World J Microbiol Biotechnol (2017) 33:122
DOI 10.1007/s11274-017-2289-3

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ORIGINAL PAPER

Overexpression of *ppc* or deletion of *mdh* for improving production of γ-aminobutyric acid in recombinant *Corynebacterium glutamicum*

Feng Shi[1,2,3] · Ming Zhang[1,2] · Yongfu Li[4]

Received: 1 February 2017 / Accepted: 18 May 2017 / Published online: 22 May 2017
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Abstract L-Glutamate decarboxylase (GAD) transforms L-glutamate into γ-aminobutyric acid (GABA). *Corynebacterium glutamicum* that expresses exogenous GAD gene(s) can synthesize GABA from its own produced L-glutamate. To enhance GABA production in recombinant *C. glutamicum* strain SH, metabolic engineering strategies were used to improve the supply of the GABA precursor, L-glutamate. Five new strains were constructed here. First, the *ppc* gene was coexpressed with two GAD genes (*gadB1* and *gadB2*). Then, the *mdh* gene was deleted in *C. glutamicum* SH. Next, *gadB1-gadB2* and *gadB1-gadB2-ppc* co-expression plasmids were transformed into *C. glutamicum* strains SH and Δ*mdh*, resulting in four recombinant GAD strains SE1, SE2, SDE1, and SDE2, respectively. Finally, the *mdh* gene was overexpressed in *mdh*-deleted SDE1, generating the *mdh*-complemented GAD strain SDE3. After fermenting for 72 h, GABA production increased to 26.3 ± 3.4, 24.8 ± 0.7, and 25.5 ± 3.3 g/L in *ppc*-overexpressed SE2, *mdh*-deleted SDE1, and *mdh*-deleted *ppc*-overexpressed SDE2, respectively, which was higher than that in the control GAD strain SE1 (22.7 ± 0.5 g/L). While in the *mdh*-complemented SDE3, GABA production decreased to 20.0 ± 0.6 g/L. This study demonstrates that the recombinant strains SE2, SDE1, and SDE2 can be used as candidates for GABA production.

Keywords γ-Aminobutyrate acid · *Corynebacterium glutamicum* · Glutamate decarboxylase · *Mdh* · Oxaloacetate · *Ppc*

Introduction

Gamma-aminobutyric acid (GABA), a non-protein amino acid, is a well-characterized inhibitory neurotransmitter in mammals and has been applied as a bioactive component in functional foods, feeds, and pharmaceuticals due to its various physiological functions, including hypotensive, anti-anxiety, tranquilising, analgesic, and diuretic effects and regulation of hormones and cells (Mohler 2012; Diana et al. 2014; Shi et al. 2016). In addition to its bioactivities beneficial to mammals, GABA also involves in acid-resistance in some bacteria such as *Listeria monocytogenes* (Karatzas et al. 2010) and environmental stress-resistance in plants (Bouche and Fromm 2004). Furthermore, GABA has been used as a major building block for the synthesis of 2-pyrrolidone and biodegradable polyamide nylon 4 (Park et al. 2013).

Naturally, GABA is synthesized from L-glutamate by L-glutamate decarboxylase (GAD) in certain species (Li and Cao 2010; Shi et al. 2016). GAD genes (*gad*) are present in *Escherichia coli* (De Biase et al. 1996), *Lactobacillus brevis* (Hiraga et al. 2008), and several other species of *Lactobacillus* as well as *Enterobacteria*. However, during

Electronic supplementary material The online version of this article (doi:10.1007/s11274-017-2289-3) contains supplementary material, which is available to authorized users.

✉ Feng Shi
shifeng@jiangnan.edu.cn

[1] State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China

[2] Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[3] Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, Wuxi 214122, China

[4] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

the production of GABA by strains of these species, L-glutamate must be added as the precursor (Zhao et al. 2016). Such bioprocess is not cost-effective, thus it is necessary to establish a new approach for sustainable industrial production of GABA. *Corynebacterium glutamicum* is a gram-positive actinobacterium widely used for the industrial production of L-glutamate and other amino acids (Hermann 2003; Leuchtenberger et al. 2005). By expressing exogenous GAD gene(s), *C. glutamicum* could be engineered to synthesize GABA from its own accumulated L-glutamate (Shi and Li 2011; Takahashi et al. 2012). Recently, through co-expressing two GAD genes (*gadB1* and *gadB2*) derived from *L. brevis* in *C. glutamicum* ATCC13032 and optimizing urea supplementation, GABA production increased to 18.7 ± 2.1 g/L (Shi et al. 2013). To improve GABA production, a more robust L-glutamate-producing strain shall be used as the host. Furthermore, L-glutamate biosynthesis must be enhanced because L-glutamate is the direct precursor of GABA.

The biosynthetic pathway of L-glutamate and GABA in recombinant *C. glutamicum* is shown in Fig. 1. L-Glutamate is synthesized from glucose via glycolysis pathway, anaplerotic pathway, first half of tricarboxylic acid (TCA) cycle, and L-glutamate dehydrogenase (GDH); among them phosphoenolpyruvate (PEP)-pyruvate-oxaloacetate (OAA) node works as the switch point for carbon flux distribution (Sauer and Eikmanns 2005). OAA supply determines the flux flowing into the TCA cycle. In addition, OAA is continuously withdrawn from TCA cycle during L-glutamate and GABA production. But OAA cannot be regenerated efficiently via the TCA cycle because GDH activity increases and 2-oxoglutarate dehydrogenase complex specific activity decreases during L-glutamate production (Hirasawa and Wachi 2016). Therefore, the anaplerotic pathway must be enhanced for efficient OAA supply and L-glutamate and GABA overproduction. In *C. glutamicum*, two anaplerotic enzymes, phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PC), mainly generate OAA, from PEP and pyruvate, respectively (Peters-Wendisch et al. 1998). Meanwhile, CO_2 released via the TCA cycle can be assimilated by these two anaplerotic reactions. However, PC, a biotin-requiring enzyme, may not function well during L-glutamate fermentation under biotin limitation (Shirai et al. 2007). Thus, PEPC may be crucial for OAA supply. In addition to OAA generation, OAA can be reduced to malate by malate dehydrogenase (MDH), the last enzyme of TCA cycle. *C. glutamicum* possesses two types of MDH, a membrane-associated malate:quinone oxidoreductase (MQO) and a cytoplasmic MDH (Molenaar et al. 2000). MQO catalyzes the oxidation of malate to OAA, whereas MDH catalyzes the opposite reaction.

In the present study, a more robust L-glutamate-producing industrial strain, *C. glutamicum* SH, was used for expressing *gadB1-gadB2* and producing GABA. To enhance OAA supply in recombinant *C. glutamicum* SH strains, *ppc* gene which encodes the PEPC was co-overexpressed with *gadB1-gadB2* and *mdh* gene which encodes the MDH was deleted. Their influence on GABA fermentation was then researched.

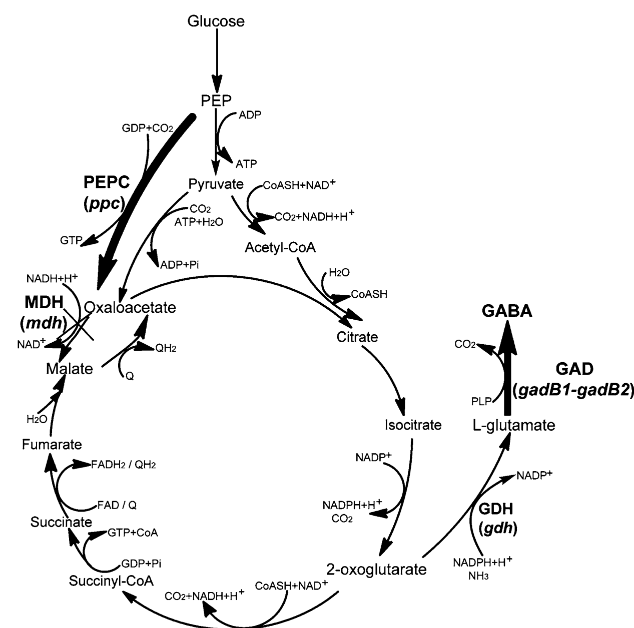

Fig. 1 Biosynthetic pathway of L-glutamate and GABA in recombinant *C. glutamicum*

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 Luria–Bertani (LB) medium at 37 °C and 200 rpm. *Corynebacterium glutamicum* SH, an L-glutamate-producing strain, was used for *mdh* gene deletion, expressing *gadB1-gadB2* and *ppc* genes and producing GABA. SH was deposited in the China General Microbiological Culture Collection (CGMCC) center with accession number CGMCC 1.581. *C. glutamicum* was grown in LBG medium (LB supplemented with 5 g/L glucose) at 200 rpm and 30 °C. When necessary, 30 µg/mL kanamycin or 15 µg/mL chloramphenicol was added to the media.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Sources
Strains		
E. coli JM 109	*E. coli* gene cloning strain	Novagen
C. glutamicum SH	Wild type *C. glutamicum*	CGMCC
SE1	*C. glutamicum* SH harbouring pJYW-4-*gadB1-gadB2*	This study
SE2	*C. glutamicum* SH harbouring pJYW-4-*gadB1-gadB2-ppc*	This study
SD	*mdh* deletion mutant of C. *glutamicum* SH	This study
SDE1	SD harbouring pJYW-4-*gadB1-gadB2*	This study
SDE2	SD harbouring pJYW-4-*gadB1-gadB2-ppc*	This study
SDE3	SD harbouring pJYW-4-*gadB1-gadB2-mdh*	This study
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)
pSD	Derived from pBluescript II SK(+) by inserting the segment *mdh*U-*loxL-kan-loxR*-*mdh*D	This study
pDTW109	Vector inserting a *cre* gene, Cm^{r}	Hu et al. (2013)
pJYW-4	A shuttle expression vector between *E. coli* and *Corynebacterium*	Hu et al. (2014)
pJYW-4-*gadB1-gadB2*	pJYW-4 harbouring *gadB1-gadB2*	This study
pJYW-4-*ppc*	pJYW-4 harbouring *ppc*	This study
pJYW-4-*gadB1-gadB2-ppc*	pJYW-4 harbouring *gadB1–gadB2-ppc*	This study
pJYW-4-*gadB1-gadB2-mdh*	pJYW-4 harbouring *gadB1–gadB2-mdh*	This study

Construction of *gadB1-gadB2* and *gadB1-gadB2-ppc* co-expression strains

The primers used in this study are listed in Table 2. Firstly, *gadB1-gadB2* genes were amplified via PCR from the plasmid pDXW-10-*gadB1-gadB2* (Shi et al. 2013) using the primer pair of B1F and B2R. The PCR product was digested with *Afl*II and *Bam*HI, and ligated into pJYW-4 (Hu et al. 2014) that was similarly digested, resulting in the plasmid pJYW-4-*gadB1-gadB2*. Secondly, *ppc* gene was PCR amplified using the primer pair of *ppc*-F and *ppc*-R from genomic DNA of *C. glutamicum* SH. The PCR product was digested with *Not*I and *Sal*I, and ligated into the pJYW-4 that was similarly digested, resulting in the plasmid pJYW-4-*ppc*. Next, the DNA fragment containing the *tac*-M promoter and *ppc* gene was PCR amplified from pJYW-4-*ppc* using primer pair of *ppc-tacM*-F and *ppc*-R, digested with *Sal*I, and

Table 2 Primers used in this study

Primers	Sequences (5′–3′)	Restriction site
B1F	GTACGG**CTTAAG**_AGAAGGAG_ATAATGGATGGCTATGTTGTATGGAA	*Afl*II
B2R	CGC**GGATCC**TTAACTTCGAACGGTGGTCTTG	*Bam*HI
ppc-F	TAA**GCGGCCGC**_AGAAGGAG_ATATACGATGACTGATTTTTTACGCG	*Not*I
ppc-R	TAA**GTCGAC**CTAGCCGGAGTTGCGC	*Sal*I
ppc-tacM-F	GCT**GTCGAC**TGGGGAAGAATTAGGCAGGC	*Sal*I
mdh-F	AGA**GGATCC**_AGAAGGAG_TTATTAAATGAATTCCCCGCAGAAC	*Bam*HI
mdh-R	GAT**GTCGAC**ATGCGTTAAAGATTAGAGCA	*Sal*I
*mdh*U-F	ATG**TCTAGA**TGCCACCAACAACCAGGTAG	*Xba*I
*mdh*U-R	GGGG**CTGCAG**AATTCATTAAACAATCCT	*Pst*I
*mdh*D-F	TAA**AAGCTT**ATGCGCGATTGGGTACAGGG	*Hind*III
*mdh*D-R	AAAT**CTCGAG**GTTGATCGCCCCGGACACTG	*Xho*I
kan-F	CTA**CTGCAG**AATACGACTCACTATAGGGCG	*Pst*I
kan-R	ACC**AAGCTT**GCGCAATTAACCCTCACTAAAG	*Hind*III

The restriction sites are indicated in boldface. The ribosomal binding sites are italicized. The sequences corresponding to the target gene are underlined

ligated into pJYW-4-*gadB1*-*gadB2* that was similarly digested, resulting in the plasmid pJYW-4-*gadB1*-*gadB2*-*ppc*. Finally, the two plasmids were transformed into *C. glutamicum* SH by the method described previously (Wang et al. 2015), generating two recombinant strains, *C. glutamicum* SH/pJYW-4-*gadB1*-*gadB2* and *C. glutamicum* SH/pJYW-4-*gadB1*-*gadB2*-*ppc*, renamed as SE1 and SE2, respectively.

Deletion of *mdh* gene in *C. glutamicum*

Based on the homologous recombination and site-specific recombination system, gene deletion in *C. glutamicum* SH was performed according to a previous study (Hu et al. 2013). First, deletion plasmid was constructed as follows. Fragment *mdh*U, located upstream of the gene *mdh*, and fragment *mdh*D, located downstream of the gene *mdh*, were amplified from the genomic DNA of *C. glutamicum* SH using the primer pairs *mdh*U-F/*mdh*U-R and *mdh*D-F/*mdh*D-R, respectively; Fragment *loxL*-*kan*-*loxR*, which contained two recognition sites (*loxL* and *loxR*) by Cre, was amplified from plasmid pDTW202 using the primer pair *kan*-F/*kan*-R. The three DNA fragments were ligated into the *Xba*I and *Xho*I restriction sites of plasmid pBluscriptII SK(+), resulting in plasmid pSD. Second, plasmid pSD was transformed into *C. glutamicum* SH to allow for homologous recombination. The recombinant strains were selected by growth on LBHIS medium (Wang et al. 2015) supplemented with 30 µg/mL kanamycin. Third, plasmid pDTW-109 was transformed into the recombinant strain to remove the *kan* gene out of the chromosome, and selected on LBHIS agar containing 15 µg/mL chloramphenicol. 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 SH Δ*mdh*, renamed SD. The strains were verified by restriction enzyme digestion or target gene amplification.

The *mdh* complemented plasmid was constructed as follow. The *mdh* gene was PCR amplified using the primer pair of *mdh*-F and *mdh*-R from genomic DNA of *C. glutamicum* SH, then digested with *Bam*HI and *Sal*I, and ligated into pJYW-4-*gadB1*-*gadB2* that was similarly digested, resulting in the plasmid pJYW-4-*gadB1*-*gadB2*-*mdh*. The three plasmids, pJYW-4-*gadB1*-*gadB2*, pJYW-4-*gadB1*-*gadB2*-*ppc*, and pJYW-4-*gadB1*-*gadB2*-*mdh* were finally transformed into the *mdh* deletion mutant, generating the new recombinant *C. glutamicum* strains, Δ*mdh*/pJYW-4-*gadB1*-*gadB2*, Δ*mdh*/pJYW-4-*gadB1*-*gadB2*-*ppc*, and Δ*mdh*/pJYW-4-*gadB1*-*gadB2*-*mdh*, renamed as SDE1, SDE2, and SDE3, respectively.

GABA fermentation of recombinant *C. glutamicum* strains in a fermentor

L-Glutamate and GABA production in recombinant *C. glutamicum* strains were evaluated in a fermentor (BioFlo 110; New Brunswick Scientific, USA) as described previously (Wang et al. 2015) with a little modification. The pH was controlled at 7.0–7.5 by adding concentrated ammonium hydroxide at 0–33 h, declined spontaneously to 5.5 at 33–36 h, and then controlled at 5.0–5.5 by adding 2 M HCl at 36–72 h. After 12 h of fermentation, glucose was fed by a peristaltic pump when the residual glucose in the medium decreased below 20 g/L. Samples were taken every 12 h to determine the optical density at 562 nm (OD_{562}), residual glucose level and amino acids concentrations. Residual glucose concentration was determined using a biosensor (Institute of Biology, Shandong Academy of Science, China). GABA and L-glutamate concentrations were assayed using reversed-phase high-performance liquid chromatography (HPLC, Agilent 1200, USA) by the method described previously (Shi et al. 2013).

Extraction of crude enzyme and assay of GAD, MDH, and PEPC activity

At 24 and 42 h of fermentation, the fermenting cells of *C. glutamicum* strains were collected, washed, and the crude enzyme was extracted as described previously by Shi et al. (2013). GAD activity was determined at 42 h by measuring the formation of GABA in a GAD reaction (Shi et al. 2013). MDH activity was determined at 24 h by monitoring the oxidation of NADH to NAD^+ accompanied by the reduction of OAA to malate in a MDH reaction (Mansouri et al. 2017). PEPC activity was determined at 24 h by monitoring the oxidation of NADH to NAD^+ in a PEPC–MDH coupling reaction (Cheng et al. 2016). The formation of GABA was analyzed using reversed-phase HPLC. The oxidation rate of NADH to NAD^+ was measured spectrophotometrically at 340 nm with a Shimadzu UV-1800 spectrophotometer. One unit (U) of enzymatic activity is defined as 1.0 µmol GABA produced (for GAD) or NADH consumed (for MDH and PEPC) in 1 min at 30 °C in the initial reaction mixture. The specific activity is expressed as U/mg or U/g of protein.

Results

To improve OAA supply and GABA production in recombinant *C. glutamicum* SH, the *ppc* gene was overexpressed and *mdh* gene was deleted. First, the *mdh* deletion plasmid pSD was constructed and then the *mdh* deletion strain SD was generated. Second, *gadB1*-*gadB2*, *gadB1*-*gadB2*-*ppc*,

and *gadB1-gadB2-mdh* co-expressing plasmids were constructed and then transformed into wild type SH and *mdh*-deleted SD stains, generating five new recombinant GAD strains, the control SE1, *ppc*-overexpressed SE2, *mdh*-deleted SDE1, *mdh*-complemented SDE3, and *mdh*-deleted *ppc*-overexpressed SDE2. The five new recombinant *C. glutamicum* strains were fermented and production of L-glutamate and GABA were investigated.

The fermentation process of recombinant *C. glutamicum* in a fermentor

The optimum pH for cell growth and GDH activity of *C. glutamicum* is 7.0–7.5, whereas that for GAD activity is 4.5–5.0. Meanwhile, more than half of GDH activity is lost at pHs lower than 6.5 (Shiio and Ozaki 1970) and nearly all of the GAD activity is lost at pHs higher than 6.0 (Shi et al. 2014). For effective production of GABA in recombinant *C. glutamicum* SH strains, pH was controlled at 7.0–7.5 during the first stage of fermentation by adding concentrated ammonium hydroxide to ensure cell growth and L-glutamate biosynthesis and later adjusted to 5.0–5.5 during the last stage of fermentation by adding 2 M HCl to ensure GAD activity and conversion of L-glutamate to GABA (Wang et al. 2015). To control the optimum pH of these two stages, recombinant *C. glutamicum* SH strains were fermented in fermentor.

During fermentation, all five strains grew fast in the first 12 h, slowly thereafter and did not grow after 36 h (Fig. 2a). Before 36 h, the growth rate of the control GAD strain SE1 and *mdh*-complemented GAD strain SDE3 was a little lower than those of *ppc*-overexpressed SE2, *mdh*-deleted SDE1, and *mdh*-deleted *ppc*-overexpressed SDE2; while after 36 h, cell concentration of SE1 became stable, whereas those of SE2, SDE1, SDE2, and SDE3 decreased slightly. Finally, cell concentrations of the five strains were nearly the same. So overexpression of *ppc* and deletion or complement of *mdh* did not influence cell growth significantly. In addition, glucose consumption patterns of all the five strains were similar. Glucose was consumed quickly and nearly completely in the first 24 h (Fig. 2b), in accordance with the fast cell growth (Fig. 2a) and quick L-glutamate biosynthesis at this stage (Fig. 3a). After 24 h, additional glucose was added when the residual glucose in the medium was lower than 20 g/L and glucose was consumed continuously and slowly thereafter, resulting in the continuous and relatively slower L-glutamate biosynthesis during 24–36 h (Fig. 3a).

Effect of *ppc* overexpression and *mdh* deletion on L-glutamate and GABA biosynthesis in recombinant *C. glutamicum*

During the fermentation of five recombinant *C. glutamicum* SH strains, the accumulation trends of L-glutamate and GABA were similar (Fig. 3). L-Glutamate accumulated quickly during 12–36 h when pH was controlled at 7.0–7.5 and then decreased quickly and converted nearly completely to GABA during 36–60 h when pH was controlled at 5.0–5.5. But their production was different. In the control GAD strain SE1, the L-glutamate concentration increased to the highest value 45.5 ± 0.7 g/L at 36 h, decreased sharply to 2.4 ± 0.8 g/L until 60 h and increased slightly during the final 12 h. Accordingly, GABA concentration increased continuously from 36 to 60 h and maintained thereafter. Finally, 22.7 ± 0.5 g/L GABA accumulated at 72 h.

In the *ppc* overexpressed GAD strain SE2, the PEPC activity increased by 5.4-fold and the GAD

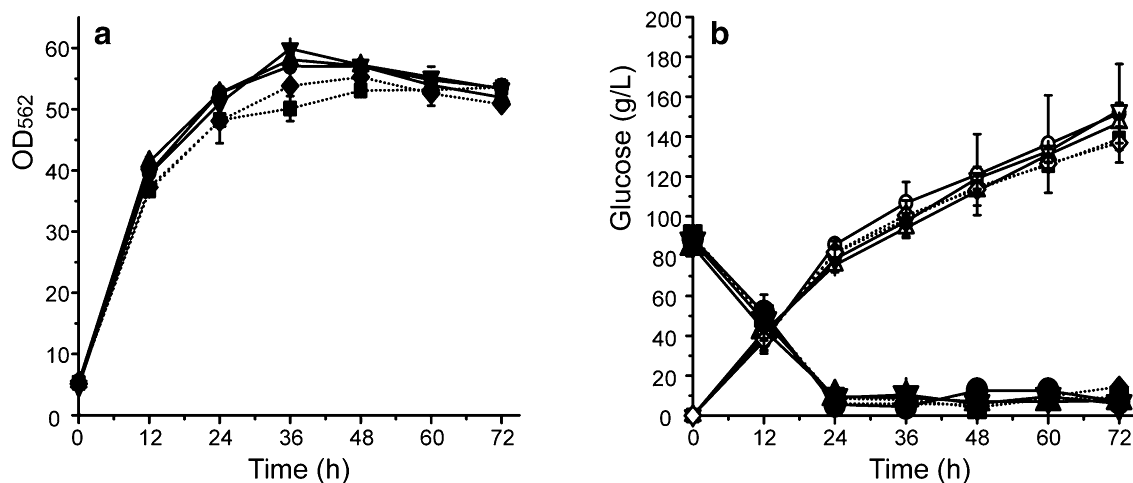

Fig. 2 The cell growth (**a**), glucose consumption (**b**, *open symbols*), and residual glucose (**b**, *closed symbols*) of recombinant *C. glutamicum* during GABA fermentation. *Squares in dotted line* SE1, *circles* SE2, *triangles* SDE1, *inverted triangles* SDE2, *diamonds in dotted line* SDE3

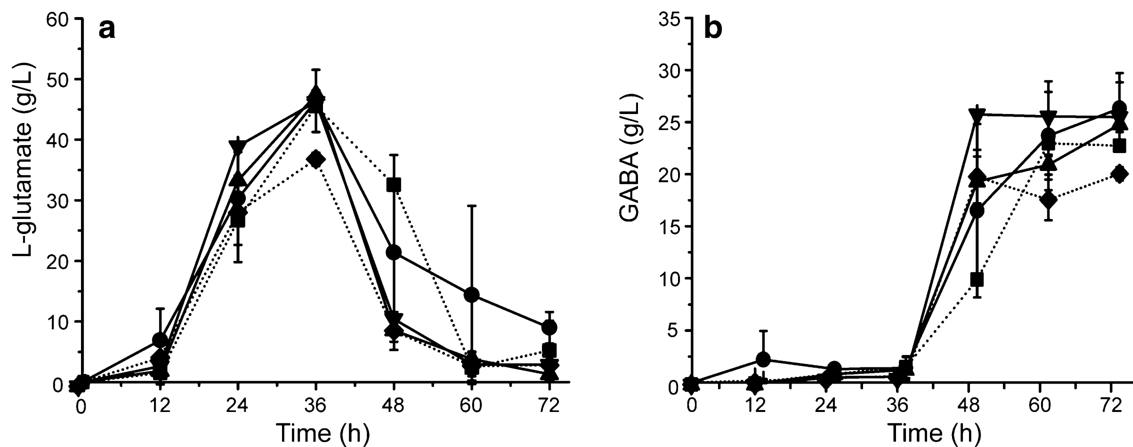

Fig. 3 Time course of L-glutamate and GABA production in recombinant *C. glutamicum*. **a** Production of L-glutamate. **b** Production of GABA. *Squares in dotted line* SE1, *circles* SE2, *triangles* SDE1, *inverted triangles* SDE2, *diamonds in dotted line* SDE3

Table 3 The PEPC, MDH, and GAD activities of the crude cell extracts of recombinant *C. glutamicum*

	PEPC activity (U/g)	MDH activity (U/g)	GAD activity (U/mg)
SE1	3.95 ± 0.03	2.21 ± 0.15	1.12 ± 0.11
SE2	25.22 ± 0.40	ND	1.07 ± 0.07
SDE1	ND	0	1.04 ± 0.04
SDE2	25.23 ± 8.42	0	1.00 ± 0.00
SDE3	ND	33.24 ± 0.59	1.01 ± 0.04

ND not determined

activity decreased slightly by 4.2% (Table 3). The L-glutamate concentration increased to the highest value 46.4 ± 5.1 g/L at 36 h, which was slightly higher than that in SE1, and decreased continuously to 9.0 ± 2.6 g/L thereafter. In accordance, GABA concentration increased continuously from 36 to 72 h and 26.3 ± 3.4 g/L GABA accumulated at 72 h, 16% higher than that in SE1, indicating that overexpression of *ppc* was benefit for GABA biosynthesis. Moreover, no significant amounts of other amino acids were detected in the final fermentation product, indicating that SE2 can be a good candidate for industrial production of GABA because of the easy downstream separation and purification of GABA.

In the *mdh* deleted GAD strain SDE1, the MDH activity decreased completely and the GAD activity decreased slightly by 7.1% (Table 3). The L-glutamate concentration increased to the highest value 47.0 ± 1.6 g/L at 36 h, which was slightly higher than that in SE1, and decreased sharply and converted completely to GABA thereafter. Accordingly, the GABA concentration increased continuously to 24.8 ± 0.7 g/L during 36–72 h, 9% higher than that in SE1, indicating that deletion of *mdh* was also benefit for GABA biosynthesis.

In the *mdh* complemented GAD strain SDE3, the MDH activity increased by 14.1-fold and the GAD activity decreased by 9.9% (Table 3). The L-glutamate concentration increased to the highest value 36.8 ± 1.1 g/L at 36 h, which was obviously lower than that in SE1, and then decreased sharply and converted nearly completely to GABA during 36–60 h and maintained thereafter. The GABA concentration increased to 20.0 ± 0.6 g/L during 36–48 h and fluctuated thereafter, 12% lower than that in SE1, indicating that complement of *mdh* was not benefit for GABA biosynthesis.

In the *mdh* deleted and *ppc* overexpressed GAD strain SDE2, the MDH activity decreased completely, the PEPC activity increased by 5.4-fold and the GAD activity decreased by 11.1% (Table 3). The L-glutamate concentration increased to the highest value 46.3 ± 0.8 g/L at 36 h, which was slightly higher than that in SE1 and was comparable to those in *ppc* overexpressed SE2 and *mdh* deleted SDE1, then decreased sharply and converted nearly completely to GABA during 36–60 h and maintained thereafter. Meanwhile, GABA concentration increased much quickly to 25.7 ± 0.4 g/L during 36–48 h and maintained at 25.7–25.5 g/L thereafter, 12% higher than that in SE1 but nearly same with those in *ppc* overexpressed SE2 and *mdh* deleted SDE1, indicating that the beneficial effects of *ppc* overexpression and *mdh* deletion on GABA biosynthesis were not synergistic.

Discussion

In this study, *ppc* was overexpressed and *mdh* was deleted in recombinant *C. glutamicum* SH expressing

gadB1-gadB2 genes. The L-glutamate concentration increased to 46.4 ± 5.1 and 47.0 ± 1.6 g/L at 36 h in the *ppc*-overexpressed GAD strain SE2 and *mdh*-deleted GAD strain SDE1, respectively, slightly higher than that in the control GAD strain SE1 (45.5 ± 0.7 g/L), whereas in *mdh*-complemented GAD strain SDE3, L-glutamate concentration decreased to 36.8 ± 1.1 g/L at 36 h (Fig. 3a), indicating that L-glutamate accumulation was improved slightly after PEPC activity increased or MDH activity lost, but weakened obviously after MDH activity increased. Meanwhile, GABA concentration increased to 26.3 ± 3.4 and 24.8 ± 0.7 g/L at 72 h in *ppc*-overexpressed SE2 and *mdh*-deleted SDE1, respectively, 16 and 9% higher than that in SE1 (22.7 ± 0.5 g/L), whereas in *mdh*-complemented SDE3, GABA concentration decreased to 20.0 ± 0.6 g/L at 72 h (Fig. 3b), indicating that GABA biosynthesis was improved obviously after *ppc* was overexpressed or *mdh* was deleted, but weakened obviously after *mdh* was complemented. In addition, there are not significant amounts of other amino acids being detected in the final fermentation product of SE2 and SDE1, indicating them as the good candidates for industrial production of GABA. However, the L-glutamate concentration at 36 h (46.3 ± 0.8 g/L) and GABA concentration at 72 h (25.5 ± 3.3 g/L) in the *mdh*-deleted and *ppc*-overexpressed GAD strain SDE2 did not increase further, suggesting that *ppc* overexpression and *mdh* deletion did not improve GABA biosynthesis synergistically.

ppc gene overexpression and *mdh* gene deletion enhanced L-glutamate production at 24 h (Fig. 3a), in accordance with the increased PEPC activity and the lost MDH activity; however, such an effect was not observed at 36 h, perhaps due to the improper condition for these enzymes and cellular metabolism. *C. glutamicum* is a moderate alkaliphile (Barriuso-Iglesias et al. 2008). Meanwhile, the optimum pH of PEPC, MDH, and GDH is 7.5, 9.5, and 7.5, respectively. The pH of fermentation broth decreased spontaneously from 7.3 to 5.5 during 33–36 h of fermentation, therefore might make these enzymes inactive and cellular metabolism slow. At the last stage of fermentation when pH was controlled at 5.0–5.5, *ppc* gene overexpression and *mdh* gene deletion enhanced GABA production at 48 h; however, such an effect was not observed at 60 or 72 h (Fig. 3b), likely due to the more serious cellular damage in SE2 and SDE1, as the cell concentration of SE2 and SDE1 decreased obviously after 48 h. Therefore, GABA should be produced as quickly as possible.

OAA is the precursor metabolite for L-glutamate and GABA biosynthesis and it is mainly supplied by anaplerotic pathway. It has been demonstrated that between the two anaplerotic enzymes, the activity of PC is diminished under biotin-limited condition, and deletion of *pyc* which encodes PC do not affect L-glutamate production under biotin limited condition (Sato et al. 2008). In the present study, L-glutamate and GABA was produced under corn steep liquor limited condition and the active component of corn steep liquor is biotin. Our recent research showed that deletion of *pyc* improved L-glutamate and GABA production under corn steep liquor limitation, partially due to the increased transcription level of *ppc* (Wang et al. 2015). Here, after *ppc* was overexpressed and PEPC activity was enhanced, L-glutamate production improved slightly and finally GABA production increased by 16%, demonstrating again the beneficial effect of *ppc* overexpression on L-glutamate and GABA biosynthesis.

On the other hand, to prevent OAA reduction and provide more OAA precursor for GABA formation, the cytosolic reductive pathway of OAA was blocked, as predicted by the analysis of a genome-scale metabolic network of *C. glutamicum* S9114 (Mei et al. 2016). After *mdh* was deleted and MDH activity lost, L-glutamate production improved a little and finally GABA production increased by 9%, demonstrating the contribution of *mdh* deletion on L-glutamate and GABA biosynthesis. But the increment of GABA production was not as high as that in *ppc* overexpressed GAD strain SE2, likely due to the fact that CO_2 released by the decarboxylation of L-glutamate to GABA as well as via the TCA cycle can be assimilated and fixed more efficiently by PEPC when *ppc* was overexpressed, but can not when *mdh* was deleted.

After simultaneous overexpression of *ppc* and deletion of *mdh*, GABA production increased only by 12% and this production was not higher than that of *ppc*-overexpressed GAD strain SE2, suggesting that the two strategies both focused on OAA supply can not act synergistically. Perhaps only the overexpression of *ppc* or deletion of *mdh* is enough to provide appropriate OAA for L-glutamate and GABA production. However, GABA produced more quickly in *ppc* overexpressed and *mdh* deleted SDE2 (Fig. 3b).

Although the production of GABA increased in SE2, SDE1, and SDE2, it was not high enough. Fermentation conditions shall also be optimized in the future. In addition, other metabolic strategies focused on the α-ketoglutarate node of L-glutamate biosynthetic pathway including deletion of *pknG* (Okai et al. 2014) and deletion of *odhA* (Wang et al. 2015) were demonstrated recently to be able to improve GABA biosynthesis in recombinant *C. glutamicum*. Furthermore, a new metabolic route for the production of GABA via putrescine was established in *C. glutamicum* (Jorge et al. 2016a, b). All these researches suggest recombinant *C. glutamicum* as the good species for the production of GABA from glucose.

Acknowledgements The authors thank the "Program of State Key Laboratory of Food Science and Technology" (Contract No. SKLF-ZZB-201405) for financial support.

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