PROCESS DEVELOPMENT FOR Γ-AMINOBUTYRIC ACID (GABA) PRODUCTION BY BIOTRANSFORMATION

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Abstract

Gamma-amino butyric acid (GABA), a neurotransmitter in humans' brains, has several physiological functions such as relieving anxiety, depression, and insomnia as well as having an anti-stress effect in humans. In recent years, the trend of adding GABA to food and drink products has been popular, especially in Japan; therefore, increased GABA production is required to support commercial demand. The objective of this study was to screen the GABA producing bacteria which could bio-transform glutamate into GABA via the glutamate decarboxylase (GAD) enzyme with whole cell reaction. Furthermore, the culture media and culture conditions that can promote high cell growth and GAD enzyme activity, as well as the process optimization, were investigated. The results showed that *L. brevis* had the highest GABA production capability (1.3 g/L). It was found that the GAD activity of the cell depended on growth as the activity was high during the mid-log and decreased during the stationary phase. The reaction was enhanced by increasing the temperature up to 40°C and adding Pyridoxal 5'-phosphate (PLP) coenzyme (the obtained productivity, final GABA concentration, and yield were 3.4 mM/h, 2.7 g GABA/L, and 0.94 mol GABA/mol glutamate, respectively). For the biotransformation reaction, it was found that the GAD activity dropped with reuse of the whole cell. The temperature at 30°C and pH in the range 3.0-5.0 were the optimal conditions for the GABA reaction. However, the GABA reaction with the cell recycling technique required a PLP addition every cycle. α-ketoglutarate was also tested as a substitute for PLP regeneration but it could not enter into the cell. The alternative process for GABA production was growth-associated production which could avoid cell death during cell reaction and showed better GABA production performance than that of cell reaction (5.1 mM/h, 16 g GABA /L, and 0.90 mol GABA/mol glutamate of yield, respectively).

Keywords: GABA, GAD, lactic acid bacteria, *L. brevis*

Introduction

γ-Aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature. It is a major inhibitory neurotransmitter in the central nervous system in the brain. In the medical field, GABA has been used to treat several diseases such as anxiety, depression, insomnia,

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and epilepsy. Besides the pharmaceutical product, GABA has been consumed widely in foods and beverages as it has an anti-stress effect. Due to the importance and benefits of GABA, the demand for GABA has increased. GABA-rich food which has been sought for supporting the increasing commercial demand includes milk, soybean, tempeh, gabaron tea, and red mold rice. Many types of tea have been reported as containing high GABA concentrations. A GABA content of 19.75 mg/100 g was found in tea (Syu *et al*., 2008) and 45.7 mg/100 g in GABA-enriched white tea (Zhao *et al*., 2011); however, the GABA concentration from natural sources is too low. Chemical and biological synthesis methods for GABA are a potential way of providing a supply. Diethyl cyanomalonate was used as starter material and changed into GABA with the multi-step chemical reaction (Cook *et al*., 2010). The preferred GABA production methods were biological synthesis methods because they are a simple, one-step reaction with a mild reaction condition and can be applied to the production of functional foods (Yang *et al*., 2008). GABA production methods have used the glutamate decarboxylase (GAD) enzyme from bacteria, plants and fungi. In recent years, the trend of GABA production through microbial biotransformation has focused on lactic acid bacteria (LAB). These bacteria have been widely researched and applied in the food industry, especially in fermented food. Several types of LAB have been reported including Lactobacillus plantarum (Ratanaburee *et al*., 2011), Lactobacillus casei (Minervini *et al*., 2009; Tsai *et al*., 2006), Lactococcus lactis (Coda *et al*., 2010), Leuconostoc mesenteroides and *Lactobacillus acidophilus* (Tsai *et al*., 2006), Lactobacillus paracasei (Komatsuzaki *et al*., 2008; Komatsuzaki *et al*., 2005; Siragusa *et al*., 2007), and *Lactobacillus brevis* (Kim *et al*., 2009). Due to the commercial demand, a GABA-producing strain with a high cell density is required as a cell factory. The culture condition was used as a medium with extra proteins. The optimal condition for GABA production was developed using inexpensive monosodium glutamate as a substrate and pyridoxal 5'-phosphate (PLP) coenzyme for the GABA-producing reaction, and the growth associated with the GABA-producing method was studied.

Materials and Methods

Chemicals and Reagents

Standard γ-Aminobutyric acid and pyridoxal 5´ phosphate was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Standard glutamate, monosodium glutamate, α-ketoglutarate, cupric sulfate ($CuSO_4 \bullet 5H_2O$), potassium dihydrogen phosphate (KH_2PO_4), dabsyl chloride, ninhydrin, phenyl methanesul fonyl fluoride (PMSF), acetic acid, acetone, 1-butanol, ethanol, hydrochloric acid, and methanol were purchased from Merck KGaA (Darmstadt, Germany). Glycerol, sodium acetate, sodium chloride, and sodium hydroxide were purchased from Scharlau Chemie, S.A. (Barcelona, Spain). 2-propanol and HPLC grade

Table 1. The composition of 5 modified MRS media for culturing *L. brevis*

Medium types	Composition (g/L)						
	Glucose	Trace element	Peptone	Meat extract	Yeast extract	Protein content	C:N
MRS	20	available	10	8	4	1X	8.6 : 2.6
iMRS	20	available	10	8	4b	1X	8.6 : 2.6
iMRS+1xY	20	available	-	-	25.05[b]	1X	8.6 : 2.6
iMRS+2xPYM	20	available	20	16	8[b]	2X	8.6 : 5.2
iMRS+2xY	20	available	-	-	50.1[b]	2X	8.6 : 5.2

Note: MRS, commercial MRS medium; iMRS, in-house prepared MRS medium; iMRS+1xY, MRS medium containing only yeast extract as nitrogen source at TN equivalent to MRS; iMRS+2xPYM, MRS medium with supplement of extra protein component in MRS medium (2×TN); iMRS+2xY, MRS medium with supplement of yeast extract (2×TN). N.B. b Bacto ® yeast extract was used with the in-house prepared MRS medium

acetonitrile were obtained from RCI Labscan Ltd. (Bangkok, Thailand). All standard chemicals and reagents used were analytical grades.

Microorganisms and Culture Condition

The 7 strains of LAB purchased from Thailand Institute of Scientific and Technological Research (TISTR) consisted of: *Lactobacillusparacasei 1463, Lactobacillus plantarum 050, Lactobacillus brevis 855, Lactococcus lactis 1401, Lactobacillus acidophilus 450, Lactobacillus casei 390, and Leuconostoc mesenteroides 053*.The culture was kept with 20% glycerol at 80°C in a 2 mL Eppendorf tube as a stock culture. The lactic acid bacteria were cultivated in de Man, Rogosa and Sharpe (MRS) medium (Merck KGaA). The inoculum used was a cell from frozen stock. The culture was cultivated at a shaking speed of 200 rpm at 30°C aerobic condition with 200 mL of cultivationmedia. At a different cell state, the culture broth was harvested by centrifugation at 5000 rpm for 15 min at 4°C. A normal saline solution was used to wash the wet cell 3 times and then the wet cell was adjusted from the optical density (660 nm) of cell concentration to 40 for GABA production.

The whole cell reaction for GAB A production The concentrated cell was incubated with monosodium glutamate (MSG) (24 g/L) and acetate buffer in the ratio 1:1:2 with a total reaction volume of 40 mL (the final MSG concentration for the reaction was 6 g/L). The reaction was performed in a flask shaken at 40°C at 200 rpm. The reaction temperature (25-60°C) and pH (3-5) were varied and are indicated in the legends for the Figures. The reaction was terminated by boiling at 95°C for 5 min. Then, the suspension was centrifuged at 10,000 rpm for 5 min. The supernatant was kept under -20°C before the GABA determination.

Modified MRS Medium Design

For the medium development in this study, modified MRS media of 5 formulae were tested including; MRS, iMRS, iMRS+1xY, iMRS+2xPYM, and iMRS+2xY. Each medium has been designed for an equivalent carbon and trace element concentration the same as the commercial MRS medium (Merck KGaA). Trace elements consisted of 2 g/L of di-potassium hydrogen phosphate, 2 g/L of di-ammonium hydrogen citrate, 5 g/L of sodium acetate, 0.2 g/L magnesium sulfate, 0.04 g/L of manganese sulfate, and 1 g/L of Tween 80. The composition was prepared as shown in Table 2.

Cell Disruption

The cell disruption was conducted by modification of the method of Hiraga (2008). *L. brevis* was the cultivation in the first MRS medium. The cell was harvested by centrifugation at 5000 rpm for 15 min at 4°C. The normal saline solution was used to wash the wet cell 3 times and then the wet cell was adjusted to the optical density (660nm) of cell concentration to 40 with a phosphate buffer of pH 7 containing protease inhibitors (EDTA and PMSF). The cells were disrupted by ultra-sonication at 4°C using a

Table 2. The comparison of performance of the resting cell reaction and growth-associated GABA production system

Factor	Resting cell reaction	Growth-associated production
Maximum GABA concentration (g/L)	2.67	16
Yield = $\frac{(mol\ product\ generate)/}{(mol\ substrate\ consume)}$	0.94	0.90
Yield = $\frac{(mol\ product\ generate)/}{(mol\ substrate\ add)}$	0.38	0.50
% Conversion	78	55
Specific productivity (g/L/h/OD_{660})	0.033	0.049

20 kHz sonicator (Sonoplus model HD 2200, BANDELIN electronic GmbH & Co. KG, Berlin, Germany), and then triton X-100 was added (1% of final concentration). The disrupted cell was left on ice for 1 h before being used.

GABA Derivatization

The sample (50 µL) was mixed with 50 µL of sodium carbonate (pH 9.0, 0.5 M) and 200 µL of dabsyl chloride (4 g/L) and then incubated at 70°C for 10 min. After that the reaction was stopped with an ice bath. Ethanol (250 µL) and monopotassium phosphate (KH_2PO_4, pH 6.8, 25mM, 250 µL) were added in the derivative solution, then the solution was filtered through a 0.45 µm cellulose acetate syringe filter and transferred to a vial before high-performance liquid chromatography (HPLC) analysis.

HPLC System

The GABA concentration in the derivative samples was measured by HPLC (Syu *et al*., 2008) with a spectrophotometric detector at 425 nm. The HPLC system consisted of a Waters 2695 separations module (Waters Corp, Milford, MA, USA), and a C-18 reversed-phase column Hypersil GOLD Thermo (Thermo Fisher Scientific, Inc., Waltham, MA, USA), with 150 mm × 4 mm i.d., 5 µm particle size, the column temperature was controlled at 30°C, the elution step was conducted with a 1 mL/min flow rate and 2 mobile phases. Mobile phase A was 99.9% of acetonitrile. Mobile phase B was 0.0045 M of acetate buffer pH4. The gradient elution profile was 30% of mobile phase A at 0-10 min. The gradient profiles were changed to 40% of mobile phase A at 11 – 20 min. and to 70% of

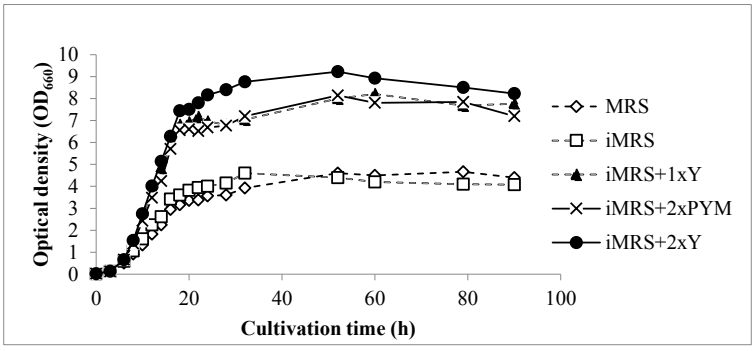

Figure 1. Growth curve of *L. brevis* in 5 designed media. Cell cultivation was carried out with 5 designed media, at a shaking speed of 200 rpm at 30°C

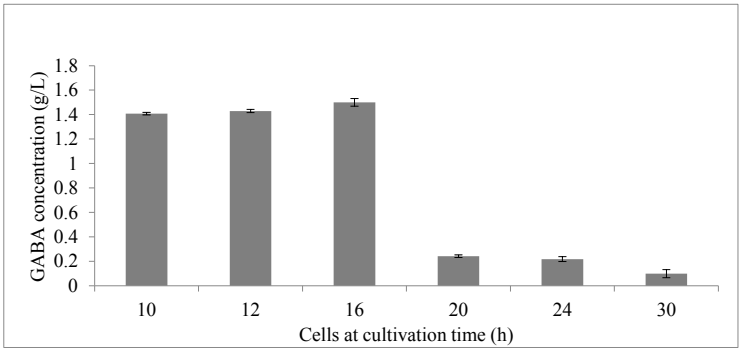

Figure 2. Effect of cell growth stage on GABA production. The reaction was carried out with a sodium acetate buffer of pH 4.4, 6 g/L of MSG, at 200 rpm shaking speed, and 18 h reaction time

mobile phase A at 21 – 25 min, respectively. The gradient profiles were changed back to 30% of mobile phase A at 26 - 35 min.

Results and Discussion

Screening of GABA-Producing Bacteria

The 7 strains of LAB purchased from TISTR were screened for their GABA-producing capability. GABA concentrations were found for all the tested LAB strains, *as follows: L. plantarum* (0.06 g/L), *L. casei* (0.01 g/L), *L.lactis* (0.01 g/L), *L. mesenteroides* (0.02 g/L), *L. acidophilus* (0.01 g/L), *L. paracasei* (0.03 g/L), and *L. brevis* (1.3 g/L). *L. brevis* was shown to have the highest GABA-production capability. The best GABA-producing strain, *L. brevis*, was further studied for cell growth. The maximum cell density reached OD660 of 4 after the stationary phase at 20 h in the growth curve (see Figure 1). In order to investigate the effect of the cell growth stage on the GABA-producing ability, *L. brevis* was grown in MRS broth at 30°C, at 200 rpm and then the cell was harvested at a different growth stage and employed in the GABA reaction. The effect of the cell growth stage is shown in Figure 2. In the GABA production, GAD enzyme activity is important. GAD enzyme activity changes during the different cell growth stages because it is an induced enzyme present in cytoplasm to resist acid and a low pH condition (Small and Waterman, 1998). The GABA concentration was relatively high in the log phase of the cell (10-20 h). The hydrogen ion accumulation is consumed by the decarboxylation reaction and generates GABA to control pH due to high adenosine triphosphate (ATP) synthesis for cell growth while GAD activity decreases after 20 h of the stationary phase. GAD enzyme activity could not be detected during the first 24 h and then increased to a maximum level at 60 h and then decreased (Huang *et al*., 2007b). Based on the results, *L. brevis* 855 was selected to be the GABA-producing microorganism for further study.

Optimization of Culture Media

Five designed media were used in cultivation for cell production, as shown in the growth curve (Figure 1). The maximum cell density (OD660) was 9.0 when using the iMRS+2xY medium with a supplement of yeast extract. The cell concentration obtained from MRS, iMRS, IMRS+1xY, and iMRS+2xPYM were 4.6, 4.6, 8.1, and 8.2, respectively. After cell cultivation, the wet cell at different cultivation times was harvested and used for the whole cell reaction of GABA production. The effect of the designed medium component on the GABA-producing capability at different growth stages is shown in Figure 3. Normally, the GABA production capability of L. brevis occurs in the log phase and drops after the stationary phase at 20 h with the MRS, iMRS, and iMRS+2xPYM culture media. However, 2 media (iMRS+1xY and MRS+2xY) can prolong the GABA production capability in the stationary phase. This would

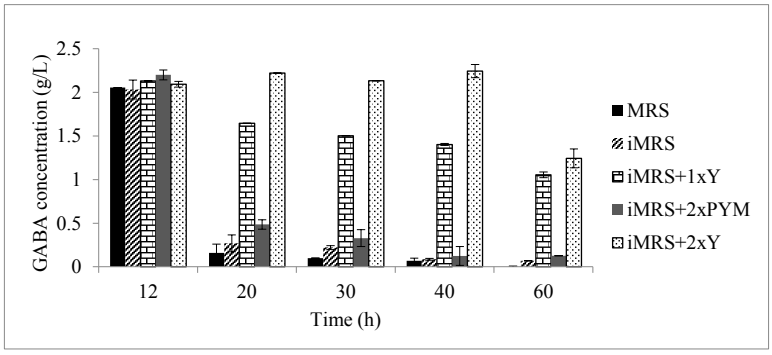

Figure 3. Effect of medium component on GABA production capability at different growth stages. Cell cultivation was carried out with 5 designed media

be due to the effect of protein (yeast extract) addition. Yeast extract is used by the health food industry as an inexpensive source of vitamins, and has long been recognized as a major source of B-complex vitamins consisting of all the B vitamins including PLP (vitamin B6). Based on the results, iMRS+2xY was selected to be the cultivation medium for high cell growth and GAD enzyme activity.

Effect of pH and Temperature on GABA Reaction

The GABA production process is catalyzed by the GAD enzyme which is affected by pH and temperature. The biochemical characteristic of the GAD enzyme is different among the microorganisms. The optimum pH and temperature of the purified GAD of *Lactobacillus brevis* CGMCC 1306 were 4.4 and 37°C, respectively (Huang *et al*., 2007a). The optimal pH 4.5 and temperature 40°C were the suitable conditions for GABA production with *S. salivarius subsp. thermophiles* (Yang *et al*., 2008). The effect of the pH of the GABA reaction on the initial velocity of the reaction is shown in Figure 4. The initial pH of the reaction mixture was adjusted to a different pH (3.0-6.0). The initial velocity remained constantly in the pH range from 3.0-5.0 and declined after pH 5.5. For the optimal temperature of the GABA reaction, it was observed that the initial velocity was enhanced when the temperature was increased from 25-40°C (Figure 4). The maximum initial velocity of the reaction was reached at 40°C. However, the initial velocity and GABA yield decreased with a further increase of the temperature. This suggested that the optimal pH and temperature for the GABA reaction are 3.0-5.0 and 40°C, respectively.

Whole Cell Reaction with Cell Recycle

The whole cell reaction with cell recycle was investigated by recycling the cell for the GABA bioconversion reaction, as shown in

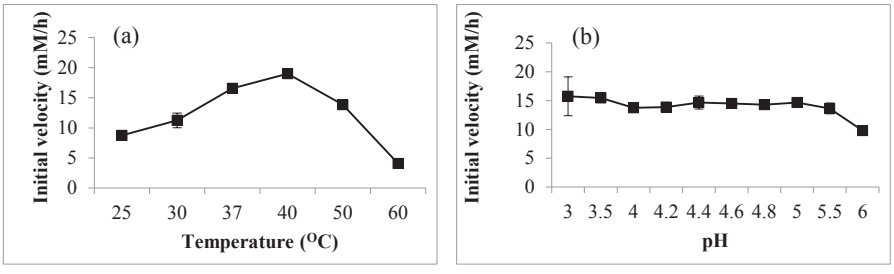

Figure 4. Effect of (a) temperature and (b) pH on initial velocity of GABA bioconversion by whole cell reaction. The reaction was carried out with a sodium acetate buffer, 6 g/L MSG, and at 200 rpm shaking speed

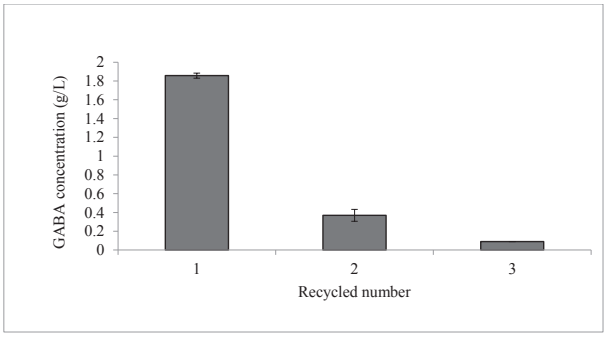

Figure 5. Effect of resting cell recycle number on GABA production of whole cell reaction. The reaction was carried out with a sodium acetate buffer pH 4.4, 6 g/L MSG, at 40°C, and at 200 rpm shaking speed for 18 h

Figure 5. The GABA concentration obtained from the third cycle largely dropped compared with the first cycle. This might be due to cell death during the GABA reaction (Figure 6). It shows that the number of viable cells drastically dropped from 3.2×10^9 to 1.6×10^2 cfu/mL during 18 h. The reaction temperature was considered to affect the physiological death of bacterial cells. The investigation of the physiological response of 4 bacteria strains with different reaction temperatures was determined by flow cytometry (Baatout *et al*., 2005). Physiological damage was observed with membrane permeability and potential, esterase activity, intracellular pH, and production of reactive oxygen species. The cell viability reduction after the GABA reaction at 3 temperatures (30, 37, and 40°C) is demonstrated in Figure 7. The death of the cell increased at high temperatures, especially at 37 and 40°C. Thus, the low temperature, i.e. at 30°C, was chosen for further study on the cell recycle technique.

Effect of Coenzyme Addition on GABA Production

Pyridoxal 5'-phosphate (PLP) is an important factor for the GAD enzyme playing an important role as a coenzyme. Many scientists have reported on its role (Blindermann *et al*., 1978; Huang *et al*., 2007a; Tsuchiya *et al*., 2003; Ueno *et al*., 1997; L. Wang *et al*., 2010; Zhang *et al*., 2007). According to the results of the experiment to investigate the effect of the resting cell recycle numbers on the whole cell reaction of GABA production, it was shown that the GABA concentration obtained from the third cycle largely dropped when compared with the first cycle (Figure 5) The effect of the

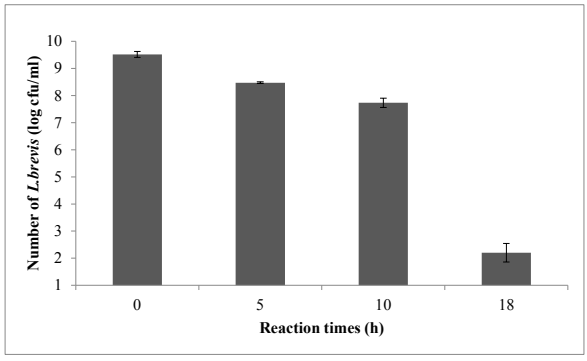

Figure 6. Viable cell count during GABA reaction of whole cell reaction. The reaction was carried out with a sodium acetate buffer pH 4.4, 6 g/L MSG, at 40°C, and at 200 rpm shaking speed

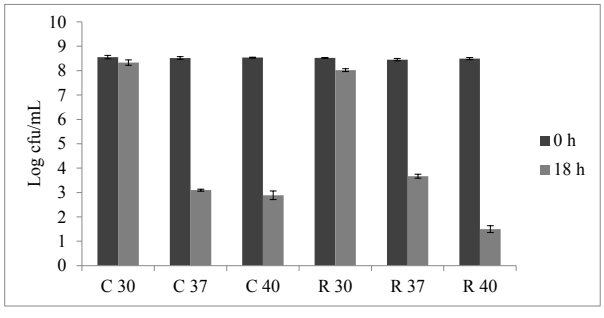

Figure 7. Effect of reaction temperature on viable cell count before and after the GABA reaction of the whole cell reaction. The reaction was carried out at 200 rpm shaking speed with a sodium acetate buffer of pH 4.4, 6 g/L MSG, and temperatures at 30°C, 37°C, and 40°C, respectively. (C is control (no reaction) and R is reaction)

coenzyme addition on the GABA production was investigated with PLP and α-kg, as shown in Figure 8. The result showed that only PLP could promote the GABA resting cell reaction. For further study, the effects of coenzyme addition with the whole cell and crude extract on GABA production are shown in Figure 9. The α-kg addition did not affect the reaction because it cannot enter into the cell which is in contrast with PLP. For retaining the reaction performance, coenzyme PLP is required for catalysis of the reaction. Every reaction cycle needed PLP to be added. This study agreed with the report by Wang (2011). The recombinant GAD was immobilized with sodium alginate and carrageenan. The activity of GAD was stable at 85% during 5 cycles and remained at 70% then dropped to 50% at 10 cycles with adding PLP at every reaction cycle. Moreover GAD has been reported for its auto deactivation mechanism (Almazov *et al*., 1985; Porter *et al*., 1985). Normally GAD requires PLP as a cofactor to help in the catalysis reaction. PLP will attach to the GAD at an active site to form an active form of enzyme. When the GAD converts glutamate to GABA at about 300,000 turnovers (Lammens *et al*., 2009; Spink *et al*., 1985), the side reaction occurred. PLP was lost from the active site and changed to pyridoxamine-5-phosphate (PMP) and then GAD became the inactive form. The regeneration of PLP from PMP can be done by transaminase reaction with α-ketoglutarate (α-kg) (Fenalti *et al*., 2007), however the α-kg addition could not improve the reaction due to it not being able to enter the cell and thus regenerate active PLP. The results show that the GABA production with the cell recycle still had problems from the cell death as well as the loss of PLP during the

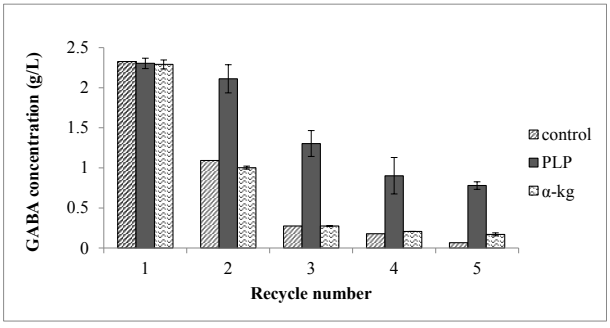

Figure 8. Effect of coenzyme addition on GABA production of the whole cell reaction. The reaction was carried out with a sodium acetate buffer of pH 4.4, 6 g/L MSG, at a temperature of 30°C, and at 200 rpm shaking speed. (Control: without coenzyme addition; PLP: 0.05 mM of PLP addition; and α-kg: 0.5 mM of α-kg addition)

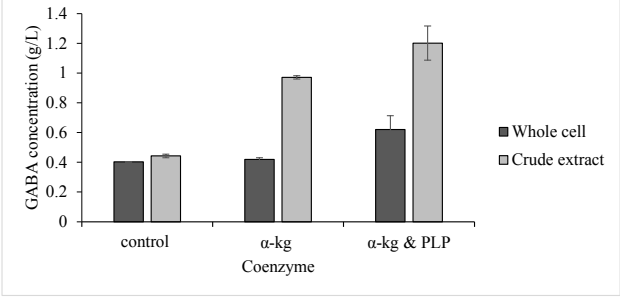

Figure 9. Effect of coenzyme addition on GABA production using the whole cell and crude extract. The reaction was carried out with a sodium acetate buffer of pH 4.4, 6 g/L MSG, at a temperature of 30°C, and at 200 rpm shaking speed for 18 h

reaction. So, growth-associated GABA production was further applied to avoid these problems.

Growth Associated GABA Production

As discussed in previous results, the disadvantage of the whole cell reaction was cell death and GAD deactivation. According to the reports on GABA production with growth-associated production (Cho *et al*., 2007; Kim *et al*., 2009; Komatsuzaki *et al*., 2005; Li *et al*., 2010), it was shown that a higher GABA concentration could be obtained in growth-associated GABA production than that in the resting cell reaction. Growth-associated GABA production was further investigated in this study. The growth-associated GABA production of *L. brevis* is shown in Figure 10. The effect of the substrate concentration on the growth of *L. brevis* is shown in Figure 10(a). The substrate (MSG) at high concentration up to 40 g/L was shown to have no inhibitive effect on cell growth and helped to maintain the pH inside the cell as well as the culture broth pH (Figure 10(b)). *L. brevis* metabolized sugars to produce organic acid which caused a decrease of the culture broth pH from 6.5 to 5.2 at 0 – 12 h. In an acidic environment, there was reduced cytoplasmic pH which induced loss of activity of the acid-sensitive enzyme and structural damage to the protein and cell membrane and then led to cell death. The negative impact of acid-sensitive glycolytic enzymes was reported to affect ATP production (Cotter and Hill, 2003). L. brevis can neutralize the pH in cytoplasm. The pH of the culture broth was changed from 5.2, to 6.0, 7.1, and 7.4 with MSG as the substrate at concentrations of 12, 20, and 40 g/L, respectively. The result in this study agreed with the report by Li *et al*. (2010) that the GAD system of *L. brevis* NCL912 acted under low pH and resulted in an increase of pH in the medium with glutamate, and maintained cell survival from the acidic condition. The time course of GABA production during cell cultivation is shown in Figure 10(c). The maximum GABA concentration was 16 g/L when adding a substrate of 40 g/L. The final product concentration and yield depended on the strains and is affected by cultivation conditions such as the medium composition

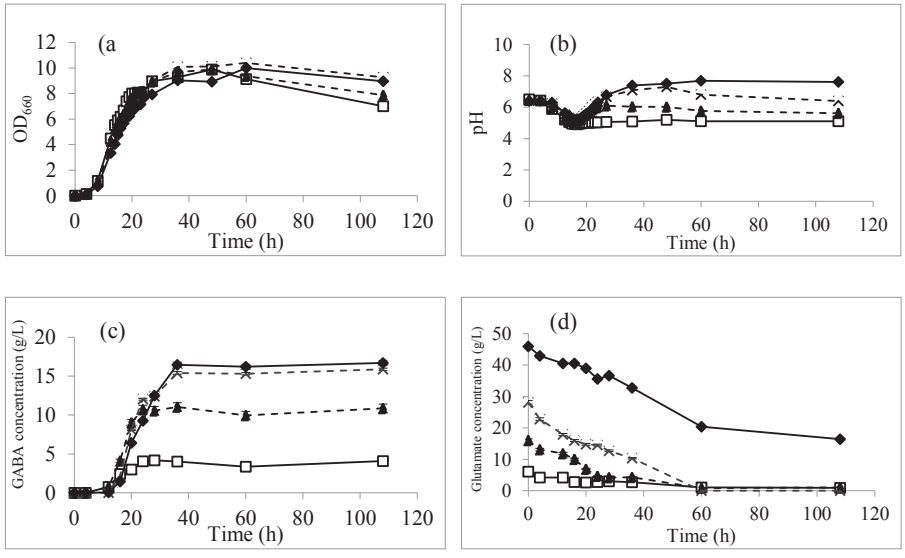

Figure 10. Effect of substrate concentration on (a) cell growth, (b) pH profile, (c) GABA production, and (d) substrate concentration during cell cultivation which was carried out with 3 additional substrate concentrations, a shaking speed of 200 rpm, at 30^{O}C. (□ : control; ▲ : 12 g/L; ×: 20 g/L; and ◆: 40 g/L)

(glucose, glutamate, soya peptone, Tween 80, and $MnSO_4 \bullet 4H_2O$) (Di Cagno *et al*., 2010; Huang *et al*., 2007b; Kim *et al*., 2009; Komatsuzaki *et al*., 2005; Li *et al*., 2010; Lu *et al*., 2008; Park and Oh, 2007). The GABA production was shown to be enhanced with increasing the MSG concentration (0 to 40 g/L). GABA occurred during the middle exponential phase (12 to 36 h) and then the GABA concentration was maintained while the substrate was gradually decreased, as shown in Figure 10(d). After 36 h, the GABA had accumulated inside the cell because the GABA transport system was closed due to the increase of the culture pH to more than 6.5. The transport of the GABA/ glutamate works under an acid condition and is inactive at a pH value higher than 6.5 (Ma *et al*., 2012). Therefore, the GABA concentration was constant after 36 h while the substrate continued to be consumed through the other transportation of the glutamate (Fernández and Zúñiga, 2006). Performance of the whole cell reaction and growth-associated GABA production system were compared for the final GABA concentration, yield, % conversion, and specific productivity (Table 2). In addition, the problems (cell death and GAD deactivation) in the resting cell reaction were lowered by the growth-associated GABA production in which the new cell was generated by growth during GABA production. Growth-associated GABA production could promote the construction of α-kg within the cell affecting PLP regeneration because the iMRS+2Y medium provided factors that L.brevis required. Then, PLP could be recycled in the reaction again. The advantage of growth-associated GABA production over the whole cell reaction mentioned above led to a better performance for GABA concentration and productivity and, meanwhile, the product yield was relatively constant.

Conclusion

In this study, potential GABA-producing bacteria were screened from the lactic acid culture collection. *L.brevis* 855 showed the highest GABA production capability when cultivated with MRS broth. The GABA production ability of the cell was changed during the different cell growth stages. The GABA yield was extremely high in the log phase and then decreased sharply during the stationary phase. The designed media, iMRS+1xY and iMRS+2xY, can maintain the GABA production capability in the stationary phase and an increased cell concentration. The optimal pH and temperature for the GABA reaction are 3.0-5.0 and 40OC, respectively. The temperature and starvation damaged the cell and caused cell death during resting of the cell recycle on the GABA production. A low temperature (30°C) could maintain cell survival. The cofactor (PLP) is important for GABA production with cell recycling. For a continuous reaction, PLP had to be added every reaction cycle because of the inactive form of PLP (PMP). In order to avoid the expense of PLP, the growth-associated GABA production was used as an alternative method which showed the high final product concentration and yield.

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