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Improved fermentative γ-aminobutyric acid production by secretory expression of glutamate decarboxylase by *Corynebacterium glutamicum*

Jingbai Wen [a,b], Jie Bao [a,*]

[a] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China*
[b] *School of Chemical and Biological Engineering, Yichun University, 576 Xuefu Road, Yichun, Jiangxi 336000, China*

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ABSTRACT

Fermentative production of γ-aminobutyric acid by the glutamate overproducing *Corynebacterium glutamicum* from cheap sugar feedstock is generally regarded as one of the most promising methods to reduce the production cost. However, the intracellularly expressed glutamate decarboxylase in *C. glutamicum* often showed feeble catalysis activity to convert glutamate into γ-aminobutyric acid. Here we tried to secretory express glutamate decarboxylase to achieve efficient extracellular decarboxylation of glutamate, thus improving the γ-aminobutyric acid production by *C. glutamicum*. We first tested glutamate decarboxylases from different sources, and the mutated glutamate decarboxylase GadBmut from *E. coli* with better catalytic performance was selected. Then, a signal peptide of the SEC translocation pathway directed the successful secretion of glutamate decarboxylase in *C. glutamicum*. The extracellular catalysis by secreted glutamate decarboxylase increased the γ-aminobutyric acid generation by three-fold, compared with intracellular catalysis. Enhancing glutamate decarboxylase expression and decreasing γ-aminobutyric acid degradation further increased γ-aminobutyric acid production by 39 %. The fed-batch fermentation of the engineered *C. glutamicum* strain reached the record high titer (77.6 ± 0.0 g /L), overall yield (0.44 ± 0.00 g/g glucose), and productivity (1.21 ± 0.00 g/L/h). This study demonstrated a unique design of extracellular catalysis for efficient γ-aminobutyric acid production by *C. glutamicum*.

1. Introduction

γ-Aminobutyric acid (GABA) is a 4-carbon non-proteinogenic amino acid with great potentials as the monomer chemical to produce biodegradable polyamide polymers (Sarasa et al., 2020; Xu et al., 2017). γ-Aminobutyric acid is currently produced by enzymatic decarboxylation of sodium glutamate substrate using glutamate decarboxylase (GAD) (Dhakal et al., 2012; Ke et al., 2018; Kook and Cho, 2013). For cost reduction, fermentative GABA production from cheap carbon sources such as glucose had been investigated by heterologous overexpression of glutamate decarboxylase in *E. coli*, lactic acid bacteria, and *Corynebacterium glutamicum* (Xu et al., 2017). *E. coli* and lactic acid bacteria produce little glutamate, and the sodium glutamate substrate must be added (Ke et al., 2018; Kook and Cho, 2013), making these species whole-cell biocatalysts instead of fermentative strains. On the other hand, *C. glutamicum* is an ideal host strain for the fermentative production of GABA because of the sufficient glutamic acid supply as the precursor substrate (Baritugo et al., 2018). However, the low active pH (4.0–5.0) of glutamate decarboxylase fails to match the neutral intracellular pH environment of *C. glutamicum* cells, leading to the low catalytic activity for efficient GABA production (Shi et al., 2014; Thu Ho et al., 2013).

Many efforts had been made to overcome the obstacle of low glutamate decarboxylase activity in *C. glutamicum*, including: (i) expanding the active pH range of glutamate decarboxylase (Baritugo et al., 2018; Choi et al., 2015; Shi et al., 2014), (ii) optimizing the expression of glutamate decarboxylase (Shi et al., 2013, 2018), (iii) enhancing the glutamate synthesis pathway (Okai et al., 2014; Shi et al., 2017; Wang et al., 2015), (iv) reducing the GABA degradation (Ni et al., 2015), and (v) applying a two-step glutamate-fermentation and GABA-conversion process (Yang et al., 2015). An alternative putrescine-based GABA synthesis pathway avoided using glutamate decarboxylase, but the cumbersome synthesis rote significantly reduced the GABA yield (Jorge et al., 2016, 2017). Unfortunately, the fermentative GABA production from these efforts was still far below the requirements for any practical application till now.

Increasing the glutamate conversion efficiency in *C. glutamicum* is critical for promising GABA production. We noticed that for all the

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* Corresponding author.
E-mail address: jbao@ecust.edu.cn (J. Bao).

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reported GABA production in *C. glutamicum*, the decarboxylation reactions were carried out inside the cells. Although intracellular catalysis is generally regarded as an efficient way, several disadvantages are encountered for this specific scenario of GABA synthesis: (i) weaker driving force for the conversion of glutamate to GABA inside the cells, because most of the intracellularly produced glutamate would be exported to the extracellular broth through the mechanosensitive channel MscCG (NCgl1221) thus causing the lower intracellular glutamate concentration than extracellular (Hoischen and Krämer, 1989; Nakamura et al., 2007); (ii) higher GABA biodegradation inside the cells (Ni et al., 2015); (iii) transmembrane transportation resistance to the transport of the substrate glutamate from outside to inside the cell, and of the GABA product in reverse (Zhao et al., 2014). These negative factors might overwhelmingly suppress the positive aspects of intracellular GABA production and restrict the catalytic performance of glutamate decarboxylase to some extent.

Taking those negative factors of intracellular GABA production into account, we attempted to move the decarboxylation reaction to the extracellular space of *C. glutamicum* to facilitate the conversion of glutamate. Extracellular expression of glutamate decarboxylase B has resulted in a more efficient conversion of glutamate into GABA in *E. coli* (Zhao et al., 2016). The glutamate conversion by *C. glutamicum* would also be benefited from the extracellular decarboxylation reaction at the points of (i) higher glutamate concentration in the extracellular broth of *C. glutamicum*; (ii) no transmembrane transportation resistance for glutamate and GABA transport; (iii) less GABA degradation in the extracellular broth. A 3-fold improvement in GABA production was obtained by the extracellular catalysis that was achieved by secretory expression of glutamate decarboxylase from *E. coli*. The fed-batch fermentation of the final strain reached the record high GABA production by *C. glutamicum*. This study paves a unique way for the fermentative GABA production by C. glutamicum from cheap sugar feedstock.

2. Materials and methods

2.1. Strain, media, and culture conditions

Strains and plasmids used in this study were listed in Table 1. *Escherichia coli* DH5α was used for plasmid construction and cultured in LB medium at 37 °C. The previously engineered *Corynebacterium glutamicum* XW6 was used as the starting strain for GABA production and cultured in CM2B medium at 30 °C (Wen and Bao, 2019). 50 μg/mL of kanamycin was added to the media if needed.

2.2. Enzymes and reagents

DNA polymerase and T4 ligase were purchased from Takara, Otsu, Japan; Restriction endonucleases were from Thermo Scientific, Wilmington, DE, USA. Seamless cloning kit HB infusion™ was purchased from Hanheng Biotech, Nanjing, China. Pyridoxal phosphate (PLP) was purchased from Aladdin, Shanghai, China. Corn steep liquor (CSL) was purchased from Xiwang Group, Zouping, Shandong, China. Other reagents used were purchased from local suppliers.

2.3. Plasmids and recombinant strains construction

The primers used for plasmids construction were shown in Supplementary Materials Table S1, and the maps of the constructed plasmids used in this study were shown in Supplementary Materials Fig. S1. The fragments of *gadA* (*COA_RS0116635*) and *gadB (COA_RS0106500)* genes were cloned from *E. coli* K12 using primers gadA-F/R and gadB-F/R and then inserted into the *Xba*I/*Sal*I of pH36mob, resulting in the pH36-*gadA* and pH36-*gadB*, respectively. The mutated GadB ($\text{GadB}^{\text{Glu89Gln}/\Delta 452\text{-}466}$) (Thu Ho et al., 2013) encoding gene was obtained by overlapping the two fragments that amplified from the *E. coli* K12 genome using primers gadBmut-F1/R1 and gadBmut-F2/R2. Then the overlapped product was inserted into the *Xba*I/*Sal*I of pH36mob, resulting in the pH36-*gadBmut*. Mutated GAD genes from *Bacillus megaterium* CICC 10055 ($\text{BmGAD}^{\text{E294R}}$, named as *gadBM* here) (Liu et al., 2016) and *Lactobacillus brevis* Lb85 ($\text{GadB1}^{\text{T17I/D294G/E312S/Q346H}}$, named as *gadBLB85* here) (Shi et al., 2014) were artificially synthesized by Shanghai Generay Biotech Co., Ltd, Shanghai, China. The sequences of the *gadBLb85* and *gadBM* were shown in the Supplementary Materials. These two fragments were then amplified using primers gadBM-F/R and gadBLB85-F/R, and then inserted into the *Sal*I/*Pst*I of pH36mob to obtain the pH36-*gadBM* and pH36-*gadBLb85* plasmids, respectively. For the plasmid co-expression of the *gadBmut* and *gadC*, these two fragments were amplified from pH36-gadBmut and the genome of *E. coli* K12 using primers

Table 1
Strains and plasmids used in this study.

Strains	**Characteristics**	**Sources**
E. coli DH5α	Host for plasmid construction	Lab stock
C. glutamicum XW6	Engineered *C. glutamicum* S9114 for cellulosic glutamate production	Wen and Bao (2019)
XW6-pH36-*gadA*	*C. glutamicum* XW6 carrying pH36-*gadA*	This study
XW6-pH36-*gadB*	*C. glutamicum* XW6 carrying pH36-*gadB*	This study
XW6-pH36-*gadBLB85*	*C. glutamicum* XW6 carrying pH36-*gadBLB85*	This study
XW6-pH36-*gadBM*	*C. glutamicum* XW6 carrying pH36-*gadBM*	This study
XW6-pH36-*gadBmut*	*C. glutamicum* XW6 carrying pH36-*gadBmut*	This study
XW6-pH36-*gadBmutgadC*	*C. glutamicum* XW6 carrying pH36-*gadBmutgadC*	This study
XW6-pH36-*Rs-gadBmut*	*C. glutamicum* XW6 carrying pH36-*Rs-gadBmut*	This study
XW6-pH36-*Ns-gadBmut*	*C. glutamicum* XW6 carrying pH36-*Ns-gadBmut*	This study
XW6-pNcgl-*Ns-gadBmut*	*C. glutamicum* XW6 carrying pNcgl-*Ns-gadBmut*	This study
XW6-pTacM-*Ns-gadBmut*	*C. glutamicum* XW6 carrying pTacM-*Ns-gadBmut*	This study
XW6-Δ*gabT*	*C. glutamicum* XW6 with *gabT* gene deleted	This study
XW6-Δ*gabP*	*C. glutamicum* XW6 with *gabP* gene deleted	This study
XW6-Δ*gabP*Δ*gabT*	*C. glutamicum* XW6 with *gabP* and *gabT* gene deleted	This study
XW6-ΔgabT-pTacM-*NsgadBmut*	XW6-Δ*gabT* carrying pTacM-*NsgadBmut*	This study
XW6-ΔgabP-pTacM-*NsgadBmut*	XW6-Δ*gabP* carrying pTacM-*NsgadBmut*	This study
XW6-ΔgabPΔgabT-pTacM-*NsgadBmut*	XW6-Δ*gabP*Δ*gabT* carrying pTacM-*NsgadBmut*	This study
Plasmids	**Characteristics**	**Sources**
pK18mobsacB	Mobilizable vector in *C. glutamicum*, kanamycin resistance, sacB	Wang et al. (2018)
pH36mob	Overexpression vector, kanamycin resistance	Lab stock
pH36-*gadA*	pH36mob carrying *gadA* from *E. coli* K12	This study
pH36-*gadB*	pH36mob carrying *gadB* from *E. coli* K12	This study
pH36-*gadBmut*	pH36mob carrying mutated *gadB* from *E. coli* K12	This study
pH36-*gadBLB85*	pH36mob carrying mutated *gadB* from *Lactobacillus brevis* Lb85	This study
pH36-*gadBM*	pH36mob carrying mutated *gadB* from *Bacillus megaterium* CICC 10,055	This study
pH36-*gadBC*	pH36mob carrying *gadBmut* and *gadC* from *E. coli* K12	This study
pH36-*RsgadBmut*	pH36mob carrying *E. coli* K12 mutated *gadB* with signal peptide of *RS04950*	This study
pH36-*NsgadBmut*	pH36mob carrying *E. coli* K12 mutated *gadB* with signal peptide of *Ncgl1289*	This study
pNcgl-*NsgadBmut*	pH36-*NsgadBmut* with H36 promoter replaced by promoter of *Ncgl1289*	This study
pTacM-*NsgadBmut*	pH36-*NsgadBmut* with H36 promoter replaced by *TacM* promoter	This study
pK18-Δ*gabT*	Plasmid for *gabT* gene knockout in the genome	This study
pK18-Δ*gabP*	Plasmid for *gabP* gene knockout in the genome	This study

gadBC-F1/R1 and gadBC-F2/R2, respectively. The two amplified fragments were then overlapped together and inserted into the *Sma*I/*Xba*I of pH36mob and resulted in pH36-*gadBC*.

The signal peptide sequence of the *cgR_0949* gene (*RS_04950* in *C. glutamicum* XW6) (Zhang et al., 2015) was amplified from *C. glutamicum* XW6 using primers RsgadB-F1/R1, and the *gadBmut* gene was amplified from the pH36-*gadBmut* plasmid using primers RsgadB-F2/R2. These two fragments were overlapped together and inserted into the *Xba*I/*Sal*I of pH36mob, resulting in the pH36-*RsgadBmut*. Similarly, the pH36-*NsgadBmut* using the signal peptide sequence of the *Ncgl1289* gene (Yim et al., 2016) from *C. glutamicum* ATCC13032 was constructed using primer pairs NsgadB-F1/R1 and NsgadB-F2/R2. TacM promoter (Zhang et al., 2015) was obtained by annealing primers pTacM-F/pTacM-NsgadB-R1, and the *NsgadBmut* fragment was amplified from pH36-*NsgadBmut* by primers pTacM-NsgadB-F2/gadBmut-R. Then, these two fragments were assembled with the backbone of pH36mob obtained by PCR using primers pH36-Vector-F/R, resulting in the pTacM-*NsgadBmut*. The fragment, including the promoter and the signal peptide sequence of the *Ncgl1289* gene, was amplified using primers Ncgl289-F/R from *C. glutamicum* ATCC13032. The *NsgadBmut* fragment was amplified from pH36-NsgadBmut by primers NCgl1289-gadB-F/gadBmut-R. Then, the two fragments together with the pH36mob backbone were assembled together and resulting in the pNcgl-*NsgadBmut*. All these constructed expression plasmids were confirmed by sequencing, the sequences of the promoters and signal peptides were shown on Supplementary Materials Table S2. The recombinant strains with corresponding genes expressed were obtained by transforming the corresponding plasmids into *C. glutamicum* XW6 cells by the electroporation method (van der Rest et al., 1999). The corresponding kanamycin-resistant recombinant strains were confirmed by colony PCR and sequencing analysis.

For the construction of plasmids for gene knockout, the upstream and downstream of homologous arms were selected to fully knockout of the complete coding sequence on the genome (Supplementary Materials Fig. S1). The upstream and downstream of the *gabT* gene were amplified from *C. glutamicum* XW6 by primers gabTup-F/R and gabTdown-F/R, respectively, and then inserted to *Eco*RI/*Xba*I and *Sal*I/*Hind*III of pK18mobsacB, resulting in the pK18-Δ*gabT* plasmid. The upstream and downstream of the *gabP* gene were amplified by primer pair gabPup-F/R and gabPdown-F/R, respectively. They were then inserted to *Sma*I/*BamHI* and *Sal*I/*Pst*I of pK18mobsacB, respectively, resulting in the pK18-Δ*gabP* plasmid. These plasmids were confirmed by sequencing analysis before use. The XW6-Δ*gabT* and XW6-Δ*gabP* strain were obtained by transforming the pK18-Δ*gabT* and pK18-Δ*gabP* plasmids into *C. glutamicum* XW6 cells by the electroporation method (van der Rest et al., 1999) and followed by two rounds of homologous recombination isolation (Schäfer et al., 1994), respectively. The pK18-Δ*gabT* plasmid was transformed into XW6-Δ*gabP* cells, and the XW6-Δ*gabP*Δ*gabT* strain was obtained by the same isolation method. Then, the secretory expression plasmid pTacM-*NsgadBmut* was transformed into XW6-Δ*gabT*, XW6-Δ*gabP*, and XW6-Δ*gabP*Δ*gabT* strain and resulted in the strains XW6-Δ*gabT*-pTacM-*NsgadBmut*, XW6-Δ*gabP*-pTacM-*NsgadBmut*, and XW6-Δ*gabP*Δ*gabT*-pTacM-*NsgadBmut*, respectively. All these strains were confirmed by PCR and sequencing analysis.

2.4. γ-Aminobutyric acid fermentation

C. glutamicum cells were cultured in CM2B ager at 30 °C for 24-36 hours, and then a single colony was picked up for seed culture as previously described (Wen et al., 2018). For the shake flask fermentation, the seed culture at 5% (v/v) inoculum ratio was inoculated into 250 mL conical flasks containing 30 mL fermentation medium (100 g/L glucose, 1 g/L KH_2PO_4, 0.6 g/L $MgSO_4$, 3 g/L urea, 2.0 mg/L $FeSO_4$, 2.0 mg/L $MnSO_4$, 25 g/L corn steep liquor and 0.1 mM pyridoxal 5′-phosphate) and cultured at 30 °C and 200 rpm shaking. The pH was adjusted to 7.0 by adding 20 % (w/w) urea solution.

The batch fermentation was performed in a 3 L fermentor (3BG-4, Baoxing Biotech, Shanghai, China) at 30 °C, 1.4 vvm aeration, and 600 rpm stirring of a single Rushton impeller. The pH was maintained at 7.0 by automatically addition of 25 % (w/v) ammonium hydroxide solution and 2 M sulfuric acid solution. The seed culture was inoculated into 800 mL of the fermentation medium at 10 % (v/v) inoculum ratio. For fed-batch fermentation, the medium reported previously (Zhang et al., 2014) with the initial glucose reduced to 100 g/L was adopted. Glucose substrate was fed into the fermentor by adding 50 mL of 279 g/L glucose solution every four h from 24 h to 5 2 h at 30 °C. The other conditions were same to that of batch fermentation. All fermentations were carried out in triplicate, and the error bars were indicated by the standard derivations of three biological replicates. The Student's *t*-test was performed for statistical analyses.

2.5. Protein samples preparation and SDS-PAGE

Samples were harvested after 48 h cultivation in shake flasks. The extracellular samples were prepared based on the acetone precipitation method (Jiang et al., 2004). The intracellular samples were prepared by ultrasonication, followed by removing cell debris, and the cell membrane samples were prepared using the extraction method as mentioned before (Tateno et al., 2007). The protein concentrations were determined by the Bradford method (Bradford, 1976). Then 10 μL of the samples were loaded for SDS-PAGE electrophoresis.

2.6. Analytical methods

Glucose, glutamic acid, and lactic acid were analyzed, as mentioned before (Wen and Bao, 2019). γ-Aminobutyric acid was analyzed by the pre-column o-phthalaldehyde (OPA) derivatization method (Koros et al., 2008) on HPLC (LC-20AT, Shimazu, Kyoto, Japan) equipped with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan) at 40 °C and 338 nm. The mobile phase consists of solution A (0.018 M sodium acetate supplemented with 0.02 % (v/v) triethylamine and 0.5 % (v/v) tetrahydrofuran, pH 7.2), and solution B (0.036 M sodium acetate solution (pH7.2): methanol: acetonitrile = 1:2:2). The total flow rate was 1.0 mL/min, solution B increased from 8% to 37 % in 15 min, and then decreased to 8% during 15−22 min.

3. Results and discussion

3.1. Screening the suitable glutamate decarboxylase for GABA synthesis

To realize the GABA production in the previously engineered *C. glutamicum* XW6 (Wen and Bao, 2019), several glutamate decarboxylase genes from different sources were first selected and overexpressed in *C. glutamicum* XW6 cells. These glutamate decarboxylase genes were selected and expressed in *C. glutamicum* XW6, including *gadA*, *gadB*, and the *gadB* mutant *gadBmut* from *E. coli* (Thu Ho et al., 2013); *gadBM* from *Bacillus megaterium* CICC 10055 (Liu et al., 2016); *gadBLB85* from *Lactobacillus brevis* Lb85 (Shi et al., 2014). Their effects on GABA production were tested in shaking flasks, and the results were presented in Fig. 1. As shown in Fig. 1, the expression of *gadBmut* resulted in the maximum GABA titer (1.54 g/L), approximately three-fold more than that by the other glutamate decarboxylase genes. This mutated glutamate decarboxylase from *E. coli* was also reported to have the potential to produce high yield GABA in *C. glutamicum* (Baritugo et al., 2018). The original GadA and GadB exhibited might exhibit lower catalytic activity in *C. glutamicum* cells due to the sharp decrease of activity at neutral pHs (Thu Ho et al., 2013). Even the mutated glutamate decarboxylase genes from *B. megaterium* CICC 10055 and *L. brevis* Lb85 have better catalytic performances at neutral pHs, their optimal temperatures were too high (Liu et al., 2016), and they might not be suitable for expression in *C. glutamicum* XW6. Therefore, the mutated GadBmut from *E. coli* that

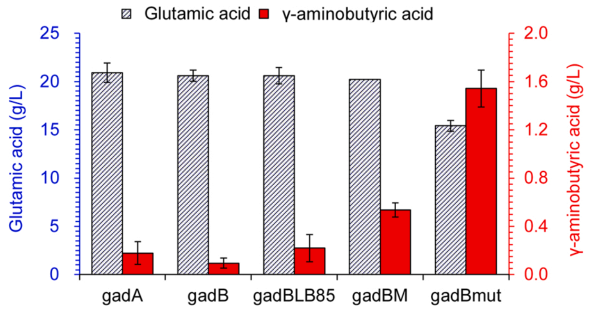

Fig. 1. GABA synthesis by overexpressing different glutamate decarboxylase genes in *C. glutamicum* XW6. The labels *gadA*, *gadB*, *gadBLB85*, *gadBM*, and *gadBmut* are indicated the *C. glutamicum* recombinants with the overexpression of corresponding genes, respectively. Fermentations were carried out in shake flasks at 30 °C, 200 rpm, pH 7.0 by adding 20 % (w/w) urea solution. Error bars were indicated by the standard deviations of three biological replicates.

showed better GABA production performance was selected and applied for the following experiments.

We also tried to enhance the transmembrane transportation by co-expressing the *gadC* gene from *E. coli* and the screened *gadBmut* gene in *C. glutamicum* XW6 to promote the transmembrane transport of glutamate (import) and GABA (export) (Supplementary Materials Fig. S2a). However, no apparent improvement in GABA accumulation was observed (Supplementary Materials Fig. S2b). We also noticed that this glutamate/GABA antiporter was only active under acidic pH conditions (Ma et al., 2012), thus promoting transmembrane transportation of glutamate and GABA under neutral pH in this study was not effective.

3.2. Secretory expression of glutamate decarboxylase in C. glutamicum

We designed a new catalytic scenario by secretory expression of glutamate decarboxylase to move the decarboxylation reaction into the extracellular space (the culture broth) of *C. glutamicum*. Two signal peptides were selected to mediate the secretion of glutamate decarboxylase, one is the signal peptide of *Ncgl1289* (Ncgl_SP) from *C. glutamicum* ATCC13032 recognized by the SEC translocation pathway (Yim et al., 2016), and the other is the signal peptide of *cgR_0494* (*CGR_RS04950* in *C. glutamicum* S9114, RS04950_SP) recognized by the TAT translocation pathway (Zhang et al., 2015) (Fig. 2a). The fragments encoding the signal peptides were separately inserted in front of the *gadBmut* gene of the expression plasmid. The plasmids were then introduced to *C. glutamicum* XW6 individually, and the two recombinants, XW6-pH36-*NsgadBmut* and XW6-pH36-*RsgadBmut*, were obtained.

The secretion effects were first tested by the SDS-PAGE analysis with the results presented in Fig. 2b. One bright single band appeared in the culture broth sample of XW6-pH36-*NsgadBmut* with the molecular weight close to the glutamate decarboxylase GadBmut (~56KDa) (Fig. 2b), suggesting that the glutamate decarboxylase was successfully secreted into the extracellular space of *C. glutamicum* through the SEC translocation pathway. On the other hand, no specific band close to the molecular weight of GadBmut was observed in the control and the recombinant XW6-pH36-*RsgadBmut* through the TAT secretion pathway (Fig. 2b). *C. glutamicum* contains both TAT and SEC translocation pathways for secretory protein expression, and many proteins can only be secreted by one of the two specific translocation pathways (Yim et al., 2016). Glutamate decarboxylase has already been successfully secreted in *E. coli* through the TAT pathway (Zhao et al., 2016), instead of SEC pathway, but its secretion in *C. glutamicum* might be only suitable for SEC pathway.

The effects of secretory glutamate decarboxylase expression on GABA synthesis were evaluated in shake flasks (Fig. 3). The secretory expression did not affect the cell growth much, and all the strains reached maximum cell growth at 60 h (Fig. 3a). The GABA titer significantly increased to 13.0 g/L, and the glutamate substrate was consumed quickly to a low level (~4 g/L) after 72 h by *C. glutamicum* XW6-pH36-*NsgadBmut* (Fig. 3b,3c). This GABA generation was approximately three-fold more than that of the control XW6-pH36-*gadBmut* strain (3.3 g/L) and eight-folds greater than that of the XW6-pH36-*RsgadBmut* recombinant through the TAT translocation pathway (1.5 g/L). This result indicates that the SEC translocation pathway secreted glutamate decarboxylase catalyzed the conversion of glutamate into GABA efficiently under the condition suitable for *C. glutamicum* growth. This extracellular GABA synthesis also resulted in about 14.6 % increase in the total glutamate and GABA accumulation (GABA + glutamate concentration in mM) at the end of the fermentation significantly ($p < 0.01$) (Fig. 3d), compared to that of control. The beneficial effects of extracellular GABA synthesis were fully demonstrated.

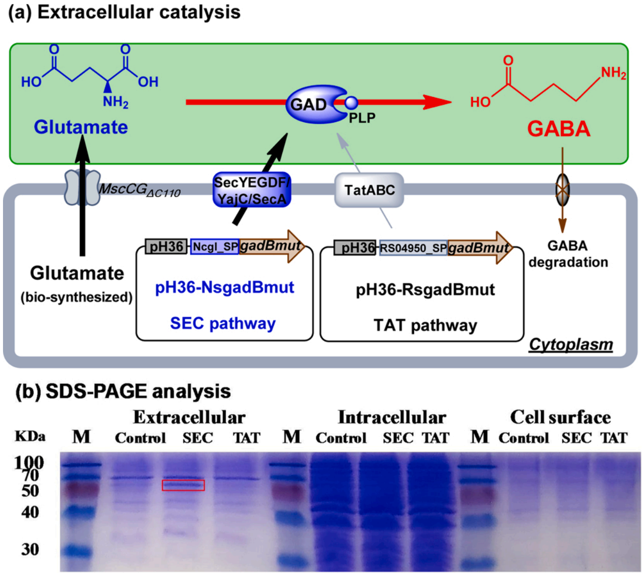

Fig. 2. Secretory expression of glutamate decarboxylase in *C. glutamicum* XW6. (**a**) Diagram of extracellular GABA conversion. GAD, glutamate decarboxylase; PLP, pyridoxal 5′-phosphate; MscCG$_{\Delta C110}$, modified glutamate secretion channel; SecYEGDF/YajC/SecA, proteins involving SEC secretion pathway; TatABC, proteins involving TAT secretion pathway. (**b**) SDS-PAGE of the extracellular expression of glutamate decarboxylase. Control, *C. glutamicum* carrying pH36-gadBmut vector without signal peptide; SEC, *C. glutamicum* carrying pH36-NsgadBmut vector with signal peptide through Sec pathway; TAT, *C. glutamicum* carrying pH36-RsgadBmut vector with signal peptide through TAT pathway. Samples were collected after cultured in shake flasks for 48 h at 30 °C, 200 rpm, and pH 7.0.

3.3. Promoter optimization and GABA degradation reduction

Two methods of optimizing the expressing promoter of glutamate decarboxylase and decreasing GABA degradation (Fig. 4) were applied to further improve the GABA accumulation. For promoter optimization, the native promoter of the *Ncgl1289* gene (PNcgl) and the strong promoter TacM (Zhang et al., 2015) were selected to construct the expressing plasmids (Fig. 4a). The constructed plasmids were transformed into the *C. glutamicum* XW6 cells, and the GABA production was tested using the H36 promoter as control. Fig. 4b showed that the GABA titer increased by 10.6 % under the control of the TacM promoter (15.4 ± 0.1 g/L, $p < 0.01$), while less GABA was accumulated under the control of the PNcgl promoter (1.50 ± 0.0 g/L, $p < 0.01$), compared to that of control (14.0 ± 0.1 g/L).

For GABA degradation in *C. glutamicum*, the extracellular GABA would be transported into the cells by GABA permease (GabP) and then converted into succinate by GABA aminotransferase (GabT) and

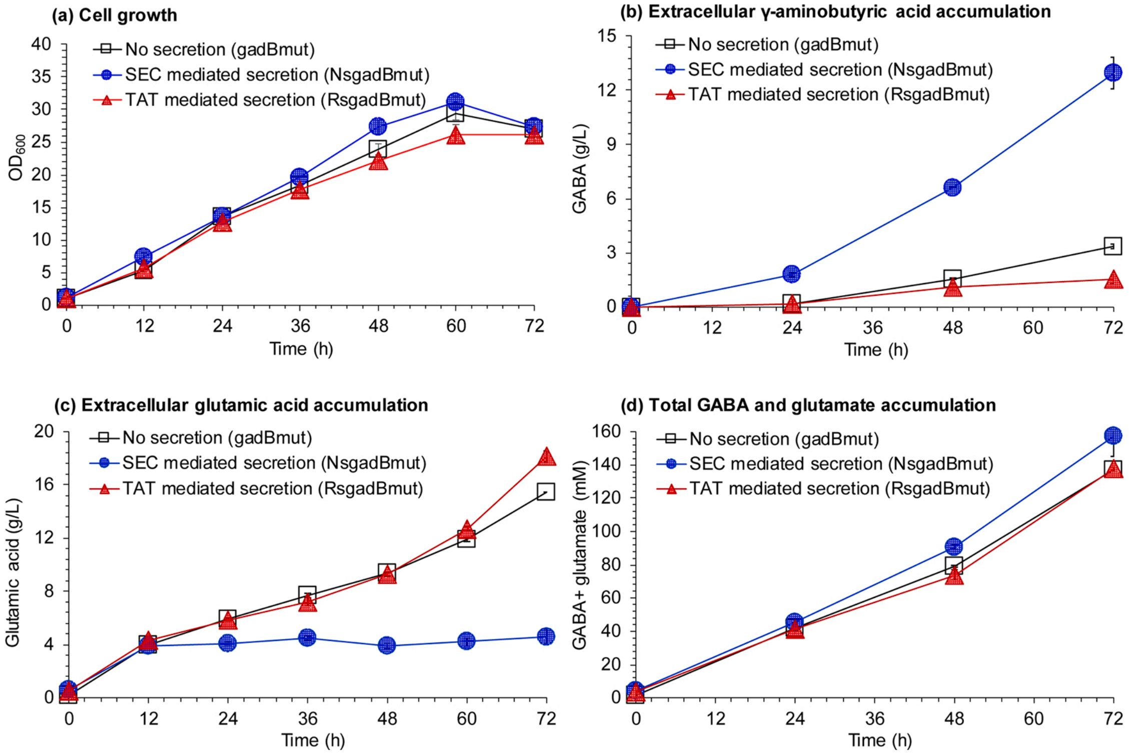

Fig. 3. GABA synthesis by extracellular expression of glutamate decarboxylase in *C. glutamicum* XW6. (**a**) Cell growth, (b) Extracellular γ-aminobutyric acid accumulation, (**c**) extracellular glutamic acid accumulation, (**d**) Total GABA and glutamate accumulation. No secretion (*gadBmut*), no glutamate decarboxylase secretion; SEC mediated secretion (*NsgadBmut*), Sec pathway mediated glutamate decarboxylase secretion; TAT mediated secretion (*RsgadBmut*), TAT pathway mediated glutamate decarboxylase secretion. Fermentations were carried out in shake flasks at 30 °C, 200 rpm, and pH was maintained at 7.0 by adding 20 % (w/w) urea solution. Error bars were indicated by the standard deviations of three biological replicates.

succinate hemialdehyde dehydrogenase (GabD) (Fig. 4a) (Ni et al., 2015). GABA decomposition is more active under neutral pH conditions of *C. glutamicum* culture; therefore, blocking the degradation pathway is an essential step for improving GABA accumulation (Ni et al., 2015). Thus, we knocked out the gene *gabP* encoding GABA permease and the gene *gabT* encoding GABA aminotransferase in *C. glutamicum* XW6 to obtain recombinants XW6-Δ*gabT*, XW6-Δ*gabP*, and the double-knockout strain XW6-Δ*gabP*Δ*gabT*. The knockout of these two genes resulted in significantly impaired cell growth when using GABA as the sole carbon and nitrogen source, and the GABA degradation was also effectively eliminated, compared to that of control (Supplementary Materials Fig. S3). This result indicates that GABA degradation can be efficiently relieved by the knockout of the two genes. Then, the effects of these genes' knockout on GABA production were tested, and improved GABA accumulation could be observed, comparing with that of control (Fig. 4b). The knockout of *gabP* showed the best GABA accumulation instead of the double knockout strain. The *gabT* gene encodes the GABA aminotransferase which catalyzes the transamination between GABA and α-oxoglutarate (Fig. 4a). The knockout of *gabT* may affect the intermediate metabolite supply to the TCA cycle thus further led to low extracellular GABA accumulation. The knockout of *gabP* was previously proved to effectively prevent the uptake of GABA (Ni et al., 2015). The results of this study also indicate that the import of the extracellular synthesized GABA can be easily blocked by the knockout of the GABA permease (GabP) encoding gene *gabP*. The *gabP* knockout recombinant strain (XW6-Δ*gabP*-pTacM-*NsgadBmut*) was selected for the final GABA fermentation and named as XW6-16.

3.4. GABA production by the engineered C. glutamicum XW6-16

GABA fermentation was conducted using the engineered *C. glutamicum* XW6-16 strain by both batch and fed-batch modes in a 3 L fermentor. For batch fermentation (Fig. 5a), the *C. glutamicum* cells grew well and GABA accumulation increased rapidly to the maximum titer of 45.0 ± 0.0 g/L after 48 h. The overall yield based on the consumed glucose was 0.44 ± 0.00 g/g and the productivity reached 0.94 ± 0.00 g·L^{-1} h^{-1} (Table 1).

The fed-batch fermentation was conducted using a previously reported medium with the initial glucose reduced to 100 g/L (Zhang et al., 2014). The highly concentrated glucose solution (50 mL of 279 g/L) was added periodically every 4 h during 24−52 h. Fig. 5b shows that the maximum cell growth was reached at 32 h then slightly reduced with the addition of glucose solution. GABA quickly accumulated to its maximum after 64 h with continuous glucose feeding. The record high GABA titer (77.6 ± 0.0 g/L), yield (0.44 ± 0.00 g/g), and productivity (1.21 ± 0.00 g·L^{-1} h^{-1}) were reached in the fed-batch fermentation (Table 2). The productivity was even close to the result of 1.34 g·L^{-1} h^{-1} that obtained by the GABA produced from the putrescine pathway (Jorge et al., 2017).

A small amount of glutamate was remained in the medium in both batch and fed-batch fermentation, this may due to the reduced activity and substrates affinity of glutamate decarboxylase under neutral pH conditions (Shi et al., 2014; Thu Ho et al., 2013). The residual glutamate in the final product may lead to a high cost of downstream separation and purification process. Thus, methods such as improve glutamate decarboxylase catalytic performance or lower the extracellular pH at the end of the fermentation should be investigated in the future to convert glutamate into GABA completely.

C. glutamicum is an ideal strain in fermentative GABA production for its super capacity on glutamate precursor supply. However, low GABA conversion efficiency severely restricted the industrial GABA production by *C. glutamicum*. Secretory expression of glutamate decarboxylase to achieve extracellular GABA catalysis by *C. glutamicum* shows great potential in solving this problem. The signal peptide of *Ncgl1289* gene successfully mediated the secretion of glutamate decarboxylase (Fig. 2)

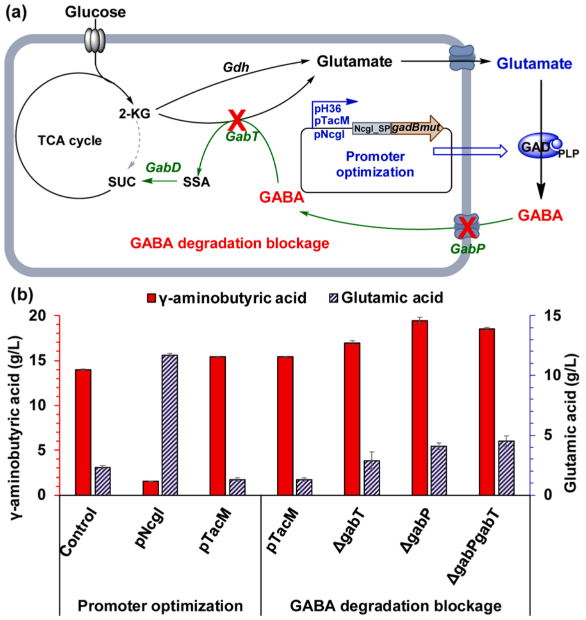

Fig. 4. Effect of promoter optimization and GABA degradation pathway knockout on GABA accumulation by *C. glutamicum*. (**a**) Metabolic diagram of GABA degradation pathway. 2-KG, 2- ketoglutarate; SUC, succinate; SSA, succinate semialdehyde; Gdh, glutamate dehydrogenase; GAD, glutamate decarboxylase. (**b**) GABA production after promoter optimization and degradation reduction. Control, XW6-pH36-*Ns-gadBmut*; pNcgl, XW6-pNcgl-*Ns-gadBmut*; pTacM, XW6-pTacM-*Ns-gadBmut*. Control for GABA degradation reduction, XW6-pTacM-*Ns-gadBmut*; Δ*gabT*, Δ*gabP*, and Δ*gabP*Δ*gabT*, knockout of the corresponding genes in XW6-pTacM-*Ns-gadBmut*. Fermentation was carried out in shake flasks at 30 °C, 200 rpm, and pH was maintained at 7.0 by adding 20 % (w/w) urea solution. Error bars were indicated by the standard deviations of three biological replicates.

and significantly improved GABA titer for more than three-folds (Fig. 3). The final strain we obtained reached the record high GABA titer, yield, and productivity compared with those reported results. This result was in accordant to the result of the extracellular expression of glutamate decarboxylase in *E. coli*, and more efficient conversion of glutamate into GABA by glutamate decarboxylase was achieved (Zhao et al., 2016). Even the GABA yields are still far from those obtained with *E. coli* or lactic acid bacteria as biocatalysts, the concept of using *C. glutamicum* as a biocatalyst for GABA production was fully demonstrated.

4. Conclusions

In this study, direct conversion of extracellular accumulated glutamate into GABA was achieved by secretory expression of glutamate decarboxylase in *C. glutamicum*. By this transition of intracellular GABA production into extracellular, a significant improvement in GABA production was successfully achieved. Fed-batch fermentation by the final strain achieved the record highest GABA production result, with the titer of 77.6 g/L, yield of 0.44 g/g, and productivity of 1.21 g·L^{-1} h^{-1}. This result demonstrated the potential of extracellular GABA production by *C. glutamicum* and lay a solid foundation for promoting industrial GABA production by *C. glutamicum*.

Credit author statement

Jie Bao - Conceived and directed the research, Wrote the manuscript.

Jingbai Wen - Conducted the experiment, Wrote the manuscript.

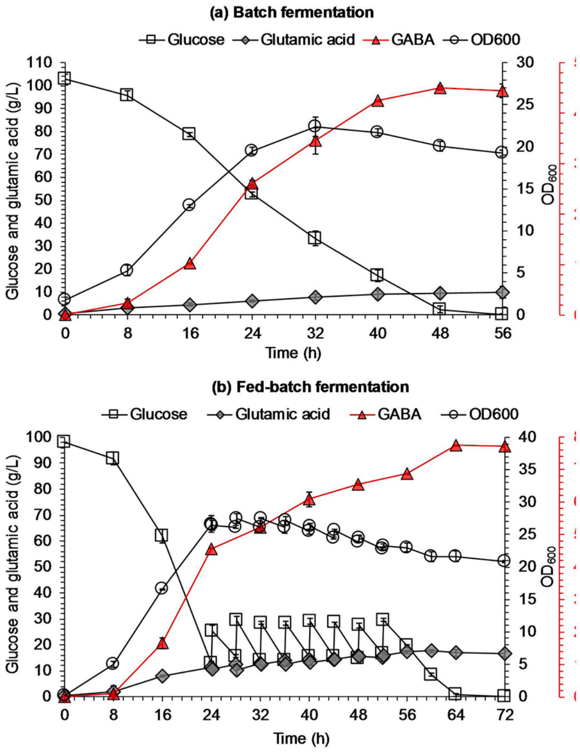

Fig. 5. GABA fermentation by the engineered *C. glutamicum* XW6-16. (**a**) Batch fermentation, (**b**) Fed-batch fermentation. The medium and the fermentation conditions were mentioned in the Materials and Method section. Error bars were indicated by the standard deviations of three biological replicates.

Table 2
Summary of the reported results for GABA production by *C. glutamicum*.

	GABA titer (g/L)	GABA yield (g/g glucose)	GABA productivity (g·L^{-1}·h^{-1})	Sources
Batch	2.7	0.02	0.03	Shi and Li (2011)
Batch	8.0	0.2	0.31	Jorge et al. (2017)
Batch	12.4	0.25	0.17	Takahashi et al. (2012)
Batch	26.5	0.21	0.44	Shi et al. (2018)
Batch	27.1	0.27	0.44	Shi et al. (2013)
Batch	31.1	0.31	0.26	Okai et al. (2014)
Batch	**45.0 ± 0.5**	**0.44 ± 0.00**	**0.94 ± 0.01**	**This study**
Fed-batch	26.3	N/A	0.37	Okai et al. (2014)
Fed-batch	29.5	0.21	0.41	Wang et al. (2015)
Fed-batch	38.6	0.32	0.54	Choi et al. (2015)
Fed-batch	63.2	0.24	1.34	Jorge et al. (2017)
Fed-batch	70.6	~0.30*	1.01	Zhang et al. (2014)
Fed-batch	**77.6 ± 0.0**	**0.44 ± 0.00**	**1.21 ± 0.00**	**This study**

* Estimated based on the initial glucose, cell mass and final GABA titer reported previously (Shi et al., 2013).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiotec.2021.03.003.

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