

## Effect of Acetic Acid on Growth and Ethanol Fermentation of Xylose Fermenting Yeast and *Saccharomyces cerevisiae*

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### ABSTRACT

Growth of some xylose fermenting yeasts, *Candida shehatae*, *Pichia stipitis* CBS5773, fusant F101 and fusant F198, was completely inhibited in xylose medium added with 0.5% v/v acetic acid which caused the reduction of pH to 4.1. Only one xylose fermenting strain, *Pachysolen tannophilus* NRRL-Y2460, showed relatively low growth and ethanol fermentation. However, in the medium added with 1.0% v/v acetic acid (pH 3.7) all of these strains were completely inhibited. When the medium was adjusted by hydrochloric acid to pH 4.1 and 3.7, all xylose fermenting strains showed almost the same growth as in the medium without pH adjustment (pH 6.2). In glucose medium added with 0.5% v/v acetic acid, various strains of *Saccharomyces cerevisiae*, M30, Sc90, N1, G/3, G/5, G/2, TJ3 and SH1089, grew with lower specific growth rate and provided lower maximal cell concentration rate than in medium without adding acetic acid (pH 6.2). All strains, except N1, produced slightly higher maximal ethanol concentration. However, all of them yielded lower ethanol production rate. Among *S. cerevisiae*, strain B120 was more sensitive to acetic acid than the others since its growth was completely inhibited by 0.5% v/v acetic acid. In glucose medium, 0.5% v/v acetic acid did the same role as in xylose medium to xylose fermenting strains. Hence, the xylose fermenting yeasts revealed higher sensitivity to acetic acid than *S. cerevisiae*.

**Key words :** acetic acid, xylose fermenting yeast, ethanol fermentation, xylose fermentation, *Saccharomyces cerevisiae*

### INTRODUCTION

Hydrolysate of lignocellulose contains not only a mixture of sugars, mainly glucose from cellulose and xylose from hemicellulose, but also some substances that exert inhibitory effects on yeast such as acetic acid, furfural and lignin derivatives (Tsao *et al.*, 1982; Olsson and Hahn-Hagerdal, 1993). Linden and Hahn-Hagerdal (1989)

indicated that xylose fermenting yeasts did not ferment well in undetoxified hydrolysate. Ferrari *et al.* (1992) reported that acetic acid in the eucalyptus wood hemicellulose acid hydrolysate caused low pH, reduced the ethanol production rate and yield of *Pichia stipitis* fermentation. Its toxicity depended not only on the concentration but also the pH of hydrolysate. Some investigators reported the inhibitory effects of acetic acid on activities of

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several yeasts. For examples; Mariorella *et al.* (1983) showed the inhibitory effects of different metabolic products of yeast including acetic acid. Pampulha and Loureiro (1989) and Ferrari, *et al.* (1992) indicated the important role of undissociate form of acetic acid which diffuse into yeast cells, causing decreased pH of cytoplasm and inhibiting the activity of some enzymes, especially endolase, phosphoglyceromutase, aldolase and triosephosphate isomerase. Phowchinda *et al.* (1995) showed the inhibitory effect of acetic on growth and fermentation activity of *Saccharomyces cerevisiae* and indicated that it was more effective on the biomass synthesis than on ethanol production. However, there were no comparative study on sensitivity to acetic acid between xylose fermenting yeast and *S. cerevisiae*. Therefore, in this study the inhibitory effect of acetic acid on growth and ethanol fermentation of some xylose fermenting yeasts and some strains of *S. cerevisiae* was demonstrated, as well as comparison on sensitivity of both yeasts to acetic acid.

## MATERIALS AND METHODS

### Yeast strains

Yeast used in this study included *Saccharomyces cerevisiae* strain Sc90, M30, N1, TJ3, B120, G/2, G/3, G/5 and SH1089 and wild strains of xylose fermenting yeast, *Candida shehatae*, *Pichia stipitis* CBS5773, *Pachysolen tannophilus* NRRL-Y2460. Fusant F101, a fusant from intergeneric protoplast fusion of *P. stipitis* CBS5773 and *S. cerevisiae* AM12, and F198, a fusant from intraspecific protoplast fusion of *P. stipitis* CBS5773 (Chomtong, 1995) were also included.

### Growth and ethanol fermentation

Inoculum was prepared by inoculation of 24 h culture of yeast cultivated on a slant YPD agar

(1% yeast extract, 2% peptone, 2% D-glucose and 1.2% agar) or YPX agar (same ingredient as YPD except D-xylose was used instead of D-glucose), depending on yeast strains, into 50 ml of YPD broth or YPX broth in 250 ml flask and incubated on a rotary shaker, 200 rpm, at room temperature for 24 h. Cells were harvested, washed twice and resuspended in distilled water.

Fermentation was carried out in 100 ml of YPD<sub>18</sub> broth (YPD broth containing 18% D-glucose) or YPX<sub>4</sub> broth (YPX broth containing 4% D-xylose) in 250 ml Erlenmeyer flask. In acetic acid treatment, pre-calculated volume of concentrated acetic acid was added prior to sterilization. For comparative study on effect of pH 1 N hydrochloric acid was used. Inoculum was added into the medium to obtain the initial cell concentration, as optical density (OD) at 660 nm, at 1.0. Incubation was performed at room temperature on a rotary shaker at 180 rpm.

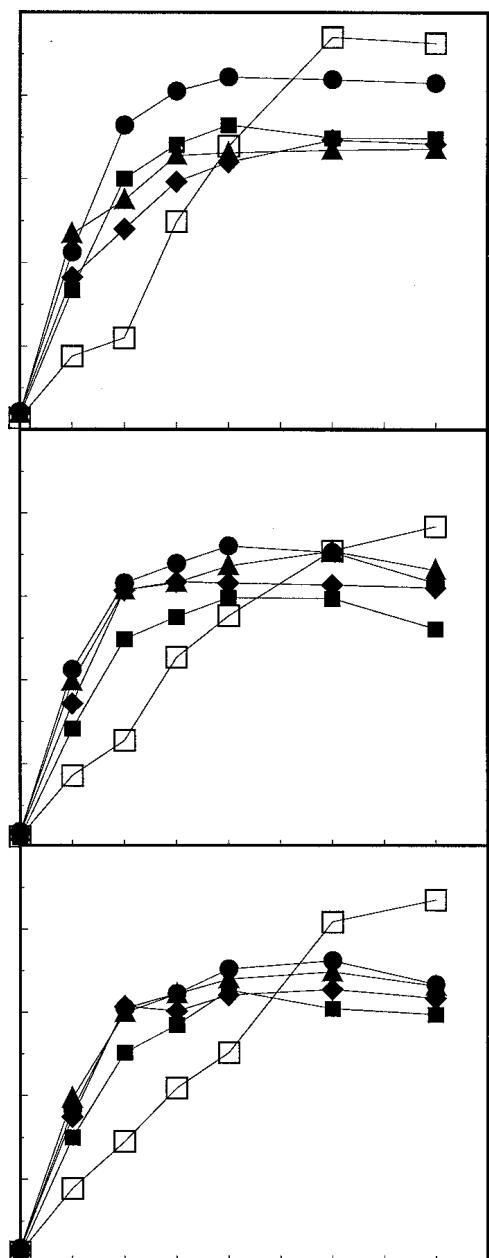
### Analyses

Ethanol was determined by gas chromatography (Shimadzu GC-9A, Japan) and propanol was used as internal standard. Cell concentration was quickly determined as OD at 660 nm by Spectrophotometer (Shimadzu model UV-240, Japan).

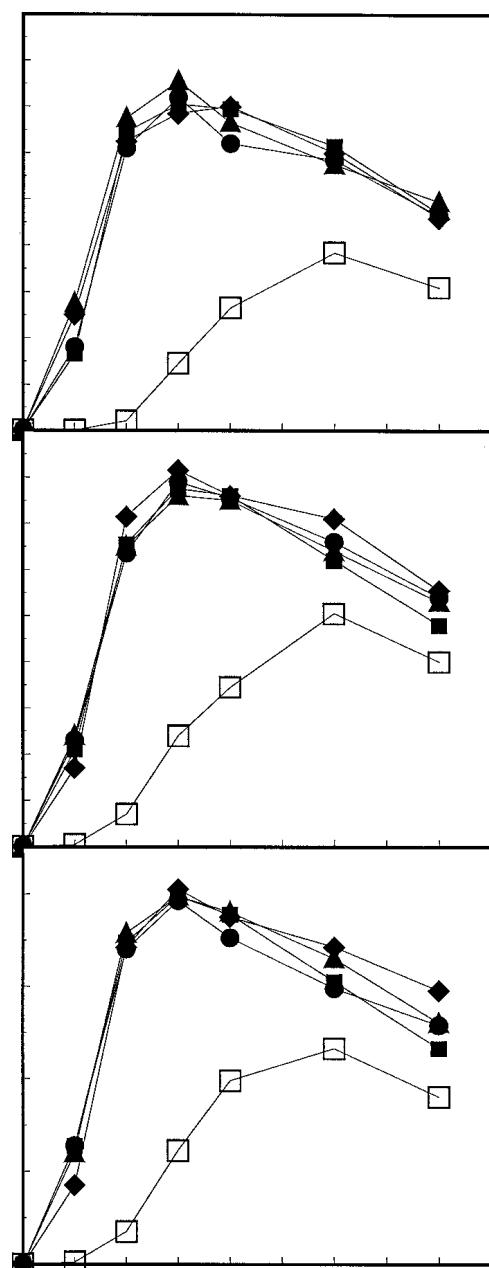
## RESULTS AND DISCUSSIONS

### Effect of acetic acid on growth and ethanol fermentation in xylose medium of xylose fermenting yeast

In xylose medium (4% xylose and pH 6.2), *P. stipitis* CBS5773 showed the highest specific growth rate ( $\mu$ ), 0.21 h<sup>-1</sup> though its maximal cell concentration measured as OD at 660 nm, was the lowest, 33.6, at 96 h (Figure 1 A). This yeast also produced the highest ethanol concentration, 1.51% w/v after 36 h, with the highest ethanol production



**Figure 1** Growth of *C. shehatae* (♦), *P. stipitis* CBS5773 (▲), *Pa. tannophilus* NRRL-Y2460 (□), F101 (■) and F198 (●) in xylose medium without (A) and with pH adjustment by HCl to pH 4.1 (B) and 3.7 (C).



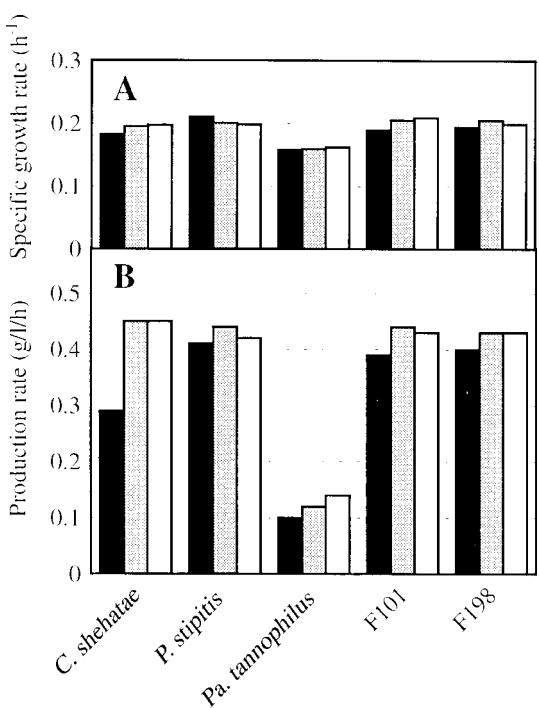
**Figure 2** Ethanol fermentation of *C. shehatae* (♦), *P. stipitis* CBS5773 (▲), *Pa. tannophilus* NRRL-Y2460 (□), F101 (■) and F198 (●) in xylose medium without (A) and with pH adjustment by HCl to pH 4.1 (B) and 3.7 (C).

rate of 0.41 g/l/h (Figure 2A). Likewise, the two fusants, F198 and F101, produced ethanol at 1.44 and 1.41% w/v after 36 h with the production rate of 0.40 and 0.39 g/l/h, respectively. *C. shehatae* accumulated 1.40 % w/v of ethanol at 48 h with low production rate of 0.29 g/l/h, while *Pa. tannophilus* NRRL-Y2460 produced only 0.77% w/v of ethanol with the lowest production rate of 0.10 g/l/h. Accordingly, *Pa. tannophilus* NRRL-Y2460 revealed the lowest specific growth rate,  $0.158 \text{ h}^{-1}$ , though its maximal OD was the highest, 47.0.

Addition of 0.5% v/v acetic acid into xylose medium resulted in reduction of pH to 4.1. Cultivation of xylose fermenting yeasts in this medium showed that only *Pa. tannophilus* NRRL-Y2460 could grow with low specific growth rate,  $0.076 \text{ h}^{-1}$ , and yielded low maximal cell concentration as OD at 29.30 after 96 h. This yeast produced maximal ethanol concentration, 0.56% w/v at 120 h resulted in production rate of 0.046 g/l/h. In 1.0% v/v acetic acid treatment causing pH reduction to 3.7, no xylose fermenting yeasts were observed in the medium. The results obviously indicated the inhibitory effect of acetic acid to growth and fermentation of xylose fermenting yeasts. These findings agreed with the report of Ferrari *et al.* (1992) on inhibitory effect of acetic acid on ethanol fermentation by *P. stipitis* in acid hydrolysate of hemicellulose in eucalyptus wood which contained various products including 3% xylose and 1% acetic acid. In addition they reported flocculation of yeast and lost of viability after inoculation, however, growth was resumed after a period of time.

To exhibit the effect of acetic acid on reduction of growth and ethanol fermentation and not from low pH caused by adding the acid, experiments were carried out in xylose medium where pH was adjusted by 1 N hydrochloric acid to the same pH level as obtained by adding acetic acid. Results showed that growth and ethanol

production by each xylose fermenting yeast in xylose medium with pH adjusted to 4.1 and 3.7 were not much different as compared to the control (Figure 1B, 1C, 2A and 2B). On the contrary, the production rate of each xylose fermenting yeast in the medium with hydrochloric acid treatment was slightly higher than in the normal xylose medium (pH 6.2), except *C. shehatae* (Figure 3B) where ethanol production rate was markedly improved from 0.29 g/l/h in normal xylose medium to 0.45 g/l/h, in both treated media. Subsequently, the specific growth rate of most xylose fermenting yeasts in hydrochloric treated media was slightly higher as compared to the control (Figure 3A). These indicated



**Figure 3** Specific growth rate (A) and ethanol production rate (B) of *C. shehatae*, *P. stipitis* CBS5773, *Pa. tannophilus* NRRL-Y2460, F101 and F198 in xylose medium without (■) and with pH adjustment by HCl to pH 4.1 (▨), and 3.7 (□).

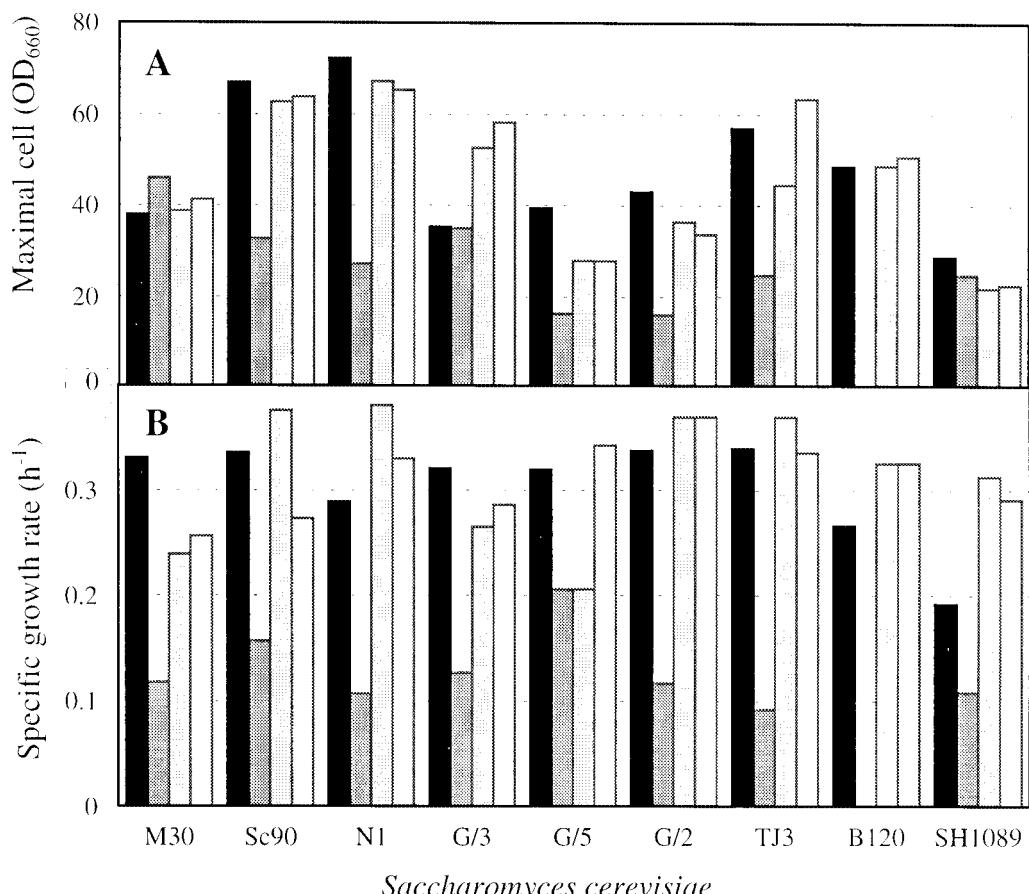
that low pH (pH 4.1 and 3.7) did not inhibit growth and ethanol fermentation of xylose fermenting yeasts, except in the case of *C. shehatae* which lower pH promoted better growth and ethanol production.

In conclusion, the results revealed that growth and ethanol fermentation of all xylose fermenting yeasts used in this study was inhibited by acetic acid not by lower pH.

#### Effect of acetic acid on growth and ethanol fermentation of *S. cerevisiae* in glucose medium

In glucose medium (pH 6.2), *S. cerevisiae* N1 and Sc90 produced relatively high cell concentration as OD at 72.20 and 66.90 after 36 and 24 h, respectively (Figure 4A). Consequently, the specific growth rates of all strains of *S. cerevisiae*, except SH1089, were relatively high and were not much different (Figure 4B).

All strains of *S. cerevisiae*, namely M30, Sc90, N1, G/3 and TJ3, except SH1089, produced high ethanol concentrations at 7.16, 7.42, 7.53,

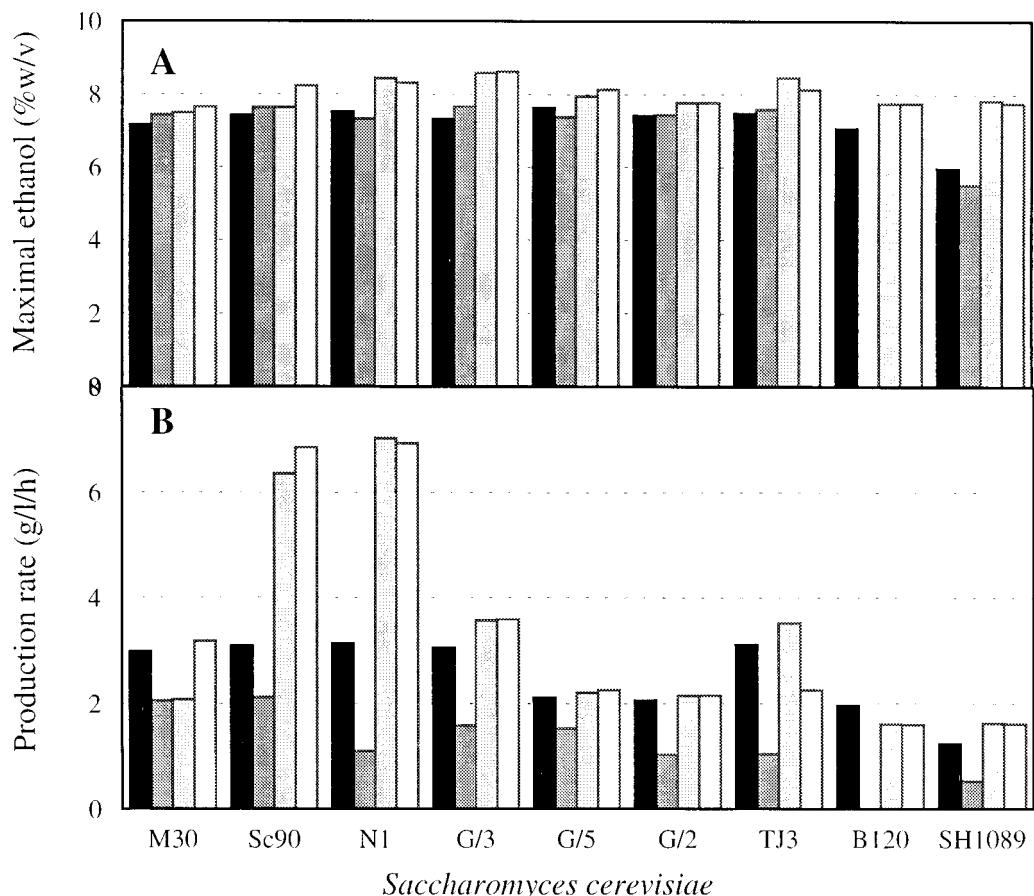


**Figure 4** Maximal cell concentration (A) and specific growth rate (B) of *S. cerevisiae* M30, Sc90, N1, G/3, G/5, G/2, TJ3, B120 and SH1089 in glucose medium without (■) and with addition of acetic acid (▨) and with pH adjustment by HCl to 4.1 (□) and 3.7 (□).

7.32 and 7.48% w/v, respectively, at fairly short fermentation time of 24 h with relatively high production rates of 2.98, 3.09, 3.13, 3.05 and 3.11 g/l/h, respectively (Figure 5B). On the other hand, ethanol produced by strains G/5, G/2 and B120 were similar at 36 h, 7.63, 7.42 and 7.07% w/v, respectively, with slightly longer incubation time of 36 h and hence their production rates were lower. For *S. cerevisiae* SH1089 lowest concentration of ethanol of 5.97% w/v was obtained after 48 h with the lowest production rate of 1.24 g/l/h.

By addition of 0.5 and 1.0% v/v acetic acid

into glucose medium, pH changes of the medium were similar to those observed in xylose medium. Likewise no growth of all *S. cerevisiae* strains were observed in medium added with 1.0% v/v. With addition of 0.5% v/v acetic acid, only *S. cerevisiae* B120 could not grow while the other strains produced comparable maximal cell concentration to those in basal glucose. However, the specific growth rates were slightly lower (Figure 4A and 4B). Though all strains, except *S. cerevisiae* N1, yielded slightly higher maximal ethanol concentration, their production rates were lower



**Figure 5** Maximal ethanol concentration (A) and production rate (B) of *S. cerevisiae* M30, Sc90, N1, G/3, G/5, G/2, TJ3, B120 and SH1089 in glucose medium without (■) and with addition of acetic acid (▨) and with pH adjustment by HCl to 4.1 (□) and 3.7 (□).

than in normal glucose medium (Figure 5B).

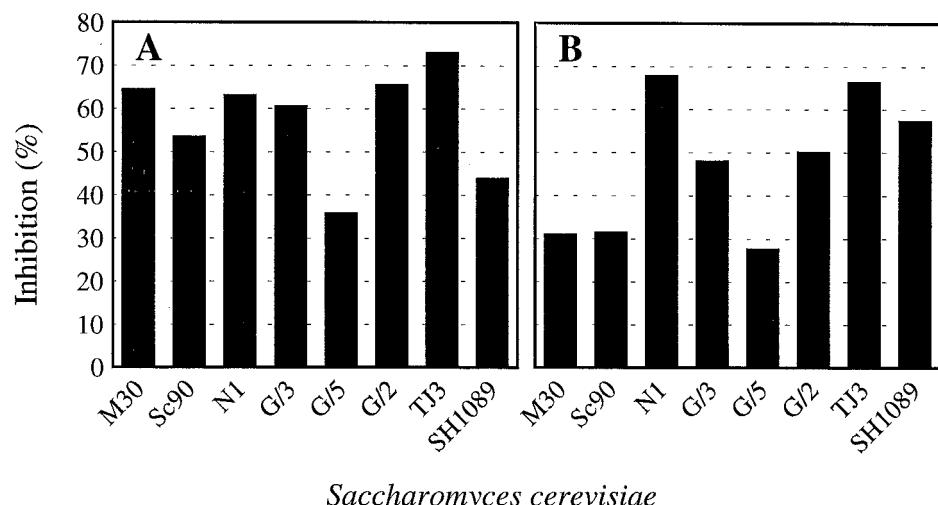
The results revealed that acetic acid at 0.5% v/v reduced maximal cell concentration but had no effect on maximal ethanol concentration of *S. cerevisiae*. However, both specific growth rate and ethanol production rate decreased. These findings confirmed the report of Phowchinda *et al.* (1995) which concluded that acetic acid inhibited the activities of *S. cerevisiae* and the inhibition was more effective on the biomass synthesis than ethanol synthesis.

Comparison on the effect of acetic acid on ethanol production rate and specific growth rate of *S. cerevisiae* in the medium added with 0.5% v/v acetic acid showed that the inhibition on production rate ranged from 67.7% to 27.5% and on specific growth rate were 72.9 to 35.6% (Figure 6). The results indicated stronger inhibition of acetic acid on specific growth rate than on production rate.

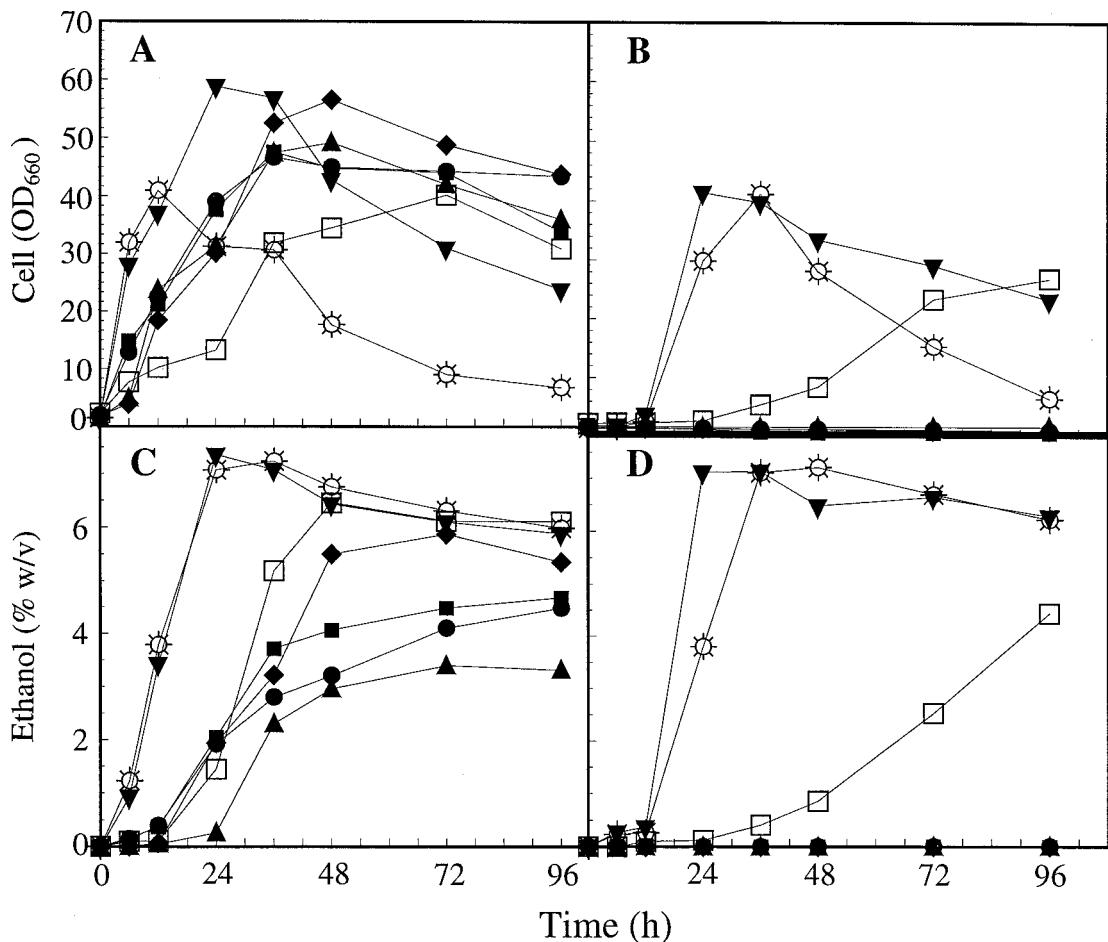
In the medium where pH was adjusted to 4.1

by hydrochloric acid specific growth rate of N1 was the highest, while G/2 showed the highest specific growth rate in medium with pH was at 3.7 (Figure 4B). As far as ethanol fermentation was concerned, all strains of *S. cerevisiae* demonstrated high performance in pH adjusted medium. At pH 4.1, N1 produced high ethanol concentration, 8.44% w/v at 12 h, with the highest production rate, 7.03 g/l/h (Figure 5), while G/3 produced the highest ethanol concentration, 8.58% w/v at 24 h, with production rate of only 3.57 g/l/h. On the contrary, B120 produced the lowest ethanol concentration, 7.75% w/v, with the lowest production rate, 1.61 g/l/h. At pH 3.7, Sc90 fermented ethanol with the highest production rate, 6.85 g/l/h, while ethanol produced was 8.23% w/v at 12 h.

Comparison on the effect of both acids revealed that growth and ethanol fermentation of *S. cerevisiae* were inhibited by acetic acid not by low pH as reported by Phowchinda *et al.* (1995).



**Figure 6** The percentage of inhibition on specific growth rate (A) and ethanol production rate (B) of various strains of *S. cerevisiae* M30, Sc90, N1, G/3, G/5, G/2, TJ3, B120 and SH1089 in glucose medium added with 0.5% v/v acetic acid comparing with the rates in medium without addition of acetic acid.



**Figure 7** Growth and ethanol fermentation of *C. shehatae* (♦), *P. stipitis* CBS5773 (▲), *Pa. tannophilus* NRRL-Y2460 (□), F101 (■), F198 (●), *S. cerevisiae* M30 (\*) and *S. cerevisiae* Sc 90 (▼) in glucose medium without (A, C) and with addition of 0.5% v/v acetic acid (B, D).

#### Comparison on effect of acetic acid on growth and ethanol fermentation of xylose fermenting yeast and *S. cerevisiae* in glucose medium

Ethanol fermentation in glucose medium without adding acetic acid (pH 6.2) by xylose fermenting yeasts was compared to two strains of *S. cerevisiae*, M30 and Sc90. The result showed that both strains of *S. cerevisiae* produced higher ethanol concentration and at the higher rate than all xylose fermenting strains (Figure 7C). Among xylose fermenting strains tested in this work, *Pa.*

*tannophilus* NRRL-Y2460 produced the highest ethanol concentration of 6.45% w/v at 48 h, with the highest production rate of 1.34 g/l/h. In contrast, *P. stipitis* CBS 5773 produced only 3.39% by weight at 72 h with production rate of 0.47 g/l/h. In addition the specific growth rate of both strains of *S. cerevisiae* was higher than those of all xylose fermenting strains (Figure 7A).

In the medium added with 0.5% v/v acetic acid, no growth and ethanol fermentation of *C. shehatae*, *P. stipitis* CBS5773, F101 and F198

were observed. *P. tannophilus* NRRL-Y2460 was the only xylose fermenting yeast that could grow and ferment ethanol (Figure 7B). However, it grew at lower specific growth rate,  $0.059\text{ h}^{-1}$ , and produced lower ethanol concentration, 4.44% by weight at 96 h, with lower production rate, 0.46 g/l/h. For *S. cerevisiae*, strain M30 produced nearly the same cell concentration as in glucose medium without adding acetic acid, while Sc90 provided much lower cell concentration (Figure 7B). However, the specific growth rates of both strains of *S. cerevisiae* were much lower than in medium without adding acetic acid.

## CONCLUSION

In conclusion, acetic acid at the concentration of 0.5% v/v completely inhibited growth of most xylose fermenting yeasts, *C. shaehatae*, *P. stipitis* CBS5773 and the two xylose fermenting fusants, F101 and F198, in medium containing glucose and xylose as a sole source of carbon. However, *Pa. tannophilus* NRRL-Y2460 relatively tolerated to acetic acid since it could grow and ferment ethanol in both media added with 0.5% v/v acetic acid. This acid plays the same role on growth and ethanol fermentation of various strains of glucose fermenting yeast, *S. cerevisiae*. Despite most strains of *S. cerevisiae* investigated in this study showed higher tolerance to acetic acid than xylose fermenting yeasts. The inhibition on growth and ethanol fermentation was proved to be the result from acetic acid and not from low pH as shown by hydrochloric acid.

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