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Improvement of γ-Aminobutyric Acid (GABA) Production Using Cell Entrapment of *Lactobacillus brevis* GABA 057

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Abstract GABA (γ-aminobutyric acid) is the principal inhibitory neurotransmitter in the brain. For the efficient production of GABA, a semicontinuous cell entrapment system using *Lactobacillus brevis* GABA 057 was optimized, including the substrate concentration, bead-stabilizing additives, and reaction conditions. The converted monosodium glutamate (MSG), which was added as a substrate for glutamic acid decarboxylase (GAD), increased from 2% (w/v) to 12% (w/v). The addition of isomaltooligosaccharide to the alginate beads also increased the stability of the entrapped *L. brevis* and GABA productivity. Consequently, when optimal conditions were applied, up to 223 mM GABA could be produced from 534 mM MSG after 48 h of reaction by using alginate-bead-encapsulated *L. brevis* GABA 057.

Key words: γ-Aminobutyric acid (GABA), entrapment, *Lactobacillus brevis* GABA 057, monosodium glutamate (MSG)

γ-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the mammalian central nervous system, is also widely distributed in nonneural tissue, including the peripheral nervous and endocrine systems [19]. Both in neural and nonneural tissue, GABA is metabolized by three enzymes: glutamic acid decarboxylase (GAD. E.C. 4.1.1.15), which produces GABA from glutamic acid, and the catabolic enzymes GABA-transaminase (GABA-T, E.C.2.6.1.19) and succinic semialdehyde dehydrogenase (SSADH, E.C.1.2.1.24). In particular, glutamate decarboxylase (GAD) with molecular sizes of 65 and 67 kDa catalyzes α-decarboxylation of L-glutamate, producing GABA [2, 21, 22]. It may also play a variety of physiological functions in nonneural tissues, including neurotransmission, the induction of hypotensive effect, diuretic effect, and tranquilizer effect; particularly with regard to sleeplessness, depression, and autonomic disorders observed during menopausal or presenium periods [12, 13]. In addition, GABA has been implicated in the regulation of neurological disorders, including seizures, Parkinson's disease, Huntington's chorea, and Alzheimer's disease [18].

Recently, increased interest has been focused on the utilization and mass production of GABA as a bioactive component for food. Yet, GABA levels are low in plants, and the role of GABA in plants remains a matter of controversy. GABA green tea, Gabaron tea, and red mold rice have all been reported to exert antihypertensive effects in human [8, 13, 14, 18, 20]. Research to increase the GABA production yield via culturing of lactic acid bacteria (LAB) with MSG has been conducted [4, 6]. In a previous study [3, 11], a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbreuckii*, isolated from commercially available yoghurt, produced a large amount of GABA. *L. plantarum*, *Enterococcus casseliflavus*, and *S. thermophilus* were also observed to produce GABA in culture filtrates, suggesting that lactobacilli-fermented extracts constitute a potentially rich GABA source. Indeed, Kim *et al.* used kimchi to isolate *L. hilgardii* K-3, which was found to produce a large amount of GABA, and Kook *et al.* [10] also used kimchi to isolate *L. sakei* B2-16 and attempted to obtain an optimal medium composition for the mass production of GABA. However, the optimal process and culture conditions for the production of GABA remain to be improved.

In this study, we have established a set of optimal conditions for the mass production of GABA by *Lactobacillus brevis* GABA 057 as a GABA-producing organism, using a sodium alginate entrapment system.

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MATERIALS AND METHODS

Strains and Culture Conditions

The *Lactobacillus brevis* GABA 057 was obtained from Bifido, Inc. (Seoul, Korea) and subcultured at 37°C for 18 h in a glucose-yeast extract polypeptone (GYP) medium [1% glucose, 1% yeast extract, 0.5% polypeptone, 0.2% sodium acetate, 20 ppm $MgSO_4 \cdot 7H_2O$, 1 ppm $MnSO_4 \cdot 4H_2O$, 1 ppm $FeSO_4 \cdot 7H_2O$, 1 ppm NaCl (pH 6.8)] under anaerobic conditions (Forma Science Inc., U.S.A.), in accordance with the specifications of Yokoyama *et al.* [23].

Cell Entrapment

The *L. brevis* GABA 057 was routinely prepared by incubating in an anaerobic system. After high-density cultivation, the strain was centrifuged at 1,500 ×*g* for 15 min, and the cell pellets were resuspended in 0.85% (w/v) saline solution. The cell suspension was then transferred to a sterile disposable bag (Seward Co., U.K.) containing 3% sodium alginate (100 ml) (Sigma Co., U.S.A.), which had been sterilized at 85°C for 30 min. The solution was homogenized using a laboratory blender (Stomacher 400, Seward Co., U.K.), and then dropped into a sterile 0.1 M $CaCl_2$ solution through a screw-pumping system (SPS), consisting of a screw, 37 multi-nozzles (18G needle), and air compressor (2.3 kg/cm^2) jointed with 5 μm, 1 μm, and 0.5 μm air filters. The beads were then gently stirred with a magnetic stirrer, hardened for 8 h in the solution, and recovered with a sieve. They were then finally washed with sterile saline prior to removal of any excess calcium ions and free cells. As a result, uniform and spherical 3% sodium alginate beads with a mean diameter (D_m) of about 2 mm were prepared without tails.

Entrapped Cell Incubation

The encapsulated beads of *L. brevis* GABA 057 50% (v/v) were added to 200 ml of GYP medium (pH 4.5) containing an appropriate amount of MSG, and incubated at 37°C for 48 h. To remove the CO_2 formed during the culture period, the culture medium was stirred at 150 rpm using an impeller. The pH of the culture media was maintained at 4.5 using 10 N HCl solution every 12 h.

Determination of Medium Additives

To compare the GABA productivity according to the medium additives, a GYP medium was prepared with glucose (50 mM or 100 mM), maltose (100 mM), ammonium sulfate (10 mM), and ammonium sulfate (5 mM), respectively. To each medium, the entrapped *L. brevis* GABA 057 and 5% of MSG were added and incubated at 37°C for 48 h.

Determination of Bead Additives

One-tenth percent of skim milk, isomaltooligosaccharide, erythritol, and pectin were added to the *L. brevis* GABA 057 alginate beads, and the GABA productivity was tested in a GYP medium containing 10% MSG, at 37°C for 48 h.

Determination of Initial pH

Each medium containing 10% MSG was tested for the production of GABA according to an initial pH of 3.5, 3.7, 4.0, 4.2, and 4.5, respectively.

Determination of Culture Temperature

The MSG concentration was increased to 12%, and the pH of the culture medium was maintained at the optimal value of 4.2. To determine the effects of temperature on the GABA production, culture temperatures of 28, 30, 32, 34, and 37°C were maintained, respectively.

Survival Rate of Encapsulated Cells

For viability count of the *L. brevis* GABA 057 in the alginate beads, 30 alginate beads were dissolved in 10 ml of 0.1 M sterile sodium citrate solution by mixing in a Stomacher for 10 min. The dissolved solution was then serially diluted in a saline solution, plated onto a trypticase-proteose peptone-yeast extract (TPY) agar, and incubated anaerobically at 37°C for 48 h. Colonies that developed in the medium were then counted.

Thin Layer Chromatography (TLC) Analysis

A qualitative analysis of GABA was performed using TLC with a cellulose F aluminum plate (Merck Co., Germany) in a solvent mixture of n-butanol-acetic acid-water (4:1:1).

High-Performance Liquid Chromatography (HPLC) Analysis

The content of GABA formed in each medium was determined via HPLC analysis. The condition of each medium was as follows; 1) FCBM (Free cell in basal medium: GYP, 37°C, pH 4.5), 2) BCBM (Bead cells in basal medium: beads in GYP, 37°C, pH 4.5), 3) BCOM (Bead cells in optimized medium: GYP with 0.6% glucose, 10 mM ammonium sulfate, 34°C, pH 4.2, and beads including 0.6% isomaltooligosaccharide). One ml of the culture broth was collected and centrifuged at 1,500 ×*g* for 15 min, and then the supernatant was filtered through a 0.45-μm filter and analyzed via HPLC (HP, U.S.A.), after PITC (phenylisothiocyanate) derivatization. GABA analysis was performed using a Hewlett Packard 1100 series HPLC with a Waters Symmetry C18 column (5 μ, 4.6×250 mm I.D.), HP 1100 series binary pump, HP 1100 series auto-sampler, column oven (46°C), and HP 1100 series UV detector (254 nm). The elution solvent system was comprised of (A) 1.4 mM NaHAc, 0.1% TEA, and 6% CH_3CN (pH 6.1) and (B) 60% CH_3CN. The column was eluted with a 50 min linear gradient of 0–100% at 1.0 ml/min (B). Each sample in duplicate was determined, and the quantities of GABA and glutamate in each sample were then calculated

using the Agilent Chem Station program and expressed as average values.

RESULTS AND DISCUSSION

Effect of Medium Additives on GABA Production

The effect of the culture medium composition and additives on the production of GABA by *L. brevis* GABA 057 was investigated. As shown in Fig. 1A, 5% of the MSG was converted into GABA within 48 h when 10 mM ammonium sulfate was added to the reaction medium. Ueno *et al.* [22] previously reported that the GAD activity of *L. brevis* IFO 12005 was increased by the addition of sulfate ions (ammonium sulfate>sodium sulfate>magnesium sulfate) in a dose-dependent manner, and suggested that the increased GAD activity was duet to an increased hydrophobic interaction between the subunits. In the present experiment, the addition of 10 mM ammonium sulfate increased the GABA production, yet no dose-dependency was observed above 10 mM (data not shown). Furthermore, Ueno *et al.* [22] also assessed the GABA conversion rates assessed according to the glucose concentration: the glucose concentration in the GYP medium, containing 10 mM ammonium sulfate and 7% MSG, was controlled from 0.2% to 1.0%. However, as shown in Fig. 1B, the addition of over 0.6% glucose as a carbon source did not increase the GABA conversion rate.

Effect of Entrapment Additives on GABA Production

To increase the GABA conversion rate by improving the cell viability and stability in the beads, the effect of a series of entrapment additives, including skim milk, isomaltooligosaccharide, erythritol, and pectin, on the production of GABA were assessed. According to a previous study [24], the concentration of each additive was adjusted to 0.6%, which produced a good stability and

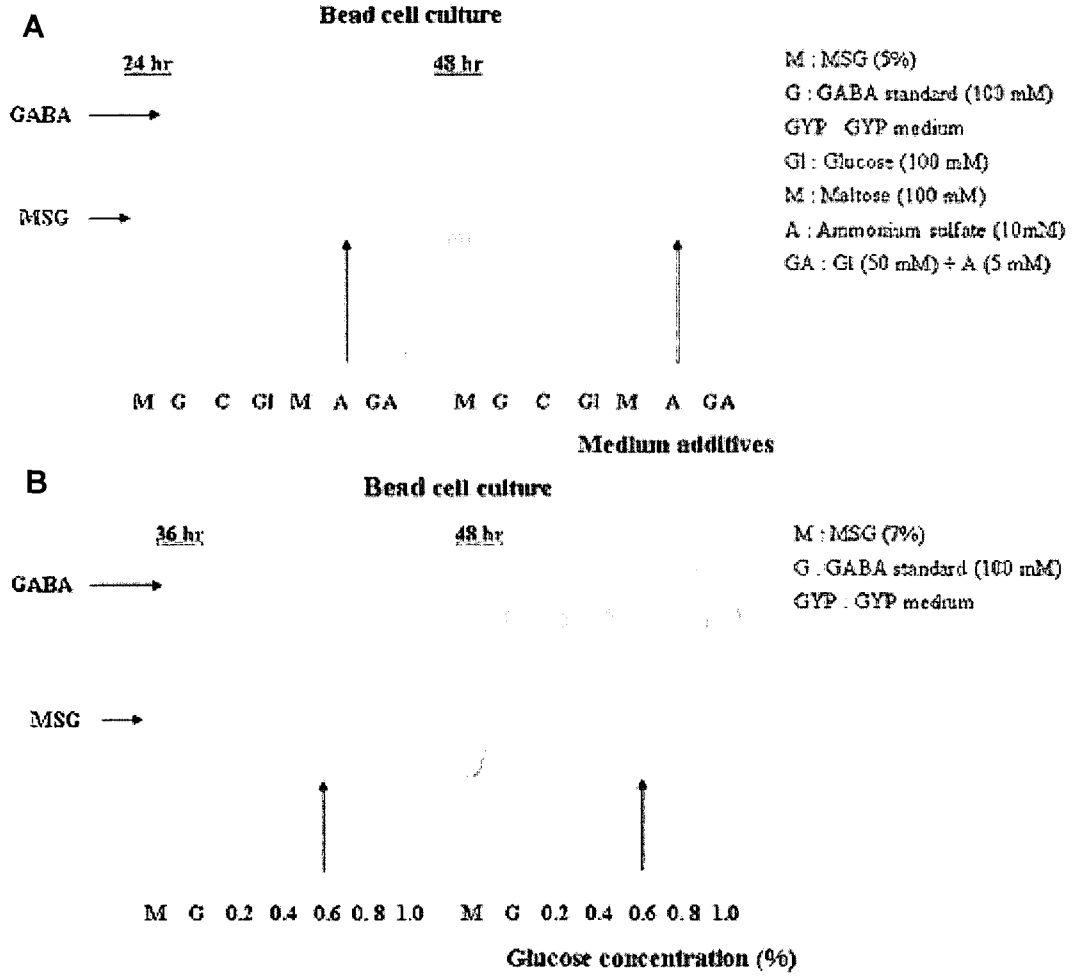

Fig. 1. The effect of media additives and glucose concentrations on the production of GABA with bead-entrapped *L. brevis* GABA 057.
A. Effect of media additives on GABA production. **B**. Effect of glucose concentrations on GABA production. One ml of the broth culture was centrifuged at 1,500 ×*g* for 15 min, and 1 μl of the supernatant was then spotted onto TLC plates. The TLC was conducted using n-butanol-acetic acid-water, and the plates were sprayed with a ninhydrin reagent.

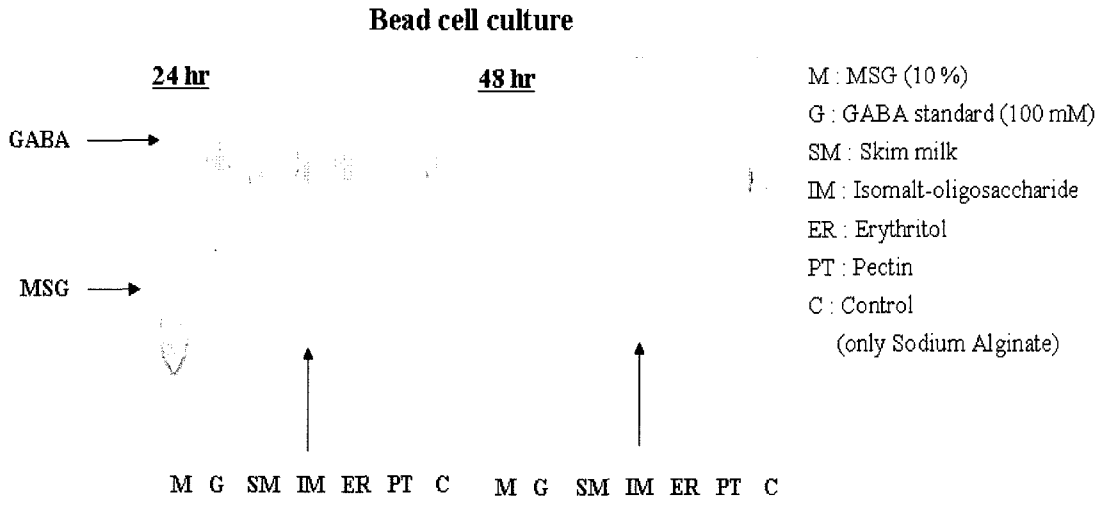

Fig. 2. Effect of entrapment additives on the production of GABA.
The test concentration for each entrapment additive was 0.6% (w/v). The control consisted of encapsulated beads containing sodium alginate without any additives.

viability for entrapped LAB. As shown in Fig. 2, the beads with 0.6% isomaltooligosaccharide were the most effective for GABA production. These beads also exhibited a higher conversion rate at 24 and 36 h than the control beads without additives. However, no improvement of GABA production due to increase of isomaltooligosaccharide concentration from 0.6 to 1.0% was observed. Therefore, the optimum additive for GABA production and the survivability of *L. brevis* GABA 057 was determined at 0.6% isomaltooligosaccharide concentration. Chen *et al.* [1] also showed that isomaltooligosaccharide improved probiotic survival in fermented milk. Isomaltooligosaccharide has been suggested to fill the pores of alginate bead; however, the mechanisms of stabilizing effect remain unclear.

Effect of Initial pH and Temperature on GABA Production

Although the GABA production yield increased, a cell outflow was clearly observed after 48 h, when the pH was controlled at 4.5 at every 12-h interval throughout the culture period (Fig. 3B, pH 4.5). For the reuse of the entrapped beads, it was vital to maintain the optimal initial pH in order to induce no damage or cell outflow. When the pH was controlled at 3.5 and 3.7, the alginate beads were dissolved in less than 12 h (Fig. 3B). However, by adjusting the initial pH at 4.0 and 4.2, no cell outflow occurred during 48 h, and 10% of the MSG was completely converted into GABA after 36 h (Fig. 3A). Therefore, the optimal initial pH for GABA production was found to be 4.2, which is consistent with previous results, where the optimal pH value for *L. brevis* GAD was found to be 4.2, and the optimum temperature was reported to be 30°C [21, 22]. In the early stages, when the MSG began to be converted into GABA, the pH of the culture medium increased rapidly from 4.5 to 6.5 during the initial 24 h (Fig. 4). When the MSG had completely been converted into GABA, the pH decreased abruptly, reaching 4.3 after 48 h. When the pH increased to 6.5, no further conversion of MSG into GABA occurred during the rest of the culture period; therefore, the pH of the culture medium was adjusted to 4.2 using a 10 N HCl solution every 12 h. The initial viable count (1.3×10^8 CFU/

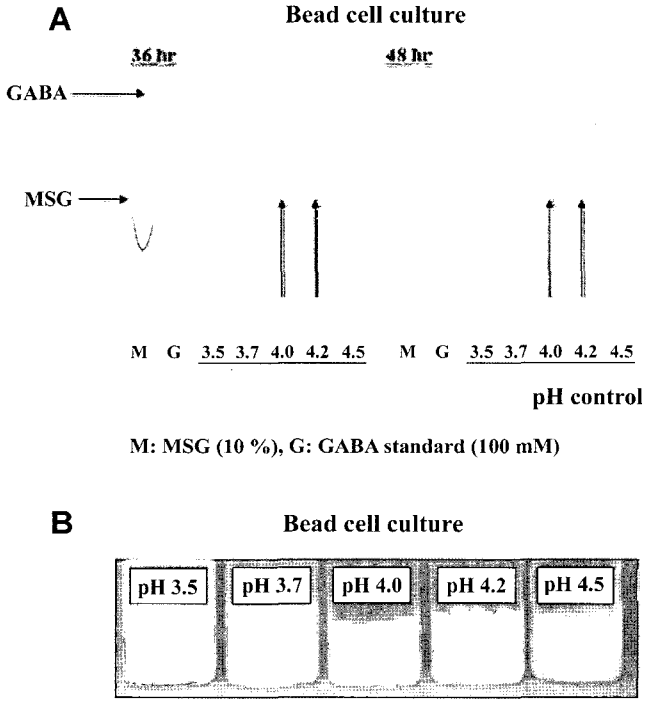

Fig. 3. Effect of initial pH on the production of GABA.
A. TLC chromatogram of GABA production according to pH. **B.** Photograph of bead and culture media after 48 h of culture.

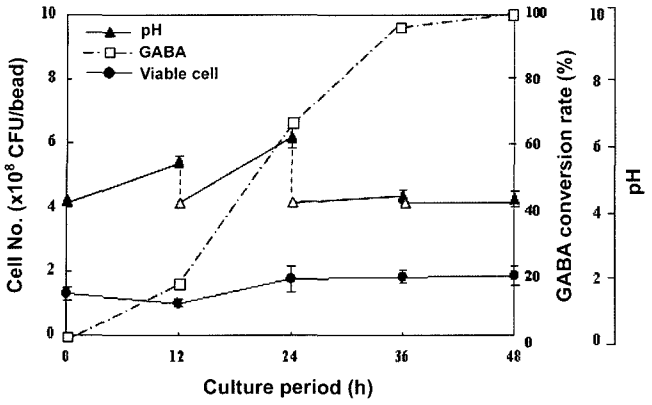

Fig. 4. Changes of cell viability and pH during GABA production.
The pH of the GYP media was optimally maintained at 4.2 at 12-h intervals (---△) throughout the culture period. One batch culture period was 48 h, and culture medium samples for analyzing the GABA conversion rate were collected at 12-h intervals, and the viable cell count was then assessed as described in Materials and Methods.

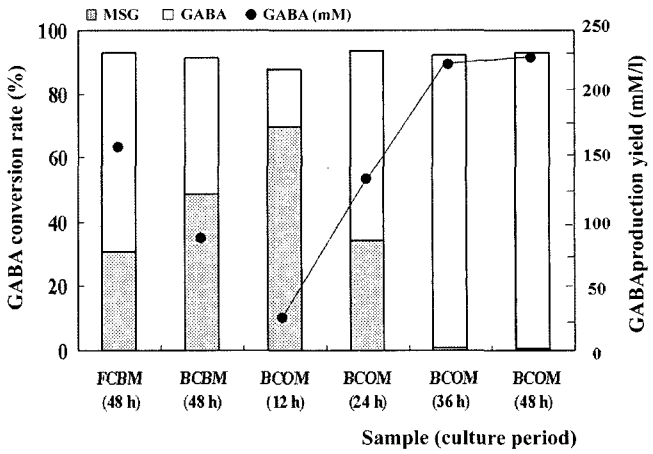

Fig. 6. Production of GABA by encapsulated *L. brevis* GABA 057 in various media.
The amount of GABA in the conditioned medium was measured for 48 h. The conditions for each medium were as follows: 1) FCBM (Free cells in basal medium: GYP, 37°C, pH 4.5), 2) BCBM (Bead cells in basal medium: bead in GYP, 37°C, pH 4.5), 3) BCOM (Bead cells in optimized medium: GYP with 0.6% glucose, 10 mM ammonium sulfate, 34°C, pH 4.2, and 0.6% isomaltooligosaccharide).

bead) remained very stable for 48 h (1.7×10^8 CFU/bead) (Fig. 4). At 34°C, 12% of the MSG was completely converted into GABA within 36 h (Fig. 5). According to Small and Waterman [16], cytoplasmic decarboxylation results in the consumption of an intracellular proton after uptake of glutamate by the specific transporter. The reaction product, GABA, is exported from the cell via an antiporter. The net result is an increase in the pH of the cytoplasm due to the removal of hydrogen ions, and a slight increase in the extracellular pH due to the exchange of extracellular glutamate for the more alkaline GABA. Komatsuzaki *et al.* [9] suggested that, when the pH of culture medium for NFRI 7412 was regulated to 5.0, GABA production was significantly increased because of enhancement of GAD activity. In the present study, GAD activity was the highest at pH 4.2, and the cell outflow occurred at pH lower than 4.0.

Amount of GABA Production

The amount of GABA produced under the various conditions was measured using HPLC analysis. As shown in Fig. 6, 534 mM MSG was converted into 227 mM GABA

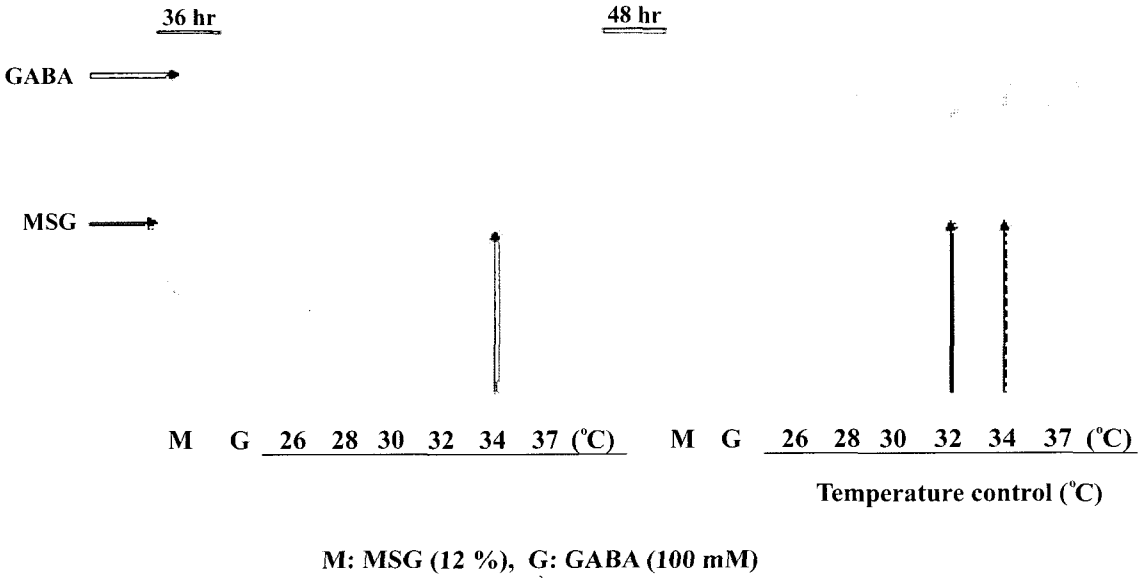

Fig. 5. Effect of temperature on the production of GABA.

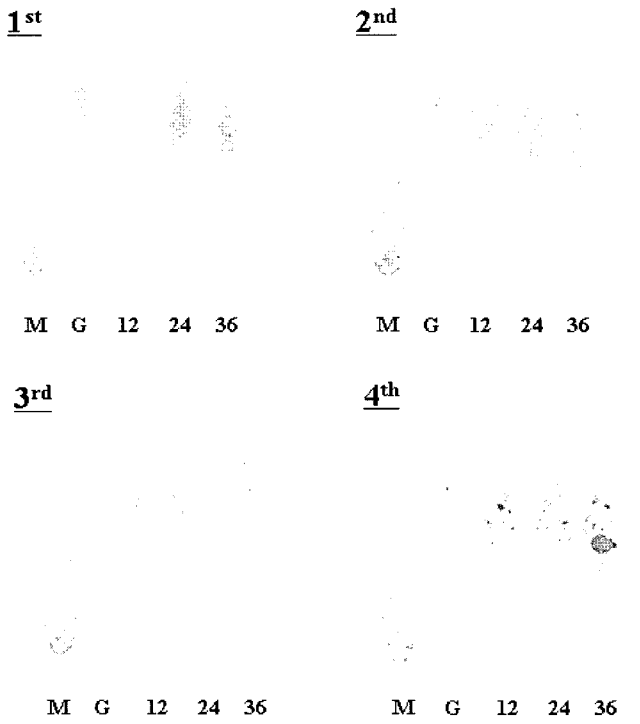

Fig. 7. GABA production in batch fermentation using recycled immobilized *L. brevis* GABA 057.
The conditions for the batch culture were as follows: BCOM (Bead cells in optimized medium: GYP with 0.6% glucose, 10 mM ammonium sulfate, 34°C, pH 4.2, and 0.6% isomaltooligosaccharide).

by the entrapped *L. brevis* GABA 057 in the optimized medium (BCOM) within 48 h, while only 83 mM was produced by the bead cells in the basal medium (BCBM) and only 164 mM produced by free cells in the basal medium (FCBM) during the same time period. In addition, under the optimized culture conditions (BCOM), the frequency of bead reuse was evaluated using a batch fermentation system. As seen in Fig. 7, it was possible to use the constructed beads up to 4 times, and 10% MSG (534 mM) was completely converted into GABA within 36 h. Yokoyama *et al.* [23] showed that 10.18 mM GABA was produced from alcohol distillery lees that contained 10.5 mM free glutamic acid, by *L. brevis* IFO12005 for 48 h. Komatsuzaki *et al.* [9] reported that *L. paracasei* NFRI 7415 required 150 h to produce 302 mM GABA from 500 mM glutamate. The productivity of the microbial metabolite is improved by manipulating the nutritional and physical factors of the growth condition. Therefore, the optimization for growth condition is an important aspect in the development of the metabolite production process by microbial cultivation [5, 7, 15, 17].

In conclusion, to develop GABA as a functional food material, we presented in the present study a high-yield production process of GABA via MSG bioconversion using *L. brevis* GABA 057 that was entrapped in alginate and isomaltooligosaccharide, and also determined the optimal conditions for this process. The results showed that 12% MSG (640.8 mM) was completely converted into GABA at 34°C and pH 4.2 within a 48-h culture period. Furthermore, the LAB entrapment system made it possible to reuse the beads up to 4 times in a batch fermentation system, because of increase of stability and viability, thereby maximizing the GABA productivity. Consequently, these results indicate that the production of GABA with *L. brevis* GABA 057 may be of great interest to the functional food industry. To the best of our knowledge, this is the first report on GABA production by entrapped lactic acid bacteria.

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