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ORIGINAL RESEARCH PAPER

Specific γ-aminobutyric acid decomposition by *gabP* and *gabT* under neutral pH in recombinant *Corynebacterium glutamicum*

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Abstract
Objectives *Corynebacterium glutamicum* that expresses the exogenous L-glutamate decarboxylase (GAD) gene can synthesize γ-aminobutyric acid (GABA). To prevent GABA decomposition in the recombinant *C. glutamicum* GAD strain, GABA uptake and the GABA shunt pathway were blocked.
Results GABA uptake is catalyzed by GABA permease encoded by *gabP*. The first reaction of the GABA shunt pathway is catalyzed by the GABA transaminase encoded by *gabT*. Initially, the effects of pH on GABA decomposition in recombinant *C. glutamicum* co-expressing two GAD genes (*gadB1* and *gadB2*) were analyzed, demonstrating that GABA could be decomposed under neutral pH. Next, the *gabP* and *gabT* were individually deleted, and the GABA production of the related GAD strains was investigated by controlling the pH of the final fermentation stage at a neutral state. During this stage, the GABA concentration of the *gabT*-deleted GAD strain decreased from 23.9 ± 1.8 to 17.7 ± 0.7 g/l. However, the GABA concentration of the *gabP*-deleted GAD strain remained at 18.6–19.4 g/l.
Conclusion This study demonstrated that GABA was decomposed under neutral pH and that the deletion of *gabP* could effectively alleviate GABA decomposition in *C. glutamicum*.

Keywords γ-Aminobutyric acid · *Corynebacterium glutamicum* · GABA decomposition · *gabP* · *gabT*

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Introduction

Gamma-aminobutyric acid (GABA) is widely distributed in nature, from microorganisms to plants and mammals, in which GABA biosynthesis and GABA catabolism, along with its export and uptake system, constitute GABA metabolism. The biosynthesis of GABA is fulfilled by the irreversible decarboxylation of L-glutamate by L-glutamate decarboxylase (GAD) in some species. Additionally, some species can take up extracellular GABA from the environment by a GABA-specific permease. The synthesized or obtained GABA can be decomposed via the GABA

shunt pathway (Feehily and Karatzas 2013), which is a pathway involving the conversion of GABA to succinic semialdehyde (SSA) by GABA transaminase (GABA-T) followed by the conversion of SSA to succinate by SSA dehydrogenase (SSADH). *Corynebacterium glutamicum* is the main producer of L-glutamate. Although no gene encoding GAD was identified in *C. glutamicum*, a GABA-T gene (*gabT*), two SSADH genes (*gabD*) and a GABA permease gene (*gabP*) were annotated and has indicated that *C. glutamicum* could metabolize GABA (Zhao et al. 2012).

By the expression of exogenous GAD gene(s), *C. glutamicum* can synthesize GABA from its own accumulated L-glutamate (Shi and Li 2011; Shi et al. 2013). However, GABA may sometimes be degraded, as indicated by the sharply decreased GABA concentration after fermentation for 72 h in *C. glutamicum* expressing *gadB* of *Escherichia coli* W3110 (Takahashi et al. 2012). The metabolic pathway of GABA in recombinant *C. glutamicum* is shown in Fig. 1. The GABA permease (GabP) identified in *C. glutamicum* is considered to be the only GABA-specific transport system in *C. glutamicum* (Zhao et al. 2012). It plays a major role in GABA uptake and is essential to *C. glutamicum* growing in the presence of GABA. GABA-T is the first enzyme of the GABA shunt pathway. It can use GABA as an amino donor to form SSA and 2-oxoglutarate as an amino acceptor to form L-glutamate. To avoid the degradation of GABA in recombinant *C. glutamicum*, the GABA shunt pathway and GABA uptake system should be blocked.

In the present study, the *gabT* and *gabP* genes were individually deleted in *C. glutamicum* ATCC13032 to prevent GABA decomposition and GABA uptake. Next, a plasmid expressing two GAD genes (*gadB1* and *gadB2*) was transformed into the deletion strains and wild-type strain. The effect of pH on GABA decomposition was investigated in the wild-type GAD strain. GABA fermentation in the *gabT*-deleted GAD strain and the *gabP*-deleted GAD strain was then investigated.

Materials and methods

Strains, media and growth conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* JM 109 was used as the host for constructing and propagating the plasmids. *E. coli* was grown in lysogeny broth (LB) at 37 °C and 200 rpm. *C. glutamicum* ATCC13032, a L-glutamate-producing strain, was used for gene deletion, expressing *gadB1*

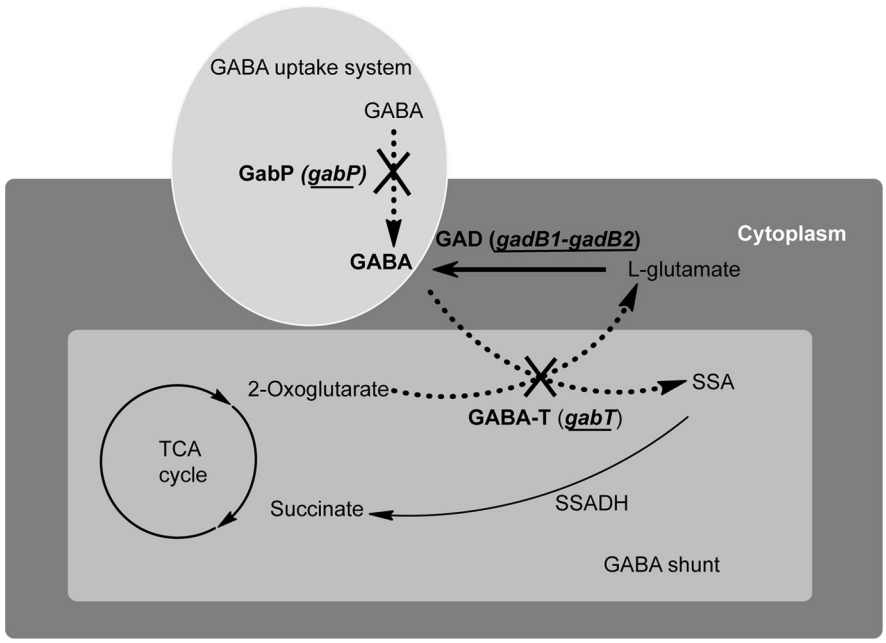

Fig. 1 The metabolic pathway of GABA in recombinant *C. glutamicum* that consists of the GABA shunt pathway and GABA uptake system. The GABA shunt pathway consists of two enzymatic steps that are catalyzed by GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). The GABA uptake system is carried out by the GABA permease (GabP) that transports GABA into the cytoplasm. *TCA* tricarboxylic acid cycle

and *gadB2* genes and producing GABA. *C. glutamicum* was grown in LBG medium (LB supplemented with 5 g glucose/l) at 200 rpm and 30 °C. Epo medium (LB supplemented with 30 g glycine/l and 0.1 % Tween 80) and LBHIS medium (5 g Tryptone/l, 5 g NaCl/l, 2.5 g yeast extract/l, 18.5 g brain heart infusion powder/l and 91 g sorbitol/l) were used for *C. glutamicum* transformation (Wang et al. 2015). When necessary, 30 μg kanamycin/ml or 15 μg chloramphenicol/ml was added to the media.

Gene deletion and construction of recombinant *C. glutamicum* ATCC13032

Based on the homologous recombination and site-specific recombination system, gene disruption in *C. glutamicum* was performed according to a previous study (Hu et al. 2013). First, the deletion plasmid was constructed. Fragment *gabT*U, located upstream of the gene *gabT*, and fragment gab*T*D, located downstream of the gene *gabT*, were amplified from the genomic DNA of *C. glutamicum* ATCC13032. Fragment *loxL-kan-loxR*, which contains two recognition sites by Cre, was amplified from plasmid pDTW202. The three DNA fragments were ligated into plasmid pBluscriptII SK(+), resulting in plasmid pSYN1 (Fig. 2a). Similarly, plasmid pSYN2, in which fragments *gabT*U and *gabT*D were replaced by *gabP*U and *gabP*D, respectively, was constructed and is shown in Fig. 2a. Second, plasmid pSYN1 or pSYN2 was transformed into *C. glutamicum* ATCC13032 to allow for homologous recombination, as illustrated previously (Wang et al. 2015). The recombinant strains were selected by growth on LBHIS media supplemented with 30 μg kanamycin/ml. Plasmid pDTW-109 was transformed into the recombinant strain to remove the *kan* gene from the chromosome and was selected on LBHIS agar containing 15 μg chloramphenicol/ml. Finally, cells were cultured at 37 °C to remove the pDTW-109 plasmid. The cells that grew on the plate without antibiotics but not on the plate with kanamycin or chloramphenicol were chosen as the mutant strain ATCC13032 Δ*gabT*, renamed as SYN101, and the mutant strain ATCC13032 Δ*gabP*, renamed as SYN102. All of the strains were verified by restriction enzyme digestion or target gene amplification.

The *gadB1* and *gadB2* coexpression plasmid (pJYW-4-*gadB1-gadB2*) constructed previously

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Sources
Strains		
JM 109	Wild type *E. coli*	Novagen
C. glutamicum ATCC13032	Wild type *C. glutamicum*	ATCC
SYN101	*gabT* deletion mutant of *C. glutamicum* ATCC13032	This work
SYN102	*gabP* deletion mutant of *C. glutamicum* ATCC13032	This work
SYN200	ATCC13032 harbouring pJYW-4-*gadB1-gadB2*	This work
SYN201	SYN101 harbouring pJYW-4-*gadB1-gadB2*	This work
SYN202	SYN102 harbouring pJYW-4-*gadB1-gadB2*	This work
Plasmids		
pBluescript II SK(+)	Cloning vector, Amp^r	Stratagene
pDTW202	Derived from pBluescript II SK(+) by inserting the segment *loxL-kan-loxR*	Hu et al. (2013)
pSYN1	Derived from pBluescript II SK(+) by inserting the segment *gabT*U-*loxL-kan-loxR-gabT*D	This work
pSYN2	Derived from pBluescript II SK(+) by inserting the segment *gabP*U-*loxL-kan-loxR-gabP*D	This work
pDTW109	Vector inserting a *cre* gene, Cm^r	Hu et al. (2013)
pJYW-4-*gadB1-gadB2*	pJYW-4 harbouring *gadB1-gadB2*	Wang et al. (2015)

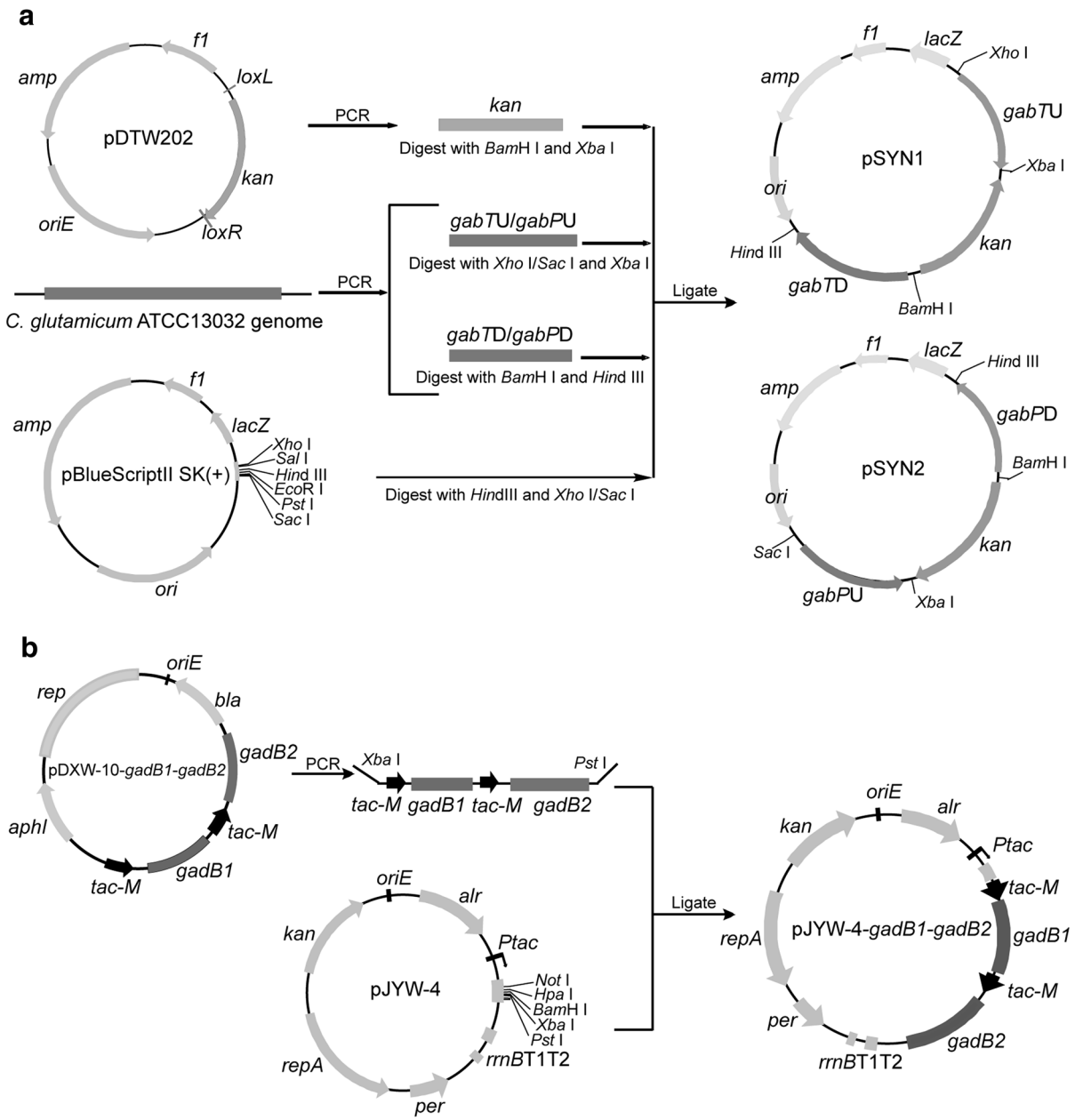

Fig. 2 Construction of plasmids. **a** Construction of the deletion plasmids pSYN1 and pSYN2; **b** construction of the expression plasmid pJYW-4-*gadB1-gadB2*

(Wang et al. 2015, Fig. 2b) was finally transformed into the deletion mutants SYN101 and SYN102 and wild-type ATCC13032, generating three new recombinant *C. glutamicum* strains, SYN101/pJYW-4-*gadB1-gadB2*, SYN102/pJYW-4-*gadB1-gadB2* and ATCC13032/pJYW-4-*gadB1-gadB2*, renamed as SYN201, SYN202 and SYN200, respectively.

Fermentation of different *C. glutamicum* strains in a fermentor

L-Glutamate and GABA production in the wild-type *C. glutamicum* strain ATCC13032, wild-type GAD strain SYN200, *gabT*-deleted GAD strain SYN201 and *gabP*-deleted GAD strain SYN202 were evaluated

by fermentation in a fermentor. First, 100 ml seed medium (25 g glucose/l, 30 g corn steep liquor/l, 8 g urea/l, 1 g $K_2HPO_4 \cdot 3H_2O$/l, 0.2 g $MgSO_4$/l, pH 7.0–7.2) was prepared in flasks at 30 °C and 110 rpm for 7 h and was then transferred to a 3 l fermentor (BioFlo 110; New Brunswick Scientific, USA) containing 1.2 l fermentation medium (100 g glucose/l, 2 g corn steep liquor/l, 2 g $K_2HPO_4 \cdot 3H_2O$/l, 0.4 g $MgSO_4$/l, 0.2 g $MnSO_4 \cdot H_2O$/l and 0.29 g $FeSO_4 \cdot 7H_2O$/l). Four g urea/l was added at the start of fermentation.

During fermentation, the temperature and aeration rates were kept at 30 °C and 1.5 vvm, respectively. The dissolved O_2 level was controlled at 30 % by coupling with the agitation speed. From 12 h of fermentation, glucose was fed by a peristaltic pump when the residual glucose in the medium decreased to below 20 g/l. The pH was controlled differently during fermentation. For the fermentation of SYN200, the pH was controlled at 7–7.5 by adding 10 % (w/v) urea at 0–36 h and at pH 5–5.5 by adding 2 M HCl at 36–60 h. However, at 60–72 h, the pH was controlled at 7.5–8 by adding 2 M NaOH (neutral sample) and at 5–5.5 by adding 2 M HCl (acidic sample). For the fermentation of ATCC13032, SYN201 and SYN202, the pH was controlled at 7–7.5 for 0–36 h, at 5–5.5 for 36–72 h and at 7.5–8 for 72–84 h. Samples were taken approx. every 12 h to determine the OD_{562} value and residual glucose. GABA and L-glutamate concentrations were assayed using reversed-phase HPLC.

Cultivation of wild-type *C. glutamicum* with GABA as the sole carbon and nitrogen source

Cells of wild-type *C. glutamicum* ATCC13032 were precultured in seed medium at 30 °C and 110 rpm for 7 h. The precultured broth was inoculated into 20 ml fermentation medium in a 500 ml Erlenmeyer flask to a final OD_{562} of 1.8 and was shaken by a reciprocating shaker at 30 °C and 110 rpm for 36 h. Next, 4 g urea/l was added at the start of fermentation and 2 g urea/l was added to the culture every 3.5 h for 10–24 h of fermentation to maintain the neutral condition. The total cells were harvested at 36 h, transferred into 20 ml GABA medium (20 g GABA/l, 2 g K_2HPO_4-$3H_2O$/l, 0.4 g $MgSO_4$/l, 0.2 g $MnSO_4 \cdot H_2O$/l and 0.29 g $FeSO_4 \cdot 7H_2O$/l, pH 7.5) with GABA as the sole carbon and nitrogen source and cultivated at 30 °C and 110 rpm for 36 h. During cultivation, the pH was adjusted to 7.5–8 every 12 h by adding 2 M NaOH. Samples were taken approx. every 12 h to determine the L-glutamate and GABA concentrations.

Results

To investigate their functions, the *gabT* and *gabP* genes of ATCC13032 were individually deleted, yielding the strains SYN101 and SYN102, respectively. Next, plasmid pJYW-4-*gadB1-gadB2* was transformed into SYN101, SYN102 and ATCC13032, generating three new recombinant strains, SYN201, SYN202 and SYN200, respectively.

Effect of pH on GABA decomposition in recombinant *C. glutamicum* SYN200

Normally, there are two stages—i.e., the L-glutamate fermentation stage and the GABA fermentation stage—during the fermentation of GABA in recombinant *C. glutamicum*. At 0–36 h (the L-glutamate fermentation stage), the pH was controlled at 7–7.5 to ensure cell growth and L-glutamate biosynthesis. After 36 h (the GABA fermentation stage), the pH was adjusted to 5–5.5 to ensure GABA synthesis because GAD exhibits optimum activity at pH 4.5–5 and significantly loses activity at near-neutral pH (Shi et al. 2014). Accompanied by the conversion of L-glutamate to GABA by GAD, a proton was consumed that might increase the pH if the pH latter was not controlled. However, under neutral to slightly alkaline pH conditions (pH 7.5–8), GabP and GABA-T of *C. glutamicum* may be active (Zhao et al. 2012; Liu et al. 2005). GabP can take up extracellular GABA into cells. GABA-T, as the first enzyme of the GABA shunt pathway, can catalyze the conversion of GABA to SSA with the concomitant conversion of 2-oxoglutarate to L-glutamate. Both the active GABA uptake and GABA decomposition system can make GABA production decrease in recombinant *C. glutamicum*. To understand whether pH influences GABA decomposition, the pH was adjusted to 7.5–8 (neutral) and 5–5.5 (acidic) at the third stage (final stage, 60–72 h) of SYN200 fermentation, and GABA production under the two conditions was compared.

During fermentation, cells grew quickly during the first 12 h and the cell concentration became stable after 24 h (Fig. 3a). Glucose was consumed quickly during the first 24 h (Fig. 3b) and was supplemented when the residual glucose in the medium was lower than 20 g/l. L-Glutamate accumulated quickly during the first 36 h, decreased sharply during 36–60 h and was almost converted to GABA at 60 h (Fig. 3c). Accordingly, the GABA concentration increased quickly from 36 to 60 h (Fig. 3c). From 60 to 72 h, glutamate decreased from 2.8 ± 2.4 to 1.2 ± 0.3 g/l, and GABA further increased from 14.9 ± 0 to 17.4 ± 1.2 g/l under acidic conditions (Fig. 3c). However, under neutral conditions, glutamate increased from 4 ± 0.3 to 7 ± 0.8 g/l, and GABA decreased from 16.5 ± 0.7 to 12.6 ± 0.8 g/l from 60 to 72 h, indicating that GABA might be decomposed under neutral conditions. Additionally, the total amounts of L-glutamate and GABA of SYN200 under the two conditions were almost invariable from 60 to 72 h (Fig. 3d).

GABA decomposition in wild-type *C. glutamicum* under neutral conditions

To confirm the decomposition of GABA under neutral conditions in *C. glutamicum*, GABA and L-glutamate concentrations were examined during the cultivation of wild-type *C. glutamicum* ATCC13032 with GABA as the sole carbon and nitrogen source. After cultivation for 36 h at pH 7.5–8, GABA decreased from 20 to 16.5 ± 0.5 g/l and L-glutamate increased from 0 to

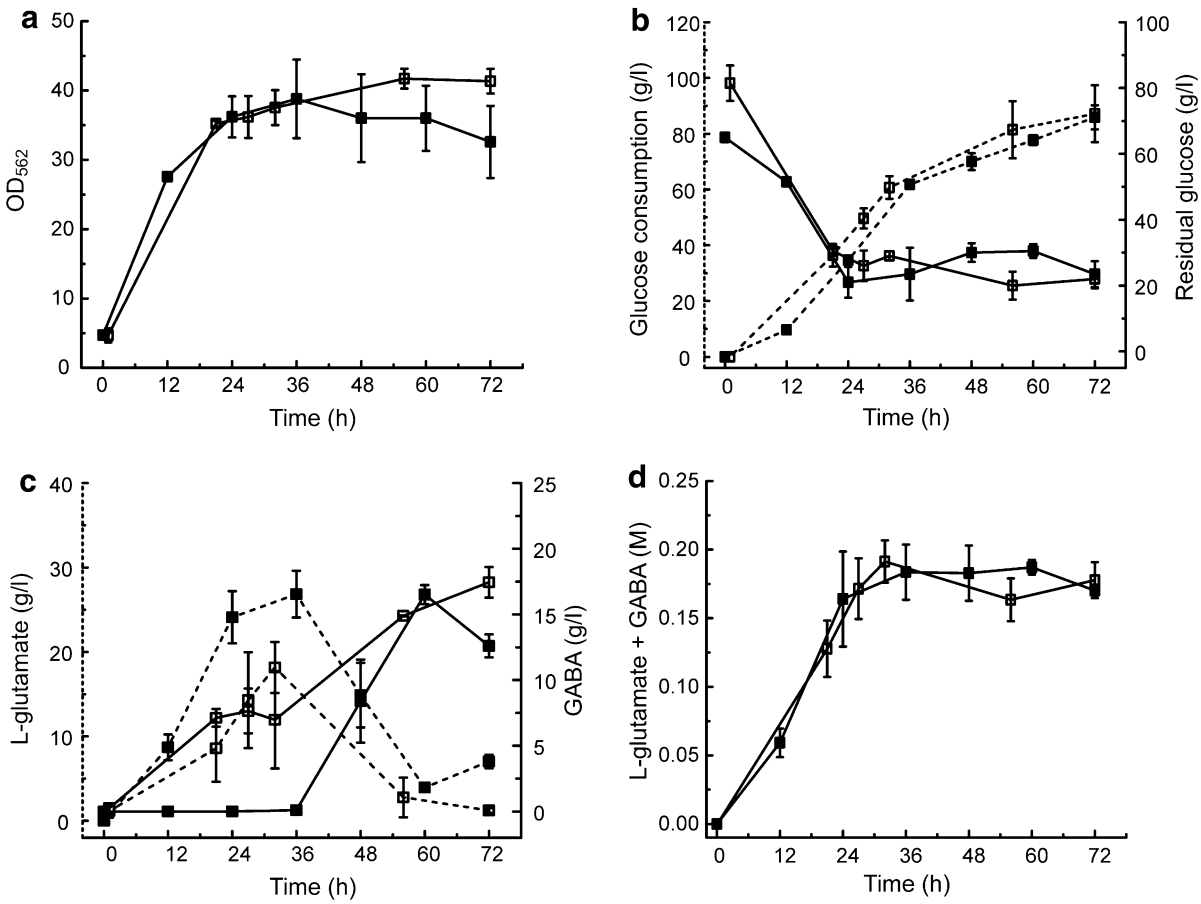

Fig. 3 Time course of GABA fermentation by recombinant *C. glutamicum* SYN200 under controlled pH. **a** Cell growth; **b** glucose consumption (*dotted lines*) and residual glucose (*solid lines*); **c** production of L-glutamate (*dotted lines*) and GABA (*solid lines*); **d** total amount of L-glutamate and GABA. *Filled squares*, pH was controlled at 7.5–8.0 from 60 to 72 h; *Open squares*, pH was controlled at 5.0–5.5 from 60 to 72 h

1.6 ± 0.2 g/l (Fig. 4), demonstrating that GABA was decomposed under neutral conditions.

Effect of the deletion of *gabT* and *gabP* on GABA decomposition in recombinant *C. glutamicum*

To understand whether the deletion of *gabT* and *gabP* alleviates GABA decomposition, the pH was adjusted to 7.5–8 (neutral) at the final stage (72–84 h) of ATCC13032, SYN201 and SYN202 fermentation, and GABA production was investigated.

During the fermentation of the wild-type *C. glutamicum* strain ATCC13032, *gabT*-deleted GAD strain SYN201 and *gabP*-deleted GAD strain SYN202, cells grew quickly during the first 12 h and the cell concentration became stable after 24 h (Fig. 5a). Glucose was consumed quickly during the first 24 h (Fig. 5b) and was supplemented when the residual glucose in the medium was lower than 20 g/l. L-Glutamate accumulated quickly during the first 36 h and decreased from 36 to 60 h in both the SYN201 and SYN202 strains; however, at 60 h, a large amount of L-glutamate remained, and only at 72 h was L-glutamate transformed nearly completely to GABA (Fig. 5c). In the strain SYN202, GABA increased continuously from 36 to 72 h and then slightly decreased from 19.4 ± 1.7 to 18.6 ± 0.6 g/l from 72 to 84 h (Fig. 5c). The GABA decrement at the final stage of SYN202 fermentation was significantly lower than that of SYN200. In the strain SYN201, GABA increased continuously from 36 to 72 h, but decreased from 23.9 ± 1.8 to 17.7 ± 0.7 g/l from 72 to 84 h; meanwhile, the L-glutamate concentration increased from 2.9 ± 0.5 to 13.7 ± 1.2 g/l during 72–84 h (Fig. 5c). The total amount of L-glutamate and GABA in SYN201 and SYN202 was almost invariable from 72 to 84 h (Fig. 5d). In the wild-type strain ATCC13032, L-glutamate accumulated during the first 36 h and was maintained at that level thereafter, while no GABA was produced during all the time.

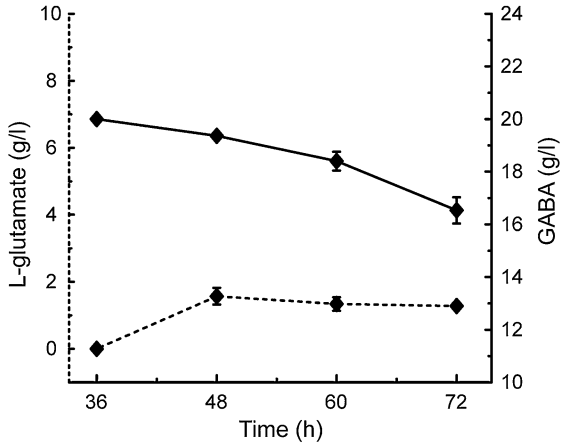

Fig. 4 Time course of the L-glutamate and GABA concentrations of wild-type *C. glutamicum* ATCC13032 with GABA as the sole carbon and nitrogen source at neutral pH. *Solid line*, GABA; *dotted line*, L-glutamate

Discussion

In this study, the effect of pH on GABA decomposition was first investigated by adjusting the pH of the final fermentation stage (60–72 h) to 7.5 to 8 and 5 to 5.5 in recombinant *C. glutamicum* ATCC13032 co-expressing two GAD genes (SYN200). GABA decreased by 3.9 ± 0.1 g/l from 60 to 72 h at pH 7.5–8.0, a level that was drastically reduced compared with the level at pH 5–5.5, which was increased by 2.6 ± 1.2 g/l (Fig. 3c), indicating that GABA could be decomposed at pH 7.5–8.

Wild-type *C. glutamicum* cannot synthesize GABA by itself, but can take up and utilize GABA as a carbon and/or nitrogen source by GABA permease and the GABA shunt pathway. GABA-T, which is the first enzyme of the GABA shunt pathway (Fig. 1), exhibits the highest activity at pH 7.8 (Liu et al. 2005). GABA permease GabP is active under neutral to slightly alkaline conditions and uptakes extracellular GABA into cells in which GABA can be further decomposed by the GABA shunt pathway (Zhao et al. 2012). In the recombinant *C. glutamicum* strain SYN200, the GABA concentration decreased when the pH was adjusted to 7.5–8 from 60 to 72 h (Fig. 3c). Meanwhile, the total amount of L-glutamate and GABA was almost constant (Fig. 3d) but the L-glutamate concentration was increased at this stage (Fig. 3c). The decrease in the GABA concentration in SYN200 may be due to GABA decomposition by the GABA shunt pathway and GABA uptake by GabP under pH 7.5–8.0. The decomposition of GABA was further confirmed in the wild-type *C. glutamicum* strain ATCC13032 under neutral pH (Fig. 4).

gabP encodes the only permease (GabP) of the GABA uptake system in *C. glutamicum* ATCC13032. GabP can take up extracellular GABA into cells. GabP of *C. glutamicum* was more active under neutral to

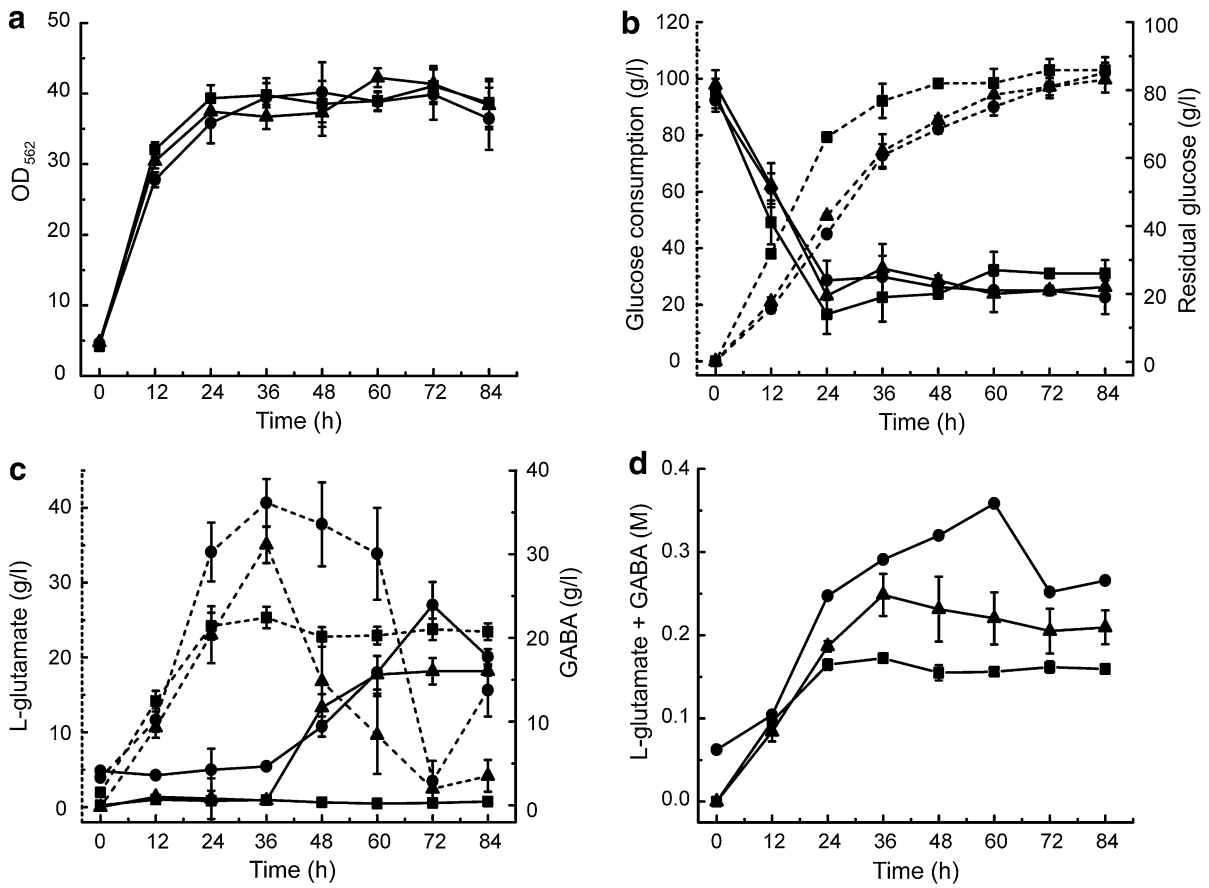

Fig. 5 Time course of GABA fermentation by recombinant *C. glutamicum* SYN201 and SYN202 and wild-type ATCC13032 under controlled pH. The pH was controlled at 7.5–8.0 from 72 to 84 h. **a** Cell growth; **b** glucose consumption (*dotted lines*) and residual glucose (*solid lines*); **c** production of L-glutamate (*dotted lines*) and GABA (*solid lines*); **d** total amount of L-glutamate and GABA. *Circles*, SYN201; *triangles*, SYN202; *squares*, ATCC13032

slightly alkaline conditions and exhibited both a high affinity and activity on GABA at pH 6.5–8.0 (Zhao et al. 2012). After the *gabP* gene was deleted, GABA concentration did not decrease when the pH was controlled at 7.5–8 (Fig. 5c), indicating that the deletion of *gabP* can effectively prevent the uptake of GABA and its entry into the GABA shunt pathway.

GABA-T and SSADH are encoded by *gabT* and *gabD* in *E. coli*. However, GABA-T and SSADH activities persist even in the *gabT*- and *gabD*-deleted strains and were induced when putrescine in the media acted as a nitrogen source (Schneider et al. 2002). PuuE and YneI, identified as the second GABA-T and second SSADH, constitute a putrescine-inducible pathway that degrades GABA to succinate (Kurihara et al. 2010). A similar result has also been confirmed in *Listeria monocytogenes* EGD-e strain with a GABA-T-encoding gene being deleted (Feehily et al. 2013). As GABA-T activity can still be detected, this suggests that an alternative GABA-T activity is present in the cells. *gabT* encodes GABA-T in *C. glutamicum* ATCC13032, which catalyzes the conversion of GABA to SSA. It is possibly the only gene that encodes GABA transaminase in *C. glutamicum* ATCC13032. However, after the *gabT* gene was deleted, the GABA concentration of SYN201 still decreased significantly when pH was controlled at 7.5–8 (Fig. 5c), indicating that the deletion of the *gabT* gene could not prevent GABA from decomposing. It can be inferred that other transaminases acting on GABA might exist in *C. glutamicum* ATCC13032. However, this remains to be studied in detail.

The decrease in the GABA concentration in *C. glutamicum* at pH 7.5–8 was effectively alleviated

by deleting the *gabP* gene. However, the decrease in GABA was not prevented in *C. glutamicum* by deleting the *gabT* gene. To further prevent GABA from decomposing, other transaminases acting on GABA will be weakened or deleted. Meanwhile, the double deletion mutant of *gabT* and *gabP* can be constructed, and its GABA concentrations during fermentation will be determined in the future. In a recent study, a *C. glutamicum* strain expressing an *E. coli* GAD mutant produced 5.9 ± 0.35 g GABA/l in flask cultivation at pH 7.0, a level that was 17-fold higher than the strain expressing wild-type GAD (Choi et al. 2015). Accordingly, the GAD mutant active in the expanded pH range could be further employed in the *gabP*-deleted strain SYN102 to provide a balanced condition for cell growth, GABA synthesis and GABA decomposition.

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