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# Biosynthesis of gama-aminobutyric acid by induced resting cells of *Lactobacillus brevis* SIIA11021

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**ABSTRACT**

*Gama-aminobutyric acid (GABA) is a natural functional amino acid. In present study, we successfully isolated Lactobacillus brevis SIIA11021 which presented a higher GABA-producing ability from Chinese traditional pickled vegetables. A cellular biotransformation process has been taken for the production of GABA, using resting cells of L. brevis SIIA11021 as enzyme systems. Firstly, L. brevis SIIA11021 was incubated anaerobically in 3 % monosodium glutamate (MSG)-containing medium to obtain cells. The L. brevis SIIA11021 cells' conversion activity of MSG to GABA was induced by MSG. Then these induced resting cells were used to build cell-factory to produce GABA by suspended in 0.2 M disodium hydrogen phosphate-citric acid conversion buffer which contained substrate (MSG) and incubated with appropriate conditions. In this process, ethanol has been added into the conversion buffer and played a key factor to the GABA yield. The effects of pH, temperature and ethanol on GABA-producing have been studied. About 134 mM of GABA has been produced at a conversion rate of 95.7 % under optimal conditions at pH 4.7, 30 $^{o}$C, supplemented with 1.3 % (v/v) ethanol for 4 h. Besides, a fed-batch fermentation has been taken, using the resting cells biotransformation process in a 3 L beaker fermentor, to produce GABA for 24 h at optimal conditions and the final yield of GABA reached 440 mM. Furthermore, real-time PCR (RT-PCR) analysis displayed that the GAD gene of L. brevis SIIA11021 expression level was induced by MSG.*

**Keywords:** Gama-aminobutyric acid (GABA); *Lactobacillus brevis*; Glutamate; Ethanol; Biotransformation

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## INTRODUCTION

Gama-aminobutyric acid (GABA) is non-protein amino acid which is widely distributed in nature [1]. It has been proved to be a major inhibitory neurotransmitter in the mammalian brain [2]. Moreover, GABA has several well-known physiological functions, such as neurotransmission, hypotensive acitivity, diuretic effects, tranquilizer effects, depression and autonomic disorders observed during the menopausal or presenium periods [3, 4]. A recent study showed that GABA was a strong secretagogue of insulin from the pancreas [5] and effectively prevents diabetic conditions [6]. So GABA has the potential to be utilized extensively in the food and pharmaceutical fields. Many GABA-enriched functional foods are currently manufactured, including GABA green tea, gabaron tea, red mold rice, tempeh-like fermented soybeans, yogurt, cheese, and fermented milk products.

Glutamate decarboxylase (GAD: EC 4.1.1.15) is a pyridoxal 5-phosphate (PLP)-dependent enzyme, which catalyses the irreversible decarboxylation of L-glutamate to γ-aminobutyric acid (GABA), associated with a glutamate-dependent acid-resistance mechanism in lactic acid bacteria (LAB) [7, 8]. GAD can be produced by many

microorganisms, such as LAB, fungi and yeasts. GAD of *Lactobacillus brevis* IFO 12005 has been purified and characterized [9], and a more detailed genetic study on this GAD has recently been performed [10].

Among the various methods to GABA-producing, the biosynthetic approach appears to be promising due to the simple reaction procedure, high catalytic efficiency, mild reaction conditions and environmental compatibility [11]. Lactic acid bacteria (LAB) are an important group of gram-positive bacteria and frequently exist in fermented food, vegetables and in the intestines of human and animals. Because LAB possess special physiological activities and are generally regarded as safe, many studies have focused on the GABA production by LAB. Several GABA-producing LAB species have been reported, including *L. brevis* [10, 12, 13], *L. lactis* and *L. delbrueckii* [14], *L. paracasei* [14, 15], *L. delbrueckii* subsp. bulgaricus [14, 16] and *L. plantarum* [14] in recent decades.

In the current study, we successfully isolated *L. brevis* SIIA11021 which exhibit a higher GABA-producing ability from Chinese traditional pickled vegetables. A cellular biotransformation process has been taken, using induced resting cells of *L. brevis* SIIA11021, for the production of GABA. Real-time PCR method has been used to analysis the expression quantity of GAD gene of *L. brevis* SIIA11021 cells which induced by various concentrations of MSG when it was cultivated in MRS broth. It can prove that the *L. brevis* SIIA11021 cells' conversion activity of MSG to GABA was induced by the initial MSG. Cell growth and cell conversion activity have also been study at the same time to seek the optimal quantity of induction substrate of MSG. Ethanol can enhance the permeabilization of cells, which add the mass transfers between cells and environment. It has been used in the resting cells bioconversion process and played as a key role for GABA production. Then a fed-batch fermentation was taken to produce GABA at optimal conditions, using the resting cells biotransformation process.

## EXPERIMENTAL SECTION

### Strain, medium and identification of isolated strain

To obtain GABA-producing LAB, 15 pickled vegetable samples were employed. About 1 mL samples were inoculated into 250 mL flasks containing 100 mL MRS broth supplemented with 2 % MSG at 30 $^{o}$C for 24 h without shaking. One milliliter aliquots of $10^7$-fold diluted enrichment culture were taken and spread onto MRS plates which supplemented with 2 % (w/v) $CaCO_3$. Calcium carbonate was used as an indicator for acid-producing strains since it dissolved when interacting with acid then a clear zone is observed [17]. LAB could easily be identified by clear and transparent circle formation because LAB produces lactic acid. MRS broth was used as seed medium. The medium of strains cultivation was composed of (g/L): glucose 20; yeast extract powder 20; soya peptone 5; beef extract 5; sodium acetate 5; $K_2HPO_4$ 2; ammonium citrate 1; Tween 80 1; $MgSO_4$ 0.2; $MnSO_4$ 0.05; monosodium glutamate 20 and pH 6.0. Morphological observations and a Gram staining test were performed on the isolated strain with the best GABA production ability. The morphology of the strain was observed under oil immersion (×1,000, magnification) at light microscope level (Olympus CX21BIM-SET6, Japan). A 16s rDNA gene fragment was amplified using a pair of primer as the forward primers: 5′- AGAGTTTGATCCTGGCTCA -3′ and the reverse primer: 5′- GGTTACCTTGTTACGACTT -3′ by PCR and then sequenced for phylogenetic analyses.

### Strains cultivation and *L. brevis* SIIA 11021 resting cells preparation

*L. brevis* SIIA11021 was cultured in the seed medium at 30 $^{o}$C and pH 6.0 for 12 h statically. Then 2 % (v/v) of seed culture was incubated in the cultivation medium which contain 3% of cell activity induction substance (MSG) at same conditions of seed culture for 24 h. *L. brevis* SIIA11021 cells were harvested by centrifugation at 8,000×g for 20 min at 4 $^{o}$C, washed three times, using 0.2 M disodium hydrogen phosphate-citric acid buffer (pH 4.5).

### GABA biotransformation by resting cells of *L. brevis* SIIA11021

The prepared resting cells were incubated 2.5 h at different temperature in 0.2 M disodium hydrogen phosphate-citric acid buffer containing 2% MSG at the pH 4.5 to observe the production of GABA as a function of temperature. To seek optimal conditions of pH and ethanol for enhancing GABA production, single-factor experiments has been employed respectively and a further optimization has been taken to determine the optimal conditions of the two key factors. Various concentrations of MSG-containing buffer were used to incubate *L. brevis* SIIA11021 resting cells at optimal conditions for 2.5 h to research the optimal MSG concentration for this biotransformation process. Then at optimal conditions with the optimal MSG concentration in the conversion buffer, the prepared resting cells were incubated up to 4 h and 1 mL of sample was withdrawn at each 0.5 h. Then these resting cells were incubated another four times at the same condition continually, 3 h at each time to study the conversion ability of *L. brevis* SIIA11021 resting cells by repeatedly used. A 3 L beaker with 1L buffer has been used, after above steps finished, for the fed-batch fermentation to produce GABA and 100 g MSG has been added averagely in five times for 24 h incubation. Biomass of each experiment was 2.0 of optical density at 600 nm. The conversion rate of MSG to GABA was calculated as follows:

$$\text{Conversion rate} = \frac{\text{Final GABA concentration (mM)}}{\text{Initial MSG concentration (mM)}} 100\%.$$

**RT-PCR for GAD gene mRNA expression quantity**

To amplify the glutamate decarboxylase-encoding Gene, we used a pair of degenerate primer as the forward primers: 5′-CGGGATCCATGGCWATGTTRTAYGGWAAA-3′ and the reverse primer: 5′-GGGAATTCTTAGTGHGTGAAYCCGTATTT-3′ [18]. The glutamate decarboxylase encoding gene was amplified using PCR and sequenced by Sangon Biotech (Shanghai) Co. Ltd. Then the homology search was performed with the BLAST algorithm utilizing the database at NCBI. *L. brevis* SIIA11021 was cultured in cell growth medium with different concentrations of MSG for 24 h and 1 mL samples were withdrawn to do real time PCR (RT-PCR) to detect the mRNA expression quantity of glutamate decarboxylase-encoding gene by Sangon Biotech (Shanghai) Co. Ltd. The gene of 16s rRNA was used as control.

**Analytical methods**

Biomass was determined by measurements of optical density at 600 nm (Mapada UV1100, China). Pre-staining paper chromatography method [19] and automatic amino acid analyzer (membrapure A300, Germany) have been used for GABA determination. Sample preparation was centrifuged at 12000×g for 5 min at 4 $^{o}$C and cell-free supernatant was withdrawn for GABA determination.

## RESULTS AND DISCUSSION

**Screening and identification of isolated strains**

It was found that 20 isolates exhibited clear zones and growth on MRS agar supplemented with $CaCO_3$. Three isolates showed GABA production from the pre-staining paper chromatography results and were confirmed as GABA producers using the automatic amino acid analyzer. The strain of SIIA11021 exhibited the highest GABA-producing ability. It was preliminarily identified by morphological observation. The gram staining result showed gram-positive of SIIA11021 and appeared as a type of brief-rods under a microscope. The colony morphology of SIIA11021 showed white, translucence and edge roughness. Phylogenetic analysis of 16s rDNA sequences indicated that SIIA11021 are closely related to the species in the *L. brevis* and showed 99.8 % similarity with *L. brevis* ATCC 14687(T). Overall, the results showed that SIIA11021 belong *L. brevis*. It was named as *L. brevis* SIIA11021 and was deposited at Microbial Resource Center of Sichuan Industrial Institute of Antibiotics.

**Optimization of pH and Ethanol on the GABA production of *L. brevis* SIIA11021 resting cells**

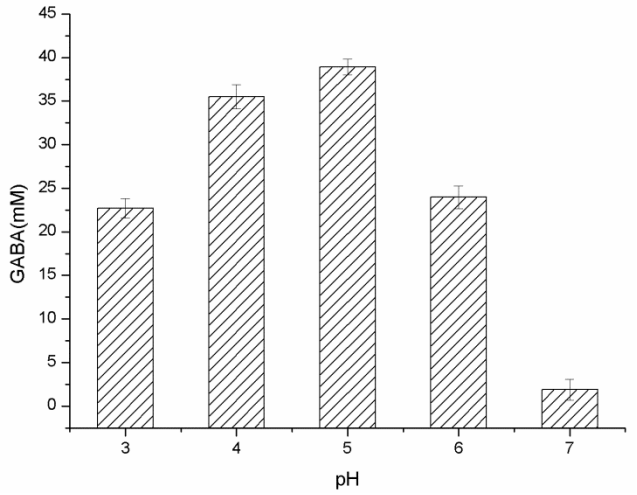


**Fig. 1 Effect of pH on the GABA production by *L. brevis* SIIA11021 resting cells. The GABA concentration (slash filled bar) were monitored at various pH in 250 mL flasks containing 100 mL conversion buffer which containing 2 g MSG at 30 $^{o}$C for 2.5 h of incubation without shaking**

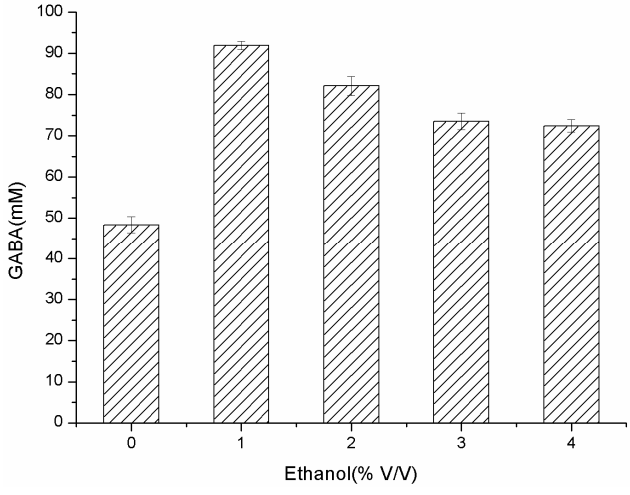


**Fig. 2 Effect of ethanol on the GABA production by *L. brevis* SIIA11021 resting cells. The GABA concentration (slash filled bar) were monitored with different concentration of ethanol in 250 mL flasks containing 100 mL conversion buffer which containing 2 g MSG at 30 $^{o}$C and pH 5 for 2.5 h of incubation without shaking**

The result of single-factor experiment of pH, as shown in Fig.1, GABA concentration increased with increase of pH from 3.0 to 5.0 for 2.5 h cultivation at 30 $^{o}$C in 2 % MSG-containing buffer. When the pH was above 5.0, the GABA concentration dropped significantly. PH was set as 5.0 in the single-factor experiment of ethanol. As shown in Fig.2, when the bioconversion buffer contained 1 % (v/v) ethanol, the yield of GABA is almost twice as much as when the

buffer didn't have ethanol. However, the GABA concentration tended to decrease when the ethanol containing above 1%. In further optimizing, using response surface methodology, the maximum production of GABA was obtained when the buffer contained 1.3 % (v/v) ethanol at pH 4.7.

**Effect of temperature on the GABA production of *L. brevis* SIIA11021 resting cells**

The effect of temperature on the GABA yield of *L. brevis* SIIA11021 resting cells was investigated. The optimum growth temperature of *L. brevis* SIIA11021 was 30 $^{o}$C, coinciding with the optimum temperature of purified GAD (EC 4.1.1.15) in *L. brevis* [9, 11, 20-21], as shown in Fig.3. Thus, a temperature of 30 $^{o}$C was chosen for optimal batch cultivations for GABA production by *L. brevis* SIIA11021.

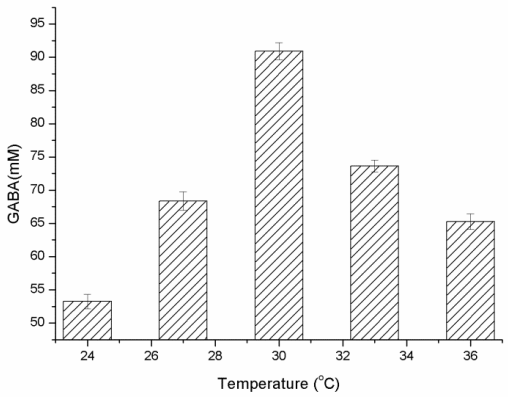


**Fig. 3 Effect of temperature on the GABA production by *L. brevis* SIIA11021 resting cells. The GABA concentration (slash filled bar) were monitored at various temperature in 250 mL flasks containing 100 mL conversion buffer which contained 2 g MSG at 30 $^{o}$C for 2.5 h of incubation without shaking**

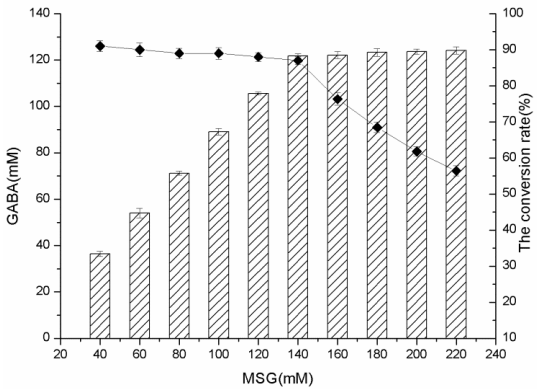


**Fig. 4 GABA production of *L. brevis* SIIA11021 resting cells with different concentration of MSG and the conversion rate of MSG. The GABA concentration (slash filled bar) and bioconversion rate (filled diamond) was monitored at various concentration of MSG in 250 mL flasks containing 100 mL conversion buffer with 1.3 %(v/v) of ethanol at 30 $^{o}$C, pH 4.7 for 2.5 h of incubation without shaking**

**The effect of substrate concentration on the GABA production of *L. brevis* SIIA11021 resting cells**

The effect of initial MSG concentration on GABA production and the rate of bioconversion by *L. brevis* SIIA11021 resting cells at an optimal level of biotransformation condition were shown in Fig.4. The yield of GABA by resting cell transformation gradually increased with increasing MSG concentration at the range of substrate concentration [(40 to 140) mM] over 2.5 h of biotransformation and almost constant when the substrate concentration increased to over 140 mM, however, the conversion rate decreased significantly.

**Time-dependent changes in GABA concentration by *L. brevis* resting cell**

GABA concentration was a function of bioconversion time in the optimal conditions. The GABA yield was greatly improved with the increase of bioconversion time at the time range of (0 to 2.5) h, as shown in Fig.5. The GABA yield increased slowly at the time range of 2.5 h to 3 h and was almost stable when the time over 3 h.

**The effect of repeatedly use *L. brevis* SIIA11021 resting cells for biotransformation on the GABA production**

Another four same experiments, like the time-dependent changes in GABA concentration by *L. brevis* resting cells, using the same cells, has been conducted to investigate the effect of GABA production of *L. brevis* SIIA11021 resting cells by repeatedly used. As shown in Fig.6, even these cells were re-used for five times, the GABA yield could be maintained 79.5 mM.

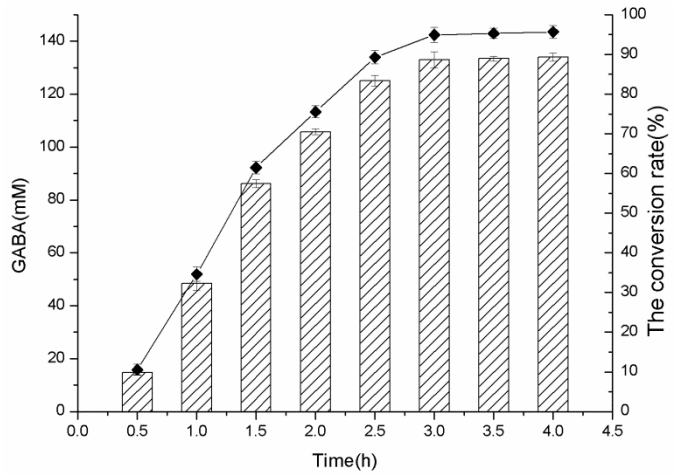


**Fig. 5 Time-dependent changes in GABA concentration and MSG conversion rate by *L. brevis* resting cells. Bioconversion process was performed at 30 °C for 4h in 250 mL flasks containing 100 mL conversion buffer(pH 4.7) with 1.3 %(v/v) of ethanol and 140 mM MSG. The GABA concentration(slash filled bar) and MSG conversion (filled diamond) rate were measured each 0.5 h**

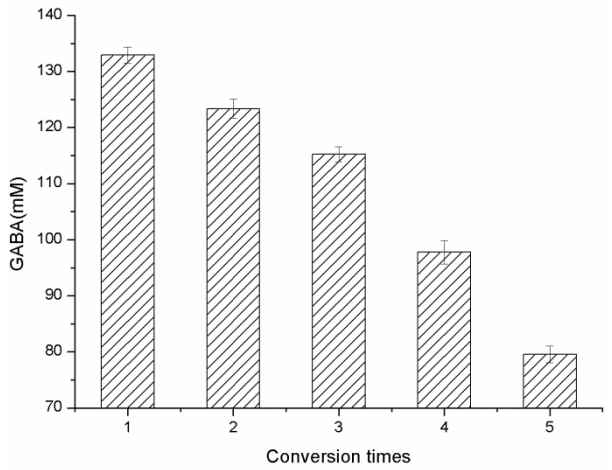


**Fig. 6 Effect of biotransformation times on the GABA production of L. brevis SIIA11021 resting cells. The resting cells were repeatedly incubated for five times at same conditions at 30 ºC for 3h in 250 mL flasks containing 100 mL conversion buffer(pH 4.7) with 1.3 %(v/v) of ethanol and 140 mM MSG. The concentration of GABA (slash filled bar) was monitored respectively**

## The effect of substrate concentration of MSG on GAD gene expression level in growing cells of *L. brevis* SIIA11021

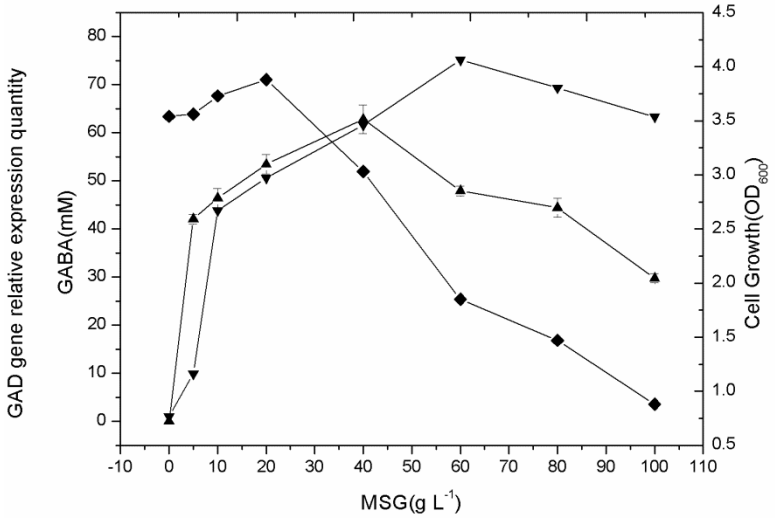


**Fig. 7 Effect of substrate concentration of MSG on L. brevis SIIA11021 GAD gene expression level in growing cells and the activity of resting cells for GABA production. The relative expression quantity of GAD gene (filled inverted triangle) and cell growth (filled square) were monitored at various MSG concentrations for 24 h of growth in 500 mL flasks containing 100 mL cultivation medium without shaking at 30 °C. The resting cells were incubated at 30 ºC for 3h in 250 mL flasks containing 100 mL conversion buffer (pH 4.5) which contained 2 g MSG. The GABA concentration is indicated by filled upright triangle**

Using the degenerate oligonucleotide primers as described above, the partial gad gene of *L. brevis* SIIA11021 was successfully cloned. Then the degenerate oligonucleotide primers was used to determine GAD gene expression level in growing cells of *L. brevis* SIIA11021 which had induced by different concentration of MSG, using RT-PCR. As shown in Fig.7, the relative expression quantity of *L. brevis* SIIA11021 GAD gene gradually increased at the range of substrate concentration[(0 to 60) g/L] and reached maximum when MSG concentration was 60 g/L. The initial sample without MSG was used as control. At the same time *L. brevis* SIIA11021 cell growth was significantly restrained when the substrate concentration was above 20 g/L. But there was a slightly increased about the cell growth when the substrate concentration was below 20 g/L. The *L. brevis* SIIA11021 resting cell activity of GABA production reached maximum when the substrate concentration was 40 g/L, stepping up when the substrate concentration was below 40 g/L and decreased above 40 g/L. The result showed that the *L. brevis* SIIA11021 resting cell conversion activity didn't absolutely fit up with the Glutamate decarboxylase expression quantity. Overall, 30 g/L of MSG concentration was added in cell growth medium for cell culture.

**Fed batch fermentation**

According to the above experiments, the fed batch fermentation has been proceeded using a 3 L beaker fermentor containing 1 L conversion buffer in optimal conditions for 24 h conversion. 100g MSG was added averagely in five times into the buffer. The final yield of GABA was 440 mM with a conversion rate of 82.3 %.

In present study, a lactic acid bacterium was successfully isolated that produced gamma-aminobutyric acid (GABA) from monosodium L-glutamate (L-MSG) at a hyper conversion rate. The 16s rDNA sequences analysis showed 99.8 % similarity with *L. brevis* ATCC 14687(T). Several LABs have been reported to show GABA-producing ability and *L. brevis* produced the highest amount of GABA (345.83 mM) [9, 22]. Most GABA-producing strains were isolated from traditional fermented foods such as kimchi [23, 24], cheese [14, 25], and paocai, etc [26]. It is clear that traditional fermented food stuffs are important isolation sources for screening GABA-producing LAB. Although many GABA-producing LAB strains have been isolated and identified, further isolation and characterization research is needed because LAB was classified as "food-grad" organisms [27], screening various types of GABA-producing LAB is important for the food industry [15].

Formerly, resting cells bioconversion was never used as a major way for GABA-producing. Normally, it was just proceeded as a recycle of growing cells fermentation. In current study, we used *L. brevis* SIIA11021 resting cells for GABA-producing. Although, there was study showing that, compared with growing cell bioconversion, resting cells resulted in both a lower yield and conversion rate in GABA production [21]. But in this study, besides shortened reaction times, non-sterile reaction conditions, convenient product separation, and fewer requirements for equipment and energy, resting cells biotransformation also has a hyper conversion rate and high GABA yield. Absolutely, resting cells biotransformation can be used as a new major way to produce GABA when the optimal conversion conditions have been controlled. Cell density controlled in resting cells biotransformation could supply a more stabilized conversion process. Because the optimal cell growth conditions do not fit the optimal GABA-synthesis conditions, a two-stage pH and temperature control strategy has been designed in growth cell biotransformation for GABA-producing [21]. However, when the bioconversion used resting cells, the conditions control has become simpler. To compare with immobilized cells, resting cells could also be re-used. But we didn't need more extra materials for immobilization and could avoid extra impurity in the production. To enhance the GABA production, several culture conditions have been optimized, such as pH and temperature which were considered as the common important factors for GABA production [28]. To the final optimize, pH of 4.7 and temperature of 30 $^{o}$C were chosen to be the two optimal culture conditions, which is almost in agreement with the findings in a previous study that utilized purified GAD from *L. brevis* TCCC13007 [23].

Ethanol is an ideal solvent about chemical permeabilization of cells, via destroying the structural and functional integrity of cytoplasmic membrane of cells [27]. It has been used successfully for permeabilizing yeast and LAB [27, 29, 30]. Because the structural and functional integrity of cytoplasmic membrane has been destroyed, the influx and efflux of small solutes became more easy, which may benefit to the biocatalytically process. George's study has showed that permeabilization of LAB cells could enhance β-galactosidase activity markedly [27]. In current study, the bioconversion buffer with different concentrations of ethanol has been used for GABA-producing. We find that the yield of GABA up to 2-fold to the initial yield when added 1 % (v/v) ethanol in the buffer. Increasing the addition of ethanol to 4 %, the result showed that the GABA yield decreased but still more than the initial yield. After further optimized, 1.32 % (v/v) of ethanol has been taken in the further fermentation.

The study of biotransformation by *L. brevis*TCCC13007 resting cells proposed a simplified Michaelis-Menten model [21]. In current study, when the substrate concentration below 140 mM, the bioconversion of GABA-producing by *L. brevis* SIIA11021 resting cells fit first order reaction. However, the GABA yield was almost constant, which the conversion process fit zero order reaction, when the substrate concentration over 140 mM. With

all optimal conditions, the reaction of GABA-producing by *L. brevis* SIIA11021 resting cells was almost stabilized after 3 h. Moreover, biotransformation of GABA-producing by *L. brevis* SIIA11021 resting cells could be repeated five times and biosynthesis activity didn't decrease markedly. To obtain optimal initial bioconversion resting cells of *L. brevis* SIIA11021, it has been found that the expression of glutamate decarboxylase gene of *L. brevis* SIIA11021 was induced by MSG and when the initial MSG concentration in the growth medium was 6 %, glutamate decarboxylase gene had the maximum expression quantity.

At last, a fed-batch fermentation, using a 3 L beaker fermentor, with optimal conditions, has been proceeded using resting cells biotransformation process. A GABA yield of 440 mM was achieved. Overall, this study lay the foundation for the new strategy for GABA-producing by resting cells.

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