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Efficient one-step preparation of γ-aminobutyric acid from glucose without an exogenous cofactor by the designed *Corynebacterium glutamicum*†

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Lactobacillus plantarum CCTCC M209102 efficiently produces γ-aminobutyric acid (GABA) from L-glutamate, in which glutamate decarboxylase and pyridoxal kinase are involved in the transformation. Pyridoxal kinase catalyzes ATP-dependent phosphorylation of pyridoxal to produce pyridoxal-5′-phosphate, which is the cofactor required for glutamate decarboxylase to biotransform GABA from L-glutamate. *Corynebacterium glutamicum* G01 is a good producer of L-glutamate from glucose. However, it cannot yield GABA from L-glutamate due to the absence of glutamate decarboxylase and pyridoxal kinase. In this work, to realize the efficient one-step preparation of GABA from glucose without exogenous pyridoxal-5′-phosphate, the metabolic module from L-glutamate to GABA based on glutamate decarboxylase and pyridoxal kinase in *L. plantarum* was grafted into *C. glutamicum*. To further improve the GABA production, the pathways to by-product pools of L-arginine, L-proline and L-lysine were blocked using the insertional mutation technique. The engineered *C. glutamicum* APLGGP carrying *arg*B::*tacgad*, *pro*B::*tacgad* and *dapA*::*tacplk* could efficiently convert glucose into GABA in one-step without an exogenous co-factor. In fed-batch cultures, the recombinant *C. glutamicum* APLGGP produced 70.6 g L^{-1} GABA at 30 °C and 70 h through a two-stage pH control strategy. To our knowledge, this is the highest reported GABA production using glucose as a substrate, and this designed *C. glutamicum* should be an excellent candidate for producing GABA on an industrial scale. This work is expected to pave the way to redesign the bioreactor for efficient one-step biosynthesis of GABA from glucose without an exogenous co-factor.

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Introduction

Gamma-aminobutyric acid (GABA), a non-protein amino acid, functions as a major inhibitory neurotransmitter in animals, and it has many physiological properties related to anti-anxiety and hypotension as tranquilizers, diuretics, and analgesics.[1] GABA can be used as a bioactive component in the food, feed, and pharmaceutical fields. Although there have been many attempts for the GABA synthesis chemically or biologically, recent special attention has been focused on GABA preparation by employing "green" and efficient bioprocesses since they have a simple reaction procedure, high catalytic efficiency, mild reaction conditions and environmental compatibility.[2]

In eukaryotes and prokaryotes, including bacteria, fungi and yeasts, glutamate decarboxylase (GAD, EC 4.1.1.15) catalyzes the conversion of L-glutamate to GABA.[3] However, GAD is a pyridoxal-5′-phosphate-dependent enzyme that catalyzes irreversible α-decarboxylation of L-glutamate to GABA with pyridoxal-5′-phosphate as a cofactor.[4] Very recently, Rowley and Whisstock's group have done excellent work on GAD structure determination and kinetic studies for regulation of GABA production.[5]

Using lactic acid bacteria harboring GAD to produce GABA-enriched food has attracted great attention since they are safe and recognized as probiotics.[6] During the GABA production by lactic acid bacteria, glutamate is added as a precursor to the fermentation medium, and some expensive nitrogen sources are supplemented for microorganism cultivation.[7,8] The fermentation method is not cost-effective, so, a new approach is required for the sustainable industrial application.[9] Kook *et al.* reported the maximum GABA production of 27.3 g L^{-1} by

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expressing *Lactobacillus plantarum* GAD gene in *Lactobacillus Sakei*.[10] *Corynebacterium glutamicum* is an important industrial producer of various amino acids with great potential. Takahashi *et al.* successfully produced GABA from glucose in one step using the recombinant *C. glutamicum* expressing *Escherichia coli* GadB.[11] As is known, the enzyme GAD is the key enzyme for the reaction from L-glutamate to GABA, the introduction of one extra copy will promote the GABA production improvement. *C. glutamicum* is an important industrial producer of various amino acids with great potential for the production of other metabolites. Shi *et al.* improved GABA production using the recombinant *C. glutamicum* coexpressing two GAD genes *gadB*1 and *gadB*2 from *Lactobacillus brevis* Lb85.[13] Through the fermentation condition optimization, they further increased GABA production to a maximum level of 27.13 g L^{-1} after 120 h flask cultivation and 26.32 g L^{-1} after 60 h fed-batch fermentation.[13] However, the expensive pyridoxal 5′-phosphate is required for GABA production. The reported methods and productivity of GABA are summarized in Table 1.

The enzyme GAD is active to produce GABA with pyridoxal 5′-phosphate as a cofactor. Pyridoxal kinase (PLK) was reported to catalyze the ATP-dependent phosphorylation reaction of pyridoxal to produce pyridoxal 5′-phosphate.[4] Although the fermentation medium, such as corn steep liquor and yeast extract, contains pyridoxal, the precursor of pyridoxal 5′-phosphate, it is not enough to support the quick biotransformation of GABA. So the introduction of the enzyme PLK involved in the formation of pyridoxal 5′-phosphate will be beneficial to the GABA biosynthesis.

In our previous work, we reported that a modified strain through multi-mutagenesis, *L. plantarum* GB 01-21, showed high GAD activity, and the engineered *E. coli* BL21 harboring *L. plantarum* GAD gene (*gad*, accession no: JN248358) yielded 204.5 g L^{-1} GABA from L-glutamate at 24 h. The mole conversion rate of the substrate reached 97.9%.[14] By analysing the metabolic pathways, L-arginine and L-proline are the main by-products obtained during the biotransformation of GABA from L-glutamate in *L. plantarum* GB 01-21 (Fig. 1). We also isolated

Table 1 The reported methods and productivity of GABA

Strains	Engineered methods	Substrates	Fermentation durations (h)	GABA (g L^{-1})	Ref.
Lactobacillus brevis NCL912	Wild-type	Sodium L-glutamate	48	35.66	**7**
L. brevis TCCC13007	Wild-type	Monosodium glutamate	66	38	**6**
Corynebacterium glutamicum	Expressing GAD gene	L-glutamate	60	26.32	**13**
Lactobacillus Sakei	Expressing GAD gene	L-glutamate	48	27.3	**10**
C. glutamicum	Expressing GAD gene	Glucose	72	12.37	**11**
C. glutamicum ATCC 13032	Expressing GAD gene	Glucose	72	2.15	**12**
Escherichia coli	Expressing GAD gene	L-glutamate	24	204.5	**14**

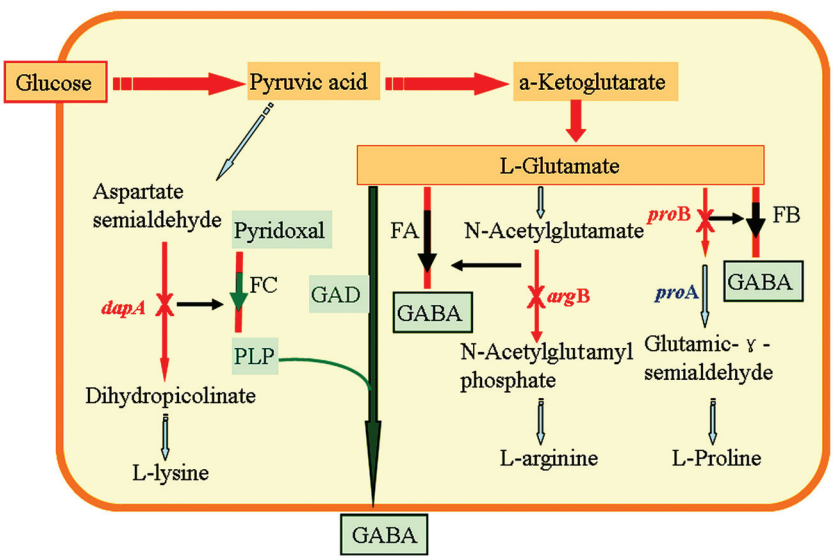

Fig. 1 The strategy for designed *C. glutamicum* to produce GABA from glucose in one-step. *Lactobacillus plantarum* CCTCC M209102 is a good producer of γ-aminobutyric acid (GABA) from L-glutamate. The glutamate decarboxylase and pyridoxal kinase are involved in the above biotransformation. Glutamate decarboxylase biotransforms L-glutamate to GABA with pyridoxal-5′-phosphate as a cofactor. Pyridoxal catalyzes ATP-dependent phosphorylation of pyridoxal to generate pyridoxal-5′-phosphate, which is supplied for the reaction by glutamate decarboxylase. *Corynebacterium glutamicum* G01 is an excellent producer of L-glutamate from glucose. However, it cannot yield GABA from L-glutamate due to the absence of glutamate decarboxylase and pyridoxal kinase. To realize the efficient one-step production of GABA from glucose, the *C. glutamicum* reactor is redesigned. The metabolic module from L-glutamate to GABA in *L. plantarum* was grafted into *C. glutamicum*, making *C. glutamicum* prepare GABA from glucose in one-step. FA, fragment of *argB::tacgad*; FB, fragment of *proB::tacgad*; FC, fragment of *plk::tacdap*A.

a GRAS (generally regarded as safe) strain, *C. glutamicum* G01, which could accumulate about 106 g L^{-1} L-glutamate from glucose.[13] L-Lysine is the main by-product obtained during the biosynthesis of L-glutamate from glucose in *C. glutamicum* G01 (Fig. 1).

In this work, to realize the efficient production of GABA from glucose in one-step without exogenous pyridoxal-5′-phosphate, we grafted the metabolic modular from L-glutamate to GABA based on GAD and PLK in *L. plantarum* GB 01-21 to *C. glutamicum* G01. To further improve the GABA production, we not only knocked out the genes involved in the by-products pathways of L-arginine, L-proline and L-lysine through insertional inactivation, but also introduced two copies of GAD genes into the engineered *C. glutamicum*. Finally, GABA production reached 70.6 g L^{-1} after 70 h of fermentation under a two-stage pH control strategy.

Results and discussion

Construction of the engineered *C. glutamicum*

Lactobacillus plantarum GB 01-21 catalyzes the biotransformation of γ-aminobutyric acid (GABA) with an excellent performance. The glutamate decarboxylase (GAD) and pyridoxal kinase (PLK) are the key enzymes involved in the conversion of L-glutamate to GABA. The enzyme GAD catalyzes L-glutamate to GABA with pyridoxal-5′-phosphate as a cofactor. Pyridoxal kinase catalyzes ATP-dependent phosphorylation of pyridoxal to produce pyridoxal-5′-phosphate, which is required by GAD.[4] Although *Corynebacterium glutamicum* G01 is an excellent producer of L-glutamate from glucose, it cannot yield GABA due to the absence of GAD and PLK. In order to produce GABA from glucose in one-step by *C. glutamicum*, the coding genes of GAD and PLK from *L. plantarum* were cloned into *C. glutamicum*. The GAD gene (*gad*) from the *L. plantarum* was inserted into the plasmid pDXW-10 to generate pDXW-*gad*. In the same way, the PLK gene (*plk*) from *L. plantarum* was cloned into the plasmid pDXW-10 to create pDXW-*plk*. The recombinant plasmids were verified by DNA sequencing.

Since L-arginine and L-proline are the main by-products during the biotransformation of GABA from L-glutamate by *C. glutamicum* G01, the pathway to by-product pools need to be blocked to increase the carbon flux from glucose to L-glutamate. Lazcano's group has reported that *N*-acetyl-L-glutamate kinase (NAGK) is involved in the arginine biosynthesis pathway, and the three enzymes, glutamate kinase (GAK), glutamate-γ-semialdehyde dehydrogenase and pyrroline-5-carboxylate reductase catalyzes the proline synthesis in bacteria.[15] L-Lysine is the other by-product during the production of L-glutamate from glucose in *C. glutamicum* G01. Dihydropicolinate synthase (DHDPS) is a key enzyme for lysine biosynthesis.[15] As described in the Experimental section, the recombination plasmids pMD-R*arg*B, pMD-*R*proB and pMD-R*dap*A containing flanks of *argB* (R*argB*), *proB* (R*proB*) and dapA (R*dapA*) were constructed.[16] So, by the homologous recombination technique, the coding genes of NAGK, GAK and DHDPS (*argB*, *proB* and *dap*A) were expected to be knocked out.

The *gad* gene with the *tac* promoter from the plasmid pDXW-*gad* was inserted into pMD-R*argB* and pMD-R*proB* to create the recombinant pMD-R*arg*B::tacgad and pMD-R*pro*B::tacgad, respectively. The gene *plk* with the *tac* promoter from pDXW-*plk* was cloned into the pMD-R*dap*A to create the plasmid pMD-*dap*A::tacplk. Then the R*arg*B::tacgad, R*pro*B::tacgad and dapA::tacplk fragments were inserted into the suicide plasmid pK18*mobsac*B, respectively, to construct the recombinant plasmids pK18-R*arg*B::tacgad, pK18-R*pro*B::tacgad and pK18-*dap*A::tacplk. After verification by restriction enzyme analysis and DNA sequencing, the recombinant plasmids pK18-R*arg*B::tacgad, pK18-R*pro*B::tacgad and pK18-*dap*A::tacplk were introduced into *C. glutamicum* G01 one by one using the electroporation method.[17] The recombinant *C. glutamicum* AG carrying pK18-R*arg*B::tacgad, *C. glutamicum* APGG carrying pK18-R*arg*B::tacgad and pK18-R*proB*::tacgad, *C. glutamicum* APLGGP carrying pK18-R*arg*B::tacgad, pK18-R*proB*::tacgad and pK18-*dap*A::tacplk were constructed. In *C. glutamicum* AG, the *gad* gene was introduced and the gene *argB* was deleted. In *C. glutamicum* APGG, the *gad* gene with two copies was introduced and the *argB* and *proB* genes were knocked out. In *C. glutamicum* APLGGP, the targets genes *gad* and *plk* involved in the biotransformation of GABA from L-glutamate were introduced, and the genes *argB*, *proB* and *dapA* involved in the by-product pathways of L-arg, L-pro and L-lys were knocked out. More important, glutamate decarboxylase gene was integrated into *C. glutamicum* APLGGP genome with two copies.

Identification of correct insertional mutation by enzyme assays

C. glutamicum is an important industrial producer of various amino acids with great potential for the production of other metabolites. In order to identify whether the insertion mutation was correctly integrated into the *C. glutamicum* genome, the specific activities of enzymes were determined using cell-free extracts of the recombinant strain *C. glutamicum* AG, APGG and APLGGP. As shown in Table 2, in the recombinant strain *C. glutamicum* AG, NAGK activity was observed, while in the parent strain *C. glutamicum* G01, GAD activity was 2.13 U mg^{-1}, but no any NAGK activity was detected. The result suggested that the enzyme GAD was expressed, while NAGK involved in the L-arg biosynthesis was successfully knocked out in the recombinant *C. glutamicum* AG strain. In the engineered *C. glutamicum* APGG, the activities of NAGK and GAK were not observed, while the GAD activity was almost improved 2-fold (Table 2) due to the introduction of an extra copy of *gad* and the deletion of NAGK and GAK genes involved in the synthesis of L-arg and L-pro. In the recombinant *C. glutamicum* APLGGP, GAD and PLK were active, but NAGK, GAK, and DHDPS involved in the by-product pathways of L-arg, L-pro and L-lys were inactive. More important, *C. glutamicum* APLGGP showed 4.2-fold PLK activity higher than the other strains: wild-type, *C. glutamicum* AG and *C. glutamicum* APGG,

Table 2 Crude enzyme activities in the recombinant strains

	Specific activities (U mg^{-1} protein)				
Strains	GAD	NAGK	GAK	DHDPS	PLK
G01	—	0.30 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.11 ± 0.01
AG	2.13 ± 0.13	—	0.23 ± 0.02	0.20 ± 0.01	0.10 ± 0.01
APGG	4.20 ± 0.25	—	—	0.21 ± 0.01	0.10 ± 0.01
APLGGP	4.18 ± 0.23	—	—	—	0.43 ± 0.03

In the recombinant *C. glutamicum* AG, the NAGK gene was knocked out and GAD gene was expressed. In the recombinant *C. glutamicum* APGG, the NAGK and GAK genes were knocked out, and two copies of GAD genes were expressed. In the recombinant *C. glutamicum* APLGGP, the genes of NAGK, GAK and DHDPS were knocked out, and PLK and two copies of GAD were expressed. GAD, glutamate decarboxylase; NAGK, *N*-Acetyl-L-glutamate kinase; GAK, glutamate kinase; DHDPS, dihydropicolinate synthase; PLK, pyridoxal kinase; —, non-detected.

making biotransformation of GABA possible without pyridoxal-5′-phosphate. These above results indicate that the key enzymes, GAD and PLK catalyzing L-glutamate to GABA were expressed, while the NAGK, GAK, and DHDPS involved in the by-products L-arg, L-pro and L-lys were successfully knocked out.

The insertional mutation had little effect on cell growth

Since L-arg, L-pro and L-lys have separate and distinct roles in the microbial metabolism, the deletion of genes involved in their synthesis may have an affect on cell growth of the recombinant *C. glutamicum*. Meanwhile, the effects of introduction of GAD and PLK into *C. glutamicum* on the cell-growth were investigated. The fermentation characteristics of the recombinants had been compared with the data from flask experiments under the same conditions. Cell growth was determined by measuring the turbidity of the culture at OD_{560} using a UV-visible spectroscopy system. Based on the three-stage division of cell growth curves (Fig. 2), the engineered *C. glutamicum* strains AG, APGG, and APLGGP grew at a similar rate as compared to the parent strain. The results suggested that the knock out of genes involved in L-arg, L-pro and L-lys biosynthesis and introduction of the key genes of *gad* and *plk* related to the biotransformation of GABA had nearly no effect on cell growth properties. The insertion mutation in *C. glutamicum* did not inhibit cell growth. The insertion mutation system based on the five enzymes, GAD, PLK, NAGK, GAK, and DHDPS would be preferable for further studies of bioconversion of GABA from glucose without an exogenous cofactor.

One-step preparation of GABA from glucose without an exogenous cofactor was realized by *C. glutamicum* APLGGP

By fermentation with glucose, GABA production was compared between the engineered *C. glutamicum* and parental strain. The results are summarized in Table 3. The strain *C. glutamicum* AG accumulated 45.8 g L^{-1} GABA with a productivity of 0.48 g L^{-1} h^{-1} in the fermented broth, while the wild-type *C. glutamicum* did not produce GABA after 80 h of fermentation (Table 3). However, the strain *C. glutamicum* AG did not

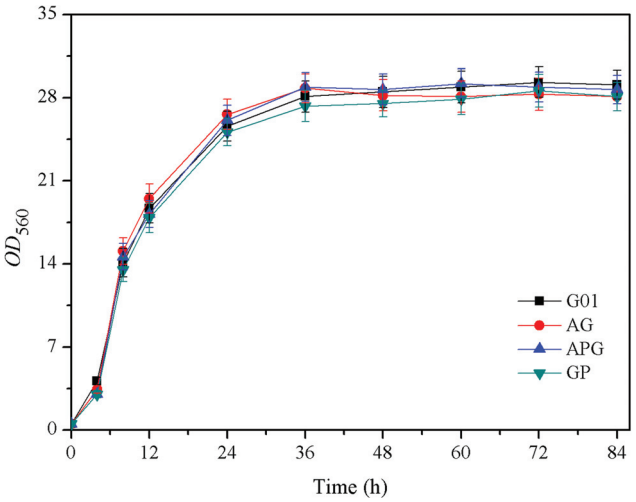

Fig. 2 Growth curves of *C. glutamicum* G01, AG, APGG and APLGGP. The engineered strains were cultivated at 37 °C in a 2.5 L flask bottle with an initial working volume of 1.0 L. Error bars represent standard deviations (n = 3).

secrete L-arg into the fermentation liquid, while the parental strain *C. glutamicum* G01 did (Table 3). These results further confirmed that *L. plantarum* GAD was active, but NAGK was inactive in the recombinant *C. glutamicum* AG. These above results demonstrated that GAD expression is a key factor for enhancing the GABA production, which is similar to the other reports.[12] More interesting, in the *C. glutamicum* AG harboring one copy of GAD coding gene, 40.7 g L^{-1} L-glutamate was detected in the broth, suggesting that much L-glutamate was not transformed to GABA.

Since the GAD enzyme is a key factor for biotransformation of GABA from glucose, one extra copy of GAD gene was introduced into the recombinant *C. glutamicum* APGG. In this recombinant strain, the by-product pathways of L-arg and L-pro were also blocked to further improve GABA yield. As shown in Table 3, the production of GABA reached 69.9 g L^{-1} in the broth of *C. glutamicum* APGG after 80 h fed-batch cultivation. Compared to *C. glutamicum* AG, the GABA production was further improved from 45.8 g L^{-1} to 69.9 g L^{-1}, and L-glutamate was almost consumed by *C. glutamicum* APGG. Meanwhile, L-arg and L-pro were not detected. The enhanced GABA yield is due to the two reasons. First, the introduction of an

Table 3 GABA production by the recombinant strains

Strains	L-Glu (g L^{-1})	L-Arg (g L^{-1})	L-Pro (g L^{-1})	L-Lys (g L^{-1})	GABA (g L^{-1})
G01	106.3 ± 3.3	5.2 ± 0.3	4.3 ± 0.2	3.1 ± 0.2	—
AG	40.7 ± 1.2	—	4.5 ± 0.3	3.3 ± 0.2	45.8 ± 1.3
APGG	0.6 ± 0.1	—	—	3.2 ± 0.2	69.9 ± 1.9
APLGGP	0.5 ± 0.1	—	—	—	70.6 ± 2.1

The recombinant *C. glutamicum* APLGGP catalyzes the biotransformation without addition of pyridoxal-5′-phosphate, while the other strains catalyze the reaction with an appropriate amount of pyridoxal-5′-phosphate. —, non-detected.

extra copy of GAD induced the higher GAD activity for the transformation of GABA from L-glutamate. Second, the inactive NAGK and GAK by insertional mutation redistributed carbon flux of glucose to the GABA pathway. Shi *et al.* also improved GABA production (27.13 g L^{-1} at 120 h) by co-expressing *gadB*1 and *gadB*2 in *C. glutamicum* ATCC13032.[13]

As the GAD requires pyridoxal-5′-phosphate as a cofactor for its activity,[4] PLK involved in the generation of pyridoxal-5′-phosphate and two copies of GAD were introduced into the recombinant *C. glutamicum* APLGGP, in which the relative genes involved in by-product pathways of L-arg, L-pro and L-lys were knocked out. The fermentation results showed that *C. glutamicum* APLGGP produced 70.6 g L^{-1} GABA from glucose without addition of pyridoxal-5′-phosphate. Compared with the fermentation results of *C. glutamicum* APGG with addition of pyridoxal-5′-phosphate, *C. glutamicum* APLGGP slightly improved GABA production without an exogenous cofactor. Thus, the one-step biotransformation of GABA from glucose was realized by the recombinant strain *C. glutamicum* APLGGP.

Enhanced production of GABA through a two-stage pH control strategy

During the biotransformation of GABA from glucose, L-glutamate is the precursor of GABA. It is very important to maintain the neutral conditions to steadily biosynthesize GABA from L-glutamate. Ammonia can not only take on this responsibility, but also can be used as the nitrogen source to promote the GABA production. Moreover, the L-glutamate dehydrogenase, which is critical for L-glutamate biosynthesis, exhibits the highest activity at pH 7.5, whereas at pH 6.5, half of its activity is lost.[18] So ammonia supplementation has a great influence on the biosynthesis of GABA from glucose. In this work, since L-glutamate is rapidly synthesized within 48 h of fermentation, ammonia was supplemented during 0–48 h of fermentation to retain the pH values at 7.3. As shown in Fig. 3A, it was found that time profiles of GABA production could be divided into two stages. At the first 48 h, pH values were constant at about 7.3, and L-glutamate was quickly accumulated, while GABA was produced very slowly. When ammonia supplement was stopped, the pH value dropped rapidly to about 5.4, GABA began to secret to the fermentation broth.

It was reported that the enzyme GAD shows activity under relatively acidic conditions.[4,13] Shi *et al.* observed that GAD exhibited a stringent pH dependence for substrate transport and functions at acidic pH, without any activity above pH 6.5.[12] Seo *et al.* found that the optimal pH of GAD activity was 5.2.[19] We further determined the effects on GABA conversion in the range of pH 4.0–5.8. As shown in Fig. 4, GAD showed the highest activity at about pH 4.8, and the conversion rate of GABA was the highest at pH 4.6–5.0. Basing on the above pH preference, a two-stage control strategy was proposed in favour of GABA formation. In the first 48 h, the pH value was controlled at about 7.3 for L-glutamate formation, and then pH was adjusted to 4.8 to promote GABA accumulation. As expected, through the strategy, the GABA production was up to 70.6 g L^{-1} after 70 h fed-batch cultivation, and the GABA formation rate was up to 1.04 g L^{-1} h^{-1} (Fig. 3B), which is the highest production report of GABA with glucose as a substrate in one-step. Thus, an excellent GABA producer from glucose was redesigned through combining the metabolic modules

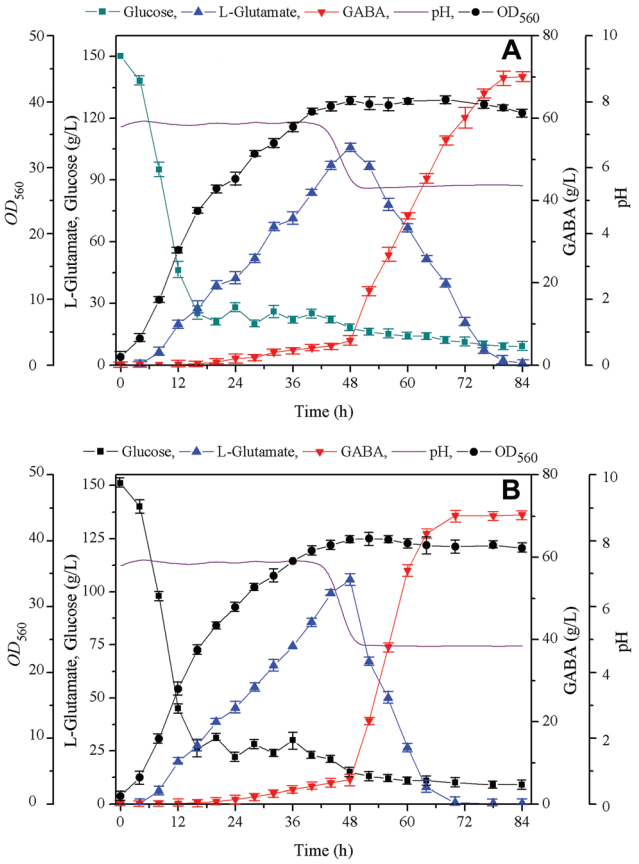

Fig. 3 Fed-batch fermentation of GABA by the recombinant *C. glutamicum* APLGGP. **(A)** pH value was controlled at 7.3 until 48 h, and was then not controlled; **(B)** pH value was controlled at 7.3 until 48 h, and then controlled at 4.8. The strains were cultured in a 5 L bioreactor at 37 °C.

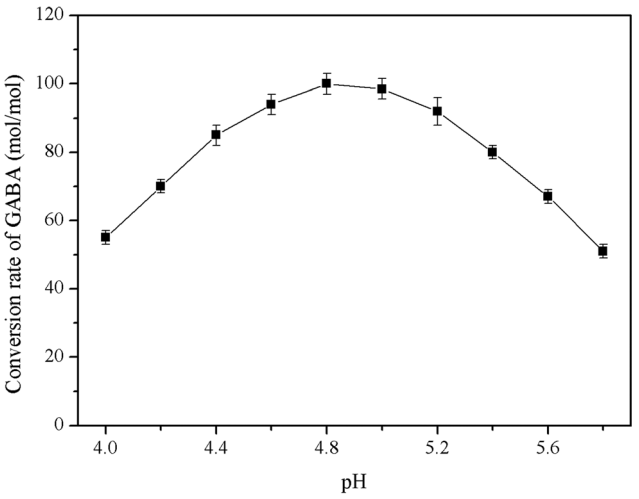

Fig. 4 Effects of pH on conversion rate of GABA.

(from L-glutamate to GABA) in *L. plantarum* and *C. glutamicum* (from glucose to L-glutamate), and blocking the by-product pathways of L-arg, L-pro and L-lys. The engineered *C. glutamicum* directly converted glucose to GABA without an exogenous cofactor through a two-stage pH control strategy.

Conclusions

An excellent producer of GABA was successfully redesigned by grafting the metabolic modular of L-glutamate from glucose in *L. plantarum* into *C. glutamicum*, making the engineered *C. glutamicum* highly yield GABA from glucose in one-step. Furthermore, the by-product pathways of L-arg, L-pro and L-lys were blocked to redistribute the metabolic flux to GABA, and a two-stage pH control strategy was proposed based on the pH preference of the key enzymes involved in the GABA synthesis. The production of GABA reached up to 70.6 g L^{-1} after 70 h. More important, *C. glutamicum* is a GRAS microorganism and its engineered strain can grow in no-selective medium. Therefore, the newly designed *C. glutamicum* system is an excellent GABA producer from glucose in food and pharmaceutical industry. This work is hoped to pave the way to redesign the bioreactor for efficient one-step preparation of GABA from glucose.

Experimental

General information

All chemicals, *i.e.*, glutamate, *N*-acetyl-L-glutamate, pyridoxal *etc.* were purchased from the Sigma-Aldrich Chemical Co. Inc. (Shanghai, China), and used without further purification. The precise determination of the amino acids was carried out on an automatic amino acid analyzer by high-performance liquid chromatography.

Organisms and growth conditions

C. glutamicum G01 has been deposited in the China Center for Type Culture Collection (CCTCC) under collection number CCTCC M2013418. *E. coli* JM109 strain was grown at 37 °C in Luria–Bertani (LB) medium with ampicillin (50 μg mL^{-1}) and/or kanamycin (50 μg mL^{-1}). *C. glutamicum* was grown in LBG medium (LB medium supplemented with 5 g L^{-1} glucose) at 30 °C with 30 μg mL^{-1} kanamycin, if necessary. The competent cells of *C. glutamicum* were cultured in the LB medium with 30 g L^{-1} glycine and 1 g L^{-1} Tween 80.

Culture conditions for L-glutamate and GABA fermentation from glucose

C. glutamicum strains were subcultured weekly and stored at 4 °C or frozen at −70 °C in 15% (v/v) glycerol. Stock and inoculum cultures were grown at 30 °C with shaking at 160 rpm in seed medium (25 g L^{-1} glucose, 20 g L^{-1} corn steep liquor, 10 g L^{-1} yeast extract, 6 g L^{-1} urea, 1.5 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 0.4 g L^{-1} $MgSO_4{\cdot}7H_2O$, pH 7.5) for 12 h. The incubated seed culture was then inoculated (4% v/v) into the fermentation medium (160 g L^{-1} glucose, 30 g L^{-1} corn steep liquor, 10 g L^{-1} yeast extract, 5.5 g L^{-1} urea, 1.5 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 0.8 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.02 g L^{-1} $MnSO_4{\cdot}4H_2O$, 0.02 g L^{-1} $FeSO_4{\cdot}7H_2O$, 200 μg L^{-1} vitamin B_1, 0.1 mmol L^{-1} pyridoxal-5′-phosphate, pH 7.5). Only for the recombinants carrying a pyridoxal kinase gene, 0.1 mmol L^{-1} pyridoxal-5′-phosphate was not added into the fermentation medium. For all the recombinant strains, 1 μmol L^{-1} isopropyl β-D-1-thiogalactopyranoside was added to the fermentation medium to induce GAD expression. Fed-batch fermentation was carried out in a 5 L bioreactor (BIOTECH-2002, Baoxing Biological Equipment Co., Shanghai, China) containing 2.0 L initial medium at 30 °C, 350 rpm and an airflow rate 0.66 vvm. Ammonia (NH_4OH) was added during the first 48 h of fermentation to maintain the alkaline environment. And then, the addition of ammonia was stopped to form an acidic fermentation environment, which was suitable for the GABA production. Glucose at 80% (w/v) was fed into the bioreactor to maintain the glucose concentration between 20 and 30 g L^{-1} for 16–44 h.

Plasmid construction and gene deletion

The glutamate decarboxylase gene (*gad*) from *L. plantarum* GB 01-21 was amplified by PCR using primers gadP1 and gadP2. The amplified *gad* gene was inserted into the *Nhe* I and *Bgl* II sites of the pDXW-10 plasmid to create pDXW-*gad*. For deletion of *argB* and *proB* in *C. glutamicum* G01 by homologous recombination, the recombination flanks of *argB* (R*argB*) and *proB* (R*proB*) were constructed and individually cloned into pMD18-T vector (Takara Co., Dalian, China), resulting in the vectors pMD-RargB and pMD-*R*proB. Recombination flanks of argB and proB were individually amplified from the *C. glutamicum* G01 chromosome by PCR as follows: flank A (445 bp) of *argB* and flank A (646 bp) of *proB* were generated with the primer pairs argBP1/argBP2 and proBP1/proBP2, respectively; flank B (529 bp) of *argB* and flank B (613 bp) of *proB* were created with the primer pairs argBP3/argBP4 and proB3/proBP4, respectively; the recombination flanks were then constructed by splicing the corresponding flanks A and B. The *Bgl* II restriction site was introduced into the splicing sites of flanks A and B. The *gad* gene, containing the *tac* promoter from the pDXW-*gad* plasmid, was then PCR-amplified using the primers tacgadP and gadP2. The amplified tac-*gad* gene was inserted into the pMD-R*argB* and pMD-R*proB* plasmids to create the pMD-R*arg*B::tacgad and pMD-RproB::tacgad plasmids, respectively. The pyridoxal kinase gene (*plk*) from *L. plantarum* GB 01-21 was amplified using primers plkP1 and plkP2. The amplified *plk* gene was inserted into the *Pst* I and Hind III sites of the pDXW-10 plasmid to create the pDXW-*plk* plasmid. The *plk* gene, containing the *tac* promoter from the pDXW-*plk* plasmid, was then amplified using the primers tacplkP and plkP2. The dihydropicolinate synthase gene (*dapA*) from *C. glutamicum* G01 was amplified using primers dapAP1 and dapAP2. The amplified *dap*A gene was cloned into the pMD18-T vector to create pMD-dapA. The amplified tac-*plk*

gene was inserted into the pMD-dapA plasmid to create the plasmid pMD-dapA::tacplk.

The pK18-R*arg*B::tacgad, pK18-RproB::tacgad and pK18-*dap*A::tacplk vectors were created by inserting a R*arg*B::tacgad, RproB::tacgad and dapA::tacplk fragments of pMD-R*arg*B::tacgad digested by *Xba* I and *Hind* III, pMD- RproB::tacgad digested by *Xba* I and *Hind* III and dapA::tacplk digested by *Xba* I and Sal I (agarose-purified, Gel DNA Purification Kit, Takara) into same restriction enzymes linearized pK18*mobsac*B, respectively. The deletion of *argB*, *proB* and *dapA* and introduction of *gad* and *plk* genes in *C. glutamicum* were preformed by homologous recombination.[20] The genetic modifications were verified by enzyme assays and gene sequencing. The primers (restriction sites were underlined) used in this work are listed in Table S1 in the ESI.† All the oligonucleotide primers were synthesized at Takara Co. (Dalian, China).

Construction of the recombinant *C. glutamicum* strains

By electroporation, the plasmid pK18-R*arg*B::tacgad was introduced into the competent cells of *C. glutamicum* G01 to generate the strain *C. glutamicum* AG, in which the *gad* gene was introduced and *arg*B was knocked out. The recombinant pK18-R*arg*B::tacgad and pK18-R*pro*B::tacgad were both introduced into *C. glutamicum* G01 to construct *C. glutamicum* APGG, in which two copies of *gad* genes were introduced, and *arg*B and *pro*B were deleted. The recombinant pK18-R*arg*B::tacgad, pK18-R*pro*B::tacgad and pK18-*dap*A::tacplk were simultaneously introduced into *C. glutamicum* G01 to create *C. glutamicum* APLGGP, in which *plk* gene and two copies of *gad* genes were introduced, and *arg*B, *pro*B and *dap*A were knocked out.

Enzyme assays

The activities of GAD, NAGK, GAK, PLK and DHDPS were assayed according to previously reported methods.[21,22] All measurements were repeated at least three times.

Abbreviations

argB	*N*-Acetyl-L-glutamate kinase gene
*dap*A	Dihydropicolinate synthase gene
DHDPS	Dihydropicolinate synthase
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
gad	Glutamate decarboxylase gene
GAK	Glutamate kinase
NAGK	*N*-Acetyl-L-glutamate kinase
PLK	Pyridoxal kinase
plk	Pyridoxal kinase gene
proB	Glutamate kinase gene.

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