.

## 3. Results

### 3.1. Growth, enzymatic activities, and end products

*Lb. reuteri* CRL 1100 grown in MRSg and MRSs broths showed differences in the growth rate and fermentation products. The highest $\mu$-value was achieved in MRSs ($\mu = 0.91\,h^{-1}$) compared to MRSg ($\mu = 0.77\,h^{-1}$), although similar colony counts (9.7 and 9.2 log cfu $ml^{-1}$, respectively) and sugar consumption (>95%) were obtained at the beginning of the stationary phase (6 h fermentation) in both culture media (Fig. 1A and B). In contrast, the microorganism was not able to use fructose as the sole carbon source (MRSf), no growth being detected after 14 days incubation (data not shown).

Table 1 shows the fermentation balance of *Lb. reuteri* CRL 1100 after 6 and 24 h incubation. Lactate was the main end product (ca. 80% carbon recovery), although the amount (45 mmol $l^{-1}$) and rate (7.3 mmol $h^{-1}$) of lactate production were higher in MRSg compared to MRSs (27 and 5.1 mmol $h^{-1}$, respectively) during the first 6 h of fermentation (Fig. 2A and B). The ratio of acetate to ethanol was 2.2 and 0.15 for MRSs and MRSg cultures, respectively, indicating that more acetate and less ethanol were produced in the presence of sucrose. This behavior was maintained throughout the fermentation period (24 h).

The release of fructose (5.6 mmol $l^{-1}$) and glucose (1.5 mmol $l^{-1}$) was only observed in MRSs cultures due to the invertase activity of *Lb. reuteri* CRL 1100, which was maximal (2 U $mg^{-1}$ proteins) after 3 h of fermentation. However, the enzyme activity decreased by 50% 1 h later when the culture pH decreased to 4.9 (Figs. 1A and 2A). The mannitol production (16.1 mM) by MRSs cultures was correlated to the fructose depletion (about 70%) up to 6 h of fermentation (Figs. 1A and 2A).

A certain uncoupling between sucrose consumed and end-products formation was also observed in 24-h-old MRSs cultures: the amount of lactate (31.1 mmol $l^{-1}$), acetate (26.7 mmol $l^{-1}$) and ethanol (11.9 mmol $l^{-1}$) exceeded the theoretical yield expected from the glucose moiety metabolism at 24 h of fermentation (Fig. 2A). The glucose and fructose moieties were almost completely consumed at the end of the fermentation period (24 h).

### 3.2. Dough fermentation

The hydrolysis of sucrose, the release of glucose and fructose, and the lactic and acetic acids production were significantly ($P<0.05$) higher in $D_{1100}$ and $DS_{1100}$ doughs with respect to Dc (Table 3). The addition of cycloheximide to doughs affected neither the indigenous

Table 1
Fermentation balance of *Lb. reuteri* CRL 1100 in MRSs and MRSg media

| Parameters | MRSs | | MRSg | |
|---|---|---|---|---|
| | 6 h | 24 h | 6 h | 24 h |
| Production (mmol $l^{-1}$) | | | | |
| Lactic acid | 27.1±0.5 | 31.1±4.2 | 45.0±0.2 | 51.0±4.3 |
| Acetic acid | 19.2±0.3 | 26.7±0.4 | 4.7±0.2 | 5.2±0.3 |
| Ethanol | 8.7±0.1 | 11.9±2.8 | 32.2±0.2 | 34.3±4.2 |
| Mannitol | 16.1±2.7 | 18.1±2.7 | 0.0±0.0 | 0.00±0.0 |
| Sugar consumption (mmol $l^{-1}$) | | | | |
| Sucrose | 27.1±0.0 | 27.8±2.8 | – | – |
| Glucose | – | – | 54.4±0.0 | 55.1±0.1 |
| Residual sugar (mmol $l^{-1}$) | | | | |
| Glucose | 0.9±0.1 | 0.0±0.0 | 1.1±0.1 | 0.5±0.1 |
| Fructose | 1.5±0.3 | 0.0±0.0 | – | – |

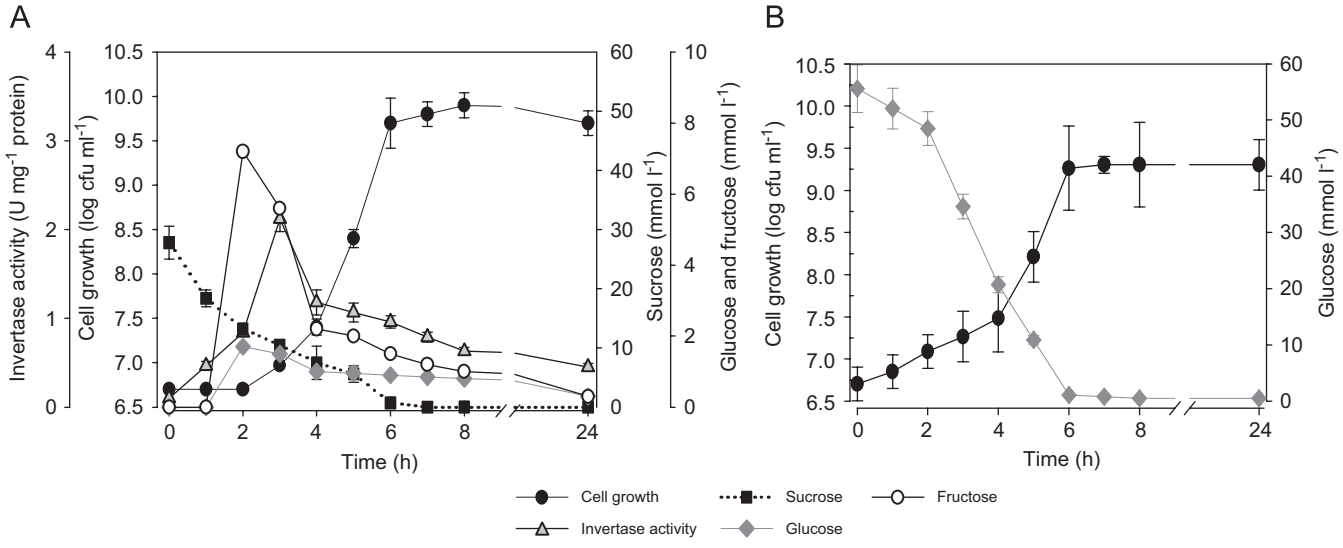


Fig. 1. Growth and invertase activity of *Lb. reuteri* CRL 1100 in MRSs (A) and MRSg (B).

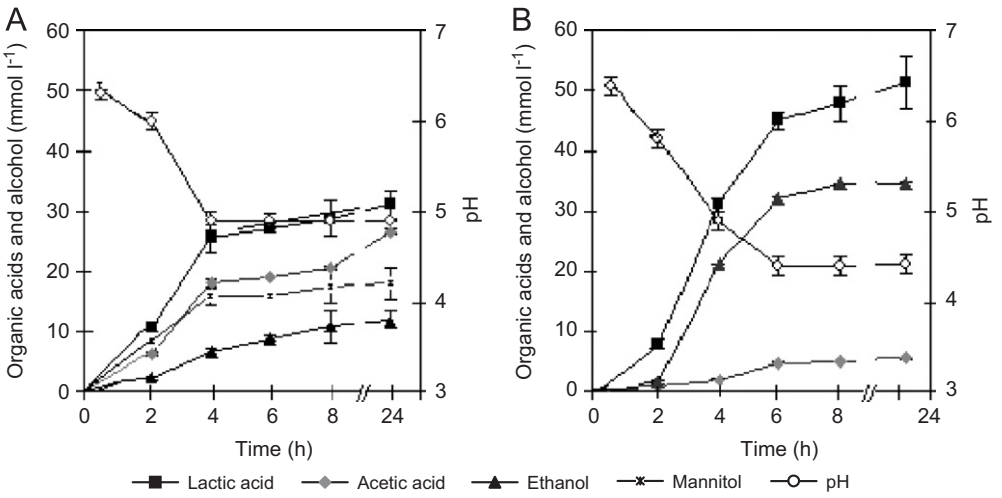


Fig. 2. Organic acids and alcohols production during *Lb. reuteri* CRL 1100 growth in MRSs (A) and MRSg (B).

Table 2
Cell growth and acid developed by *Lb. reuteri* CRL 1100 in doughs with and without sucrose

| Dough | Cell count (log cfu $g^{-1}$ dough) | | | pH | | | Titratable acidity (ml of NaOH 0.1 N $g^{-1}$ dough) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 6 h | 24 h | 0 h | 6 h | 24 h | 0 h | 6 h | 24 h |
| Dc[a] | 2.9±0.2 | 3.9±0.1 | 4.6±0.3 | 6.2±0.2 | 6.0±0.1 | 5.7±0.2 | 0.2±0.0 | 0.2±0.0 | 0.3±0.2 |
| $D_{1100}$[b] | 6.6±0.1 | 7.9±0.3 | 9.4±0.4 | 6.2±0.3 | 4.7±0.3 | 3.9±0.3 | 0.2±0.0 | 0.5±0.0 | 1.1±0.0 |
| $DS_{1100}$[c] | 6.6±0.2 | 8.1±0.3 | 10.5±0.3 | 6.2±0.2 | 4.6±0.2 | 3.8±0.3 | 0.2±0.0 | 0.6±0.0 | 1.4±0.0 |

[a]Dc: dough control.
[b]$D_{1100}$: dough fermented by *Lb. reuteri* CRL 1100.
[c]$DS_{1100}$: sucrose-supplemented dough.

Table 3
Fermentation balance of *Lb. reuteri* CRL 1100 during dough fermentation

| Parameters | Dc[a] | | $D_{1100}$[b] | | $DS_{1100}$[c] | |
|---|---|---|---|---|---|---|
| | 6 h | 24 h | 6 h | 24 h | 6 h | 24 h |
| Production (mmol 100 $g^{-1}$) | | | | | | |
| Lactic acid | 0.0±0.0 | 0.02±0.1 | 2.5±0.4 | 7.7±0.4 | 3.5±0.3 | 11.6±0.3 |
| Acetic acid | 0.0±0.0 | 0.05±0.2 | 1.1±0.3 | 1.6±0.0 | 1.4±0.2 | 2.8±0.0 |
| Ethanol | 0.0±0.0 | 0.01±0.1 | 0.0±0.0 | 4.1±0.0 | 0.0±0.0 | 2.6±0.0 |
| Consumption (mmol 100 $g^{-1}$) | | | | | | |
| Sucrose | 0.1±0.2 | 0.2±0.2 | 0.6±0.2 | 1.0±0.1 | 3.4±0.1 | 3.5±0.2 |
| Increases (mmol 100 $g^{-1}$) | | | | | | |
| Glucose | 0.5±0.3 | 1.1±0.1 | 1.0±0.1 | 2.6±0.2 | 1.8±0.1 | 2.8±0.2 |
| Fructose | 0.1±0.0 | 0.1±0.0 | 0.5±0.3 | 0.8±0.2 | 2.0±0.1 | 3.1±0.1 |
| FQ[d] | 0.0±0.0 | 0.4±0.1 | 2.3±0.3 | 4.8±0.2 | 2.5±0.1 | 4.1±0.2 |

Mean±S.D. of three independent essays.
[a]Dc: dough control.
[b]$D_{1100}$: dough fermented by *Lb. reuteri* CRL 1100.
[c]$DS_{1100}$: dough with sucrose fermented by *Lb. reuteri* CRL 1100.
[d]FQ = lactate/acetate molar ratio.

LAB (2.9 log cfu $g^{-1}$) nor growth of *Lb. reuteri* CRL 1100 but inhibited the indigenous yeast microflora (Table 2).

The presence of sucrose ($DS_{1100}$) enhanced the *Lb. reuteri* CRL 1100 growth in more than one log unit (10.5 log cfu $g^{-1}$ dough) during dough fermentation. A 1.5 and 1.7 folds higher lactic and acetic acids production, respectively, and a lower (1.6 folds) ethanol formation were determined in $DS_{1100}$ with respect to $D_{1100}$ (Table 3). The

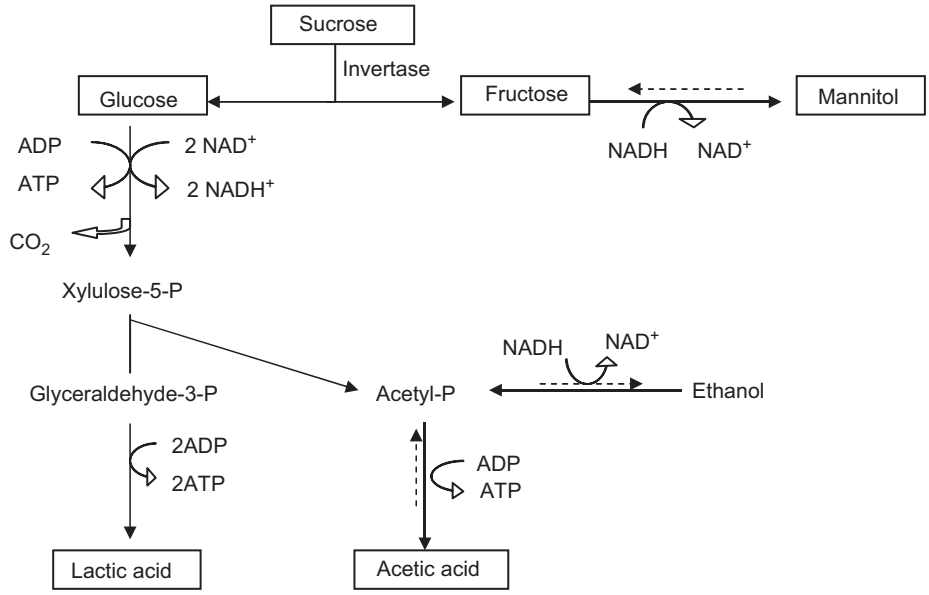


Fig. 3. Fermentation of sucrose by *Lb. reuteri* CRL 1100.

pH decreased from 6.2 to 3.8 in both types of doughs, while titratable acidity was higher (27%) in $DS_{1100}$ compared to $D_{1100}$ after 24 h (Table 1).

The invertase activity of *Lb. reuteri* CRL 1100 hydrolyzed 0.6 and 3.4 mmol 100 $g^{-1}$ of sucrose in $D_{1100}$ and $DS_{1100}$, respectively, releasing glucose (1.8 mmol 100 $g^{-1}$) and fructose (2 mmol 100 $g^{-1}$) during the first 6 h of fermentation; these monosaccharides were absent in the dough before fermentation (Table 3). The concentration of maltose (2.4 mmol 100 $g^{-1}$) increased by 1.4 (Dc), 1.9 ($D_{1100}$), and 2.5 ($DS_{1100}$) folds after 24 h of fermentation.

The addition of sucrose to the wheat doughs increased the acetic and lactic acid production by *Lb. reuteri* CRL 1100 without changing the FQ (FQ = lactate/acetate molar ratio). However, the FQ (2.3) was clearly enhanced in fermented doughs ($D_{1100}$ and $DS_{1100}$) compared to Dc (FQ = 0.02) after 6 h of fermentation.

## 4. Discussion

Sourdoughs contain a diverse microbiota including LAB and yeasts (De Vuyst and Neysens, 2005). *Lb. reuteri*, an obligated heterofermentative LAB, is a predominant species in type II sourdough fermentations, in which yeasts are generally added as leavening agents (Müller et al., 2000; Dal Bello et al., 2005). The yeast growth mainly depends on the ability to ferment sucrose through the invertase activity (Verstrepen et al., 2004). Such β-fructofuranosidase enzyme is rare in LAB (Tieking et al., 2003).

In this study, the ability of *Lb. reuteri* CRL 1100 to ferment sucrose (due to the invertase activity) was put in evidence both in wheat doughs and culture medium. This activity was not detected in MRSg broth indicating that it is inducible by sucrose in contrary to the yeasts invertase (Ostergaard et al., 2000; Alves Araújo et al., 2007). The *Lb. reuteri* CRL 1100 invertase was dependent on the environmental pH and the concentration of the hydrolysis products (glucose and fructose). A decrease ($P<0.05$) in activity was found at concentrations of glucose and fructose higher than 1.5–2.0 mmol 100 $g^{-1}$ dough and at pH values below 5.0, in agreement with Cuezzo de Ginés et al. (2000).

The glucose and fructose moieties released from the sucrose hydrolysis were metabolized by different ways. Glucose was fermented by the phosphoketolase pathway, while fructose was mainly used as electron acceptor to produce mannitol, thus changing the metabolism from the ethanol to the acetate routes (Fig. 3). The stoichiometric mannitol production at a ratio of 1 mannitol:1 fructose obtained up to 6 h of fermentation indicates that *Lb. reuteri* CRL 1100 used fructose mainly as an external electron acceptor. The reduction of fructose to mannitol allows the regeneration of $NAD^+$ and the cells to gain ATP via the acetate kinase reaction by leading acetylphosphate to acetate instead of ethanol (Stolz et al., 1995). The use of fructose as electron acceptor was reported for *Leuconostoc mesenteroides* (Erten, 1998) and *Lb. sanfranciscensis* (Korakli and Vogel, 2003).

The capacity of fructose to regenerate $NAD^+$ was also confirmed by the higher acetate and the lower ethanol production in MRSs compared to MRSg cultures. The shift in the metabolism from the ethanol to the acetate routes (Fig. 3) produces one mole of ATP per mole of glucose consumed (Stolz et al., 1995), a fact that would explain the higher cell viability of *Lb. reuteri* CRL 1100 in MRSs medium (Fig. 1A). Like MRSs cultures, the fermentation balance of *Lb. reuteri* CRL 1100 in $DS_{1100}$ also resulted in lower (63%) ethanol and higher (75%) acetate production. Although mannitol could not be detected in the doughs, these results would indicate that

fructose is being used as electron acceptor during dough fermentation (Gobbetti et al., 1995; Stolz et al., 1995).

After sucrose hydrolysis, the ratio of fructose to glucose in MRSs cultures was 1.7. Under this condition of fructose excess, about 60% of the sugar is used as electron acceptor and large amount of mannitol (16.1 mmol $l^{-1}$) is produced. However, the depletion of both glucose and fructose after 10 h of fermentation would induce *Lb. reuteri* CRL 1100 to ferment fructose by the heterofermentative pathway (Fig. 1A). The steady formation of organic acids and ethanol between 10 and 24 h, which exceeded the theoretical yield from glucose, and the lack of mannitol formation in this period would support this observation. Zaunmuller et al. (2006) reported the presence of two different routes for fructose metabolism (phosphoketolase/mannitol pathway) in *L. mesenteroides* and *Oenococcus oeni*, which would be advantageous for the cells by increasing the ATP yield.

The hydrolysis of starch by indigenous amylases of flour or alpha-amylase activity of the microorganisms can enhance the maltose concentration during the dough fermentation (Mathewson, 2000). LAB with amylase activity, such as *L. mesenteroides* BELL 17 (Lefebvre et al., 2002), *Lactobacillus fermentum* Ogi E1 (Calderon Santoyo et al., 2003) and *Lactobacillus plantarum* A6 (Sanni et al., 2002), have been isolated from the tropical fermented amylaceous foods. The increase of maltose in $D_{1100}$ and $DS_{1100}$ may be attributed to alpha-amylase activity of *Lb. reuteri* CRL1100 (unpublished), which sequentially is fermented by this strain giving glucose (Ginés et al., 1997). Maltose-negative yeasts can further use glucose during the dough fermentation (Gobbetti, 1998).

*Lb. reuteri* CRL 1100 would be useful as starter culture for type II sourdough fermentation. Its invertase activity would act in a synergic or a complementary way together with the yeasts invertase increasing the sugar availability during wheat dough fermentation. This increase in soluble carbohydrates might also contribute, through the Maillard or other amino acid reactions, to the optimal bread flavor and taste (Martinez-Anaya et al., 1990; Onno and Roussel, 1994). Besides, the doughs fermented with *Lb. reuteri* CRL 1100 had optimal FQ, a criterion of bread quality (Schieberle, 1996; Rosenquist and Hansen, 1998).

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