Urease production by Streptococcus thermophilus

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Abstract

In order to identify potential alternative sources of urease for the removal of urea from alcoholic beverages, 205 strains of lactic acid bacteria belonging to 27 different species were screened for urease production. Only Streptococcus thermophilus produced urease. Cell permeabilization with toluene allowed to increase activity significantly. Optimal pH for urease activity in whole and permeabilized cells and of cell free extracts differed slightly, but was in the range 6.0–7.0. Significant activity was retained at pH 3.0 and 8.0, and, for cell free extracts, at pH 4.0 in the presence of ethanol. Urease production was evaluated in fermentations with pH control (5.25–6.5) and without pH control. Very little urease was produced in absence of urea, which at 5 g/l slowed growth significantly in fermentations without pH control, but prevented a decrease in pH below 5.1 and resulted in higher final biomass. Optimal pH for growth was between 6.0 and 6.5 but specific urease activity was higher for fermentations at low pH at the beginning of the exponential phase. However, a higher total urease activity was obtained at pH 6.0 and 6.5 because of higher biomass. Potential technological applications of urease production by S. thermophilus are discussed.

Keywords: Urease; Streptococcus thermophilus; Cell permeabilization; Fermentation; Urea in alcoholic beverages

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.15) catalyzes the hydrolysis of urea to ammonia and carbamate, which spontaneously hydrolyzes to give a further molecule of ammonia and carbonate. Urease production is widespread in prokaryotes: all bacterial ureases are nickel metalloproteins and share significant sequence homology among them and with eucaryotic ureases (Mobley et al., 1995). Regulation of the expression of the urease operon varies in different species. In oral lactic acid bacteria (LAB), like Streptococcus salivarius, it is affected by the presence of urea, pH and sugar availability (Chen and Burne, 1996; Chen et al., 1998). This is in good agreement with the dual role of ureases in response to acid stress and nitrogen metabolism (Chen et al., 2000). In the human pathogen Helicobacter pylori urease is the most important factor contributing to the maintenance of intracellular and periplasmic pH near neutrality under acid stress (Pflock et al., 2006) and is essential for colonization of several animal models.

Acid ureases with pH optima between 2.0 and 4.5 (Mobley et al., 1995) are produced by some species of lactic acid bacteria (Kakimoto et al., 1989, 1990a, b; Yamazaki et al., 1990) and corynebacteria (Miyagawa et al., 1999) and are potentially useful for the reduction of urea content in wine and in other alcoholic beverages, like sake, thus reducing the risk of accumulation of ethyl carbamate (ETC; Kodama, 1996; Butzke and Bisson, 1997; Fidaleo et al., 2006). ETC is a potential carcinogenic agent which is spontaneously produced in wine and other alcoholic beverages in reactions between urea or other compounds containing carbamyl groups (citrulline and carbamyl phosphate) and ethanol (Ough et al., 1988). An acid urease produced by Lactobacillus fermentum is commercialized by several producers (Fidaleo et al., 2006) and the European Commission has expressed a positive opinion on its use in wine (http://europa.eu.int/comm/food/fs/sc/scr/out19_en.html).

Among urease-producing species, Streptococcus thermophilus is particularly interesting. It is a moderately...
thermophilic, food-grade, industrially important microorganism which is used as a starter in the production of fermented milks and cheese and is commonly isolated from artisanal cheeses and natural starter cultures (Parente and Cogan, 2004). Urease production is common in *S. thermophilus* (Mora et al., 2002) and, although it is important for acid stress resistance (Mora et al., 2002) and, although it is important for acid stress resistance (Mora et al., 2005), it slows pH decrease in milk and cheese due to the production of ammonia (Mora et al., 2004; Monnet et al., 2004). The urease operon of *S. thermophilus* has been recently characterized (Mora et al., 2004) and it has been found to be similar to that of the taxonomically related *S. salivarius* (Chen and Burne, 1996; Chen et al., 1998).

In order to find alternatives to the commercially available acid ureases, we screened lactic acid bacteria obtained from several food sources. A preliminary study was then conducted on urease-producing *S. thermophilus* to obtain information on conditions for urease production and urease activity as a function of pH.

2. Methods

2.1. Microbial strains

Strains of lactic acid bacteria (205) isolated from milk and dairy products (130), from sourdoughs (41) or obtained from culture collections (34), belonging to different species of the genera *Enterococcus* (3 *E. faecalis* strains, 2 *E. faecium*, 2 *E. pseudoaoactum*), *Lactobacillus* (1 *L. alimentarius*, 2 *L. brevis*, 7 *L. casei*, 2 *L. corynformis*, 10 *L. curvatus*, 1 *L. farciminis*, 3 *L. fermentum*, 11 *L. helveticus*, 22 *L. paracasei*, 6 *L. paraplantarum*, 2 *L. pentosus*, 25 *L. plantarum*, 6 *L. rhamnosus*, 2 *L. sakei*, 1 *L. zeae*), *Lactococcus* (29 *Lc. lactis*), *Leuconostoc* (9 *Le. mesenteroides*), *Pediococcus* (1 *P. acidilactici* and 1 *P. pentosaceus*), *Streptococcus* (5 *S. macedonicus*, 43 *S. thermophilus*, 5 *S. salivarius*), *Weissella* (3 *W. cibaria*, 1 *W. confusa*, 1 *W. viridescens*) were used in this study. All strains were maintained as freeze-dried stocks in reconstituted (11%, w/v) Skim Milk (Oxoid Ltd., Basingstoke, Hampshire, England), containing 0.1% (w/v) ascorbic acid (Riedel-de Haën, Sigma-Aldrich, Milan, Italy) and their identity was confirmed by SDS–PAGE of whole-cell proteins using the procedure described in Piraino et al. (2006).

2.2. Screening for urease activity

All strains were grown in modified MRS broth (Oxoid) (MRSU) containing urea (5 g/l, Riedel-de Haën, Sigma-Aldrich, Milan, Italy) and nickel sulphate (0.05 g/l, Carlo Erba, Milan, Italy), both added as filter (0.2 μm) sterilized solutions, for 16 h at 37 °C (*Enterococcus*, *L. fermentum*, *L. paracasei*, *L. zeae*), 42 °C (*L. helveticus*, *Streptococcus*) or 30 °C (all other species). Cells were harvested by centrifugation (12,000g, 5 min, 4 °C), washed twice and resuspended in sterile 0.85% (w/v) NaCl, and used in the phenol red assay (Mora et al., 2004). Colour change was evaluated after incubation (2 h) at 37 °C.

2.3. Urease activity of whole cells and cell-free extracts

After cultivation in MRSU for 16 h at 42 °C in anaerobiosis (Genex-box jars with Generbag anaer, bio-Mérieux, Marcy l’Étoile, France), biomass of urease-positive *S. thermophilus* strains was collected as described above, washed twice in 50 mmol/l potassium phosphate buffer, pH 7 (PB7), resuspended in the same buffer and standardized to an optical density at 650 nm (OD650) of 1. Whole cells were used as such or lysed by sonication in ice for 5 min (power MS 73/D, cycle 50%, pulse 10 s) using a Bandelin Sonopuls HD 200 (Bandelin Electronics, Berlin, Germany) apparatus with an UW 200 probe. Cells and cell debris were removed by centrifugation (12,000g, 5 min, 4 °C) and protein content of cell-free extracts (CFEs) was measured using the Bradford reagent (Sigma) against a calibration curve obtained with bovine serum albumin, fraction V (Sigma). Cell suspension or CFEs (50 μl) were mixed with 50 μl of 200 mmol/l urea in PB7 and incubated at 37 °C for 15 min. The reaction was stopped by addition of 10 μl of trichloroacetic acid (AppliChem Inc., Cheshire, CT, USA; TCA, 33%, w/v). N-NH4+ content was measured (after removal of cells by centrifugation, if applicable) using a colorimetric method (Strickland and Parson, 1968). A standard curve was obtained with NH4Cl in the range 0.1–20 mg N-NH4+ /l. One μkat (μkat) was defined as the amount of enzyme required to release 1 μmol N-NH4+ /s.

2.4. Effect of pH on urease activity of whole and permeabilized cells and on CFEs

Permeabilized cells of *S. thermophilus* Y3 were obtained using a modification of the technique described by Krishnan et al. (2000). Washed standardized (OD650 = 1) cells were resuspended in PB7 and an equal volume of 10% (v/v in water) toluene was added. The suspension was incubated for 2 min at 30 °C with shaking (100 rpm, Unimax 2010, Heidolph Instruments, Milan, Italy). The permeabilized cells were recovered by centrifugation (12,000g, 5 min), washed twice in PB7 and used immediately for urease assay. Fifty microliters of whole or permeabilized cell suspensions or CFEs were added to 50 μl of 50 mmol/l sodium citrate buffers (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5) or 50 mmol/l potassium phosphate buffers (pH 6.0, 6.5, 7.0, 7.5, 8.0) containing 200 mmol/l urea. After incubation at 37 °C for 15, 30 and 60 min the reaction was stopped by addition of 10 μl of 33% (w/v) TCA. Urease activity was measured as described above.

2.5. Urease production in stirred tank fermentations

Fermentations were carried out in MRSU in 1 l (working volume), stirred (100 rpm, using magnetic bars) with
fermenters (Applikon, Schiedam, the Netherlands) at 42 °C without pH control (initial pH 6.8) and at controlled pH (5.25, 5.5, 6.0, or 6.5, by automatic addition of sterile 6 mol/l NaOH or 2 mol/l HCl), using a 5% (v/v) inoculum with an overnight MRSU culture. Fermentation was carried in MRS without urea as a control, inoculated (5%, v/v) with an overnight MRS culture. pH, temperature, amount of base and acid were recorded at 10 min intervals using Bio-Xpert software (version 1.1, Applikon). Dissolved oxygen concentration was neither controlled nor measured. Samples (10 ml) were obtained aseptically and used to measure biomass, urease activity, N-NH₄⁺ and glucose concentration. Biomass were estimated indirectly from OD at 650 nm using a standard curve. A linear relationship (R² > 0.99) was found between cell dry weight or cell numbers (estimate by plate counts in MRS agar) and OD₆₅₀ in the range 0.02–0.50. Urease activity and N-NH₄⁺ concentration were measured, respectively, on permeabilized cells and in supernatants only as described above. Residual concentration of glucose in the supernatants was measured by DNSA method (Miller, 1959). Growth was modeled using a structured dynamic model with DMFit v 2.0 (Baranyi and Roberts, 1994).

2.6. Comparison between urease activity of S. thermophilus Y3 and a commercial urease preparation

The urease activities of whole, permeabilized cells and cell lysates of S. thermophilus Y3 collected during stationary phase in the fermentation in MRSU at pH 6.0, were compared with the activity of the commercial acid urease preparation (Enzeco®, New York, NY, USA) from L. fermentum. Urease activity was measured after 30 min incubation at 37°C as described above in 50 mmol/l potassium phosphate buffer pH 6.0, and in 100 mmol/l sodium acetate buffer, pH 4.0, containing 12.5% (v/v) ethanol. Both buffers contained a final concentration of 200 mmol/l urea.

3. Results

Out of 205 strains belonging to 27 species of LAB screened for urease production only 34 S. thermophilus strains (79% of the strains belonging to this species) scored positive. Urease activity was measured for whole cells, culture broth supernatants and cell lysates of the urease-positive strains in a quantitative assay. The activity was always intracellular (no measurable activity was found in cell-free supernatants; data not shown). A large variability was observed and urease activity of whole cells and lysates were not significantly correlated (Fig. 1). One of the fastest growing strains, which showed a urease activity of both cells and lysates higher than the 75th percentile, S. thermophilus Y3, was chosen for further study.

The activity of whole and toluene permeabilized cells and cell-free extracts was evaluated as a function of pH (3.5–8.0). Activity was measured after 15, 30 and 60 min. The results after 60 min incubation are shown in Fig. 2 because this provided the highest values for whole and permeabilized cells, while differences in activity with cell lysates were smaller. No change in pH of the buffers was observed even after 60 min incubation values of apparent optimal pH were 6.0, 7.0 and 6.5 for whole cells, permeabilized cells and CFEs, respectively. At pH 3 values of urease activity were 22%, 37% and 20% of that
measured at optimal pH for whole, permeabilized cells and CFEs, respectively. At pH 8.0 values of the residual activity were 63%, 58% and 70%, of the optimum activity, respectively. To evaluate if buffer composition may have affected the results, we compared the activity of cell lysates at pH 6.0 in the following buffers: 50 mmol/l potassium phosphate, 50 mmol/l sodium phosphate-citrate, 50 mmol/l MOPS and no significant differences were found among the urease levels measured in these buffers (data not shown).

Growth and urease activity of *S. thermophilus* Y3 were initially compared in M17 with 0.5% glucose or lactose and in MRS, the substrate used in the screening. Urea and nickel sulfate were used at 5 and 0.05 g/l, respectively. Although growth was best in lactose M17, urease production was always higher in MRS (from 14% to 100%, depending on time of sampling), which was therefore used in all further experiments. Growth (Fig. 3a) and urease activity of permeabilized cells of *S. thermophilus* (Fig. 3b) were evaluated in MRS without (MRS) and with urea (MRSU) in fermentations without pH control and in MRSU with pH control (5.25–6.5). The scale of the graphs is limited to 12 h since growth ceased before this time for all fermentations except that carried out at pH 5.25. In the fermentation without pH control in MRS, growth ceased after 3.5 h when biomass concentration (as CDW cell dry weight) was 0.83 g/l and pH was 4.7. A limited cell lysis followed, and pH decrease and glucose consumption continued until the fermentation was stopped. Final pH and glucose concentration (Fig. 3c) were 4.44 and 2.6 g/l, respectively. Specific urease activity was always below 0.04 μkat/g CDW. No net ammonia production and consumption was observed and ammonia concentration was always close to the initial concentration present in

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**Fig. 3.** Growth (a), urease activity of toluene-permeabilized cells (b), residual glucose (c) and net N-NH₄⁺ release (d) in fermentations with *S. thermophilus* Y3 in MRS broth without pH control (○), and in MRSU (see Section 2) without pH control (△), and with pH controlled at 6.5 (□), 6.0 (●), 5.5 (▲) or 5.25 (■) by automatic addition of NaOH and HCl. In (a) continuous lines show the predicted growth with the Baranyi and Roberts (1994) model (see text) calculated with DMFit v 2.0. In all other graphs continuous curves were obtained by distance-weighted least-square smoothing.
MRS (0.266 g N-NH$_4^+$ /l, Fig. 3d). When urea was added, pH decreased to 5.2 in 5 h and then stabilized. The stationary phase was reached in 7 h and final biomass concentration was 1.27 g/l (1.5 times higher than without urea) while maximum cell number was 8.7 x 10$^8$ cfu/ml (2.7 times higher than that without urea). Final pH was 5.3 and glucose was completely consumed. Release of ammonia paralleled cell growth, and final N-NH$_4^+$ concentration was consistent with the hydrolysis of at least 16% of the urea. However, growth in MRSU was significantly slower than in MRS: maximum specific growth rate ($\mu_{max}$) in MRS ad MRSU estimated with the D-model (see Section 2) were 1.80 ± 0.10 h$^{-1}$ (estimate ± standard error) and 0.82 ± 0.03 h$^{-1}$, respectively. The D-model calculated on CDW values provided an excellent fit for all fermentations ($R^2 > 0.99$) and the estimated $\mu_{max}$ values were within 5–15% of those calculated from cell counts or estimated by a simple linear fit of the linear portion of the growth curve (data not shown). A linear relationship ($R^2 > 0.99$) existed between biomass and glucose consumption until cells entered in the stationary phase, with calculated biomass yields ($Y_{X/S}$) of 0.097 ± 0.006 and 0.092 ± 0.0008 g CDW/g glucose for growth in MRS and MRSU without pH control, respectively. However, upon entry in the stationary phase, cell yield rapidly decreased due to continued glucose consumption and to the onset of autolysis. To evaluate the effect of pH on urease production, further fermentations were carried out at controlled pH (6.5, 6.0, 5.5, 5.25) with 5 g/l urea. The results are shown in Fig. 3. Growth was faster at pH 6.0, with a calculated $\mu_{max}$ of 1.12 ± 0.03 h$^{-1}$, and a maximum biomass concentration of 2.15 g CDW/l. Biomass yield ($Y_{X/S}$, 0.111 ± 0.004 g/g) and maximum viable cell number (5.1 x 10$^9$ cfu/ml) were also highest in fermentation at pH 6.0. Growth was only slightly slower at pH 6.5 ($\mu_{max}$ 0.98 ± 0.02 h$^{-1}$), with comparable yield (0.099 ± 0.009 g/g) but lower maximum biomass concentration and viable cell number (1.5 g CDW/l, and 1.4 x 10$^9$ cfu/ml, respectively). Growth was significantly inhibited at pH 5.5 and 5.25 ($\mu_{max}$ 0.45 ± 0.01 and 0.37 ± 0.02 h$^{-1}$, respectively), with reduced maximum biomass concentration (1.52 and 0.80 g CDW/l), and viable cell numbers (2.30 x 10$^9$ and 1.7 x 10$^9$ cfu/ml). Although the biomass yield for the fermentation at pH 5.5 (0.0093 ± 0.002 g/g) was similar to that measured at pH 6.0 and 6.5, $Y_{X/S}$ was greatly reduced at pH 5.25 (0.040 ± 0.001 g/g). With the exception of fermentation at pH 6.0, cells entered the stationary phase when residual glucose concentration was still higher than 5 g/l. Ammonia production was correlated with total urease activity. The total ammonia released at the end of fermentation, assuming that urea hydrolysis was the only source of ammonia and that ammonia uptake was negligible, accounted for 40% of initial urea concentration at pH 6.0 and 6.5 and for 22% and 10% at pH 5.5 and 5.25, respectively. At the end of the exponential growth phase the ammonia released exceed that of acid production and acid was pumped to control pH rather than alkali.

### Table 1

<table>
<thead>
<tr>
<th>Urease source</th>
<th>Urease activity$^a$</th>
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<tbody>
<tr>
<td>pH 6.0, 50 mmol/l potassium phosphate buffer</td>
<td>pH 4.0, 100 mmol/l sodium acetate buffer, 12.5% (v/v) ethanol</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> Y3, whole cells</td>
<td>0.093 ± 0.006</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> Y3, permeabilized cells</td>
<td>0.255 ± 0.008</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> Y3, cell-free extracts</td>
<td>0.484 ± 0.003</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em>, permeabilized cells</td>
<td>0.563 ± 0.001</td>
</tr>
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</table>

$^a$µkat/g CDW for whole and permeabilized cells, µkat/mg protein for cell free extract.

The results of the comparison between urease activity of *S. thermophilus* Y3 and a commercial urease preparation (Enzeco®) are shown in Table 1. Activity of whole and permeabilized cells of *S. thermophilus* Y3 was reduced to 22% in the presence of ethanol at pH 4, while cell-free extracts conserved 50% of their original activity. In contrast, *L. fermentum* urease activity at pH 4 and in the presence of ethanol was 78% of that shown at pH 6.

### 4. Discussion

Although urease production is widespread among prokaryotes (Mobley et al., 1995), with the exception of *S. thermophilus* (Mora et al., 2002, 2004) and *S. salivarius* (Chen and Burne, 1996; Chen et al., 1996, 1998, 2000) only a few species of lactic acid bacteria have been tested for urease activity and found positive (Kakimoto et al., 1989, 1990a, b; Yamazaki et al., 1990). Acid urease activity has been found in *L. fermentum* (Kakimoto et al., 1990a), *Lactobacillus reuteri* (Kakimoto et al., 1989), and *Streptococcus mitior* (Yamazaki et al., 1990). Urease activity was always intracellular, as for the large majority of urease-producing prokaryotes (Mobley et al., 1995). Our results are in good agreement with this findings although none of the five *S. salivarius* strains we tested was urease positive. This may be either associated to the source of isolation (all strains had been isolated from milk) or to their inability to produce urease under the conditions of the screening, during growth or assay (pH in the phenol red assay was 7.2). In fact, urease production in *S. salivarius* is regulated in a complex fashion and both low pH and high carbohydrate content are needed (Chen et al., 1998) to induce expression. Some *S. thermophilus* strains also
scored negative in the screening: urease negative *S. thermophilus* strains are not infrequent (Mora et al., 2002) and can occur by spontaneous mutation (Monnet et al., 2004).

The variability in urease activity of *S. thermophilus* found in this study is in agreement with previous findings by Mora et al. (2002). However, the reasons for the lack of correlation between activity of whole cells and cell lysates are not clear. Differences in urea transport across the cell membrane may be a contributing factor.

Permeabilization of *S. thermophilus* cells by toluene significantly increased urease activity and was more convenient than obtaining cell lysates. Cell permeabilization has been used for the study of other, technologically important, enzymatic activities in lactic acid bacteria (Krishnan et al., 2000; Exterkate, 2006). The optimal pH for urease activity in *S. thermophilus* is in good agreement with that of *S. salivarius* urease heteroergously expressed in *Escherichia coli* (Chen et al., 1996) but is much higher than that of acid ureases of *L. fermentum* and *L. reuteri* which was claimed to be 2.0 (Kakimoto et al., 1989, 1990b). However, it is worth noticing that the optimum pH of *L. fermentum* urease strongly depended on urea concentration and shifted from approximately 2.7 to 5.7 as urea concentration was decreased from 5 to 0.005 g/l (Kodama, 1996). Although *S. thermophilus* urease is not an acid urease, the relatively high residual activity at low pH may be promising for its use in wine and other alcoholic beverages.

The results of the effect of pH on *S. thermophilus* growth are in good agreement with the results of Vaningelgem et al. (2004), who compared growth and exopolysaccharide production in *S. thermophilus* in a milk medium as a function of pH and temperature and found that growth was optimum at pH 6.2. However, in their study no reduction of cell yield was observed at low pH: this may be related to a significantly higher value of $p_{\text{max}}$ at the lowest pH tested (0.95 h$^{-1}$ at pH 5.1 compared to 0.37 h$^{-1}$ at pH 5.25 in our study) and to differences in the media used. Glucose consumption without growth at low pH may be attributed to maintenance requirements related to the conservation of intracellular pH homeostasis (van de Guchte et al., 2002). The pattern of urease production found in our study is similar to that found by Mora et al. (2005), who also observed a peak in specific activity early in the exponential growth phase and slower growth in the presence of urea. Mora et al. (2005), based on analysis of ureA1 activity and on detection of urease in electrophoresis gels, concluded that the regulation of urease production is significantly different in *S. thermophilus* compared to *S. salivarius*. Urease activity was found to be higher at pH 6 (the lowest pH tested) than at pH 7, and no effect of high carbohydrate levels or of Ni was found. We found that maximum specific urease activity was highest at pH 5.5 (3.30 μkat/g CDW, compared to 2.1, 2.2 and 1.32 at pH 6.5, 6.0 and 5.25, respectively). This is in agreement with the results of Chen and Burne (1996), who showed that in chemostat cultures of *S. salivarius* specific urease activity increases at pH 5.5, and that higher specific growth rates and higher glucose concentration increase urease activity. The decrease in specific activity we observed relatively early in the exponential phase at pH 6.0 and 6.5 may be due to the effect of carbohydrate concentration rather to a decrease in specific growth rate, but a decrease in specific urease activity occurred at pH 5.5 even when residual glucose concentration was still higher than 15 g/l. However, the effects of growth rate and carbohydrate concentration are confounded in batch fermentations and further experiments in chemostat culture and measurement of transcription of urease genes are needed to clarify this. We did not test the effect of urea concentration on urease activity. Urea has been used at concentrations varying between 0.1 and 1 g/l for *S. thermophilus* and *S. salivarius* (Mora et al., 2004, 2005; Chen et al., 1996), while urea at 5 g/l was used to study urease production by *L. fermentum* (Kakimoto et al., 1990b). We found that urease activity was very low but measurable in fermentations without urea, thus confirming that urease production is constitutive in *S. thermophilus* (Mora et al., 2005), as in *S. salivarius* (Sissons et al., 1990) and many other procaryotes (Mobley et al., 2005). Addition of 5 g/l urea increased urease activity of *S. thermophilus* Y3 approximately 60 times in fermentations without pH control in MRS, while Mora et al. (2005) found that maximum specific urease activity increased from 0.6 μkat/g protein to 3.9 μkat/g protein when 0.5 g/l urea were added to M17 containing 2% lactose, and further increased to 8.5 μkat/g protein when pH was controlled to 6.0. These differences may be due to the effect of the culture medium, urea concentration or to the differences in behavior of the strains used.

We have shown for the first time that *S. thermophilus* urease does have significant residual activity at low pH (3–4) and in the presence of ethanol. A commercial acid urease preparation from *L. fermentum* made of dried, ethanol-permeabilized cells, is currently used for the degradation of urea in sake and wine (Fidaleo et al., 2006; Kodama, 1996) in order to prevent the accumulation of the potentially carcinogenic ethyl carbamate. To be used for this purpose, urease should be active at the low pH and low urea concentrations and in the presence of inhibitors found in alcoholic beverages (Kodama, 1996; Fidaleo et al., 2006). An additional requirement may be a low heat tolerance to allow its inactivation by pasteurization (Miyagawa et al., 1999) and the ability to retain sufficient activity at temperatures used for storage of wines and alcoholic beverages. We did not test the effect of temperature on urease activity. The optimum for *S. salivarius* urease (whose genes share a very high homology with *S. thermophilus* urease) is 60 °C, and the enzyme retains 10–25% activity in the range of temperature, 10–25 °C (Chen et al., 1996). The same is true for the commercially available acid urease of *L. fermentum* (Kakimoto et al., 1990b; Kodama, 1996).
5. Conclusions

Urease production by *S. thermophilus* has been regarded as detrimental for its activity as starter culture in yoghurt and in cheese production (Mora et al., 2004; Monnet et al., 2004) because milk acidification is delayed by the presence of urea. However, growth in the presence of urease, although slower, results in higher cell numbers and may reduce cell damage at acid pH, thus resulting in higher activity. Moreover, urea can be used to keep pH constant during lactic acid fermentation. A similar approach has been used for *Lactobacillus casei* (Peeva and Peev, 1997) by addition of exogenous urease. We did not study in detail urease regulation; however, our results are only in partial agreement with those of Mora et al. (2005) and may suggest that urease regulation is indeed similar to that in *S. salivarius*. As to the potential application of *S. thermophilus* urease in the removal of urea from alcoholic beverages, further study is needed to improve fermentation conditions, specific activity of permeabilized cells and stability of the enzyme under conditions of pH, temperature and presence of inhibitors prevailing in wine and sake (Kodama, 1996; Fidaleo et al., 2006).

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