J Ind Microbiol Biotechnol (2015) 42:1157–1165
DOI 10.1007/s10295-015-1645-2

FERMENTATION, CELL CULTURE AND BIOENGINEERING

Two-step production of gamma-aminobutyric acid from cassava powder using *Corynebacterium glutamicum* and *Lactobacillus plantarum*

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Received: 28 April 2015 / Accepted: 15 June 2015 / Published online: 27 June 2015

Abstract Production of gamma-aminobutyric acid (GABA) from crop biomass such as cassava in high concentration is desirable, but difficult to achieve. A safe biotechnological route was investigated to produce GABA from cassava powder by *C. glutamicum* G01 and *L. plantarum* GB01-21. Liquefied cassava powder was first transformed to glutamic acid by simultaneous saccharification and fermentation with *C. glutamicum* G01, followed by biotransformation of glutamic acid to GABA with resting cells of *L. plantarum* GB01-21 in the reaction medium. After optimizing the reaction conditions, the maximum concentration of GABA reached 80.5 g/L with a GABA productivity of 2.68 g/L/h. This is the highest yield ever reported of GABA production from cassava-derived glucose. The bioprocess provides the added advantage of employing nonpathogenic microorganisms, *C. glutamicum* and *L. plantarum*, in microbial production of GABA from cassava biomass, which can be used in the food and pharmaceutical industries.

Keywords Gamma-aminobutyric acid · Cassava · *Corynebacterium glutamicum* · *Lactobacillus plantarum*

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Introduction

Gamma-aminobutyric acid (GABA) may lower blood pressure [8, 35], increase the activity of choline acetyltransferase in the brain [31] and is a component of pharmaceuticals and foods [16]. In recent years, microbial GABA production biotechnologies have received growing attention because of GABA's broad applications in the pharmaceutical and functional foods industries [10, 22, 24, 25].

Lactic acid bacteria (LAB) are of interest for GABA biosynthesis because of their probiotic status in humans and animals [28]. During the biosynthesis of GABA by LAB, the fermentation medium must be supplemented with the precursor glutamate [14, 17, 37]. However, the bioprocess is uneconomical because of the purification of glutamic acid, thus it is necessary to establish a new approach for sustainable industrial production of GABA [36]. Glutamate decarboxylase (GAD, EC 4.1.1.15, encoded by *gad*) catalyzes the conversion of L-glutamate to GABA [2, 20]. *Corynebacterium glutamicum* is a nonpathogenic bacterium [6] and a well-known glutamic acid producer. Takahashi et al. [29] successfully developed a one-step strategy for producing GABA from glucose by cloning the *gad* gene from *E. coli* into *C. glutamicum*. The maximum titer achieved of GABA was 12.37 ± 0.88 g/L. The production of GABA was later enhanced (31.1 ± 0.41 g/L) by improving the flux of 2-oxoglutarate to glutamic acid [22]. Shi et al. [26] reported that co-expression of two GAD genes *gadB*1 and *gadB*2 using recombinant *C. glutamicum* could enhance GABA production to 27.13 g/L under the optimal fermentation conditions [25]. However, the GABA yield and productivity remained prohibitively low for commercial viability. Furthermore, the relatively high cost of conventional substrates (L-glutamate or sugar) is one of main factors influencing the economic viability of

GABA production. Cassava, a readily available starch crop biomass, is relatively inexpensive and is not affected by food and feed shortage concerns [3, 32]. Cassava has been widely used in the microbial production of bulk chemicals such as 2,3-butanediol [32], acetone–butanol [7], ethanol [3, 21], and glutamic acid [1, 9]. However, there is little information available on the microbial production of GABA from cassava.

Corynebacterium glutamicum, generally recognized as safe (GRAS) microorganism, is widely used in the industrial production of glutamate and other amino acids [15]. Recently, we isolated a GRAS and high glutamate producer, *C. glutamicum* G01, through multi-mutagenesis and metabolic evolution, capable of achieving a maximum titer of 119 g/L L-glutamate from glucose (data not published). We also obtained the modified strain *Lactobacillus plantarum* GB01-21 through multi-mutagenesis, and showed that it had high GAD activity [19]. Inspired by these advances, a safe biotechnological route for producing GABA from pretreated cassava was designed using two GRAS microorganisms, *C. glutamicum* G01 and *L. plantarum* GB01-21. After optimizing the reaction conditions, a high concentration of GABA was achieved from cassava crop biomass.

Materials and methods

Microorganisms

Corynebacterium glutamicum G01 was isolated from soil and *L. plantarum* GB01-21 was obtained through multi-mutagenesis as described in our previous study [19]. *C. glutamicum* G01 and *L. plantarum* GB01-21 were deposited in the China Center for Type Culture Collection (CCTCC) under collection number CCTCC M2013418 and CCTCC M209102, respectively.

Preparation of liquefied cassava powder

Commercially available amylase (20,000 U/mL) and glucoamylase (100,000 U/mL) were purchased from KDN Biotech Group and Sigma-Aldrich, respectively. Liquefied cassava was prepared as described in previous studies [3, 32]. The liquefied cassava mash was used as the medium for glutamate production.

Production of glutamic acid from liquefied cassava powder

Stock and inoculum cultures were grown at 30 °C with shaking at 160 rpm in medium containing (g/L): glucose 60, urea 5, yeast extract 15, $MgSO_4$ 0.2, $K_2HPO_4{\cdot}3H_2O2$, $FeSO4{\cdot}7H_2O$ 0.02, $MnSO_4{\cdot}H_2O$ 0.02, biotin 5×10^{-6}, and thiamine HCl 8×10^{-5}, pH 7.0. The composition of the fermentation medium was the same as the seed culture medium except that liquefied cassava powder was used as the sole carbon source instead of pure glucose. *C. glutamicum* G01 was incubated for 12 h in 500-mL flasks containing 100 mL of the seed medium. The incubated seed culture was then inoculated (6 % v/v) into the fermentation medium. Simultaneous saccharification and fermentation (SSF) and fed-batch SSF were performed as described in a previous study [32]. For SSF, the liquefied solution of cassava powder was added to maintain an appropriate glucose concentration, and the SSF was carried out at 30 °C and pH 7.0 (addition of 4 M H_3PO_4 or 6 M KOH). In fed-batch SSF, liquefied cassava powder (500 g/L) was added to maintain the glucose concentration between 20 and 40 g/L.

Preparation of gamma-aminobutyric acid from glutamic acid by *L. plantarum* GB01-21

The seed medium was designed as follows (g/L): glucose 10, peptone 10, yeast extract 5, sodium acetate 2, monosodium glutamate 10, $MgSO_4$ 0.2, $MnSO_4$ 0.2, and pH 7.0. The cultivation medium contained (g/L): corn steep liquor 20, glucose 25, yeast extract 15, sodium acetate 2, $MgSO_4$ 0.2, $(NH_4)_2SO_4$ 0.5, MSG 20. The initial pH was adjusted with 4 M NaOH to pH 7.0. Culture conditions optimization was conducted in 250-mL Erlenmeyer flasks that contained 80 mL of the medium. The flasks were inoculated with 6 % (v/v) seed culture grown at 30 °C for 12 h, and incubated under static conditions in an incubator. The batch fermentation was carried out in a 5-L fermentor (BIOTECH-2002, Baoxing Biological Equipment Co., Shanghai, China) under the following conditions: medium volume 3 L, inoculum size 6 % (v/v), temperature 30 °C, pH 7.0, agitation speed 100 rpm. The pH was kept constant at 7.0 by addition of ammonia. After incubation for 36 h, cells were harvested by centrifugation at $8000 \times g$ for 15 min and washed twice with 20 mM acetate buffer (pH 4.8) and then resuspended in the same buffer.

Analytical methods

The glucose concentration was determined as previously described [34]. GABA and glutamate were determined by high-performance liquid chromatography (HPLC) [37]. Optical density (OD) was assayed at 600 nm by an SP-2100 UV spectrophotometer (Hangzhou Chiheng Technology Co., Ltd. China), and was converted to dry cell weight (DCW) using the following equations: for *C. glutamicum* G01, DCW (g/L) = $0.395 \times OD_{600}$ nm; for *L. plantarum* GB01-21, DCW (g/L) = $0.332 \times OD_{600}$ nm.

Results

Enzymatic hydrolysis of cassava powder

Cassava powder as a source of starch generally requires pretreatment when used as a substrate for microbial fermentation. Amylase and glucoamylase were used for its hydrolysis. The liquefaction was carried out in 250-mL Erlenmeyer flasks by adding appropriate dosage of α-amylase to the suspension (pH 6.0) and incubated at 95 °C for 120 min in a water bath (100 rpm). The liquefied cassava mash was then saccharified by adding appropriate dosage of glucoamylase at pH 4.5 and 60 °C. The resulting saccharified cassava solution was used as the initial carbon source in the fermentation process. The optimal concentrations of amylase (for liquefaction) and glucoamylase (for saccharification) were 100 and 90 U/g cassava powder, respectively. After liquefaction and saccharification, the rate of hydrolysis of starch in cassava powder and reducing sugar concentration were about 88 % and 60 g/L, respectively. For SSF and fed-batch SSF, the liquefied solution was autoclaved and the glucoamylase filtrated by the 0.22 mm filtration membrane was added to the medium with the inoculum for fermentation.

Production of L-glutamic acid from cassava powder by *C. glutamicum* G01

The effect of the cassava powder concentration on glutamic acid production was investigated in 250-mL Erlenmeyer flasks by varying the initial cassava powder concentrations from 150 to 300 g/L at an initial pH of 7.0, 30 °C, and 200 rpm. As listed in Table 1, the highest DCW (8.2 ± 0.28 g/L) and maximum glutamic acid production (80.6 ± 3.6 g/L) were obtained with an initial cassava powder concentration of 200 g/L. When the initial cassava powder concentration was above 200 g/L, the glucose consumption, cell growth and glutamic acid production were all suppressed. To improve glutamic acid production, fed-batch SSF was adopted as described in a previous study [32]. When the sugar concentration was reduced to about 20–30 g/L, glucoamylase and liquefied solution of cassava powder (500 g/L) were added into the bioreactor. For the first 6 h of cultivation, the consumption rate of sugar was lower than the saccharification rate; sugar concentration was, therefore, gradually increased, followed by a sharp decline due to the rapid consumption of sugar. Under these conditions, the maximum glutamic acid concentration was about 99.7 g/L after 40 h with a productivity of 2.49 g/L/h (Fig. 1a).

Table 1 Effect of cassava powder concentration on glutamic acid production in SSF

Cassava powder concentration (g/L)	DCW (g/L)	Residual glucose (g/L)	Glutamic acid (g/L)
150	7.3 ± 0.22	2.5 ± 2.1	73.7 ± 2.8
200	8.2 ± 0.28	15.8 ± 3.5	80.6 ± 3.6
250	7.5 ± 0.25	64.1 ± 4.3	74.2 ± 3.4
300	5.1 ± 0.18	125.4 ± 6.5	50.5 ± 2.5

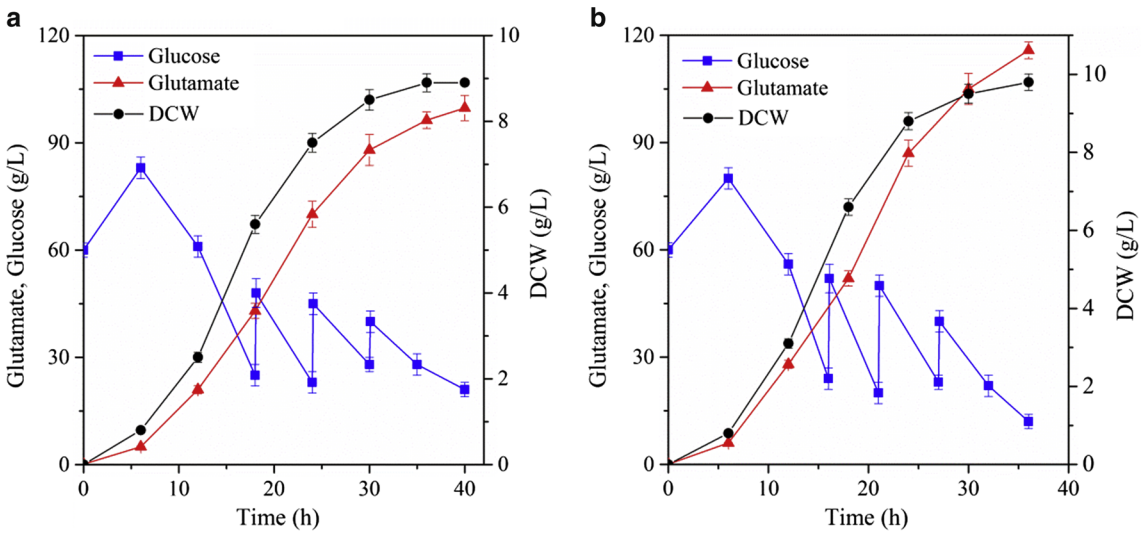

Fig. 1 Time profiles of glutamic acid production by *C. glutamicum* G01 in fed-batch SSF. **a** Temperature and agitation speed were separately controlled at 30 and 500 rpm during the whole process; **b** temperature and agitation speed wereset at the multi-step operation (see Table 2)

Table 2 A multi-stage culture parameter (temperature and agitation speed) combination control strategy for glutamic acid production (the other two parameters pH and aeration rate were maintained at 7.0 and 2.0, respectively, during the all over process)

Time (h)	Temperature (°C)	Agitation speed (rpm)
0–6	30	450
6–12	30	500
12–24	32	550
24–30	32	600
30–36	34	600

Previously, Kishimoto et al. [12] had reported that temperature shift-up was capable of improving not only the yield but also the specific production rate of glutamic acid [5]. L-Glutamic acid fermentation is a well-known typical aerobic fermentation process. In our preliminary tests, we had found that temperature shift-up was favorable for glutamic acid production. In addition, we had also observed that dissolved oxygen level controlled at 25–35 % by adjusting agitation speed was crucial for glutamic acid formation. Inspired by this information and based on the results of our preliminary tests, a multi-stage culture parameter (temperature and agitation speed) combination control strategy, aimed at enhancing glutamate production by *C. glutamicum* G01, was designed (see Table 2). The time course for glutamate production from the SSF process using the multi-stage culture parameters strategy is shown in Fig. 1b. The maximum titer of glutamate reached 116.2 g/L after 36 h with a productivity of 3.23 g/L/h from the fed-batch SSF using the multi-stage culture parameter control strategy. The results also showed that cell growth and glutamate production were both higher than that obtained from the constant parameters process (Fig. 1). We concluded that this multi-stage culture parameter control strategy could considerably improve glutamate concentration and increase glutamate productivity.

Optimization of nutrition conditions for GAD production by *L. plantarum* GB01-21

The effects of the carbon source (10 g/L of glucose, sucrose, glycerol, lactose or soluble starch) on cell growth and GAD production were first determined. The highest DCW (1.82 ± 0.12 g/L) and GAD activity (48.2 ± 2.66 U/mL) were obtained with glucose (Fig. 2). In addition, the effect of glucose concentration on GAD production was investigated by varying the initial glucose concentrations from 5 to 25 g/L, and the highest GAD activity was found at 10 g/L of glucose.

Subsequently, the effects of the organic and inorganic nitrogen sources (2 g/L nitrogen, including peptone, beef extract, corn steep liquor, soybean meal, yeast extract, urea, sodium nitrate, ammonium sulfate, ammonium acetate, ammonium nitrate and ammonium citrate) on cellular biomass and GAD production were also tested. When an organic nitrogen source was used, the cellular biomass and GAD production were superior than when an inorganic nitrogen source was used. As shown in Fig. 3, more than 51.2 ± 2.72 U/mL GAD was obtained when soybean meal or yeast extract was used as a sole nitrogen source. The highest DCW (1.86 ± 0.13 g/L) and GAD production (53.1 ± 2.46 U/mL) were observed with the yeast extract, however, the highest activity of GAD per DCW (3.13×10^4 U/g) was obtained when soybean meal was employed as the nitrogen source. These results indicated that soybean meal and yeast extract could also provide some factors to improve cell growth. For further

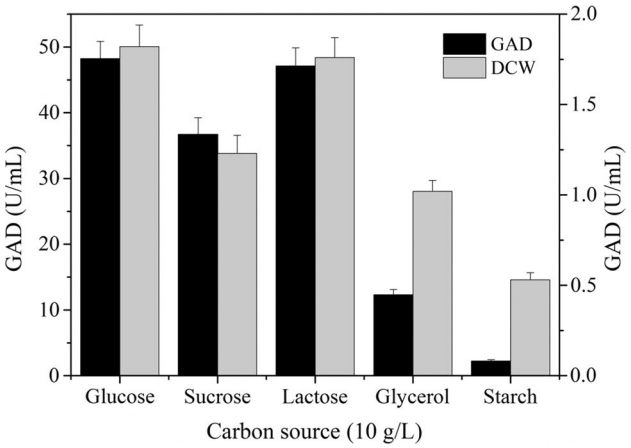

Fig. 2 Effect of carbon source on cell growth and GAD production by *L. plantarum* GB01-21

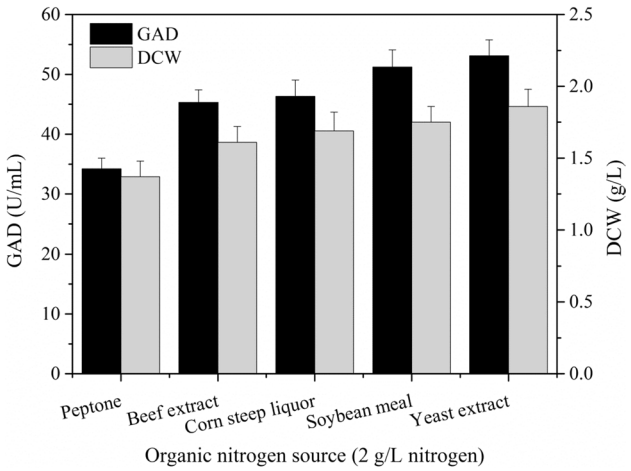

Fig. 3 Effect of organic nitrogen source on cell growth and GAD production by *L. plantarum* GB01-21

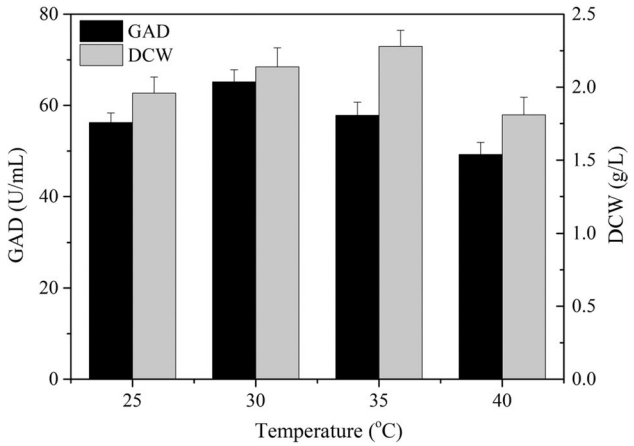

Fig. 4 Effect of temperature on cell growth and GAD production by *L. plantarum* GB01-21

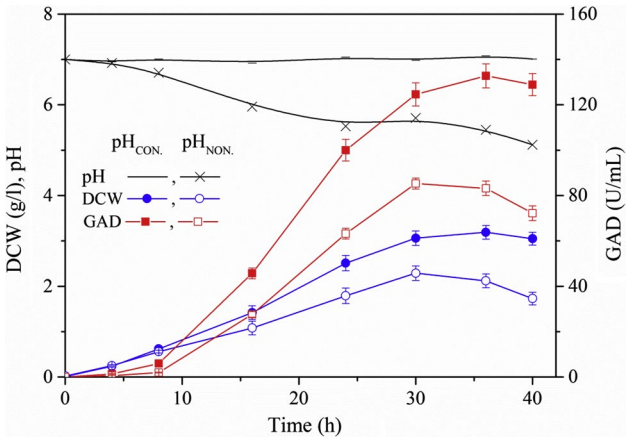

Fig. 5 Effect of pH on GAD production by *L. plantarum* GB01-21

enhancement of GAD production, a mixed nitrogen source containing 15 g/L soybean meal and 5 g/L yeast extract was tested. The mixed nitrogen source provided the highest DCW (2.07 ± 0.14 g/L) and GAD activity (64.5 ± 2.72 U/mL), which was an increase of 11.3, 21.5 and 26.0 %, 20.8 % higher than when soybean meal and yeast extract were used as the sole nitrogen source, respectively.

Optimization of culture conditions for GAD production by *L. plantarum* GB01-21

The effect of temperature on cell growth and GAD production were investigated by conducting four batch fermentations at temperatures varying from 25 to 40 °C. From the results obtained, it was apparent that the cell growth and GAD production by *L. plantarum* GB01-21 were affected by temperature (Fig. 4). The optimal temperature for GAD production was 30 °C, while that for cell growth was 35 °C.

Subsequently, the effects of the initial pH (5.5–8.0) on cell growth and GAD production were investigated by carrying out six batch fermentations at 30 °C. The highest DCW (2.16 ± 0.14 g/L) and GAD production (65.1 ± 2.81 U/mL) were obtained at pH 7.0. Nevertheless, GAD production was nearly constant within a narrow range of pH from 7.0 to 7.5; it decreased noticeably when the pH was beyond this range. The comparison between flask cultures performed at different initial pHs, suggested that pH 7.0 was more suitable for GAD production. Batch processes without pH control (initial pH 7.0) and with the pH maintained at 7.0 were then carried out in a 5-L fermenter at 30 °C (Fig. 5). In the batch fermentation without pH control, the pH sharply dropped during the first 24 h to about 5.5, after which a consecutive gradual drop of pH occurred. These results are in agreement with a previous study which reported that the pH of a MRS medium inoculated with the GABA-producing lactic acid bacteria *L. paracasei* dropped from 6.5 at the beginning of the fermentation to about 4.5 within 50 h [13]. In addition, the results corroborate a report where the pH of a *Lactobacillus brevis* GABA100 fermentation dropped from 6 to 4.7 after 48 h [11]. When the pH was maintained at 7.0, the cell growth was higher than that without pH control. The highest GAD activity (132.8 U/mL) was obtained when the pH was maintained at 7.0 compared with the GAD activity (85.3 U/mL) obtained without pH control. The results above revealed that pH 7.0 was the most favorable for GAD production.

Efficient preparation of GABA from glutamic acid by *L. plantarum* GB01-21

After 36 h of culture under the optimal conditions, the biocatalyst *L. plantarum* GB01-21 was harvested by centrifugation for use as the catalyst for the glutamic acid conversion. The *C. glutamicum* G01 catalyzed glucose conversion medium was then harvested by centrifugation and the glutamic acid (about 116 g/L) in the supernatant was used as the substrate for *L. plantarum* GB01-21.

The biocatalytic conditions were optimized in our previous study [19, 30]. Under the optimal conditions (0.15 M acetic acid-sodium acetate buffer (pH 4.8), 35 °C, cell density 15 g/L, 200 rpm), the final concentration of GABA was 80.5 g/L with a mass yield of 0.69 g/g after 30 h of biocatalysis in a 5-L stirred bioreactor. The biocatalyst retained over 90 % of its residual activity.

GABA production was also conducted with 15 g/L of cells and 116 g/L of glutamic acid from the glutamic acid fermentation solution to investigate the biocatalytic properties of the recovered cells. The cells were recovered three times and resuspended in the glutamic acid fermentation solution for GABA production. The results of the transformation achieved by the recovered cells are shown

Table 3 Repeated use of *L. plantarum* GB01-21 cells for GABA production

Repeated use times	Consumed glutamic acid (g/L)	GABA (g/L)	Productivity (g/L/h)	Yield (g/g)
Original	116 ± 2	80.5 ± 2.3	2.68 ± 0.06	0.69 ± 0.02
First	102 ± 2	69.8 ± 1.8	2.33 ± 0.05	0.69 ± 0.02
Second	81 ± 3	55.5 ± 1.4	1.85 ± 0.05	0.68 ± 0.02
Third	48 ± 3	32.8 ± 1.5	1.08 ± 0.05	0.68 ± 0.03

in Table 3. After 30 h of biocatalysis, the productivity of GABA decreased from 2.68 to 1.08 g/L/h, and the maximum titer of GABA decreased from 80.5 to 32.8 g/L, with increasing recovery times. However, about 0.68 g/g of GABA was obtained for each of the three cycles.

Discussion

In microbial fermentations, the costs of the carbon sources to the total production cost are significant. However, most research into GABA production have focused on using pure L-glutamic acid or its monosodium salt as the starting material [10, 14, 25, 37]. The cost of materials makes up a large part of the microbial GABA production cost, while biomass starting materials can help to reduce the cost of fermentation. Therefore, the development of a fermentation method based on raw materials, other than L-glutamic acid or its monosodium salt, is important for reducing the costs of GABA production. In this work, a safe biotechnological route was proposed to produce GABA from crop biomass cassava powder using two GRAS microorganisms *C. glutamicum* G01 and *L. plantarum* GB01-21. To enhance the efficiency of the GABA production, the culture and reaction conditions were investigated in detail.

First, we optimized the operating parameters for the production of L-glutamic acid from cassava powder by *C. glutamicum* G01. Cell growth and glutamic acid production were suppressed at the high initial concentration (>300 g/L) of cassava powder. This inhibition probably resulted from a decrease in water activity that affects the metabolic rates [32]. Fed-batch fermentation can help to avoid the influence of high concentration substrate limitation and inhibition by controlling the substrate concentration below the toxic level [33, 34]. In this study, the glutamic acid production was also improved under fed-batch SSF. Controlling temperature and the DO level is important for successful glutamic acid fermentation [5, 23]. To enhance the production of glutamate, we designed a multi-stage culture parameter (temperature and agitation speed) combination control strategy. Under the optimal conditions, the maximum titer of glutamate reached 116.2 g/L, with a productivity of 3.23 g/L/h by fed-batch SSF. The proposed multi-stage temperature and agitation speed combination control strategy and the fed-batch SSF were successful at improving glutamate production. To date, *Brevibacterium* sp. G012 and *C. glutamicum* were employed for glutamic acid synthesis from cassava whey hydrolysate, but the highest concentration of glutamate achieved was only 9.8 g/L. Jyothi et al. [9] has also tried to utilize cassava starch factory residues for the production of glutamic acid using *Brevibacterium divaricatum*, but the maximum glutamate production was only about 4 g/L. Thus, the 116 g/L-glutamate obtained as a result of this research was superior to the previous reports.

GAD catalyzes the conversion of L-glutamate to GABA. The cell growth and GAD production conditions are highly affected by culture conditions and medium composition [13, 17]. However, there is no general defined medium for GAD production by all microbial strains because every microorganism has its own special nutritional requirements depending on its environment. In the present investigation, glucose was found to be suitable for GAD production with *L. plantarum* GB01-21, soybean meal, and yeast extract stimulated GAD production. Li et al. [17] also found that soya peptone affected GAD and GABA production. Soybean meal, the by-product obtained after extracting most of the oil from soybeans, which are rich in protein and energy, is a cheap nutrient source available on a large scale that can help to reduce the cost of GAD production. Therefore, soybean meal could be used as the sole organic nitrogen source for GAD production with industrial applications. Subsequently, the operating conditions for the production of GAD were also investigated. First, the effect of temperature on the production of GAD was observed. The production of GAD was accelerated as the temperature increased until high temperatures were reached which resulted in thermal inactivation [24]. Experiments conducted at variable pH indicated that GAD production by *L. plantarum* GB01-21 was pH dependent with an optimal pH range of 7.0–7.5. A pH maintained at 7.0 was the most favorable for GAD production, which was higher than that (pH 5.0) for *Lb. paracasei* NFRI 7415 [13].

A highly effective method for the production of GABA has been achieved via the conversion of monosodium glutamate using an engineered glutamate decarboxylase from *Escherichia coli* [10]. *Lactobacillus* species are generally the most applicable microorganisms for GABA production because they are probiotic to humans and animals. Previously, several strategies have been utilized to improve

Table 4 The current progress in GABA production

Strains	Engineered methods	Substrates	Fermentation conditions	Organic nitrogen source	GABA		References
					Titer (g/L)	Productivity (g/L/h)	
Corynebacterium glutamicum	Expressing GAD mutant	Glucose	One-step, fed-batch	P	38.6	0.536	[4]
C. glutamicum	Expressing GAD and disrupting *pknG*	Glucose	One-step, batch	SP	31.1	0.259	[21]
C. glutamicum	Expressing *gadB*1 and *gadB*2	Glucose	One-step, fed-batch	CSL	26.32	0.439	[24]
C. glutamicum	Expressing GAD and disrupting *argB*, *proB* and *dapA*	Glucose	One-step, fed-batch	CSL, YE	70.6	1.01	[35]
Lactobacillus brevis	Wild type	Sodium glutamate	Two-step, fed-batch	P, BE,YE	61	0.904	[37]
Escherichia coli	Expressing GAD mutant	Glutamic acid	IPTG induction, one-step, fed-batch	NR	103.1	34.3	[10]
E. coli	Expressing GAD	Sodium glutamate	IPTG induction, one-step, fed-batch	P, CSL	204.5	8.52	[30]
C. glutamicum and *L.plantarum*	Wild type	Cassava powder	Two-step, fed-batch	YE, SM	80.5	2.68	This work

P peptone, *BE* beef extract, *YE* yeast extract, *SM* soybean meal, *SP* soy peptone, *CSL* corn steep liquor, *NR* not reported

GABA production. Zhang et al. [37] established a model system for the conversion of monosodium glutamate to GABA using *Lactobacillus brevis* TCCC13007 resting cells, which could achieve a maximum concentration of 61 g/L GABA. Li et al. [18] optimized fermentation operating conditions for *L. brevis* NCL912 to obtain 35.7 g/L GABA. Kook et al. [14] introduced an extra copy of GAD into *L.sakei* B2-16, and the GABA titer increased to 27.3 g/L; however, the GABA precursor L-glutamate or monosodium glutamate had to be added during the process. Preparation of GABA from glucose has been reported by Takahashi et al. [29], Shi et al. [25, 26], Okai et al. [22], Zhang et al. [36], and Choi et al. [4] using engineered *C. glutamicum* (expressing exogenous GAD). However, the GABA yield and productivity were prohibitively low for commercial production. The highest concentration of GABA obtained herein was 80.5 g/L. To our knowledge, this is the highest yield of GABA obtained by GRAS microorganisms. Significantly, the results were achieved by employing cassava, a relatively inexpensive and available starch crop biomass. More importantly, the strains *C. glutamicum* and *L. plantarum* employed in this work are widely used in medicine, animal feed, and as food supplements.

Although we successfully presented a process of GABA production from cassava as a raw material with industrial applications, cassava requires pretreatment with amylase and glucoamylase for hydrolysis, which ultimately increases the total cost of production. Consequently, in the preliminary experiment, we constructed an amylase secreting *C. glutamicum* to avoid addition of extra amylase hence lower the production cost, however, the amylase production was very low resulting in an inefficient GABA production. Future studies will focus more on developing the strategies to amylase production with *C. glutamicum*. Furthermore, it is generally known that one-step (microorganism) process is generally preferred for industrial applications. Engineering of *C. glutamicum* for GABA production by *gad* overexpression could achieve this goal. Some investigators had successfully developed a one-step strategy for producing GABA from glucose by cloning the *gad* gene into *C. glutamicum* [25, 29], but the GABA yield and productivity remained prohibitively low for commercial production (Table 4). One factor that limits GABA formation is low level of GAD in engineered *C. glutamicum*, assumed to be the rate-limiting factor in the conversion of glutamic acid into GABA. While in this work, we could increase cells addition to improve the GAD level. Alternatively, the pH may limit the GAD reaction, because GAD shows its full activity around pH 4–4.8, but completely loses its activity at or above pH 6 [10, 27, 30], while pH 7.0 favors glutamic

acid production. Therefore, expending the pH range of GAD will be a promising way for one-step production of GABA from glucose or other low-cost biomass materials. While using the two-step strategy presented in this work, the conflict of reaction pH could be ignored, because glutamic acid production and GABA formation were not performed in the same system.

In summary, preparation of GABA from cassava, a relatively inexpensive and available starch crop biomass, was realized through a two-stage fermentation strategy using *C. glutamicum* G01 and *L. plantarum* GB01-21. The maximum concentration of GABA reached 80.5 g/L with a GABA productivity of 2.68 g/L/h, which is the highest reported GABA yield from cassava or glucose. Since *C. glutamicum* and *L. plantarum* are GRAS microorganisms, they are excellent candidates for the microbial production of GABA from glucose for the pharmaceutical and functional food industries on a commercial scale.

Acknowledgments This work was supported by the High-tech Research and Development Programs of China (2015AA021004), the National Natural Science Foundation of China (31400082), the Research Project of Chinese Ministry of Education (113033A), the Fundamental Research Funds for the Central Universities (JUSRP11544), the Priority Academic Program Development of Jiangsu Higher Education Institutions,the 111 Project (111-2-06),and the Jiangsu province "Collaborative Innovation Center for Advanced Industrial Fermentation" industry development program.

References

1. Akpan I, Ikenebomeh M, Doelle H (1998) Glutamic acid production from cassava whey by *Brevibacterium* sp. G012. Trop Sci 38:147–150
2. Capitani G, De Biase D, Aurizi C, Gut H, Bossa F, Gruetter G (2003) Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. EMBO J 22:4027–4037
3. Choi G-W, Moon S-K, Kang H-W, Min J, Chung B-W (2009) Simultaneous saccharification and fermentation of sludge-containing cassava mash for batch and repeated batch production of bioethanol by *Saccharomyces cerevisiae* CHFY0321. J Chem Technol Biotechnol 84:547–553. doi:10.1002/jctb.2077
4. Choi JW, Yim SS, Lee SH, Kang TJ, Park SJ, Jeong KJ (2015) Enhanced production of gamma-aminobutyrate (GABA) in recombinant *Corynebacterium glutamicum* by expressing glutamate decarboxylase active in expanded pH range. Microb Cell Fact 14:21. doi:10.1186/s12934-015-0205-9
5. Choi S-U, Nihira T, Yoshida T (2004) Enhanced glutamic acid production of *Brevibacterium* sp. with temperature shift-up cultivation. J Biosci Bioeng 98:211–213. doi:10.1016/s1389-1723(04)00268-3
6. George M (2001) Burgey's manual of systematic bacteriology, 2nd edn. Springer, New York
7. Gu Y, Hu S, Chen J, Shao L, He H, Yang Y, Yang S, Jiang W (2009) Ammonium acetate enhances solvent production by *Clostridium acetobutylicum* EA 2018 using cassava as a fermentation medium. J Ind Microbiol Biotechnol 36:1225–1232. doi:10.1007/s10295-009-0604-1
8. Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y (2004) Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. Brit J Nutr 92:411–417. doi:10.1079/BJN20041221
9. Jyothi AN, Sasikiran K, Nambisan B, Balagopalan C (2005) Optimisation of glutamic acid production from cassava starch factory residues using *Brevibacterium divaricatum*. Process Biochem 40:3576–3579. doi:10.1016/j.procbio.2005.03.046
10. Kang TJ, Ho NAT, Pack SP (2013) Buffer-free production of gamma-aminobutyric acid using an engineered glutamate decarboxylase from *Escherichia coli*. Enzyme Microb Technol 53:200–205. doi:10.1016/j.enzmictec.2013.04.006
11. Kim JY, Lee MY, Ji GE, Lee YS, Hwang KT (2009) Production of γ-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. Int J Food Microbiol 130:12–16. doi:10.1016/j.ijfoodmicro.2008.12.028
12. Kishimoto M, Alfafara C, Nakajima M, Yoshida T, Taguchi H (1989) The use of the maharanobis and modified distances for the improvement of simulation of glutamic acid production. Biotechnol Bioeng 33:191–196
13. Komatsuzaki N, Shima J, Kawamoto S, Momose H, Kimura T (2005) Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. Food Microbiol 22:497–504. doi:10.1016/j.fm.2005.01.002
14. Kook M-C, Seo M-J, Cheigh C-I, Lee S-J, Pyun Y-R, Park H (2010) Enhancement of γ-amminobutyric acid production by *Lactobacillus sakei* B2–16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. J Korean Soc Appl Biol Chem 53:816–820. doi:10.3839/jksabc.2010.123
15. Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1–8. doi:10.1007/s00253-005-0155-y
16. Li H, Cao Y (2010) Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino Acids 39:1107–1116. doi:10.1007/s00726-010-0582-7
17. Li H, Qiu T, Gao D, Cao Y (2010) Medium optimization for production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912. Amino Acids 38:1439–1445
18. Li H, Qiu T, Huang G, Cao Y (2010) Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. Microb Cell Fact 9:85
19. Liu T, Yang T, Zhang S, Xia H, Rao Z (2010) Screening, identification and primary optimizing of a strain producing γ-aminobutyric acid from L-glutamic acid. J Food Sci Biotechnol 29:742–747
20. Masuda K, X-f Guo, Uryu N, Hagiwara T, Watabe S (2008) Isolation of marine yeasts collected from the Pacific Ocean showing a high production of γ-aminobutyric acid. Biosci Biotechnol Biochem 72:3265–3272. doi:10.1271/bbb.80544
21. Nitayavardhana S, Shrestha P, Rasmussen ML, Lamsal BP, van Leeuwen J, Khanal SK (2010) Ultrasound improved ethanol fermentation from cassava chips in cassava-based ethanol plants. Bioresour Technol 101:2741–2747. doi:10.1016/j.biortech.2009.10.075
22. Okai N, Takahashi C, Hatada K, Ogino C, Kondo A (2014) Disruption of pknG enhances production of gamma-aminobutyric acid by *Corynebacterium glutamicum* expressing glutamate decarboxylase. AMB Express 4:20. doi:10.1186/s13568-014-0020-4
23. Roy D, Chatrerjee S (1989) Production of glutamic acid by *Arthrobacter globiformis*: influence of cultural conditions. Folia Microbiol 34:11–24
24. Seo MJ, Nam YD, Lee SY, Park SL, Yi SH, Lim SI (2013) Expression and characterization of a glutamate decarboxylase

from *Lactobacillus brevis* 877G producing gamma-aminobutyric acid. Biosci Biotechnol Biochem 77:853–856. doi:DN/JST.JSTAGE/bbb/120785

25. Shi F, Jiang J, Li Y, Xie Y (2013) Enhancement of gamma-aminobutyric acid production in recombinant *Corynebacterium glutamicum* by co-expressing two glutamate decarboxylase genes from *Lactobacillus brevis*. J Ind Microbiol Biotechnol 40:1285–1296. doi:10.1007/s10295-013-1316-0
26. Shi F, Li Y (2011) Synthesis of γ-aminobutyric acid by expressing *Lactobacillus brevis*-derived glutamate decarboxylase in the *Corynebacterium glutamicum* strain ATCC 13032. Biotechnol Lett 33:2469–2474. doi:10.1007/s10529-011-0723-4
27. Shukuya R, Schwert G (1960) Glutamic acid decarboxylase, part I. Isolation procedures and properties of the enzyme. J Biol Chem 235:1649–1652
28. Siragusa S, De Angelis M, Di Cagno R, Rizzello C, Coda R, Gobbetti M (2007) Synthesis of γ-amino butyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Environ Microbiol 73:7283–7290
29. Takahashi C, Shirakawa J, Tsuchidate T, Okai N, Hatada K, Nakayama H, Tateno T, Ogino C, Kondo A (2012) Robust production of gamma-amino butyric acid using recombinant *Corynebacterium glutamicum* expressing glutamate decarboxylase from *Escherichia coli*. Enzyme Microb Tech 51:171–176. doi:10.1016/j.enzmictec.2012.05.010
30. Tian L, Xu M, Rao Z (2012) Construction of a recombinant *Escherichia coli* BL21/pET-28a-lpgad and the optimization of transformation conditions for the efficient production of γ-aminobutyric acid. Chin J Biotechnol 1:65–75
31. Tujioka K, Thanapreedawat P, Yamada T, Yokogoshi H, Horie K, Kim M, Tsutsui K, Hayase K (2014) Effect of dietary gamma-aminobutyric acid on the nerve growth factor and the choline acetyltransferase in the cerebral cortex and hippocampus of ovariectomized female rats. J Nutr Sci Vitaminol Tokyo 60:60–65
32. Wang A, Xu Y, Ma C, Gao C, Li L, Wang Y, Tao F, Xu P (2012) Efficient 2,3-butanediol production from cassava powder by a crop-biomass-utilizer, *Enterobacter cloacae* subsp. dissolvens SDM. PLoS One 7:e40442. doi:10.1371/journal.pone.0040442
33. Yang T-W, Rao Z-M, Zhang X, Xu M-J, Xu Z-H, Yang S-T (2013) Fermentation of biodiesel-derived glycerol by *Bacillus amyloliquefaciens*: effects of co-substrates on 2,3-butanediol production. Appl Microbiol Biotechnol 97:7651–7658. doi:10.1007/s00253-013-5048-x
34. Yang T, Rao Z, Zhang X, Lin Q, Xia H, Xu Z, Yang S (2011) Production of 2,3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J Basic Microbiol 51:650–658. doi:10.1002/jobm.201100033
35. Yoshimura M, Toyoshi T, Sano A, Izumi T, Fujii T, Konishi C, Inai S, Matsukura C, Fukuda N, Ezura H, Obata A (2010) Antihypertensive effect of a gamma-aminobutyric acid rich tomato cultivar 'DG03-9' in spontaneously hypertensive rats. J Agric Food Chem 58:615–619. doi:10.1021/jf903008t
36. Zhang R, Yang T, Rao Z, Sun H, Xu M, Zhang X, Xu Z, Yang S (2014) Efficient one-step preparation of [gamma]-aminobutyric acid from glucose without an exogenous cofactor by the designed *Corynebacterium glutamicum*. Green Chem 16:4190–4197. doi:10.1039/C4GC00607K
37. Zhang Y, Song L, Gao Q, Yu S, Li L, Gao N (2012) The two-step biotransformation of monosodium glutamate to GABA by *Lactobacillus brevis* growing and resting cells. Appl Microbiol Biotechnol 94:1619–1627