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BIOTECHNOLOGY and BIOENGINEERING

# Enhancement of Pyruvate Production by Osmotic-Tolerant Mutant of *Torulopsis glabrata*

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**ABSTRACT:** Pyruvate production by *Torulopsis glabrata* was used as a model to study the mechanism of product inhibition and the strategy for enhancing pyruvate production. It was found that the concentration of cell growth and pyruvate deceased with the increase of NaCl and sorbitol concentrations. To enhance the osmotic stress resistance of the strain, an NaCl-tolerant mutant RS23 was screened and selected through a pH-controlled continuous culture with 70 g/L NaCl as the selective criterion. Compared with the parent strain, mutant RS23 could grow well on the medium containing 70 g/L NaCl or 0.6 mol/L sorbitol. Pyruvate concentration by the mutant strain RS23 reached 94.3 g/L at 82 h (yield on glucose 0.635 g/g) in a 7-l fermentor with 150 g/L glucose as carbon source. Pyruvate concentration and yield of mutant RS23 were 41.1% and 11.1% higher than those of the parent strain, respectively. The strategy for enhancing pyruvate production by increasing osmotic stress resistance may provide an alternative approach to enhance organic acids production with yeast.

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**KEYWORDS:** *Torulopsis glabrata*; pyruvate; NaCl-tolerance

## Introduction

Pyruvic acid, an important α-oxocarboxylic acid, locating at the vital junction of cell metabolism, plays a central role in energy and carbon metabolism (Yonehara and Miyata, 1994). Pyruvic acid is currently manufactured for use as a food additives, nutriceuticals, and weight-control supplement. Pyruvic acid can also be used as a starting material in the biosynthesis of pharmaceuticals, crop protection agents, polymers, and cosmetics (Li et al., 2001a). Pyruvate can be produced by four different processes: (i) chemical synthesis (Ai and Ohdan, 1995; Howard and Fraser, 1932; Sugiyama et al., 1992); (ii) fermentation process (Li et al., 2001a,b; Yokota et al., 1994); (iii) enzymatic process (Burdick and Schaeffer, 1987; Eisenberg et al., 1997); and (iv) resting cells (Izumi et al., 1982; Ogawa et al., 2001; Schinschel and Simon, 1993). Among these approaches, fermentation production of pyruvate from sustainable, low-cost carbon source (such as glucose) has merits in terms of both high yield and the high purity of the product (Bruno et al., 2004).

Fermentation pyruvate production uses primarily two microorganisms, a multi-vitamin auxotroph of the yeast *Torulopsis glabrata* (Li et al., 2001a) and a lipoic acid auxotroph of *Escherichia coli* (Yokota et al., 1994). Of these two strains, the direct fermentation approach with *T. glabrata* could obtain the highest pyruvate concentration (69 g/L), along with the highest yield of 0.636 g/g and the highest productivity, and this offers a high industrial potential in the future (Li et al., 2002). High concentration and high yield as well as high productivity are the objectives to realize a cost-effective biotechnological production of pyruvate. In order to further improve the competitiveness of fermentation pyruvate production, the final pyruvate concentration must be further increased. However, it is found that high extracellular pyruvate concentrations above 45 g/L may cause significant inhibition of microbial pyruvate synthesis (Bruno et al., 2004; Li et al., 2001b; Zeli et al., 2003). Similar physiological phenomenon was also observed in the fermentation production of other organic acids (Buche, 1991; Jin and Yang, 1998; Kertes and King, 1986; Kwon et al., 2001; Van der Wielen and Luyben, 1992; Vandák et al., 1997), all of them showed end-product inhibition (Schügerl, 2000; Van der Wielen and Luyben, 1992). Product inhibition slows down the reaction rate

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when product concentrations are high, thus, the microbial production capacity cannot reach its maximum theoretical level (Schügerl, 2000; Schügerl and Hubbuch, 2005). During organic acid fermentation, the production of acid results in pH decrease which subsequently inhibits cell growth and acid accumulation in turn. In addition, the toxicity of acids, especially at low pH when the acid is in its undissociated state, also inhibits cell growth and acid accumulation (Schügerl, 2000). However, for pyruvate production, the product inhibition is not directly caused by low pH and the toxicity of pyruvic acid. In this case, the pH of culture broth is maintained at 5.5 and pyruvic acid was in the form of pyruvate sodium. According to the result of Hua et al. (1999), about 70 g/L pyruvic acid is accumulated by *T. glabrata* in a medium containing 100 g/L glucose. In his case, as up to 0.8 mol/L NaOH was fed into the fermentation broth to control pH, a high osmotic stress caused by high concentration of $Na^+$ could occur. Then, under this condition, it is conceivable that high osmotic stress may become the major inhibitory for cell growth and organic acid fermentation in turn. However, very few research works investigating the effect of osmotic stress on organic acid fermentation have been reported.

In this study, with the target of further increasing the pyruvate concentration, the influence of osmotic stress on pyruvate production by *T. glabrata*, was carefully investigated, and the NaCl-tolerant mutants were screened. Our results showed that increasing the osmotic tolerance of strain could further enhance pyruvate production. Furthermore, the strategy of increasing *T. glabrata* osmotic tolerance to enhance pyruvate production may be applied to the other organic acids fermentation processes.

## Materials and Methods

### Microorganism

A multi-vitamin-auxotrophic yeast *T. glabrata* CCTCC M202019, screened by our laboratory in a previous study, was used as the working and parent strain (Liu et al., 2004).

### Media

Medium composition for seed culture (medium A) contained (g/L): 30 glucose, 10 peptone, 1 $KH_2PO_4$, and 0.5 $MgSO_4 \cdot 7H_2O$. For slant culture, 20 g/L agar was added to medium A. The fermentation medium (medium B) contained (g/L): 6 sodium acetate, 7 $NH_4Cl$, 5 $KH_2PO_4$, 0.8 $MgSO_4 \cdot 7H_2O$, 4 mg/L nicotinic acid, 15 μg/L thiamine · HCl, 100 μg/L pyridoxine · HCl, 10 μg/L biotin, and 50 μg/L riboflavin. In plate cultures, 5 g/L $CaCO_3$ was added. The initial pH of all media was adjusted to 5.0. Vitamin stock solutions were filter-sterilized prior to addition to the medium. $CaCO_3$ was sterilized by dry-heat sterilization at 160°C for 30 min before being added to the medium.

### Culture Conditions

#### *Batch Cultures*

The seed culture inoculated from a slant was cultivated in a 500 mL flask containing 50 mL medium A on a reciprocal shaker for 24 h. Fermentation was carried out in 500 mL flasks containing 50 mL medium B, or in a 7-l jar fermentor (KF-7 l, Korea Fermentor Co., Inchon, Korea) with 4 L medium B. The inoculum's size was 10% (v/v). In flask cultures, the medium was buffered by 40 g/L $CaCO_3$. In the fermentor cultures, pH was automatically controlled at 5.0 with 8 mol/L NaOH. The flask cultures were grown for 48 h and the rotation rate was controlled at 200 rev/min. All experiments were done in triplicate. All cultivations were carried out at 30°C.

#### *Continuous Cultures*

A 2-l jar fermentor (KF-2 l) with 1.2 L working volume was used. The fermentor was equipped with a pH electrode in the culture vessel, connected to an automatic pH controller (Fig. 1). The nutrient pump of the fermentor was

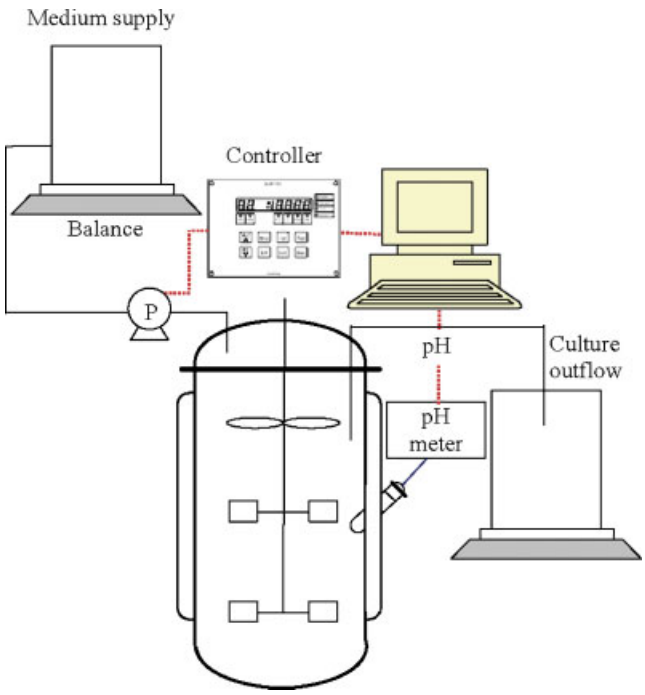


**Figure 1.** Chemostat (schematic) that automatically controls culture density with a pH-meter. The nutrient pump of the chemostat, connected to the automatic pH controller, was switched on ($D > \mu$) and off ($D = 0$) in response to the pH of the culture, which was therefore kept near a critical value, $pH_c$, resulting in a continuous culture system. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]



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regulated to provide a dilution rate, $D$, of 0.5 $h^{-1}$, higher than the highest growth rate of *T. glabrata* used (0.48 $h^{-1}$ for *T. glabrata* in fermentation medium at the middle of logarithmic phase at 30°C). The pump was connected to the pH controller so that when growth increased and the pH fell below 3 ($pH_c = 3$), the pH controller switched on the nutrient pump and the culture was diluted with fresh medium, increasing the pH. When, after dilution, the pH rose above 5, the pH controller switched off the nutrient pump ($D = 0$). This feedback system results in a "pH-controlled continuous" culture. The NaCl concentration was initially 30 g/L and was raised gradually to 70 g/L. The culture vessel with 1.2 L of the appropriate medium was inoculated with 15 mL of an early-stationary-phase culture and kept at 30°C, the agitation speed and aeration rate were controlled at 200 rev/min and 0.5 vvm, respectively.

### Mutagenesis of *T. glabrata* CCTCC M202019 and Selection of Mutants

To isolate NaCl-tolerance mutants, *T. glabrata* grown in the fermentation medium for 12 h was harvested by centrifugation (6,000*g*) and washed twice with 0.1 mol/L potassium phosphate buffer (pH 7.0). Cells were treated with nitrosoguanidine (NTG; 100 mg/L) after reaching a density of $5 \times 10^8$ cells/mL. After 60 min of the NTG-treatment, cells were extracted and centrifuged at 6,000*g*, and then washed with sterile saline (0.85% NaCl, w/v). The NTG-treated cell was inoculated into the fermentation medium with 30 g/L NaCl and the pH-controlled continuous culture was activated once the cells reached the early stationary phase. When pH in the fermentation broth fell to 3.0 with 16.7 g/L pyruvic acid produced (Table I), a fresh medium containing 40 g/L NaCl was fed at the dilution rate of 0.5 $h^{-1}$, higher than the highest growth rate (0.48 $h^{-1}$ for *T. glabrata* in fermentation medium at 30°C) of *T. glabrata*. When pH rose above 5, the pH controller switched off the nutrient pump ($D = 0$). Then the cell growth increased and the pH fell below pH 3.0, with the consequence that the pH controller switched on the nutrient pump automatically and the culture was diluted with fresh medium containing 50 g/L NaCl. When pH rose above 5 again, the pH controller switched off the nutrient pump ($D = 0$). The NaCl concentration was initially 30 g/L and was raised gradually to 70 g/L. This process was repeated, until a cell growth balance was obtained at the fermentation medium containing 70 g/L NaCl. To isolate colonies, samples were taken periodically, each sample was centrifuged at 6,000*g*, followed by washing with sterile saline (0.85% NaCl, w/v) and again centrifuged. The cell pellets were diluted to $10^{-4}$, $10^{-5}$, and $10^{-6}$ and spread onto fermentation medium plates containing 70 g/L NaCl or 0.6 mol/L sorbitol. Incubation was conducted at 30°C for 48 h. Colonies appeared on plates containing NaCl or sorbitol were replicated onto $CaCO_3$ plates containing 70 g/L NaCl to select putative mutants. The NaCl-tolerant mutant should have higher $R$-value as defined below:

$$R = \frac{\text{The diameter of transparent circle(mm)}}{\text{The diameter of colony(mm)} \times \text{The time of zone form(h)}}$$

The pyruvate producing ability and genetic stability of the selected mutants were subsequently examined. For genetic stability test, mutants were incubated in fermentation medium containing 70 g/L NaCl and evaluated for 20 consecutive generations.

**Table I.** The concentration of growth and pyruvate at different stage of continuous culture.

| | Cell concentration/(g/L) | | Pyruvate concentration/(g/L) | |
|---|---|---|---|---|
| Concentration of NaCl (g/L) | Initial of culture | End of culture | Initial of culture | End of culture |
| 30 | 0.36 | 3.1 | 0.6 | 16.7 |
| 40 | 0.34 | 3.6 | 0.7 | 18.3 |
| 50 | 0.41 | 3.8 | 0.5 | 17.5 |
| 60 | 0.52 | 4.3 | 0.4 | 18.7 |
| 70 | 0.62 | 4.6 | 0.5 | 19.4 |

### Analytical Methods

Pyruvate concentration was determined by the enzymatic assay described previously (Li et al., 2002). Glucose was assayed enzymatically using a commercial test kit supplied by Merck (Merckotest 14365, Darmstadt, Germany). Cell concentration was measured using a spectrophotometer (Biospe-1601; Shimadzu Co., Kyoto, Japan) at 660 nm after an appropriate dilution. The optical density ($OD_{660}$) value was converted to dry cell weight (DCW) according to a predetermined calibration line ($OD_{660}$:DCW (g/L) = 1:0.23).

## Results

### Effect of Osmotic Stress on the Growth of *T. glabrata* and Pyruvate Production

The effects of NaCl on the growth of *T. glabrata* and pyruvate production were presented in Figure 2. The highest level of DCW (11.7 g/L) and the highest pyruvate concentration (42.2 g/L) were observed without NaCl addition. However, the concentrations of cell and pyruvate decreased, along with the increase of the NaCl concentration in culture broth. When 50 g/L NaCl was added to the culture broth at the start of the fermentation, as shown in Figure 2A, the concentrations of cell (3.6 g/L) and pyruvate (2.8 g/L) decreased 79.3% and 93.2%, respectively, as compared with the control (without NaCl addition). The effect of NaCl addition time on the growth of *T. glabrata* (Fig. 3A) and the pyruvate production (Fig. 3B) was also investigated in detail (Fig. 3). Addition of 50 g/L NaCl at the beginning of the fermentation inhibited the growth of *T. glabrata* and the pyruvate production, and the inhibitory effect increased

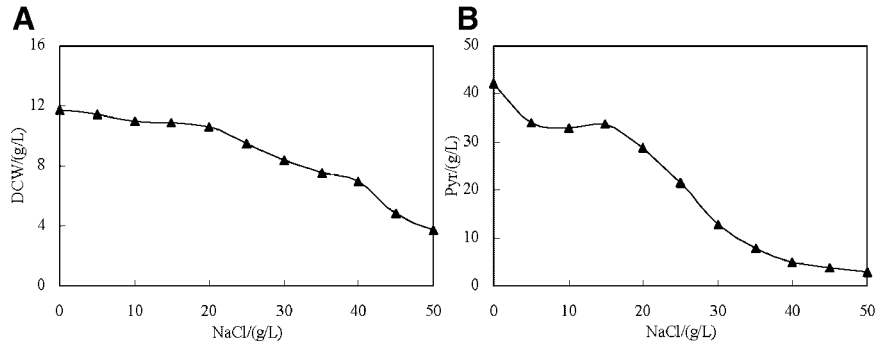


**Figure 2.** Effect of NaCl on the *Torulopsis glabrata* growth (**A**) and pyruvate production (**B**). NaCl was supplemented to the fermentation medium at the initial of fermentation.

significantly with the NaCl addition amount when the addition time was fixed. On the other hand, less inhibitory effects were observed on addition of NaCl at a later fermentation phase (Fig. 3).

The similar physiological changes were observed when adding sorbitol, an osmotic stress inducer. The concentrations of cell and pyruvate were decreased with the increase of the sorbitol concentration in culture broth (Fig. 4). All these results demonstrated that the growth of *T. glabrata* and pyruvate production could be adjusted and controlled by fermentation medium osmolality, and cell growth and pyruvate production would decrease under a higher osmolality. In other words, the pyruvate production could be enhanced if the tolerance of *T. glabrata* to high osmotic stress was increased.

### Isolation of Osmotic-Tolerance Mutants in a pH-Controlled Continuous Culture

The resistance of *T. glabrata* to NaCl was checked in fermentation medium which containing high concentration of NaCl, and the result showed that the wild-type strain ceased to grow when the concentration of NaCl reached 40 g/L.

The pH-controlled continuous culture was then initiated with the feeding medium containing 30 g/L NaCl. The NaCl concentration in the feeding medium was manually adjusted up to a level of 70 g/L, stepwise in a 10 g/L increment, to bring up the NaCl level in the fermentation medium gradually. The NaCl-tolerant mutants were bred according to the methods described earlier (Materials and Methods). About 400 colonies capable of growing on plates containing 70 g/L NaCl or 0.6 mol/L sorbitol were selected, and then replicated onto the $CaCO_3$ plates containing 100 g/L glucose and 70 g/L NaCl. From the colonies formed, 20 strains with higher *R*-value were chosen. The pyruvate producing ability of these 20 mutants was examined in the culture containing 150 g/L glucose as carbon source. One mutant, RS23, exhibiting the highest pyruvate concentration (more than 80 g/L), was eventually selected. This mutant was genetic-stable over generations and therefore, it was chosen as the working strain for further study.

### Effect of NaCl on Pyruvate Production by Parent Strain and Mutant RS23

The effects of 70 g/L NaCl on the pyruvate production by parent strain and mutant strain RS23 was investigated in

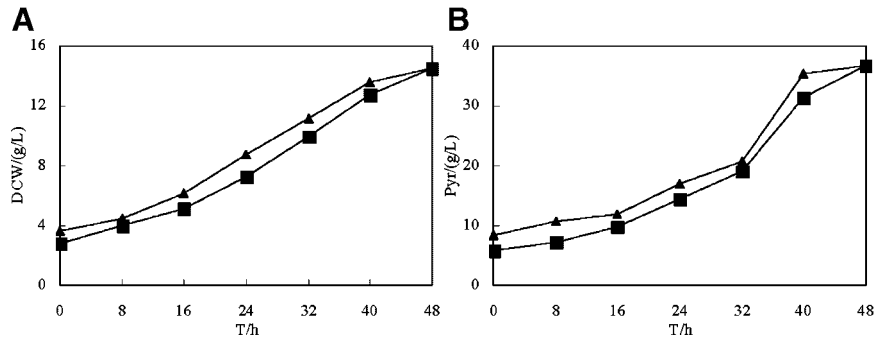


**Figure 3.** Effect of NaCl addition time on the cell growth (**A**) and pyruvate production (**B**) (▲50 g/L; ■ 60 g/L).



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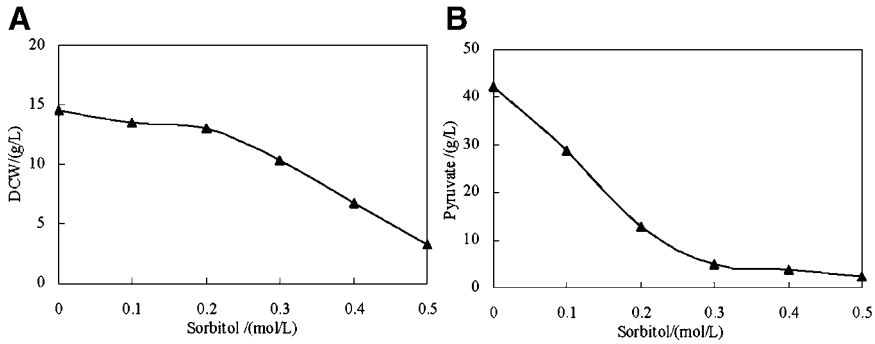


**Figure 4.** Effect of sorbitol on the *T. glabrata* growth (**A**) and pyruvate production (**B**).

medium containing 100 g/L glucose (Fig. 5). Without NaCl addition, growth rate and final cell concentration of the mutant RS23 were 32.5% higher than those of the parent strain (Fig. 5A). Moreover, the pyruvate concentration and the pyruvate yield were 36.5% and 33.9% higher than those of the parent strain, respectively. When 70 g/L NaCl was added to the fermentation media, the final cell concentration of the parent strain decreased by 77%, compared with that of the control (without NaCl addition). However, the final cell concentration of the mutant RS23 did not vary much regardless NaCl addition or not. For the pyruvate production, the final pyruvate concentrations of both parent strain and mutant strain RS23 decreased when 70 g/L NaCl was supplemented. For the parent strain, the pyruvate concentration was only 10.4% of that of the control (41.3 g/L, without NaCl addition). On the other hand, 30.2 g/L pyruvate could still be accumulated by the mutant RS23 when growing on the media containing 70 g/L

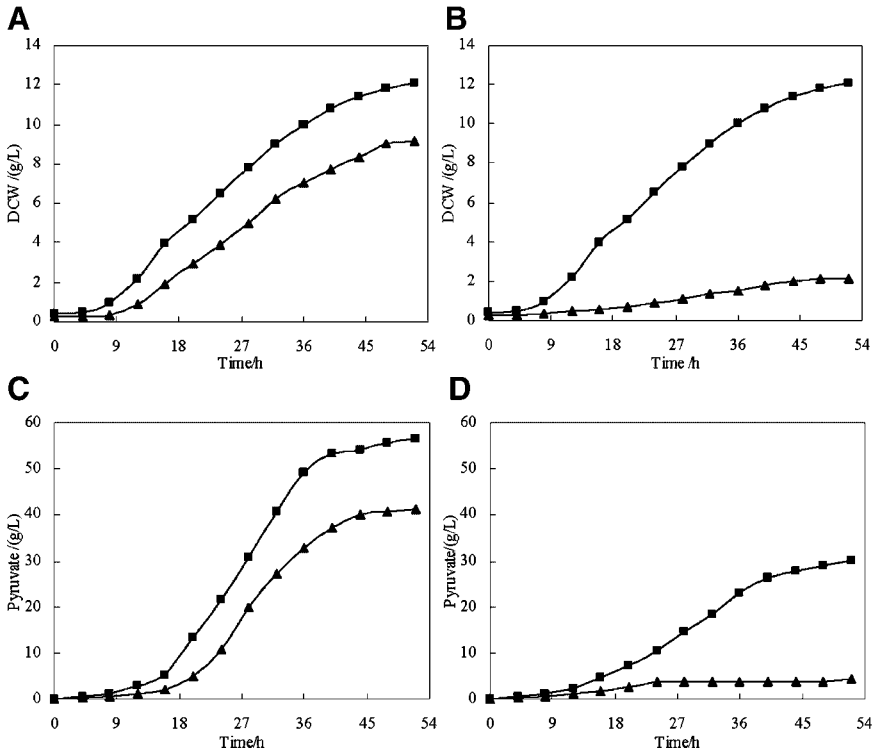


**Figure 5.** Effect of NaCl (70 g/L) on pyruvate production by mutant strain and parent strain (**A** and **C**: without NaCl; **B** and **D**: 70 g/L NaCl). ■, mutant strain RS23; ▲, parent strain CCTCC M202019.

NaCl. These results suggested that the pyruvate production ability of *T. glabrata* could be enhanced by increase of the strain's NaCl tolerance.

### Comparison of Parent Strain and Mutant RS23 at 150 g/L Glucose

Figure 6 showed the time course of pyruvate production by parent strain and mutant RS23 with 150 g/L glucose in initial fermentation medium, and the results indicated that significant changes took place in carbon metabolism of the mutant strain RS23. First, the length of lag phase decreased 75%, as compared with that of the parent strain (16 h). Second, the final cell concentration reached 13.8 g/L, which was 23.2% higher than that of the parent strain. Third, the highest pyruvate concentration of 94.3 g/L was achieved at 82 h, which was 41.1% higher than that of parent strain. Moreover, the pyruvate production rate of the mutant strain RS23 was 72.1% higher than that of the parent strain. Fourth, for the parent strain, glucose consumption stopped at 100 h with a high residual glucose concentration of 33.3 g/L and the glucose consumption rate was 44.3% lower than that of the mutant strain. Fifth, as illustrated in Table II, the pyruvate yield on glucose of the mutant strain RS23 was 11.1% higher than that of the parent strain. Sixth, the glycerol concentration of 2.3 g/L was also detected in fermentation broth with the parent strain as shown in Table II, while no glycerol was detected in the fermentation broth using mutant strain RS23.

## Discussion

In organic acid fermentation, alkali materials (such as NaOH, $NH_3$, $CaCO_3$) are often used to regulate pH to maintain it within the optimal range. As a consequence, an osmotic stress increase in fermentation broth is always observed if the addition of alkali materials continues in order to neutralize the organic acid formed. In pyruvate fermentation by *E. coli* and *T. glabrata* (Li et al., 2001a; Yokota et al., 1994), NaOH was used to maintain pH at an optimal level. As $Na^+$ concentration in fermentation broth increased along with pyruvate continuous accumulation, the osmotic stress in fermentation broth increased subsequently. Under this condition, the major concern or question would be that, the further pyruvate accumulation is affected by the pyruvate self-inhibition, or the higher osmotic stress caused by $Na^+$, or the combinational effect of both. However, few research works regarding on these for not only pyruvate fermentation but also other organic acids production have been reported. This study has clearly demonstrated that the growth and the pyruvate production capability of *T. glabrata* decreased with high osmotic stress caused by $Na^+$ in fermentation broth (Figs. 2–4).

In order to alleviate the osmotic stress inhibition in organic acid fermentations and to further increase the yield and productivity of organic acid, a lot of metabolic and process engineering techniques have been developed (Roffler, 1984). The process engineering strategies mainly involved with the in situ product removal systems, such as precipitation, solvent extraction (Schügerl, 1994), ion exchange (Vaccari et al., 1993), ultrafiltration membranes, electrodialysis (Bruno et al., 2004; Nomura et al., 1987), and crystallization (Takamatsu and Ryu, 1988). For example, in order to enhance the pyruvate production by *E. coli*, a fully integrated continuous process with electro-dialysis was developed to separate pyruvate from fermentation broth, so that a high pyruvate concentration of 79 g/L was achieved (Bruno et al., 2004). However, the integrated in situ product removal processes are still waiting for broad industrial application because of their higher complexity, and thus there is a need for detailed process knowledge of the application (Schügerl and Hubbuch, 2005).

Besides the integrated processes, an alternative process is to breed the feedback resistant mutant (Yun et al., 2005). This approach could be simplified by the introduction of high-throughput screening techniques as a tool in early process development. These approaches promise a high level of acceptance in industry and have been proven to be successful in biotechnological production, such as amino

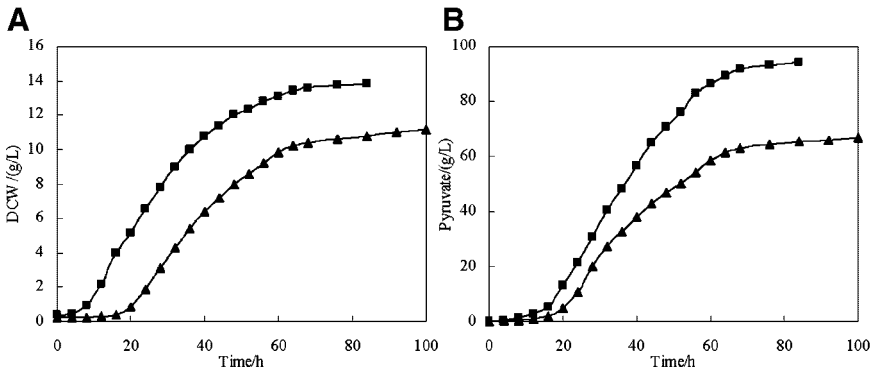


**Figure 6.** Comparison of pyruvate production by mutant strain and parent strain with 150 g/L glucose. ■, mutant strain RS23; ▲, parent strain CCTCC M202019.



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**Table II.** Comparison of parent strain and mutant strain RS23 at 150 g/L glucose.

| Parameters | Strain | | |
|---|---|---|---|
| | Parent strain (A) | Strain RS23 (B) | Change(%) $[(B/A) - 1] \times 100$ |
| Fermentation time (h) | 100 | 82 | −18 |
| The amount of glucose consumed (g/L) | 116.7 | 148.3 | 27 |
| Maximum dry cell weight (g/L) | 11.2 | 13.8 | 23.2 |
| Maximum pyruvate concentration (g/L) | 66.8 | 94.3 | 41.1 |
| Yield of pyruvate to glucose (g/g) | 0.572 | 0.635 | 11.1 |
| The amount of glucose consumed per cell (g/g) | 10.4 | 10.7 | 2.8 |
| The glucose consumption rate (g/(L · h)) | 1.16 | 1.80 | 55.7 |
| The amount of pyruvate production per cell (g/g) | 5.96 | 6.83 | 14.5 |
| Pyruvate productivity (g/(L · h)) | 0.668 | 1.15 | 72.1 |

acids (Kazutoyo et al., 1995) and organic acids. However, for organic acid fermentation, research papers involved with breeding mutant strains to tolerate high osmotic stress or high concentration of salt were seldom available. In this study, the mutant strain RS23, which could tolerate 70 g/L NaCl or 0.6 mol/L sorbitol, was bred with the maximum pyruvate capacity of 94.3 g/L.

This study demonstrated that, the product inhibition in organic acid fermentation caused by high osmotic stress could be eliminated, and pyruvate production could be significantly increased by enhancing *T. glabrata* osmotic stress tolerance through a pH-controlled continuous culture. In addition, increasing the pyruvate production in *T. glabrata* through enhancing osmotic-tolerance may provide an alternative approach for effective production of other organic acids.

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