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Biosynthesis of Polyamide 4, a Biobased and Biodegradable Polymer

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Polyamide 4, which is composed of repeating unit of γ-aminobutyric acid (GABA), is a biobased and biodegradable polymer since it can be synthesized from renewable material instead of fossil-based material. GABA is produced by decarboxylation of glutamate (Glu) using glutamate decarboxylase (GAD: EC 4.1.1.15), which is produced by some microorganisms. In this study, enzymatic conversion of GABA from glutamate by *Lactococcus lactis* and *Escherichia coli* cell and chemical polymerization of GABA to polyamide 4 were revealed. The results show that GAD activity of *E. coli* was higher than that of *L. lactis*. The treatment of *E. coli* cell by heating and sonication increased the GAD activity and conversion rate of glutamate to GABA was up to 70.5%. The optimum temperature for this conversion is 37ºC. On the other hand, chemical synthesis of polyamide 4 was catalyzed by heating GABA at 215ºC for 2 minutes.

Key words: polyamide 4, biodegradable polymer, γ-aminobutyric acid, glutamate decarboxylase

Much fossil fuel and its related resources have been and are being consumed to produce many kinds of products for human life. However, future reliance on fossil fuels has been questioned due to emerging concerns about greenhouse gas (GHG) emissions, particularly carbon dioxide (CO_2) and its potential contribution to global climate change (GCC) (Hansen *et al*. 2000; Woodruff *et al*. 2006). Recent study predicted that by 2100 the global average surface air temperature will be 1.4-5.8°C higher than in 1961-1990. For that reason, it is important to take into account full implementation of plans to reduce fossil fuel use (Hebert 2005).

Consequently, there is a need to develop a bio-based product by using renewable materials as an alternative material to substitute fossil-based products. The one of bio-based products is biopolymers. Utilization of biopolymers not only reduces the consumption of fossil-based material but also solves the disposal problem due to non-degradable petroleum based polymer as means of reducing the environment impact. Furthermore, the exploitation of abundant biomass resources to develop biopolymer is one of the solutions of solid waste accumulation problem. Representatives of biobased-biodegradable polymers are poly D-β-hydroxybutylate (PHB), poly L-lactide (PLA), well known as biodegradable plastic (Tokiwa and Calabia 2006) and poly amino acid (Obst and Steinbuchel 2004).

The other biobased-biodegradable polymer which has different structure is polyamide 4 also well known as nylon 4. Polyamide 4 is composed of repeating unit of γ-aminobutyric acid (GABA). Kawasaki *et al*. 2005 reported that polyamide 4 can be biodegraded in an activated sludge. On the other hand, polyamide 4 has good prospect as a novel bio-based polymer because it is synthesized from 2-pyrrolidone, a lactam of GABA, which can be made by means of decarboxylation of glutamate. This synthesis was catalyzed by glutamate decarboxylase (GAD). The synthetic route for biodegradable polyamide 4 was shown in Fig 1. At first, biomass is saccharified to produce glucose. Then it was fermented by coryneform bacteria (eg: *Corynebacterium glutamicum*) to produce glutamate. Using microbial GAD, the glutamate was decarboxylated to become GABA. Finaly, the monomer unit of GABA was heated to produce 2-pyrrolidone and chemically polymerized to produce polyamide 4.

This paper described the study on biosynthesis of polyamide 4. It covers the synthesis of GABA using microbial GAD of *Lactococcus lactis* and *Escherichia coli* under different conditions and the chemical synthesis of polyamide 4 from GABA.

MATERIALS AND METHODS

Bacterial Strain. A wild type *L. lactis* NBRC 100933 and *E. coli* NBRC 3806 were obtained from National Institute of Technology and Evaluation-Biological Resource Center (NBRC), Japan. *L. lactis* NBRC 100933 was grown in medium no. 310 containing 10 g l^{-1} peptone, 10 g l^{-1}meat extract, 5 g l^{-1} yeast extract, 20 g l^{-1} glucose, 1 g l^{-1} Tween 80, 2 g l^{-1} K_2HPO_4, 5 g l^{-1} CH_3COONa, 2 g l^{-1} $C_6H_{14}N_2O_7$, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.05 g l^{-1} $MnSO_4 \cdot nH_2O$, pH 6.0-6.5. *E. coli* NBRC 3806 was grown in medium no. 802, which contains 10 g l^{-1} polypepton, 2 g l^{-1} yeast extract, 1 g l^{-1} $MgSO_4 \cdot 7H_2O$, diluted in distilled water.

Rapid Glutamate Decarboxylase (GAD) Assay. One ml of seed culture of *L. lactis* NBRC 100933 growing in medium no. 310 was transferred to 50 ml of TYGG medium (5 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} glucose and 10 mM glutamic acid) in 300 ml flask and incubated for 24 h at 30°C in a shaker on 180 rpm. The cells were harvested by centrifugation at 1 800 xg for 20 min at 4°C and washed once with PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.3). GAD activity assay was carried out using GAD reagent (Rice *et al*.1993). The GAD reagent consisted of 1 g l^{-1} L-glutamic acid, 0.05 g l^{-1} bromocresol green (colorimetric indicator), 90 g l^{-1} of NaCl and 3 ml of TritonX-100 per liter. After the wash step, the cells were transferred to a test tube, 1 ml of

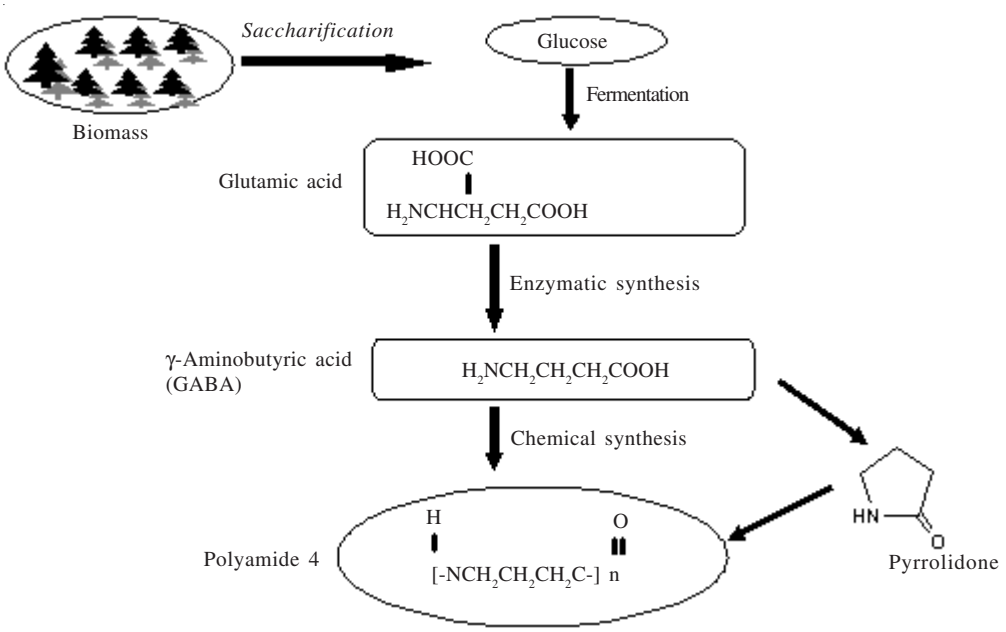

Fig 1 The biosynthetic route of polyamide 4 synthesis, a biobased and biodegradable polymer.

GAD reagent was added, mixed immediately, and vortexed vigorously for 30s. The tube was then incubated in a water bath at 35°C and observed hourly for 4 h. A distinct change from yellow to blue was considered as a positive response of GAD activity. The similar method for GAD activity assay was also applied for of *E. coli* NBRC 3806.

GAD Activity Assay. The determination of GAD activity assay of *L. lactis* NBRC 100933 was referred to the method of Nomura *et al*. (1999) with minor modification. The cell suspension of *L. lactis* NBRC 100933 was frozen at -50°C and lyophilized. For the enzyme assay, the cell powder was suspended in 1 ml of mM sodium acetate buffer (pH 4.7). The reaction mixture for GAD activity assay consisted of 50 µl of 4 mM glutamate in 0.1 M sodium acetate buffer (pH 4.7), 25 µl of 0.4 mM pyridoxal phosphate (PLP), 10 µl cell powder suspension and 15 µl of distillated water. The reaction was carried out for 8 h at 30°C. One katal of GAD was defined as the amount of enzyme required to produce 1 mol GABA s-1 at 30°C and pH 4.7.

The cell preparation of *E. coli* NBRC 3806 and measurement of its GAD activity were carried as refered by Plokhov *et al*. (2000). *E. coli* NBRC 3806 was grown in a □□□□□□ □□□□□□□□□□ 10.0 □ □□ glucose, 0.2 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$, 1.0 g l^{-1} (NH_4) Cl and 20 g l^{-1} peptone in distillated water (pH 7). After incubation for 48 h, the cell was harvested by centrifugation at 40 000 xg for 20 min at 4°C. The obtained wet cell biomass was frozen at -20°C. Five milligrams of wet cells were introduced to 12.5 ml 0.9% NaCl and stirred with a magnetic stirrer for 15 min. This suspension was then divided into 2 equal portions. The first portion was incubated for at 53°C for 45 min and sonication at 1 min, while the second portion was not treated by heating and sonicated. Furthermore, both portions were centrifuged at 5 000 xg for 10 min. The pellets finally were diluted in 2.5 ml 50 mM sodium acetate buffer (pH 4.7). These solutions were used for GAD activity assay.

The reaction mixture for GAD activity assay of *E. coli* NBRC 3806 consisted of 50 µl 4 mM glutamate in 0.1 M sodium acetate buffer (pH 4.7), 25 µl 0.4 mM pyridoxal phosphate (PLP), 10 µl enzyme solution as prepared above and 15 ml of distilled water. The reaction was carried out at various incubation times at 30°C and terminated by the addition of 30µl 30% NaOH solution. The enzyme activity was expressed in units. One unit was defined as the amount of enzyme required to produce 1 µmole GABA in 1 min at 37°C. GAD specific activity was expressed in U mg^{-1} cell (wet weight).

GABA Analysis by High Performance Liquid Chromatography (HPLC). Standard sample contained 2.5 µM and 5 µM of Glu-GABA. A 20 µl of the sample was dried using vacuum system. Then, it was added with 20 µl TEA solution (ethanol:H_2O:triethylamine=2:2:1) and evaporated by vacuum system for 10 min. The dried sample then was added by 20 µl of PITC solution (ethanol:H_2O:TEA:PITC = 7:1:1:1) and vortexed for 20 min at 25°C. Furthermore, the sample was dried using vacuum system and dissolved in 200 µl phase A solution (6% acetronitrile in 60 mM sodium acetate buffer pH 6.0). The solution was then filtered using 0.45 µm filter.

HPLC was performed using reverse-phase column Wakosil PTC (200 × 4.0 mm) at 1 ml min^{-1} flow rate. Two mobile phase were employed, 6% acetronitrile in 60 mM sodium acetate buffer at pH 6.0 and 60% acetronitrile. Detection was conducted at ultra violet spectrum (λ = 254 nm).

Preparation of Pyrrolidone from GABA. 2-Pyrrolidone, a monomer of polyamide 4 is a lactam that is produced by the cyclization of GABA. It is synthesized by dehydration of GABA. A sum of 10 mmol GABA were placed in round-bottom flask equipped with magnetic stirrer. The GABA was then heated at high temperature on oil bath under inert atmosphere until gas-bubbling stops. The products were analyzed with the Jeol ECA-500 NMR spectrometer in D2O solution.

Polymerization of Pyrrolidone. The method of polymerization of pyrrolidone was referred to Kawasaki *et al*. (2005). A mixture of 2-Pyrrolidone (17 g, 200 mmol) and sodium (138 mg, 6 mmol) were put in round-bottom flask equipped with magnetic stirrer. The mixture was heated to 50°C using oil bath under reduced pressure. After the sodium was reacted completely with 2-pyrrolidone, sebacoyl chloride (583.5 mg, 1.5 mmol) was added into the flask and was maintained at 50°C under reduced pressure for about 3 h. The polymerization mixture was dissolved in formic acid and precipitated in acetone followed by washing with water and with methanol. NMR spectrum was obtained from chloroform/ formic acid solution (95/9%) (v/v).

RESULTS

Production of GABA by Microbial GAD of *Lactococcus lactis* and *Escherichia coli*. The results of the rapid GAD activity assay of *L. lactis* and *E. coli* was shown in Fig 2. The tube containing *E. coli* cell in GAD reagent show a darker blue color, suggesting that *E. coli* has higher GAD activity than that of *L. lactis*. GAD activity of *E. coli* NBRC 3806 cells with heating and sonication treatment was 4.15 U mg^{-1} cell, (wet weight) and the GAD activity without treatment was 3.81 U mg^{-1} cell (wet weight).

Furthermore, we attempted to synthesize GABA using *L. lactis* and *E.coli* on medium TYG. The results showed that after 16 h incubation the production of GABA using *E. coli* (678.1 mg l^{-1}) was higher than that using *L. lactis* (178.8 mg l^{-1}) (Fig 3). This result seems to be corresponding with the previous result of rapid GAD activity assay. However, the addition of 10 mM glutamate on TYG medium had no effect in enhancing GABA production. On the other hand, the rate and level of *E. coli* grown on TYG medium was also higher than those of *L. lactis* (Fig 4).

To optimize the enzymatic production of GABA by GAD of *E. coli*, it is important to reveal the substrate specificity of the enzyme. Fig 5 shows the conversion of various glutamate-containing substrates to GABA by *E. coli*. The result shows that the highest conversion (70.5%) is obtained using L-glutamic acid. The lowest conversion (3.82%) was obtained on L-glutamate HCl substrate.

The effect of temperature on the reaction rate to produce GABA was also studied. The result showed that the reaction

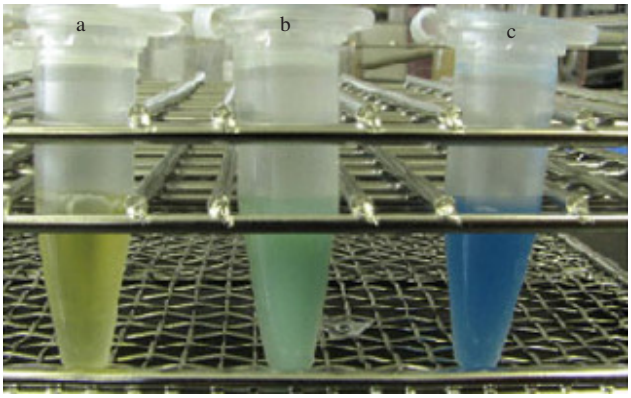

Fig 2 Rapid GAD assay. a, GAD reagent as a control; b, GAD reagent with *Lactococcus lactis* cell and c, GAD reagent with *Escherichia coli* cell.

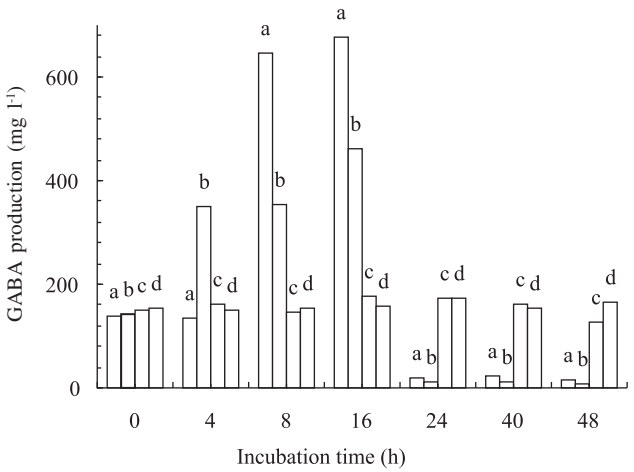

Fig 3 GABA production by *Lactococcus lactis* and *Escherichia coli* on TYG medium with addition of 10 mM glutamate. a, *E. coli* on TYG medium; b, *E. coli* on TYG medium with addition of 10mM glutamate; c, *L. lactis* on TYG medium; d, *L. lactis* on TYG medium with addition of 10 mM glutamate.

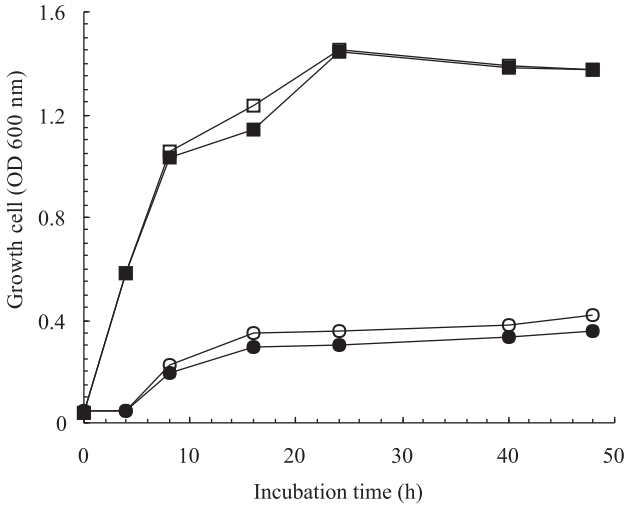

Fig 4 Cell growth of *Lactococcus lactis* and *Escherichia coli* on TYG medium with addition of 10 mM Glutamate. ■, *E. coli* on TYG medium; □, *E. coli* on TYG medium with addition of 10 mM Glutamate; ●, *L. lactis* on TYG medium; ○, *L. lactis* on TYG medium with addition of 10 mM glutamate.

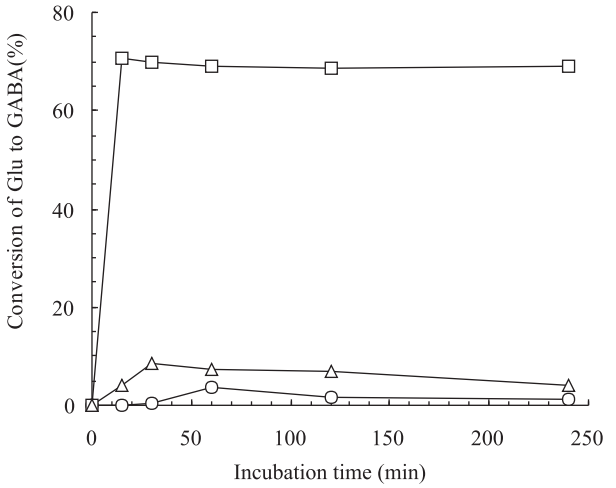

Fig 5 Glutamate-containing substrate specificity of GAD of *Escherichia coli*. -○-, L-Glu; -□-, L-Glu HCl; -△-, Na L-Glu.

rate on incubation at 30°C, 37°C and 50°C were similar. On the other hand, the conversion rate of glutamate to GABA was decreased when the reaction was carried out at 70°C (Fig 6).

Chemical Synthesis of Polyamide 4. The chemical synthesis of polyamide 4 was initiated by preparation of pyrrolidone, a monomer unit of polyamide 4. The gas-bubbling in a flask containing GABA stopped when it was heated on 215°C for 2 min. Fig 7 shows 1H NMR spectra of the product and GABA. The spectrum displays characteristics signal for the methylene protons (b, c, a) of GABA at chemical shifts of 1.8, 2.2 and 2.9 ppm (Fig 7a), and characteristics signal for the methylene protons (b, c, a) of pyrrolidone at chemical shifts of 2.1, 2.3 and 3.3 ppm (Fig 7b).

Furthermore, the crude polyamide 2 was washed with methanol and applied in 1H NMR analysis. The spectra illustrate that washing the crude polyamide 4 with methanol effectively removes the remaining pyrrolidone (Fig 8).

DISCUSSION

GABA is an important ubiquitous non-protein amino acid in both prokaryotic and eukaryotic organisms and is

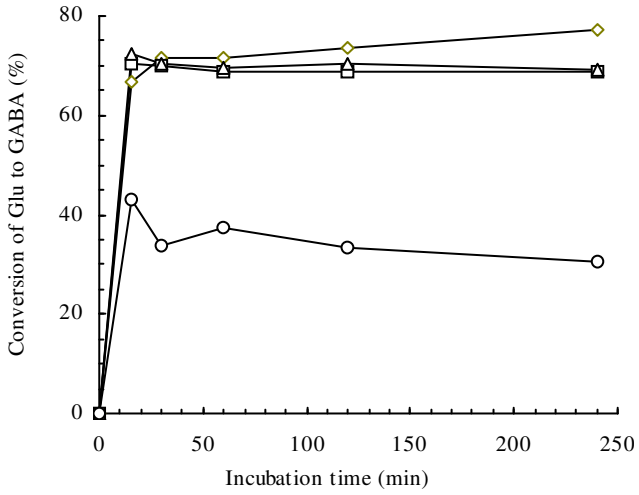

Fig 6 Effect of temperature on the conversion of glutamate to GABA. ◇, 30°C; □, 37°C; △, 50°C; ○, 70°C.

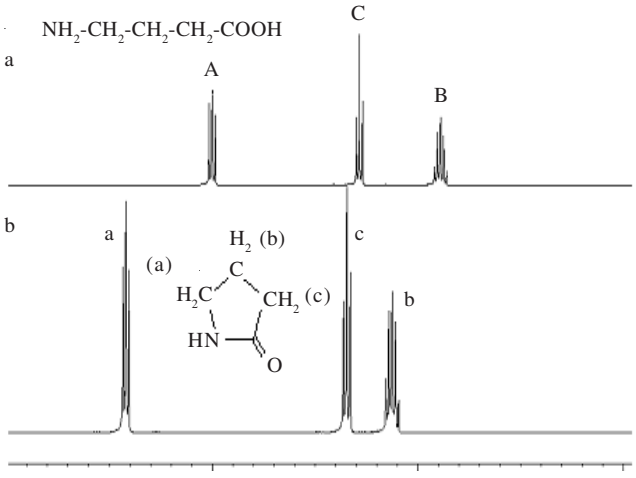

Fig 7 ^{1}H NMR spectra analysis of GABA (a) and pyrrolidone (b) as the results of heating of GABA.

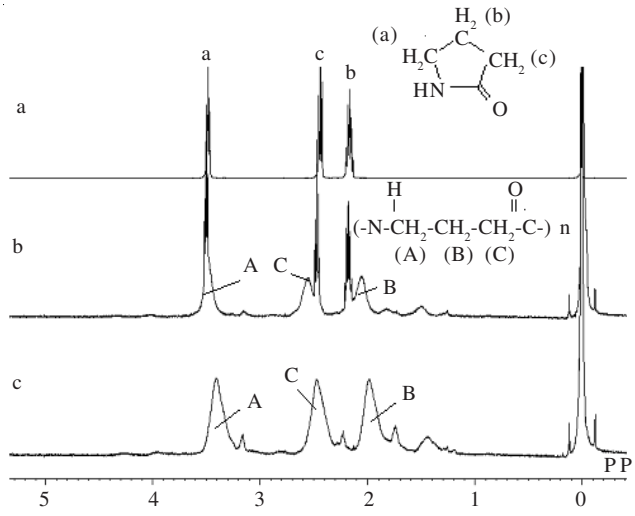

Fig 8 ^{1}H NMR spectra analysis of pyrrolidone (a), crude polyamide 4 (b) and polyamide 4 after washing with methanol (c).

produced by GAD (EC: 4.1.1.15) from glutamic acid. GABA is representative depressive neurotransmitter in the sympathetic nervous system and has been proved to be effective for lowering the blood pressure of experimental animals and humans. Furthermore, GABA-enriched food is also used as dietary supplement and nutraceutical to help treat sleeplessness, depression and autonomic disorders, chronic alcohol-related symptoms, and to stimulate immune □□□□□ (□□□□□ □□ *al*. 2006). Many studies were conducted to produce GABA especially for the nutraceutical purpose (Nomura *et al*. 1998; Kono and Himeno 2000; Aoki *et al*. 2003; Park *et al*. 2004; Park and Oh 2006).

It is very important to develop the method to produce GABA in massive production. The utilization of microbial GAD in transformation of glutamic acid to GABA seems to be promising method in the future. A lactic acid bacterium is well known in producing GAD that is used in cheese industry to increase the content of GABA in cheese production. However, in this study, *E. coli* had higher activity of GAD than that of *L. lactis* when glutamic acid was used as a substrate. GAD is an intracellular enzyme and for that reason the increase of the cell wall permeability controls the substrate access to the enzyme. For that reasons, it seems that the heating and sonication treatment affecting in the cell wall permeability is a one method for increasing the GAD activity of *E. coli* as shown in the results of this study.

On the other hand, cyclization of GABA to produce pyrrolidone a monomer unit of polyamide 4 and polymerization of pyrrolidone is also play important role in biosynthesis of polyamide 4. In this study, heating of GABA on 215°C for 2 min is appropriate for cyclization of GABA. Furthermore, the chemical polymerization of pyrrolidone was successfully achieved using sebacoyl chloride (0.75 mol %) as initiator reaction, followed by washing the crude polyamide 4 with methanol.

This study clearly demonstrated that polyamide 4, the biobased and biodegradable polymer can be biochemically synthesized. This material has a good prospect in the future since the development of bio-based product by using renewable materials as an alternative material to substitute fossil-based products is needed.

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