J Ind Microbiol Biotechnol (2006) 33: 469–474
DOI 10.1007/s10295-006-0091-6

ORIGINAL PAPER

Tara Graves · Neelakantam V. Narendranath
Karl Dawson · Ronan Power

Effect of pH and lactic or acetic acid on ethanol productivity by *Saccharomyces cerevisiae* in corn mash

Received: 8 March 2005 / Accepted: 27 January 2006 / Published online: 21 February 2006

Abstract The effects of lactic and acetic acids on ethanol production by *Saccharomyces cerevisiae* in corn mash, as influenced by pH and dissolved solids concentration, were examined. The lactic and acetic acid concentrations utilized were 0, 0.5, 1.0, 2.0, 3.0 and 4.0% w/v, and 0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/v, respectively. Corn mashes (20, 25 and 30% dry solids) were adjusted to the following pH levels after lactic or acetic acid addition: 4.0, 4.5, 5.0 or 5.5 prior to yeast inoculation. Lactic acid did not completely inhibit ethanol production by the yeast. However, lactic acid at 4% w/v decreased ($P < 0.05$) final ethanol concentration in all mashes at all pH levels. In 30% solids mash set at pH ≤ 5, lactic acid at 3% w/v reduced ($P < 0.05$) ethanol production. In contrast, inhibition by acetic acid increased as the concentration of solids in the mash increased and the pH of the medium declined. Ethanol production was completely inhibited in all mashes set at pH 4 in the presence of acetic acid at concentrations ≥0.8% w/v. In 30% solids mash set at pH 4, final ethanol levels decreased ($P < 0.01$) with only 0.1% w/v acetic acid. These results suggest that the inhibitory effects of lactic acid and acetic acid on ethanol production in corn mash fermentation when set at a pH of 5.0–5.5 are not as great as that reported thus far using laboratory media.

Keywords Corn mash · Lactic acid · Acetic acid · pH · Dry solids · *Saccharomyces cerevisiae*

Introduction

The weak organic acids, lactic and acetic, are of utmost concern to fuel alcohol producers because they are potential inhibitors of yeast growth and metabolism. Lactic acid is produced by contaminating lactic acid bacteria as a result of carbohydrate metabolism. Minor quantities of acetic acid are produced by *Saccharomyces cerevisiae* during alcoholic fermentation, but toxic concentrations may be produced by lactic acid bacteria and/or acetic acid bacteria [7]. Unlike most beverage alcohol operations, pure culture conditions in the fuel ethanol industry are generally not practiced [13]. As a result, the emergence of bacterial contaminants in industrial fuel ethanol fermentation is, in many cases, inevitable. When present in significant numbers, these contaminants can produce lactic and/or acetic acids at concentrations which may be toxic to the yeast.

A considerable amount of research has been conducted on the inhibitory effects of lactic and acetic acids on yeast growth and metabolism. The majority of these studies have been carried out in minimal or synthetic laboratory media [6, 9, 10, 17]. In one such study using chemically defined minimal media, it was reported that concentrations as low as 0.2–0.8% w/v of lactic acid or 0.05–0.1% w/v of acetic acid stressed the yeast, as evidenced by decreased growth rates and reduced rates of glucose consumption and ethanol production [7]. Minimal media, however, are devoid of particulate materials and thus are not representative of practical, industrial media.

In the United States, fuel ethanol is manufactured primarily from corn mash, a much more complex matrix than standard laboratory growth media. It has been established that yeast growth is more rapid and the biomass produced is greater in media containing complex ingredients, when compared to growth in minimal medium [17]. Complex media provide increased levels of nutrients and other non-nutritional components, which promote yeast growth and survival [16]. The added buffering capacity of corn mash could offer some protection to yeast against acid-induced stress. The inhibitory concentrations of these weak organic acids in such a system are affected by a number of factors, such as pH and the concentration of dissolved solids in the medium. It is possible that the inhibitory effects of lactic and

T. Graves · N. V. Narendranath (✉) · K. Dawson · R. Power
Alltech Biotechnology Center, 3031 Catnip Hill Pike, Nicholasville, KY 40356, USA
E-mail: nnarendranath@gmail.com
Tel.: +1-605-9653588
Fax: +1-605-9652203

acetic acids in a complex medium, such as corn mash, thus may be less than those observed in a minimal medium. The objective of the current study, therefore, was to examine the effects of pH and lactic or acetic acid on ethanol production by *S. cerevisiae* in industrially relevant corn mash media with varying solids contents.

Materials and methods

Microorganism and growth conditions

The strain of *S. cerevisiae*, S001 (yeast code 362-83-1, Alltech, Nicholasville, KY) used in these studies is an industrial strain widely used for the production of fuel ethanol.

Yeast, from single colony isolates, was cultivated in 50 ml of sterile YM broth in an orbital shaker (200 rpm) at 30° C for 24 h. An aliquot (10 ml) of this 24 h culture was inoculated to 1 L of sterile YM broth and grown for an additional 18 h. Cultures were then harvested by centrifugation (4000*g* for 30 min) at 4 °C. The cell pellets were re-suspended in 40 ml of sterile de-ionized water. An appropriate quantity (1.6 ml for 20% solids mash, 2.0 ml for 25% solids mash and 2.4 ml for 30% solids mash) of the suspension was added to each flask in order to achieve an inoculation rate of one million cells/% dry solids/ml mash.

Mashing of corn and fermentation

Liquefied mash (with approximately 20, 25 or 30% dry solids) was prepared using corn (US #1) purchased from a local supplier (Thompson & Shearer, Nicholasville, KY). Corn was ground using a hammer mill (Model No. 9506TF, Bliss Industries, Inc., Ponca City, OK) fitted with a #4 screen. To prepare the mash, ground corn (920 g for 20% solids, 1,149 g for 25% solids and 1,379 g for 30% solids) was slowly added to tap water (3,080 ml for 20% solids, 2,851 ml for 25% solids and 2,621 ml for 30% solids) at 60 °C. The slurry was continuously mixed during the cooking phase using a Silverson Homogenizer (Model L4RT). Following the addition of corn, 0.33 ml of α-amylase (High TDS, 145,000 amylase units/ml, Alltech) per 100 g of corn was added to reduce viscosity and prevent starch retrogradation. The slurry was heated to 85 °C, and held at this temperature for 20 min. The slurry was then autoclaved at 121 °C for 20 min. After autoclaving, the mash was cooled to 85 °C and held at this temperature for 1 hr (with continous stirring) with 0.67 ml of α-amylase added per 100 g corn. The mash was then cooled to room temperature. Water lost during autoclaving was made up with sterile water. The antibiotic product Lactoside 247 (Alltech) was added (5 μg/ml) to prevent bacterial contamination.

The mash was divided into 440 g batches (one batch per treatment, each treatment run in duplicate) and adjusted to one of the following pH values: 4.0, 4.5, 5.0 or 5.5. The pH values chosen for the study are those typically encountered in fuel ethanol plants. Adjustments in pH were made using either 8 M KOH or concentrated H_2SO_4, after acid (lactic or acetic) additions. Lactic acid (85%) and acetic acid (glacial) were added to the mash, prior to pH adjustment, to achieve concentrations of 0, 0.5, 1.0, 2.0, 3.0 and 4.0% w/v, and 0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/v, respectively. Suitable controls with sterile de-ionized water (to account for dilutions caused by the addition of acids) were included. Amyloglucosidase (Allcoholase II L400, Alltech) was used for saccharification of the dextrins at an addition rate of 0.08% the weight of grain. Urea at 0.016% by weight of mash (0.032 g/200 g mash), was used as the nitrogen source. All treatments were performed in duplicate and fermentation was monitored for either 72 or 96 h (72 h for 20% solids mash, and 96 h for 25 and 30% solids mash). The temperature was maintained at 30 °C throughout fermentation in an incubator shaker. Samples were withdrawn for analysis from each flask at 6, 18, 48, 72 and 96 h. Fermentation rates were calculated from the linear portions of the curves generated as ethanol was synthesized over time during the initial 18 h of fermentation.

Assay methods

HPLC analysis

Concentrations of ethanol were determined by high-performance liquid chromatography (HPLC). The samples collected were centrifuged (4000*g* for 15 min) and the supernatant filtered (0.20 μm filter) prior to analysis. A 20 μl portion of a sample or a standard solution was injected onto a Bio-Rad HPX-87H Aminex ion exclusion column coupled to a refractive index detector (Model 2410, Waters Chromatographic Division, Milford, MA). The column was operated at 65 °C and sulfuric acid (0.002 M) was used as the mobile phase at a flow rate of 0.6 ml/min. The data were processed by Millennium Software (Waters Chromatographic Division).

Experimental design and Statistical analysis

Two full factorial experiments (6 acid concentrations × 4 pH levels × 3 mash solids concentrations) were conducted separately for lactic acid and acetic acid, respectively. The concentrations of lactic and acetic acids used in the study were chosen on the basis that the higher concentrations of the acids (individually) would inhibit the metabolic activity of the yeast [6]. All final ethanol and fermentation rate data were analyzed using the General Linear Model (GLM) procedure of SAS (Cary, NC).

Results

Effect of lactic acid on ethanol production by *S. cerevisiae*

Lactic acid did not completely inhibit ethanol production by yeast all the corn mashes. Rates of fermentation were not affected ($P > 0.05$), even when the yeast were exposed to high lactic acid levels, increased osmotic stress (i.e., in the corn mash with 25 and 30% solids) and low pH. Under all circumstances, the rates of ethanol synthesis decreased by no more than 1.01 g/l/h in all mashes containing various concentrations of lactic acid (Fig. 1).

Final ethanol quantities were reduced ($P < 0.05$) in all mashes containing 4% w/v lactic acid at all pH values (Fig. 1). At pH 4, where maximum inhibition was observed, 4% w/v lactic acid decreased final ethanol concentrations by 0.50, 1.03 and 2.07% v/v in the 20, 25 and 30% solids mashes, respectively. In 30% solids mash, where osmotic stress on the yeast was elevated, lactic acid at 3% w/v reduced ($P < 0.05$) ethanol production when the pH of the mash was ≤ 5.

Effect of acetic acid on ethanol production by *S. cerevisiae*

Acetic acid inhibited ethanol production by yeast as the concentration of solids in the mash increased and the pH of the medium declined. At all pH values, addition of 1.6% w/v acetic acid to the mashes significantly decreased ($P < 0.01$) ethanol synthesis rates (Fig. 2). The lowest concentration of acetic acid which stressed the yeast, as assessed by reductions in ethanol production rates, decreased from 1.6 to 0.4% w/v as the initial pH of the mashes declined from 5.5 to 4.0, respectively. Furthermore, the rates of ethanol production decreased by 50% with the addition of 0.4% w/v acetic acid to the 20% solids corn mash set at pH 4, whereas 98% reductions in the rates were observed in the mashes containing 25 and 30% solids. Ethanol synthesis rates were accelerated ($P < 0.05$) by small concentrations of acetic acid

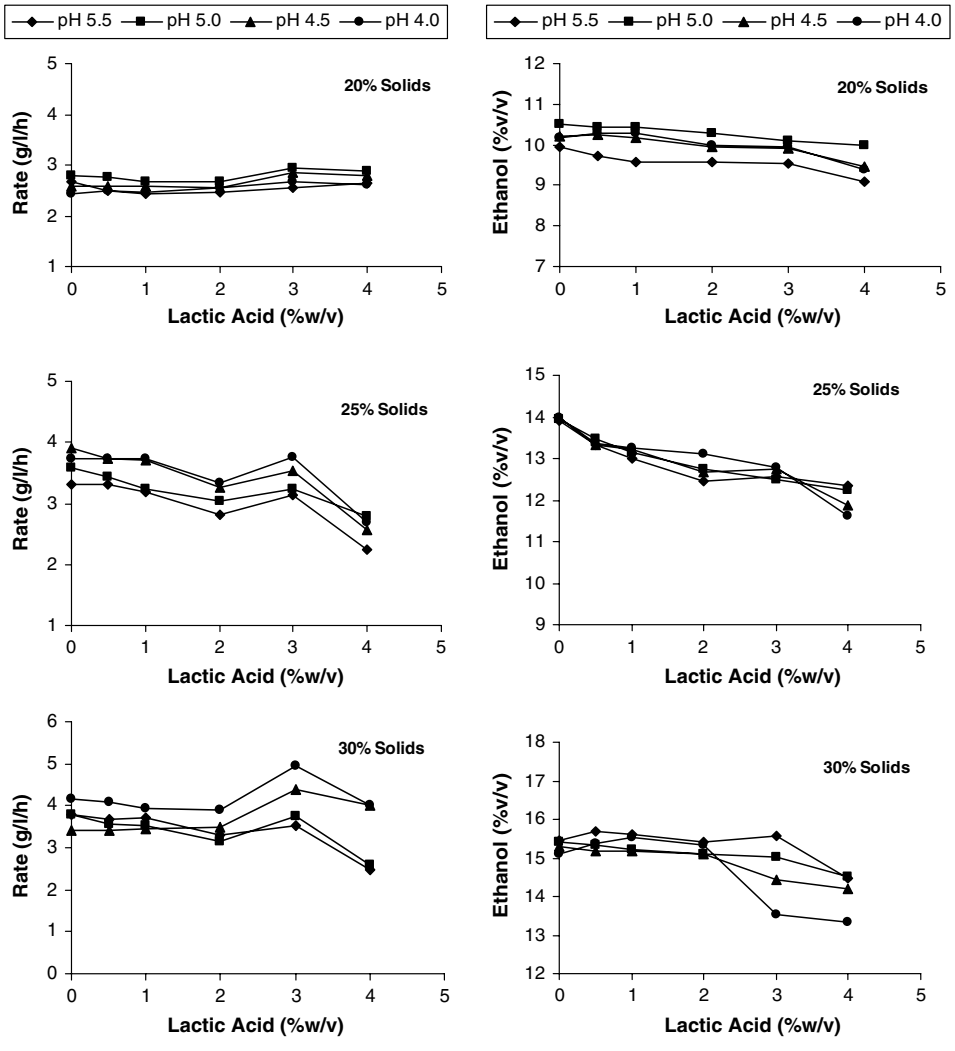

Fig 1 Rates of ethanol synthesis and final ethanol concentrations produced by *S. cerevisiae* during fermentation of corn mash with varying levels of solids containing increasing concentrations of lactic acid adjusted to different pH values at 30 °C. Coefficient of variation among duplicate fermentations was < 5%

(≤ 0.2% w/v) in all the mashes compared to controls (with no added acetic acid) adjusted to all pH values.

The yeast were resistant to high levels of acetic acid (0.8% w/v) at higher set pH values (≥5), as relatively high concentrations of ethanol were produced under these conditions (Fig. 2). As the pH of the corn mash was lowered to 4.5 and below, less acetic acid was required to substantially reduce final ethanol levels or completely inhibit ethanol production. In the 30% solids mash set at pH 4, final ethanol concentrations decreased ($P < 0.01$) even when 0.1% w/v acetic acid was added (Fig. 2). The addition of small concentrations of acetic acid (≤ 0.2% w/v) resulted in increased ($P < 0.05$) final ethanol levels. This effect occurred primarily in the 20% solids mash and was less apparent as the osmotic pressure exerted on the yeast increased (i.e., as the concentration of dissolved solids in the corn mash increased to 30%).

Discussion

Growth inhibition of *S. cerevisiae* by lactic acid or acetic acid has previously been established [2, 4, 5, 8, 12]. In the present work, the inhibitory effects of lactic or acetic acid on ethanol production by *S. cerevisiae* were studied in corn mash, the substrate most commonly used for the production of fuel ethanol in the USA. The results showed that the concentrations of lactic and acetic acids which hindered ethanol synthesis by yeast were considerably higher than the reported results obtained using minimal media where the pH was not adjusted after adding lactic or acetic acids [7]. The concentration of undissociated acid in the minimal medium used in that study was 87 and 94% for lactic acid and acetic acid, respectively, whereas in the present study, <42% of lactic acid and <85% of acetic acid (as determined by the Henderson–Hasselbach equation) was in the undissociated state, under all circumstances. Thomas et al. [17] demonstrated that *S. cerevisiae* tolerated higher concentrations of lactic or acetic acids when the initial pH of the medium was increased after acid addition. Other authors have demonstrated that *S. cerevisiae* can tolerate relatively high concentrations of lactic or acetic acids in pH-corrected chemically defined media [9, 15].

Abbott and Ingledew [1] recently illustrated that the presence of particulate materials had a major influence

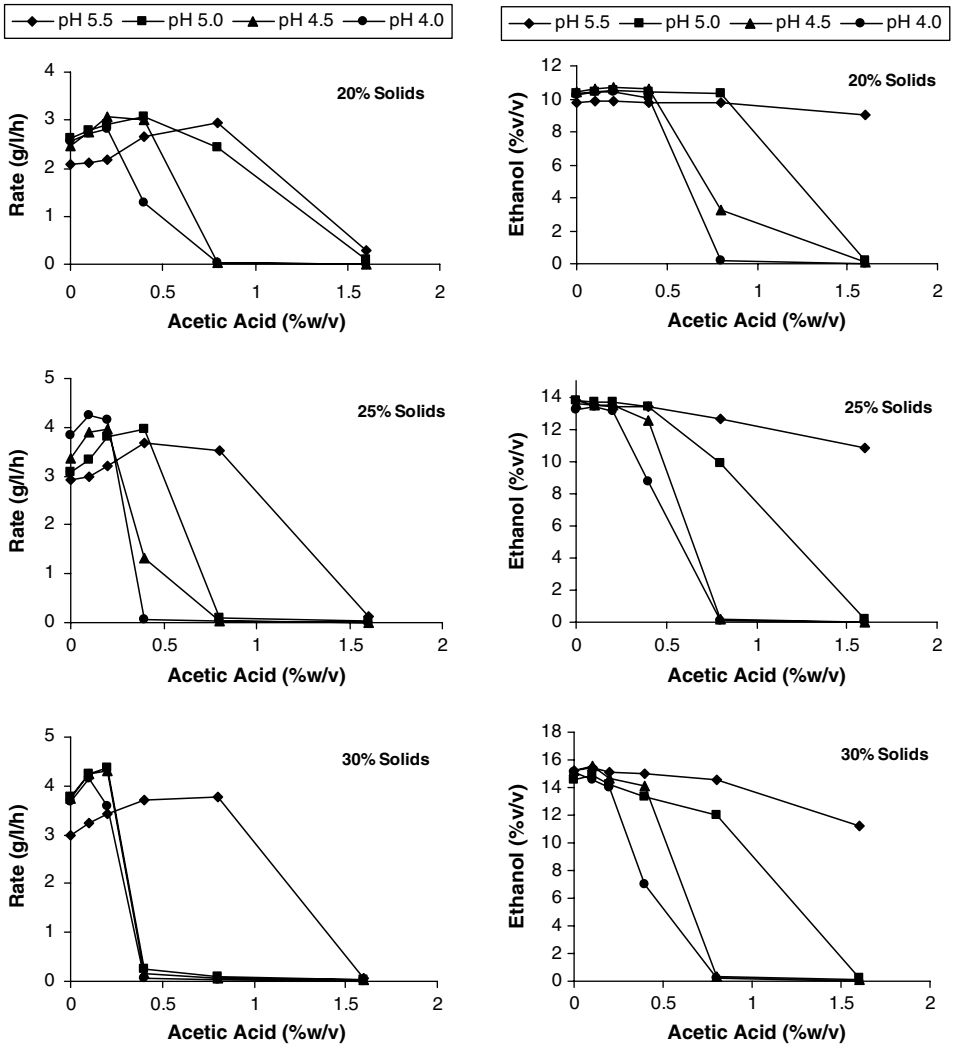

Fig 2 Rates of ethanol synthesis and final ethanol concentrations produced by *S. cerevisiae* during fermentation of corn mash with varying levels of solids containing increasing concentrations of acetic acid adjusted to different pH values at 30 °C. Coefficient of variation among duplicate fermentations was <6%

on the buffering capacity of whole corn mash. Given that particulate materials improve buffering, the buffering capacity of corn mash should increase with higher solids where increased particulate matter is present. Moreover, certain components of complex media may also improve the tolerance of yeast to various stresses. Previous studies have shown that the addition of peptone and yeast extract improved a yeast strain's tolerance to osmotic stress and elevated temperature [14]. When exposed to a particular stress, yeast cells often trigger an adaptive response resulting in a transient resistance to the same stress or a different stress, a phenomenon known as cross-protection [3]. Therefore, it is reasonable to assume that particular components in complex medium, such as corn mash, may induce resistance to acid-stressed conditions, either independently or as a result of a different stress.

The apparent increased toxicity of acetic acid in corn mash, when compared to lactic acid, was due to the increased concentration of the undissociated molecule (Fig. 3). Similar observations in other media were reported by other authors [7, 12, 17]. Acetic acid (pK_a 4.74) has a higher pK_a value than lactic acid (pK_a 3.86). At a pH below the pK_a value, a weak acid will exist largely in the undissociated state, a form in which they are potent microbial growth inhibitors [11]. Although, acetic acid was more inhibitory to ethanol production by yeast than lactic acid, low concentrations of acetic acid stimulated ethanol production rate by yeast as reported by other authors [4, 9, 10, 15, 17]. In the presence of acetic acid, yeast cells require increased ATP production to facilitate maintenance of the internal pH [4]. Glycolytic flux increases as a result of the need for increased ATP levels, thereby enhancing the rate of ethanol formation [9].

Interestingly, data obtained from this study suggest that when corn mash is set to an initial pH of 5.0–5.5, the concentrations of lactic or acetic acid required to inhibit yeast growth and fermentation are considerably higher than those reported thus far in the literature based on work using chemically defined media. Corn mash also possibly provides some degree of protection to the yeast against the toxic effects of these acids.

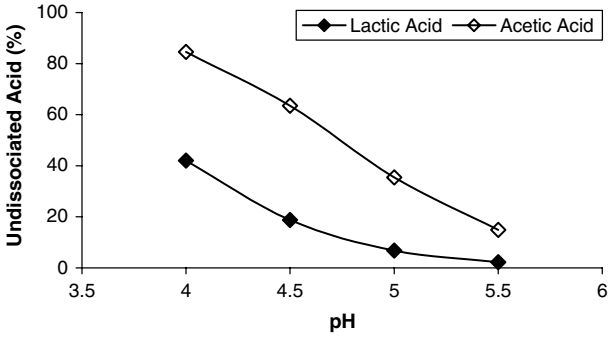

Fig 3 Amount of lactic and acetic acids in the undissociated form as influenced by medium pH

However, it is a routine practice in the ethanol industry to set mash pH at 4.0–4.5 or sometimes even less than 4.0 (in plants that practice continuous fermentation) as a measure to control lactic acid bacterial contamination. At these pH levels, the inhibition of yeast by lactic or acetic acids is enhanced. During growth, it is important for the yeast to maintain a constant intracellular pH for the normal functioning of the glycolytic enzymes. Therefore, raising the external pH closer to the desired intracellular pH places less stress on the cells. In media containing organic acids, raising the pH to a value higher than the pK_a value for the acid results in a decrease in the inhibitory effect of the organic acid on yeast growth and metabolism. At a higher set pH of 5.0–5.5 the difference between internal and external pH values (ΔpH) is smaller which results in a reduced inhibition of yeast growth since the accumulation of undissociated acids within the cell is a function of ΔpH. Work investigating the interactions between pH, lactic and acetic acids on ethanol production by yeast in corn mash is currently underway, as are studies on the effects of additional environmental stress factors, such as temperature.

References

1. Abbott DA, Ingledew WM (2004) Buffering capacity of whole corn mash alters concentrations of organic acids required to inhibit growth of *Saccharomyces cerevisiae* and ethanol production. Biotech Lett 26:1313–1316
2. Bayrock DP, Ingledew WM (2004) Inhibition of yeast by lactic acid bacteria in continuous culture: nutrient depletion and/or acid toxicity? J Ind Microbiol Biotechnol 31:362–368
3. Carmelo V, Santos R, Viegas CA, Sa-Correia I (1998) Modification of *Saccharomyces cerevisiae* thermotolerance following rapid exposure to acid stress. Int J Food Microbiol 42:225–230
4. Garay-Arroyo A, Covarrubias AA, Clark I, Nino I, Gossett G, Martinez A (2004) Response to different environmental stress conditions of industrial and laboratory Saccharomyces cerevisiae strains. Appl Microbiol Biotechnol 63:734–741
5. Halm M, Hornbaek T, Arneborg N, Sefa-Dedeh S, Jespersen L (2004) Lactic acid tolerance determined by measurement of intracellular pH of single cells of Candida krusei and Saccharomyces cerevisiae isolated from fermented maize dough. Int J Food Microbiol 94:97–103
6. Maiorella B, Blanch HW, Wilke CR (1983) By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. Biotech Bioeng 25:103–121
7. Narendranath NV, Thomas KC, Ingledew WM (2001) Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J Ind Microbiol Biotechnol 26:171–177
8. Narendranath NV Thomas KC, Ingledew WM (2001) Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J Am Soc Brew Chem 59:187–194
9. Palmqvist E, Grage H, Meinander NQ, Hahn-Hagerdal B (1999) Main and interaction effects of acetic acid, furfural, and *p*-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol Bioeng 63:46–55
10. Pampulha ME, Loureiro-Dias MC (2000) Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 184:69–72

11. Piper P, Calderon CO, Hatzxanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiol 147:2635–2642
12. Savard T, Beaulieu C, Gardner NJ, Champagne CP (2002) Characterization of spoilage yeasts isolated from fermented vegetables and inhibition by lactic, acetic and propionic acids. Food Microbiol 19:363–373
13. Skinner KA, Leathers TD (2004) Bacterial contaminants of fuel ethanol production. J Ind Microbiol Biotechnol 31:401–408
14. Stewart GG, D'Amore T, Panchal CJ, Russell I (1988) Factors that influence the ethanol tolerance of brewer's yeast strains during high gravity wort fermentations. Master Brew Assoc Am 25:47–53
15. Taherzadeh MJ, Niklasson C, Liden G (1997) Acetic acid—-friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*? Chem Eng Sci 52:2653–2659
16. Thomas KC, Hynes SH, Ingledew WM (1994) Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae*. Appl Environ Microbiol 60:1519–1524
17. Thomas KC, Hynes SH, Ingledew WM (2002) Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids. Appl Environ Microbiol 68:1616–1623