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Improving the stability of glutamate fermentation by *Corynebacterium glutamicum* via supplementing sorbitol or glycerol

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Abstract

Background: *Corynebacterium glutamicum* is widely used in glutamate fermentation. The fermentation feature of the strain varies sometimes. These variations may lead to the reduction in the ability of the strain to resist environmental changes and to synthesize glutamate, resulting in abnormal glutamate fermentations.

Results: In the abnormal glutamate fermentations, glutamate accumulation stopped after glucose feeding and the final glutamate concentration was at a lower level (50 to 60 g/L). The $r_{NAD}{}^{+}/r_{NADH}$ ratio was lower than that in normal batch which was reflected by lower oxidation-reduction potential (ORP) value. The abnormal fermentation performance was improved when glucose was co-fed with sorbitol/glycerol at a weight ratio of 5:1 or adding 10 to 15 g/L of sorbitol/glycerol in the initial medium. Under these conditions, glutamate synthesis continued after substrate (s) feeding and final glutamate concentration was restored to normal levels (≥72 g/L). r^{+}_{NAD}/r_{NADH} ratio, ORP, and pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and cytochrome c oxidase (CcO) activities were maintained at higher levels.

Conclusions: Sorbitol and glycerol were not used as carbon sources for the fermentation. They were considered as effective protective agents to increase cells' resistance ability against environmental changes and maintain key enzymes activities.

Keywords: Enzyme activity; Fermentative stability; Glutamate fermentation; Oxidation-reduction potential; Protective substance; $r_{NAD}{}^{+}/r_{NADH}$

Background

L-Glutamate is mainly used as a flavor enhancer in food industry and nutrient in pharmaceutical industry. The annual production has exceeded 2.2 million tons by fermentation with *Corynebacterium glutamicum* [1]. The biosynthetic pathway of glutamate includes complex enzymatic reactions, as shown in Figure 1a. The enzymes effectively convert substrate (such as glucose) into glutamate only when they are well coordinated with coenzymes NAD^+ or NADH. Intracellular levels of NAD^+ and NADH significantly affect the catalytic efficiency of the enzymes [2]. The ratio of NAD^+/NADH in vivo is a key factor affecting energy transfer and redox state of the cells, and the optimal value at different stages during glutamate fermentation usually varied [3]. The metabolic flux distribution can be altered by variation of NAD^+/NADH ratio or $r_{NAD}{}^{+}/r_{NADH}$ ratio in glutamate fermentation [4].

NAD^+/NADH ratio is indirectly reflected by oxidation-reduction potential (ORP) [5,6], which represented the redox state of the cells. The optimal ORP range corresponding to different fermentation processes is different. For example, maximum lysine yield was obtained when ORP was controlled between the range of −230 and −210 mV, while the preferable ORP range was −275 to −225 mV in homoserine and valine fermentations [6,7]. The redox state of cells is changed if certain auxiliary substances (such as sorbitol and glycerol) are supplemented and intracellular NAD^+/NADH ratio can be varied correspondingly [8]. These auxiliary substrates are usually non-repressive

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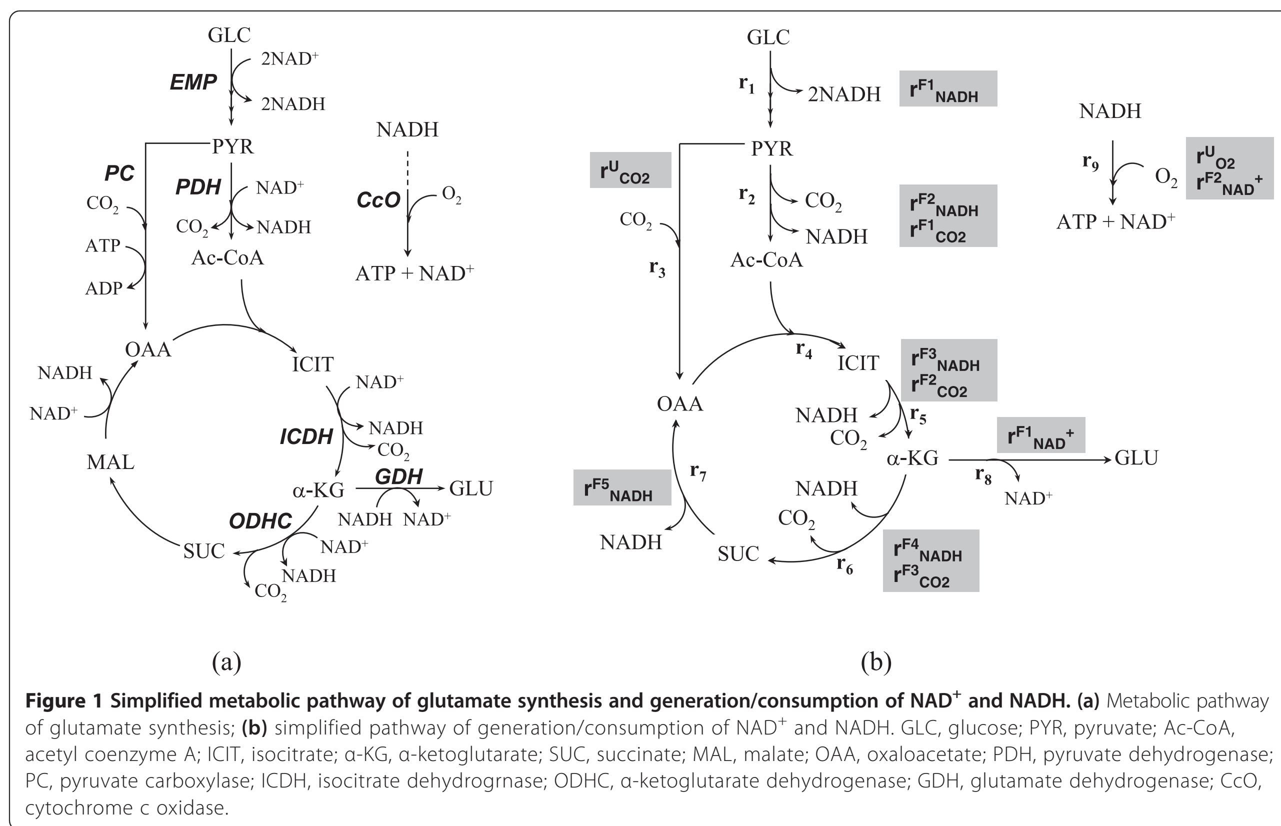


Figure 1 Simplified metabolic pathway of glutamate synthesis and generation/consumption of NAD^+ and NADH. (a) Metabolic pathway of glutamate synthesis; **(b)** simplified pathway of generation/consumption of NAD^+ and NADH. GLC, glucose; PYR, pyruvate; Ac-CoA, acetyl coenzyme A; ICIT, isocitrate; α-KG, α-ketoglutarate; SUC, succinate; MAL, malate; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; ICDH, isocitrate dehydrogrnase; ODHC, α-ketoglutarate dehydrogenase; GDH, glutamate dehydrogenase; CcO, cytochrome c oxidase.

carbon sources. They can protect cells against stress in their living environment, enhance cell viability, and reduce the metabolic burden [9-12]. It was reported that the production of alkaline polygalacturonate lyase and lipase increased by 1.85-fold and 8.7-fold, respectively, when the strategy of methanol/sorbitol co-feeding was adopted [10,13]. Arruda and Felipe found that xylitol productivity could be increased by 35% when glycerol was added in the medium [14]. Therefore, fermentation with mixed carbon sources was considered as an effective way to enhance the targeted metabolite productions.

C. glutamicum used in industry is usually stored at 4°C for a short time and replaced regularly. In this way, production fluctuation resulting from strain change can be avoided. However, sometimes the fermentation characteristics of the strain vary, resulting in decreased glutamate synthesis ability and resistance to environmental changes. In such a case, fermentation performance becomes abnormal and glutamate production also ends at a very low level. Glutamate production fluctuated in a large range, and fermentation stability decreased greatly. Frequent rejuvenation is a common solution to this problem. But it is a costly, time-consuming, and troublesome procedure. Furthermore, glutamate is a low value-added product. It is more economical to adopt a simple way with low operation cost to maintain the fermentative stability. The strategy of feeding mixed substrates has been applied in other fermentations. This was an effective method to decrease cell mortality, maintain the enzyme activity, and promote targeted metabolite production [9-11]. Similar studies with regard to glutamate fermentation are also important, but few have been reported. In this study, sorbitol or glycerol was either co-fed with glucose or added in the initial medium, aiming at protecting the cells against environmental change/stress and stabilizing glutamate productions. Meanwhile, the theoretical mechanism was interpreted. The results gained in this study will provide some useful information and reference to the glutamate fermentation industry in terms of stabilizing the glutamate production.

Methods

Strain and culture condition

C. glutamicum ATCC13032 was used in this study. The seed microorganism was grown in a shaker at 32°C and 200 r/min for 8 to 10 h in liquid medium containing (in g/L) glucose 25, K_2HPO_4 1.5, $MgSO_4$ 0.6, $MnSO_4$ 0.005, $FeSO_4$ 0.005, corn slurry 25, and urea 2.5 (separately sterilized). Initial pH was adjusted to 7.0 to 7.2. The medium for jar fermentation contained (in g/L) glucose 140, K_2HPO_4 1.0, $MgSO_4$ 0.6, $MnSO_4$ 0.002, $FeSO_4$ 0.002, thiamine 5.0×10^{-5}, corn slurry 15, and urea 3.0 (separately sterilized).

The fed-batch fermentation was implemented in a 5-L fermentor (BIOTECH-5BG, Baoxing Co., Shanghai, China) equipped with on-line DO/pH/ORP electrodes. Initial medium volume was 3 L, and air aeration rate was 1.33 vvm. The temperature was maintained at 32°C during the entire fermentation period (about 34 to 36 h). pH was maintained at 7.0 to 7.2 by automatically pumping in 25% (*w*/*v*) ammonia water. Dissolved oxygen (DO) was controlled at 20% of air saturation by manually adjusting agitation rate. Concentrated glucose (50%, *w*/*v*) was fed when glucose concentration was lower than 20 g/L. Sorbitol or glycerol was supplemented by the following two methods:

Method #1: 50% (*w*/*v*) sorbitol or glycerol solution was co-fed with the addition of concentrated glucose solution. The feeding ratio of 1:5 (*w*/*w*) sorbitol/glycerol versus glucose was applied.
Method #2: 5 to 15 g /L sorbitol or glycerol was added into the initial medium before inoculation. Only glucose was fed when glucose concentration was lower than 20 g/L.

Analytical and measurement methods

Cell concentration was assayed by spectrophotometer at 620 nm (OD_{620}). Glucose and glutamate concentrations were measured by a biosensor (SBA-40C, Shandong Science Academy, Jinan, China). The concentrations of sorbitol and glycerol were analyzed by HPLC (Hitachi Chromaster Organizer, Hitachi, Ltd, Chiyoda-ku, Japan) equipped with an ion exclusion column (Aminex HPX-87H, 300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) and a differential refractive index detector at 30°C. The mobile phase was 0.005 mol/L H_2SO_4 at a flow rate of 0.6 mL/min [10]. Two electronic balances (JA1102, Haikang Co., Shanghai, China) were connected to the computer and used to monitor the feeding amount of glucose and sorbitol or glycerol solution. O_2 and CO_2 partial pressure in exhaust gas were on-line measured by a gas analyzer (LKM2000, Lokas Co., Daejeon, Korea). CO_2 evolution rate (CER), O_2 uptake rate (OUR), and respiratory quotient (RQ) were then calculated by standard formula.

Enzyme activity assay

The activities of pyruvate dehydrogenase (PDH) and isocitrate dehydrogenase (ICDH) were analyzed by the methods reported [15,16]. Cytochrome c oxidase (CcO) was assayed using the kit for bacteria (Genmed Scientifics Inc., Wilmington, DE, USA). The enzyme activity was expressed as U/mg-DCW, where 1 U was the quantity of dry cell converting 1 μmol NAD^+ per minute. The relative enzymatic activity (REA) was used for comparison and interpretation. REA before feeding glucose/mixed carbon sources (18 h) was set as the unit (1), and REA after feeding glucose/mixed carbon sources (26 h) was described by Eq. (1).

$$\mathrm{REA}_{\mathrm{aft}}(k) = \frac{E_{\mathrm{aft}}(k)}{E_{\mathrm{bef}}(k)} \tag{1}$$

Where $E_{\mathrm{bef}}(k)$ and $E_{\mathrm{aft}}(k)$ referred to the activities of *k*-th enzyme (PDH, ICDH, and CcO) before and after glucose/mixed carbon sources feeding.

Modeling and calculation of r_{NAD^+}/r_{NADH} ratio

The glutamate synthesis pathway was depicted according to the map reported [16], shown in Figure 1a. NADH and NAD^+ were generated or consumed to run the entire fermentation, and they were closely associated with glucose consumption, glutamate synthesis, CO_2 release, and O_2 consumption rates. Therefore, the generation or consumption rates of NADH and NAD^+ could be determined by a couple of measurable reaction rates, such as glucose consumption rate (r_{GLC}), glutamate formation rate (r_{GLU}), CER, and OUR. The metabolic pathways of NADH and NAD^+ were simplified as Figure 1b based on the following assumptions:

(1) The main products were glutamate and CO_2, because the concentrations of other byproducts (lactate, acetate, and other amino acids) were very low.
(2) Pentose phosphate (PP) pathway was ignored because it was not related to generation/consumption of NADH or NAD^+.
(3) The metabolic flux into the two reaction branches at pyruvate node followed the ideal condition, namely $r_2 = r_3$ in Figure 1b [16]. Therefore, the glyoxylate shuttle was ignored.
(4) The intermediate carbon metabolites were in pseudo-steady-state, and the net accumulation of them was 0. But it was not applied for NADH and NAD^+. r_{NAD^+}/r_{NADH} ratio was closely and positively associated with the NAD^+/NADH ratio, while r_{NAD^+} actually represented NADH consumption rate ($r^{(C)}_{NADH}$) and r_{NADH} represented NADH formation rate ($r^{(F)}_{NADH}$). NADH formation rate ($r^{(F)}_{NADH}$) could differ with its consumption rate ($r^{(C)}_{NADH}$), which led the variation in r_{NAD^+}/r_{NADH} ratio.
(5) Glutamate fermentation was a non-growth associated process; the cell concentration in production phase basically stayed at a constant level or declined slightly. So, we used the volume reaction rate to replace the specific reaction rate for convenience purpose.

The simplified metabolic pathway (Figure 1b) contains nine reactions shown in the Appendix, which covers the basic reactions occurring in EMP pathway, tricarboxylic acid (TCA) cycle, CO_2 fixing reaction, respiratory chain,

and glutamate synthesis. According to the assumptions and simplifications above, the rates of all the reactions were coupled as follows.

$$r_1 = r_{GLC} \quad (2)$$

$$r_2 = r_3 = r_4 = r_5 = 0.5r_1 \quad (3)$$

$$r_6 = r_7 = r_5 - r_8 \quad (4)$$

$$r_8 = r_{GLU} \quad (5)$$

$$r_9 = 2r_{O2} = 2\ \mathrm{OUR} \quad (6)$$

The generation/consumption rates of NADH and NAD^+ as well as $r_{NAD}{}^{+}/r_{NADH}$ ratio at a specified time t could be calculated by the equations of (7) to (16).

$$r^{F1}_{NADH}(t) = 2r_1(t) = 2r_{GLC}(t) \quad (7)$$

$$r^{F2}_{NADH}(t) = r_2(t) = r^{F1}_{CO_2}(t) \quad (8)$$

$$r^{F3}_{NADH}(t) = r_5(t) = r^{F2}_{CO_2}(t) \quad (9)$$

$$r^{F4}_{NADH}(t) = r_6(t) = r^{F3}_{CO_2}(t) \quad (10)$$

$$r^{F5}_{NADH}(t) = r_7(t) = r_6(t) \quad (11)$$

$$r^{F1}_{NAD^+}(t) = r_{GLU}(t) \quad (12)$$

$$r^{F2}_{NAD^+}(t) = r_9(t) = 2r^{U}_{O_2}(t) = 2\mathrm{OUR}(t) \quad (13)$$

$$r^{U}_{CO_2}(t) = r_3(t) \quad (14)$$

$$\mathrm{CER}(t) = r^{F1}_{CO_2}(t) + r^{F2}_{CO_2}(t) + r^{F3}_{CO_2}(t) - r^{U}_{CO_2}(t) \quad (15)$$

$$\begin{aligned}\frac{r_{NAD^+}}{r_{NADH}}(t) &= \frac{r^{F}_{NAD^+}(t)}{r^{F}_{NADH}(t)} = \frac{r^{F1}_{NAD^+}(t) + r^{F2}_{NAD^+}(t)}{r^{F1}_{NADH}(t) + r^{F2}_{NADH}(t) + r^{F3}_{NADH}(t) + r^{F4}_{NADH}(t) + r^{F5}_{NADH}(t)} \\ &= \frac{r_{GLU}(t) + 2\mathrm{OUR}(t)}{2r_{GLU}(t) + r^{F1}_{CO_2}(t) + r^{F2}_{CO_2}(t) + r^{F3}_{CO_2}(t) + r_7} \\ &= \frac{r_{GLU}(t) + 2\mathrm{OUR}(t)}{2r_{GLU}(t) + \mathrm{CER}(t) + r_3 + r_7} = \frac{r_{GLU}(t) + 2\mathrm{OUR}(t)}{2r_{GLU}(t) + \mathrm{CER}(t) + 2r_3 - r_8} \\ &= \frac{r_{GLU}(t) + 2\mathrm{OUR}(t)}{3r_{GLU}(t) + \mathrm{CER}(t) - r_{GLU}(t)}\end{aligned} \quad (16)$$

Where $r^{F}_{NAD}{}^{+}(t)$ is the NAD^+ formation rate (mmol/L/h), $r^{F}_{NADH}(t)$ NADH formation rate (mmol/L/h), $r^{U}_{O2}(t)$ O_2 uptake rate (mmol/L/h), $r^{F}_{CO2}(t)$ CO_2 evolution rate (mmol/L/h), $r^{U}_{CO2}(t)$ CO_2 uptake rate (mmol/L/h), $r_{GLC}(t)$ glucose consumption rate (mmol/L/h), and $r_{GLU}(t)$ glutamate production rate (mmol/L/h).

Results and discussion

Fermentation performance of normal and abnormal batches

Final glutamate production and cell concentration were two factors reflecting the fermentation performance. In 'normal' fermentation, final glutamate concentration and the maximum cell concentration (OD_{620}) were more than 70 g/L and 50, respectively. Otherwise, fermentations were categorized into 'abnormal.' Glutamate fermentation was non-growth associated, and glutamate was accumulated when cell growth had almost ceased (after 10 h). At this moment, about 1/3 glucose in the initial medium was consumed. Additional glucose was supplemented during the main glutamate production phase at 18 to 20 h. In the normal batch, glutamate concentration still increased after glucose was fed, although glutamate accumulation rate became slow. However, glutamate production stopped after glucose feeding in the abnormal batch, and the final glutamate concentration was around 50 g/L. In this case, cell growth and glutamate production before glucose feeding were almost the same as those in the normal fermentation (Figure 2a,b). There was no significant difference in the changing trend of RQ between the normal and abnormal batches. However, the changing pattern of ORP in the abnormal batch differed from that of normal batch significantly (Figure 2c,d). ORP was maintained in the normal range (−75 to −85 mV) before 20 h (glucose was fed at 18 h) and decreased slowly to a lower level (−120 mV) after 20 h. Generally, $r_{NAD}{}^{+}/r_{NADH}$ ratio was closely and positively associated with the NAD^+/NADH ratio. It was shown that in vivo $r_{NAD}{}^{+}/r_{NADH}$ ratio was at a lower level

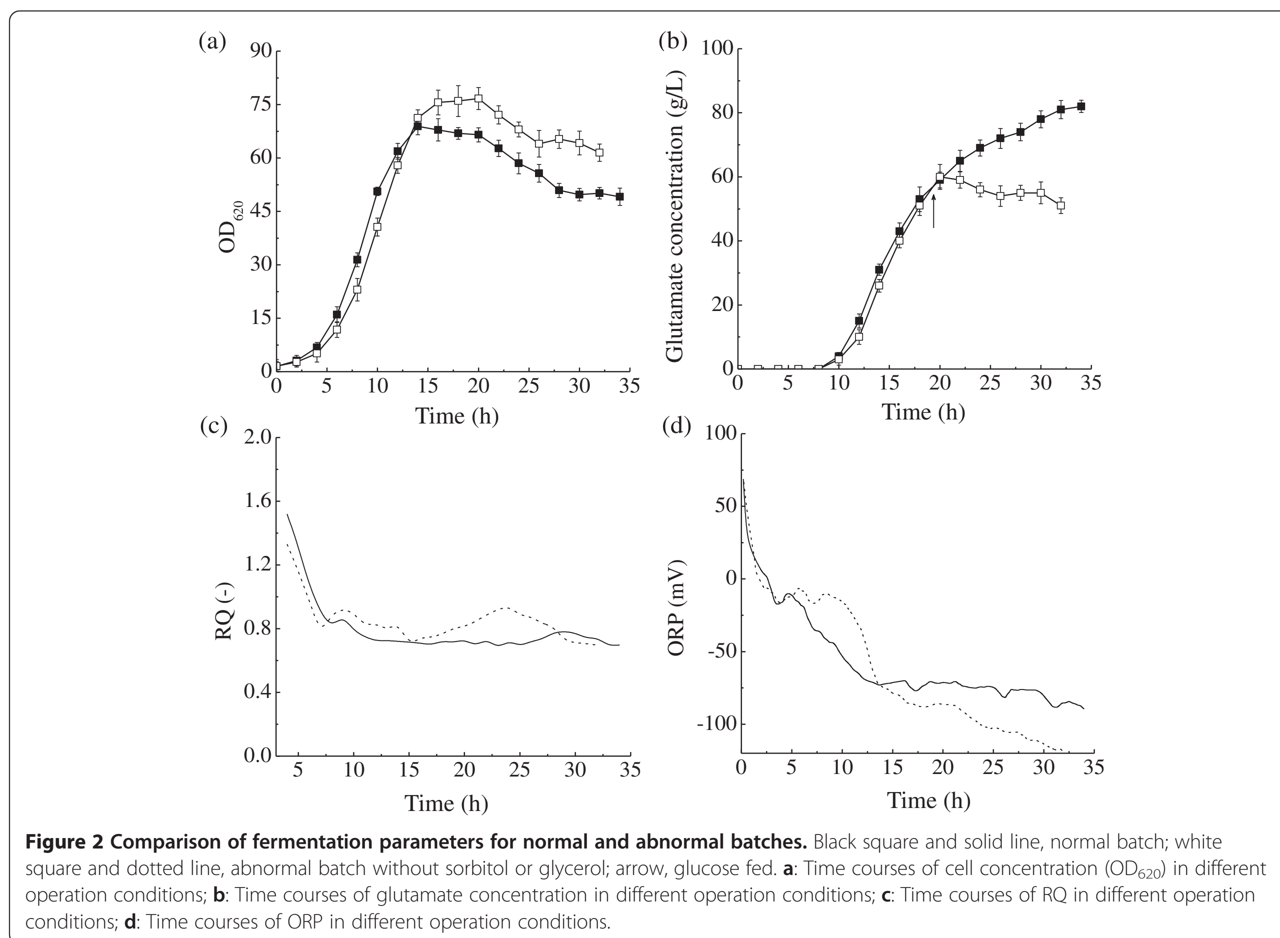


Figure 2 Comparison of fermentation parameters for normal and abnormal batches. Black square and solid line, normal batch; white square and dotted line, abnormal batch without sorbitol or glycerol; arrow, glucose fed. **a**: Time courses of cell concentration (OD_{620}) in different operation conditions; **b**: Time courses of glutamate concentration in different operation conditions; **c**: Time courses of RQ in different operation conditions; **d**: Time courses of ORP in different operation conditions.

and NADH was excessive in abnormal fermentation after 20 h, which was verified by the lower r_{NAD^+}/r_{NADH} ratio calculated in Table 1. Therefore, the improper r_{NAD^+}/r_{NADH} ratio after glucose feeding might be the reason for the non-accumulation of glutamate in the abnormal batch [17].

The abnormal performance appeared after glucose was fed, and it was not contaminated. It was speculated that abnormal fermentation was due to the change of the characteristics of strain which led to the following results: (1) glutamate production ability decreased and (2) fermentation environment changed after glucose was fed and the strain failed to adapt to the change, resulting in intracellular abnormal metabolism and stoppage of glutamate synthesis. It has been reported that some osmoregulators (such as trehalose or betaine) are either

Table 1 r_{NAD^+}/r_{NADH} ratio at different instants under different operation conditions

Time (h)	Fermentation batches					
	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Batch #6
14	1.11	0.85	1.10	0.78	1.03	0.90
16	1.11	0.92	1.13	0.85	0.94	0.94
18	1.02	0.83	1.15	0.83	0.98	0.90
20	0.94	0.66	1.10	0.89	0.93	0.88
22	0.93	0.58	1.08	0.84	0.97	0.87
24	0.89	0.56	1.15	0.95	0.89	0.91
26	0.93	0.59	1.09	0.96	0.94	0.86
28	0.72	0.64	0.95	0.82	0.76	0.82

Batch #1, normal batch (control); *batch #2*, abnormal batch without sorbitol or glycerol; *batch #3*, sorbitol co-fed with glucose; *batch #4*, 15 g/L sorbitol added in the initial medium; *batch #5*, glycerol co-fed with glucose; *batch #6*, 10 g/L glycerol added in the initial medium.

produced by the microorganisms or taken up from the medium in lysine production by *C. glutamicum* in response to a hyperosmotic shock [18,19]. They can protect cells against environmental shock/stress. Sorbitol has the same effect. When glutamate accumulation ceased and the apparent fermentation parameters (OUR, CER, etc.) declined after glucose feeding for a period of 2 h, then 'abnormal' fermentation status was concluded. Twelve and 2 g/L sorbitol were added at 26 and 32 h, respectively; glutamate concentration increased gradually and reached 72 g/L at 36 h with a 2-h fermentation period extend, as shown in Figure 3.

Therefore, the abnormal fermentation was due to the decrease of resistance ability in response to the environmental alterations. In addition, the permeability of cell membrane increased to secrete glutamate extensively in the production phase, and the glucose addition easily brought about shock or stress in the living environment. The carbon flux distribution in vivo was adjusted, and consequently, the metabolism of NAD^+ and NADH was changed. On the other hand, PDH and ICDH required NAD^+ as the coenzyme and less NAD^+ amount restricted the enzymes' catalytic actions. As a result, the metabolic flux was redistributed and a series of abnormal effects arose.

Cells might be more tolerant to the environmental change if some osmoregulators, such as trehalose or betaine, were added before or at the same time when the environmental shock/stress occurred. However, trehalose and betaine are expensive. Sorbitol and glycerol were cheaper, and they were also efficient environmental shock/stress protective reagents. Hence, fermentation performance when co-feeding sorbitol/glycerol with glucose or adding sorbitol/glycerol in the initial medium was studied.

Fermentation performance in presence of sorbitol

The fermentation performance in presence of sorbitol is shown in Figure 4. The cell growth patterns did not change much despite the sorbitol supplement methods adopted. Glutamate production did not cease and final glutamate concentration reached 73 and 77 g/L at 34 h, respectively, when co-feeding sorbitol with glucose or adding sorbitol in the initial medium. No difference in RQ before 20 h in the batches with/without sorbitol was observed. RQ decreased continuously after 20 h in the presence of sorbitol (RQ was about 0.4 at 34 h). Lower RQ was favorable for glutamate accumulation and indicated that less glucose proceeded beyond the α-KG node in the TCA cycle [20]. In these cases, less NADH was accumulated in vivo and it was desirable for maintaining cellular activities [21]. Less NADH accumulation implied that $r^{(F)}_{NADH}$ is less than $r^{(C)}_{NADH}$, leading to a higher r_{NAD^+}/r_{NADH} ratio as well as a higher ORP levels. Under the condition of co-feeding sorbitol with glucose, ORP was maintained at a normal level (−75 to −85 mV) after feeding the mixed carbon sources. When 15 g/L sorbitol was added in the initial medium, ORP always decreased in the production phase, but it was much higher than that in the abnormal batch without sorbitol. r_{NAD^+}/r_{NADH} ratio was more than 0.8 after 20 h when co-feeding sorbitol with glucose or adding sorbitol in the initial medium, as shown in Table 1. The deterioration of the fermentation performance was reversed in the presence of sorbitol. This might be because the resistance to environmental change was enhanced and NADH consumption was returned to normal. The r_{NAD^+}/r_{NADH} ratio was returned to normal level in the presence of sorbitol, and higher ORP values supported this fact indirectly.

Fermentation performance in presence of glycerol

The fermentation performance when co-feeding glycerol with glucose or adding glycerol in the initial medium is shown in Figure 5. Similar fermentation curves and performance as those in the presence of sorbitol were obtained. The final glutamate concentration also reached

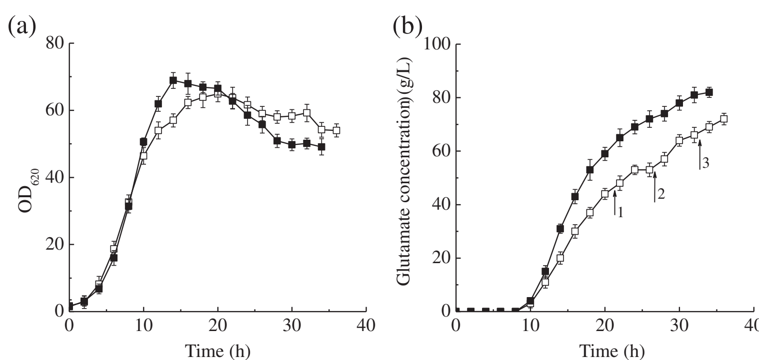


Figure 3 Changing patterns of cell and glutamate concentrations under the condition of supplementing sorbitol when glutamate accumulation ceased. Black square, normal batch; white square, abnormal batch with addition of sorbitol; arrow 1, glucose fed; arrow 2 and arrow 3, adding 12 and 2 g/L sorbitol, respectively. **a**: Time courses of cell concentration (OD_{620}) in different operation conditions; **b**: Time courses of glutamate concentration in different operation conditions.

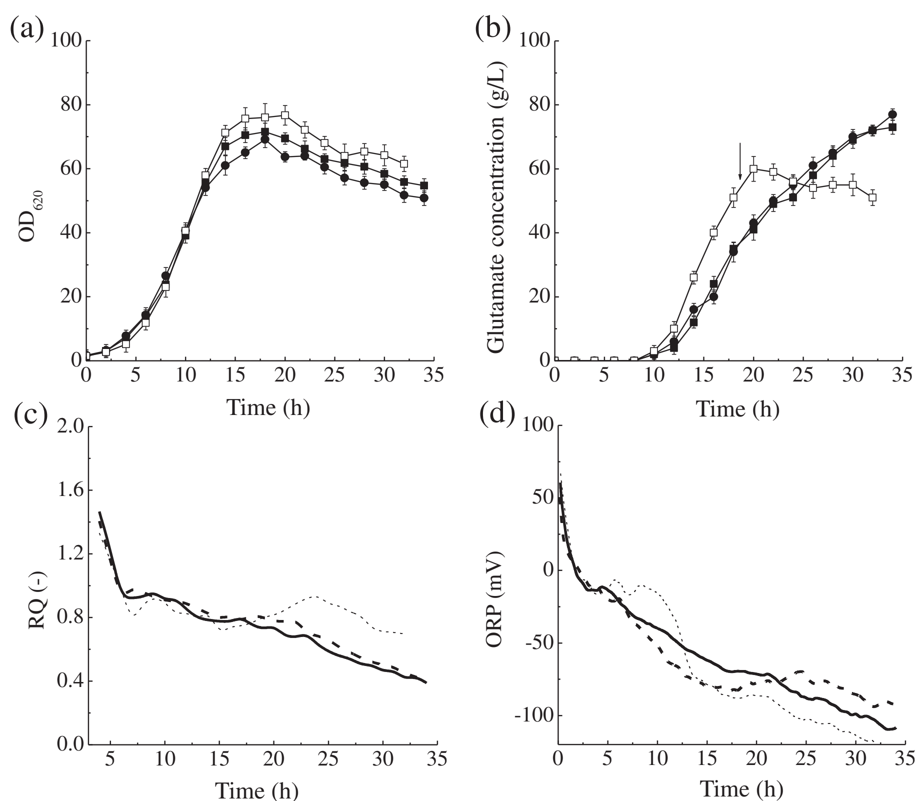


Figure 4 Comparison of fermentation parameters with/without sorbitol. White square and dotted line, abnormal batch without sorbitol; black square and dashed line, sorbitol co-fed with glucose; black circle and solid line, 15 g/L sorbitol added in the initial medium; arrow, glucose fed. **a**: Time courses of cell concentration (OD_{620}) in different operation conditions; **b**: Time courses of glutamate concentration in different operation conditions; **c**: Time courses of RQ in different operation conditions; **d**: Time courses of ORP in different operation conditions.

normal levels at 34 h (72 and 76 g/L, respectively) when co-feeding glycerol with glucose or adding glycerol in the initial medium. The improvements in glutamate production were also closely associated with higher $r_{\mathrm{NAD}^+}/r_{\mathrm{NADH}}$ ratio (more than 0.8) as shown in Table 1, which was also reflected by higher ORP values (Figure 5) and lower RQ (0.6 to 0.7) in the late production phase.

From the results above, it could be concluded that abnormal glutamate fermentations could be restored to normal by supplementing the media with sorbitol or glycerol, especially when sorbitol or glycerol was added in the initial medium. It has been reported that sorbitol and glycerol could be assimilated by yeast and *Escherichia coli* to increase the targeted product yield by effectively providing the required energy [22,23]. Furthermore, both sorbitol and glycerol were used as effective protective agents of cell viability and enzymes due to their hygroscopicity, freezing tolerance, and oxidation resistance. The major role that sorbitol and glycerol played in the restoration of abnormal glutamate fermentation was analyzed subsequently.

Investigation of the role of sorbitol and glycerol during glutamate fermentation

The concentrations of sorbitol and glycerol were assayed, and results are shown in Figure 6. The results indicated that sorbitol and glycerol were hardly utilized by *C. glutamicum* when co-feeding them with glucose or adding them in the initial medium. When sorbitol or glycerol was co-fed with glucose, sorbitol and glycerol did not reduce after feeding; instead, they were gradually accumulated in the broth. While only a small portion of sorbitol or glycerol could be consumed when they were added in the initial medium. In summary, sorbitol and glycerol were not assimilated by *C. glutamicum* but functioned as protectants to improve the tolerance of the strain in response to the disturbance of the living environment.

The glutamate biosynthesis pathway is composed of many enzymatic reactions in which citrate synthase (CS), PDH, and ICDH are the key enzymes directing carbon flux towards TCA cycle (Figure 1a). Their activities are repressed by excessive NADH. Lower $r_{\mathrm{NAD}^+}/r_{\mathrm{NADH}}$ ratio indicates that NADH was more than NAD^+ in the

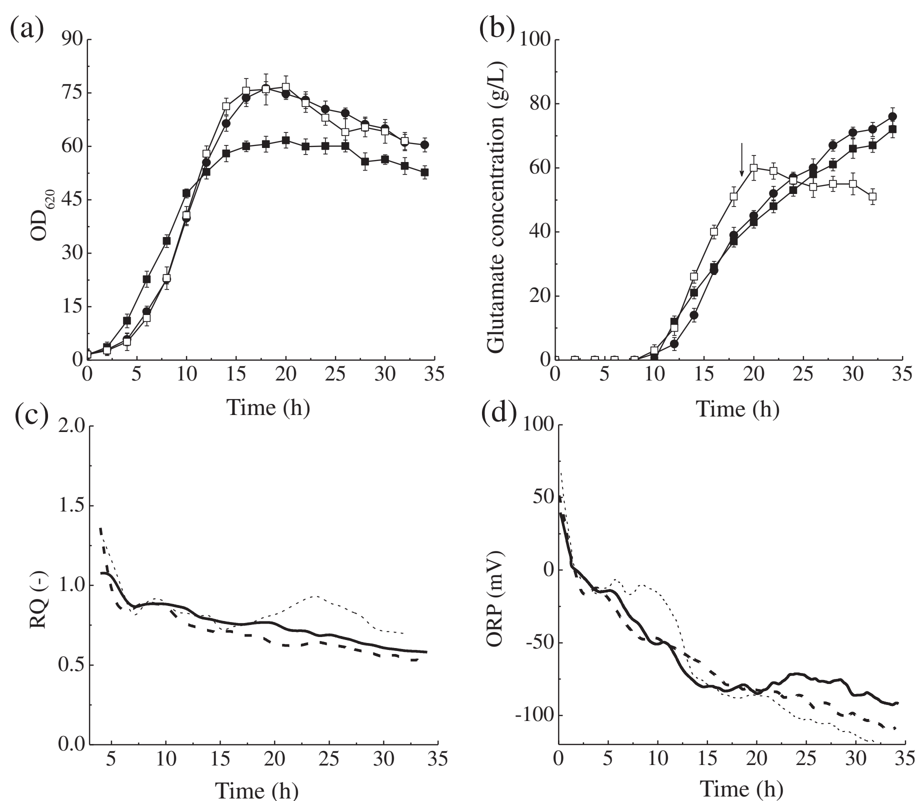


Figure 5 Comparison of fermentation parameters with/without glycerol. White square and dotted line, abnormal batch without glycerol; black square and dashed line, glycerol co-fed with glucose; black square and solid line, 10 g/L glycerol added in the initial medium; arrow, glucose fed. **a**: Time courses of cell concentration (OD_{620}) in different operation conditions; **b**: Time courses of glutamate concentration in different operation conditions; **c**: Time courses of RQ in different operation conditions; **d**: Time courses of ORP in different operation conditions.

cytoplasm. PDH and ICDH activities could be limited by the insufficiency of NAD^+. CcO is the key enzyme catalyzing the transformation of proton (H^+) from NADH to O_2 through the respiratory chain [24]. During this period, NADH was consumed and NAD^+ was regenerated. The activity of CS was difficult to measure, so the activities of PDH, ICDH, and CcO before and after glucose addition (also mixed substrates) under different operation conditions were analyzed. The relative activity of each enzyme before feeding glucose (also mixed substrates, 18 h) was defined as 1. The REA after feeding (26 h) are shown in Table 2. In the abnormal fermentation without sorbitol or

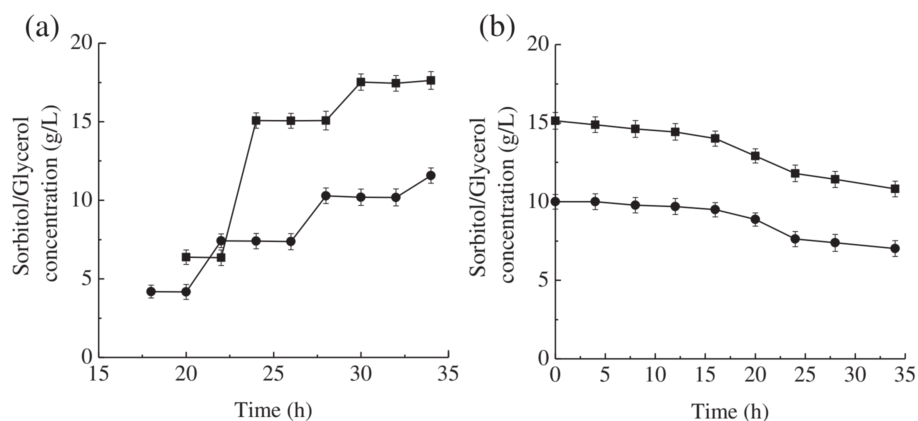


Figure 6 Time courses of sorbitol and glycerol concentrations under different supplementing conditions. (a) Sorbitol or glycerol co-fed with glucose; **(b)** sorbitol or glycerol added in the initial medium. Black square, sorbitol; black circle, glycerol.

Table 2 Relative enzymatic activities of PDH, ICDH, and CcO after substrate(s) feeding (26 h) in different batches

Key enzyme	Fermentation batches					
	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Batch #6
PDH	0.84 ± 0.057	0.58 ± 0.043	0.72 ± 0.052	0.72 ± 0.036	0.76 ± 0.078	0.72 ± 0.061
ICDH	1.18 ± 0.051	0.72 ± 0.039	0.97 ± 0.061	0.92 ± 0.032	1.24 ± 0.076	1.27 ± 0.092
CcO	0.50 ± 0.063	0.00	1.50 ± 0.089	0.79 ± 0.043	0.75 ± 0.057	0.25 ± 0.04

Batch #1, normal batch (control); *batch #2*, abnormal batch without sorbitol or glycerol; *batch #3*, sorbitol co-fed with glucose; *batch #4*, 15 g/L sorbitol added in the initial medium; *batch #5*, glycerol co-fed with glucose; *batch #6*, 10 g/L glycerol added in the initial medium.

glycerol, the activities of PDH and ICDH decreased by 42 and 28%, respectively, and CcO was inactive after glucose feeding. In the presences of sorbitol or glycerol, the inactivation of these enzymes was relieved. The activity of PDH decreased by 20 to 30%, and ICDH activity was not significantly affected. The activity of CcO was almost maintained at a higher level. It was concluded that the repression of the key enzymes directing glucose into glutamate synthesis was relieved by addition of sorbitol or glycerol. Accompanied by the recovery of NAD^+ regeneration, the metabolic flux was shifted into the normal pathway of glutamate synthesis. Consequently, glutamate accumulated continuously after glucose feeding and a failed fermentation could be avoided.

Sorbitol and glycerol served as shield materials during glutamate fermentation. The activity of key enzymes could be properly maintained when supplementing sorbitol or glycerol. The r_{NAD^+}/r_{NADH} ratio was increased, and ORP was maintained around the normal range. The stability of glutamate fermentation was improved efficiently by adding sorbitol or glycerol, and the improvement was more obvious when sorbitol/glycerol was added in the initial medium.

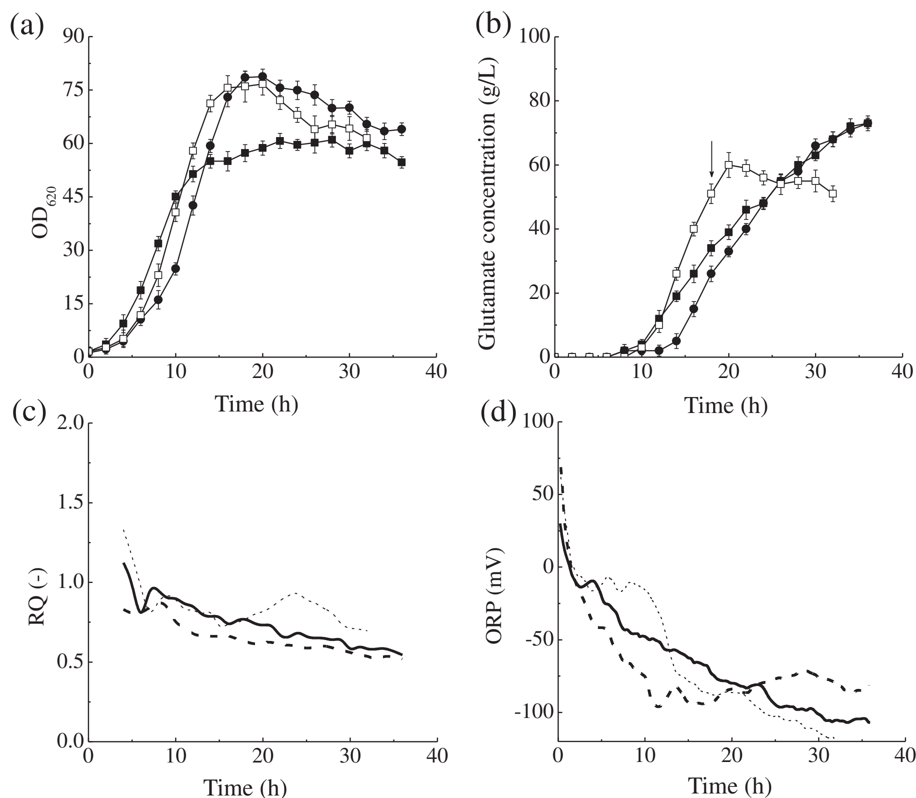


Figure 7 Glutamate fermentation performance when less amount of sorbitol or glycerol was added in the initial medium. White square and dotted line, abnormal batch without sorbitol or glycerol; black square and dashed line, 5 g/L sorbitol added in the initial medium; black circle and solid line, 4 g/L glycerol added in the initial medium; arrow, glucose fed. **a**: Time courses of cell concentration (OD_{620}) in different operation conditions; **b**: Time courses of glutamate concentration in different operation conditions; **c**: Time courses of RQ in different operation conditions; **d**: Time courses of ORP in different operation conditions.

Feasibility analysis in industry

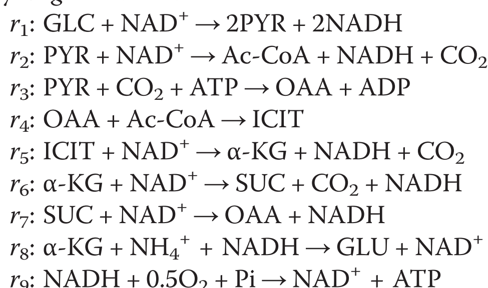
A likely failed fermentation could be restored to normal when co-feeding sorbitol or glycerol with glucose or adding them in the initial medium. However, glutamate is a low value-added product, and the supplementing amount of sorbitol or glycerol should be minimized to save the raw-material cost in industry. Therefore, the cell growth and glutamate production were analyzed with less sorbitol or glycerol (4 to 5 g/L) addition in the initial medium, as shown in Figure 7. In these cases, cell growth was not affected and glutamate synthesis did not stop after feeding glucose and glutamate concentration ended at 74 g/L at 36 h. It was verified again that sorbitol and glycerol functioned mainly as protectants, and their protective effect strengthened with increasing concentration of shield materials [8].

Conclusions

The fermentation features of *C. glutamicum* changed during preservation process and glutamate accumulation stopped after glucose feeding, leading to an abnormal fermentation. This abnormal fermentation performance could be restored to normal by co-feeding sorbitol or glycerol with glucose or adding them in the initial medium. Restoration was more effective when sorbitol or glycerol was added in the initial medium. Glutamate fermentation stability was also improved efficiently. In these cases, sorbitol and glycerol were used as protective agents. When sorbitol or glycerol was added, the adaptive capability of cells to environmental change was promoted and the activities of PDH/ICDH/CcO could be maintained. The usage efficiency of NADH was improved, and r_{NAD^+}/r_{NADH} ratio increased to normal level which was reflected by higher ORP value. These results provided theoretical basis and feasibility for stabilizing glutamate fermentation in its industrial production.

Appendix

Simplified metabolic reactions in glutamate fermentation by *C. glutamicum*:



r_1: $GLC + NAD^+ \rightarrow 2PYR + 2NADH$

r_2: $PYR + NAD^+ \rightarrow Ac\text{-}CoA + NADH + CO_2$

r_3: $PYR + CO_2 + ATP \rightarrow OAA + ADP$

r_4: $OAA + Ac\text{-}CoA \rightarrow ICIT$

r_5: $ICIT + NAD^+ \rightarrow \alpha\text{-}KG + NADH + CO_2$

r_6: $\alpha\text{-}KG + NAD^+ \rightarrow SUC + CO_2 + NADH$

r_7: $SUC + NAD^+ \rightarrow OAA + NADH$

r_8: $\alpha\text{-}KG + NH_4^+ + NADH \rightarrow GLU + NAD^+$

r_9: $NADH + 0.5O_2 + Pi \rightarrow NAD^+ + ATP$

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YC carried out the experiments, performed the statistical analysis, and drafted the manuscript. Z-NH was involved in performing the experiments. ME helped carry out the experiments and revised the manuscript. Z-PS conceived the idea, participated in its design and coordination, and helped in drafting of the manuscript. All authors read and approved the final manuscript.

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