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RESEARCH PAPER

Improvement of Pyruvate Production Based on Regulation of Intracellular Redox State in Engineered *Escherichia coli*

MaohuaYang and Jianmin Xing

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Abstract The commercial demand for pyruvate has been expanding. However, some challenges need to be overcome in the microbial production of pyruvate, such as low glucose consumption caused by excessive accumulation of NADH. In this study, weakening or block of the TCA cycle, overexpression of foreign NADH oxidase, and carbon sources with different oxidation state was attempted to decrease NADH accumulation in engineered strain YP211. Results showed that blocking or weakening TCA cycle could not lower the intracellular redox state in strain YP211.Overexpressing NADH oxidase from *Lactococcus lactis* significantly decreased the intracellular NADH content and increased the consumption rate of glucose. However, the yield of pyruvate did not increase significantly. Compared with glucose as carbon source, sodium gluconate with a higher oxidation state resulted in a significant decrease of $NADH/NAD^+$, and the concentration and yield of pyruvate increased by 62 and 6%, respectively. In the fed-batch fermentation, the yield of pyruvate increased to 0.78 g/g gluconate, and the concentration of pyruvate reached 78.8 g/L. It was suggested that sodium gluconate was a more ideal carbon source for strain YP211, which could effectively decrease NADH content and improve the pyruvate production.

Keywords: *Escherichia coli*, pyruvate, redox state, NADH oxidase, sodium gluconate

MaohuaYang*, Jianmin Xing
Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100-190, PR China
Tel: +86-010-8254-4980; Fax: +86-010-6255-0913
E-mail: mhyang@ipe.ac.cn

1. Introduction

As a chemical, pyruvate is used as a raw material for drug, agrochemical, chemical, and food industries [1,2]. The commercial demand for pyruvate has been expanding. In cells, pyruvate occupies a key place in the metabolic network, and there are several pathways for its degradation. Unlike the end products of metabolic pathways, such as acetic acid and lactic acid, it is difficult to accumulate pyruvate under natural conditions [3].

Through the alteration of the microbial metabolic networks, the accumulation of pyruvate can be achieved in cells. In this area, a variety of high-pyruvate-yield strains have been built. CGSC7916, constructed by Tomar A *et al.* by inactivating the *aceF* and *ppc* genes, had a pyruvate yield of 0.78 g/g glucose and a volumetric productivity of 1.2 g/L/h [4]. Zhu *et al.* removed the genes *aceEF*, *pfl*, *poxB*, *pps*, *ldhA*, *atpFH*, *and arcA* from *E. coli* to obtain strain ALS1059. After fed-batch fermentation, the concentration of pyruvate reached 90 g/L, and pyruvate yield and production efficiency reached 0.68 g/g glucose and 2.1 g/L/h, respectively [5]. Causey TB *et al.* retained pyruvate dehydrogenase complex fully and deleted the genes *focA-pflB*, *frdBC*, *ldhA*, *atpFH*, *adhE*, *sucA*, *poxB and ackA* in *E. coli.* From the fermentation of the resulting strain, the pyruvate conversion rate reached 0.75 g/g glucose, and the maximum concentration of pyruvate reached 66 g/L [6]. In our previous work, genes *ldhA*, *pflB*, *pta*, *ackA*, *poxB*, *ppc*, and *frdBC* were knocked out from *E. coli* MG1655 to get strain YP211 for producing pyruvate [7]. When glucose was used as a carbon source in cultivation, the production of lactic acid, formic acid, acetic acid, succinic acid, and other carboxylic acids was effectively controlled. As the main product, pyruvate reached a concentration of 60 g/L. However, after 20 h of culture, the glucose consumption rate decreased rapidly and even

stopped, and the concentration of pyruvate could not be further increased. This was a common and serious problem in production of pyruvate by microbial fermentation [8].

It was found that the accumulation of pyruvate was mainly due to the blocking of the acetic acid pathways. Compared with conversion to acetic acid, acetyl CoA converting to CO_2 in the TCA cycle produces more NADH. The accumulation of NADH and the strengthening of oxidative phosphorylation result in the formation of even more ATP. Previous studies have shown that excessive accumulation of NADH and ATP inhibited both glucose consumption and cell growth [9,10]. To verify this, transcriptional differences between strain YP211 and the wild-type were compared, and it was found that the transcription of genes in the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation pathway were enhanced [7]. For example, the transcriptional intensities of genes *gltA*, *icd*, *suc*, *cyoB*, and *atpH* were increased by 2.5, 3.9, 3.8, 43.7, and 3.2 times, respectively. At the same time, the transcription of genes related to the glycolsis were down-regulated in YP211. For example, the transcription of genes *pgi*, *pfkB*, and *fbaB* was decreased to 0.3, 0.3, and 0.1-fold, respectively.

Regulation of the redox state in the cells, specifically lowering the NADH/NAD^+ ratio, could provide a solution to the above problems. As TCA cycle is the primary way of producing NADH in aerobic growth, decreasing the amount of carbon entering the TCA cycle could potentially decrease the amount of NADH. In addition, oxidation of NADH to NAD^+ and H_2O by NADH oxidase is another way to reduce the ratio of NADH/NAD^+ [11]. Moreover, carbon sources with different oxidation states would produce different amounts of NADH in metabolism. Oxidative carbon sources can produce less NADH than reduced carbon sources. In this study, to strengthen glycolysis and produce more pyruvate, it was tried to weaken or block of the TCA cycle, express foreign NADH oxidase, and compare three carbon sources to decrease NADH production in strain YP211. From the results, using high oxidation carbon source could effectively decrease NADH content and improve the pyruvate production. This was the first report on the production of pyruvate using gluconate as carbon source.

2. Materials and Methods

2.1. Strains and plasmids

Strains and plasmids constructed in this study are listed in Table 1. Restriction enzymes were purchased from Takara Biotechnology (Dalian, China). PCR purification kits, gel extraction kits were from Axygen (Union City, CA, USA). Uni-seamless cloning and assembly kits were from Transgen (Beijing, China). Primers were synthesized by Sangon Biotechnology (Shanghai, China).

2.2. Genetic manipulation

A modified method based on tandem repeat primers and homologous recombination was developed for substitution and deletion of *E. coli* chromosomal genes [12]. Primers were designed to amplify the *cat-sacB* cassette from plasmid pEASY-*cat-sacB* using PCR and Pfu polymerase (Supplementary Table 1).

2.3. Construction of plasmid pTrc99A-*noxE*

The *noxE* gene was amplified by PCR using *Lactococcus. Lactis* DNA as template. The forward primer *noxE*-F contained an EcoRI restriction site at the beginning of the amplified fragment and a KpnI restriction site at the end of the amplified fragment (Supplementary Table 1). The resulting PCR product was gel isolated, digested with EcoRI and KpnI, and ligated into the pTrc99A expression vector with Uni-seamless cloning and assembly kit.

2.4. Media and fermentation

During plasmid and strains construction, cultures were grown in LB broth or on LB plates. Assessments of the

Table 1. Sources and characteristics of strains and plasmids used in this study

	Relevant characteristics	Sources
Strains		
YP211	*E. coli* MG1655, Δ*ldhA*Δ*pflB*Δ*pta-ackA*Δ*poxB*Δ*ppc*Δ*frdBC*	Lab collection
YP212	YP211, replacing ATG, the start codon of *gltA*, with GTG	This study
YP213	YP211, Δ*sucA*	This study
YP214	YP211, pTrc99A-*noxE*	This study
Plasmids		
pKD46	Red recombinase, temperature-conditional replicon	Lab collection
pEASY-*cat-sacB*	T-easy vector with *cat-sacB* cassette	Transgen
pTrc99A	Expression vector	Invitrogen
pTrc99A-*noxE*	pTrc99A containing *Lactococcus. Lactis noxE* gene	This study

engineered strains were conducted in flask with M9 medium containing about 20 g/L glucose or sodium gluconate or glycerol at 37°C and 200 rpm. Two gram $CaCO_3$ was added to control the pH. Fed batch fermentation was conducted in a 5.0 L fermenter (BioFlo/Celli Gen115, New Brunswick Scientific) with M9 medium. During the experiments, sodium gluconate was added to about 40 g/L from a sterile 50% stock, when the remaining sodium gluconate was below 10 g/L. 20% NaOH were used to control pH. Samples were stored at -20ºC for subsequent analysis.

2.5. Analytical methods

Organic acids and carbon sources concentrations were determined by using a HPLC (1200 series, Agilent Technologies) equipped with a Bio-Rad HPX-87H column with 5 mM H_2SO_4 as the mobile phase (0.6 mL/min, 50ºC) [13]. NADH and NAD^+ concentrations were measured using NAD^+/NADH Quantitation Kit (Sigma-Aldrich). The activity of NADH oxidase (NOX) and citrate synthase was measured using NADH oxidase activity Assay Kit and citrate synthase activity Assay Kit, respectively (Suzhou Comin Biotechnology Co., Ltd).

3. Results and Discussion

3.1. Weakening the expression of citrate synthase gene (*gltA*)

In cells, citrate synthase catalyzes acetyl coenzyme A and oxaloacetate to produce citrate. This enzyme controls the entrance to the TCA cycle, and is the rate-limiting step of the TCA cycle [14]. Compared to the wild-type, the intensity of transcription of the *gltA* gene in YP211 increased 2.5 times. In order to decrease the overall strength of the TCA cycle, the activity of citrate synthase was turned down, specifically, that was weakening the translation of *gltA* mRNA. Given the preference of *E. coli* for start codons, ATG, the start codon, was replaced by a codon GTG with lower activity in strain YP212 (Fig. 1). We determined the activity of citrate synthase in cells under exponential growth, and found that, compared to YP211, the activity of citrate synthase in YP212 decreased from 15.1 to 10.4 U/g protein, illustrating a decrease in the translation of *gltA*.

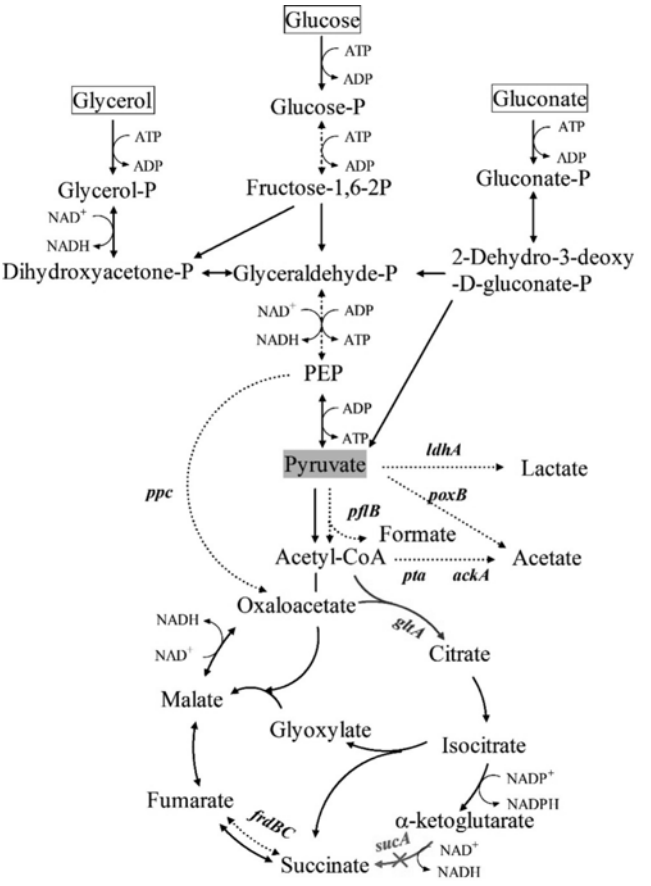

Fig. 1. Biochemical pathways involved in pyruvate accumulation in *E. coli* and schematic showing differences in oxidation of glucose, gluconate, and glycerol. The dotted lines indicate metabolic steps that have been blocked in the previous strain YP211. Gene *sucA* was deleted and the start codon of gene *gltA* was replaced in this study.

Table 2. Comparison of metabolic parameters of *E. coli* strains used in this study

	YP211[a]	YP212	YP213	YP214	YP211[b]	YP211[c]
OD600	3.7 ± 0.2	3.8 ± 0.2	3.8 ± 0.0	5.2 ± 0.3	4.5 ± 0.1	3.5 ± 0.1
Pyruvate (g/L)	10.4 ± 0.5	10.1 ± 0.2	10.5 ± 0.1	11.5 ± 0.3	16.8 ± 0.9	3.1 ± 0.1
Pyruvate production rate (g/L/h)	0.43 ± 0.02	0.42 ± 0.01	0.44 ± 0.00	0.48 ± 0.01	0.70 ± 0.04	0.13 ± 0.00
Substrate consumption (g/L)	15.1 ± 0.2	15.5 ± 0.2	15.6 ± 0.1	17.0 ± 0.02	23.1 ± 0.0	4.8 ± 0.2
Substrate consumption rate (g/L/h)	0.63 ± 0.01	0.65 ± 0.01	0.65 ± 0.00	0.71 ± 0.01	0.96 ± 0.00	0.2 ± 0.01
Pyruvate yield (g/g substrate)	0.69 ± 0.02	0.65 ± 0.01	0.67 ± 0.01	0.68 ± 0.01	0.73 ± 0.04	0.64 ± 0.02
NADH/NAD^+	0.32 ± 0.05	0.35 ± 0.05	0.30 ± 0.05	0.13 ± 0.01	0.23 ± 0.05	0.43 ± 0.03
NAD(H) (μmol/g/biomass)	9.10 ± 0.13	9.49 ± 0.10	8.10 ± 0.07	9.78 ± 0.09	7.10 ± 0.13	9.30 ± 0.15

[a]Unless stated otherwise, the results were from batch fermentation with glucose as carbon source at 24 h. The initial carbon source was about 20 g/L.
[b]The results were from batch fermentation with sodium gluconate as carbon source.
[c]The results were from batch fermentation with glycerol as carbon source.

However, it was found that the cell growth and the ratio of NADH/NAD$^+$ were unaffected, and the yield of pyruvate did not increase (Table 2). As was found with YP211, cells barely consumed glucose after 24 h, even though the leftover glucose had a concentration of 4.1 g/L (Fig. 2).

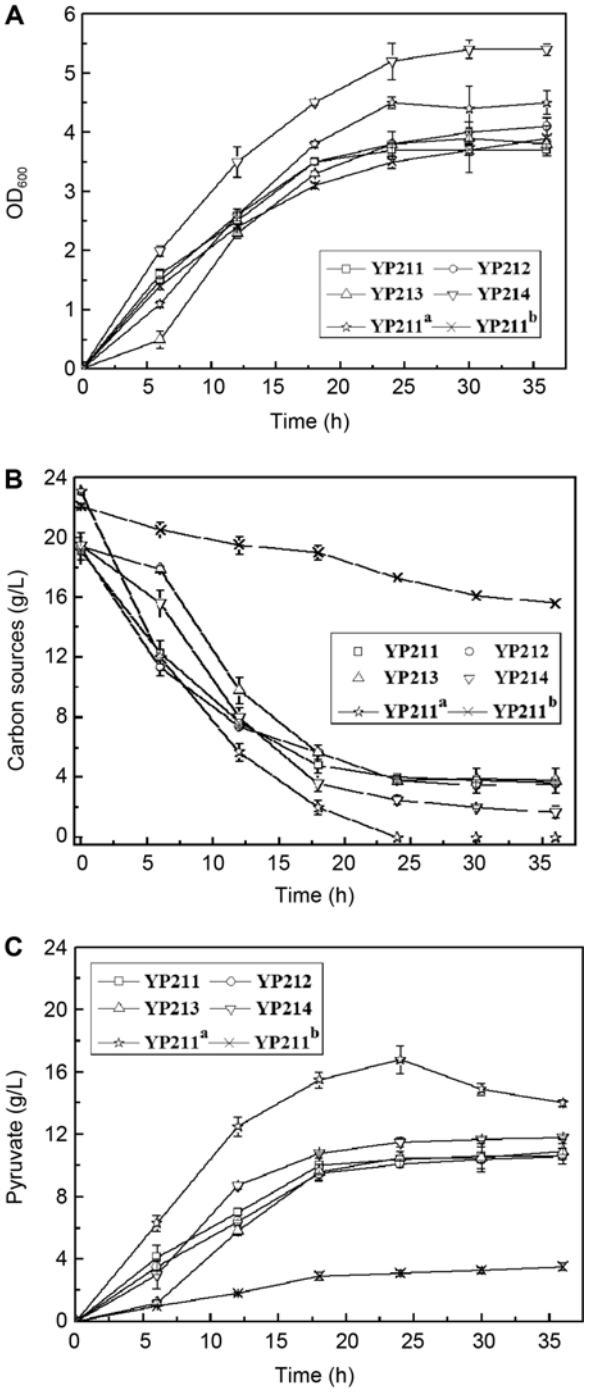

Fig. 2. Production of pyruvate by flask-fermentation with engineered strains. A, growth; B, residual glucose, gluconate or glycerol; C, pyruvate. YP211, YP212, YP213, YP214, using glucose as carbon source; YP211^a, using sodium gluconate as carbon source; YP211^b, using glycerol as carbon source.

This result showed that decreasing the activity of citrate synthase had very little effect on the ratio of NADH/NAD$^+$ and pyruvate production in YP211.

3.2. Removal of the gene of 2-oxoglutarate decarboxylase (*sucA*)

Gene *sucA* is responsible for encoding the enzyme that catalyzes the conversion of 2-ketoglutarate to succinyl-CoA, while accompanied by the oxidation of NADH to NAD$^+$ [15]. Compared to the wild-type, the transcription of *sucA* was increased by 4.6 times in strain YP211. To decrease the strength of the TCA cycle, the gene *sucA* was deleted to interrupt the TCA pathway in strain YP213. The growth curve showed that the growth of YP213 was severely inhibited in the early stage, and its rate of growth was significantly lower than YP211. Compared to YP211, the OD, concentration of pyruvate and the consumption of glucose were found to have decreased by 69, 88, and 78%, respectively, after 6 h (Fig. 2). However, as cultivation progressed, the growth of YP213 was found to have quickly recovered. At 24 h, the cell mass and concentration of pyruvate was at around the same value as those of YP211 (Table 2), which may have been a result of the activation of 2-oxoglutarate decarboxylase isozyme or the glyoxylate cycle in the later stage of fermentation. In any case, there was no significant decrease in ratio of NADH/NAD$^+$ and increase in production of pyruvate in strain YP213. These results suggested that blocking or weakening TCA cycle was not an ideal method to reduce the intracellular redox state in strain YP211.

3.3. Overexpression of NADH oxidase from *Lactococcuslactis*

In *E. coli* wild-type, overexpression of NADH oxidase could decrease the NADH/NAD$^+$ ratio and thereby strengthened the glycolysis. Expression of NADH oxidase also could lead to a decrease in the production of lactic acid and ethanol [16], and an increase in the production of 2, 3-butanediol [17], 1-butanol [18]. In this study, gene *noxE* from *L. lactis* was amplified and overexpressed in plasmid pTrc99A. Strain YP214 with plasmid pTrc99A-*noxE* was cultured by the same methods, in addition to the need for IPTG (1mM) to induce expression of the gene *noxE*. Compared to YP211, the NADH/NAD$^+$ ratio of YP214 were found to have decreased from 0.32 to 0.13 (Table 2). From the results of fermentation, OD reached 5.2 at 24 h in YP214, a 41% increase over that found for YP211, and the rate of consumption of glucose was also found to have increased by 13%, and the remaining glucose was 2.5 g/L. As previous studies have reported [19,20], when NADH was converted to NAD$^+$, glycolysis was stimulated, which was beneficial to the growth of *E. coli*. The concentration of pyruvate increased by 11%, reaching 11.5g/L. These

results showed that overexpression of NADH oxidase can decrease the amount of NADH and increase the amount of pyruvate produced. However, the overall yield of pyruvate did not increase, reaching only 0.68 g/g glucose, similar to that found for YP211. The reason might be that more carbon sources were transformed into biomass.

3.4. Comparison of three carbon sources with different oxidation state

Glucose is a kind of carbon source with a low oxidation state. During the glucose metabolism in YP211, a large quantity of NADH was accumulated, which resulted in the inhibition of glycolysis. The use of a suitable carbon source, such as alginate [21], can effectively increase the glycolysis. In this study, it was attempted to use sodium gluconate as carbon source. The carboxyl group in sodium gluconate has a higher oxidation state than the hydroxyl group in glucose. As a control, glycerol was selected as the third carbon source, since glycerol has a lower oxidation state than glucose. We compared the differences in metabolism of the three carbon sources.

The consumption rate of sodium gluconate reached 0.96 g/L/h, which were 1.5 times that of glucose and 4.8 times that of glycerol. Moreover, after 24 h, it was found that only sodium gluconate was entirely consumed. Comparing with glucose, the production of pyruvate from gluconate increased by 62% and to a concentration of 16.8 g/L (Table 2). The pyruvate yield reached 0.73 g/g gluconate and increased by 6%. Glycerol was the most unsuitable carbon source for pyruvate production. The pyruvate concentration was only 3.1 g/L. Fig. 1 showed the metabolic pathways of three carbon sources [22]. During the oxidation of 1 mol sodium gluconate to 2 mol pyruvate, only 1 mol ATP and 1 mol NADH were produced. Compared to glucose, the amount of ATP and NADH produced had halved. In the formation of 2 mol pyruvate from glycerol, 2 mol ATP and 4 mol NADH were produced.

$$\text{Glucose}+2\text{Pi}+2\text{ADP}+2\text{NAD}^+ \rightarrow 2\text{Pyruvate}+2\text{ATP}+2\text{NADH}+2\text{H}^+ +2\text{H}_2\text{O}$$

$$\text{Gluconate}+\text{Pi}+\text{ADP}+\text{NAD}^+ \rightarrow 2\text{Pyruvate}+\text{ATP}+\text{NADH}+\text{H}^+ +\text{H}_2\text{O}$$

$$2\text{Glycerol}+2\text{Pi}+2\text{ADP}+4\text{NAD}^+ \rightarrow 2\text{Pyruvate}+2\text{ATP}+4\text{NADH}+4\text{H}^+ +4\text{H}_2\text{O}$$

The experimental results verified the above. Maximum NADH was produced during glycerol metabolism, and the intercellular NADH/NAD^+ ratios was 0.43. During the conversion of gluconate to pyruvate, the minimum NADH was produced, and the NADH/NAD^+ ratios reached 0.23. Compared with glycerol and gluconate, the amount of NADH from glucose metabolism was in the middle level. The results showed that the carbon source with high oxidation state was more favorable for the production of pyruvate. Therefore, for stain YP211, sodium gluconate was a suitable carbon source.

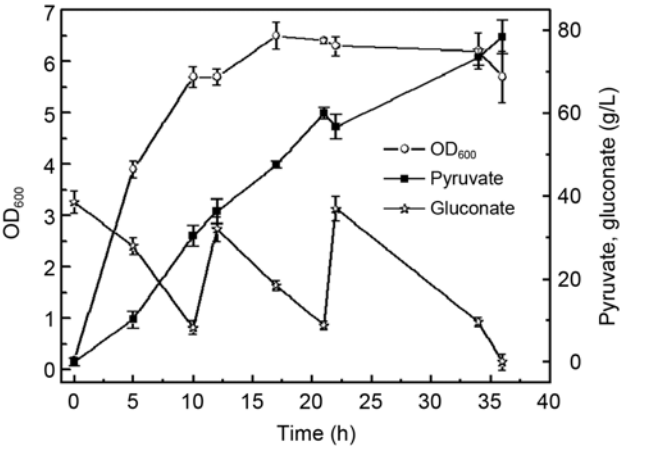

Fig. 3. Results of fed-batch fermentation using strain YP211 with sodium gluconate as carbon source.

3.5. Production of pyruvate by fed-batch cultivation with sodium gluconate as carbon source

The fed-batch fermentation using sodium gluconate as carbon source was carried out in a 5 L fermentor. The results showed that the rate of consumption of sodium gluconate remained at a relatively high level and reaching 2.6 g/L/h. After 36 h of fermentation, the concentration of pyruvate reached 78.8 g/L with a yield of 0.78 g/g glucose and an increase of 13% comparing with glucose as carbon source (Fig. 3). Succinate and 2-ketoglutarate both maintained at a lower level. Due to the continuous secretion of pyruvate from cells, NaOH or other base must be added to regulate the pH of the fermentation system. As sodium gluconate contained sodium ions, the decrease in pH occurred at a far slower rate than when glucose was used as a carbon source, and the need for a base was decreased. In this study, we used NaOH as the pH regulator. It was found that, compared with glucose as carbon source, usage of NaOH decreased by 60%, and the final sodium concentration decreased to 0.6 M from 1.0 M. In the fermentation system, the high salt content may also be the cause of metabolic arrest. The above results indicated that the use of sodium gluconate as a carbon source could effectively solve the problems presented in the later stage of fermentation of YP211.

4. Conclusion

In this study, to solve the problem posed by a standstill in

glucose consumption in the late stages of fermentation, it was attempted to decrease the production of NADH by weakening the activity of key enzyme in the TCA cycle and interrupting the TCA cycle. However, the results obtained were not as originally expected. Expressing foreign NADH oxidase significantly increased the rate of consumption of glucose and increased the cell mass, but the resulting yield of pyruvate did not increase significantly. When sodium gluconate with a higher oxidation state than glucose was used as the carbon source, the yield was increased by 13%, reaching 0.78 g/g gluconate. In the fed-batch fermentation, the cells consumed 96 g/L of sodium gluconate and produced 78.8 g/L pyruvate. The use of sodium gluconate as a carbon source could effectively solve the problems presented and sodium gluconate was a more ideal carbon source for strain YP211.

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