



## Article

# pH Auto-Sustain-Based Fermentation Supports Efficient Gamma-Aminobutyric Acid Production by *Lactobacillus brevis* CD0817

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**Abstract:** Gamma-aminobutyric acid (GABA) plays a role in several physiological functions. GABA production by lactic acid bacteria has attracted considerable interest; however, there is need to improve production. This study aimed to develop a pH auto-sustain (PAS)-based GABA fermentation process for *Lactobacillus brevis* CD0817, with L-glutamic acid (solubility ~6.0 g/L and isoelectric point 3.22) as the substrate. Firstly, we determined the optimum levels of vital factors affecting GABA synthesis using Erlenmeyer flask experiments. The results showed that optimal levels of sugar, yeast extract, Tween-80, manganese ion, and temperature were 5.0 g/L, 35.0 g/L, 1.0 g/L, 16.0 mg/L, and 30.0 °C, respectively. The added L-glutamic acid (650 g per liter of medium) mostly existed in the form of solid powder was slowly released to supply the substrate and acidity essential for GABA production with the progress of fermentation. Based on the optimizations, the PAS-based GABA fermentation was performed using a 10 L fermenter. The PAS-based strategy promoted GABA synthesis by the strain of up to 321.9 ± 6.7 g/L after 48 h, with a productivity of 6.71 g/L/h and a substrate molar conversion rate of 99.6%. The findings suggest that the PAS-based fermentation is a promising method for GABA production by lactic acid bacteria.

**Keywords:** gamma-aminobutyric acid; *Lactobacillus brevis* CD0817; culture condition optimization; pH auto-sustain-based GABA fermentation



**Citation:** Jia, M.; Zhu, Y.; Wang, L.; Sun, T.; Pan, H.; Li, H. pH Auto-Sustain-Based Fermentation Supports Efficient Gamma-Aminobutyric Acid Production by *Lactobacillus brevis* CD0817. *Fermentation* **2022**, *8*, 208. <https://doi.org/10.3390/fermentation8050208>

Academic Editor: Yi-huang Hsueh

Received: 24 March 2022

Accepted: 29 April 2022

Published: 3 May 2022

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## 1. Introduction

Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid that is extensively distributed in nature [1,2] and functions as the major inhibitory neurotransmitter in mammals [3,4]. GABA is also associated with the auditory cortex [5] and psychosis states [6]. Furthermore, GABA possesses therapeutic effects on liver injury [7], hepatic encephalopathy [8], and mechanical pain [9] and also possesses antidiabetic and hypotensive properties [10]. Therefore, GABA has been widely used in the food and pharmaceutical industries [11]. Moreover, GABA is a precursor for the synthesis of biodegradable polyamide 4 [12–14].

Over the past three decades, GABA production by lactic acid bacteria (LAB) has been extensively studied because of the safe status of LAB [15–17]. Generally, a glutamic acid decarboxylase (GAD) system in the LAB cell is responsible for synthesizing GABA [18]. The system consists of glutamate/GABA antiporter (GadC) and glutamate decarboxylase (GadA/GadB). The mechanism and process of GABA synthesis by the GAD system is as follows: (i) glutamate is imported into the cell by GadC; (ii) the imported glutamate is decarboxylated to GABA by intracellular GadA/GadB, simultaneously releasing one

molecule of carbon dioxide; and (iii) the final product, GABA, is exported out of the cell by GadC.

Many LAB with GAD activity, including lactobacilli and lactococci strains, have been screened and identified [19–21]. A few LAB strains exhibit potential application for industrial GABA production. However, the production efficiency of LAB-sourced GABA still needs to be enhanced. Researchers have been striving to seek strategies for elevating GABA bioconversion efficiency. Up to date, several strategies have been employed to improve GABA synthesis by LAB strains. These strategies can vary substantially in experiments but fall into two types: (i) modern biotechnology-dependent strategies and (ii) traditional fermentation optimizations [22–25]. As a modern strategy, the genetic improvement based on understanding the cell physiology could effectively enhance GABA production by LAB strains [26]. For instance, Wu and Shah [27] demonstrated that early acidification of the fermentation environment can partly ameliorate the inhibitory effect of oxygen on GABA synthesis by *L. brevis* NPS-QW-145. Additionally, Gong et al. [28] found deleting the *global nitrogen regulator (glnR)* gene increased the amount of GABA synthesized by *L. brevis* ATCC367 from 26.3 to 284.7 g/L.

Traditional optimization has also been proved to be an effective way to elevate the GABA production of LAB strains. For instance, the traditional optimizations have led to a 0.41, 0.31, and 10.29-fold increase in GABA production by *L. brevis* TCCC 13007 [29], *L. brevis* K203 [30], and *L. buchneri* WPZ001 [31], respectively. During GABA fermentation, an acidic environment is essential for activating the GAD system [32]. In the traditional method, which involves the use of monosodium L-glutamate (MSG) as substrate, the environment is progressively alkalized as fermentation progresses because of the consumption of one proton with each decarboxylation. Therefore, acidic agents are fed into the culture broth to maintain the acidic environment essential for GAD activity [33]. This process results in energy and resource wastage, and the introduced acid radical may inhibit the decarboxylation process [34].

In contrast, L-glutamic acid has a relatively low solubility (~6.0 g/L) and isoelectric point (3.22), making it an ideal substrate for GABA fermentation. Clearly, even if a massive amount of L-glutamic acid is added at once to the medium, it shows little inhibitory effect on GABA production as the L-glutamic acid mostly exists in solid powder form. Additionally, the dissolution of L-glutamic acid synchronizes with substrate consumption, thus maintaining the acidity of the fermentation broth.

Although many studies have been performed to improve GABA production by different LAB strains, an optimized method is yet to be developed for each strain. The aim of this study was to develop an optimized pH auto-sustain (PAS)-based fermentation method for efficient GABA production by *L. brevis* CD0817 [35], using L-glutamic acid as the substrate.

## 2. Materials and Methods

### 2.1. Reagents

Beef extract 01-009 and tryptone 01-002 were purchased from Aoboxing Biotech Co., Ltd. (Beijing, China). Yeast extracts FM405, FM408, FM803, FM818, FM828, and FM888; beef extract 81001536; bovine heart extract 81001494; bovine liver extract 81001539; bovine bone peptone FP328; tryptone FP318; soy peptone FP410; and yeast peptone FP103 were purchased from Angel Yeast Co., Ltd. (Wuhan, China). The other nitrogen sources were obtained from Shuangxuan Microbe Culture Medium Products Factory (Beijing, China). L-glutamic acid was obtained from Dragon Biotech Co., Ltd. (Emeishan, China). MSG was purchased from Lanji Technology Development Co., Ltd. (Shanghai, China). 3,5-dinitrosalicylic acid reagent was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). The reagents used for high-performance liquid chromatography were of chromatographic grade, and other chemicals used in this work were of analytical grade.

## 2.2. Strain, Starting Media, and Preparation of Inoculum

*L. brevis* CD0817, a robust GABA producer, was previously isolated from fecal samples from a healthy adult [35]. The strain was stored in 50% glycerol at  $-80\text{ }^{\circ}\text{C}$  and was resuscitated by culturing at  $32\text{ }^{\circ}\text{C}$  for 2 d following streaking on a plate.

The starting seed medium contained 25.0 g/L of yeast extract, 10.0 mg/L of  $\text{MnSO}_4\cdot\text{H}_2\text{O}$ , 28.0 g/L of MSG, 2.0 g/L of Tween-80, and 50.0 g/L of glucose and was maintained at a pH of 5.0. The starting fermentation medium contained yeast extract (25.0 g/L),  $\text{MnSO}_4\cdot\text{H}_2\text{O}$  (25.0 mg/L), Tween-80 (2.0 g/L), and glucose (25.0 g/L). Just before inoculation, sterilized L-glutamic acid solid powder was added in the amount of 650 g per liter of fresh fermentation broth. The added L-glutamic acid mostly existed in the form of solid powder due to its low solubility. L-glutamic acid, glucose, and the remaining components were separately sterilized at  $121\text{ }^{\circ}\text{C}$  for 20 min and mixed together before use [35].

We successively optimized the starting fermentation medium, fermentation temperature, glucose level of the starting seed medium, and seed age. The preparation of the seed medium varied with the advancing optimization. For optimizing the fermentation medium and temperature, the inocula were prepared at  $32\text{ }^{\circ}\text{C}$  using the starting seed medium. For optimizing the glucose level of the seed medium, the inocula were made at  $32\text{ }^{\circ}\text{C}$ . For optimizing the seed age, the inocula were prepared at  $30\text{ }^{\circ}\text{C}$  using the optimized seed medium. The optimized seed medium was identical to the starting seed medium, with the exception of the glucose being reduced to 10 g/L. The cells of *L. brevis* CD0817 were cultivated in a ZHWY-2102C rotary shaker (Zhicheng Inc., Shanghai, China) under constant rotation (100 rpm) and could be used as the inoculum when the absorbance at 600 nm ( $A_{600}$ ) reached 3.0–6.0.

## 2.3. Optimization Experiments

Single-factor experiments were performed to determine the optimal levels of key factors affecting GABA synthesis one by one. Once a key factor was optimized, its optimized level was thereafter incorporated into the fermentative formula and kept fixed in the following experiments. The starting fermentation medium, fermentation temperature, glucose level of the starting seed medium, and seed age were successively optimized. For the fermentation medium, levels of the glucose, nitrogen source,  $\text{Mn}^{2+}$ , and Tween-80 were sequentially optimized. To achieve this, fermentation experiments were performed using a 250 mL Erlenmeyer flask (Sichuan Shuniu Co., Ltd., Chongzhou, China). The fermentation was initiated by transferring seed culture (inoculum size, 10%, *v/v*) into the flask containing 100 mL fermentation medium supplemented with 65 g solid powder L-glutamic acid. The fermentation was conducted at  $32\text{ }^{\circ}\text{C}$  for 96 or 72 h to optimize level of glucose/nitrogen source or of  $\text{Mn}^{2+}$ /Tween-80, respectively, in the fermentation medium. The fermentation was conducted for 72 h to optimize the fermentation temperature. The fermentation was conducted at  $30\text{ }^{\circ}\text{C}$  for 60 h to optimize the seed medium glucose level or seed age. On the premise of assuring experiment quality, the stepwise reduction of fermentation time enhanced research efficiency. Each Erlenmeyer flask experiment was conducted in triplicate. Samples were aseptically withdrawn every 12 h to determine GABA content and  $A_{600}$ .

## 2.4. Fermentation Trial

The optimized seed medium contained 25.0 g/L yeast extract, 10.0 mg/L  $\text{MnSO}_4\cdot\text{H}_2\text{O}$ , 28.0 g/L MSG, 2.0 g/L Tween-80, and 10.0 g/L glucose and was maintained at a pH of 5.0. The optimized fermentation medium contained yeast extract (35.0 g/L),  $\text{MnSO}_4\cdot\text{H}_2\text{O}$  (50.0 mg/L), Tween-80 (1.0 g/L), and glucose (5.0 g/L). Just before inoculation, 650 g of sterilized L-glutamic acid solid powder per liter of fermentation medium was added. The seed culture was made as described above. The seed culture (inoculum size, 10%, *v/v*) was inoculated in a 10 L fermenter containing 3.0 L of the optimized fermentation medium supplemented with 1950.0 g of solid powder L-glutamic acid and incubated at  $30.0\text{ }^{\circ}\text{C}$  under constant agitation (50 rpm) for 48 h. The fermenter was equipped with EasyFerm Plus 120 pH electrodes (Hamilton, Bonaduz, Switzerland) and two layers of six-flat-bladed

impellers. The fermentation was performed under sealed conditions, and the cover of the fermentation tank was fitted with a silicone pipe to serve as an exhaust for carbon dioxide. The exhaust pipe was submerged in water to prevent air from entering the tank. Samples were aseptically withdrawn every 2 h to determine the GABA and residual sugar content and cell growth. All the samples were stored at  $-20\text{ }^{\circ}\text{C}$  for further analysis. The experiment was repeated three times.

### 2.5. Analytic Procedures

The GABA concentration of the culture broth was determined by high-performance liquid chromatography. Briefly, after a 5 min treatment in a boiling bath, a sample of the culture broth was centrifuged at  $8000\times g$  for 3 min using a Sorvall ST 8R centrifuge (Thermo Fisher Scientific, Waltham, USA). Thereafter, the supernatant ( $20.0\text{ }\mu\text{L}$ ) was subjected to derivatization at  $25\text{ }^{\circ}\text{C}$  for 5.0 min by mixing with  $20.0\text{ }\mu\text{L}$  of the derivatization reagent and  $100.0\text{ }\mu\text{L}$  borate buffer. Approximately  $50\text{ }\mu\text{L}$  of the derived sample was injected into an Agilent 1200 system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent Eclipse XDB-C18 column ( $4.6\times 150\text{ mm}$ ,  $5\text{ }\mu\text{m}$ ). The high-performance liquid chromatography conditions were as follows: mobile phase flow rate  $0.8\text{ mL/min}$ , column temperature  $30\text{ }^{\circ}\text{C}$ , and detection wavelength  $338\text{ nm}$ . All data were expressed as the means  $\pm$  standard deviations.

The amino acid derivatization reagent was freshly prepared by dissolving  $10.0\text{ mg}$  of *o*-phthalaldehyde and  $10\text{ }\mu\text{L}$  of 2-mercaptoethanol in  $2.5\text{ mL}$  of acetonitrile. The borate buffer was prepared by adding  $4.9\text{ g}$  of boric acid into  $100\text{ mL}$  of ultrapure water, pH was adjusted to 10.4 using NaOH, and the solution was further diluted to  $200.0\text{ mL}$ . The mobile phase was prepared as follows:  $2.7\text{ g}$  of sodium acetate trihydrate and  $200.0\text{ }\mu\text{L}$  of triethylamine were dissolved in  $0.9\text{ L}$  of ultrapure water, pH was adjusted to 7.3 using acetic acid, the solution was diluted to a final volume of  $1\text{ L}$ , and then mixed with  $250.0\text{ mL}$  acetonitrile [36].

Cell biomass was expressed as  $A_{600}$  value. The cells were recovered by centrifugation at  $5000\times g$  for 5 min using a Sorvall ST 8R centrifuge and resuspended in an appropriate volume of 0.8% NaCl according to the dilution factors of different samples. Finally, cell growth was monitored by determining the  $A_{600}$  value on an UV1200B ultraviolet-visible spectrophotometer (Mapada Instruments, Shanghai, China).

Residual glucose was quantitated by 3,5-dinitrosalicylic acid method, according to the manufacturer's instructions. Briefly,  $2\text{ mL}$  of 3,5-dinitrosalicylic acid reagent was added into a tube containing  $1\text{ mL}$  of 10-fold diluted samples, mixed, heated for 5 min in a boiling water bath, and immediately cooled with cold running water. Thereafter,  $9\text{ mL}$  distilled water was added and mixed, and the  $A_{540}$  value of the mixture was determined using the UV1200B spectrophotometer.

### 2.6. Calculations

The substrate molar conversion rate ( $k$ ) was calculated using the formula below:

$$k = \frac{147.1 C_{GABA} V}{103.1 m_{Glu}} \times 100\%$$

where  $C_{GABA}$  is concentration of GABA (g/L),  $m_{Glu}$  is mass of consumed L-glutamic acid (g), 147.1 is molar mass of L-glutamic acid (g/mol), 103.1 is molar mass of GABA (g/mol), and  $V$  is volume (L). It should be noted that  $1\text{ L}$  of fresh medium increased to  $1.41\text{ L}$  after L-glutamic acid was depleted.

### 2.7. Statistical Analysis

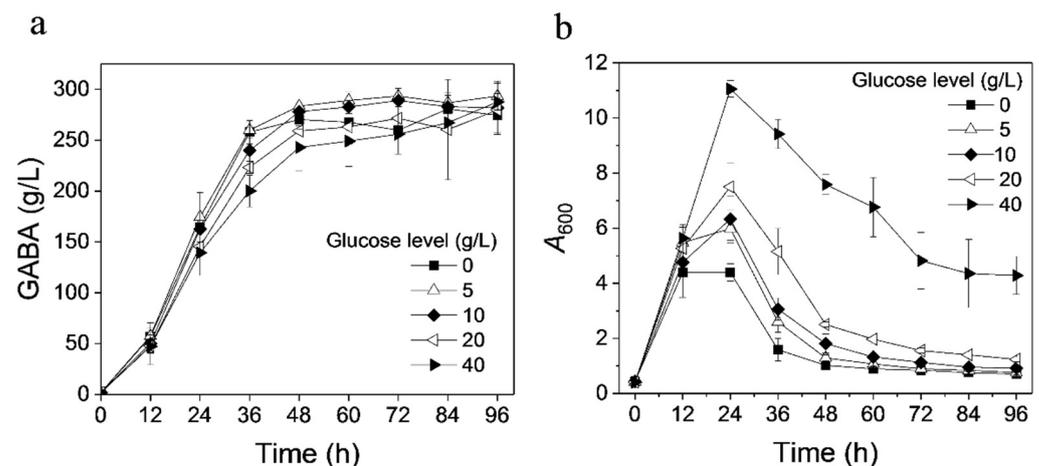
All statistical analyses were performed using SPSS statistics V22.0 software (International Business Machines Corporation, Armonk, NY, USA), and a post hoc test was performed using Tukey's test at  $p < 0.05$ . All charts and graphs were plotted using the

Origin 2017 software (OriginLab, Northampton, MA, USA). Data were represented as means  $\pm$  standard deviations ( $n = 3$ ).

### 3. Results and Discussion

#### 3.1. Effect of Glucose on GABA Production

Glucose is an ideal carbon source for GABA-synthesizing LAB [28,37]. Therefore, we determined the optimal glucose concentration for efficient GABA production. The results indicated that glucose concentration had little effects on the trend of GABA synthesis (Figure 1), that is, GABA was rapidly produced from 0 to 36 h across all the concentrations (0–40.0 g/L), plateauing afterward. However, GABA content varied with glucose concentration, with 5.0 g/L of glucose yielding the highest GABA concentration ( $283.5 \pm 23.0$  g/L) after 48 h of fermentation. Higher concentrations of glucose (10.0–40.0 g/L) instead slightly reduced GABA production (Figure 1). *Lactobacillus brevis* is a microaerophilic, obligately heterofermentative lactic acid bacterium that uses the phosphoketolase pathway to produce a mixture of lactic acid, ethanol, acetic acid, and CO<sub>2</sub> as products of glucose fermentation [38,39]. The decrease in GABA concentration at higher glucose concentration could be attributed to the effects of the metabolites, such as lactic acid, ethanol, and acetic acid. Moreover, a mildly acidic (around 5.0) [40,41] environment is favorable for maximizing GAD activity, whereas extremely low pH (below 4.0) inhibits GAD activity [42,43]. High content of glucose generates massive amounts of organic acids and, thus, may inhibit GAD activity by further acidifying the environment [34,44]. However, the exact mechanism of glucose in GABA production remains poorly understood and requires further studies.



**Figure 1.** The effect of glucose on (a) GABA production and (b) cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask at 32.0 °C. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). There was no significant difference in the 48 h GABA concentration of the tested glucose levels ( $p = 0.084$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

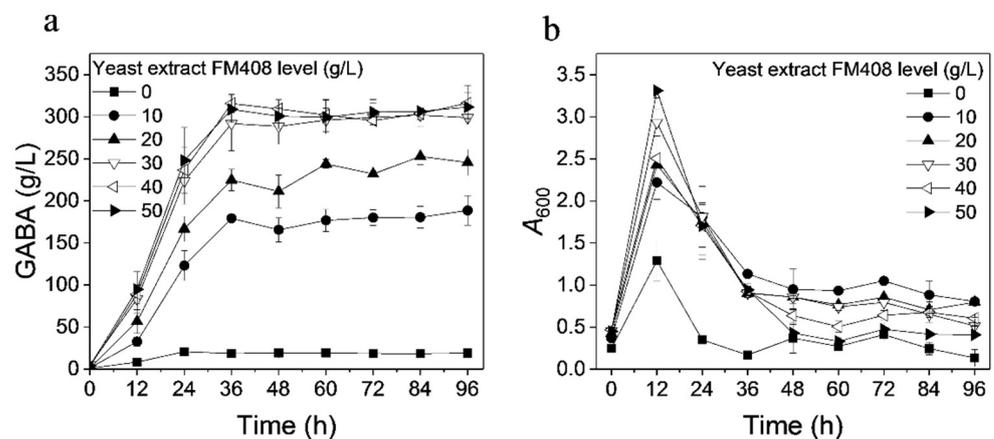
Additionally, the LAB strain examined in this study could grow and produce GABA in a glucose-free medium (Figure 1), indicating that a nitrogen source could also provide some essential carbons for microbial growth. The carbons mainly comprised peptides, amino acids, and nucleotides as the product label indicated. Overall, 5.0 g/L of glucose supported optimal GABA synthesis by *L. brevis* CD0817, which was lower than that ( $\geq 10.0$  g/L) required by other strains of *L. brevis* for efficient GABA production [28,34,37,44].

#### 3.2. Effect of Nitrogen Source on GABA Production

Several studies have shown that the nitrogen level has a considerable influence on GABA production by LAB [29,34,37]. For instance, tryptone, yeast extract, and whey powder are the best nitrogen sources for *L. brevis* HYE1 [45], *L. plantarum* EJ2014 [46], and

*L. brevis* A3 [47], respectively; and a mixture of peptone, meat extract, and yeast extract enhanced GABA production by *L. brevis* CRL 2013 [22]. Therefore, the identification of the right nitrogen source and its optimal level is essential in enhancing GABA production [37,46,47]. In the present study, we examined the effects of 37 nitrogen sources on GABA production. The results indicated that the nitrogen source remarkably modulated GABA synthesis, as a GABA titer difference of up to 26.0-fold was observed among the nitrogen sources examined (Figure S1). Among which, yeast extract FM408 yielded the highest GABA output ( $239.4 \pm 6.5$  g/L), whereas soy peptones 02-19 yielded the lowest output ( $9.2 \pm 0.5$  g/L). Therefore, yeast extract FM408 was employed for further study.

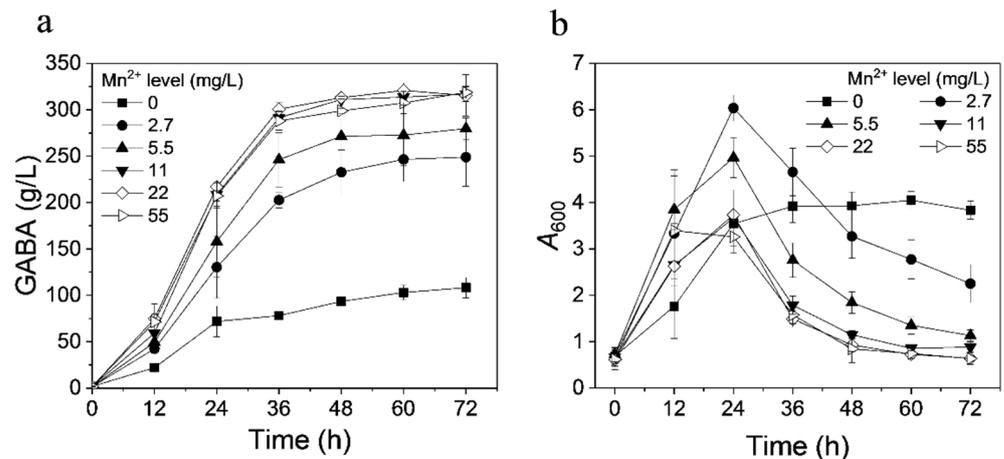
Further analysis indicated an increase in GABA production with increase in yeast extract FM408 concentration from 0 to 30.0 g/L (Figure 2). Although higher concentrations (40.0–50.0 g/L) of yeast extract FM408 increased the cell biomass, it did not increase GABA production further (Figure 2). To guarantee efficient production of GABA, the dosage of yeast extract FM408 was suggested to be 35.0 g/L, slightly higher than 30.0 g/L. The glutamate ( $1.85 \pm 0.05$  g/L) introduced by 35.0 g/L of yeast extract FM408 had a minimal effect on the bioprocess.



**Figure 2.** The effect of yeast extract FM408 on (a) GABA production and (b) cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask at 32.0 °C. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). The difference in 48 h GABA concentrations was not significant between 40 g/L and 50 ( $p = 0.979$ ) or 30 g/L of yeast extract FM408 ( $p = 0.573$ ), but it was significant between 40 g/L and 20 ( $p = 0.000$ ) or 0 g/L of yeast extract FM408 ( $p = 0.000$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

### 3.3. Effect of Ions on GABA Production

The effects of several compounds including  $MnSO_4$ ,  $MgSO_4$ ,  $K_2SO_4$ ,  $NaCl$ ,  $CuSO_4$ ,  $ZnSO_4$ ,  $FeSO_4$ ,  $Ba(OH)_2$ ,  $CoSO_4$ , and  $NiSO_4$  on the GABA production were individually evaluated. The results demonstrated that only  $MnSO_4$  had a positive effect on GABA synthesis (data not shown). Therefore, the effect of  $MnSO_4$  concentration on GABA production by *L. brevis* CD0817 was assessed. As shown in Figure 3a,  $Mn^{2+}$  was an indispensable factor for efficient GABA production. Overall, 11–55 mg/L  $Mn^{2+}$  supported peak GABA production; however, maximal cell growth occurred at 2.7–5.5 mg/L  $Mn^{2+}$  (Figure 3b). We speculated that the high GABA content resulting from 11–55 mg/L  $Mn^{2+}$  may have suppressed cell growth. Compared with the  $Mn^{2+}$ -free control, 11–55 mg/L  $Mn^{2+}$  increased GABA production by 2.3-fold. To assure the need of the strain for  $Mn^{2+}$ , 16 mg/L (slightly higher than 11 mg/L) of  $Mn^{2+}$  (equal to 50.0 mg/L  $MnSO_4 \cdot H_2O$ ) was adopted.



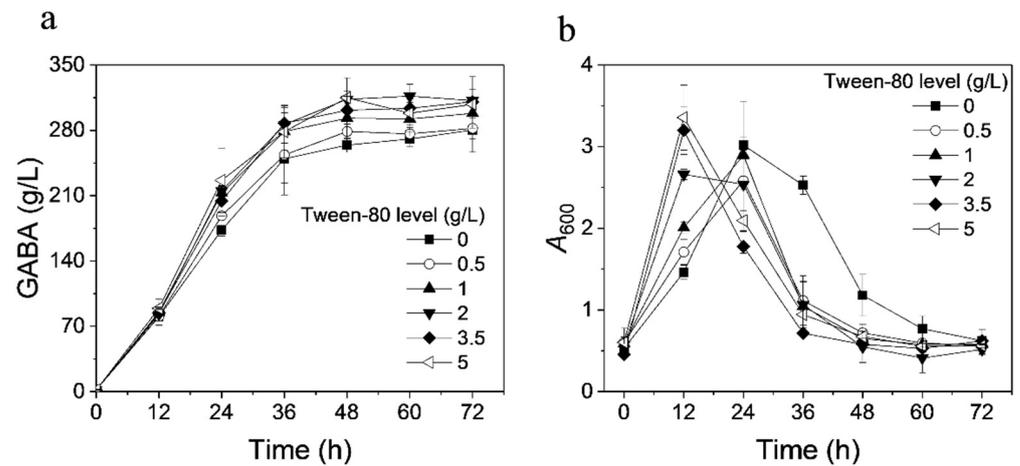
**Figure 3.** The effect of manganese ion on (a) GABA production and (b) cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask at 32.0 °C. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). The difference in 48 h GABA concentrations was not significant between 22 mg/L and 11 ( $p = 1.000$ ), 55 ( $p = 0.936$ ), or 5.5 mg/L of  $Mn^{2+}$  ( $p = 0.159$ ), but it was significant between 22 mg/L and 2.7 ( $p = 0.003$ ) or 0 mg/L of  $Mn^{2+}$  ( $p = 0.000$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

Ions can affect GABA production in a strain-specific manner [41]. For instance,  $Mn^{2+}$ ,  $Cl^-$ , and  $Ca^{2+}$  plus  $Na^+$  have been shown to significantly enhance GABA production by *L. brevis* NCL912 [34], *Lactococcus lactis* NCDO 2118 [48], and *L. brevis* K203 [30,37], respectively. Additionally, GADs of *Streptococcus salivarius* ssp. *thermophilus* Y2, *L. zymae*, and *L. sakei* A156 were activated in vitro by  $Ba^{2+}$  [49],  $Mg^{2+}$  [50], and  $Co^{2+}$  plus  $Zn^{2+}$  [40], respectively. Generally, some ions can improve GAD activity by acting as cofactors [51]. Hence, it is necessary to screen and optimize ions for a specific GABA-producing LAB strain.

### 3.4. Effect of Tween-80 on GABA Production

Tween-80 has been widely used as an enhancer in the manufacture of lactic acid bacterial biologics, such as GABA [16,37], bacteriocins [52], and riboflavin [53]. Therefore, the effects of different concentrations (0–5.0 g/L) of Tween-80 on GABA production were evaluated in this study. The results showed that GABA production increased with increase in Tween-80 concentration from 0 to 1.0 g/L (Figure 4a), which was similar to the previous findings [22,34,54]. However, higher concentrations of Tween-80 did not significantly affect GABA synthesis. Therefore, 1.0 g/L of Tween-80 was selected for further experiments.

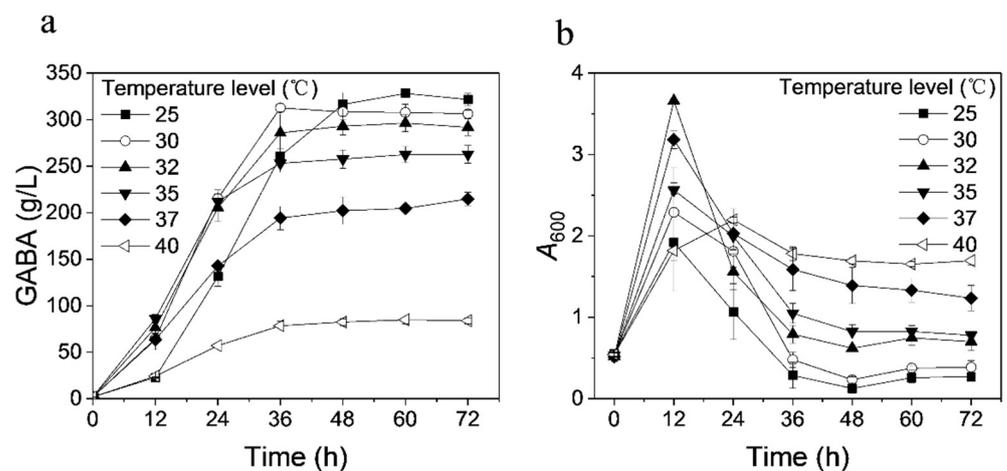
As an oleate, Tween-80 is a growth-stimulating factor for LAB strains [52]. Accordingly, the cell biomass of *L. brevis* CD0817 increased with increase in Tween-80 concentration in this study (Figure 4b). Increased cell biomass may contribute to GABA production. Moreover, as a non-ionic surfactant, Tween-80 may support oleic acid uptake by cells, which is then converted to cyclopropane fatty acid (a membrane fluidity enhancer) [52]. These findings indicated that Tween-80 may elevate GABA production by improving cell membrane permeability [34]. However, further studies should investigate whether Tween-80 can help maintain the acidic pH by promoting glucose metabolism.



**Figure 4.** The effect of Tween-80 on (a) GABA production and (b) cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask at 32.0 °C. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). There was no significant difference in 48 h GABA concentration between 5 g/L and 2 ( $p = 1.000$ ), 3.5 ( $p = 0.907$ ), 1 ( $p = 0.587$ ), or 0.5 g/L of Tween-80 ( $p = 0.143$ ), but there was a significant difference between 5 and 0 g/L of Tween-80 ( $p = 0.024$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

### 3.5. Effect of Temperature on GABA Production

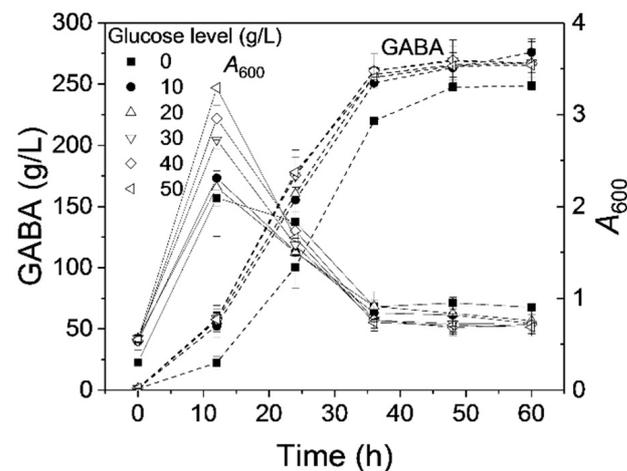
In the present study, we examined the effect of temperature (25–40 °C) on GABA synthesis by *L. brevis* CD0817. Generally, temperature significantly regulated GABA generation (Figure 5), with peak GABA production during the first 48 h achieved at 30 °C. However, 25 °C was slightly more suitable than 30 °C for GABA production by *L. brevis* CD0817 after 48 h, which could be because GAD activity is generally more stable under lower temperatures [49]. Additionally, temperatures above 30 °C significantly inhibited GABA production (Figure 5). Overall, 30 °C was the optimal temperature for GABA synthesis by *L. brevis* CD0817, which was similar to findings in *L. brevis* strains CRL 1942 [55] and RK03 [37]. Therefore, 30 °C was selected for further experiments.



**Figure 5.** The effect of fermentation temperature on (a) GABA production and (b) cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). There was no significant difference in 48 h GABA concentration between 25 °C and 30 °C ( $p = 0.926$ ) or 32 °C ( $p = 0.136$ ), but there was a significant difference between 25 °C and 35 °C ( $p = 0.000$ ), 37 °C ( $p = 0.000$ ), or 40 °C ( $p = 0.000$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

### 3.6. Effect of Glucose in Seed Medium on GABA Production

The glucose level of the starting seed medium for *L. brevis* CD0817 was up to 50.0 g/L [35]. The findings of this study have shown that 5.0 g/L of glucose in the fermentation medium was the optimal level for GABA production. Therefore, it was necessary to reduce the glucose level of the seed medium for an efficient fermentation process. To achieve this, we examined the effect of the glucose level of the seed media on GABA fermentation efficiency. The results indicated that inoculum prepared using 10.0 g/L glucose resulted in an almost identical GABA titer to those prepared using higher levels of glucose (Figure 6), indicating that 10.0 g/L glucose was appropriate for seed medium preparation.

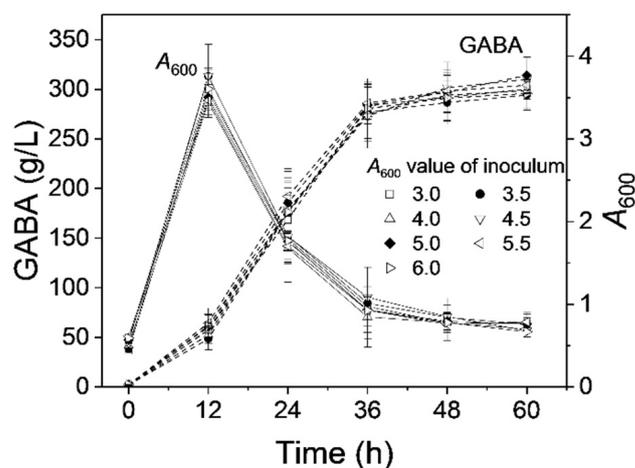


**Figure 6.** The effect of seed medium glucose content on GABA production and cell growth. Inocula prepared using seed media with different levels of glucose were used for the fermentation experiment, which was performed in a 250 mL Erlenmeyer fermentation flask at 30.0 °C. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). There was no significant difference in 48 h GABA concentration between the different glucose levels examined ( $p = 0.267$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

### 3.7. Effect of Seed Age on GABA Production

Next, we examined the effect of seed age on GABA production. GABA fermentation was initiated using seeds with  $A_{600}$  values ranging from 3.0 to 6.0 ( $4.8\text{--}9.7 \times 10^8$  CFU/mL). The data showed that seed age exhibited marginal effects on either GABA synthesis or strain growth (Figure 7), reflecting flexibility of inoculation.

Certain factors influence GABA production by LAB in a strain-specific manner [16,37]. The results of the present study showed that glucose, yeast extract, Tween-80,  $Mn^{2+}$ , and temperature are key factors influencing GABA production by *L. brevis* CD0817, with optimal levels of 5.0 g/L, 35.0 g/L, 1.0 g/L, 16.0 mg/L, and 30 °C, respectively. Therefore, the seed medium was reconstituted to contain 10.0 g/L of glucose, 28.0 g/L of MSG, 35.0 g/L of yeast extract, 1.0 g/L of Tween-80, and 50.0 mg/L of  $MnSO_4 \cdot H_2O$ , and pH was maintained at 5.0. Additionally, the reconstituted fermentation medium was composed of glucose (5.0 g/L), yeast extract (35.0 g/L), Tween-80 (1.0 g/L), and  $MnSO_4 \cdot H_2O$  (50.0 mg/L). L-glutamic acid solid powder was added in the amount of 650 g per liter of fermentation medium.



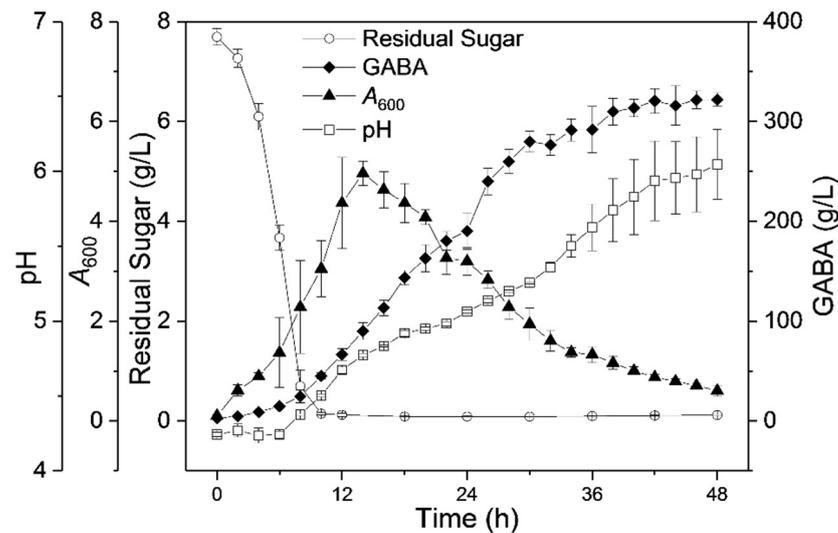
**Figure 7.** The effect of seed age on GABA production and cell growth. This was evaluated using a 250 mL Erlenmeyer fermentation flask at 30.0 °C. Fermentations were performed using inocula with different  $A_{600}$  values, which were prepared from the seed media containing 10 g/L glucose. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). There was no significant difference in 48 h GABA concentration between the different treatments (inocula different  $A_{600}$  value;  $p = 0.979$ ). GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

### 3.8. PAS-Based GABA Bioprocess

Based on the results of the single-factor experiments, a PAS-based GABA fermentation bioprocess was prepared for *L. brevis* CD0817, and the efficiency of the bioprocess was evaluated. The findings of the single-factor experiments also showed that rapid GABA production occurred from 0 to 36 h (Figures 1–7). Therefore, the PAS-based bioprocess was operated within a 48 h culture period.

The PAS-based fermentation process was performed using 3.0 L of the optimized fermentation medium, 10.0% ( $v/v$ ) of inoculum size, and 1950.0 g of L-glutamic acid solid powder at 30 °C and 50 rpm. As shown in Figure 8, rapid cell growth occurred immediately after inoculation and decreased considerably after 14 h. GABA production increased external osmolarity that triggered water loss of bacteria. A negative effect on cell growth occurred when dehydration reached a physiologically unsustainable level at high GABA content. The high osmotic pressure arising from high content of GABA was assumed to cause the decline of cell growth [48,56]. Glucose was depleted within 10 h, but cell growth continued till 14 h, which could be attributed to carbon supply by yeast extract. GABA production during the first 6 h of the fermentation process was slow, followed by rapid production till 38 h, after which the production plateaued. The final GABA titer was  $321.9 \pm 6.7$  g/L, with a productivity of 6.71 g/L/h and a substrate conversion ratio of 99.6%. The substrate was depleted within 48 h. Compared with the unoptimized process [37], the optimized process increased GABA production by 28.0%.

In the unoptimized process, the starting seed medium and the starting fermentation medium, described in the Section 2, were used to make the seed medium and conduct fermentation at 32 °C, respectively. The other parameters of the unoptimized process were identical to those of the optimized process. By analyzing the parameters and the results between the two fermentations, we attribute the variation of GABA contents to the differences in the glucose level and culture temperature, which have been discussed in the Section 3.1. “Effect of glucose on GABA production” and Section 3.5. “Effect of temperature on GABA production”, respectively.



**Figure 8.** pH auto-sustain-based GABA fermentation in a 10 L fermenter. The fermentation experiment was conducted at 30.0 °C and 50 rpm. Each liter of fermentation medium was supplemented with 650 g solid powder L-glutamic acid. Cell growth was expressed as  $A_{600}$ . Vertical bars represent standard deviations of means ( $n = 3$ ). The 48 h GABA concentration was  $321.9 \pm 6.7$  g/L. GABA, gamma-aminobutyric acid;  $A_{600}$ , absorbance at 600 nm.

The fresh fermentation medium saturated by L-glutamic acid had an extremely low pH (3.3). The pH was enhanced to 4.2 after inoculating the 150.0 mM MSG-based seed culture. The pH was maintained at 4.2 for the first 6 h of fermentation, followed by a gradual increase to approximate 6.0. The increase in pH followed a similar trend with GABA formation, which could be attributed to the consumption of  $H^+$  during decarboxylation [16,44,57]. Clearly, it is almost impossible to always maintain the optimal pH (around 5.0) required for decarboxylation during the PAS-based fermentation. However, the broth remained acidic due to the organic acids metabolized from carbons and the PAS activity of the substrate. Even if the substrate was depleted, the organic acids would prevent the final pH from exceeding 7.0. As one control, we conducted a non-pH-controlled MSG-based GABA fermentation (initial pH 5.0), which was identical to the PAS-based method with the exception of substituting 0.75 M MSG for L-glutamic acid. The results indicated that the pH was gradually increased, up to ~7.5 and ~8.0 at 48 and 72 h, respectively; meanwhile the decarboxylation rate was progressively decreased (data not shown). These data implied that the GAD-induced cell can decarboxylate the substrate even in a weakly alkaline environment, although its decarboxylation ability is decreased with the increase of pH. It, thus, could be concluded that the suboptimal acid environment should guarantee a satisfactory GABA bioconversion during the PAS-based fermentation, which was confirmed by the high GABA titer of 321.9 g/L (Figure 8). Overall, these findings indicated that L-glutamic acid was necessary for maintaining the low pH of the medium. However, whether L-glutamic acid is involved in other functions deserves further investigation.

Compared with this process, MSG is used as the substrate in the traditional GABA fermentation process. MSG has a neutral isoelectric point because of the substitution of sodium ion ( $Na^+$ ) on the  $\alpha$ -carboxyl group for a hydrogen ( $H^+$ ). Park et al. [46] found that high levels of MSG can inhibit GABA production by introducing large amounts of  $Na^+$ . Meanwhile, the alkalization of the environment attributed to the decarboxylation also exerted a negative effect on the GABA formation. In such a scenario, supplying an acid solution is necessary to maintain mild acidity [28]. However, the introduced acid radical can inhibit GABA synthesis and reduce the quality of the fermented product [42,43,58]. The PAS-based fermentation process developed in this study possesses some advantages over the MSG-based process. For instance, the substrate inhibitory effect on GABA production

observed in MSG-based process was negligible in the PAS-based one. A brief comparison of the PAS-based GABA fermentation with the MSG-based ones is shown in Table 1.

**Table 1.** Comparison of the effects of fermentation methods on gamma-aminobutyric acid production by lactic acid bacteria.

Strain	Method	Substrate	Titer (g/L)	Conversion Rate (%)	Productivity (g/L/h)	Reference
<i>Lactobacillus brevis</i> CD0817	pH auto-sustain	L-glutamic acid	321.9	99.6	6.7	This study
<i>Lactobacillus brevis</i> ATCC 367ΔglnR	pH-controlled	MSG	284.7	NS	NS	[28]
<i>Lactobacillus brevis</i> D17	pH-controlled	MSG	177.7	NS	4.9	[25]
<i>Lactobacillus brevis</i> 9530: pNZ8148-gadBC	pH-controlled	MSG	104.4	NS	NS	[44]
<i>Lactobacillus buchneri</i> WPZ001	NS	MSG	75.5	NS	NS	[31]
<i>Lactobacillus brevis</i> RK03	NS	MSG	62.5	93.3	NS	[37]

MSG: monosodium L-glutamate; NS: not specified; pH-controlled refers to pH-controlled fermentation in which pH was controlled by the feeding of an acid agent.

#### 4. Conclusions

In the present study, a PAS-based GABA fermentation process was developed for efficient GABA production by *L. brevis* CD0817, with L-glutamic acid as the substrate. After 48 h of fermentation, GABA titer reached 321.9 g/L with a productivity of 6.71 g/L/h and a substrate conversion rate of 99.6%. Overall, these findings demonstrate the need for LAB strain-specific fermentation methods to maximize GABA production.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fermentation8050208/s1>: Figure S1: The effect of nitrogen sources on GABA production.

**Author Contributions:** Conceptualization, investigation, writing—original draft preparation, M.J.; supervision, Y.Z.; data collection and analysis of GABA production, L.W.; data acquisition and interpretation of  $A_{600}$  value, T.S. and H.P.; funding acquisition and writing—review and editing, H.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** We are grateful to the National Natural Science Foundation of China (grant Nos. 32160014 and 31570070), State Key Laboratory of Food Science and Technology, Nanchang University (grant No. SKLF-ZZB-202118), and Jiangxi Provincial Department of Science and Technology (grant No. 20171BCB23019) for financial support.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in this article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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