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ORIGINAL PAPER

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Efficient pyruvate production by a multi-vitamin auxotroph of *Torulopsis glabrata*: key role and optimization of vitamin levels

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Abstract A multi-vitamin auxotroph, *Torulopsis glabrata* strain WSH-IP303, which can use ammonium chloride as a sole nitrogen source for pyruvate production, was selected. To optimize pyruvate yield and productivity, a simple but useful, orthogonal design method, was used to investigate the relationship between thiamine, nicotinic acid, pyridoxine, biotin, and riboflavin. Thiamine was confirmed to be the most important factor affecting pyruvate production. When the concentration of thiamine was 0.01 mg/l or 0.015 mg/l, glucose consumption was improved by increasing the nicotinic acid concentration. When the concentrations of nicotinic acid, thiamine, pyridoxine, biotin, and riboflavin were 8.0, 0.015, 0.4, 0.04, and 0.1 mg/l, respectively, pyruvate concentration and yield reached 52 g/l and 0.52 g/g, respectively, in a 48-h flask culture. By employing a combination of the optimum vitamin concentrations, a batch culture was conducted in a 2.5-l fermentor with an initial glucose concentration of 112 g/l; and the pyruvate concentration reached 69 g/l after 56 h (yielding 0.62 g/g).

Introduction

Pyruvate is not only a useful organic acid (Yonehara and Miyata 1994) but is also a good model product for studying the regulatory mechanism of glycolysis (Yokota et al. 1994a). Yokota et al. (1994b) provide a good example of such a study, in which 30 g pyruvate/l (yield to glucose, 0.6 g/g) was produced after 24 h in a culture of an F_1-ATPase-defective mutant, *Escherichia coli* strain TBLA-1. Yokota et al. (1997) also physiologically demonstrated that the enhancement of pyruvate productivity was related to the improved activities of the phosphotransferase system and some glycolytic enzymes in this strain. Although that strain showed excellent ability in pyruvate production (Yokota et al. 1994b), it seems that only the vitamin-auxotroph of *Torulopsis glabrata* has been used for the industrial production of pyruvate (Miyata and Yonehara 1996).

For *T. glabrata*, the difficulty in getting a high yield seems to be the only drawback in using it as a pyruvate producer. For example, in the report of Miyata and Yonehara (1996), 67.8 g pyruvate/l was achieved after 63 h in a fed-batch culture, but the yield to glucose was only 0.49 g/g. Hua et al. (1999) used the metabolic flux analysis method to analyze the effect of dissolved oxygen on pyruvate fermentation by *T. glabrata* strain IFO 0005. In that case, a pyruvate yield higher than 0.8 g/g could be achieved in the typical pyruvate production phase. However, the overall yield (0.53 g/g) was still not good, even though a thiamine-addition strategy was applied in a fed-batch culture.

As pyruvate is located at a key junction of metabolism (Hua et al. 1999), it is necessary to inhibit the activities of enzymes which are responsible for the degradation of pyruvate and to accelerate the glycolysis, in order to get high yield and high productivity. For the multi-vitamin-auxotrophic yeast, *T. glabrata*, a suitable concentration of each vitamin in the medium is very important for efficient pyruvate production. However, so far, the effects of vitamins on pyruvate production have only been examined using single-factor experiments with polypeptone (Yonehara and Miyata 1994) or soybean hydrolyzate plus ammonium sulfate (Miyata and Yonehara 1996) as nitrogen source. Polypeptone contains a certain quantity of vitamins, which can be deduced from the result that *T. glabrata* strain IFO 0005 grew well in a medium supplemented with 30 g polypeptone/l but free of vitamins (Hua and Shimizu 1999; Hua et al. 1999). *T. glabrata* strain WSH-IP12, a multi-vitamin-auxotrophic yeast selected by ourselves, also grew well in a medium supplemented with 5 g peptone/l but free of vita-

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mins (Li et al. 2000). As the category and quality of peptone always vary, it is difficult to quantitatively elucidate the vitamin requirement of *T. glabrata* when peptone was used as a nitrogen source.

Therefore, it was necessary for us to develop a defined medium, in which the organic nitrogen source was replaced by an inorganic nitrogen source, to check the vitamin demand of *T. glabrata* in detail. *T. glabrata* strain WSH-IP12 could grow in a medium supplemented with an inorganic compound as the nitrogen source, such as ammonium sulfate (Li et al. 2000). However, the pyruvate production was rather low, compared with the case where peptone was supplied. Thus, we selected an excellent pyruvate producer from *T. glabrata* WSH-IP12 which could use ammonium chloride as a sole nitrogen source for cell growth and pyruvate production. This characteristic made it possible to investigate in detail the effect of each vitamin on the production of pyruvate, because no complex compounds existed in the defined medium. A simple but useful experimental, orthogonal design method [which was used in optimizing the medium for glutathione production (Li et al. 1999)] was applied to check the relationship of each vitamin to each other and to optimize their concentrations.

Materials and methods

Microorganism

T. glabrata strain WSH-IP12, isolated by our laboratory and used elsewhere (Li et al. 2000), was a wild-type strain and was used as the parent strain here.

Media

The seed medium consisted of (per liter): 20 g glucose, 5 g peptone (biochemical grade, Sino-American Biotechnology Co., Shanghai, China), 1 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$. The complete medium (CM) consisted of (per liter): 10 g glucose, 3 g NH_4Cl, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 mg nicotinic acid, 0.05 mg thiamine·HCl, 0.2 mg pyridoxine·HCl, 0.03 mg biotin, 0.015 mg riboflavin, and 5 g $CaCO_3$. The minimal medium (MM) composition was the same as CM, except that MM was free of vitamins; and 20 g agar/l was used to solidify the medium when necessary. The fermentation medium consisted of (per liter): 100 g glucose, 7 g NH_4Cl, 3 g KH_2PO_4, 0.8 g $MgSO_4 \cdot 7H_2O$, and 40 g $CaCO_3$. The concentration of each vitamin in the fermentation medium was added according to the design of each experiment. The initial pH of all media was adjusted to 5.0. In the fermentor, the pH was automatically controlled at 5.0 with 8 M NaOH solution. All vitamins were sterilized by microfiltration; and $CaCO_3$ was sterilized by dry-heat sterilization at 160 °C for 30 min before being added to the medium. Flask culture was done for 48 h.

Cultivation

The cultivation was done at 30 °C. The culture from a slant was inoculated into a 500-ml flask with 50 ml seed medium and cultivated for 12 h on a reciprocal shaker. It was then inoculated either into a 500-ml flask containing 50 ml fermentation medium or into a 2.5-l jar fermentor (KF-2.5 l, Korea Fermentor Co., Inchon, Korea) containing 1.5 l fermentation medium. The inoculum size was 10% (v/v). In fermentor culture, the agitation speed was controlled at 700 rpm in the first 16 h and then switched to 500 rpm. The air flow rate was 1.5 l/min.

Mutagenesis of *T. glabrata* strain WSH-IP12 and mutants obtained

Cells grown in the seed medium for 12 h were harvested by centrifugation (6,000 *g*) and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). Then, 1.0 g (wet weight) washed cells was added to 100 ml 0.1 M potassium phosphate buffer (pH 7.0) containing 10 g ethyl methanesulfonate (EMS)/l and shaken at 30 °C for 1 h. EMS-treated cells were centrifuged at 6,000 *g* and then washed with a solution of 9 g NaCl/l. The cells were harvested, diluted to 10^{-4}, 10^{-5}, and 10^{-6} to spread onto a CM plate, and then incubated at 30 °C for 48 h. The colonies which produced a large transparent circle on the CM plate were replicated onto MM to verify the genetic characteristic of vitamin-auxotrophy. Those colonies which were normal colonies with a large transparent circle on CM, but were very small colonies on MM, were checked for pyruvate-producing ability.

Analyses

Pyruvate concentration was assayed enzymatically with lactic acid dehydrogenase (Lamprecht and Heinz 1984). The standard reaction mixture for the assay consisted of 300 µmol potassium phosphate buffer (pH 7.0), 0.05 unit lactic acid dehydrogenase, 0.3 µmol NADH, and an appropriate volume of diluted fermentation broth in a final volume of 3.0 ml. The decrease in NADH concentration, measured by the change in absorbence at 340 nm, was proportional to the amount of pyruvate reduced; and the concentration of pyruvate was expressed as free pyruvic acid. Glucose concentration was measured by the 3,5-disalicylic-acid spectrometric method (Li et al. 1998). Cell concentration was measured using a spectrophotometer (UV-120-02; Shimadzu Co., Kyoto, Japan) at 660 nm after an appropriate dilution. The optical density (OD_{660}) value was converted to dry cell weight (DCW) using the equation that OD_{660}=1.0 is equal to 0.23 g DCW/l. Ethanol concentration in the fermentor culture was detected with an ethanol monitor (Katakura et al. 1998).

Orthogonal design method

Orthogonal design is a type of experimental design method which uses the Taguchi parameter design methodology (Montgomery 1991). It is easy to investigate the influence of controlled factors in a multivariable system using this method. In this study, an $L_{16}(4^5)$ orthogonal array was used. The $L_{16}(4^5)$ orthogonal array is a table of integers whose column elements (1–4) represent the four levels of the column factors, as shown in Table 1. Each row of the orthogonal array represents a run, i.e., a specific set of factor levels to be tested. The $L_{16}(4^5)$ orthogonal array will accommodate five factors at four levels each in 16 runs. Intuitive analyses and statistical calculations were carried out according to the above methods.

Table 1 Factors and levels in the $L_{16}(4^5)$ orthogonal array

Factor (and name of vitamin)	Level (and concentration of vitamin; mg/l)			
	1	2	3	4
A Nicotinic acid	2.0	4.0	6.0	8.0
B Thiamine	0.01	0.02	0.03	0.04
C Pyridoxine	0.1	0.2	0.3	0.4
D Biotin	0.01	0.02	0.03	0.04
E Riboflavin	0.0	0.05	0.1	0.15

Table 2 Pyruvate-producing ability of mutants with ammonium chloride as a sole nitrogen source. Medium contained (per liter): 0.03 mg thiamine, 8 mg nicotinic acid, 0.03 mg biotin, 0.3 mg pyridoxine, and 0.15 mg riboflavin

Pyruvate production (g/l)	Number of mutants
10–17	4
18, 19	8
20, 21[a]	10
22–24	12
25–29	13
30	8

[a] Pyruvate production by the parent strain, WSH-IP12, is 21 g/l

Table 3 Genetic stability of eight mutants with ammonium chloride as sole nitrogen source. *WSH-IP12* is the parent strain

Strains	Pyruvate production (g/l)		
	First generation	Second generation	Third generation
WSH-IP12	22	22	21
WSH-IP303	35	35	35
WSH-IP312	33	33	32
WSH-IP1225	34	33	33
WSH-IP1226	35	30	28
WSH-IP1228	35	31	29
WSH-IP1220P	37	33	30
WSH-IP1236P	35	33	30
WSH-IP214	34	31	27

Results

Mutagenesis to produce mutants which can assimilate ammonium chloride as sole nitrogen source

After EMS treatment, 54 mutants were obtained, which exhibited large transparent circles on a CM plate supplemented with ammonium chloride as sole nitrogen source. Table 2 shows the amount of pyruvate produced by these mutants. Among them, eight strains produced more than 31 g pyruvate/l, which was 35% higher than the amount produced by the parent strain, WSH-IP12. One of the mutants, strain WSH-IP303, not only produced 60% more pyruvate than the parent strain, but also showed strong genetic stability through the generations (Table 3). Therefore, it was chosen to be used in the following study.

Nitrogen-assimilating ability of strain WSH-IP303

As shown in Fig. 1, the ability of *T. glabrata* strain WSH-IP303 to assimilate inorganic nitrogen compounds is stronger than its ability to assimilate organic material. Among the nitrogen sources tested, cells grown in media supplemented with either ammonium chloride or urea (with a nitrogen concentration of 1.8 g/l) produced comparable amounts of pyruvate, which were about 30% higher than in the case of peptone. For the convenience of nitrogen determination, ammonium chloride was chosen as the nitrogen source for strain WSH-IP303 in the further study; and the optimum concentration was 7 g/l (containing 1.8 g nitrogen/l).

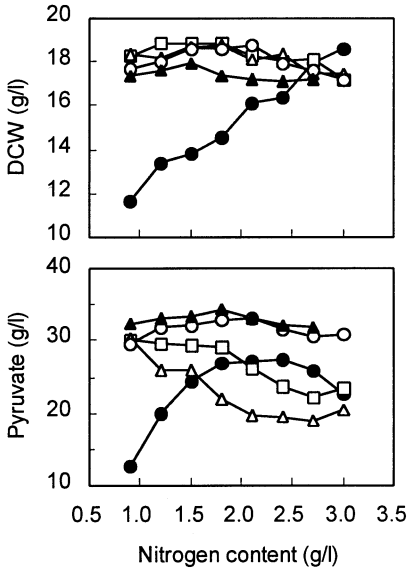

Fig. 1 The nitrogen-assimilating ability of *Torulopsis glabrata* strain WSH-IP303. Nitrogen compounds with the same nitrogen content were added to the culture. Peptone contained 12% (w/w) nitrogen. The fermentation medium contained (per liter): 0.03 mg thiamine, 8 mg nicotinic acid, 0.03 mg biotin, 0.3 mg pyridoxine, and 0.15 mg riboflavin. △ Peptone, □ $(NH_4)_2SO_4$, ● $(NH_4)_2HPO_4$, ▲ urea, ○ NH_4Cl

Effects of vitamins on over-production of pyruvate by strain WSH-IP303

Based on the above results, a defined medium was developed, with ammonium chloride as sole nitrogen source. Because there are five vitamins in the medium (nicotinic acid, thiamine, pyridoxine, biotin, and riboflavin), an $L_{16}(4^5)$ orthogonal design experiment was applied to optimize the vitamin levels; and the results are shown in Table 4. The intuitive analysis, based on statistical calculations using the data in Table 4, is shown in Fig. 2.

The range analysis of orthogonal design experiments gave two optimum combinations of vitamin concentrations, as follows (per liter): (1) to obtain a high concentration of pyruvate, 0.02 mg thiamine, 2 mg nicotinic acid, 0.04 mg biotin, 0.4 mg pyridoxine, and 0.1 mg riboflavin, (2) to obtain a high yield of pyruvate to glucose, 0.01 mg thiamine, 2 mg nicotinic acid, 0.04 mg biotin, 0.4 mg pyridoxine, and 0.1 mg riboflavin. Therefore, a set of experiments was performed to check the optimum combinations. Furthermore, since increasing the concentration of nicotinic acid stimulated glucose consumption but reduced pyruvate production (Fig. 2), we also checked the synergistic effect of thiamine and nicotinic acid, with the other three vitamin concentrations unal-

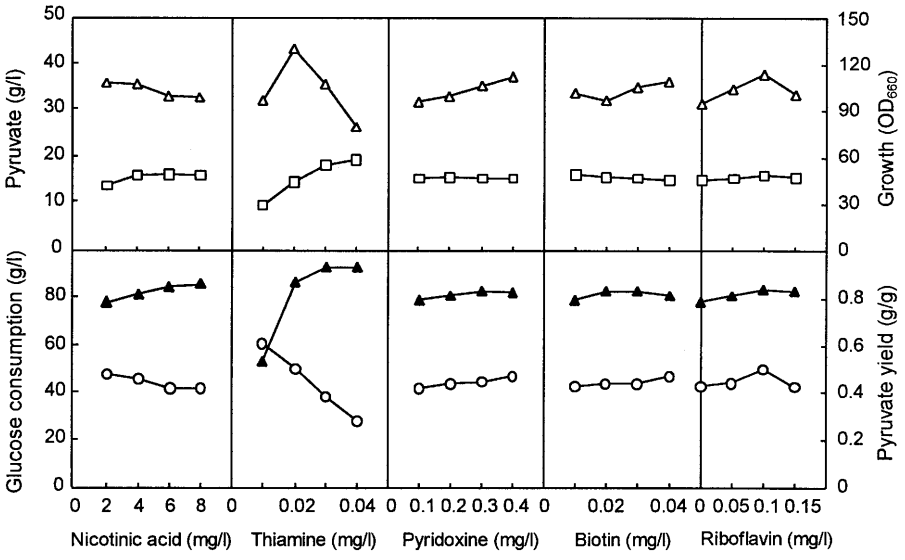

Fig. 2 Intuitive analysis of the orthogonal design experiments. △ Pyruvate, □ cell growth, ▲ glucose consumption, ○ pyruvate yield

Table 4 Results of $L_{16}(4^5)$ orthogonal design experiments. Each datum is the mean value of two identical flask samples. The concentration range of each vitamin was determined according to the results of preliminary experiments (data not shown), given in Table 1

Factor					Cell growth (OD_{660})	Pyruvate production (g/l)	Glucose consumption (g/l)	Yield (g pyruvate/g glucose)
A	B	C	D	E				
Level								
1	1	1	1	1	27	31	56	0.55
1	2	2	2	2	40	39	80	0.49
1	3	3	3	3	47	42	96	0.44
1	4	4	4	4	51	32	98	0.33
2	1	2	3	4	31	32	59	0.54
2	2	1	4	3	43	48	89	0.54
2	3	4	1	2	58	36	98	0.37
2	4	3	2	1	61	27	98	0.28
3	1	3	4	2	30	31	57	0.54
3	2	4	3	1	48	46	97	0.47
3	3	1	2	4	58	29	98	0.30
3	4	2	1	3	63	27	98	0.28
4	1	4	2	3	29	35	58	0.60
4	2	3	1	4	48	41	97	0.42
4	3	2	4	1	57	35	98	0.36
4	4	1	3	2	60	20	98	0.20

Table 5 Effect of thiamine and nicotinic acid concentrations on pyruvate production. Each datum is the mean value of two identical flask samples. The concentrations of pyridoxine, biotin, and riboflavin were (per liter): 0.4 mg, 0.04 mg, and 0.1 mg, respectively

Thiamine (mg/l)	Nicotinic acid (mg/l)	Cell growth (OD_{660})	Residual glucose (g/l)	Pyruvate production (g/l)	Yield (g pyruvate/ g glucose)
0.01[a]	2[a]	30	39.0	38	0.62
0.01	4	32	32.0	40	0.59
0.01	8	32	25.0	44	0.59
0.01	10	34	18.0	48	0.58
0.01	12	36	14.0	46	0.53
0.02[b]	2[b]	44	8.4	48	0.52
0.02	8	50	0.2	44	0.44

[a] Optimum combination for producing high yield of pyruvate to glucose
[b] Optimum combination for producing high concentration of pyruvate

Table 6 Determination of the critical concentrations of nicotinic acid and thiamine

Thiamine (mg/l)	Nicotinic acid (mg/l)	Cell growth (OD_{660})	Residual glucose (g/l)	Pyruvate production (g/l)	Yield (g pyruvate/ g glucose)
0.015	8	47	0.2	52	0.52
0.015	10	48	0.2	48	0.48

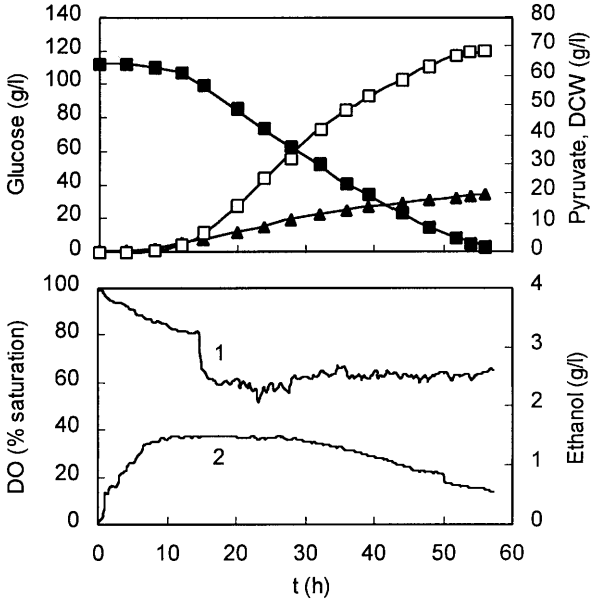

Fig. 3 Time-course of batch fermentation process for strain WSH-IP303. Initial glucose concentration was 112 g/l. *1* Dissolved oxygen (*DO*), *2* ethanol concentration. ■ Glucose concentration, □ pyruvate concentration, ▲ dry cell weight (*DCW*), *t* Time

tered (Table 5). Pyruvate concentration and yield, in the media supplemented with the two optimum combinations of vitamin concentrations for obtaining either high pyruvate concentration or high yield, were 48 g/l (yield, 0.52 g/g) and 0.62 g/g (concentration, 38 g/l), respectively. Based on the results in Table 5, another combination, 0.015 mg thiamine/l and 8 mg nicotinic acid/l, was tested (Table 6). Both high pyruvate concentration (52 g/l) and high yield (0.52 g/g) were achieved. However, pyruvate yield decreased by 7.6% when the nicotinic acid concentration was further increased to 10 mg/l.

Batch culture process with suitable combination of vitamins in a jar fermentor

A batch culture, in which the initial concentration of thiamine, nicotinic acid, pyridoxine, biotin, and riboflavin were 0.015, 8, 0.4, 0.04, and 0.1 mg/l, respectively, was conducted in a 2.5-l jar fermentor (Fig. 3). Pyruvate concentration reached 69 g/l after 56 h, achieving a yield to glucose of 0.62 g/g. In the process, the ethanol concentration was no more than 1.6 g/l.

Discussion

With respect to cell growth and pyruvate production, *T. glabrata* IFO 0005 could assimilate inorganic nitrogen compounds (such as ammonium sulfate), but not as the sole nitrogen source (Miyata and Yonehara 1996). In the present report, strain WSH-IP303 (which has the ability to assimilate ammonium chloride as sole nitrogen source and to provide both good cell growth and satisfactory pyruvate production) was selected after EMS treatment of *T. glabrata* strain WSH-IP12. By orthogonal design experiments, two optimum combinations of vitamins were identified which obtained either high pyruvate concentration or high yield. Pyruvate concentration and yield, in media supplemented with the corresponding optimum combinations of vitamins, were 48 g/l and 0.62 g/g respectively, which were almost as high as the highest concentration (48 g/l) and yield (0.61 g/g) in the orthogonal design experiments.

Thiamine was found to be the most important factor affecting both cell growth and glucose consumption for pyruvate production. When the thiamine concentration was 0.01 mg/l, a high pyruvate yield was achieved and the glucose consumption rate was relatively low. Under thiamine deficiency, pyruvate accumulates due to the decreased activity of the pyruvate dehydrogenase complex (Yokota et al. 1994a; Hua et al. 1999). However, the pentose phosphate pathway might also be affected, because thiamine is also a cofactor of transketolase (Yokota et al. 1994a). The impaired pentose phosphate pathway decreases the rate of NADPH synthesis, leading to a slow glucose consumption rate because the decrease in NADPH inhibits the synthesis of cell materials (Hua et al. 1999). I.e., a low concentration of thiamine is a bottleneck, preventing the accumulation of pyruvate from accelerating. Increasing the thiamine concentration leads to an increased activity of both the pyruvate dehydrogenase complex and pyruvate decarboxylase, the enzymes responsible for the breakdown of pyruvate to acetyl-CoA and acetaldehyde, respectively. At the same time, the pentose phosphate pathway is also activated (Hua et al. 1999). Thus, it is easy to understand the phenomenon that increasing the concentration of thiamine greatly improved cell growth and glucose consumption but reduced pyruvate production (Fig. 2).

Nicotinic acid also affected cell growth. However, compared with thiamine, increasing the concentration of nicotinic acid contributed more to the improvement of the glucose consumption rate. As shown in Table 5, glucose consumption and pyruvate production in the medi-

um supplemented with 0.01 mg thiamine/l increased by 35% and 29%, respectively, while the pyruvate yield decreased by no more than 4% when the nicotinic acid concentration was increased from 2 mg/l to 10 mg/l. This may be due to the fact that nicotinic acid is a component of NAD, a cofactor of glycolysis (Miyata and Yonehara 1996). However, when the nicotinic acid concentration was further increased above 10 mg/l, the pyruvate yield decreased, which may be due to the fact that NAD is also a cofactor of the pyruvate dehydrogenase complex. In contrast, when the thiamine concentration was increased from 0.01 mg/l to 0.02 mg/l, glucose consumption was stimulated. However, pyruvate concentration and yield decreased obviously when the nicotinic acid concentration was increased from 2 mg/l to 8 mg/l. This indicated that the balance between pyruvate accumulation and degradation easily breaks at such a critical thiamine concentration.

The effects of the other three vitamins (biotin, pyridoxine, and riboflavin) on the production of pyruvate by strain WSH-IP303, were not significant. Thus, the synergistic effect of thiamine and nicotinic acid on the accumulation and breakdown of pyruvate can be examined in medium supplemented with the optimal concentrations of biotin, pyridoxine, and riboflavin. High yield (0.52 g/g) and high productivity (52.8 g/l) of pyruvate were achieved in a flask culture within 48 h, by using suitable vitamin concentrations. Furthermore, the highest ethanol concentration for a batch culture in a 2.5-l fermentor was only 1.6 g/l, which was lower than the ethanol concentration (4.0 g/l) when using organic nitrogen material as a nitrogen source (Miyata and Yonehara 1996; Hua et al. 1999). From the viewpoint of ethanol formation, it is presumed that the pyruvate decarboxylase activity of strain WSH-IP303 was also reduced due to thiamine limitation.

Although there are many reports related to the fermentative production of pyruvate by *T. glabrata*, this is the first report of using ammonium chloride as sole nitrogen source. An efficient pyruvate production was easily achieved in batch culture by applying a suitable concentration of vitamins. Further improvement in productivity with fed-batch culture may be anticipated.

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