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Short Communication

Stepwise metabolic engineering of a plasmid-free *Corynebacterium glutamicum* for efficient production of γ-aminobutyric acid (GABA) by co-utilizing lignocellulosic feedstock-derived sugars

Jie Wang, Yingying Xu, Zhuolin Song, Bin Zhang *, Jie Bao *

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

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ABSTRACT

γ-aminobutyric acid (GABA) can be synthesized through plasmid-based expression of glutamate decarboxylase in L-glutamic acid producing *Corynebacterium glutamicum* strain. However, the addition of antibiotic to maintain the expression plasmid during the fermentation not only increases production and recovery costs, but also poses potential food safety hazards. In this study, a plasmid-free GABA producing *C. glutamicum* strain was constructed from *C. glutamicum* GJ04 chassis, which can produce L-glutamate by co-utilizing lignocellulose-derived glucose and xylose. Secretory glutamate decarboxylase was integrated into the genome of *C. glutamicum* GJ04 in three copies by replacing *ldh*, *gabT*, *gabD* genes. The metabolic flux in engineered *C. glutamicum* was further fine-tuned by knocking out *aceA* and *gabP* genes to enhance GABA production. The recombinant strain *C. glutamicum* GJ09 can produce 44.3 ± 3.8 g/L GABA from 15 % (w/w) solids loading corncob residues hydrolysate with the yield and productivity of 0.45 g/g and 0.74 g/L/h. The highest GABA titer reached 63.4 g/L by fed-batch fermentation using corncob residues-derived syrup. This study provided a robust and plasmid-free *C. glutamicum* strain by stepwise metabolic engineering for industrial production of GABA from lignocellulosic feedstocks.

γ-aminobutyric acid (GABA) is a non-protein amino acid, and has a wide range of applications in the fields of medicine, food, cosmetics, agriculture, and biodegradable materials (Wang et al., 2024a). Microbial production of GABA offers a scalable and cost-effective alternative compared with chemical synthesis and plant enrichment (Han et al., 2023). Generally, GABA is synthesized from glutamate via an irreversible decarboxylation reaction catalyzed by glutamate decarboxylase (GAD) (Su et al., 2021). *C. glutamicum* is a non-pathogenic bacterium, and commonly used in the industrial production of L-glutamate (Wang et al., 2024b). By the heterologous expression of GAD in *C. glutamicum*, GABA can be synthesized from sugars de-novo (Takahashi et al., 2012).

A variety of low-cost alternative carbon sources have been tested to produce GABA (Baritugo et al., 2018; Hasegawa et al., 2020; Lai et al., 2021). However, most of these GABA-producing *C. glutamicum* strains harbor free plasmid (Yao et al., 2022), antibiotics are thus introduced as a selective pressure. The utilization of antibiotic not only increases production and recovery costs, but also poses potential food safety hazards (Lv et al., 2024).

Our previous study constructed a GABA producing strain using glucose by plasmid expression of secretory GAD in *C. glutamicum* (Xu et al., 2025). However, the antibiotic kanamycin needed to be added during the fermentation to maintain the plasmid stability. To further develop the plasmid-free GABA-producing *C. glutamicum* strain for industrial application using low-cost lignocellulose feedstock, GAD and xylose assimilation pathway should be integrated into the genome. The strains, plasmids and primers used in this study were listed in Table S1 and S2.

An engineered *C. glutamicum* GJ04 with the ability to co-utilize glucose and xylose was firstly constructed from wild *C. glutamicum* S9114 by integrating gene cluster *xylAB* (xylose isomerase and xylulokinase) from *E. coli* BL21, deleting C-terminal 110 amino acids of glutamate channel protein MscCG, down-regulating the activity of α-oxoglutarate dehydrogenase, and overexpressing the pentose transporter *araE* (Jin et al., 2020). The obtained *C. glutamicum* GJ04 strain can produce over 60 g/L glutamate, which provides a favorable chassis for GABA production.

A plasmid, pTacM-*NsgadBmut*, was then constructed to express GAD for GABA production in *C. glutamicum* GJ04, consisting of the strong promoter TacM (Zhang et al., 2015), signal peptide of *Ncgl1278* from *C. glutamicum* ATCC13032 recognized by SEC translocation pathway

* Corresponding authors.
E-mail addresses: binzh@ecust.edu.cn (B. Zhang), jbao@ecust.edu.cn (J. Bao).

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(Shi et al., 2017), and GAD mutant (*gadB*$^{Glu89Gln/\Delta452–466}$) from *E. coli* K12 (Baritugo et al., 2018). All the used plasmid profiles and construction protocols were showed in Figs. S1–S7. The GAD mutant (*gadB*$^{Glu89Gln/\Delta452–466}$) from *E. coli* has high activity at a wide pH range between 5.0 and 7.0 (Son et al., 2022), which matches the optimal culture pH (7.0) of *C. glutamicum*. Our previous study revealed that the secretory expression did not affect the cell growth much, the extracellular catalysis by secreted GAD increased the GABA generation compared with intracellular catalysis (Wen et al., 2024). The expression of pTacM-*NsgadBmut* led to the GABA production of 9.6 ± 0.4 g/L from glucose in flask (Fig. 1). Further attempts were made to integrate *gadBmut* gene in the genome of *C. glutamicum* GJ04 to avoid the usage of antibiotic.

The lactate dehydrogenase (*ldh*) gene was then replaced by pTacM-*NsgadBmut* to form a one-copy recombinant *C. glutamicum* GJ05 (Fig. 1**a**). However, the GABA titer was only 1.4 ± 0.2 g/L (Fig. 1**b**). qRT-RCR was conducted to measure the expression of *gadBmut* in *C. glutamicum*. The protocol of qRT-RCR was showed in Supplementary materials. The results of qRT-RCR showed that the expression level of *gadBmut* in *C. glutamicum* GJ05 was only 2.9 % of that in plasmid-expressing strain (Fig. 1**c**). In fact, the copy number of *gadB* in genome should be optimized. Yao et al. (2022) optimized the copies ratio of glutamic acid decarboxylase and glutamic dehydrogenase for GABA production. Wen et al. (2024) triply inserted *gadB* into the genome of *C. glutamicum* to improve the GABA fermentation performance. The *gadBmut* gene was further integrated at different loci to increase its transcription level and GABA production. The GABA biodegradation pathway in *C. glutamicum* involved GABA transaminase (*gab*T), succinate-semialdehyde dehydrogenase (*gab*D) and permease (*gab*P) (Shi et al., 2017). *gabT* was replaced by pTacM-*NsgadBmut* to form a two-copy recombinant *C. glutamicum* GJ06 (Fig. 1**a**). The GABA titer increased to 6.1 ± 0.1 g/L by *C. glutamicum* GJ06 (Fig. 1**b**). *gab*D gene was then replaced by pTHP7-*NsgadBmut*. In this expression cassette, a bicistronic vector HP7 (62 bp) was inserted between promoter TacM and *NsgadBmut* to enhance the secreted expression of *gadBmut* (Fig. 1**a**). The vectors with bicistronic expression patterns have strong compatibility for expressing various heterogeneous proteins in high yield (Sun et al., 2020), and had been verified in our previous study for GABA production (Wen et al., 2024). The titer increased to 6.6 ± 0.3 g/L by three-copy recombinant *C. glutamicum* GJ07 (Fig. 1**b**). The transcriptional level of *gadB* in GJ07 increased to 19.1 ± 2.2 % of that in plasmid-expressing strain (Fig. 1**c**). Although the GABA production of *C. glutamicum* GJ07 was still lower than that of the plasmid-expressing strain, the metabolic burden caused by multiple-copy integration resulted in poor cell growth and failure to continue integration.

The metabolic flux in *C. glutamicum* GJ07 was fine-tuned to enhance GABA production. The *aceA* gene, encoding isocitrate lyase, involved in glyoxylate shunt was knocked out in *C. glutamicum* GJ07 to form GJ08

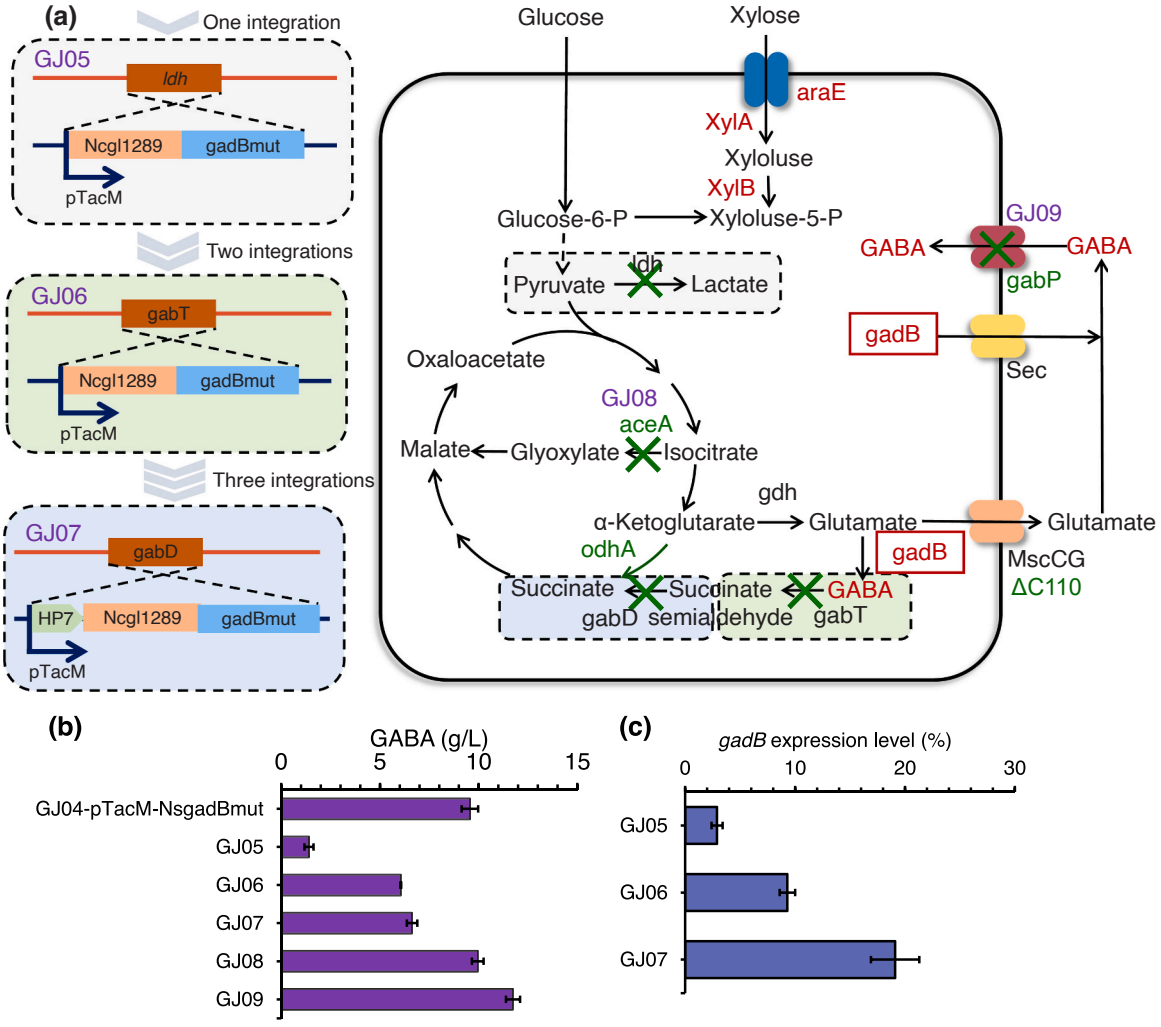

Fig. 1. Construction of the plasmid-free recombinant strain by multiple-copy integration of secretory glutamate decarboxylase (*gadBmut*) into the genome of *C. glutamicum* GJ04. (a) Schematic diagram of multiple-copy integration of *gadBmut* and fine-tuning of metabolic flux for GABA production. (b) GABA production in flasks. (c) Expression levels of *gadBmut* in recombinant strains. The cells were harvested after 24 h's culture for qRT-PCR.

strain (Fig. 1**a**). The knockout of *aceA* gene can re-direct more isocitrate to α-ketoglutarate and be used for glutamate production. The results showed that the GABA titer reached 10.0 ± 0.3 g/L by *C. glutamicum* GJ08 (Fig. 1**b**). *gabT* gene was further knocked out to block the degradation of GABA (Fig. 1**a**). The generated recombinant strain GJ09 produced 11.7 ± 0.4 g/L GABA, which is similar to that of plasmid-expressing strain.

We evaluated the GABA fermentation performance of engineered strains *C. glutamicum* GJ04-pTacM-*NsgadBmut* and *C. glutamicum* GJ09 using both refined mixed sugars and corncob residues-derived sugars in a 3 L fermenter (Fig. 2). In the case of *C. glutamicum* GJ04-pTacM-*NsgadBmut*, nearly all glucose and xylose were consumed, resulting in a GABA titer of 45.5 ± 2.6 g/L at 72 h, with a yield of 0.32 g/g sugars and a productivity of 0.63 g/L/h (Fig. 2**a**). The recombinant strain *C. glutamicum* GJ09 showed a stronger GABA production capacity compared to the plasmid-expressing strain. The highest GABA titer reached 55.1 ± 0.7 g/L at 60 h with the yield and productivity of 0.39 g/g sugars and 0.92 g/L/h (Fig. 2**b**). The OD_{600} value of *C. glutamicum* GJ09 was lower than that of plasmid-expressing strain, indicating that more carbon flux was redirected from cell growth to GABA biosynthesis in recombinant strain.

The industrial corncob residues were hydrolyzed at 15 % (w/w) solids loading for 48 h with the cellulase dosage of 6 mg protein/g. The corncob residues hydrolysate contained 86.6 g/L glucose and 12.0 g/L xylose was used for GABA fermentation (Fig. 2**c**). The highest GABA titer reached 44.3 ± 3.8 g/L at 60 h by *C. glutamicum* GJ09 with the yield and productivity of 0.45 g/g and 0.74 g/L/h. All the sugars were consumed, and the cell growth in corncob residues hydrolysate was better than that in synthetic medium. The corncob residues hydrolysate was further centrifuged to remove the solids, and evaporated to prepare the concentrated syrup containing 778 g/L glucose and 120 g/L. The fed-batch fermentation was conducted by feeding the corncob residues-derived syrup at 36 h, 48 h, and 70 h (Fig. 2**d**). The final GABA titer and yield reached 63.4 g/L, meanwhile, all the glutamate and xylose were consumed. Despite the fed-batch fermentation increased the final GABA titer (63.4 g/L) and productivity (0.88 g/L/h) compared with the batch fermentation, GABA yield of fed-batch fermentation was only 0.42 g/g. The ability of sugars metabolism of the engineered strain decreased at the late-stage of fed-batch fermentation, and there was 41.2 g/L residual glucose at 72 h. The consumed sugars were disproportionate to the growth of DO_{600} and GABA titer at the late-stage of fed-batch fermentation, suggesting that the sugars were consumed to maintain the cell activity instead of GABA generation and cell growth at the late-stage of fed-batch fermentation. A nitrogen-limited medium (10 g/L CSL and 5 g/L $(NH_4)_2SO_4$) was adopted in this study for GABA fermentation (see Supplementary materials), because that a high organic nitrogen source was preferred for cell growth but not for GABA generation (Xu et al., 2025). This phenomenon possibly attributed to the depletion of nutrients at the late-stage of fermentation.

In conclusion, a recombinant *C. glutamicum* GJ09 strain was constructed by multiple-copy integration of secretory GAD into the genome of L-glutamate-producing chassis, fine-tuning the metabolic flux of glyoxylate shunt and knocking out GABA biodegradation pathway. The engineered *C. glutamicum* GJ09 can efficiently produce GABA from lignocellulosic feedstock. The GABA titer reached 44.3 g/L and 63.4 g/L by batch and fed-batch fermentation. This study provided a robust and plasmid-free *C. glutamicum* strain for the industrial production of GABA from non-food lignocellulosic feedstocks.

CRediT authorship contribution statement

Zhuolin Song: Investigation, Methodology. **Yingying Xu:** Methodology, Resources. **Jie Bao:** Writing – review & editing, Supervision, Funding acquisition. **Bin Zhang:** Writing – review & editing,

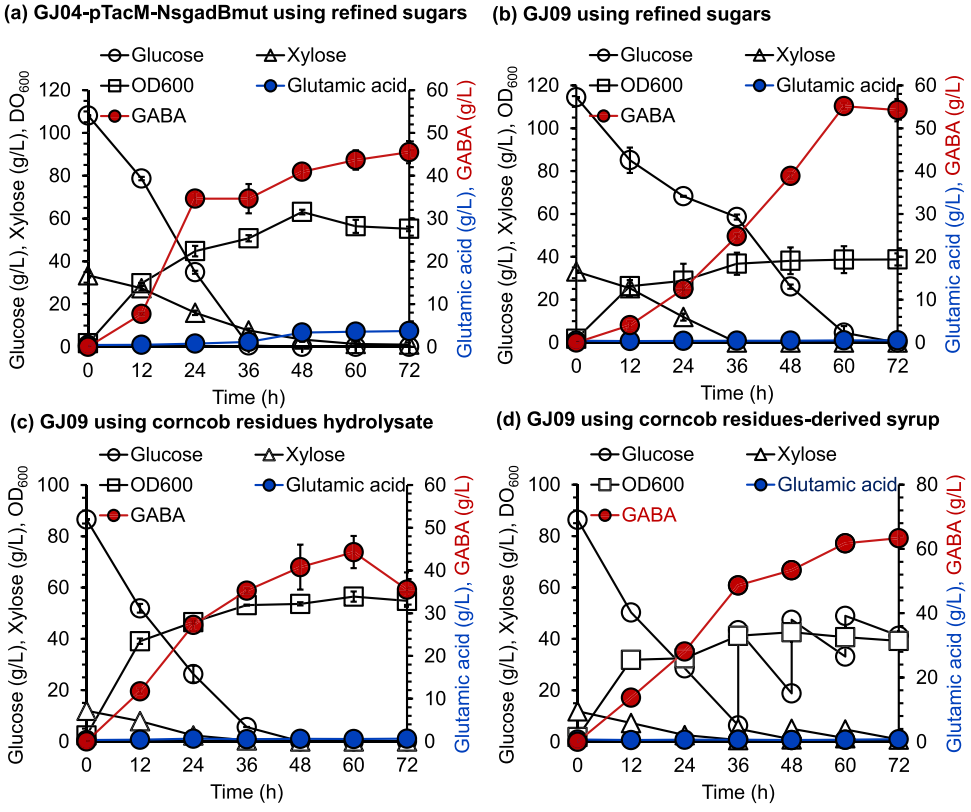

Fig. 2. GABA fermentation evaluations of engineered strains. (a) *C. glutamicum* GJ04-pTacM-*NsgadBmut* using refined sugars. (b) *C. glutamicum* GJ09 using refined sugars. (c) *C. glutamicum* GJ09 using 15 % (w/w) solids loading of corncob residues hydrolysate. (d) Fed-batch fermentation using corncob residues-derive syrup. Conditions: pH 30 °C, 1.4 vvm, 600 rpm for 72 h. Total three-time feedings were conducted at 36 h, 48 h, and 60 h.

Visualization, Formal analysis. **Jie Wang:** Writing – original draft, Investigation, Data curation.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2025.07.023.

Data availability

Data will be made available on request.

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