Enhancing glutamine production by optimising the GS-GOGAT pathway in *Corynebacterium glutamicum* and NH_4^+ -limited fermentation

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Abstract

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Keywords: L-glutamine, *Corynebacterium glutamicum*, GS-GOGAT pathway, glutamine synthase, NH_4^+ -limited fermentation process

L-glutamine is a neutral amino acid of γ -carboxylamidation of L-glutamate. It is one of the 20 basic amino acids that compose biological proteins. It plays an important role in maintaining human function and life activities. Its content in the human body is very high, accounting for 61% of free amino acids[1-2]. In recent years, with the in-depth study of L-glutamine, it has been widely used in medicine, health food, feed, and other products[2]. As a potential new drug, L-glutamine is mainly used in the clinical treatment of gastrointestinal ulcers[3], relief for exercise fatigue[4], improvement of brain nerve function[5], and regulation of immunity of the body[6]; exogenous L-glutamine can stimulate immunoglobulin secretion and promote immune system reconstruction, such as in burns, AIDS, and arthritis as well as other immune system recovery[7]. The potential value of L-glutamine in cancer treatment lies in reducing to reduce the side effects of chemotherapy and radiotherapy[8]. With in-depth research of the physiological function and application range of L-glutamine, the demand and production of L-glutamine are increasing. In particular, the medicinal demand is very large; so, it has a broad market prospect.

The glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway is an important metabolic pathway between glutamate and glutamine. There are two ways for microorganisms to absorb NH_4^+ [9-10]; (1) the glutamate dehydrogenase (GDH) pathway: α -ketoglutarate and NH_4^+ produce glutamate under the catalysis of GDH, and (2) the GS-GOGAT pathway: glutamate and NH_4^+ produce glutamine under the catalysis of GS, while one molecule of glutamine and one molecule of α -ketoglutaric acid produce two molecules of glutamate under the catalysis of GOGAT. With a sufficient nitrogen source, microorganisms mainly rely on the GDH pathway to absorb NH_4^+ . In the absence of nitrogen sources, the GS-GOGAT pathway is the main pathway for microorganisms to absorb NH_4^+ [11-12]. Martin Teschet *et al.* [10] showed that when the concentration of NH_4^+ in the culture medium increased from 1 to 90 mmol/L, the GDH enzyme activity in wild-type *Corynebacterium glutamicum* showed little change and remained at a high level, while the enzyme activity of GS and GOGAT decreased to less than 10% of the original activity. This shows that the activity of GS, the key enzyme in glutamine fermentation, is regulated by the concentration of NH_4^+ . Ertan *et al.* [9] showed that when the concentration of NH_4^+ in the culture decreased from 100 to 10 mmol/L, the enzyme activity of GS increased by two times. Tesch [13] showed that 10 mmol/L is a critical concentration of NH_4^+ ; if NH_4^+ is lower than 10 mmol/L, then the absorption of NH_4^+ by microorganisms mainly depends on the GS-GOGAT pathway. Schulz *et al.* [14] explored the effects of carbon and nitrogen sources on the activities of GS and GOGAT in *C. glutamicum* ATCC13032. They found that under the conditions of a sufficient carbon source and lack of a nitrogen source, the activity of GS and GOGAT increased by five and seven times, respectively. When both carbon and nitrogen sources were lacking, the activity of GS decreased by three times and that of GOGAT was not affected.

GS in *C. glutamicum* is affected by adenosine acylation[15]. AMP binds to the tyrosine residues on the peptide chain of GS in a covalent manner to produce GS (AMP)[16]. Under the influence of adenosine acylation, the catalytic properties of GS change significantly, and the activity of catalysing glutamate and NH_4^+ to produce glutamine decreases significantly[12]. However, GS in *Bacillus subtilis* and *Lactobacillus acidophilus* is not affected by adenosine acylation[17].

The main problems faced by L-glutamine fermentation include the following: (1) the GS-GOGAT pathway in *C. glutamicum* make glutamate and glutamine mutual transformation, which leads to high content of glutamate as a by-product, increased difficulty of glutamine extraction, and high costs. (2) Glutamine fermentation usually uses a large amount of NH_4^+ , which reduces the enzyme activity of GS by adenosine acylation, and the bacterial growth activity is also affected by high concentrations of NH_4^+ . As a result, the osmotic pressure becomes too high and bacterial growth activity weakens. These problems make it difficult

for the production capacity and sugar-acid conversion rate of L-glutamine to reach the ideal effect.

In this study, to address the main problems of L-glutamine fermentation, the GS-GOGAT pathway of *C. glutamicum* was optimised by knocking out GOGAT genes *NCgl0181* and *NCgl0182* and glutaminase genes *NCgl2395* and *NCgl2500*. A copy of the GS gene *glnA_{bsu}* from *B. subtilis* and a copy of the glutamine synthase gene *glnA_{lcb}* from *L. acidophilus* were integrated at genomic *NCgl0182* and *NCgl2500* sites. Furthermore, the GS genes *glnA_{bsu}* and *glnA_{lcb}* from *B. subtilis* and *L. acidophilus* were overexpressed using pXT01 with the strong promoter *tuf*. Based on the effect of the above genetic modification and the characteristics of the GS-GOGAT pathway, an NH_4^+ -limited fermentation process was designed, which effectively improved the activity of GS and bacterial activity and production capacity of glutamine. A schematic diagram of the GS-GOGAT pathway optimisation is shown in Fig. 1.

Fig. 1 The GS-GOGAT pathway and its optimisation methods

1. Materials and methods

1.1 Materials

1.1.1 Strains and plasmids

The strains and plasmids used in this study are shown in Table 1.

Table 1 strains and plasmids

Strain	Characteristic
GM34	GM34
G-1	GM34[?] <i>NCgl0181</i>
G-2	GM34[?] <i>NCgl2395</i>
G-3	GM34[?] <i>NCgl0181</i> [?] <i>NCgl2395</i>
G-4	GM34 [?] <i>NCgl0181</i> [?] <i>NCgl2395</i> <i>NCgl0182::Ptuf-glnA_{bsu}</i>
G-5	GM34 [?] <i>NCgl0181</i> [?] <i>NCgl2395</i> <i>NCgl0182::Ptuf-glnA_{bsu}</i> <i>NCgl2500::Ptuf-glnA_{lcb}</i>
G-6	GM34 [?] <i>NCgl0181</i> [?] <i>NCgl2395</i> <i>NCgl0182::Ptuf-glnA_{bsu}</i> <i>NCgl2500::Ptuf-glnA_{lcb}</i>
G-7	GM34 [?] <i>NCgl0181</i> [?] <i>NCgl2395</i> <i>NCgl0182::Ptuf-glnA_{bsu}</i> <i>NCgl2500::Ptuf-glnA_{lcb}</i>
Plasmid	Characteristic
pK18 <i>mobsacB</i>	Km ^R , suicide vector
pK18 <i>mobsacB</i> -[?] <i>NCgl0181</i>	Km ^R , carrying homologous arm of [?] <i>NCgl0181</i>
pK18 <i>mobsacB</i> -[?] <i>NCgl2395</i>	Km ^R , carrying homologous arm of [?] <i>NCgl2395</i>
pXT01	Cm ^R , <i>tuf</i> promoter, <i>E. coli</i> <i>C. glutamicum</i> shuttle cloning vector
pXT01- <i>glnA_{bsu}</i>	Cm ^R , carrying <i>glnA_{bsu}</i> fragment
pXT01- <i>glnA_{lcb}</i>	Cm ^R , carrying <i>glnA_{lcb}</i> fragment

pK18*mobsacB*-*NCgl0182*::*PtufglnA_{bsu}* Km^R, carrying *NCgl0182* and *Ptuf-glnA_{bsu}* fragments
pK18*mobsacB*-*NCgl2500*::*PtufglnA_{lcb}* Km^R, carrying *NCgl2500* and *Ptuf-glnA_{lcb}* fragments

1.1.2 Primer

According to the genes published by the KEGG, *NCgl0181*, *NCgl0182*, *NCgl2500*, and *NCgl2395* of *C. glutamicum* ATCC 13032; *bsu-glnA* of *B. subtilis* subsp. *subtilis* 168; and *lcb-glnA* of *Lactobacillus casei* BL23 were studied. PCR amplification primers were designed according to the upstream and downstream nucleotide sequences of the above genes, as shown in Table 2.

Table 2 Primer sequence

Primer name	Primer sequence(5'-3')
N0up-s	ACGACGGCCAGTGCCAAGCTTCTATCTTCACGCACCATCATTTACA
N0up-a	GTGAAGCAATACCACCGAAGTAGTTGATTGAGAAGCCATCACAACCAAAC
N0down-s	GTTTGGTTGTGATGGCTTCTGAATCAACTACTTCGGTGGTATTGCTTCAC
N0down-a	GGTACCCGGGGATCCTCTAGATTTCAGCAGAAATCGAGCCATAAGAC
N2up-s	ACGACGGCCAGTGCCAAGCTT GACATCATAAATGGTGGCTTTTGAG
N2up-a	AGCTGTTTGAATATTTTTACGCCGC GTAGGCAAATGGCTTGGAATACTT
N2down-s	AAGTATTTCCAAGCCATTTGCCTACGCGGCGTAAAAATATTCAAACAGCT
N2down-a	GGTACCCGGGGATCCTCTAGA CTGCAACAATCAAAGGCAACATAAG
<i>bsu-glnA</i> -s	CTGCAGGTCGACTCTAGAGGATCCATGGCAAAGTACACTAGAGAAGATATCG
<i>bsu-glnA</i> -a	CGCCAAAACAGCCAAGCTGAATTCTTAATACTGAGACATATACTGTTTCGCGT
<i>lcb-glnA</i> -s	CTGCAGGTCGACTCTAGAGGATCCATGGGCGATGAAGCCGTCATCGAGC
<i>lcb-glnA</i> -a	CGCCAAAACAGCCAAGCTGAATTCTGCCTTGCATCGCGAATTGTATTAA
N0182up-s	cgagctcggtaccgggggatcc ACCCACAAAGGATTCATCAAATACTC
N0182up-a	CCTTCGGATCTAAACGATCTGTAAAC ATCCAGCGGTTCTCCATTTTGTATT
N0182 <i>tuf</i> -s	AATACAAAATGGAGAACCCTGGATGTAAACAGATCGTTTAGATCCGAAGG
N0182 <i>glnA</i> -a	GTGTTGACAGCAAATTTACGTTCACTTAATACTGAGACATATACTGTTTCGCGT
N0182down-s	ACGCGAACAGTATATGTCTCAGTATTAAGTGAACGTAAATTTGCTGTCAACAC
N0182down-a	TTGTAAAACGACGGCCAGTGCC ACTGCTACAGGGAGTGCAGTTTCAC
N2500 up-s	cgagctcggtaccgggggatcc AAAATCGGTGTCATTACCTTCCCAG
N2500 up-a	CCTTCGGATCTAAACGATCTGTAAACCAGTGGTGTGTTCTCTACAACGAG
N2500 <i>tuf</i> -s	CTCGTTGTAGAGAACAACACCACTGGTTAACAGATCGTTTAGATCCGAAGG
N2500 <i>glnA</i> -a	ATCTGCTGACCCTTTTCCAAAGTGT TTACCAATTCATGTAGCGTTGGC
N2500down-s	GCCAACGCTACATGAATTGGTAA AACTTTGGAAAAGGGTCAGCAGAT
N2500down-a	TTGTAAAACGACGGCCAGTGCC GAACAGCTCCAGGCCATCAATAGAT

1.1.3 Fermentation culture medium of L-glutamine

The seed culture medium was glucose 25 g/L, corn pulp dry powder 15 g/L, soybean meal hydrolysate 15 mL/L, K₂HPO₄·3H₂O, and MgSO₄·7H₂O 1 g/L.

The fermentation culture medium was K₂HPO₄·3H₂O 1.8 g/L, VB1 0.1 mg/L, soybean meal hydrolysate 10 mL/L, corn pulp dry powder 4 g/L, MnSO₄·H₂O 10 mg/L, FeSO₄ 10 mg/L, ZnSO₄ 5 mg/L, and MgSO₄·7H₂O 1 g/L.

1.2 Method

1.2.1 Construction of the *NCgl0181* knockout strain

(1) Construction of the *pK18mobsacB*[?]*NCgl0181* vector

The genome of *C. glutamicum* GM34 was used as the template, and N0up-s, N0up-a, N0down-s, and N0down-a were used as the upstream and downstream homologous arm amplification primers of *NCgl0181* (in which the 5' ends of N0up-s and N0down-a were added into the linear vector homologous sequences of restriction endonuclease XbaI and HindIII, and N0up-an and N0down-s had an overlapping region of 25bp). Upstream and downstream homologous arms were amplified using PCR. Using the amplified upstream and downstream homologous arms as templates and N0up-s and N0down-a as primers, overlapping PCR was carried out to obtain the overlapping fragment *[?]**NCgl0181*.

The linear vector was obtained by double restriction endonuclease digestion at the XbaI and HindIII sites of *pK18mobsacB*. The linear vector and overlapping fragment *[?]**NCgl 0181* were recombined and introduced into *Εσσηερισηηα ζολι ΔΗ5α*-competent cells, and then coated on 0.05 mg/mL kanamycin-resistant plate medium. Single colonies carrying the recombinant plasmids were screened and cultured in BHI liquid medium, and the plasmids were extracted.

(2) *NCgl0181* knockout

The *pK18mobsacB**[?]**NCgl0181* was transformed into GM34-competent cells by electroporation. The bacteria were coated on 0.01 mg/mL kanamycin-resistant plate medium, and the single colonies with single crossover were screened.

A single colony with a single crossover was placed in BHI liquid medium and cultured at 32°C. Cells were cultured for 2, 4, and 6 h, and 50 µL of fermentation broth was coated on the BHI liquid medium plate containing 15% sucrose, and the single colony was screened for double crossover.

1.2.2 Construction of the *NCgl2395* gene knockout strain

The method used was the same as that provided in 1.2.1.

1.2.3 Integrating a copy of *glnA_{bsu}* at the genome *NCgl0182* site

The method used was the same as that provided in 1.2.1.

1.2.4 Integrating a copy of *glnA_{lcb}* at the genome *NCgl2500* site

The method used was the same as that provided in 1.2.1.

1.2.5 Overexpression of the heterologous GS gene by pXT01

1.2.5.1 Overexpression of *glnA_{bsu}* using pXT01

(1) Construction of pXT01-*glnA_{bsu}*

The genome of *B. subtilis* was used as a template, and *bsu-glnA-s* and *bsu-glnA-a* were used as upstream and downstream amplification primers (the 5' ends of upstream and downstream amplification primers were added to the linear vector homologous sequences of restriction endonucleases BamHI and EcoRI). The *glnA_{bsu}* fragment with a length of 1335 bp was amplified by PCR.

The linear vector was obtained by double restriction endonuclease digestion at the BamHI and EcoRI sites of pXT01. The linear vector was recombined with the *glnA_{bsu}* fragment and introduced into *E. ζολι ΔΗ5α*-competent cells coated on 0.0005 mg/mL chloramphenicol-resistant plate medium. Single colonies carrying recombinant plasmids were screened and cultured in BHI liquid medium, and the plasmids were extracted.

Construction of the overexpression *glnA_{bsu}* strain

pXT01-*glnA_{bsu}* was transformed into GM34-competent cells by electroporation, and the bacteria were coated on a 0.0001 mg/mL chloramphenicol-resistant plate medium, and single colonies were screened for the successful introduction of pXT01-*glnA_{bsu}*.

1.2.5.2 Overexpression of *glnA_{lcb}* using pXT01

The method used was the same as that provided in 1.2.5.1.

1.2.6 Strain culture method

The strain was inserted into the bevel culture medium from the bacteria preservation tube of 20% glycerol for activation, and the culture conditions were 32°C for 12 h.

Three 1 L triangular flasks were used as the primary seed shaking flask, and the bacteria on the bevel culture medium were inserted into the primary seed shaking flask. Shaker culture conditions were 32°C, 12 h, pH 7.0, and 220 rpm/min.

Secondary seed culture was carried out in a 5 L fermenter. First, 300 mL of the primary seed liquid was poured into a 5 L fermenter for secondary seed culture. The culture medium volume was 2 L, and the culture conditions were as follows: 34°C, pH 7.0, dissolved oxygen 30–50%, culturing until OD₆₀₀ reached 40.

Fermentation culture was carried out in a 5 L fermenter. The inoculation amount was 20%, the culture medium system was 3 L, and the culture conditions were 34°C, pH 7.0, and dissolved oxygen 30–50%.

1.2.7 NH₄⁺-limited fermentation process

NH₄⁺ is a key factor in glutamine fermentation. NH₄⁺ is needed to catalyse the production of glutamine by GS, but a high concentration of NH₄⁺ affects bacterial growth and reduces the activity of GS. Under a low concentration of NH₄⁺, the activity of GS is high; however, if the concentration of NH₄⁺ is too low, it reduces the production of glutamine. To solve the contradiction of NH₄⁺ concentration during fermentation and improve the activity of GS, an NH₄⁺-limited fermentation process was designed, and two groups of experiments were carried out with the following different (NH₄)₂SO₄ feeding strategies: (1) (NH₄)₂SO₄ was fed uniformly 0, 2, and 4 h after fermentation. (2) The following three (NH₄)₂SO₄ feeding strategies were used: uniformly fed, intermittently fed, and gradient-fed. The glutamine fermentation yield, glutamate content, NH₄⁺ concentration, and GS activity obtained using the above fermentation strategies were analysed, and the most suitable NH₄⁺-limited fermentation process was obtained.

1.2.8 Calculation of the sugar-acid conversion rate

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where SA is the sugar-acid conversion rate (%), ρ is the mass concentration of L-glutamine (g/L), V is the total volume of fermentation broth (L), and m is the total sugar consumption quality.

1.2.9 Determination of glutamine production by high performance liquid chromatography

Pre-treatment of the fermentation broth: The fermentation broth was centrifuged at 13,000 r/min for 2 min, and the supernatant was obtained.

Derivative method: 200 μ L of derivative buffer, 10 μ L of sample, and 300 μ L of derivative agent were added to the ep tube, mixed well, and bathed at 65°C for 1 h, and then the temperature was reduced to 25°C. Next, 690 μ L of constant volume buffer was added, and the solution was mixed well.

The Agilent C18 (15 $\times$ 4.6 mm, 3.5 μ m) chromatographic column was used with a mobile phase of 4.1 g/L sodium acetate solution and 50% acetonitrile. Other conditions were a derivative agent 2,4-dinitrofluorobenzene, a column temperature of 33°C, a flow rate of 1 mL/min, and a detection wavelength of 360 nm.

1.2.10 Determination of the NH₄⁺ concentration in fermentation broth

The method used was the same as that provided in 1.2.9.

1.2.11 Determination of GS activity

GS activity was determined using a GS kit (Suzhou Keming Biotechnology Co., Ltd.). In the presence of ATP and Mg^{2+} , GS catalyses NH_4^+ and glutamate to synthesise glutamine, which is further converted to γ -glutamyl hydroxamic acid, and the complex formed with iron under acidic conditions has a maximum absorption peak at 540 nm, which was determined by a multifunctional enzyme labelling instrument (TECAN, Infinite 200PRO). The unit of enzyme activity was defined as 1 μ mol γ -glutamyl hydroxamic acid produced per hour in every mL reaction system per g of tissue.

2 Results and discussion

2.1 Effect of optimising the GS-GOGAT pathway on glutamine fermentation

2.1.1 Effect of knocking out *NCgl0181* and *NCgl2395* on glutamine fermentation

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Fig. 2 Effects of knocking out *NCgl* 0181 and *NCgl* 2395 genes on bacterial growth (OD_{600})

As shown in Fig. 2, the growth of the original strains GM34, G-1, and G-2 was roughly the same. Due to the use of the biotin sub-appropriate process, the biotin content was limited, and the logarithmic growth phase of the four strains ended in approximately 14 h, and the highest OD_{600} was approximately 102. In the later stage of fermentation, the OD_{600} decreased slightly due to biotin deficiency and bacterial ageing. Under the same culture medium and method, the bacterial growth was basically the same, which indicated that *NCgl0181* and *NCgl2395* were not necessary for the growth and metabolism of GM34, and that their knockout had no adverse effect on bacterial growth.

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Fig. 3 Effect of knocking out the *NCgl* 0181 and *NCgl* 2395 genes on glutamine and glutamate production

As shown in Fig. 3, the glutamine yield of GM34 was 48.2 g/L, and the by-product glutamate was as high as 30.4 g/L. The yield of glutamine and glutamate increased and decreased alternately after 20 h of fermentation, which was due to the mutual transformation of glutamine and glutamate through the GS-GOGAT pathway. After glutamate is converted to glutamine, glutamine is converted to glutamate through GOGAT and glutaminase. As a result, glutamate cannot be completely converted to glutamine, resulting in low glutamine yield and high by-product glutamate content. To solve this problem, it is necessary to knock out the GOGAT genes *NCgl0181* and *NCgl0182* and the glutaminase genes *NCgl2395* and *NCgl2500*.

The glutamine production of G-1 and G-2 reached 51.5 and 53.8 g/L, which was 6.8% and 11.6% higher than that of GM34, and the glutamate content was 27.3 and 24.0 g/L, which was 10.2% and 21.1% lower than that of GM34. The alternating increase and decrease of glutamine and glutamate yield still existed, but it was weakened; the yield of glutamine increased slightly and the content of the by-product glutamate decreased slightly, suggesting that knockout of *NCgl0181* or *NCgl2395* alone weakens the metabolic pathway for the conversion of glutamine to glutamate. Furthermore, *NCgl0181* and *NCgl2395* were knocked out simultaneously, and the strain G-3 was constructed. The glutamine yield of G-3 reached 68.2 g/L, which was 41.5% higher than that of GM34; and the content of glutamate was 19.3 g/L, which was 36.5% lower than that of GM34. The alternating increase and decrease of glutamine and glutamate yield were not obvious. This shows that when *NCgl0181* and *NCgl2395* are knocked out at the same time, the resulting glutamine is hardly converted to glutamate, and more glutamate is converted to glutamine. However, the content of glutamate in the fermentation broth remained very high, which is speculated to be due to the low activity

of GS, and the glutamate produced in the cells cannot be converted into glutamine in time. To further increase the yield of glutamine and reduce the content of glutamate, it is necessary to further optimise the GS-GOGAT pathway and enhance the activity of GS.

2.1.2 Effect of the overexpression of the heterologous GS gene on glutamine fermentation

GS in *C. glutamicum* is affected by adenosine acetylation. The principle of action is that AMP combines covalently with tyrosine residues on the peptide chain to form GS (AMP), and the adenylation site is Tyr405. Once adenosine acylation occurs, the activity of GS catalysing the conversion of glutamate to glutamine changes. There is no adenylation site in the GS of *B. subtilis* and *L. acidophilus*, thus, it is not affected by adenosine acylation.

In *C. glutamicum* GM34, the GOGAT gene has *NCgl0182* in addition to *NCgl0181*, and the glutaminase gene has *NCgl2500* in addition to *NCgl2395*. In this study, a copy of the GS gene *glnA_{bsu}* from *B. subtilis* and a copy of *glnA_{lcb}* from *L. acidophilus* were integrated into the genomic *NCgl0182* and *NCgl2500* sites of strain G-3, respectively, and the strong promoter *tuf* was used to express *glnA_{bsu}* and *glnA_{lcb}*. Strains G-4 and G-5 were constructed. Furthermore, pXT01 with the strong promoter *tuf* was used to overexpress *glnA_{bsu}* and *glnA_{lcb}*, respectively, and the strains G-6 and G-7 were constructed. The effects of *glnA* from these two sources on glutamine production and GS activity were analysed.

2.1.2.1 Effect of integrating a copy of *glnA_{bsu}* and *glnA_{lcb}* on glutamine fermentation

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Fig. 4 Effect of integrating a copy of *glnA_{bsu}* and *glnA_{lcb}* on bacteria growth (OD₆₀₀)

As shown in Fig. 4, there was no significant difference in OD₆₀₀ between G-3, G-4, and G-5, indicating that the integration of *glnA_{bsu}* and *glnA_{lcb}* on the genome had no adverse effect on bacterial growth.

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Fig. 5 Effect of integrating a copy of *glnA_{bsu}* and *glnA_{lcb}* on glutamine and glutamate production

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Fig. 6 Effect of integrating a copy of *glnA_{bsu}* and *glnA_{lcb}* on glutamine synthetase (GS) activity

As shown in Fig. 5 and 6, the glutamine production of G-4 and G-5 reached 71.6 and 74.8 g/L, which was 5.0% and 9.7% higher than that of G-3; the content of glutamate was 15.7 and 13.5 g/L, which was 18.7% and 30.1% lower than that of G-3; the GS activity of G-4 and G-5 was significantly higher than that of G-3, and the GS activity of G-5 was slightly higher than that of G-4. This proved that *glnA_{bsu}* and *glnA_{lcb}* can be expressed in *C. glutamicum* and play a positive role in the production of glutamine. The increase in GS activity results in more conversion of glutamate to glutamine and reduces the production of glutamate by-products. The alternating increase and decrease of glutamine and glutamate production does not exist because *NCgl0181*, *NCgl0182*, *NCgl2395*, and *NCgl2500* genes are knocked out, and the glutamine produced in the cells is no longer converted to glutamate. The GS activity of G-3 and G-5 was relatively stable in the early stages and increased slightly in the later stages of the fermentation process. It

is speculated that this is because, with the progression of the fermentation process, NH_4^+ is consumed and the NH_4^+ concentration in the later stage of fermentation will be greatly reduced, so that the GS activity will increase to some extent.

2.1.2.2 Effect of the overexpression of the heterologous GS gene by pXT01 on glutamine fermentation

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Fig. 7 Effect of the overexpression of the heterologous GS gene by pXT01 on bacteria growth (OD_{600})

As shown in Fig. 7, the bacterial growth of G-5, G-6, and G-7 was roughly the same, and the OD_{600} of G-5 was slightly higher than that of G-6 and G-7 in the later stage of fermentation. This shows that the overexpression of these two heterologous GS genes in *C. glutamicum* did not reduce bacterial activity.

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Fig. 8 Effect of the overexpression of the heterologous GS gene by pXT01 on the production of glutamine and glutamate

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Fig. 9 Effect of the overexpression of the heterologous GS gene by pXT01 on glutamine synthetase activity

As shown in Fig. 8 and 9, the glutamine production of G-6 and G-7 reached 84.3 and 80.8 g/L, which was 12.7% and 8.0% higher than that of G-5, and the content of glutamate was 9.9 and 10.6 g/L, which was 26.7% and 21.5% lower than that of G-5. The GS activity of G-5, G-6, and G-7 fluctuated in the middle and early stages of fermentation and increased slightly in the later stages. Among them, the GS activity of G-6 and G-7 was significantly higher than that of G-5, and G-6 was the highest of the three. The glutamine yield of G-6 was the highest and the glutamate content was the lowest. This indicated that GS activity significantly affected glutamine fermentation. The higher the GS activity, the more glutamate was converted to glutamine; thus, the higher the glutamine yield, the less glutamate produced. Among them, the GS gene *glnA_{bsu}* from *B. subtilis* had higher enzyme activity in *C. glutamicum*, and the production performance of G-6 was better than that of G-7. The following fermentation experiments were carried out using G-6.

2.2 Effect of the NH_4^+ -limited fermentation process on glutamine fermentation

2.2.1 Effect of the feeding start time of $(\text{NH}_4)_2\text{SO}_4$ on glutamine fermentation

Through the above genetic modification to optimise the GS-GOGAT pathway, the yield of glutamine was greatly increased, and the content of glutamate was greatly decreased. Although the heterologous GS gene is not affected by adenosine acylation, the enzyme activity is inhibited by high concentrations of NH_4^+ , thus, it cannot fully play its catalytic role. To further increase the yield of glutamine, it is necessary to optimise the glutamine fermentation process, explore the effect of NH_4^+ concentration on glutamine fermentation, and regulate the NH_4^+ concentration. In common glutamine fermentation, the addition of a large amount of NH_4^+ inhibited not only the bacterial activity, but also the activity of GS. In this study, an NH_4^+ -limited fermentation process was designed in which 350 g of $(\text{NH}_4)_2\text{SO}_4$ (dissolved in 700 mL of water) was fed

uniformly at the beginning of 0, 2, and 4 h of fermentation until the end of fermentation, and the differences among the three were compared.

Because the side chain of glutamine contains an amide group, the amide group is not protonated within the physiological pH range, so the side chain of glutamine is not charged. Glutamate contains two carboxyl groups, and the carboxyl group on the side chain also dissociates completely at around pH 7, so the molecule of glutamic acid is negatively charged. The pH of the fermentation broth was adjusted with 10% NaOH. When measuring the concentration of NH_4^+ in fermentation broth, the measured value is the concentration of NH_4^+ ionised by $(\text{NH}_4)_2\text{SO}_4$.

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Fig. 10 Effect of fermentation at 0, 2, and 4 h, beginning with uniform flow and disposable addition of $(\text{NH}_4)_2\text{SO}_4$ on bacterial growth (OD_{600})

As shown in Fig. 9, the number of bacteria in the three uniform feeding strategies was higher than that when 350 g of $(\text{NH}_4)_2\text{SO}_4$ was added to the medium at one time. Among them, the number of bacteria was the highest when $(\text{NH}_4)_2\text{SO}_4$ was added at 4 h, and the highest value of OD_{600} reached 121.8, which was 24.8% higher than that for the one-time addition of culture medium $(\text{NH}_4)_2\text{SO}_4$.

The number of bacteria reflects the growth activity of the bacteria. In the logarithmic growth phase of fermentation, it can be seen that the accumulation of bacteria is faster and the bacterial activity is better under the strategy of uniform feeding $(\text{NH}_4)_2\text{SO}_4$. This is because adding a large amount of $(\text{NH}_4)_2\text{SO}_4$ at one time makes the concentration of NH_4^+ in the fermentation broth very high and has an inhibitory effect on bacteria growth, while the uniform addition of $(\text{NH}_4)_2\text{SO}_4$ keeps the concentration of NH_4^+ in the fermentation process at a low level. In particular, the logarithmic growth phase is an important stage of bacteria growth, and the accumulation of more bacteria in the logarithmic growth phase will lead to higher acid production efficiency in the acid production period.

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Fig. 11 Effect of fermentation at 0, 2, and 4 h, beginning with uniform flow and disposable addition of $(\text{NH}_4)_2\text{SO}_4$ on glutamine yield and glutamate content

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Fig. 12 Effects of fermentation at 0, 2, and 4 h, beginning with uniform flow and disposable addition of $(\text{NH}_4)_2\text{SO}_4$ on NH_4^+ concentration and glutamine synthase activity

Combined with Fig. 11 and 12, it can be seen that the glutamine yield was the highest and the glutamate content was the lowest when $(\text{NH}_4)_2\text{SO}_4$ was fed at 0 h of fermentation. The yield of glutamine reached 91.4 g/L and the content of glutamate reached 7.3 g/L. The time of glutamine production was earlier than that of the other three strategies, reaching 1.8 g/L at 4 h, and almost no yield at 4 h for the other three strategies. The NH_4^+ concentrations of the three uniform feeding strategies were almost the same at 0 h and different at 4 h, and the NH_4^+ concentrations were 7.8, 6.4, and 5.1 mmol/L, respectively. This showed

that the concentration of NH_4^+ in the early stage of fermentation affects the formation time of glutamine, and too low a concentration of NH_4^+ will delay the formation time of glutamine.

The GS activity of the three strategies of uniform feeding $(\text{NH}_4)_2\text{SO}_4$ was much higher than that of adding $(\text{NH}_4)_2\text{SO}_4$ at one time. When 350 g of $(\text{NH}_4)_2\text{SO}_4$ was added to the medium at one time, the NH_4^+ concentration reached 5453 mmol/L at 0 h of fermentation. The NH_4^+ concentration of the uniform feed $(\text{NH}_4)_2\text{SO}_4$ strategy was not higher than 20 mmol/L throughout the fermentation process, indicating that a high concentration of NH_4^+ in the fermentation broth inhibits GS activity. The GS activity in the whole fermentation process was at a low level, thus, affecting glutamine synthesis.

The NH_4^+ concentration of the three uniform feed $(\text{NH}_4)_2\text{SO}_4$ strategies was near 10 mmol/L or lower than 10 mmol/L 20 h before fermentation, and their GS activity was at a high level, with almost no difference and only a small fluctuation. After 24 h of fermentation, the concentration of NH_4^+ began to increase and the activity of GS began to decrease. At the end of 36 h of fermentation, the concentration of NH_4^+ reached more than 16 mmol/L, and GS activity was the lowest in the fermentation process.

There are two reasons for the decrease in GS activity in the middle and later stages of fermentation. First, with the ageing of bacteria, the rate of NH_4^+ consumption slows, and the concentration of NH_4^+ increases gradually, which inhibits the activity of GS. Second, the bacteria ageing in the middle and later stages of fermentation will decrease the expression ability of a variety of enzymes, and the activity of GS will also be affected. It can be concluded that controlling the concentration of NH_4^+ in the fermentation broth is the key to glutamine fermentation.

To sum up, the best effect was found by evenly feeding $(\text{NH}_4)_2\text{SO}_4$ at 0 h of fermentation, which was used in the following experiments.

2.2.2 Effects of intermittent and gradient feed $(\text{NH}_4)_2\text{SO}_4$ strategies on glutamine fermentation

It can be seen from Fig. 11 and 12 that the activity of GS is at a high level when the concentration of NH_4^+ is less than 10 mmol/L in the early stage of fermentation at 0–4 h, but too little NH_4^+ is not conducive to the conversion of glutamate to glutamine, and even delays the glutamine production time. At about 12–20 h after fermentation, when the NH_4^+ concentration was around 10 mmol/L, GS activity reached the highest, and the glutamine acid production rate also reached the peak value. Although the strategy of uniform feeding of $(\text{NH}_4)_2\text{SO}_4$ greatly improved the GS activity, the NH_4^+ concentration increased gradually in the middle and late stage of fermentation, and the NH_4^+ concentration increased from around 10 to over 16 mmol/L, while the GS activity decreased gradually. In view of the shortcomings of uniform feeding of $(\text{NH}_4)_2\text{SO}_4$, this study also designed two strategies: intermittent feeding and gradient feeding of $(\text{NH}_4)_2\text{SO}_4$, both of which fed 350 g of $(\text{NH}_4)_2\text{SO}_4$ (dissolved in 700 mL of water) at the beginning of fermentation at 0 h.

The intermittent feeding method: 0–4 h uniform feed 140 mL $(\text{NH}_4)_2\text{SO}_4$, 4–8 h stop feeding, and so on until the end of fermentation. The gradient feeding method: 0–12 h uniform feed 200 mL $(\text{NH}_4)_2\text{SO}_4$, 12–24 h uniform feed 350 mL $(\text{NH}_4)_2\text{SO}_4$, and 24–36 h uniform feed 150 mL $(\text{NH}_4)_2\text{SO}_4$.

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Fig. 13 Effects of three $(\text{NH}_4)_2\text{SO}_4$ flow addition methods on bacterial growth (OD_{600})

As shown in Fig. 13, the growth of bacteria in the three $(\text{NH}_4)_2\text{SO}_4$ feeding modes was roughly the same, which indicates that the $(\text{NH}_4)_2\text{SO}_4$ feeding mode had no influence on the growth of bacteria.

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Fig. 14 Effects of three $(\text{NH}_4)_2\text{SO}_4$ flow addition methods on glutamine yield and glutamate content

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Fig. 15 Effect of three $(\text{NH}_4)_2\text{SO}_4$ flow methods on NH_4^+ concentration and activity of glutamine synthase in fermentation fluid

As shown in Fig. 14, the glutamine yields of intermittent and gradient feeding strategies reached 95.6 and 98.7 g/L, which were 4.6% and 8.0% higher than those of uniform feeding. Glutamate content is 6.7 and 4.5 g/L, which is 8.2% and 38.4% lower than that of uniform feeding. As shown in Fig. 15, the NH_4^+ concentration in the fermentation broth of the intermittent feeding strategy fluctuates greatly, ranging from 10 to 14 mmol/L, and the GS activity in the early stage of fermentation is not much different from that of the uniform feeding strategy. After 36 h of fermentation, the final concentration of NH_4^+ reached 13.7 mmol/L, which was lower than 16.2 mmol/L of the uniform feeding strategy, and the GS activity was 37.4% higher than that of the uniform feeding strategy. The NH_4^+ concentration in the fermentation broth was stable at about 10 mmol/L from 4 h to the end of fermentation, with little fluctuation. After 36 h of fermentation, only the final concentration of NH_4^+ in the gradient feeding strategy was approximately 10 mmol/L. The GS activity was at a high level throughout the process, which was higher than 4000 $\mu\text{mol/h/g}$ fresh weight, and decreased slightly in the later period, but was always higher than the uniform and intermittent feeding strategies in the middle and later period of fermentation, especially in the later period. According to the characteristics of acid production and NH_4^+ consumption rates of bacteria in glutamine fermentation, gradient feeding of $(\text{NH}_4)_2\text{SO}_4$ into culture medium can maintain the NH_4^+ concentration in the fermentation broth at about 10 mmol/L, and GS activity is at a high level, which is superior to the other two feeding methods and is most suitable for glutamine fermentation. To sum up, the NH_4^+ -limited fermentation process adopts the strategy of gradient feeding of 350 g of $(\text{NH}_4)_2\text{SO}_4$.

2.3 Effects of optimising the GS-GOGAT pathway and NH_4^+ -limited fermentation process on the sugar-acid conversion rate

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Fig. 16 Comparison of the sugar-acid conversion rate of glutamine fermentation in this study

Note: 0, 2, and 4 h on the x-axis represent uniform feeding $(\text{NH}_4)_2\text{SO}_4$ at 0, 2, and 4 h after the beginning of fermentation.

As shown in Fig. 16, the sugar-acid conversion rate of GM34 was the lowest (32.5%). Because it produces a large amount of the by-product glutamate, the conversion efficiency of glucose to glutamine was very low. By optimising the GS-GOGAT pathway, the glutamate content was greatly reduced, and the sugar-acid conversion rates of G-6 and G-7 reached 37.1% and 37.3% respectively. Using $(\text{NH}_4)_2\text{SO}_4$ uniform, intermittent, and gradient feeding strategies, the content of glutamate was further decreased, the yield of glutamine was further increased, and the sugar-acid conversion rate reached 40.8%, 40.5%, and 41.2%. Among them, the $(\text{NH}_4)_2\text{SO}_4$ gradient feeding strategy with higher glutamine yield and lowest glutamate content had the highest sugar-acid conversion rate, which was 26.7% higher than that of the original strain GM34.

3 Conclusion

To increase the glutamine yield and reduce glutamate content, the GS-GOGAT pathway was optimised by knocking out GOGAT genes *NCgl0181* and *NCgl0182* and glutaminase genes *NCgl2395* and *NCgl2500*, and overexpressing glutamine synthase genes *glnA_{bsu}* and *glnA_{lcb}* from *B. subtilis* and *L. acidophilus*. The strain G-6 with the strongest glutamine production capacity was obtained, and the NH_4^+ -limited fermentation process was designed. As a result, the best feeding strategy of gradient feed $(\text{NH}_4)_2\text{SO}_4$ was obtained. The enzyme activity of GS was improved by controlling the concentration of NH_4^+ in the fermentation broth. The yield of glutamine was increased from 48.2 to 98.7 g/L, the content of glutamate was reduced from 30.4 to 4.5 g/L, the activity of GS was greatly increased, and the sugar-acid conversion rate was increased from 32.5% to 41.2%. Wu Sifang of the Hubei University of Technology screened L-glutamine high-yield strain SH44 from the original strain *C. glutamicum* S9114 by using the methods of γ -ray-diethyl sulfate- γ -ray compound mutagenesis and sulpha-resistant directional breeding, and the yield of L-glutamine reached 55.3 g/L. Wu Sifan *et al.* optimised the type and concentration of nitrogen sources, metal ions, and CaCO_3 of the culture medium, so that the average L-glutamine production of the strain SH77 reached 53.0 g/L and the highest was 56.2 g/L, which is the highest yield of L-glutamine reported to date. In this study, compared with mutagenesis and resistance-oriented breeding, the method of genetic modification was more purposeful and can improve the production capacity of the strain more obviously. Compared with the traditional fermentation medium optimisation, the NH_4^+ -limited fermentation process not only combines the characteristics of the optimised GS-GOGAT pathway, but also detects the GS activity and NH_4^+ concentration in real-time. These two factors are the key parameters of L-glutamine fermentation. The relationship between these factors and their influence on glutamine production were analysed, the best $(\text{NH}_4)_2\text{SO}_4$ feeding strategy was designed, and the NH_4^+ -limited fermentation process was obtained, which provides a new idea for L-glutamine industrialisation.

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