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Production of γ-aminobutyric acid using corncob residue as carbohydrate feedstock by engineered *Corynebacterium glutamicum*

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ABSTRACT

This study investigated *Corynebacterium glutamicum* for GABA production using corncob residue, a cheap carbon source, as carbohydrate feedstock instead of grains. Current fermentative production of γ-aminobutyric acid (GABA) mainly relies on starch and sugar feedstocks. Therefore, the use of cheap carbon sources can effectively reduce the production costs of GABA. This study validated the feasibility of an available non-food lignocellulose feedstock, corncob residue, for GABA fermentation. An engineered *C. glutamicum* was constructed by secretory expression of glutamate decarboxylase gadBmut and knock-out the GABA biodegradation gene. The fermentation parameters in shake flasks and bioreactors were optimized including the sugar concentration, nitrogen levels, cofactor levels and aeration rate, and fermentation mode. The fed-batch fermentation of the concentrated corncob residue hydrolysate achieved a record high GABA production of 93.15 g/L from lignocellulosic feedstock with a yield of 0.43 g/g glucose. The results provided technical support for the industrial production of GABA from low-cost lignocellulosic carbohydrates.

1. Introduction

The production of γ-aminobutyric acid (GABA) from carbohydrate feedstocks typically involves two steps: the synthesis of glutamic acid from carbohydrates and the consequent decarboxylation of glutamic acid to GABA [1,2]. The commonly used fermentation strains include *Escherichia coli*, lactic acid bacteria, and *C. glutamicum* [3–10]. Among these, *C. glutamicum* offers a distinct advantage in glutamate synthesis, and is then used for GABA synthesis by heterologous expression of glutamate decarboxylase [11–15]. The availability of inexpensive carbohydrate feedstocks is the most important factor on the economics of GABA production. Waste biomass feedstocks such as rice bran, empty fruit bunch (EFB), and date pomace had been tested to produce GABA by enzymatic hydrolysis of the cellulose in these feedstocks into fermentable sugars. However, GABA yields were too low to meet the demands of industrial production [16–20].

Corncob residue (CCR) is a high cellulose-containing solid corncob residue after extraction of the hemicellulose (xylan) fraction [21]. Compared to conventional lignocellulosic feedstocks such as corn stover, wheat straw and rice straw, corncob residue is an available industrial byproduct with a high cellulose content, easy hydrolysis, and virtually free of aldehydes and phenolic inhibitors [22]. These properties make corncob residues an optimal choice for production of biobased-products with high added value. Corncob residue has been applied for the production of ethanol [23–25], microbial lipid [26,27], 2,3-butanediol [28], but no reports on the production of GABA.

In this study, an engineered strain was constructed by effectively expressing glutamate decarboxylase through secretory expression for GABA synthesis using a glutamate-producing *C. glutamicum* strain as the starting strain [29]. Corncob residues were hydrolyzed to fermentable sugars as a carbohydrate feedstock for GABA fermentation using the constructed *C. glutamicum* strain. A satisfactory conversion of corncob residue to GABA was achieved. This study provided important technical support for the low-cost production of GABA using cellulosic biomass carbon sources.

2. Materials and methods

2.1. Feedstock

Corncob residue (CCR) was obtained from Shandong Longlive Biotech Co., Jining, Shandong, as the residue left after acid hydrolysis of xylan from corncob. The moisture content was 62 % (w/w) and the composition based on the dry matter (w/w) was 82.08 % of cellulose

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and 5.43 % of hemicellulose according to the two-step acid hydrolysis method of the National Renewable Energy Laboratory (NREL) [30,31].

Corn steep liquor (CSL) was purchased from Shandong Xiwang Group, Zouping, Shandong. Pyridoxal 5′-phosphate (PLP) was purchased from Aladdin, Shanghai, China. Other reagents used were purchased from Titan Tech, Shanghai, China.

Cellulase Cellic CTec2 was purchased from Novozymes (China), Beijing, China. The cellulase activity was measured as 256 FPU per milliliter according to the method in NREL LAP-006 and the cellobiose activity was 4653.3 CBU per milliliter [32]. The protein concentration was 86.3 g proteins/L according to the Bradford method [33].

2.2. Strains and media

The previously engineered *C. glutamicum* XW6 was used as a host bacterium [29]. The activation medium contained 5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl, and 17 g/L agar powder. The synthetic medium contained 120 g/L of glucose, 1 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4$, 3 g/L of urea, 2 mg/L of $FeSO_4$, 2 mg/L of $MnSO_4$, 30 g/L of corn steep liquor (CSL, dry base), 0.1 mM PLP with the initial pH 7.0. The nutrients added to corncob residue hydrolysate contained 1 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4$, 3 g/L of urea, 2 mg/L of $FeSO_4$, 2 mg/L of $MnSO_4$, 10 g/L of CSL, and 24.7 mg/L of PLP.

For shake flask fermentations, strains were grown in activation medium at 30 ℃ for 48 hours and then a single colony was picked for seed culture. The seed culture at 5 % (v/v) inoculum ratio was inoculated into 250 mL flasks containing 30 mL of corn steep liquor synthetic medium and cultivated at 30 ℃ and 200 rpm shaking. The pH was adjusted to 7.0 by adding 200 g/L NaOH.

The batch fermentation was performed in a 3 L fermenter (3BG-4, Baoxing Biotech, Shanghai, China) at 30 ℃, 1.4 vvm aeration, and 600 rpm. The pH was maintained at 7.0 by automatically addition of 25 % (w/v) ammonium hydroxide solution and 196 g/L sulfuric acid solution. The seed culture was inoculated at 10 % (v/v) inoculum ratio.

Fed-batch fermentation was performed in the same 3 L fermenter. The glucose was fed by adding the high-sugar hydrolysate and the concentrated hydrolysate to the fermenter from 24 h to 60 h. The culture conditions are the same as for batch fermentation.

2.3. Preparation of corncob residue hydrolysate and concentrated hydrolysate

Corncob residue was hydrolyzed by adding 4 mg of total cellulase proteins per gram of dry corncob residue matter at the solid content from 10 % to 30 % (w/w) at 50 °C, 200 rpm for 48 hours in a 5 L fermenter (Baoxing Biotech, Shanghai, China). The hydrolysate obtained was centrifuged at 8000 rpm for 15 minutes to remove solid residues. The supernatant was then sterilized at 115 °C for 20 minutes and filtered through filter paper to remove residual solids.

Three corncob residue hydrolysates were prepared under the different corncob residue solids loadings. The low-sugar corncob residue hydrolysate was prepared using corncob residue with a solids content of 15 %. The medium-sugar hydrolysate was prepared using corncob residue with a solids content of 20 %. The high-sugar hydrolysate was prepared using corncob residue with a solids content of 30 %.

The concentrated hydrolysate was prepared using the high-sugar hydrolysate by vacuum rotary evaporator (LC-RE-301, Shanghai Li-Chen Bang Xi Instrument Tech Co.) to the glucose concentration of 600 g/L.

The corncob residue hydrolysates were supplemented with nutrients and used for subsequent shake flask or bioreactor fermentations. The nutrients added included 1 g/L of KH_2PO_4, 0.6 g/L of $MgSO_4$, 3 g/L of urea, 2 mg/L of $FeSO_4$, 2 mg/L of $MnSO_4$, and 30 g/L of CSL (dry base), 24.7 mg/L of PLP.

2.4. Analytical methods

Glucose and xylose concentrations were determined using high-performance liquid chromatography (HPLC) from Shimadzu Corporation (Japan), model LC-20D with a differential refractometer RID-10A. The chromatographic column used was Aminex HPX-87H (300 mm × 7.8 mm) from Bio-Rad, Hercules, USA. The HPLC conditions were at 65 °C, column temperature with a mobile phase of 5 mM H_2SO_4, and a flow rate of 0.6 mL/min.

Glutamate was determined using the biosensor analyzer SBA-90 developed by Shandong Academy of Sciences, Jinan, Shandong, China. γ-Aminobutyric acid was analyzed using the pre-column O-phthalaldehyde derivatization method [34]. The concentration was determined using high-performance liquid chromatography (HPLC). The HPLC concentration determination was conducted using the HPLC from Shimadzu Corporation (Japan), model SPD-20A with a UV detector, with a column temperature of 40 °C and a detection wavelength of 338 nm. A gradient elution method was used with a total flow rate of 1.0 mL/min. The HPLC gradient elution method was performed according to a previous study [29].

3. Results and discussion

3.1. Construction of recombinant strains

The glutamate-producing strain *C. glutamicum* XW6 was used as the starting host [35] for metabolic engineering to obtain the high GABA

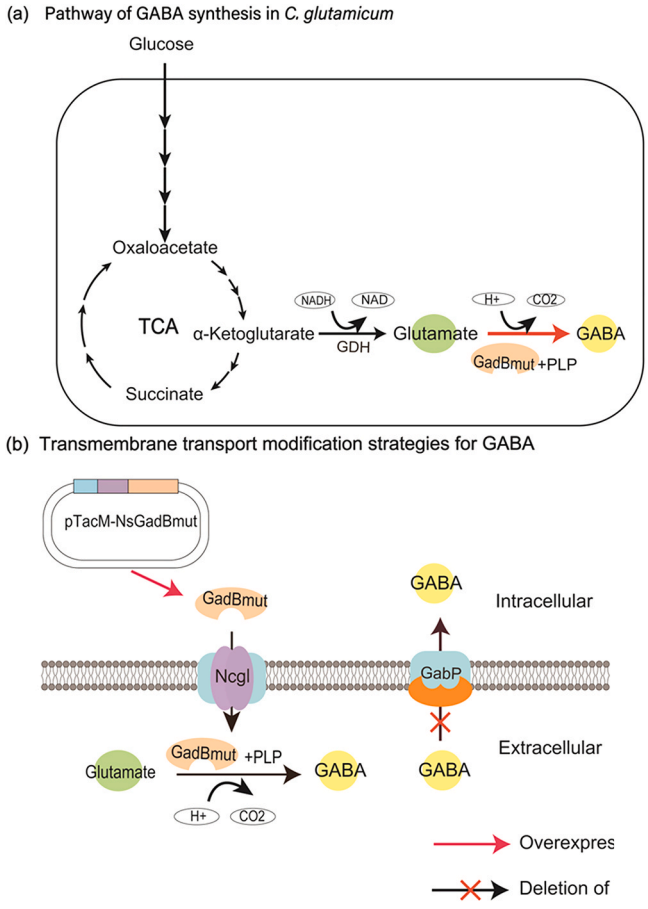

Fig. 1. Metabolic modification of *C. glutamicum* cells for GABA production. (a) Pathway of GABA synthesis in *C. glutamicum*; (b) Transmembrane transport modification strategies for GABA. Red crosses, gene knockout; Red arrow, gene overexpression. GadBmut, glutamic acid decarboxylase. GabP, GABA permease. NcgI, secretory expressed protein. PLP, pyridoxal 5′-phosphate.

producing strain. Fig. 1 showed the three metabolic modification steps of *C. glutamicum* XW6, including the steps: (i) Construction of GABA synthesis pathway by heterologous expression of glutamate decarboxylase gadBmut with the encoded gene origin from *E. coli* K12 under the control of TacM promoter, resulting in a GABA-producing strain XW6-pH36-gadBmut; (ii) Secretory expression of glutamate decarboxylase gadBmut into the extracellular space by the signal peptide *Ncgl1289* [36], resulting in the strain XW6-pH36-NsgadBmut; (iii) Knock-down the GABA biodegradation gene gabP encoding GABA transaminase to stop the conversion of succinate to GABA [37]. The finally obtained engineered strain was defined as *C. glutamicum* XY24 for the consequent GABA fermentations.

3.2. Improving the GABA fermentation efficiency in shake flasks using corncob residue

The preliminary screening of GABA fermentation parameters by *C. glutamicum* XY24 were carried out using corncob residue hydrolysate as the carbohydrate feedstock in shake flasks (Fig. 2). Nitrogen source is an important factor in GABA fermentation efficiency. *Corynebacterium glutamicum* is an amino acid heterotrophic bacterium and its cell growth is more favorable with organic nitrogen source such as corn steep liquor with high amino acid content. On the other hand, the syntheses of glutamic acid and the consequent GABA requires ammonia ion as the direct nitrogen uptake for amination of α-ketoglutarate. Fig. 2(a) shows that the sole inorganic nitrogen source (ammonia sulfate) was not preferred for both the cell growth and GABA generation; the sole organic nitrogen source (CSL with high amino acid content) promoted the cell growth but not favorable for GABA generation. When the mixture of both inorganic nitrogen source and the relatively decreased organic nitrogen source (CSL) were used, the cell growth was curbed and the GABA generation was increased. To meet the need cell growth and metabolism of *C. glutamicum*, the combination of corn steep liquor and ammonium sulfate is required. Therefore, the GABA titers were used to represent the generation of GABA approximately. PLP is the key cofactor in GABA production, however, Fig. 2b indicates that the varying PLP concentration only slightly affected the GABA generation, suggesting that the supply of PLP was not the limiting factor on GABA production. The initial sugar concentration in corncob residue hydrolysate was prepared by hydrolyzing different corncob residue solids in the hydrolysis step. Fig. 2c showed that an approximately 30 g/L increase in the initial sugar hydrolysate (from 100 g/L to 130 g/L) led to a double increase of GABA generation, indicating the higher initial sugar concentration in the hydrolysate was a crucially important factor. On the other hand, the higher initial sugar concentration in the corncob residue hydrolysate required an excessively high solid content, which led to a lower sugar recovery yield in the enzymatic hydrolysis step. Overall, the use of corn steep liquor (CSL) and $(NH_4)_2SO_4$ under the high initial sugar concentration and the moderate PLP concentration were the most beneficial parameters for GABA production.

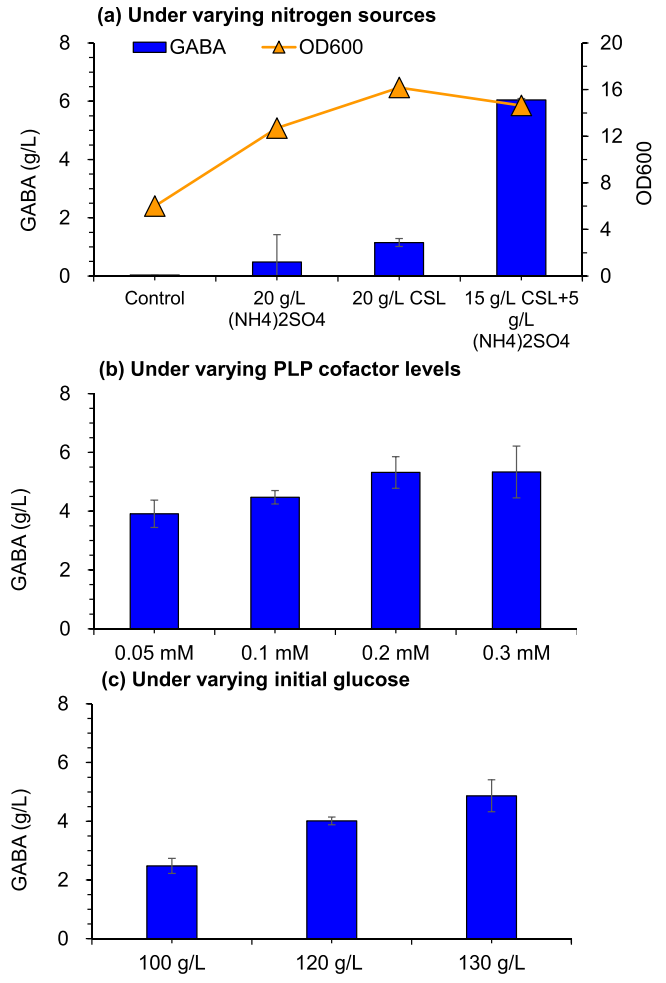

Fig. 2. Optimizing GABA fermentation parameters using corncob residue hydrolysates in the flask. All the fermentations were conducted at 30 ℃, pH 7.0 with 200 rpm in flasks and 200 rpm in bioreactor. Nutrient salts except the changes in (a) and (b) were described in the Section 2.2. (a) nitrogen sources in shake flasks; (b) PLP cofactor addition in shake flasks; (c) initial glucose concentration in shake flasks. The low-, medium-, and high-sugar corncob residue hydrolysates were described in the Section 2.2.

3.3. Production of GABA using corncob residue feedstock in bioreactors

The GABA fermentation performance of *C. glutamicum* XY24 using corncob residue hydrolysates was conducted in bioreactors with well-controlled pH and sufficient oxygen supply, following the initial investigation of GABA fermentation parameters in shake flasks.

Fig. 3 showed the GABA fermentation in 1 L bioreactor under changing aeration rate using low-sugar corncob residue hydrolysate. The result showed that the higher aeration rate led to the higher GABA generation, indicating the dissolved oxygen level was crucially important for GABA production. Notably, the GABA titer reached 27 g/L in the bioreactor compared to led to only 6 g/L of GABA in the shake flask (Fig. 2) due to the stable pH at 7.0 and sufficient dissolved oxygen supply by strong agitation in the fermenter.

The nitrogen source concentration significantly impacts the GABA generation and its effect was further investigated in a 3 L bioreactor using the synthetic medium with varied nitrogen concentration by supplementing different levels of CSL and $(NH_4)_2SO_4$ (Fig. 4). The flasks and the fermentation parameters in Fig. 4 were the same except the nitrogen sources used. Fig. 4a showed that the rich nitrogen source

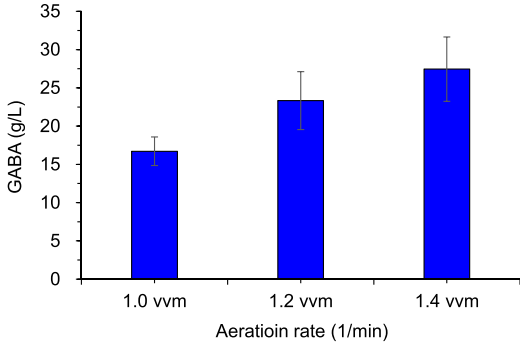

Fig. 3. Effect of aeration rate on GABA fermentation using corncob residue hydrolysates in 1 L bioreactor. The term "vvm" indicates the air rate (L/min) per liter of fermentation broth.

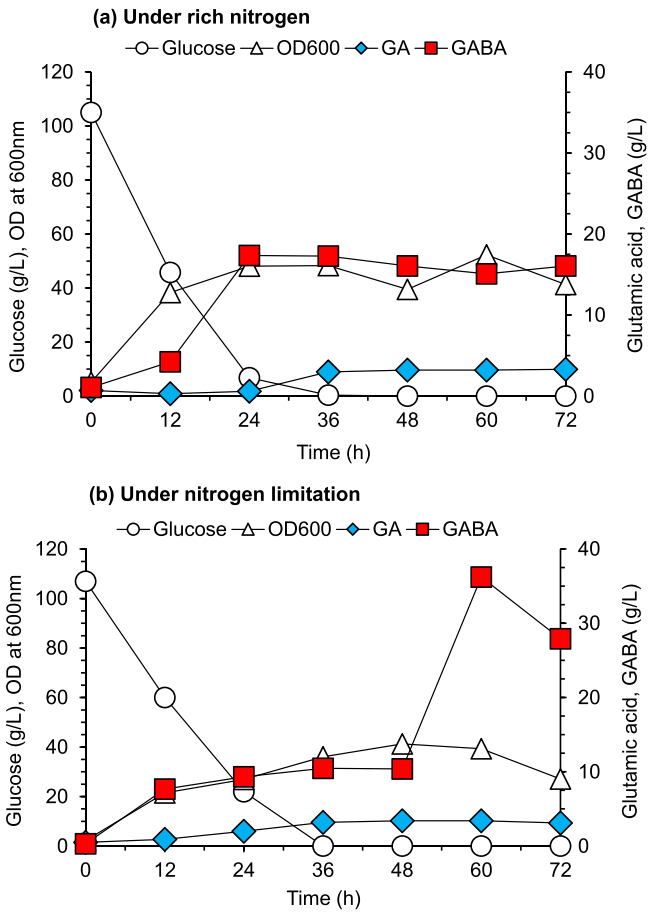

Fig. 4. Effect of corn steep liquor (CSL) concentration on GABA production in the synthetic medium in a 3 L fermenter at 30 ℃, 600 rpm, 1.4 vvm, pH 7.0. (a) Rich nitrogen condition by adding 30 g/L CSL and 5 g/L $(NH_4)_2SO_4$; (b) limited nitrogen condition by adding 10 g/L CSL and 5 g/L $(NH_4)_2SO_4$. Nutrient supplementation in hydrolysate as described in the Section 2.2.

(30 g/L CSL and 5 g/L $(NH_4)_2SO_4$) led to excessive cell growth (the OD600 was 50) but the lower GABA yield (17 g/L), perhaps the sugar was consumed to maintain the cell activity instead of GABA generation. Fig. 4b showed that under nitrogen-limited condition by reducing CSL to 10 g/L from 30 g/L, the cell growth was reduced, and the GABA generation was significantly increased to 36 g/L, approximately twice that in the rich nitrogen condition. The result reveals that a high organic nitrogen source was preferred for cell growth but not for GABA generation. A moderate nitrogen level is a determinant factor in the production of GABA using corncob residue hydrolysate by balancing the cell growth and GABA generation.

The changing initial sugar concentrations of corncob residue hydrolysate were generated by hydrolyzing the solids loading from 15 %, 20 %, to 30 % according to the Method in Section 2.3. Fig. 5 showed that for the low-sugar hydrolysate, the glucose was consumed within 24 hours but the GABA generation was limited; with the increased initial sugar concentration to the medium-sugar hydrolysate, the glucose was consumed within 48 hours and the GABA generation reached a maximum of 73 g/L with the yield of 0.6 g GABA per gram of glucose consumed; when the initial sugar concentration was further increased to the high-sugar hydrolysate, both the glucose consumption and cell growth were greatly inhibited, while the maximum GABA was reduced to 60 g/L, possibly due to the higher inhibitor concentration such as acetic acid. The GABA fermentation performance in bioreactors generally agreed well with that in shake flasks, providing the practical and optimal fermentation parameters for further applications.

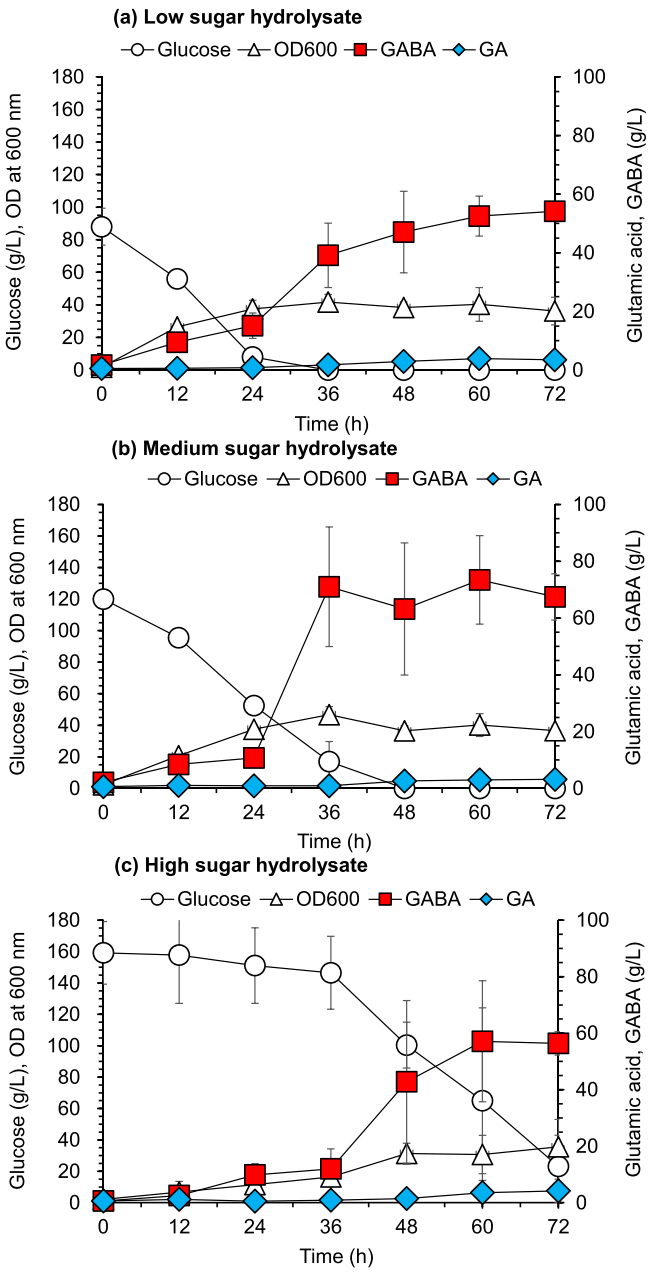

Fig. 5. GABA production using corncob residue hydrolysate under different initial sugar concentrations. The fermentation was conducted in 3 L fermenter. (a) Low-sugar corncob residue hydrolysate; (b) medium-sugar corncob residue hydrolysate; (c) high-sugar corncob residue hydrolysate. Conditions: 30 ℃, 600 rpm, 1.4 vvm, pH 7.0. Nutrient supplementation was under the nitrogen-limited condition as described in the Section 2.2.

3.4. Fed-Batch fermentation for GABA production using corncob residue feedstock

To further increase the GABA production level using corncob residue, the fed-batch fermentation was conducted by adding more sugar into the fermentation broth to increase the carbohydrate level. Fig. 6a showed that the high-sugar hydrolysate (160 g/L of glucose) was used to fed-batch the GABA fermentation with the medium-sugar hydrolysate. The supplemented glucose was mostly used to maintain the cell density but the GABA generation was not significantly increased compared to batch fermentation due to the dilution factor of the fermentation broth by the relatively low sugar concentration. To maintain the high sugar concentration during the fed-batch, the concentrated corncob residue

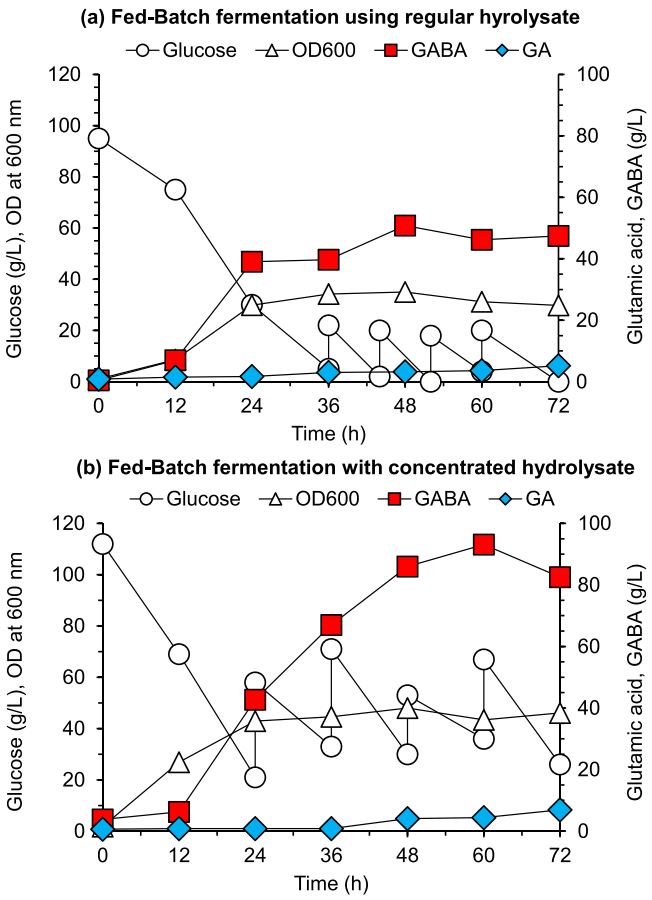

Fig. 6. Fed-Batch fermentation for GABA production using corncob residue hydrolysates at different sugar concentrations in 3 L fermenter. (a) Fed-batch using high-sugar corncob residue hydrolysate; (b) fed-batch using concentrated corncob residue hydrolysate. The fermentation was carried out in 3 L fermenter at 30 ℃, 600 rpm, 1.4 vvm, pH 7.0. Nutrient supplementation in hydrolysate under nitrogen-limited conditions as described in the Section 2.2.

hydrolysate (~600 g/L of glucose) was used. Fig. 6b showed that the fed-batch of the concentrated hydrolysate maintained a relatively high sugar level and cell density. Finally, the GABA production reached the maximum of 93.15 g/L with the yield of 0.43 g per gram of sugar consumed.

Fig. 6(a) used the regular hydrolysate with a relatively low sugar concentration, therefore the increase of GABA production was limited in the fed-batch operation because the supplementation diluted the fermentation broth. Fig. 6(b) used the concentrated hydrolysate with much higher sugar concentration, therefore the fed-batch did not result in the dilution of either sugar concentration or the GABA concentration. As a result, the GABA production was significantly increased.

Jorge et al. constructed a pathway for the production of GABA from putrescine, and the yield of GABA after fed-batch fermentation was 63.2 g/L from glucose, but the theory yield was low due to the long metabolic pathway [38]. Lai et al. used defatted rice bran for GABA biosynthesis and investigated the effect of different enzymatic conditions on GABA production by Lactobacillus brevis, with the highest yield of 8.43 g/L [17]. Baritugo et al. heterologous expressed the xylAB gene in *C. glutamicum* and produced 35.47 g/L of GABA using empty fruit bunch (90 g/L glucose and 10 g/L xylose) [18]. Wei et al. achieved GABA production by *C. glutamicum* using low-value glycerol through dynamic modulation, and the final recombinant strain could produce 45.6 g/L GABA [19]. Studies have also investigated the feasibility of fermentation for the production of GABA, using whey powder and monosodium glutamate [39], contaminant food bio-product like dairy sludge and soybean meal [40], coffee grounds [41], But the production of GABA in all the above studies was at a low level, which was difficult to meet the demand of industrial production (Table 1).

In the current study, *E. coli* produced GABA in a whole-cell catalytic manner, which requires exogenous incorporation of glutamate and is therefore expensive to produce. A few studies used recombinant *E. coli* fermentation to produce GABA, but all of them used glucose or yeast extract as the carbon source, which is not only costly but also the production of GABA is very low, which is difficult to meet the demand of industrial production. In contrast, although lactic acid bacteria have endogenous glutamic acid decarboxylase, most of the studies still used glucose as a carbon source, and the few studies that used cheap agricultural by-products as a carbon source had very low GABA yields. The corncob residue used in this study not only solved the problem of high cost of carbon source, but also achieved high GABA production compared to other similar studies, so it has more potential for application. A record high GABA production of 93.15 g/L with the yield of 0.43 g/g was reached by fermentation parameter optimization and fed-batch fermentation.

Either the intracellularly expressed or the extracellularly transported glutamate decarboxylase were used under the neutral pH ~7, which was not favorable for glutamate decarboxylase. However, the results in Figs. 4, 5 and 6 show that the residual glutamic acid in the fermentation broth was only 1–5 g/L after the conversion to GABA, suggesting that the enzyme activity of glutamic acid decarboxylase was not the limiting factor.

4. Conclusion

Production of GABA from corncob residue feedstock was investigated using the engineered *C. glutamicum* XY24. The fermentation parameters in shake flasks and bioreactors were optimized including the sugar concentration, nitrogen levels, cofactor levels and aeration rate, and fermentation mode. The fed-batch fermentation of the concentrated corncob residue hydrolysate reached 93.15 g/L of GABA generation with a yield of 0.43 g/g glucose. The results provided technical support for the industrial production of GABA from low-cost lignocellulose. However, strain *C. glutamicum* XY24 still carries the expression plasmid, and in future research, glutamic acid decarboxylase can be integrated into the genome for expression, and the recombinant bacteria will be more suitable for industrial applications. In addition, enhanced expression of glutamate pathway genes by metabolic engineering modifications may further increase GABA production.

In the background of persistent energy and environmental concerns, biomass waste has great potential as a sustainable carbon resource for the production of biobased fuels, platform chemicals and non-food

Table 1
Comparison of the production of different bacterial strains for GABA production by fermentation.

Host strain	Feedstock	Production of GABA	Reference
Lactobacillus brevis VTCC-B397	Rice bran	8.43 g/L	[17]
Lactobacillus brevis A3	Whey powder and monosodium glutamate	553.5 ppm	[39]
Lactobacillus brevis PML1	Dairy sludge and soybean meal	300 ppm	[40]
Levilactobacillus brevis PML1	Spent coffee grounds	492 mg/L	[41]
C. glutamicum H36GD1852	Empty fruit bunch	35.47 g/L	[18]
C. glutamicum G7–1	Glycerol	45.6 g/L	[19]
C. glutamicum XY24	Corncob residue hydrolysate	93.15 g/L	This study
E. coli	Glucose	6.16 g/L	[42]
E. coli Nissle 1917	Yeast extract	17.9 g/L	[43]

controversial composites as an alternative to traditional petroleum resources. Corn cob, being an agricultural residue, has been used to extract hemicellulose for the preparation of value-added chemicals (e.g., xylose, furfural, and xylitol). Currently, about 23 million tons of corn cob residue (CCR) are generated annually from industrial furfural production. However, CCR is mostly underutilized, resulting in the loss of valuable biological resources and environmental pollution problems, therefore, the present study on corncob residue provides an option for low-carbon sustainable production of GABA.

CRediT authorship contribution statement

Jie Bao: Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Bin Zhang:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Ying-Ying Xu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

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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Data availability

The source data supporting the findings of this study are available within the paper.

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