# Enhancement of Pyruvate Production by *Torulopsis glabrata* through Supplement of Oxaloacetate as Carbon Source

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**Abstract** The capability of utilizing a TCA cycle intermediates as the sole carbon source by the multi-vitamin auxotrophic yeast *Torulopsis glabrata* CCTCC M202019 was demonstrated with plate count method. It is indicated that *T. glabrata* could grew on a medium with one of the TCA cycle intermediates as the sole carbon source, but more colonies were observed when glucose, acetate and one of the TCA cycle intermediates coexisted in the medium. Among the intermediates of the TCA cycle examined in this study, cell growth was improved by supplementing oxaloacetate. Further investigation showed that the presence of acetate was necessary when oxaloacetate was supplemented. By supplementing with 10 g/L of oxaloacetate in pyruvate batch fermentation, dry cell weight increased from 11.8 g/L to 13.6 g/L, and pyruvate productivity was enhanced from 0.96 $gL^{-1}h^{-1}$ to 1.19 $gL^{-1}h^{-1}$ after cultivation of 56 h. The yield of pyruvate to glucose was also improved from 0.63 g/g to 0.66 g/g. These results indicate that under vitamins limitation, the productivity and yield of pyruvate could be enhanced *via* an increase of cell growth by the supplementation of oxaloacetate.

*Keywords*: *Torulopsis glabrata*, pyruvate, TCA cycle intermediate, acetate, oxaloacetate

## INTRODUCTION

Pyruvate, the end-product of glycolysis, is an important intermediate in the metabolism of carbohydrates by yeast [1]. As pyruvate is located at the branch-point between respiratory and fermentative carbon metabolism, a flux distribution at the level of pyruvate is of crucial importance for cell growth and biosynthesis of biological macromolecules by *Torulopsis glabrata*. The multi-vitamin auxotrophic yeast, *T. glabrata* CCTCC M202019, is the only microorganism being used in the commercial fermentative production of pyruvate [2]. In which, the activities of enzymes that are responsible for further conversion of pyruvate could be minimized by limiting the concentrations of thiamine, nicotinic acid, pyridoxine, and biotin in the medium (Fig. 1) [3,4]. The limitation of carbon flux from pyruvate into the TCA cycle by adding sub-optimal concentrations of vitamins resulted in a declined cell growth of *T. glabrata* due to the weak TCA cycle activity [5,6]. Although in these studies a higher yield of pyruvate to glucose was obtained, pyruvate productivity was low due to poor cell growth. However, pyruvate productivity may be improved by increasing TCA cycle activity and cell growth.

The intermediates of the TCA cycle can be used as carbon sources for growth by yeast and bacteria [7-11]. Some yeast can utilize malate as a sole carbon source and an energy source. The proton transport system, which transports TCA cycle intermediates across the plasma membrane into the cytoplast, has been found in *Candida sphaerica*, *Candida utili* and *Saccharomyces cerevisiae* [12-16]. In taxonomy of yeast, *T. glabrata* belongs to one of the class of *Candida*, and it was assumed that *T. glabrata* may utilize TCA cycle intermediates for cell growth; thus, if cell growth is enhanced by the addition of TCA cycle intermediates, *T. glabrata* will metabolize more glucose to form pyruvate (Fig. 2). This study was performed to investigate the influence of TCA cycle intermediates on cell growth and pyruvate production by *T. glabrata*.

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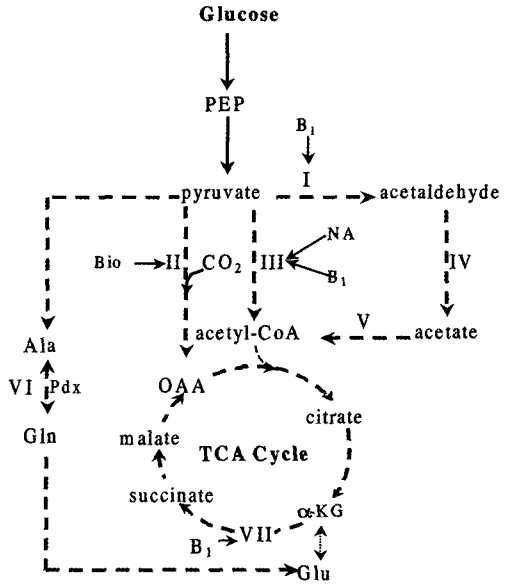
## MATERIALS AND METHODS

### Microorganism

A multi-vitamin auxotrophic yeast *Torulopsis glabrata* CCTCC M202019 which could use $NH_4Cl$ as a sole nitrogen source and acetate as supplemental carbon source [2] was used in this study.

### Media

The medium for seed preparation contained: 30 g glucose, 10 g peptone, 1 g $KH_2PO_4$ and 0.5 g $MgSO_4 \cdot 7H_2O$. 20 g of agar per liter was added into the seed medium for




I Pyruvate decarboxylase; II Pyruvate carboxylase; III Pyruvate dehydrogenase complex; IV Aldehyde dehydrogenase; V Acetyl-CoA synthase; VI Transaminase; VII α-Ketoglutarate dehydrogenase
$B_1$: Thiamin; NA: Nicotinic acid; Bio: Biotin; Pdx: Pyridoxine; OAA: Oxaloacetate ----➤ Weaken metabolic pathway; ——➤ Normal metabolic pathway

**Fig. 1.** Metabolic pathway of pyruvic acid in *T. glabrata* CCTCC M202019.

slant culture. The fermentation medium consisted of the following: 100 g glucose, 6 g sodium acetate, 7 g $NH_4Cl$, 5 g $KH_2PO_4$, 0.8 g $MgSO_4 \cdot 7\ H_2O$, 6 g sodium acetate, 4 mg nicotinic acid, 15 μg thiamine·HCl, 100 μg pyridoxine·HCl, 10 μg biotin and 50 μg riboflavin per liter. Malate, succinate, α-ketoglutarate and oxaloacetate were added according to the experiment design. 5 g/L of $CaCO_3$ and 20 g/L of agar were added into the fermentation medium for plate cultures. $CaCO_3$ was sterilized by dry-heat sterilization at 160°C for 40 min before being added into the medium. The initial pH values of all media were adjusted to 5.0. All vitamins were filter-sterilized prior to addition to the medium.

### Cultivation Conditions

For the preparation of seed culture, one loop of slant culture was inoculated into 50 mL of seed medium in a 500-mL flask and incubated for 24 h at 30°C and 200 rpm on a reciprocal shaker.

For plate cultivation, the seed culture was harvested by centrifugation (6,000 × *g*) and washed three times with 0.1 M potassium phosphate buffer (pH 7.5). 1.0 g of pellet cells was resuspended into 100 mL of 0.1 M potassium phosphate buffer (pH 7.5) and diluted to $10^{-1}$, $10^{-2}$, ..... and $10^{-7}$. The last two dilutes were spread onto plates containing different carbon source in fermentation medium. The plates were incubated at 30°C for 48 h and the number of colonies on the plates was counted.

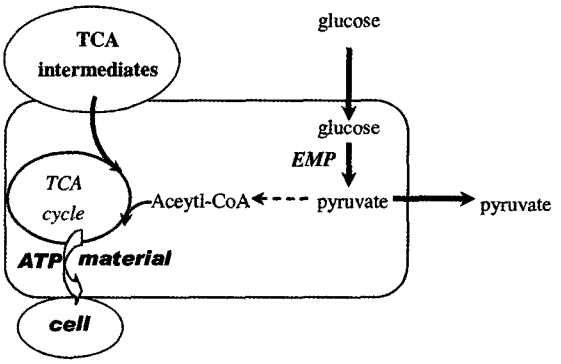



**Fig. 2.** Schematic representation of glucose and TCA-cycle intermediates in *T. glabrata*.

For flask cultures, 5 mL of the seed culture was inoculated into 500-mL flasks containing 50 mL of the fermentation medium. The pH of the culture broth was buffered by adding 40 g/L of $CaCO_3$ before inoculation. The cultivation was conducted for 48 h at 30°C and 200 rpm on a reciprocal shaker.

For fermentor cultures, 10% (v/v) of the seed medium was inoculated into a 7-L jar fermentor (KF-7 L, Korea Fermentor Co., Inchon, Korea) containing 4 L fermentation medium. The pH was automatically controlled at 5.0 with 8 M NaOH solution. The agitation speed was 300 rpm and the aeration rate was 4 L/min. The temperature was controlled at 30°C.

### Analytical Methods

Pyruvate concentration was determined by enzymatic assay method described by LamprechtW and Heinz F [17]. Glucose concentration was measured with the 3,5-dinitrylsalicylic acid spectrometric method [18]. Cell growth (dry cell weight, DCW) was monitored by measuring the optical density (OD) at 660 nm after an appropriate dilution. The $OD_{660}$ value was converted to dry cell weight (DCW) by using the following equation: 1 $OD_{660}$ = 0.23 g DCW/L.

## RESULTS AND DISCUSSION

### Effect of TCA Cycle Intermediates on Growth of *T. glabrata*

The effect of carbon sources (glucose, acetate and TCA cycle intermediates) on cell growth of *T. glabrata* was examined. As shown in Fig. 3, with acetate or glucose as the sole carbon source, only 8 and 30 colonies were observed on the plate, respectively. However, with glucose and acetate as carbon sources, 36 ± 2 colonies were obtained. It was interesting to find that all four examined TCA cycle intermediates including malate, succinate, α-ketoglutarate and oxaloacetate could be used as energy and carbon sources for the growth of *T. glabrata*. Moreover, more colonies were observed when glucose,

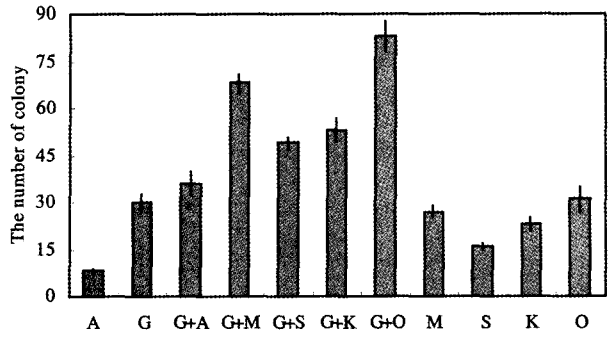



**Fig. 3.** Effect of TCA cycle intermediates on *T. glabrata* CCTCC M202019 growth A: Acetate; GA: Glucose and acetate; M: Malate; S: Succinate; K: α-ketoglutarate; O: Oxaloacetate.

acetate and one of the TCA cycle intermediates were used as co-substrates. This suggests that cell growth of *T. glabrata* could be enhanced by adding one of the TCA cycle intermediates, in which oxaloacetate showed the best.

## Effect of TCA Cycle Metabolites on Pyruvate Batch Fermentation

The effect of TCA cycle intermediates on pyruvate batch fermentation was investigated. Dry cell weight (DCW) of *T. glabrata* cultured on the medium by adding 10 g/L of malate, 15 g/L of α-ketoglutarate and 10 g/L of oxaloacetate as a supplemental carbon source were 20.2%, 9.4% and 15.7%, respectively, higher than that on the medium with only 100 g/L of glucose and 6 g/L of acetate (Fig. 4a). With the addition of TCA cycle intermediates, glucose consumption rates were improved. Pyruvate concentration was increased by 17.7% with the addition of oxaloacetate (Fig. 4b and c). These results indicate that the addition of TCA cycle intermediates can strengthen glucose metabolism, moreover cell growth and pyruvate production. The yields of dry cell weight to glucose consumed ($Y_{x/s}$) with glucose and one of the TCA cycle intermediates as carbon sources were 8% to 43.4% higher than that on glucose medium, while the yields of pyruvate to glucose were increased by 8.7% to 17.4% (Table 1).

It is further demonstrated that, among the four TCA-cycle intermediates, oxaloacetate was an ideal supplemental carbon source for the improvement of cell growth, glucose consumption and pyruvate production (Fig. 4 and Table 1). Metabolic flux analysis of *T. glabrata* showed that the flux of pyruvate carboxylation reaction, controlled by pyruvate carboxylase, increases significantly with the presence of a large amount of $Ca^{2+}$ in the medium, resulting in the acculumation of α-ketoglutarate (about 6 g/L) even under biotin limitation [17]. Pyruvate was converted into oxaloacetate through the pyruvate carboxylation reaction in cytoplast, while α-ketoglutarate was formed in mitochondria. The current study suggests that the strain *T. glabrata* possesses the oxaloacetate transport system, which can transport oxaloacetate from the cytosol to mitochondria. Within the mitochondria, oxaloacetate was used to build up the pools of TCA cycle intermediates [19]. This indicates that, with the addition of oxaloacetate in the medium, extracellular oxaloacetate could be transported into cytoplast rapidly, and the formation of oxaloacetate from pyruvate carboxylation reaction was decreased with the increase of the oxaloacetate pool in cytosol [20]. As a result, the carbon flux from pyruvate into the TCA cycle drops, leading to an increase in the yield of pyruvate to glucose and pyruvate production.

**Table 1.** Effect of TCA cycle intermediates on $Y_{x/s}$ and $Y_{p/s}$

| | Glucose | Glucose and α-ketoglutarate | Glucose and oxaloacetate | Glucose and malate |
|---|---|---|---|---|
| $Y_{x/s}$ | 0.46 ± 0.02 | 0.50 ± 0.01 | 0.66 ± 0.03 | 0.64 ± 0.01 |
| $Y_{p/s}$ | 0.57 ± 0.01 | 0.64 ± 0.02 | 0.67 ± 0 | 0.62 ± 0.01 |

$Y_{x/s}$ is the yield of dry cell weight to glucose consumed.
$Y_{p/s}$ is the yield of pyruvate to glucose consumed.

The effect of oxaloacetate concentration on cell growth and pyruvate production was further investigated to determine the optimal oxaloacetate concentration (Fig. 5). Dry cell weight remained almost constant with the increase of oxaloacetate concentration, while the yield of pyruvate to glucose consumed ($Y_{p/s}$) and pyruvate concentration increased and reached their maximal values of 0.68 g/g and 45.3 g/L, respectively with the addition of 10 g/L of oxaloacetate. However, their values decreased with a further increase of oxaloacetate concentration.

## Effect of Thiamin and Acetate on Cell Growth

Although the addition of oxaloacetate improved cell growth of *T. glabrata*, the presence of acetyl-CoA is necessary for the condensation of acetyl-CoA and oxaloacetate to form citroyl-CoA in the TCA cycle [21]. Providing enough acetyl-CoA to combine with oxaloacetate is therefore important under thiamin limitation.

Two pathways exist in *T. glabrata* for the formation of acetyl-CoA. One is the direct oxidation of pyruvate to acetyl-CoA catalysed by the mitochondrial pyruvate dehydrogenase (PDH) complex. The other pathway is an indirect route with the involvement of pyruvate decarbonxylase (PDC), alcohol dehydrogenase and acetyl-CoA synthase, which has been frequently referred to as the PDH bypass. During glucose respiratory growth, the PDH complex is primarily responsible for the conversion of pyruvate into acetyl-CoA. However, a small flux through PDH bypass is essential to provide cytosolic acetyl-CoA for lipid synthesis. As a cofactor of the PDH complex and PDC, thiamin plays an important role in cell growth of *T. glabrata*. Compared to the control (medium A in Fig. 6), very poor growth was observed with glucose as the carbon source under thiamin deficiency (medium B in Fig. 6). When 10 g/L of oxaloacetate was supplemented into medium B, growth was not restored (medium I in Fig. 6). Partial growth of *T. glabrata* CCTCC M202019 was observed when 6 g/L of acetate was added to medium B

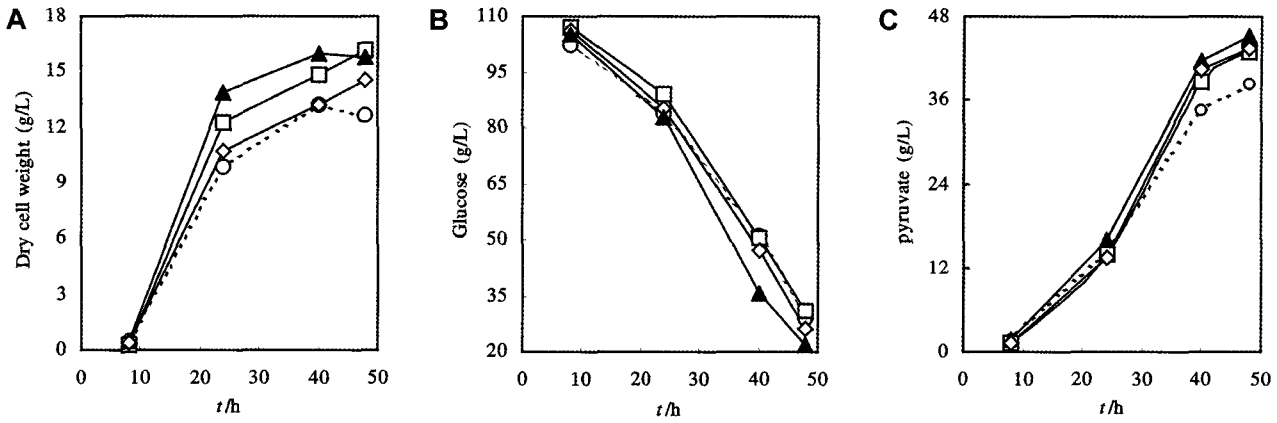



**Fig. 4.** Effect of TCA cycle metabolites on pyruvate batch fermentation ○ Glucose and acetate; ◇ Glucose, acetate and α-ketoglutarate; ▲ Glucose, acetate and oxaloacetate; □ Glucose, acetate and malate.

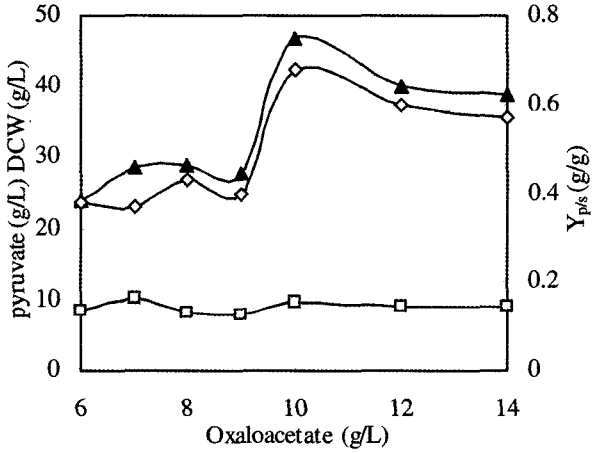
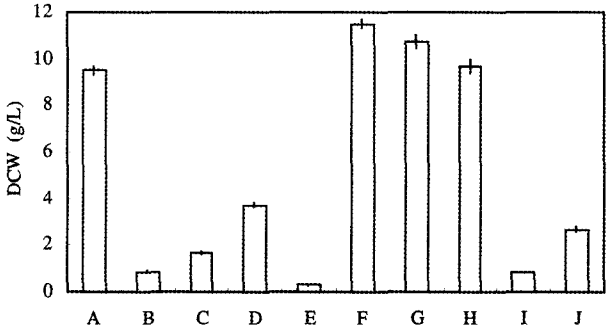



**Fig. 5.** Effect of oxaloacetate concentration on pyruvate batch fermentation ▲ pyruvate; ◇ $Y_{p/s}$; □ DCW.




The concentrations of acetate and oxaloacetate were 6 g/L and 10 g/L, respectively.

A : Control medium (contains glucose, thiamin, Biotin, nicotinic acid, and Pyridoxine);
B : Medium A without $B_1$;
C : Medium B supplemented with acetate;
D : Medium C supplemented with oxaloacetate;
E : Medium A without glucose, supplemented with acetate;
F : Medium A supplemented with acetate and oxaloacetate;
G : Medium A supplemented with acetate;
H : Medium A supplemented with oxaloacetate;
I : Medium A without $B_1$ but supplemented with oxaloacetate;
J : Medium contains acetate and oxaloacetate, no vitamin.

**Fig. 6.** Growth of *T. glabrata* on different media.

(medium C in Fig. 6). This indicates that *T. glabrata* can assimilate acetate to acetyl-CoA for the demand of cell growth. When 10 g/L of oxaloacetate was supplemented to medium C, dry cell weight was 20% higher than that in medium C (medium D in Fig. 6). When 10 g/L of oxaloacetate was added into medium A, but without a simultaneous addition of acetate, enhanced cell growth was not obtained (medium H in Fig. 6). Cell growth on medium A supplemented with 6 g/L of acetate (medium G in Fig. 6) was about 21% higher than that in medium A. Cell growth was increased by 25.4% when 10 g/L of oxaloacetate and 6 g/L of acetate were added into medium A (medium F in Fig. 6). With acetate as the sole carbon source, very poor growth (DCW 0.28 g/L) was observed (medium E in Fig. 6), and cell growth was improved to 2.66 g/L by supplementing 10 g/L of oxaloacetate to medium E (medium J in Fig. 6). All of these results indicate that the enhancement of oxaloacetate on cell growth of *T. glabrata* was based on the fact that the strain could convert acetate into cytosolic acetyl-CoA by the PDH complex bypass pathway.

## Pyruvate Fermentation in 7-L Fermentor with the Addition of Oxaloacetate

Time courses of pyruvate fermentation on fermentation medium with or without 10 g/L of oxaloacetate are presented in Fig. 7. When 10 g/L of oxaloacetate was added to the fermentation medium, a higher growth rate was obtained, and dry cell weight was increased by 15%. Pyruvate concentration reached 66.7 g/L at 56 h with yield of pyruvate to glucose consumed ($Y_{p/s}$) and pyruvate productivity at 0.66 g/g and 1.19 $gL^{-1}h^{-1}$, respectively. Without the addition of oxaloacetate, however, pyruvate concentration was 65.3 g/L at 68 h with the yield of pyruvate to glucose consumed ($Y_{p/s}$) and pyruvate productivity at 0.63 g/g

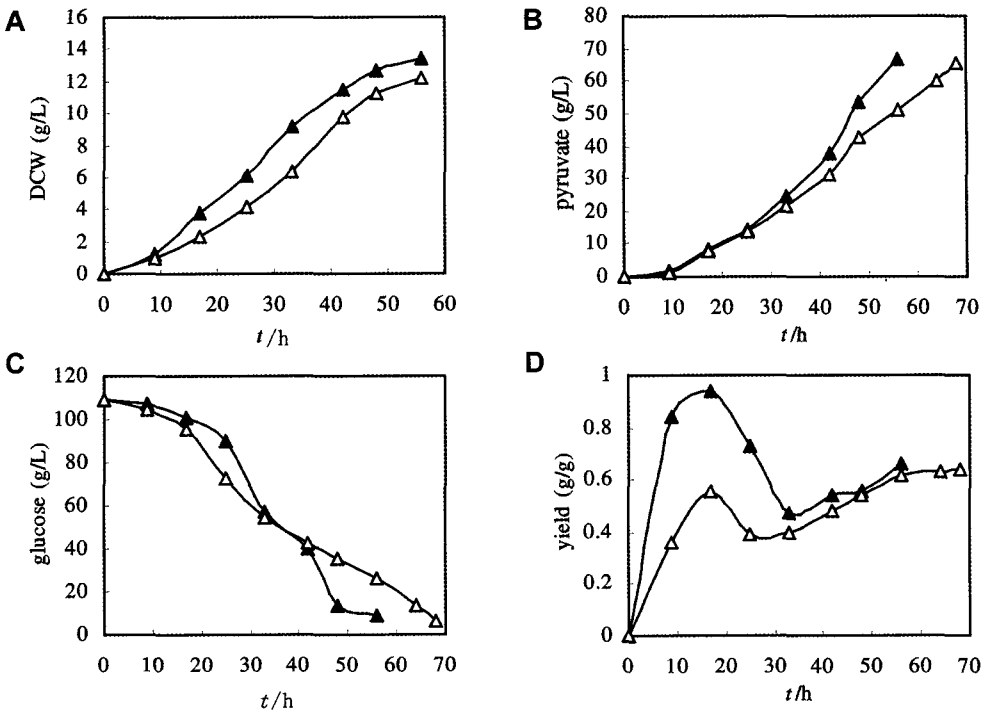



**Fig. 7.** Effect of oxaloacetate on the production of pyruvate in 7-L fermentor ▲ Medium contains oxaloacetate Δ Medium without oxaloacetate.

and 0.96 $gL^{-1}h^{-1}$, respectively. In this study, the strategy of adding TCA-cycle intermediates proved to be a way to increase the productivity of pyruvate.

## CONCLUSION

This research has proven that strain *Torulopsis glabrata* CCTCC M202019 could utilize the TCA cycle intermediates as carbon and energy source for growth. Of these TCA cycle intermediates, oxaloacetate was propitious to cell growth and pyruvate production. When 10 g/L of oxaloacetate was present in fermentation medium, the yield of cell growth (DCW), the productivity of pyruvate and the yield of pyruvate to glucose consumed was achieved at 13.6 g/L, 1.19 $gL^{-1}h^{-1}$ and 0.66 g/g, respectively, after cultivation of 56 h.

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## REFERENCES

[1] Pronk, J. T., H. Y. Steensma, and J. P. Van Dijken (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* 12: 1607-1633.

[2] Liu, L. M., Y. Li, H. Z. Li, and J. Chen (2004) Manipulating the pyruvate dehydrogenase bypass of a multi-vitamin auxotrophic yeast *Torulopsis glabrata* enhanced pyruvate production. *Lett. Appl. Microbiol.* 39: 199-206.

[3] Hua, Q. and K. Shimizu (1999) Effect of dissolved oxygen concentration on the intracellular flux distribution for pyruvate fermentation. *J. Biotechnol.* 65: 135-147.

[4] Miyata, R. and T. Yonehara (1996) Improvement of fermentative production of pyruvate from glucose by *Torulopsis glabrata* IFO 0005. *J. Ferment. Bioeng.* 82: 475-479.

[5] Liu, L. M., Y. Li, G. C. Du, and J. Chen (2002) Progress in biotechnological production of pyruvic acid (In Chinese). *Chinese J. Biotechnol.* 18: 651-655.

[6] Li, Y., J. Chen, and S. Y. Lun (2001) Efficient pyruvate production by a mulit-vitamin auxotroph of *Torulopsis glabrata*: key role and optimization of vitamin levels. *Appl. Microbiol. Biotechnol.* 55: 680-685.

[7] Xu, D. P., C. P. Madrid, M. Rohr, and C. P. Kubcek (1989) Influence of type and concentration of the carbon source on citric acid production by *Aspergillus niger*. *Appl. Microbial. Biotechnol.* 30: 553-558.

[8] Green, L. S. and D. W. Emerich (1997) *Bradyrhizobium japonicum* does not require α-ketoglutarate dehygenase for growth on succinate or malate. *J. Bacteriol.* 179: 194-201.

[9] Antoun, H., L. M. Bordeleau, and R. Sauvageau (1984) Utilization of tricarboxylic acid cycle intermediates and symbiotic efficiency in *Rhizobium meliloti*. *Plant. Soil.* 77: 29-38.

[10] Barnett, J. A. and H. L. Kornberg (1960) Utilization by yeast of acids of the tricarboxylic acid cycle. *J. Gen. Mi-*

*crobiol.* 23: 65-82.
[11] Barnett, J. A., R. W. Payne, and D. Yarrow (1990) *Yeast Characteristics and Identification*. University Press Cambridge, UK.
[12] Corte, R. M., C. Leao, and N. Van U (1989) Transport of L(-)malic acid and other dicarbonxylic acids in the yeast *candidas sphaerica*. *Appl. Microbiol. Biotechnol.* 31: 551-555.
[13] Corte, R. M. and C. Leao (1990) Transport of L(-) malic acid and other dicarbonxylic acids in the yeast *Hansenula anomala*. *Appl. Microbiol. Biotechnol.* 56: 1109-1113.
[14] Cassio, F. and C. Leao (1993) A comparative study on the transport of L(-)malic acid and other short chain carboxylic acids in the yeast *Candida utilis*: Evidence for a general organic acid permease. *Yeast* 9: 743-752.
[15] Rodriguez, S. and R. J. Thornton (1990) Factors influencing the utilization of L-malate by yeasts. *FEMS Microbiol. Lett.* 72: 17-22.
[16] Salmon, J. M. (1987) L–malic acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta* 901: 30-40.
[17] Lamprecht, W. and F. Heinz (1984) Pyruvate. pp. 570-577. In: Bergmeyer, H. U. (ed.). *Methods of Enzymatic Analysis*. VCH, Weinheim, Germany.
[18] Miller, G. (1951) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
[19] Levente, K. and P. K. Christian (2003) *Aspergillus niger* citric acid accumulation: Do we understand this well working black box? *Appl. Microbiol. Biotechn.* 61: 189-196.
[20] Palmieri, L., A. Vozza, G. Agrimi, V. De Marco, M. J. Runswick, F. Palmieri, and J. E. Walker (1999) Identification of the yeast mitochondrial transporter for oxaloacetate and sulfate. *J. Biol. Chem.* 74: 22184-22190.
[21] Gombert, A. K., M. Moreira dos Santos, B. Christensen, and J. Nielsen (2001) Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. *J. Bacteriol.* 183: 1441-1451.

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