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# Enhancement of pyruvate productivity in *Torulopsis glabrata*: Increase of $NAD^+$ availability

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**Abstract**

This study aimed at increasing the pyruvate productivity from a multi-vitamin auxotrophic yeast *Torulopsis glabrata*, by increasing the availability of $NAD^+$. We examined two strategies for increasing availability of $NAD^+$. To supplement nicotinic acid (NA), the precursor of $NAD^+$; and to increase the activity of alcohol dehydrogenase integrating with addition acetaldehyde as exterior electron acceptor. The addition of 8 mg $l^{-1}$ NA to the fermentation medium resulted in a significant increase in the glucose consumption rate (48.4%) and the pyruvate concentration (29%). An ethanol-utilizing mutant WSH-13 was screened and selected after nitrosoguanidine mutagenesis of the parent strain *T. glabrata* CCTCC M202019. Compared with the parent strain, the alcohol dehydrogenase activity of the mutant WSH-13 increased about 110% and the mutant could utilize ethanol as the sole carbon source for growth (1.8 g $l^{-1}$ dry cell weight). When growing with glucose, the addition of 4 mg $l^{-1}$ acetaldehyde to the mutant WSH-13 culture broth led to a significant increase in the glucose consumption rate (26.3%) and pyruvate production (22.5%), but the ratio of NADH/$NAD^+$ decreased to 0.22. Acetaldehyde did not affect the glucose and energy metabolism at high dissolved oxygen (DO) concentration. However, at lower DO concentration (20%), maintaining the acetaldehyde concentration in the mutant culture broth at 4 mg $l^{-1}$ caused an increased $NAD^+$ concentration but a decreased NADH concentration. As a consequence, the pyruvate production rate, the pyruvate yield on glucose and the pyruvate concentration were 68, 44 and 45% higher, respectively, than the corresponding values of the control (without acetaldehyde). The strategy for increasing the glycolytic flux and the pyruvate productivity in *T. glabrata* by increasing the availability of $NAD^+$ may provide an alternative approach to enhance the metabolites productivity in yeast.


*Keywords:* *Torulopsis glabrata*; $NAD^+$ available; Pyruvate; Productivity

## 1. Introduction

In order to increase the productivity of fermentation products from biomass feedstock, it is extremely important to increase the flux through glycolytic

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pathway (Schmid et al., 2001). Metabolic engineering has focused on over-expressing a specific rate-limiting enzyme in the glycolytic pathway and thereby to release a metabolic bottleneck (Pritchard and Kell, 2002; San et al., 2002). However, individual or combinational over-expression of genes encoding the key glycolytic enzymes, in both yeast (Schaaff et al., 1989) and bacterial cells (Ruijter et al., 1997) does not increase the glycolytic flux (Davies and Brindle, 1992). Indeed, cofactors play an essential role in a large number of biochemical reactions and the production of different fermentation products (San et al., 2002). The cofactor levels and the availability of the cofactor forms can become limiting step in cofactor-dependent production systems. Therefore, cofactor manipulations can potentially become a powerful tool for improvement of process productivity.

Nicotinamide adenine nucleotide ($NAD^+$), one of the key cofactors in the metabolic networks, functions as a cofactor in over 300 oxidation–reduction reactions (Foster et al., 1990; Lopez de Felipe et al., 1998). It is generally considered that NADH/$NAD^+$ cofactor pair plays a central role in glucose catabolism where glucose is oxidized using $NAD^+$ as a cofactor and $NAD^+$ is simultaneously converted into the reduced form of NADH with the equivalent amount. Studies had demonstrated that cytosolic NADH/$NAD^+$ is an important cofactor of glycolytic flux in yeast (Luttik et al., 1998), thus, a lack of $NAD^+$ would force glycolysis to stop. In order to increase the glycolytic flux, it is crucial for constant supply of $NAD^+$; and NADH produced in glycolysis, fatty acid oxidation, and the citric acid cycle must be oxidized to $NAD^+$ to achieve a redox balance (Van Dijken and Scheffers, 1986).

The regeneration of $NAD^+$ from NADH by yeast cells can occur in two separate ways (Li et al., 2002). Under aerobic growth, $NAD^+$ regeneration is through the mitochondrial electron transfer chain (ETC), in which oxygen is used as the final electron acceptor, and a large amount of ATP was produced. While under anaerobic growth and in the absence of an alternate oxidizing agent, the regeneration of $NAD^+$ could happen by alcohol fermentation pathway with acetaldehyde as the exogenous electron acceptor. In this case, acetaldehyde acts as the substrate, and alcohol dehydrogenase catalyzes the oxidation of NADH to $NAD^+$ (Stanley et al., 1997) (Fig. 1). In yeast, anaerobic fermentation allows the yeast to continue energy production and to survive in the environment of oxygen absence, meanwhile to produce ethanol and carbon dioxide from pyruvate.

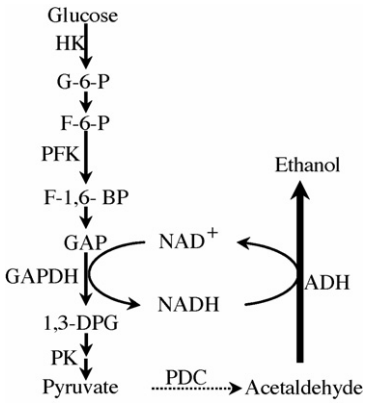


Fig. 1. Relationship of glycolytic pathway and $NAD^+$ regeneration.

Pyruvate is an important raw material widely used in chemical, pharmaceutical, and agrochemical industries (Li et al., 2001a). The multi-vitamin auxotrophic yeast, *Torulopsis glabrata*, is the only microorganism being used in the commercial fermentation production of pyruvate (Li et al., 2001a). As pyruvate is located at a key junction of metabolism (Pronk et al., 1996), it is a good model for studying the regulatory mechanism of glycolysis (Yokota et al., 1994). In previous studies (Li et al., 2002), a high pyruvate yield on glucose ($0.797\,g\,l^{-1}$) but a low pyruvate production rate ($0.91\,g\,(l\,h)^{-1}$) were achieved under a relatively high dissolved oxygen concentration (DO, 85%). Metabolic flux analysis revealed that the NADH produced from glycolytic pathway is mainly oxidized via the electron transfer chain (Hua and Shimizu, 1999). This leads to the regeneration of a large amount of ATP, which subsequently retards the glycolytic flux. When the DO decreases from 85 to 50%, a higher glucose consumption rate ($1.97\,g\,(l\,h)^{-1}$) is achieved as compared with the case of under high DO. But the pyruvate yield on glucose decreases to $0.483\,g\,g^{-1}$ due to a large amount of glycerol production. Metabolic flux analysis revealed that glycerol production is an alternative pathway to oxidize excessive NADH that is not fully oxidized by ETC under low DO (Hua and Shimizu, 1999; Li et al.,

2002). In order to increase the pyruvate productivity of *T. glabrata*, a hypothesis was developed that: an increase in the availability of $NAD^+$ integrating with a lower intracellular ATP level was expected to increase the glycolytic flux. When the DO was decreased from 85 to 50%, NADH could not be fully oxidized by ETC, resulted in a decrease of the amount of ATP production through oxidative phosphorylation. The excessive NADH also could not be oxidized through fermentative pathway because the activity of pyruvate decarboxylase in *T. glabrate* was limited. As a result, a NADH fully oxidation strategy with high pyruvate yield on glucose was proposed: increasing the activity of alcohol dehydrogenase then feeding acetaldehyde as an exogenous electron acceptor (Fig. 1).

In this study, we attempted to increase the productivity of pyruvate in *T. glabrata*. To achieve this target, we used a novel approach to increase the availability of intracellular $NAD^+$ through biochemical method, that was, to increase the activity of a biologically active NADH-dependent alcohol dehydrogenase (ADH) and to add electron acceptor to oxidize the excessive NADH at lower DO concentration. In the presence of acetaldehyde, the excessive NADH was oxidized to $NAD^+$, and a lower intracellular ATP and higher pyruvate productivity could be achieved. The new system would allow the cells subject to a lower ratio of NADH/$NAD^+$ and ATP level, and therefore, a higher pyruvate productivity under lower DO could be obtained.

## 2. Materials and methods

### 2.1. Microorganism

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

### 2.2. Media

Medium composition for seed culture (medium A) contained: 3% glucose, 1% peptone, 0.1% $KH_2PO_4$ and 0.05% $MgSO_4{\cdot}7H_2O$. For slant culture, 2% agar was added to medium A. The fermentation medium (medium B) contained: 10% glucose, 0.6% sodium acetate, 0.7% $NH_4Cl$, 0.5% $KH_2PO_4$, 0.08% $MgSO_4{\cdot}7H_2O$, 4 mg $l^{-1}$ nicotinic acid, 15 μg $l^{-1}$ thiamine HCl, 100 μg $l^{-1}$ pyridoxine HCl, 10 μg $l^{-1}$ biotin, and 50 μg $l^{-1}$ riboflavin. In plate cultures, 5 g $l^{-1}$ $CaCO_3$ 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.

### 2.3. Culture conditions

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. Fermentations was carried out in a 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 $l^{-1}$ $CaCO_3$. In the fermentor cultures, the pH was automatically controlled at 5.0 with 8 M NaOH solution. The flask cultures were grown for 48 h and the rotation rate was controlled at 200 rpm. All experiments were done in triplicate. All cultivations were carried out at 30 °C.

### 2.4. Dissolved oxygen measurement and control

The dissolved oxygen concentration in the culture medium is monitored in situ by a DO probe (Mettler Toledo, Inc., USA). It was calibrated using the standard two-point reference technique, with 100% corresponding to air-saturation in fresh medium before the inoculation of cells at 30 °C, and 0% corresponding to the probe reading following equilibration with pure nitrogen.

Batch cultures were performed in a baffled 7-l fermentor (KF-7 l, Korea Fermentor Co., Inchon, Korea) equipped with two six-blade turbines. The cultures were maintained at 30 °C and sparged with nitrogen and air at a constant total flow rate of 3 l $min^{-1}$. The DOT was measured with DO probe and controlled at a constant value by a proportional-integral-derivative (PID) algorithm that varied the agitation rate. PID constants were determined from process reaction curves and following the Ziegler–Nichols criteria.

Constant DOT values, in the range of 20–85% (with respect to air saturation), were tested. A constant agitation rate was used for each culture but was different (in the range of 150–700 rpm) for the various DOT tested.

### 2.5. Mutagenesis of T. glabrata CCTCC M202019 and selection of mutants

*T. glabrata* grown in the fermentation medium for 12 h was harvested by centrifugation ($6000 \times g$) and washed twice with 0.1 M $l^{-1}$ potassium phosphate buffer (pH 7.5). Cells were treated with nitrosoguanidine (NTG; 100 mg $l^{-1}$) after reaching a density of $5 \times 10^8$ cells $ml^{-1}$. After 60 min the NTG-treated cells were sedimented and re-suspended in fresh medium and grown 24 h for recovery. Aliquots were inoculated into minimal medium plus 5 g $l^{-1}$ ethanol as sole carbon source and allowed to grow at 30 °C for 48 h. Samples from flasks showing growth were extracted and centrifuged at $6000 \times g$, followed by washing with sterile saline (0.85% NaCl, w/v) and again centrifuged. Cell pellets were diluted to $10^{-4}$, $10^{-5}$ and $10^{-6}$ and spread onto alcohol indicator agar and incubated at 30 °C for 48 h. Alcohol indicator plates (AIP) were used to detect ADH activity of yeast colonies. On AIP, ADH activity is indicated by reduction of the indicator 2,3,5-triphyl tetrazolium chloride, then produced red colonies. Red colonies that appeared on alcohol indicator plates were picked and re-purified. The specific activity of alcohol dehydrogenase and genetic stability of the selected mutants were subsequently examined. For genetic stability test, mutants were incubated in fermentation medium containing ethanol as sole carbon source selection for 20 consecutive generations (Clark and Cronan, 1980).

### 2.6. Analytical methods

Pyruvate concentration was determined by the enzymatic assay described previously (Li et al., 2001b). 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 $l^{-1}$) = 1:0.23].

### 2.7. Measurement of the concentration of acetaldehyde

The extracellular acetaldehyde concentration was measured by extracting aliquots of approximately 0.5 ml of the cell suspension through a Dyna Gard filter (microgon) into a 2-ml syringe. A 200 μl of the filtrate was immediately transferred to a cuvette containing 800 μl 100 mM potassium phosphate, pH 6.8, alcohol dehydrogenase (1000 U $ml^{-1}$, Boehringer) and 10.7 mM NADH (disodium salt, Boehringer). The time between filtration and addition of the sample to the cuvette was 4–6 s. The absorbance at 340 nm was measured before and after addition of the filtrate. The acetaldehyde concentration was calculated by reference to acetaldehyde standards.

### 2.8. Estimation of intracellular metabolites concentration

Intracellular metabolite concentrations were measured by using an in vitro procedure based on inactivation of metabolism followed by metabolite extraction directly in the cell sample. A 50 ml cell samples were removed from the culture, frozen immediately in liquid nitrogen for 60 s, and stored at −20 °C.

The concentration of intracellular ATP was measured by HPLC (Agilent 1100 series, column stable-C18, Thermo electron corporation, MA, USA) (Sato et al., 2000; Stanley, 1986). For extracting ATP, 10 ml of 0.6 M $HClO_4$ was added to the cell pellets and mixed thoroughly with a magnetic stirrer for 10 min. The mixture was centrifuged for 10 min at $10{,}000 \times g$ to collect the supernatant. Another 10 ml of 0.6 M $HClO_4$ was added to the pellets, mixed thoroughly for 10 min, and the supernatant was collected after centrifugation. Two portions of the supernatant were combined in a 25 ml volumetric flask and made up to 25 ml with 0.6 M $HClO_4$. Ten milliliter of the prepared solution was taken and the pH adjusted to 7.0 with 0.8 M potassium hydroxide. After being kept at 4°C for 30 min, crystal $KClO_4$ was removed from the solution by filtration (pore size = 0.22 μm) and then diluted to 25 ml

with phosphate buffer (pH 7.0) before HPLC column application. The injection volume for the HPLC analysis was 10 μl. A mixture of 80% 10 mM $KH_2PO_4$ (pH 7.0) and 20% methanol was used as mobile phase with a flow rate of 1.2 ml $min^{-1}$. The wavelength of the UV detector was set at 260 nm and the column temperature was controlled at 25 °C.

NADH and $NAD^+$ are acid and alkali labile, respectively. Therefore, measurement of both oxidized and reduced forms requires that extraction be carried out in separate samples.

For extracting the NADH, 20 ml cell sample were freeze-dried for 24 h and transferred to 20 ml solution containing 50 mM KOH, 30% ethanol and 22 mM borate (pH 10). Extracted samples were left on ice for 30 min before pH being adjusted to 9.0–9.4 with 3 M HCl under vigorous agitation. After centrifugation at 4 °C for 10 min at 10,000 × *g*, the supernatant was immediately tested for NADH without neutralizing to avoid destruction of NADH by locally too high concentrations of acid. The NADH present in the alkaline extract was measured in a reaction mixture containing 200 μl of triethanolamine buffer (500 mM, pH 7.0, containing 15 mM $MgSO_4$ and 4 mM EDTA), 300 μl of alkaline extract, 480 μl of $H_2O$, 20 μl of pyruvate (200 mM), and 20 μl of lactate dehydrogenase (2000 U $ml^{-1}$) to initiate the pyruvate-dependent oxidation of NADH (Karp et al., 1983).

For extracting the $NAD^+$, 20 ml cell sample were freeze-dried for 24 h and transferred to 20 ml 70% (v/v) cold ethanol containing 10 mM potassium phosphate buffer, pH 6.5, and extracted for 30 min at room temperature (25 °C). During the extraction procedure, the pH was kept below 7.0 with 0.5 M HCl under vigorous agitation to avoid $NAD^+$ degradation. After centrifugation for 10 min (4 °C and 10,000 × *g*), the supernatant was immediately used for metabolite concentration measurements. $NAD^+$ was assayed by using 200 μl of pyrophosphate buffer (250 mM, pH 8.8, containing 12 g of semicarbazide per liter), 300 μl of extract, 490 μl of $H_2O$, and 10 μl of alcohol dehydrogenase (3000 U $ml^{-1}$) (Karp et al., 1983).

All metabolite concentrations were obtained relative to cell dry weight, and were expressed as mmol $g^{-1}$ DCW. And all values of metabolites concentrations were mean values of at least three independent extraction procedures.

### 2.9. Assays of intracellular enzymes

#### 2.9.1. Assay of key enzymes in pyruvate dehydrogenase complex bypass

Four milliliter cells harvested when the middle exponential phase was reached ($OD_{660}$ = 6–8) were washed with 0.85 (w/v) ice-cold saline and resuspended in 4 ml 0.1 M ice-cold potassium phosphate buffer (pH 7.5). Four milliliter of cell suspension added with 2 g of glass-beads with 0.7 mm diameter was ultrasonicated at 0 °C for 30 s repeatedly for four times (total 120 s) (ACX 400 sonicator, 20 kHz, Sonic and Materials Inc., Newton, MA, USA). Cell debris was removed by centrifugation at 6000 × *g* and 4 °C for 10 min. The supernatant containing 2–4 mg of protein per ml was used as cell-free extract (CFE). Protein concentrations were measured by the Lowry method. All values of enzyme assay were the means of results of at least three independent measurements.

The reaction mixture (2.0 ml) for $NAD^+$-dependent alcohol dehydrogenase assay comprised 60 mM sodium pyrophosphate buffer (pH 8.5), 0.7 M ethanol, 0.5 mM $NAD^+$ in a 50 mM glycine/NaOH buffer, and CFE. To allow the temperatures of the solutions to reach 30 °C, solutions in the cuvettes were allowed to stand for 10 min. The assay was initiated by addition of 0.2 ml CFE and terminated after 60 s by addition of 0.3 ml of 1 M KOH. One unit of specific activity was defined as 1 μmol of NADH formed per minute per milligram of protein under the given conditions (Miyata and Yonehara, 1999).

Enzyme assay for ADH was performed at 30 °C, and the increase in absorbance at 340 nm was monitored. The activity was calculated from the linear slope of increasing absorption of NAD(P)H by using $\varepsilon = 6220$ $M^{-1}$ $cm^{-1}$.

PDC activity was assayed as developed by Flikweert et al. (1996). The reaction mixture (1.5 ml) comprised 50 mM citrate buffer (pH 6.0), 0.07 mM thiamine pyrophosphate, 12 mM sodium pyruvate, and CFE. The reaction mixture was incubated at 30 °C for 20 min, afterwards the reaction was stopped by the addition of 1 ml of 0.8 mM 2,4-dinitrophenyl-hydrazine dissolved in 2 M hydrochloric acid. The reaction product was left further 15 min to convert the produced acetaldehyde into its hydrazone. Upon addition of 2 ml of methanol, the 2,4-dinitrophenylhydrazone of acetaldehyde and its derivatives were dissolved and

determined by high-performance liquid chromatography (HPLC) under the following conditions: column, Capcel pack $C_{18}$ (Interactive Chromatography, San Jose, CA, USA); mobile phase, 30 g $l^{-1}$ acetate aqueous solution:methanol (1:2); flow rate, 1.0 ml $min^{-1}$; injection volume, 5 μl; detection, UV (365 nm); temperature, 30 °C. A reaction mixture excluding pyruvic acid was used as a control.

*2.9.2. Assay of key enzymes in glycolytic pathway*

Hexokinase (HK) activity was assayed according to the method of Barnard with minor modification (Barnard, 1975). The reaction mixture (3 ml) consisted of 0.1 M triethanolamine (pH 7.6), 2 mM $MgCl_2$, 8 mM ATP, 15 mM glucose, 0.3 mM NADP and 2 units of glucose-6-phosphase dehydrogenase. The mixture was equilibrated at 30 °C for 5 min and the reaction was started by adding 0.1 ml CFE. The reaction was monitored by measuring the increase in absorbance at 340 nm due to NADPH formation. One unit of hexokinase activity is defined as the formation of 1 μmol of glucose-6-phosphate per minute at 30 °C.

Phosphofructokinase (PFK) activity was determined according to the method by Davies and Brindle with minor modification (Davies and Brindle, 1992). The reaction mixture (3.5 ml) contained: 5.2 mM glycyl-glycine (pH 7.6), 2 mM $MgCl_2$, 8 mM ATP, 3 mM fructose-6-phosphast, 0.3 mM NADH, 1 unit of aldolase, 7 units of triosephosphate isomerase and 1 unit of glycerol-3-phosphate dehydrogenase. The mixture was equilibrated at 30 °C for 5 min and the reaction was started by adding 0.2 ml CFE. The rate of NADH oxidation was measured at 340 nm and 30 °C. One unit of phosphofructokinase is defined as the production of 1 μmol of fructose-1,6-bisphosphate per minute per milligram of protein under the assay conditions.

The reaction mixture (3.0 ml) for the determination of pyruvate kinase (PK) consisted of 0.1 M Tris–HCl buffer (pH 7.6), 1.5 mM EDTA, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADH, 360 units of lactate dehydrogenase, 3 mM neutralized ADP and 5 mM phosphoenolpyruvate. The reaction was started by adding 0.2 ml CFE after incubation of the mixture for 5 min at 30 °C. Activity of PK is expressed as micromoles of pyruvate formed per minute per milligram of protein (Aust et al., 1975).

## 3. Results

### *3.1. Effect of nicotinic acid concentration on pyruvate productivity in T. glabrata*

Nicotinic acid (NA) were defined as the vitamin precursors of nicotinamide adenine nucleotide ($NAD^+$) (Fig. 2), which is soluble and transportable, and could be used for elevating the intracellular $NAD^+$ level (Berrios-Rivera et al., 2002). $NAD^+$ was the cofactor of glycolysis and the citric acid cycle, without $NAD^+$ the Krebs Cycle and glycolysis would be shut down quickly. For the multi-vitamin auxotroph, *T. glabrata*, a suitable concentration of NA in the medium is very important for efficient pyruvate production. Weak growth (3.04 g $l^{-1}$ DCW) was occurred without NA in the fermentation medium (Table 1). With the increases of NA concentration in the fermentation medium, the glucose consumption rate and the pyruvate concentration increased, but the pyruvate yield on glucose decreased. This may be due to the fact that $NAD^+$ also was a factor of the pyruvate dehydrogenase complex (PDHC), which catalyzed the reaction of converting pyruvate to acetyl-coenzyme A, the starting substance for the tricarboxylic acid cycle. With the increase of NA concentration, more pyruvate was transferred to acetyl-CoA. The highest level of the glucose consumption rate (2.01 g $(l\,h)^{-1}$) and the pyruvate

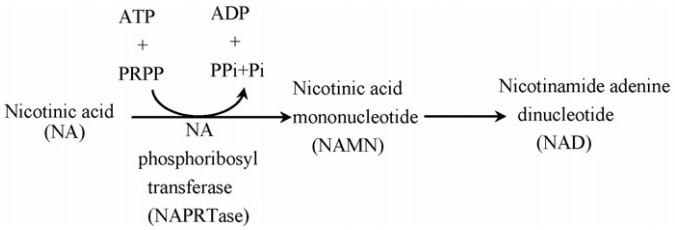


Fig. 2. The $NAD^+$ synthesis pathway from nicotinic acid (NA).

Table 1
Effect of nicotinic acid concentration on pyruvate production by *T. glabrata*

| Parameters | Nicotinic acid ($mg\,l^{-1}$) | | | |
|---|---|---|---|---|
| | 0 | 4 | 8 | 20 |
| Maximum dry cell weight ($g\,l^{-1}$) | $3.04 \pm 0.2$ | $6.53 \pm 0.2$ | $8.74 \pm 0.3$ | $9.45 \pm 0.2$ |
| Total consumed glucose ($g\,l^{-1}$) | $27.9 \pm 1.3$ | $71.5 \pm 1.6$ | $96.8 \pm 1.0$ | $99.4 \pm 1.2$ |
| Maximum pyruvate concentration ($g\,l^{-1}$) | $12.2 \pm 0.6$ | $37.2 \pm 0.8$ | $48.4 \pm 0.6$ | $41.7 \pm 0.5$ |
| The pyruvate yield on glucose ($g\,g^{-1}$) | $0.43 \pm 0.04$ | $0.52 \pm 0.02$ | $0.48 \pm 0.03$ | $0.42 \pm 0.02$ |
| Fermentation time (h) | 60 | 53 | 48 | 38 |
| The glucose consumption rate ($g\,(l\,h)^{-1}$) | $0.465 \pm 0.02$ | $1.35 \pm 0.03$ | $2.01 \pm 0.02$ | $2.61 \pm 0.03$ |
| Pyruvate productivity ($g\,(l\,h)^{-1}$) | $0.20 \pm 0.01$ | $0.70 \pm 0.02$ | $1.00 \pm 0.02$ | $1.10 \pm 0.02$ |
| Intracellular $NAD^+$ concentration ($mg\,g^{-1}$ DCW) | $13.1 \pm 1.2$ | $24.5 \pm 1.8$ | $28.6 \pm 2.2$ | $35.46 \pm 1.6$ |

concentration ($46.4\,g\,l^{-1}$) was achieved when adding $8\,mg\,l^{-1}$ NA to the fermentation medium, which was, respectively, 48.4 and 29% higher than those of the control ($4\,mg\,l^{-1}$ NA). In addition, as shown in Table 1, a higher intracellular $NAD^+$ level was also achieved. Those results indicated that, a large quantity of $NAD^+$ synthesized with the aid of NA was crucial for both the overproduction of pyruvate and the promotion of glycolysis.

### 3.2. Isolation of mutant capable of using ethanol as sole carbon source

2,3,5-Triphenyl tetrazolium chloride (TTC) is the prototype of a subclass of tetrazolium salts that has been proved to be useful in biological work. TCC in its oxidized state is soluble in water and appears colourless or faint yellow in solution. Under reduced state, it is converted to deep red. The substance in its reductive form is insoluble in water, so the reductive state is essentially irreversible. Since the development of the red colour is dependent on reduction of the dye, TTC can be used as a redox indicator. The medium with TTC serving as the alcohol indicator has been developed. On alcohol indicator plates, ADH activity is indicated by reduction of the indicator TCC and red colonies appearance. Red colonies indicate complete oxidation of ethanol to acetyl-CoA.

Our previous work had shown that the wild-type *T. glabrata* is unable to grow with ethanol as carbon source. Cultures of the wild-type treated with the mutagen NTG and mutants capable of growing with ethanol as sole carbon source were isolated after enrichment of them in liquid culture as described in Section 2. Approximately 2500 red colonies were obtained on alcohol indicator plates. They were then replicated onto alcohol indicator plates containing alcohol dehydrogenase inhibitor pyrazole ($100\,mg\,l^{-1}$). Among 2500 red colonies examined, 97 red colonies were able to grow on ethanol with pyrazole. The specific activities of ADH of these 97 selected mutants were examined, of which 18 mutants showed increased specific activity of ADH (60–108%), as compared with that of the parent strain. One mutant, WSH-13, exhibited the highest activity of alcohol dehydrogenase (about 110% higher than that of the parent strain), was selected. This mutant was also genetic-stable over 20 generations, so that it was chosen as a working strain for further study.

### 3.3. Comparison of the mutant and the parent strain

No growth occurred when growing the parent strain with ethanol as sole carbon source. However, for the mutant strain WSH-13, the cell concentration reached $1.8\,g\,l^{-1}$ (dry cell weight, DCW) under the same condition. Moreover, no difference was detected when the parent and the mutant strains were grown on glucose in flask, the rates of glucose consumption and pyruvate production, the rate and yield of growth, the intracellular $NAD^+$ level of the mutant were identical to the corresponding values of the parent strain. The results implied that, under the condition of the lower activity of PDC, increase of the ADH activity did not increase the glycolytic flux and modify the energy metabolism. When growing on glucose, the effect of pyrazole, a typical inhibitor of alcohol dehydrogenase, on the specific activity of ADH of the parent strain and the mutant WSH-13 was investigated. The activity of ADH in the parent strain did not change much regardless of

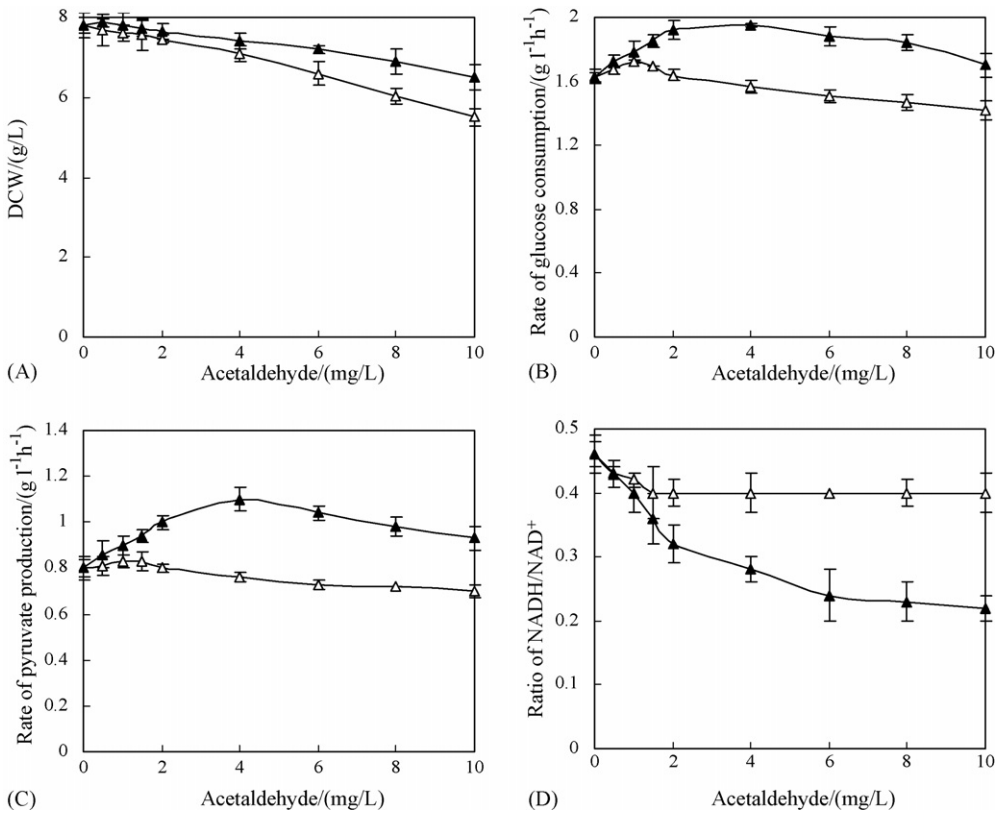


Fig. 3. (A–D) Effect of the concentration of acetaldehyde on the availability of $NAD^+$ and pyruvate production in the parent (△) and mutant strain (▲).

the inhibitor amount used. However, for the mutant strain WSH-13, the activity of ADH decreased with the increase in the inhibitor's concentration, ADH activity reached the lowest level when 10 mM of pyrazole was supplemented to the mutant strain culture broth.

### *3.4. Effects of acetaldehyde concentration on cell growth*

When growing the strains with glucose as sole carbon source, some major differences in using the parent and the mutant strains were observed if acetaldehyde, an electron acceptor, was added to the parent and the mutant culture broth. As illustrated in Fig. 4, the growth of the parent and the mutant strains decreased along with the increase of the acetaldehyde concentration. The highest rates of glucose consumption ($2.06\ g\ (l\ h)^{-1}$) and pyruvate production ($0.98\ g\ (l\ h)^{-1}$) were observed when adding $4\ mg\ l^{-1}$ acetaldehyde to the mutant WSH-13 culture broth, which were, respectively, 26.3 and 22.5% higher than those of the control (without acetaldehyde). However, the rates of glucose consumption and pyruvate production in parent strain did not change much regardless of acetaldehyde amount added (Fig. 3B and C). Similar change was observed for the ratio of $NADH/NAD^+$ (Fig. 3D). The ratio of $NADH/NAD^+$ decreased from 0.46 (the control) to 0.22 along with the increase of the acetaldehyde concentration in the mutant WSH-13 culture broth. In addition, the results also indicated that the optimal concentration of acetaldehyde was $4\ mg\ l^{-1}$.

### *3.5. Effect of acetaldehyde on glucose metabolism under different DO condition*

In our previous study, higher pyruvate yield on glucose ($0.797\ g\ g^{-1}$) was achieved under higher DO concentration (85%), but the glucose consumption rate was

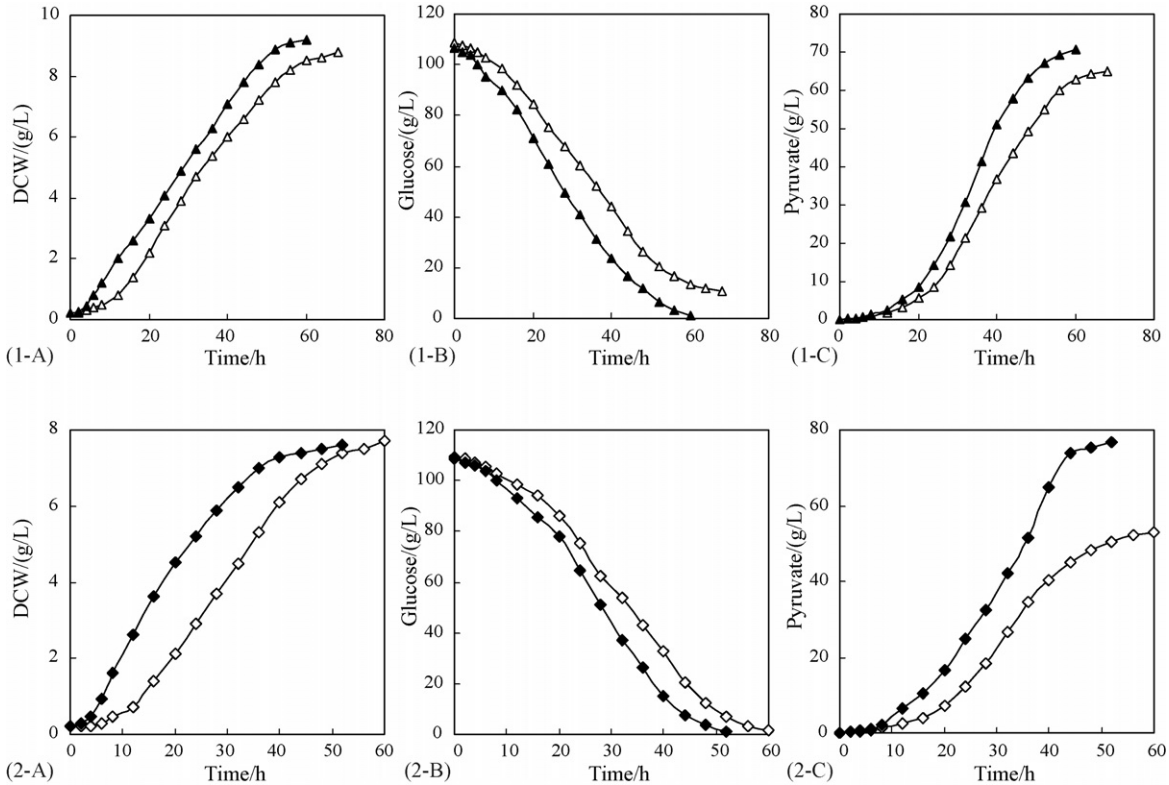


Fig. 4. Effect of acetaldehyde on the pyruvate production with the mutant strain WSH-13 at DO = 50% (1) and DO = 20% (2); (▲) acetaldehyde; (△) without acetaldehyde; (■) acetaldehyde; (□) without acetaldehyde.

lower (1.14 g (l h)$^{-1}$) (Li et al., 2002). On the other hand, glucose consumption was enhanced under lower DO concentration (50%), but the pyruvate yield on glucose decreased (to 0.483 g g$^{-1}$). The unification of relatively high concentration, high yield, and high productivity of pyruvate was achieved by applying a two-stage oxygen supply control strategy, combining the advantages of high yield under high DO concentration and high glucose consumption rate under low DO concentration. However, the two-stage strategy improved pyruvate productivity at the expense of pyruvate yield, indicating that it is very difficult to achieve the highest pyruvate productivity and the highest pyruvate yield simultaneously.

Fig. 4 showed the time courses of pyruvate production by the mutant WSH-13 under different DO concentrations (DO 50 and 20%) with or without acetaldehyde addition. The length of lag phase decreased 70% when acetaldehyde was added to the culture broth at the beginning of fermentation for all different DO concentrations cases. At higher DO concentration (85%), there is no difference in rates of glucose consumption and pyruvate production with acetaldehyde addition. However, with the DO concentration was 20%, if maintaining the acetaldehyde concentration in the culture broth at a level of 4 mg l$^{-1}$, the glucose consumption rate, the pyruvate production rate, the pyruvate yield on glucose and the concentration of pyruvate were 16.2, 68, 44 and 45% higher, respectively, than the corresponding values of the control (without acetaldehyde) (Fig. 4A2–C2). In addition, the glucose consumption rate, the pyruvate production rate, the pyruvate yield on glucose and the concentration of pyruvate in the mutant strain WSH-13 increased 76.2, 15.8, −5.0% (negative increase) and 70.1% (with respect to the case of DO 85%) and 6, 10.8, 11.6 and 19.3% (with respect to the case of two-stage oxygen supply), respectively, as compared with those of the high DO concentration

and the two-stage oxygen supply cases when using the parent strains. For the case of 50% DO, the glucose consumption rate, the pyruvate production rate, the pyruvate yield on glucose and the concentration of pyruvate in the mutant strain WSH-13 increased 30.5, 31.2, 7.1 and 8.4% (with respect to the case of the control, without acetaldehyde) (Fig. 4A1–C1) and 59.3, 6.3, −10% (negative increase) and 44.8% (with respect to the case of DO 85%). However, no change was observed between the case of 50% DO with acetaldehyde and the case of two-stage oxygen supply strategy (Table 2).

Furthermore, an interesting result was obtained when two-stage oxygen supply strategy was applied to the mutant strain with 4 mg $l^{-1}$ acetaldehyde in the culture broth, when DO from 85% at the first 16 h then adjusted to 50% (Table 2), the glucose consumption rate, the pyruvate production rate, the pyruvate yield on glucose and the concentration of pyruvate were 14.8, 14.2, 0 and 6.5% (with respect to the case of DO 50% and supplement acetaldehyde) and 10.7, 16.1, 5.3 and 8.35% (with respect to the case of Li et al., 2000), respectively. On the other hand, when DO at 85% for the first 16 h then adjusted to 20% (Table 2), the glucose consumption rate, the pyruvate production rate, the pyruvate yield on glucose and the concentration of pyruvate were 13.9, 18.9, 4.2 and 3.1% (with respect to the case of DO 20% and supplement acetaldehyde) and 21.5, 41.9, 16.3 and 14.2% (with respect to the case of Li et al., 2000), respectively, as compared with those of the DO 50 and 20% with acetaldehyde addition case using the mutant strain and the two-stage oxygen supply cases when using the parent strains.

As shown in Table 2, energy metabolism analysis revealed that at the higher DO concentration (DO 85%), the intracellular ATP level and the ratio of NADH/$NAD^+$ stay identically, regardless of acetaldehyde addition or not. However, for the case of lower DO concentration (DO 20%) with acetaldehyde addition, the intracellular ATP level and the ratio of NADH/$NAD^+$ decreased 12.6 and 63.9%, respectively. No glycerol was detected. The intracellular ATP level of lower DO concentration (DO 20%) with acetaldehyde was 49.5% lower than that of higher DO concentration (DO 85%). Other changes also occurred in the glucose metabolism as shown in Table 2. With acetaldehyde addition, the specific activity of PFK, GADPH and PK, the key enzymes of the glycolytic pathway

Table 2
Effect of acetaldehyde on glucose consumption under differ DO concentration

| Parameters | DO 85% | | DO 50% | | DO 20% | | 85–50% | 85–20% | Reference (Li et al., 2002) |
|---|---|---|---|---|---|---|---|---|---|
| | C | A | C | A | C | A | | | |
| Glucose consumed (g $l^{-1}$) | 89.7 ± 1.2 | 91.2 ± 0.7 | 97.8 ± 1.7 | 105.3 ± 1.3 | 107.4 ± 1.5 | 108.3 ± 1.2 | 112.6 ± 1.3 | 106.3 ± 1.2 | 109.2 |
| Culture time (h) | 76 | 76 | 68 | 56 | 60 | 52 | 52 | 45 | 56 |
| Glucose consumption rate (g (l h)$^{-1}$) | 1.18 ± 0.02 | 1.20 ± 0.01 | 1.44 ± 0.03 | 1.88 ± 0.02 | 1.79 ± 0.03 | 2.08 ± 0.02 | 2.16 ± 0.03 | 2.3 ± 0.05 | 1.95 |
| Pyruvate concentration (g $l^{-1}$) | 66.4 ± 1.6 | 66.5 ± 1.8 | 65.1 ± 2.1 | 70.6 ± 1.7 | 52.8 ± 1.9 | 76.9 ± 2.3 | 75.2 ± 1.6 | 79.3 ± 1.8 | 69.4 |
| Pyruvate yield on glucose (g g $^{-1}$) | 0.74 ± 0.02 | 0.73 ± 0.2 | 0.665 ± 0.01 | 0.670 ± 0.01 | 0.491 ± 0.02 | 0.71 ± 0.03 | 0.670 ± 0.02 | 0.742 ± 0.02 | 0.636 |
| Pyruvate production rate (g (l h)$^{-1}$) | 0.87 ± 0.02 | 0.87 ± 0.02 | 0.96 ± 0.03 | 1.26 ± 0.03 | 0.88 ± 0.03 | 1.48 ± 0.04 | 1.44 ± 0.03 | 1.76 ± 0.04 | 1.24 |
| Final ethanol concentration (g $l^{-1}$) | N | N | N | 1.36 ± 0.3 | N | 1.78 ± 0.4 | 1.53 ± 0.3 | 2.42 ± 0.3 | 1.6 |
| Final glycerol concentration (g $l^{-1}$) | N | N | 4.0 ± 0.4 | N | 7.6 ± 0.7 | N | N | N | 5.3 |
| The intracellular ATP level (mg g$^{-1}$ DCW) | 36.84 ± 2.7 | 37.42 ± 3.4 | 33.53 ± 1.5 | 29.17 ± 1.3 | 21.32 ± 2.1 | 18.64 ± 1.7 | 26.46 ± 1.6 | 17.14 ± 1.2 | N |
| The ratio of NADH/$NAD^+$ | 0.29 | 0.28 | 0.57 | 0.31 | 0.83 | 0.30 | 0.32 | 0.31 | N |
| PFK (U mg$^{-1}$ protein) | 1.07 ± 0.08 | 1.06 ± 0.06 | 1.13 ± 0.04 | 1.24 ± 0.05 | 1.86 ± 0.12 | 2.29 ± 0.07 | 1.26 ± 0.05 | 2.29 ± 0.07 | N |
| GADPH (U mg$^{-1}$ protein) | 2.38 ± 0.13 | 2.36 ± 0.15 | 2.34 ± 0.07 | 2.57 ± 0.16 | 2.36 ± 0.11 | 2.68 ± 0.13 | 2.57 ± 0.20 | 2.68 ± 0.13 | N |
| PK (U mg$^{-1}$ protein) | 0.29 ± 0.01 | 0.29 ± 0.01 | 0.32 ± 0.02 | 0.33 ± 0.01 | 0.35 ± 0.03 | 0.41 ± 0.04 | 0.36 ± 0.02 | 0.41 ± 0.04 | N |
| PDC (U mg$^{-1}$ protein) | 9.07 ± 0.24 | 9.08 ± 0.17 | 9.12 ± 0.36 | 9.04 ± 0.23 | 9.05 ± 0.27 | 9.07 ± 0.32 | 9.05 ± 0.23 | 9.05 ± 0.28 | N |
| ADH (U mg$^{-1}$ protein) | 2.83 ± 0.21 | 2.79 ± 0.13 | 2.85 ± 0.17 | 2.87 ± 0.22 | 2.87 ± 0.14 | 2.95 ± 0.20 | 2.90 ± 0.30 | 2.92 ± 0.25 | N |

*Abbreviations:* (C) control (no acetaldehyde addition), (A) acetaldehyde addition, (N) no detected; (PFK) phosphofructokinase, (GADPH) glyceraldehydes-3-phosphate dehydrogenase, (PK) pyruvate kinase, (PDC) pyruvate decarboxylase, (ADH) alcohol dehydrogenase.

increased 23.1, 13.5 and 7.89%, respectively. Similar energy and carbon metabolism changes were observed for the case of DO 50%.

## 4. Discussion

*T. glabrata*, a multi-vitamin-auxotrophic yeast, could accumulate high concentration of pyruvate under vitamin limiting conditions, was used as a good model system to study the relationship between the glycolytic flux and the cofactor level in eukaryotic cell (Li et al., 2001b). Previous studies had demonstrated that a lower NADH/$NAD^+$ ratio, a higher $NAD^+$ availability, and a lower ATP level might be beneficial for accelerating the glycolysis in *T. glabrata* (Liu et al., 2005). An ideal condition for high glycolytic flux is that: $NAD^+$ is continued to supply for glycolytic pathway and ATP is maintained at very low level. However, the increase of the carbon flux in the glycolytic pathway could also lead to an increased NADH production. The excessive NADH must be regenerated to $NAD^+$, a co-enzyme of the glycolytic pathway. At higher DO concentration, the excessive NADH was oxidized to $NAD^+$ through electron transfer chain and a large amount of ATP was produced. However, the glycolytic flux would be retarded at high intracellular ATP level (Table 2). In the case of lower oxygen supply, the $NAD^+$ regeneration may occur in fermentative pathway (ethanol production or glycerol production). During fermentative metabolism, acetaldehyde reduced to ethanol as NADH oxidized to $NAD^+$. Alternatively, NADH can be oxidized by reduction of dihydroxyacetone phosphate to glycrol-3-phosphate. The latter compound can then be either dephosphorylated to yield glycerol or re-oxidized to dihydroxyacetone phosphate under aerobic conditions via a mitochondrial, respiratory-chain-linked glycerol-3-phosphate dehydrogenase. The formation of ethanol and/or glycerol could lead to a decreased pyruvate yield on glucose.

Among $NAD^+$ regeneration pathways, $NAD^+$ production through alcohol dehydrogenase has been proven to be the efficient one where acetaldehyde is reduced by the addition of two electrons and two hydrogen ions supplied by NADH, which is reduced to $NAD^+$. But for *T. glabrata* CCTCC M202019, the carbon flux catalyzed by alcohol dehydrogenase was limited because the activity of PDC was diminished, and thus the $NAD^+$ regeneration ability was vanished. As a result, no ethanol but a large amount of glycerol was detected under low DO concentration (Table 2). In our study, in order to decrease the carbon flux for glycerol formation and to increase the pyruvate yield on glucose under the condition of partial oxidation of NADH to $NAD^+$ through ETC, a novel approach of continuous $NAD^+$ supply was adopted. This approach was based on increasing the activity of alcohol dehydrogenase and supplement of acetaldehyde. With acetaldehyde as the substrate, alcohol dehydrogenase catalyzed the oxidation reaction from NADH to $NAD^+$, and this was not involved with the ATP production. Flikweert had demonstrated that controlled feeding of acetaldehyde partially restores glycolytic capacity in a pyruvate decarboxylase negative *Saccharomyces cerevisia* strain (Flikweert, 1999).

Significant physiological changes in *T. glabrata* were observed when 4 mg $l^{-1}$ acetaldehyde was supplemented. First, for all different DO concentrations, the length of lag phase decreased when acetaldehyde was present. At the beginning of the fermentation, the cell was transferred to a new environment and facing a stress condition, intracellular acetaldehyde concentration was depleted by diffusion, and then slowing the $NAD^+$ regeneration and limiting the growth. Therefore, the lag phase was observed. The length of lag phase diminished was likely due to the fact that the supplement acetaldehyde could replenish the intracellular acetaldehyde pool and then restore the cellular redox balance. Similar physiological changes were also observed in *S. cerevisiae* and *Zymomonas mobilis* under different stress conditions (Barber et al., 2000; Stanley et al., 1997). Second, the addition of acetaldehyde did not affect the rates of glucose consumption and pyruvate production under higher DO concentration. This was because that, under high DO concentration (DO 85%), NADH was fully oxidized to $NAD^+$ through ETC with oxygen as final acceptor, no NADH oxidation occurred through fermentative pathway. Therefore, the addition of acetaldehyde did not change the ratio of NADH/$NAD^+$, the rates of glucose consumption and the pyruvate production.

However, for the lower DO concentration (20%), NADH production from glycolysis could not be oxidized by ETC and was accumulated in cell. And some glycerol was detected in the culture broth. This was because that, in order to achieve a redox balance

under the condition of less oxygen supply, the cell had to oxidize the excessive NADH to $NAD^+$ through the formation of glycerol. Under this circumstance, with the addition of acetaldehyde, higher pyruvate concentration, higher pyruvate yield on glucose and higher pyruvate productivity were achieved simultaneously. Furthermore, a decreased intracellular ATP level (12.6%) and a decreased ratio of NADH/$NAD^+$ (63.9%) were also found. Our previous work revealed that the majority of intracellular ATP is synthesized when NADH oxidized to $NAD^+$ through oxidative phosphorylation pathway (Liu et al., 2005). Partial oxidation of NADH to $NAD^+$ through ETC results in a decreasing intracellular ATP level. With acetaldehyde addition, the excessive NADH was redirected to the alcohol dehydrogenase involved fermentation pathway and oxidized to $NAD^+$, and thus the ratio of NADH/$NAD^+$ decreased. In addition, no glycerol was detected in the culture broth here. On the other hand, an increase of the activities of key enzymes in the glycolytic pathway also occurred. Those results indicated that, the increased availability of $NAD^+$ could increase the glycolytic flux and the pyruvate productivity.

In this work, we had clarified that the changes in carbon metabolism pathway with increasing the availability of $NAD^+$ in *T. glabrata*. With the increase of the availability of $NAD^+$ under a lower DO concentration, high glycolytic flux, high pyruvate concentration, high pyruvate yield on glucose and high pyruvate productivity, in combination with increased activities of key enzymes in glycolytic pathway were achieved. All results have demonstrated that, for the first time, enhancing the availability of $NAD^+$ through increasing the activity of ADH and adding acetaldehyde as exterior electron acceptor could increase the glycolytic flux and the metabolites productivity. Furthermore, the strategy for increasing the glycolytic flux and the pyruvate productivity in *T. glabrata* by increasing the availability of $NAD^+$ may provide an alternative approach to enhance the metabolites productivity in yeast.

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