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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Enhancement of γ-aminobutyric acid production in recombinant *Corynebacterium glutamicum* by co-expressing two glutamate decarboxylase genes from *Lactobacillus brevis*

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Abstract γ-Aminobutyric acid (GABA), a non-protein amino acid, is a bioactive component in the food, feed and pharmaceutical fields. To establish an effective single-step production system for GABA, a recombinant *Corynebacterium glutamicum* strain co-expressing two glutamate decarboxylase (GAD) genes (*gadB1* and *gadB2*) derived from *Lactobacillus brevis* Lb85 was constructed. Compared with the GABA production of the *gadB1* or *gadB2* single-expressing strains, GABA production by the *gadB1*–*gadB2* co-expressing strain increased more than twofold. By optimising urea supplementation, the total production of L-glutamate and GABA increased from 22.57 ± 1.24 to 30.18 ± 1.33 g L^{-1}, and GABA production increased from 4.02 ± 0.95 to 18.66 ± 2.11 g L^{-1} after 84-h cultivation. Under optimal urea supplementation, L-glutamate continued to be consumed, GABA continued to accumulate after 36 h of fermentation, and the pH level fluctuated. GABA production increased to a maximum level of 27.13 ± 0.54 g L^{-1} after 120-h flask cultivation and 26.32 g L^{-1} after 60-h fed-batch fermentation. The conversion ratio of L-glutamate to GABA reached 0.60–0.74 mol mol^{-1}. By co-expressing *gadB1* and *gadB2* and optimising the urea addition method, *C. glutamicum* was genetically improved for de novo biosynthesis of GABA from its own accumulated L-glutamate.

Keywords γ-Aminobutyric acid · *Corynebacterium glutamicum* · Glutamate decarboxylase · Co-expression · Extracellular pH

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Introduction

γ-Aminobutyric acid (GABA), a four-carbon non-protein amino acid, is widely distributed in nature from microorganisms to plants and animals [14]. This amino acid is a well-characterised inhibitory neurotransmitter in animals that has various physiological functions, including hypotensive, anti-anxiety, tranquilising, analgesic and diuretic functions [4, 17], and can be applied to treat various neurological disorders [31]. Recent research has indicated that GABA may improve the protein synthesis rate in the brain [29] and suppress carcinomas, such as small airway-derived lung adenocarcinomas [22]. GABA has been considered a bioactive component in the food, feed and pharmaceutical fields. In addition to its bioactivities beneficial to animals, GABA functions as an important component in the acid resistance systems of certain bacteria, such as *Escherichia coli* [28], *Lactococcus lactis* [21] and *Listeria monocytogenes* [9], and is required for the normal oxidative stress tolerance in *Saccharomyces cerevisiae* [2]. GABA is synthesised by glutamate decarboxylase (GAD), a pyridoxal 5′-phosphate-dependent enzyme that catalyses irreversible α-decarboxylation of L-glutamate to GABA [30]. GAD is widely distributed in eukaryotes and prokaryotes [14]. Because many lactic acid bacteria (LAB) are

probiotic to humans and animals, LAB would be the most suitable microorganisms for GAD gene cloning and GABA production [15, 18, 25].

Although GABA production using LAB with higher GAD activity is acceptable for food applications, GABA production requires addition of L-glutamate or monosodium glutamate as the precursor [16, 27] and expensive nitrogen sources for cultivating LAB [15, 35]. This type of production system is not cost-effective for industrial applications. It is important to find a cost-effective and convenient approach for synthesising GABA. Considering that *Corynebacterium glutamicum*, a non-pathogenic Gram-positive bacterium, overproduces the precursor of GABA, i.e. L-glutamate [34], GABA production by engineered *C. glutamicum* over-expressing the GAD gene from LAB warrants investigation. *C. glutamicum* is an important industrial microorganism because of its high productivity of vitamins, L-glutamate and other amino acids [5, 13]. The complete genome of *C. glutamicum* ATCC 13032 was sequenced by Kalinowski et al. [8] and Ikeda and Nakagawa [7]. The completed deciphering contributed to significant improvements in bioprocessing and downstream technology, as well as in molecular biology [11]. In *C. glutamicum* ATCC 13032, neither gene encoding GAD was identified, and GABA production was not reported. Recently, by expressing the GAD gene from *Lactobacillus brevis* Lb85, we succeeded in engineering *C. glutamicum* ATCC 13032 to produce GABA spontaneously, but production was low (2.15 ± 0.16 g L^{-1}) [23]. A later study indicated that, by expressing the GAD gene from *E. coli* W3110, *C. glutamicum* ATCC 13032 was able to produce 12.37 ± 0.88 g L^{-1} of GABA [26].

Two genes encoding GAD, namely *gadB1* and *gadB2*, were identified in *L. brevis* Lb85. The transcriptional regulator gene *gadR* and the L-glutamate/GABA antiporter gene *gadC* are upstream of *gadB2*. In our previous research, *gadB1*, *gadB2*, *gadCB2* and *gadRCB2* were introduced into *C. glutamicum* ATCC 13032 separately by an inducible expression vector (pDXW-8) [23]. The four recombinant strains were able to synthesise GABA using endogenous L-glutamate, with the strain ATCC 13032/pDXW-8-*gadB2* yielding a slightly higher production level than the strain ATCC 13032/pDXW-8-*gadB1*, and the strain ATCC 13032/pDXW-8-*gadRCB2* generating the highest production level (2.15 g L^{-1}). Such GABA production was not high, and the production should be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) because pDXW-8 is an inducible expression vector with a weaker *tac* promoter and a *lacI* repressor gene. In this study, to improve GABA production in recombinant *C. glutamicum*, a constitutive expression strain co-expressing *gadB1* and *gadB2* was constructed and compared with the *gadB1* and *gadB2* single-expressing strains. The urea addition strategy during the L-glutamate fermentation stage was researched, and using the optimal urea supplementation method, GABA production reached 27.13 ± 0.54 g L^{-1} after 120 h of fermentation.

Materials and methods

Strains, media and growth conditions

The bacterial strains and plasmids used in this study are summarised in Table 1. *L. brevis* Lb85 exhibiting GABA production capacity was used for amplification of *gadB1* and *gadB2* genes. *L. brevis* was grown under static conditions in de Man–Rogosa–Sharpe (MRS) medium (10 g tryptone, 5 g yeast extract, 5 g beef extract, 5 g glucose, 3 g sodium acetate, 2 g citric acid diamine, 2 g $K_2HPO_4 \cdot 3H_2O$, 0.1 g $MgSO_4$, 0.04 g $MnSO_4$ L^{-1}) at 30 °C. *E. coli* DH5α was used as the host for constructing and propagating the plasmids. *E. coli* BL21(DE3) was used as the host for expressing the *gadB1* and *gadB2* genes derived from *L. brevis* Lb85. *E. coli* was grown in Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl L^{-1}, pH 7.2) at 37 °C and 200 rpm with 50 mg L^{-1} kanamycin, as required. *C. glutamicum* ATCC 13032, an L-glutamate-producing strain, was used to express the GAD genes derived from *L. brevis* Lb85 for GABA production. *C. glutamicum* was grown in LBG medium (LB supplemented with 5 g L^{-1} glucose) at 200 rpm and 30 °C with 30 mg L^{-1} kanamycin, as required.

Construction of *gadB1*- and *gadB2*-expressing plasmids and strains

For expression of *gadB* genes in *C. glutamicum*, *gadB1*, *gadB2* and *gadB2* plus the upstream L-glutamate/GABA antiporter gene *gadC* and the transcriptional regulator gene *gadR* (*gadRCB2*) were amplified from the genomic DNA of *L. brevis* Lb85. The primers were designed using the *L. brevis* Lb85 *gadB1* and *gadRCB2* gene sequence [23] and are listed in Table 2. The 1,407-bp *gadB1* gene was amplified using the gadB1(+) and gadB1(−) primer pair and ligated into the *Eco*RI and *Not*I restriction sites of the vector pDXW-10 to produce the plasmid pDXW-10-*gadB1*. pDXW-10 is a shuttle expression vector carrying a stronger *tac*-M promoter for cloning between *E. coli* and *Corynebacterium* [33]. The 1,440-bp *gadB2* gene was amplified using the gadB2(+) and gadB2(−) primer pair and ligated into the *Nhe*I and *Pst*I restriction sites of pDXW-10 to produce the plasmid pDXW-10-*gadB2*. Subsequently, the *gadB2* gene with the *tac*-M promoter sequence was amplified from the plasmid pDXW-10-*gadB2* using the tac(+) and gadB2(−) primer pair. The

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
L. brevis Lb85	*L. brevis* capable of producing GABA	[23]
E. coli DH5α	Wild-type *E. coli*	Novagen
DH5α/pDXW-10-*gadB1*	DH5α harbouring pDXW-10-*gadB1*	This work
DH5α/pDXW-10-*gadB2*	DH5α harbouring pDXW-10-*gadB2*	This work
DH5α/pDXW-10-*gadB1–gadB2*	DH5α harbouring pDXW-10-*gadB1–gadB2*	This work
DH5α/pDXW-10-*gadRCB2*	DH5α harbouring pDXW-10-*gadRCB2*	This work
E. coli BL21(DE3)	*E. coli* strain for expressing target gene(s)	Novagen
BL21/pET28a-*gadB1*	BL21(DE3) harbouring pET28a-*gadB1*	This work
BL21/pET28a-*gadB2*	BL21(DE3) harbouring pET28a-*gadB2*	This work
C. glutamicum ATCC 13032	Wild-type *C. glutamicum*	ATCC
ATCC 13032/pDXW-10	ATCC 13032 harbouring pDXW-10	This study
ATCC 13032/pDXW-10-*gadB1*	ATCC 13032 harbouring pDXW-10-*gadB1*	This work
ATCC 13032/pDXW-10-*gadB2*	ATCC 13032 harbouring pDXW-10-*gadB2*	This work
ATCC 13032/pDXW-10-*gadB1–gadB2*	ATCC 13032 harbouring pDXW-10-*gadB1–gadB2*	This work
ATCC 13032/pDXW-10-*gadRCB2*	ATCC 13032 harbouring pDXW-10-*gadRCB2*	This work
Plasmids		
pDXW-10	A shuttle vector between *E. coli* and *Corynebacterium*	[33]
pDXW-10-*gadB1*	pDXW-10 harbouring *gadB1*	This work
pDXW-10-*gadB2*	pDXW-10 harbouring *gadB2*	This work
pDXW-10-*gadB1–gadB2*	pDXW-10 harbouring *gadB1–gadB2*	This work
pDXW-10-*gadRCB2*	pDXW-10 harbouring *gadRCB2*	This work
pET-28a(+)	*E. coli* expression vector, Kan^r	Novagen
pET28a-*gadB1*	pET-28a(+) harbouring *gadB1*	This work
pET28a-*gadB2*	pET-28a(+) harbouring *gadB2*	This work

tac-M-*gadB2* amplification product was ligated into the *Not*I and *Pst*I restriction sites of the plasmid pDXW-10-*gadB1* to produce the co-expression plasmid pDXW-10-*gadB1–gadB2*. The 3,785-bp *gadRCB2* gene sequence was amplified using the gadRCB2F and gadB2R primer pair and ligated into the *Nhe*I and *Hin*dIII restriction sites of pDXW-10 to produce the plasmid pDXW-10-*gadRCB2*. All the plasmids transferred into competent *E. coli* DH5α cells were confirmed by restriction enzyme digestion and target gene amplification.

The constructed plasmids pDXW-10-*gadB1*, pDXW-10-*gadB2*, pDXW-10-*gadB1–gadB2* and pDXW-10-*gadRCB2* were isolated from *E. coli* and transformed into electro-competent *C. glutamicum* ATCC 13032 according to the protocol of Xu et al. [32], yielding *gadB1*-expressing strain *C. glutamicum* ATCC 13032/pDXW-10-*gadB1*, *gadB2*-expressing strain *C. glutamicum* ATCC 13032/pDXW-10-*gadB2*, *gadB1–gadB2* co-expressing strain *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2* and *gadRCB2*-expressing strain *C. glutamicum* ATCC 13032/pDXW-10-*gadRCB2*. All plasmids from the positive transformants were verified by target gene amplification.

For expression of *gadB* genes in *E. coli*, *gadB1* was amplified from the genomic DNA of *L. brevis* Lb85 using the gadB1(E+) and gadB1(E−) primer pair and ligated into the *Nde*I and *Eco*RI restriction sites of the vector pET-28a(+) to produce the plasmid pET28a-*gadB1*. Similarly, *gadB2* was amplified from the genomic DNA of *L. brevis* Lb85 using the gadB2(E+) and gadB2(E−) primer pair and ligated into the *Nhe*I and *Sac*I restriction sites of the vector pET-28a(+) to produce the plasmid pET28a-*gadB2*. The plasmids pET28a-*gadB1* and pET28a-*gadB2* were transformed into *E. coli* BL21(DE3), yielding *E. coli* BL21/pET28a-*gadB1* and BL21/pET28a-*gadB2* strains. The use of pET-28a(+) enabled us to fuse a 6xHis tag to the N-terminal of the GadB1 and GadB2 proteins and allowed IPTG-inducible expression of *gadB1* and *gadB2* genes in *E. coli* BL21(DE3).

Expression of *gadB1* and *gadB2* in *E. coli* and purification of GadB1 and GadB2

The recombinant *E. coli* BL21/pET28a-*gadB1* and BL21/pET28a-*gadB2* were cultivated for 12 h, transferred to

Table 2 Primers for amplifying GAD genes from *L. brevis*

Primer	Oligonucleotide sequence (5'-3')	Restriction site
gadB1(+)	CCG**GAATTC***AGAAGGAG*ATATACCATGGCTATGTTGTATGGAAAACACAC	*Eco*RI
gadB1(−)	AAATAT**GCGGCCGC**ATGAGATGAACGATTGATGATTAGTGC	*Not*I
gadB2(+)	CTA**GCTAGC***AGAAGGAG*ATATAGGATGAATAAAAACGATCAGGAAACACAG	*Nhe*I
gadB2(−)	ATCT**CTGCAG**TTAACTTCGAACGGTGGTCTTG	*Pst*I
tac(+)	AAATAT**GCGGCCGC**TCGGAAGCTGTGGTATGG	*Not*I
gadRCB2F	CTA**GCTAGC***AGAAGGAG*ATATACCATGGAATCCAGAACTAAGGAAAGG	*Nhe*I
gadB2R	GAT**AAGCTT**TTAACTTCGAACGGTGGTCTTG	*Hin*dIII
gadB1(E+)	GACCGCT**CAT****ATG**GCTATGTTGTATGGAAAAC	*Nde*I
gadB1(E−)	CGT**GAATTC**TTAGTGCGTGAACCCGTATT	*Eco*RI
gadB2(E+)	CTA**GCTAGC**AATAAAAACGATCAGGAAACACAGC	*Nhe*I
gadB2(E−)	T**GAGCTC**TTAACTTCGAACGGTGGTCTTG	*Sac*I

The restriction sites are in boldface. The sequences corresponding to the genomic DNA of *L. brevis* are underlined, and the start and stop codons are boxed. The Shine–Dalgarno sequences are in italics

400 mL LB broth, and grown at 37 °C until the optical density (OD_{600}) reached 0.6. IPTG was added to final concentration of 1 mM, and cultivation was continued at 30 °C and 200 rpm for 5 h. The cells were collected, washed, and resuspended in lytic buffer [300 mM NaCl, 10 mM imidazole, 50 mM phosphate buffer saline (pH 8.0), 1 mM phenylmethylsulphonyl fluoride (PMSF)] to wet cell concentration of 0.1 g mL^{-1} and disrupted using sonication. A total of 10 mg mL^{-1} Triton X-100 was added to the sonicated cell suspension. The GadB1 protein was extracted, purified by nickel-chelating affinity chromatography, and dissolved in 20 mM sodium acetate buffer (pH 4.6).

GABA fermentation by recombinant *C. glutamicum* in shake flask

For GABA production in shake flask, recombinant *C. glutamicum* cells were precultured in seed medium (25 g glucose, 30 g corn steep liquor, 8 g urea, 1 g $K_2HPO_4{\cdot}3H_2O$, 0.2 g $MgSO_4$ L^{-1}, pH 7.0–7.2) supplemented with 30 mg L^{-1} kanamycin at 30 °C and 110 rpm for 7 h. The preculture broth was inoculated into 20 mL fermentation medium containing 30 mg L^{-1} kanamycin in a 500-mL Erlenmeyer flask to final optical density (OD_{562}) of 2.0 and shaken by a reciprocating shaker at 30 °C and 110 rpm for 84 h. The fermentation medium contained 100 g glucose, 4 g corn steep liquor, 4 g urea, 2 g $K_2HPO_4{\cdot}3H_2O$, 0.4 g $MgSO_4$, 0.2 g $MnSO_4$ and 0.29 g$FeSO_4{\cdot}7H_2O$ L^{-1}, and was initially adjusted to pH 7.0–7.2. Generally, at 10, 17 and 24 h of fermentation, 4 g L^{-1} urea was added to the culture to maintain the neutral condition; this fermentation method is defined as the general fermentation method.

To determine the effect of the urea supplementation method on GABA production, four methods of urea addition were carried out during the first 24 h of fermentation, retaining the initial urea concentration at 4 g L^{-1} and the total urea amount at 16 g L^{-1}. In three methods, urea was added during 10–24 h of fermentation three times (4 g L^{-1} every 7 h, denoted as $4 + 3 \times 4$), four times (3 g L^{-1} every 4.5 h, denoted as $4 + 4 \times 3$) or five times (2.4 g L^{-1} every 3.5 h, denoted as $4 + 5 \times 2.4$). In the fourth method, urea was added to the broth six times during 6–24 h of fermentation (2 g L^{-1} every 3.5 h, denoted as $4 + 6 \times 2$). One-time addition of urea (12 g L^{-1}) at 10 h of cultivation (denoted as $4 + 1 \times 12$) and addition of the total amount of urea (16 g L^{-1}) at the starting point of cultivation (denoted as $16 + 0$) were conducted as control methods. Fermentation was performed on a reciprocating shaker at 110 rpm and 30 °C.

GABA fermentation by recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2* in fermentor

For GABA production in the fermentor, 100 mL seed culture was prepared in flasks at 30 °C for 8 h and transferred to a 3-L fermentor (BioFlo 110; New Brunswick Scientific, USA) containing 1.2 L fermentation medium. The temperature and aeration rate were kept at 31 °C and 1.5 vvm, respectively. The dissolved oxygen level was controlled at 20 % in the first 10 h and 30 % thereafter by coupling with the agitation speed. During the 8–22 h of fermentation, the pH was controlled at 7.5–8.0 by automatically adding 40 % urea solution, whereas after 36 h, the pH was adjusted to 4.8–5.3 by adding 6 M HCl. Samples were taken every 4 h to determine the OD_{562}

value and residual glucose. From 28 h of fermentation, glucose was fed by a peristaltic pump when the residual glucose in the medium was lower than 20 g L^{-1}.

Determination of pH level as well as extracellular and intracellular L-glutamate and GABA concentrations during fermentation

During fermentation, an appropriate volume of culture broth was harvested and centrifuged at 12,000 rpm and 4 °C for 5 min. The supernatant was used for measuring the pH as well as the L-glutamate and GABA concentrations in the fermentation broth. The precipitate was washed twice and used to determine the intracellular L-glutamate and GABA concentrations. pH was measured directly by a pH electrode (Mettler-Toledo, Germany). The L-glutamate and GABA concentrations were assayed using reversed-phase high-pressure liquid chromatography (HPLC, 1200 series; Agilent Technologies, USA). The cell concentration was determined by measuring the OD_{562} value with a UV-1800 spectrophotometer (Shimadzu, Japan). To determine the intracellular L-glutamate and GABA concentrations, the washed precipitate was resuspended in anidentical volume of phosphate buffer (20 mM, pH 7.2) and boiled for 15 min; the cell debris was discarded by centrifugation at 12,000 rpm for 10 min [20], and the supernatant was used for HPLC analysis. Before analysis, the supernatant sample was treated with trichloroacetic acid to precipitate proteins. The clear filtrate was used directly for HPLC assay. HPLC separation and quantisation were performed on a Thermo ODS C_{18} column (250 mm × 4.0 mm, USA) by the *o*-phthalaldehyde pre-column derivatisation method [12]. The L-glutamate and GABA were detected at 338 nm, and the spectra were recorded online. The intracellular concentration was calculated with the intracellular volume of 1.6 mL g^{-1} dry weight [10]. The conversion ratio ofL-glutamate to GABA was calculated as the mol of GABA divided by the total mol of L-glutamate and GABA.

Extraction of crude enzyme from recombinant *C. glutamicum* and determination of GAD activity

The cells in the fermentation broth were harvested, washed once with chilled distilled water, and suspended in phosphate-buffered saline (20 mM, pH 7.2). After incubation with 1 g L^{-1} lysozyme at 37 °C for 2 h, the cells were disrupted by ultrasonication at 4 °C. The supernatant (cell extract) was recovered and designated as crude enzyme after lysed cell debris was removed by centrifugation. The protein concentration of the enzyme solution was determined according to the Bradford method, with bovine serum albumin as standard.

Enzyme solution (<0.1 mL) was added to a reaction mixture containing 0.4 mL substrate buffer (0.2 M Na_2HPO_4–citric acid buffer, pH 4.8 and 10 mM sodium glutamate) and incubated at 37 °C for 60 min. The reaction was terminated by immediately boiling for 10 min. After centrifugation at 12,000 rpm for 10 min, the amount of GABA in the supernatant was determined by HPLC. One unit (U) of enzyme activity was defined as 1.0 μmol GABA produced in 1 min at 37 °C in the initial reaction mixture (0.5 mL), and the specific activity was expressed as U mg^{-1} or U g^{-1} of protein. The optimum pH for purified GadB1 was assayed over a pH range of 3.6–6.2, and 0.2 M sodium acetate buffer was used instead of 0.2 M Na_2HPO_4–citric acid buffer, pH 4.8.

Results

To improve GABA production in recombinant *C. glutamicum*, the constitutive expression vector pDXW-10, with a stronger *tac*-M promoter, was used for the expression of *L. brevis* Lb85 *gadB* genes in *C. glutamicum*, and the production of GABA was examined.

GABA production by recombinant *C. glutamicum* strains

First, GABA production was compared between *C. glutamicum* strains expressing only *gadB1*, only *gadB2*, and *gadRCB2*, and co-expressing *gadB1–gadB2*. These recombinant strains were fermented by the general fermentation method. After 84 h of fermentation, the strain ATCC 13032/pDXW-10-*gadB1–gadB2* could accumulate 4.02 ± 0.95 g L^{-1} of GABA in the fermented broth, higher than the strains ATCC 13032/pDXW-10-*gadB1* (0.69 ± 0.40 g L^{-1}), ATCC 13032/pDXW-10-*gadB2* (0.74 ± 0.16 g L^{-1}) and ATCC 13032/pDXW-10-*gadRCB2* (2.41 ± 1.33 g L^{-1}); no GABA was detected in the control strain, ATCC 13032/pDXW-10. This result indicated that *L. brevis* Lb85 *gadB* genes were expressed and exhibited glutamate decarboxylase activity in recombinant *C. glutamicum* by pDXW-10, and the *gadB1–gadB2* co-expression exhibited better activity. The ATCC 13032/pDXW-10-*gadB1–gadB2* strain was used as the predominant strain for GABA production.

Effect of urea supplementation method on GABA production

L-Glutamate is the precursor of GABA. Urea supplementation has a great influence on the biosynthesis of L-glutamate because urea is not only the nitrogen source but also the basic reagent required for maintaining the neutral

condition for L-glutamate biosynthesis in *C. glutamicum*. The L-glutamate dehydrogenase, which is critical for L-glutamate biosynthesis, exhibits the highest activity at pH 7.5 [24], whereas at pH 6.5, half of its activity is lost. Urea was supplemented during 10–24 h of fermentation because L-glutamate is synthesised within 36 h of fermentation. The accumulated L-glutamate can then be converted into GABA by the expressed GAD protein. To understand the possible influence of urea supplementation on pH variation, GAD expression and GABA production, four types of urea addition method were compared for recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*. The total addition of urea was maintained at 12 g L^{-1} in all four methods. One-time addition of urea (12 g L^{-1}) at 10 h of cultivation and addition of the total amount of urea (16 g L^{-1}) at the cultivation starting point were conducted as control methods.

Initially, when urea was supplemented three times, with 4 g L^{-1} each time (denoted as $4 + 3 \times 4$), the fermentation broth pH level decreased to 5.67 at 24 h just before the last supplementation, the GAD activity of the crude cell extract was 114.3 U g^{-1} at 60 h of fermentation, and after 84 h, the L-glutamate and GABA concentration in the fermentation broth reached 18.55 ± 2.19 and 4.02 ± 0.95 g L^{-1}, respectively (Fig. 1). When urea was added four times, with 3 g L^{-1} each time (denoted as $4 + 4 \times 3$), the

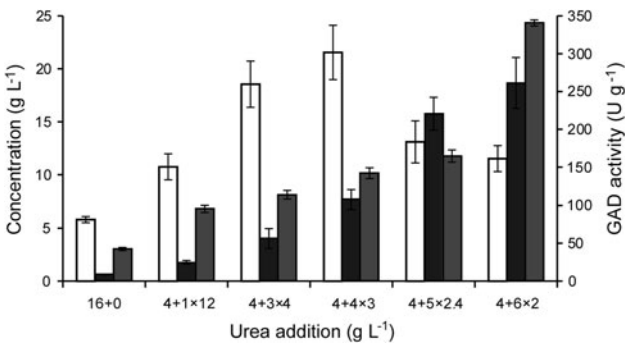

Fig. 1 Effect of urea supplementation method on L-glutamate and GABA production by recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*. Cells were precultured in seed medium for 7 h at 30 °C and 110 rpm, inoculated into fermentation medium, and cultivated at 30 °C and 110 rpm for 84 h. By three methods, urea was supplemented during 10–24 h of fermentation in three times (4 g L^{-1} every 7 h, denoted as $4 + 3 \times 4$), four times (3 g L^{-1} every 4.5 h, denoted as $4 + 4 \times 3$) or five times (2.4 g L^{-1} every 3.5 h, denoted as $4 + 5 \times 2.4$). In the fourth method, urea was supplemented during 6–24 h of fermentation in six times (2 g L^{-1} every 3.5 h, denoted as $4 + 6 \times 2$). In the control methods, one-time addition of urea at 10 h of cultivation (12 g L^{-1}, denoted as $4 + 1 \times 12$) and addition of the total amount of urea at the starting point of cultivation (16 g L^{-1}, denoted as $16 + 0$) were conducted. After cultivation, L-glutamate (*white bars*) and GABA (*black bars*) concentrations in the fermented broth were analysed. GAD activities from crude cell extracts of 60-h cultivations (*grey bars*) were assayed. Averages of three independent experiments are provided

fermentation broth pH level remained at 7.33 at 24 h, the GAD activity of the crude cell extract increased to 142.0 U g^{-1} at 60 h, and after 84 h, the L-glutamate and GABA production increased to 21.55 ± 2.57 and 7.70 ± 0.95 g L^{-1}, respectively. When urea was supplemented five times (2.4 g L^{-1} each time, denoted as $4 + 5 \times 2.4$) and six times (2 g L^{-1} each time, denoted as $4 + 6 \times 2$), the fermentation broth pH level was 7.13 and 6.26, respectively, at 24 h, the GAD activity of the crude cell extract increased to 164.6 and 340.7 U g^{-1}, respectively, at 60 h, and after 84 h, the L-glutamate concentration decreased to 13.10 ± 1.99 and 11.51 ± 1.23 g L^{-1}, respectively, whereas the GABA concentration increased to 15.76 ± 1.55 and 18.66 ± 2.41 g L^{-1}, respectively. In the control methods, where 12 g L^{-1} of urea was added in one time at 10 h of cultivation (denoted as $4 + 1 \times 12$) and all the 16 g L^{-1} of urea was added at the cultivation starting point (denoted as $16 + 0$), the fermentation broth pH level decreased to 5.37 and 5.30, respectively, at 24 h, the GAD activity of the crude cell extract decreased to 95.6 and 42.4 U g^{-1}, respectively, at 60 h, and after 84 h, the L-glutamate production was 10.74 ± 1.23 and 5.81 ± 0.30 g L^{-1}, respectively, and the GABA production was 1.76 ± 0.18 and 0.62 ± 0.05 g L^{-1}, respectively (Fig. 1), being much lower than the values produced using the urea supplementation methods with three to six additions. The more frequently urea was supplemented, the higher the GAD activity and GABA conversion ratio obtained, and the more GABA was produced. The conversion ratio of L-glutamate to GABA increased to 0.70 mol mol^{-1}, and the GABA productivity increased by 3.6-fold compared with the initial fermentation method ($4 + 3 \times 4$). Addition of urea six times with 2 g L^{-1} each time was adopted in subsequent fermentation.

Time course of GABA fermentation in recombinant *C. glutamicum* strains

In our previous study, we found that L-glutamate was generally synthesised within 36 h of fermentation, whereas GABA began to accumulate after 36 h and increased continuously thereafter [23]; thus 0–36 h can be regarded as the L-glutamate fermentation stage, and after 36 h can be regarded as the GABA fermentation stage. At the GABA fermentation stage, GAD must be activated. GAD can only exhibit activity in a relatively acid condition because the purified GadB1 protein after being expressed in *E. coli* BL21(DE3) exhibited optimum activity at pH 4.6, whereas at pH 5.8, approximately 85 % of its activity was lost (data not shown). GadB2 was expressed as an inclusion body in *E. coli* BL21(DE3). An additional GadB2 protein from *L. brevis* IFO12005 was reported to exhibit optimum activity at pH 4.5–5.0 [6]; at pH 5.8, approximately 80 %

of its activity was lost. Apart from the L-glutamate fermentation stage, the pH level in the GABA fermentation stage will influence GABA biosynthesis. At the same time, the accumulation of L-glutamate and the conversion of L-glutamate to GABA will change the pH level. To elucidate the relationship between GABA biosynthesis and pH fluctuation, the time course of L-glutamate concentration, GABA concentration and extracellular pH level at the GABA fermentation stage were investigated.

During the fermentation of recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*, the L-glutamate concentration reached the highest values (27.55–28.17 g L^{-1}) at 36–48 h and continuously reduced thereafter, whereas GABA began to be synthesised before 36 h, and its concentration continuously increased (Fig. 2a). The GAD activity of the crude cell extracts at pH 4.8 remained at 172–341 U g^{-1} during 24–84 h of fermentation (Table 3), with the activity at 60 h being the highest (340.7 ± 4.0 U g^{-1}). The expressed GadB1 and GadB2 proteins were not visible in the crude cell extract of fermentation broth by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After 84 h of fermentation, the L-glutamate concentration decreased to 15.19 ± 0.71 g L^{-1}, whereas the GABA concentration increased to 19.38 ± 0.17 g L^{-1}. The conversion ratio of L-glutamate to GABA reached 0.65 mol mol^{-1}, and the total amount of L-glutamate and GABA reached 0.29 M. The strain grew rapidly and consumed much more glucose (75.5 g L^{-1}) before 36 h of fermentation, indicating that the carbon source glucose was mainly required at the L-glutamate fermentation stage. After 36 h, the strain grew slowly and consumed little glucose (Fig. 2b). During this GABA fermentation stage, 84 mM of extracellular L-glutamate was reduced, but 159 mM of GABA was formed, indicating that biosynthesis of L-glutamate did not cease after 36 h. With the conversion of L-glutamate to GABA, the pH level of the fermentation broth changed. It was lowest at 36–48 h (pH 5.56–5.53), in accordance with the highest L-glutamate and lowest GABA concentration at this period, then increased quickly until 60 h (pH 6.24) and slowly thereafter, in accordance with the decrease of the L-glutamate and increase of the GABA concentration at this period, because L-glutamate is an acidic [isoelectric point (p*I*) 3.22] molecule and GABA is a neutral (p*I* 7.19) molecule. Because the time course of the extracellular pH level during the GABA fermentation stage was in accordance with the conversion of L-glutamate to GABA, the pH level could be used as an indicator to reflect GABA synthesis in recombinant *C. glutamicum*.

Continuous accumulation of extracellular GABA might be observed because of delayed export of intracellular GABA synthesised previously. The intracellular concentrations of L-glutamate and GABA during fermentation were therefore determined. In *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*, the intracellular concentration of L-glutamate decreased during 36–60 h, similar to the extracellular concentration, but increased obviously thereafter (Fig. 2c). The relatively higher concentration of intracellular L-glutamate than extracellular L-glutamate indicates that such retarded export of L-glutamate would occur. The intracellular concentration of GABA continuously increased during 36–60 h, suddenly declined at 72 h, and later increased to 15.14 ± 0.25 g L^{-1}. The nearly identical level of intracellular and extracellular GABA concentrations indicated that the synthesised GABA could be transported out of cells almost simultaneously.

During the fermentation of the control strain *C. glutamicum* ATCC 13032/pDXW-10, the L-glutamate

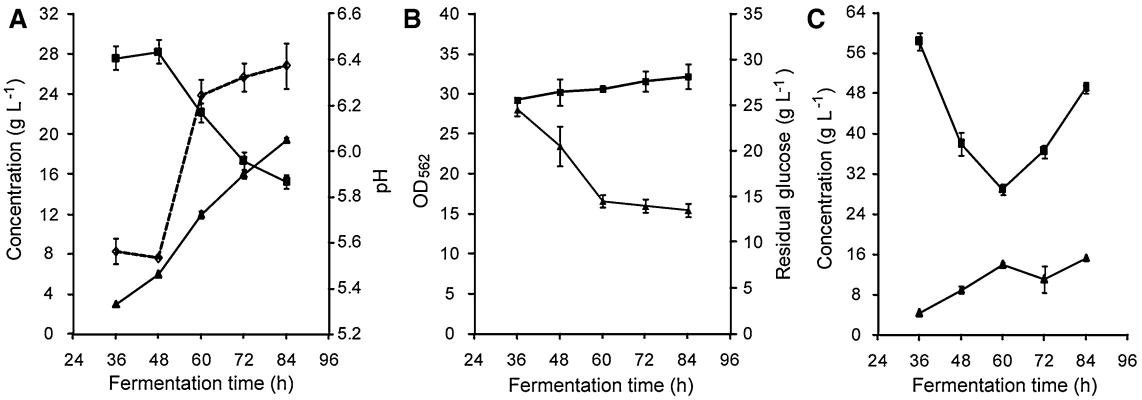

Fig. 2 Time courses of GABA production during the GABA fermentation stage by recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*: **a** extracellular L-glutamate concentration (*solid line with squares*), GABA concentration (*solid line with triangles*) and pH level (*dotted line with diamonds*); **b** cell growth (*squares*) and glucose consumption (*triangles*); **c** intracellular L-glutamate concentration (*squares*) and GABA concentration (*triangles*). Cells were cultivated as described in Fig. 1 by supplementing urea six times. At each time point, the fermentation broth was collected and assayed for L-glutamate, GABA, pH, OD_{562} and glucose. Each point represents the average of three independent experiments

Table 3 GAD activity from crude cell extract of recombinant *C. glutamicum*

GAD activity (U g^{-1})	24 h	36 h	48 h	60 h	72 h	84 h
ATCC 13032/pDXW-10-*gadB1–gadB2*	171.6 ± 3.5	188.3 ± 2.9	259.1 ± 6.4	340.7 ± 4.0	256.6 ± 7.1	210.7 ± 2.9
ATCC 13032/pDXW-10-*gadB2*	151.7 ± 2.6	172.1 ± 4.1	186.3 ± 3.7	194.2 ± 5.6	136.7 ± 2.3	130.9 ± 3.1

Cells cultivated in fermentation medium at 30 °C for a certain time were collected, and GAD activities from the crude cell extracts were assayed as described in "Materials and methods" section. Averages of three independent experiments are shown

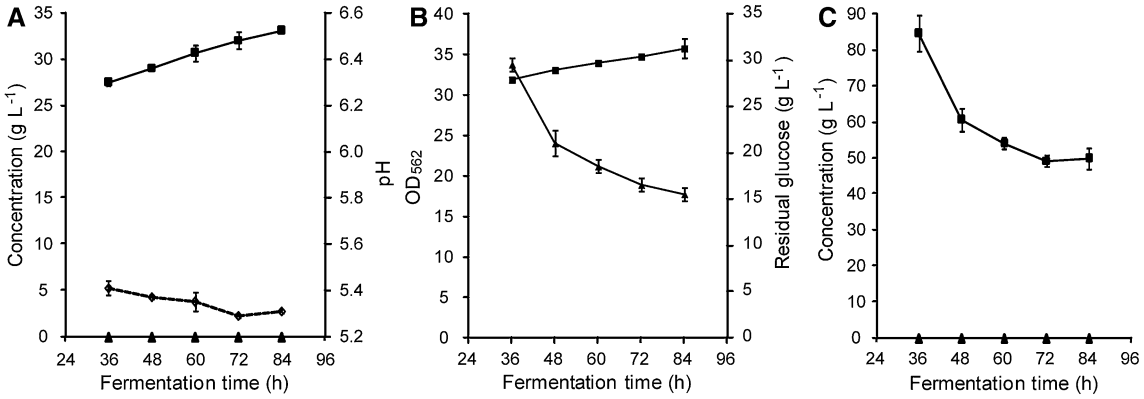

Fig. 3 Time courses of L-glutamate production during the later period of fermentation by the control strain *C. glutamicum* ATCC 13032/pDXW-10: **a** extracellular L-glutamate concentration (*solid line with squares*), GABA concentration (*solid line with triangles*) and pH level (*dotted line with diamonds*); **b** cell growth (*squares*) and glucose consumption (*triangles*); **c** intracellular L-glutamate concentration (*squares*) and GABA concentration (*triangles*). Cells were cultivated, and fermentation broth was collected and assayed as described in Fig. 2. Each point represents the average of three independent experiments

concentration in the fermentation broth reached a similar level (27.49 ± 0.40 g L^{-1}) at 36 h, but increased slightly thereafter to 33.05 ± 0.10 g L^{-1}, whereas the GABA concentration could not be detected at any time (Fig. 3a). The pH level of the fermentation broth decreased slightly from 5.41 to 5.29 over 36–72 h and remained at 5.29–5.31. This strain exhibited a similar growth rate and glucose consumption pattern (Fig. 3b) to ATCC 13032/pDXW-10-*gadB1–gadB2*. The intracellular L-glutamate reached the highest concentration (84.57 ± 5.01 g L^{-1}) at 36 h and decreased continuously to approximately 50 g L^{-1} (Fig. 3c), which was consistently much higher than the intracellular L-glutamate concentration of the *gadB1–gadB2* co-expressing strain (Fig. 2c), most likely because of the continuous conversion of L-glutamate to GABA in the *gadB1–gadB2* co-expressing strain. This concentration was consistently much higher than the extracellular L-glutamate concentration, especially early on (36 h), demonstrating the retarded export of L-glutamate. Simultaneously, the intracellular GABA concentration could not be detected at any time, as was the case for the extracellular GABA concentration (Fig. 3a), indicating that the control strain could not produce GABA.

The progression of the L-glutamate and GABA concentrations and the fermentation broth pH level of recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB2* was similar to that of ATCC 13032/pDXW-10-*gadB1–gadB2*, but the variation range was much smaller (Fig. 4a). Its growth pattern and glucose consumption pattern (Fig. 4b) were similar to those of ATCC 13032/pDXW-10-*gadB1–gadB2*. The L-glutamate concentration decreased to 18.57 ± 1.54 g L^{-1}, and the GABA concentration increased to 11.16 ± 0.68 g L^{-1}. The conversion ratio of L-glutamate to GABA reached 0.46 mol mol^{-1}, a much lower level than that in the *gadB1–gadB2* co-expressing strain, demonstrating that the *gadB1–gadB2* co-expressing strain is more efficient for single-step production of GABA. The GAD activity from the crude cell extracts remained at 131–194 U g^{-1} during 24–84 h of fermentation, which was lower than that of the ATCC 13032/pDXW-10-*gadB1–gadB2* strain (Table 3).

Prolongation of GABA fermentation time in ATCC 13032/pDXW-10-*gadB1–gadB2*

After fermentation for 84 h of recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2*, the GABA concentration reached nearly 20 g L^{-1} and continued to increase. Prolonged fermentation was performed, and the GABA accumulation was investigated.

During fermentation, this strain completed growth at 36 h but continued to consume 15 g L^{-1} of glucose thereafter (Fig. 5a). At 120 h, the optical density of the fermentation broth was 34.1 ± 1.3, and approximately

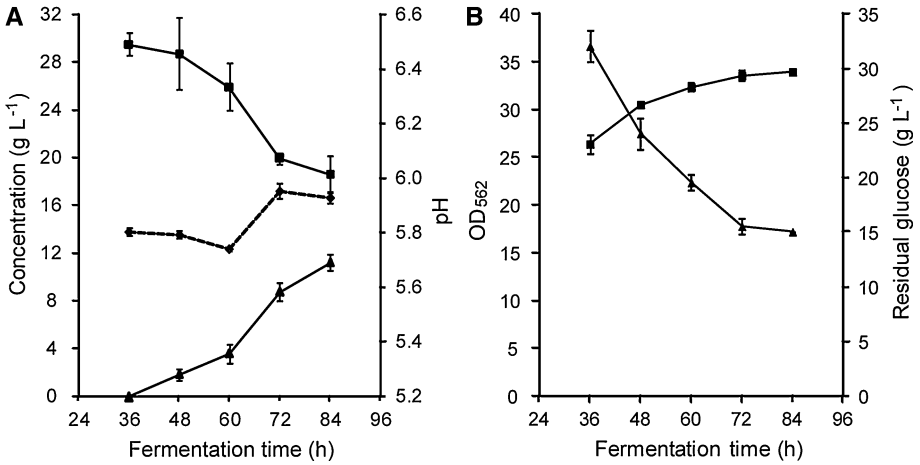

Fig. 4 Time courses of GABA production during the GABA fermentation stage by recombinant *C. glutamicum* ATCC 13032/pDXW-10-*gadB2*: **a** extracellular L-glutamate concentration (*solid line with squares*), GABA concentration (*solid line with triangles*) and pH level (*dotted line with diamonds*); **b** cell growth (*squares*) and glucose consumption (*triangles*). Cells were cultivated, and fermentation broth was collected and assayed as described in Fig. 2. Each point represents the average of three independent experiments

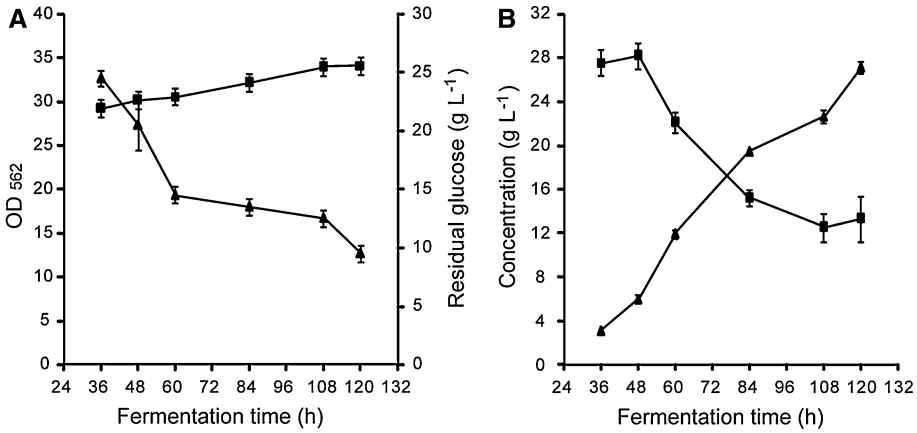

Fig. 5 GABA production by *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2* under optimised condition: **a** cell growth (*squares*) and glucose consumption (*triangles*); **b** extracellular L-glutamate (*squares*) and GABA (*triangles*) production. Cells were cultivated, and fermentation broth was collected and assayed as described in Fig. 2. Averages of three independent experiments are provided

9.5 ± 0.7 g L^{-1} of glucose was not consumed. The GABA concentration in the fermentation broth increased continuously after 84 h, despite a relatively lower yield (0.216 g L^{-1} h^{-1}) than before 84 h (0.342 g L^{-1} h^{-1}) (Fig. 5b). At 120 h, the GABA concentration reached 27.13 ± 0.54 g L^{-1}, and the L-glutamate concentration decreased slightly to 13.27 ± 2.10 g L^{-1}. The conversion ratio of L-glutamate to GABA reached 0.74 mol mol^{-1}, and the total amount of L-glutamate and GABA reached 0.35 M. With extension of the fermentation time, the GABA production increased further.

GABA production of ATCC 13032/pDXW-10-*gadB1–gadB2* in fermentor

C. glutamicum ATCC 13032/pDXW-10-*gadB1–gadB2* was then evaluated in the fermentor. During 8–22 h of fermentation, the pH was controlled at 7.5–8.0 by adding 40 % urea solution, whereas after 36 h, the pH was adjusted to 4.8–5.3 by adding 6 M HCl. The cells grew quickly in the first 12 h, and after 24 h, the cell concentration became stable (Fig. 6a). Glucose was consumed quickly in the first 24 h and was fed after 28 h when the residual glucose in the medium was lower than 20 g L^{-1}. There was no GABA production in the first 24 h, which was similar to the shake flask cultivation. The L-glutamate concentration increased to the highest value (43.64 g L^{-1}) at 36 h and reduced continuously thereafter, whereas the GABA concentration increased continuously during 24–60 h, and after 60 h, it became stable (Fig. 6b). At 60 h of fermentation, the GABA concentration in the fermentation broth reached 26.32 g L^{-1}; the total amount of L-glutamate and GABA reached 0.42 M, and the conversion ratio of L-glutamate to GABA reached 0.60 mol mol^{-1}.

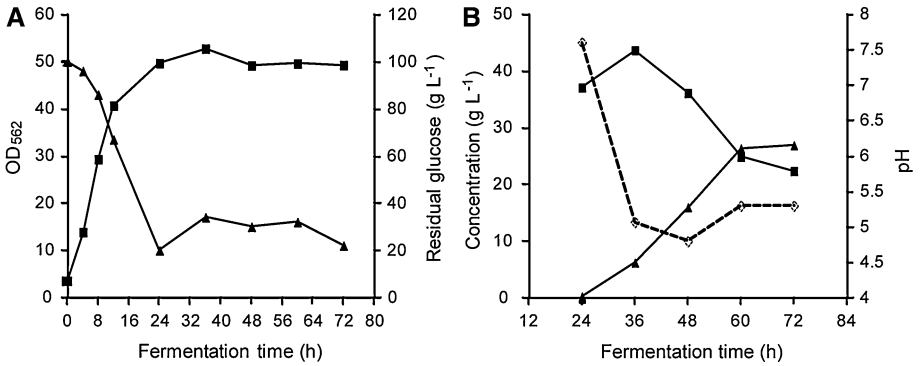

Fig. 6 Fed-batch production of GABA by *C. glutamicum* ATCC 13032/pDXW-10-*gadB1–gadB2* in fermentor: **a** cell growth (*squares*) and residual glucose (*triangles*), **b** L-glutamate production (*solid line with squares*), GABA production (*solid line with triangles*) and pH level (*dotted line with diamonds*). Cells were cultivated, and fermentation broth was collected and assayed as described in Fig. 2

Discussion

In this study, an enhanced single-step GABA production system using a recombinant *C. glutamicum* strain co-expressing two glutamate decarboxylase genes (*gadB1* and *gadB2*) was established. Through optimisation of the urea addition method, the GABA concentration increased from 4.02 ± 0.95 to 19.38 ± 0.17 g L^{-1} after 84 h of fermentation in a shake flask, and after 120 h of fermentation, the production reached 27.13 ± 0.54 g L^{-1}. With cultivation in a fermentor, the GABA concentration reached 26.32 g L^{-1} after 60 h of fermentation. In a recent study, a recombinant *C. glutamicum* strain expressing *gadB* of *E. coli* W3110 accumulated 12.37 ± 0.88 g L^{-1} of GABA after 72 h of fermentation but decreased sharply thereafter [26]. Compared with the sole expression of the *gadB2* gene of *L. brevis* Lb85 or the *gadB* gene of *E. coli* W3110, co-expression of the *gadB1* and *gadB2* genes of *L. brevis* Lb85 contributed significantly to the accumulation of GABA. This accumulation does not require addition of the GABA precursor L-glutamate.

In recombinant *C. glutamicum*, the fermentation conditions for L-glutamate biosynthesis and GABA conversion are notably different, with the former occurring in a neutral condition [1], and the latter occurring in an acidic condition. Generally, L-glutamate is accumulated first and GABA conversion occurs subsequently. The pH level of the fermentation broth at the first fermentation stage is maintained at approximately 7.0 to ensure L-glutamate biosynthesis. With the accumulation of acidic L-glutamate, the pH level decreases accordingly, thus the urea supplementation strategy is adopted during the first 24 h of fermentation because urea can be hydrolysed to ammonia in *C. glutamicum* by the abundant intracellular urease. The released ammonia can neutralise the generated acidic L-glutamate and maintain the pH level. After 24 h, with the L-glutamate concentration increasing to nearly its highest level, the pH level is left to decline automatically and GABA biosynthesis can begin.

Considering that urea supplementation has a great influence on the production of L-glutamate, as well as on the variation of the pH level and the resulting production of GABA, various urea supplementation methods were investigated in this study. By adding urea 3–6 times during 6–24 h of fermentation, for a total amount of 12 g L^{-1}, the pH level during the L-glutamate fermentation stage was maintained in a more suitable range. At the same time, the *gadB* expression was enhanced. At 60 h of fermentation, the in vitro GAD activity of the crude cell extract at pH 4.8 increased to 340.7 ± 4.0 U g^{-1}. After 84 h of fermentation, the total amount of L-glutamate and GABA increased to 30.18 ± 1.33 g L^{-1}, the GABA production increased to 18.66 ± 2.41 g L^{-1} (Fig. 1), and the conversion ratio of L-glutamate to GABA increased to 0.70 mol mol^{-1}, which was much higher than for the general fermentation method or control methods. This result illustrates that frequent supplementation of urea during the L-glutamate fermentation stage results in an appropriate fluctuation of the pH level and efficient L-glutamate accumulation and subsequent GABA conversion.

At the GABA fermentation stage, the pH level varied regularly, being lowest at the beginning, markedly elevated thereafter, and weakly changed at the end (Figs. 2a, 4a). The variation range in the *gadB1–gadB2* co-expressing strain was larger than that in the *gadB2* single-expressing strain, in accordance with its higher GAD expression (Table 3) and higher conversion ratio of L-glutamate to GABA (0.65 mol mol^{-1}) and GABA yield (0.342 g L^{-1} h^{-1}). The pH variation during the GABA fermentation stage can be used as an indicator to reflect the progress of GABA biosynthesis. The varied pH would influence the L-glutamate biosynthesis and GABA conversion. After 108 h, with the increased pH level, L-glutamate began to accumulate again and thus improved the GABA biosynthesis in the *gadB1–gadB2* co-expressing strain (Fig. 5a). The co-expression of *gadB1* and *gadB2*

and the sole expression of *gadB2* in *C. glutamicum* indicate the potential for conferring acid resistance to this microorganism because the pH level in the recombinant strains was not as acidic as for the control strain, and after 36 h of fermentation, the pH level increased in the recombinant strains (Figs. 2a, 4a) but decreased in the control strain (Fig. 3a). A novel L-glutamate producer, *Pantoea ananatis*, which is able to grow in acidic pH, was isolated and sequenced recently [3]. It might be suitable for single-step GABA production following appropriate construction.

In *C. glutamicum*, the L-glutamate secretion or transport system has a significant effect on the L-glutamate production [19]. The control strain ATCC 13032/pDXW-10 showed retarded export of L-glutamate (Fig. 3). In recombinant *C. glutamicum*, L-glutamate does not need to be transported from cells so rapidly, because it is converted to GABA by the intracellular enzymes GadB1 and GadB2, and the GABA is secreted. Compared with the extracellular concentrations, the constantly higher level of intracellular L-glutamate concentration and nearly identical level of intracellular GABA concentration (Fig. 2) indicate the retarded export of L-glutamate and almost simultaneous export of GABA in ATCC 13032/pDXW-10-*gadB1–gadB2*, although a GABA uptake system $GabP_{Cg}$ should exist in *C. glutamicum*, as discovered recently by Zhao et al. [36].

In this study, by modifying the urea supplementation strategy, an improved single-step GABA production system was obtained using a recombinant *C. glutamicum* strain co-expressing *gadB1* and *gadB2* genes from *L. brevis* Lb85. This system could effectively and continuously convert its own accumulated L-glutamate into GABA. Considering that the bioprocessing and downstream technology of L-glutamate production by *C. glutamicum* have matured sufficiently, the recombinant strain has great potential and superiority for industrial application if an L-glutamate-overproducing strain is used as the host bacterium. Because *C. glutamicum* is a microorganism that is generally recognised to be safe, the de novo GABA fermentation system could be applied for production of GABA as a component of functional foods and pharmaceuticals.

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References

1. Asakura Y, Kimura E, Usuda Y, Kawahara Y, Matsui K, Osumi T, Nakamatsu T (2007) Altered metabolic flux due to deletion of *odhA* causes L-glutamate overproduction in *Corynebacterium glutamicum*. Appl Environ Microbiol 73:1308–1319
2. Coleman ST, Fang TK, Rovinsky SA, Turano FJ, Moye-Rowley WS (2001) Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. J Biol Chem 276:244–250
3. Hara Y, Kadotani N, Izui H, Katashkina JI, Kuvaeva TM, Andreeva IG, Golubeva LI, Malko DB, Makeev VJ, Mashko SV, Kozlov YI (2012) The complete genome sequence of *Pantoea ananatis* AJ13355, an organism with great biotechnological potential. Appl Microbiol Biotechnol 93:331–341
4. Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y (2004) Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats. Br J Nutr 92:411–417
5. Hermann T (2003) Industrial production of amino acids by coryneform bacteria. J Biotechnol 104:155–172
6. Hiraga K, Ueno Y, Oda K (2008) Glutamate decarboxylase from *Lactobacillus brevis*: activation by ammonium sulfate. Biosci Biotechnol Biochem 72:1299–1306
7. Ikeda M, Nakagawa S (2003) The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl Microbiol Biotechnol 62:99–109
8. Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Kramer R, Linke B, McHardy AC, Meyer F, Mockel B, Pfefferle W, Puhler A, Rey DA, Ruckert C, Rupp O, Sahm H, Wendisch VF, Wiegrabe I, Tauch A (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol 104:5–25
9. Karatzas KA, Brennan O, Heavin S (2010) Intracellular accumulation of high levels of gamma-aminobutyrate by *Listeria monocytogenes* 10403S in response to low pH: uncoupling of gamma-aminobutyrate synthesis from efflux in a chemically defined medium. Appl Environ Microbiol 76:3529–3537
10. Kennerknecht N, Sahm H, Yen MR, Patek M, Saier MH, Eggeling L (2002) Export of L-isoleucine from *Corynebacterium glutamicum*: a two-gene-encoded member of a new translocator family. J Bacteriol 184:3947–3956
11. Kirchner O, Tauch A (2003) Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J Biotechnol 104:287–299
12. Koros A, Varga ZS, Molnar P (2008) Simultaneous analysis of amino acids and amines as their *o*-phthalaldehyde-ethanethiol-9-fluorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography. J Chromatogr A 1203:146–152
13. Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1–8
14. Li HX, Cao YS (2010) Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino Acids 39:1107–1116
15. Li HX, Qiu T, Gao DD, Cao YS (2010) Medium optimization for production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912. Amino Acids 38:1439–1445
16. Li HX, Qiu T, Huang GD, Cao YS (2010) Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. Microb Cell Fact 9:85
17. Okada T, Sugishita T, Murakami T, Murai H, Saikusa T, Horino T, Onoda A, Kajimoto O, Takahashi R, Takahashi T (2000) Effect of the defatted rice germ enriched with GABA for sleeplessness, depression, autonomic disorder by oral administration. J Jpn Soc Food Sci 47:596–603
18. Park KB, Oh SH (2007) Cloning, sequencing and expression of a novel glutamate decarboxylase gene from a newly isolated lactic acid bacterium, *Lactobacillus brevis* OPK-3. Bioresour Technol 98:312–319
19. Rehm N, Burkovski A (2011) Engineering of nitrogen metabolism and its regulation in *Corynebacterium glutamicum*: influence

on amino acid pools and production. Appl Microbiol Biotechnol 89:239–248
20. Sanchez B, Champomier-Verges MC, Collado Mdel C, Anglade P, Baraige F, Sanz Y, de los Reyes-Gavilan CG, Margolles A, Zagorec M (2007) Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype longum. Appl Environ Microbiol 70:6450–6459
21. Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J (1998) A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. Mol Microbiol 27:299–310
22. Schuller HM, Al-Wadei HA, Majidi M (2008) Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. Carcinogenesis 29:1979–1985
23. Shi F, Li YX (2011) Synthesis of gamma-aminobutyric acid by expressing *Lactobacillus brevis*-derived glutamate decarboxylase in the *Corynebacterium glutamicum* strain ATCC13032. Biotechnol Lett 33:2469–2474
24. Shiio I, Ozaki H (1970) Regulation of nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Brevibacterium flavum*, a glutamate-producing bacterium. J Biochem 68:633–647
25. Siragusa S, De Angelis M, Di Cagno R, Rizzello CG, Coda R, Gobbetti M (2007) Synthesis of γ-amino butyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Environ Microbiol 73:7283–7290
26. Takahashi C, Shirakawa J, Tsuchidate T, Okai N, Hatada K, Nakayama H, Tateno T, Ogino C, Kondo A (2012) Robust production of gamma-amino butyric acid using recombinant *Corynebacterium glutamicum* expressing glutamate decarboxylase from *Escherichia coli*. Enzym Microb Tech 51:171–176
27. Tamura T, Noda M, Ozaki M, Maruyama M, Matoba Y, Kumagai T, Sugiyama M (2010) Establishment of an efficient fermentation system of gamma-aminobutyric acid by a lactic acid bacterium, *Enterococcus avium* G-15, isolated from carrot leaves. Bio Pharm Bull 33:1673–1679
28. Tramonti A, De Canio M, Delany I, Scarlato V, De Biase D (2006) Mechanisms of transcription activation exerted by GadX and GadW at the *gadA* and *gadBC* gene promoters of the glutamate-based acid resistance system in *Escherichia coli*. J Bacteriol 188:8118–8127
29. Tujioka K, Ohsumi M, Horie K, Kim M, Hayase K, Yokogoshi H (2009) Dietary gamma-aminobutyric acid affects the brain protein synthesis rate in ovariectomized female rats. J Nutr Sci Vitaminol 55:75–80
30. Ueno H (2000) Enzymatic and structural aspects on glutamate decarboxylase. J Mol Catal 10:67–79
31. Wong CG, Bottiglieri T, Snead OC (2003) GABA, gamma-hydroxybutyric acid, and neurological disease. Ann Neurol 54:S3–S12
32. Xu DQ, Tan YZ, Huan XJ, Hu XQ, Wang XY (2010) Construction of a novel shuttle vector for use in *Brevibacterium flavum*, an industrial amino acid producer. J Microbiol Methods 80:86–92
33. Xu DQ, Tan YZ, Shi F, Wang XY (2010) An improved shuttle vector constructed for metabolic engineering research in *Corynebacterium glutamicum*. Plasmid 64:85–91
34. Yao WJ, Deng XZ, Zhong H, Liu M, Zheng P, Sun ZH, Zhang Y (2009) Double deletion of *dtsR1* and *pyc* induce efficient L-glutamate overproduction in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 36:911–921
35. Zhang Y, Song L, Gao Q, Yu SM, Li L, Gao NF (2012) The two-step biotransformation of monosodium glutamate to GABA by *Lactobacillus brevis* growing and resting cells. Appl Microbiol Biotechnol 94:1619–1627
36. Zhao Z, Ding JY, Ma WH, Zhou NY, Liu SJ (2012) Identification and characterization of γ-aminobutyric acid uptake system $GabP_{Cg}$ (NCgl0464) in *Corynebacterium glutamicum*. Appl Environ Microbiol 78:2596–2601