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Biosynthesis of γ-aminobutyrate by engineered *Lactobacillus brevis* cells immobilized in gellan gum gel beads

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γ-Aminobutyrate (GABA) is an important chemical in pharmaceutical field. The use of lactic acid bacteria as biocatalysts for the conversion of L-monosodium glutamate (MSG) into GABA opens interesting perspectives for the production of this functional compound. In this work, an engineered GABA high-producing strain *Lactobacillus brevis* $GadA_{\Delta C14}$ was constructed by overexpressing a C-terminally truncated GadA mutant, which is active in expanded pH range. After comparison with agar and κ-carrageenan, gellan gum was selected as the optimal immobilization support, and the properties of *L. brevis* $GadA_{\Delta C14}$ cells encapsulated in this hydrogel were examined. The optimum pH and temperature of immobilized cells were found to be 40°C and pH 4.4, respectively. It was also observed that operational and thermal stabilities of the cells were increased with immobilization. After ten consecutive reaction cycles, the total amounts of GABA produced by the immobilized cells summed up to 87.56 g/L under the optimum experimental conditions. Taken together, the improved stability and good usability make the immobilized *L. brevis* $GadA_{\Delta C14}$ cells more valuable for industrial applications.

[**Key words:** *Lactobacillus brevis*; GABA; Glutamate decarboxylase; Gellan gum; Immobilization]

γ-Aminobutyrate (GABA), a major inhibitory neurotransmitter in the mammalian central nervous system, has been reported to posses hypotensive, diuretic, tranquilizing and growth-promoting effects (1–3). Owing to these beneficial functions, many attempts to synthesize food-grade GABA by biological methods have been developed to meet its increasing commercial demand. In recent years, a variety of microorganisms such as bacteria, fungi and yeast have been reported for their ability to produce GABA (4). Particularly, the most interesting and practical group of these organisms is lactic acid bacteria (LAB), which could catalyze the irreversible α-decarboxylation of L-glutamate (Glu) via a glutamate decarboxylase system (Gad system) in a proton-consuming reaction, thus contributing to pH homeostasis within the cells and resulting in a stoichiometric release of GABA (5,6). Meanwhile, LAB are the main microorganisms used for production of recognized fermented food products and biologically active supplements (7). It is clear that biosynthesis of GABA by LAB for the manufacturing of food can make full use of the health-promoting properties of GABA and LAB themselves (8,9).

In our previous work, a LAB strain *Lactobacillus brevis* CGMCC1306 isolated from raw milk has shown a great promise and potential in large-scale fermentation for the production of GABA (10,11). Molecular genetic analysis demonstrated that the Gad system of this strain comprises a transcriptional regulator (GadR), a glutamate/GABA antiporter (GadC) and two biochemically identical isoforms of glutamate decarboxylase, GadA and GadB. The GadA and GadB exhibited optimum activity at pH 4.8 and pH 4.4, respectively (12,13). However, similar to the GADs in *Escherichia coli* and *Listeria monocytogenes*, the two isoforms sharply lose their activities as pH rises, and show no activities at near-neutral pH (12–14). Specifically, GADs catalyze the decarboxylation reaction intracellularly, i.e., in an environment with an overall neutral pH (15). Taken together, one of the key obstacles involved in the improvement of GABA production by *L. brevis* is how to deal with the discrepancy of optimal pH between the activity of intracellular GADs and cell growth.

Earlier, crystal structures of *E. coli* GAD isoforms (GadA and GadB) have been determined (16,17). The features are remarkably given that C-termini of GADs were mostly unstructured at acidic pH, but underwent the formation of well-structured plug that blocks the binding of Glu to active site at neutral pH (18). This observation shed new light on the correlation between the C-terminal structural feature and catalytic performance of GAD, and several interesting features regarding the structural changes in GAD upon pH shifts were presented therein (14,19–21). Accordingly, expanding the active pH range of intracellular GAD by protein engineering might be a feasible approach to reduce the negative effects of weakly acidic and neutral conditions on the use of *L. brevis* cells for production of GABA.

During the past few years, several strategies have been developed for the efficient production of GABA by *L. brevis*, including resting and immobilized cells technologies, batch, fed-batch as well as mixed fermentation techniques (5,9,22,23). Compared to planktonic cell fermentation, the use of immobilized microbial cells

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in production processes is advantageous to enhance the stability of the biocatalyst and to facilitate its recovery and reuse (23). It is therefore conceivable that the use of immobilized *L. brevis* cells may provide a solution towards a reduction in the cost of GABA production. To address the above issues, in this paper, a recombinant *L. brevis* strain was constructed based on a nisin-controlled gene expression system (NICE) for enhanced production of GABA by overexpressing a C-terminally truncated GadA mutant, which is active in expanded pH range (14,24). Subsequently, a simple but efficient method for immobilization of the engineered *L. brevis* strain in gellan gel beads was further developed, and its advantages on GABA productivity and yield has also been determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this work are listed in Table S1. *E. coli* strains were aerobically grown with shaking at 250 rpm in Luria–Bertani (LB) broth at 37°C. *Lactococcus lactis* strains were grown in M17 medium (Hopebiol, Qingdao, China) supplemented with 0.5% glucose (GM17) at 30°C. *L. brevis* strains were generally cultured in MRS medium (Hangzhou Microbial Reagent Co., Ltd., China) at 37°C without agitation. Selective media contained 7 μg/mL erythromycin or 10 μg/mL chloramphenicol for selection of *L. brevis*, and 20 μg/mL chloramphenicol was used for the selection of *E. coli*. In fermentation experiments, *L. brevis* cells were grown in glucose yeast extract peptone (GYP) medium as described previously (10) with minor modifications (g/L): glucose, 20; yeast extract, 15; peptone, 5; CH_3COONa, 3; $FeSO_4 \cdot 7H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.001; $MnSO_4 \cdot 4H_2O$, 0.02; L-monosodium glutamate (MSG), 72.75. Under the pH-controlled batch fermentations, pH was adjusted and maintained by automatic addition of 3 mol/L NaOH or 3 mol/L H_2SO_4.

Construction of expressing plasmids and strains For expression of GadA and its mutant in *L. brevis*, the wild-type *gadA* (Accession number: GU987102) and C-terminal truncated GadA mutant genes were amplified by PCR using pET28a-*gadA* as the template with primers *gadA*-F and *gadA*-R and with *gadA*-F and $gadA_{\Delta 14}$-R, respectively. To delete the last 14 C-terminal amino acid residues of the peptide chain, the TTC codon between 1363 and 1365 bp in *gadA* gene was replaced with the stop codon (TAA) through primers design and PCR. The information on primers is listed in Table S2. After digestion with the enzymes *Pst*I and *Xba*I, the gene fragments were ligated into the nisin-inducible expression vector pNZ8148 to generate pNZ8148-*gadA* and pNZ8148-$gadA_{\Delta C14}$, respectively. Subsequently, the recombinant plasmids were further transformed into *L. brevis* 9530 to form *L. brevis* GadA and *L. brevis* $GadA_{\Delta C14}$, respectively. All plasmids were constructed in *E. coli* MC1061 and then transformed into *L. brevis* by electroporation as described in our previous work (11).

Expression of wild type and C-terminal truncated GADs in *L. brevis* Nisin induction was carried out essentially as described by Pavan et al. (25). Overnight grown cultures of the recombinant *L. brevis* strains in GYP broth supplemented with 7 μg/mL erythromycin and 10 μg/mL chloramphenicol were inoculated at a level of 2% into fresh media. After incubating at 37°C for 12 h, cultures were induced with 5 ng/mL of nisin, and subsequently incubated for two days at 37°C. Following nisin induction of *L. brevis* CK, *L. brevis* GadA and *L. brevis* $GadA_{\Delta C14}$, GABA concentration and cell-bound GAD activity assays were completed to compare the productive performances of the three strains. Moreover, the expression of wild-type and mutant GadA ($GadA_{\Delta C14}$) in *L. brevis* was confirmed by SDS-PAGE analysis as described in our previous work (11).

GABA and cell-bound GAD activity assay Cell-bound GAD activity was determined by measuring the amount of GABA formed at 37°C in a reaction mixture containing 0.1 mg (dry cell weight)/mL cell biomass, 0.2 mol/L sodium acetate buffer (pH 4.8) and 60 mmol/L MSG. One unit (U) of GAD activity was defined as the amount of cells that produced 1 μmol of GABA in 1 min under the above conditions. Specific activity was defined as U/mg dry cell weight cells. The concentrations of Glu and GABA were determined by reversed phase high-performance liquid chromatography as described by Marquez et al. (26).

Preparation and characterization of immobilized cells of *L. brevis* The gellan gum, agar and κ-carrageenan were used as support matrix for cells immobilization in this work. The interphase technique described by López et al. was used to form gel beads from agar and κ-carrageenan (27). Similarly, the ionotropic method described by Woodward was used to form gel beads from gellan gum (28). 1 g of gellan gum was dissolved in 90 mL of deionized double-distilled water by using magnetic stirrer. Subsequently, 10 mL of cell suspension (OD_{600} = 50) were added to the carrier solution. The gellan gel and cell suspension were mixed at a ratio of cell dry weight to dry polymers powder of 0.27 (wt/wt). The immobilized cells were formed by extruding the mixture through a 20 mL glass syringe with an 21 G hypodermic needle into 0.2 mol/L $CaCl_2$ and letting it harden for 1 h. The morphology of cells immobilized in gel beads was determined using a scanning electron microscope (SEM) (SU70; Hitachi, Japan). Mean diameter was calculated by measurement of diameter of 20 randomly chosen beads. The breakage of beads was determined as the quotient of the number of broken beads divided by the total number of beads.

Biotransformation of MSG to GABA by resting and immobilized cells of *L. brevis* To optimize the reaction conditions for the biotransformation of MSG to GABA by resting cells of *L. brevis*, nisin-induced engineered cells were harvested after 24 h of cultivation via centrifugation at 6000× *g* for 15 min at 4°C, washed twice with 0.05 mol/L sodium phosphate buffer (pH 6.8), and then resuspended in 0.1 mol/L Na_2HPO_4-citric acid buffer at 2.7 g dry cell weight per liter. Subsequently, 25 mL of mixture containing various concentrations of MSG in 50 mL Erlenmeyer flask was used with variations as follows. The pH value was from 3.6 to 5.6 and temperature range was from 20°C to 50°C. The mixture was shaken at 150 rpm. To document the dominant effect of temperature on the activity, the thermal stability of biocatalyst has also been determined. The immobilized and free cells were incubated for 30 min at different temperatures, and then put into the 0.1 mol/L Na_2HPO_4-citric acid buffer, respectively, containing 120 mmol/L MSG at pH 4.4 and 40°C.

During the decarboxylation reaction, samples were withdrawn periodically and subjected for GABA analysis. Similarly, 67.5 mg (dry cell weight) of the immobilized cells was added to 25 mL reaction mixture. The GABA transformation experiments were performed under the same reaction conditions as described above. In the recycling experiments, after each transformation batch, the free and immobilized cells were collected by centrifugation and filtration, respectively, and then were washed twice with Na_2HPO_4-citric acid buffer to remove the soluble impurities. After the washing buffer was removed, 25 mL of fresh buffer containing MSG was added to repeat the cycle. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Comparison of GAD activity of *L. brevis* strains In our previous work, a C-terminal truncated mutant of GadA derived from *L. brevis* CGMCC1306, $GadA_{\Delta C14}$, was constructed according to the monomer homology model of wild-type GAD deduced from the *E. coli* GadB structure and exhibited higher activity at pH values above 5.6 (14). To document the progress of this mutant to cell-bound GAD activity, the wild-type *gadA* and mutant genes were cloned into pNZ8148, and subsequently transformed into strain *L. brevis* 9530 (11) to form *L. brevis* GAD and *L. brevis* $GAD_{\Delta C14}$, respectively. The *gadA* and mutant genes were under the control of the nisin-inducible *nisA* promoter (Fig. S1A). After induction with 5 ng/mL nisin, the samples of recombinant *L. brevis* strains that grown in GYP fermentation media under acidic conditions (pH 5.2) were collected periodically during the cultivation for extraction or determination of cell-bound GAD activity. Analysis of cell extracts by SDS-PAGE revealed that the protein bands were consistent with the predicted molecular mass of recombinant GadA and $GadA_{\Delta C14}$ (Fig. S1B). It is evident that both the GadA and mutant $GadA_{\Delta C14}$ were well expressed under *nisA* promoter with high solubility.

To evaluate whether overexpression of GadA and $GadA_{\Delta C14}$ could improve GABA productivity, cell-bound GAD activity of *L. brevis* at different growth phases was further assayed. As shown in Fig. 1, the three strains showed similar trends in the GAD activity during the course of batch fermentation. Increased cell-bound GAD activity was observed during the exponential growth phase, and then a slow decrease was found from the start of the stationary (30 h). The specific cell-bound GAD activity of *L. brevis* peaked during the late exponential growth phase (24 h), and the highest activities for *L. brevis* CK, *L. brevis* GadA and *L. brevis* $GadA_{\Delta C14}$ were 0.692 ± 0.017, 0.928 ± 0.041 and 1.177 ± 0.081 U/mg_{DCW}, respectively. Obviously, both of the two engineered strains revealed higher GAD activities than that of wild strain. Moreover, the recombinant strain harboring pNZ9530/pNZ8148-$gadA_{\Delta C14}$ system achieved a preferable result in the increased GAD activity to that of other strains. As expected, expanding the active pH range of GAD has the advantage of favoring the decarboxylation reaction mediated by *L. brevis* cells.

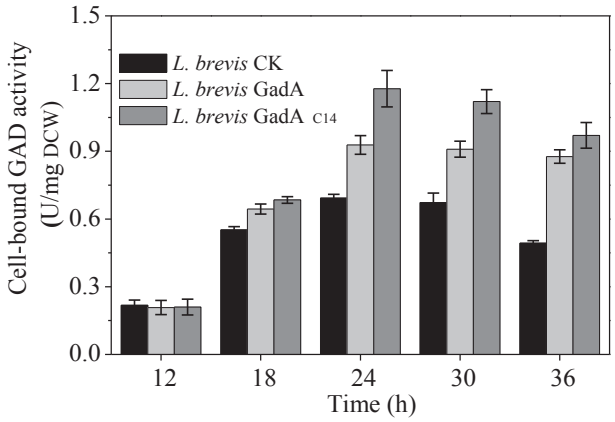

FIG. 1. The cell-bound GAD activities of *L. brevis* strains during the course of batch fermentation with pH controlled at 5.2. *L. brevis* CK (closed columns), *L. brevis* GadA (light gray columns), *L. brevis* $GadA_{\Delta C14}$ (dark gray columns). One unit (U) of GAD activity was defined as the amount of cells that produced 1 μmol of GABA in 1 min under the conditions as described in materials and methods. Specific activity was defined as U/mg dry cell weight cells.

Improvement of GABA biosynthesis by overexpressing the C-terminally truncated GAD in *L. brevis* To confirm that overexpressing of the C-terminally truncated GadA mutant contributes to higher GABA productivity, the GABA production using *L. brevis* CK and *L. brevis* $GadA_{\Delta C14}$ in batch culture were further investigated. As shown in Fig. 2A, the pH profile of *L. brevis* $GadA_{\Delta C14}$ under the uncontrolled pH condition gradually decreased from initial value of 6.8 to less than 5.3 within 24 h of cultivation. Subsequently, the fermentation pH started to increase and reached 8.3 at 60 h. The time at which the pH of the culture medium began to increase coincided with that of GABA production during the fermentation process. After 60 h of fermentation, approximately 20.36 g/L of GABA was obtained from 72.75 g/L of MSG at a conversion rate of 45.9%. Similar results have also been observed for strain *L. brevis* CK, while the GABA content (18.15 g/L) and the final pH value (7.9) in culture medium were relatively lower than that of *L. brevis* $GadA_{\Delta C14}$. In addition, it is noteworthy that the direct conversion of MSG to GABA by *L. brevis* was initiated in late exponential growth phase when the culture pH was relatively lower than initial pH, as was the case for *L. lactis* (29) and *L. brevis* NCL912 (30).

In contrast with the observations under uncontrolled pH condition, the GABA production by *L. brevis* under controlled pH condition (pH 5.2) was initiated in the early exponential growth phase, and GABA content increased rapidly during the exponential growth phase whereas the MSG content decreased sharply, as shown in Fig. 2B. As expected, relatively higher GABA yield and conversion rate of *L. brevis* $GadA_{\Delta C14}$ (42.27 g/L, 95.34%) than *L. brevis* CK (34.79 g/L, 78.44%) was also observed at 60 h of cultivation. At the same time, the GABA volumetric productivity of 2.18 g/L/h obtained in cultivation at pH 5.2 for *L. brevis* $GadA_{\Delta C14}$ was obviously higher than that obtained for the control strain (1.74 g/L/h) during the exponential growth phase (12–30 h). Moreover, it should be mentioned that the final cell density (OD_{600}) of *L. brevis* CK and *L. brevis* $GadA_{\Delta C14}$ reached 6.5 and 5.8, respectively, and increased by 80.6% and 81.3% as compared to the two strains cultured under uncontrolled pH conditions.

Selection of immobilization supports Immobilized microbial cells are frequently used for preparation of a series of metabolic products in the food and pharmaceutical industries. It can be advantageous to use immobilized *L. brevis* cells for GABA production, thus avoiding separation and purification of the enzyme,

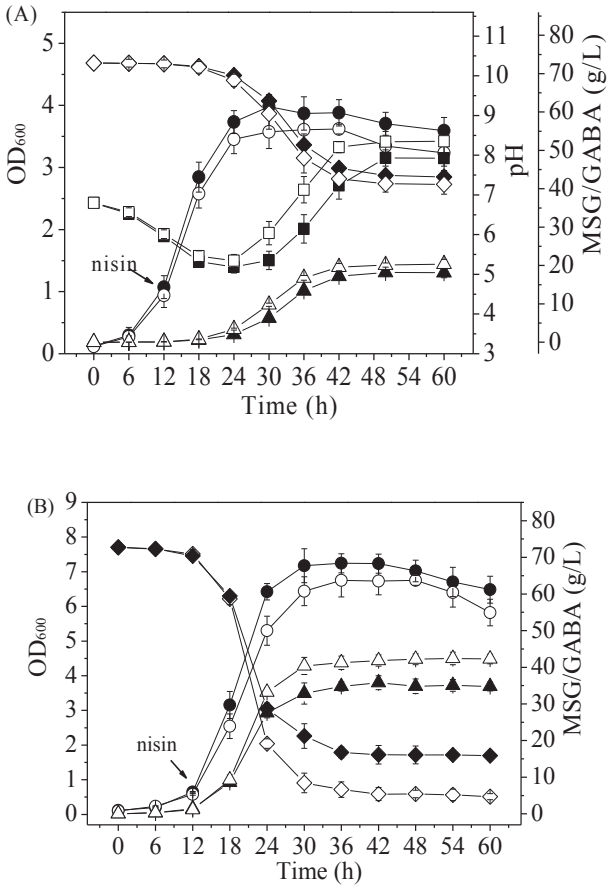

FIG. 2. Time courses of cell growth, GABA production and residual MSG in batch fermentation under uncontrolled (A) and controlled pH conditions (B). The OD_{600} (circles), GABA (triangles), MSG (diamonds) and pH (squares) value of *L. brevis* CK (solid) and *L. brevis* $GadA_{\Delta C14}$ (hollow) grown in the fermentation medium for over 60 h at 37°C were measured. Data are presented as mean ± SD values from three independent experiments.

simplifying the product purification process, and enhancing long-term operation stability (23). Among various immobilization methods, encapsulation is one of the most promising strategies realized under mild conditions, commonly with minimum denaturation of the biocatalysts (31). Furthermore, in search of suitable matrices for the encapsulation of cells, naturally occurring hydrogels, like gellan gum, agar and κ-carrageenan have been found to be the top candidates in terms of the biocompatibility and processability (32–34). Thus, the three gel polymers were selected as the support matrix in this work.

After immobilization, the physical properties of encapsulated cells were analyzed. Results are shown in Table 1. All gel beads were spherical and homogeneous, and showed strong resistance to breakage. Subsequently, to investigate the influence of encapsulation on GAD activity, the biotransformations of MSG to GABA by free and immobilized cells in the 0.1 mol/L Na_2HPO_4-citric acid buffer containing 120 mmol/L MSG at pH 4.8 and 37°C were performed. As shown in Fig. 3, more than 9.13 g/L of GABA could be effectively produced in 5 h by immobilized cells when gellan gum served as the immobilization support. However, the GABA conversion rates of cells immobilized by κ-carrageenan and agar were relatively lower, and the generated contents of GABA were 7. 95 g/L and 7.41 g/L after 5 h of incubation, respectively. It is clear that the GAD activity of immobilized *L. brevis* $GadA_{\Delta C14}$ was affected by the nature of the entrapping carrier, and the gellan gum showed to entrap more GAD activity than that of agar and κ-carrageenan. Such

TABLE 1. Properties of gel beads prepared with different polymers.

Polymer	Concentration (%)	Bead-forming procedure	Immobilization temperature (°C)	Form	Bead size without cells mm ± S.D.	Bead size with cells mm ± S.D.	Breakage (%)
Agar	2	Interphase	45	Spherical	2.63 ± 0.21	3.04 ± 0.17	0
κ-Carrageenan	2	Interphase	55	Spherical	2.61 ± 0.23	2.93 ± 0.14	0
Gellan gum	1	Ionotropic	20	Spherical	2.34 ± 0.18	2.61 ± 0.15	0

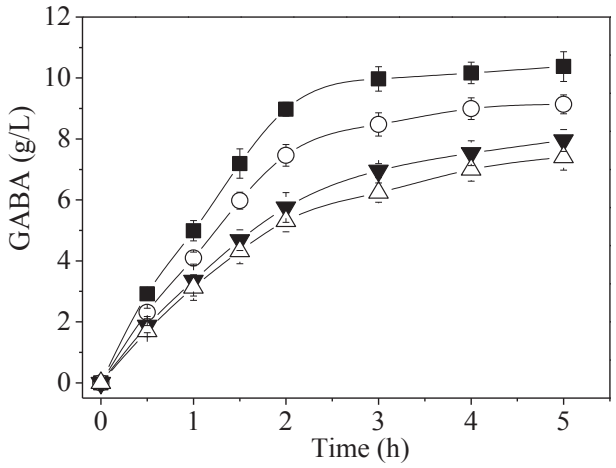

FIG. 3. The biotransformations of MSG to GABA by free and immobilized cells in different supports. Free cells (solid squares), cells immobilized by gellan gum (open circles), cells immobilized by κ-carrageenan (solid triangles), cells immobilized by agar (open triangles). Before immobilization, cells of *L. brevis* $GadA_{\Delta C14}$ were grown into late exponential growth phase (24 h) under the controlled pH conditions (pH 5.2) to maximize GAD activity. The biotransformation was performed in the 0.1 mol/L Na_2HPO_4-citric acid buffer containing 120 mmol/L MSG at pH 4.8 and 37°C. The same amounts of cells (the cell dry weight was 67.5 mg) were used in all experiments.

phenomenon has also been observed in the degradation of carbazole by *Arthrobacter* sp. and *Sphingomonas* sp. immobilized in these gel polymers (32,33). In addition, it is important to note that the conversion ability of MSG into GABA was reduced when the cells were immobilized as compared to free cells. This common feature may be attributed to the mass transfer limitation of bioavailable concentration in the inner spaces of the beads in contrast to that in the bulk liquid (35). Despite of this shortcomings, gellan gum was selected as the most suitable support in the subsequent experiments.

Effects of pH, temperature and substrate concentration on the biotransformation of MSG to GABA To achieve the optimal conditions for maximum yield of GABA, the effects of operational pH, temperature and substrate concentration on the GAD activity of free and immobilized *L. brevis* $GadA_{\Delta C14}$ cells were performed. As shown in Fig. 4A, the highest activity of immobilized cells was achieved at pH 4.4, entirely consistent with that of free cells. However, the immobilized cells retained 52.1% of its maximum activity at pH 3.6 and 93.4% at pH 4.0, respectively, whereas free cells retained only 39.8% and 84.6% of its maximum activity at the same pH, respectively. It was apparent that the immobilized cells were less pH-sensitive from pH values comprised between 3.6 and 4.4, probably owing to the microenvironment provided by the gel networks which may shield cells from the effects of H^+ ions (36).

Similarly, the temperature optimum for free cells and their immobilized form is shown in Fig. 4B. At low temperatures, the activities of both free and immobilized cells increased as temperature raised, with the same maximum value being observed at 40°C, above which the activities tended to decrease. However, the immobilization resulted in slower decline in GAD activity. To further document the dominant effect of temperature on the activity, the thermal stability of biocatalyst has also been determined as described in materials and methods. As shown in Fig. 4C, there was no obvious differences occurred for the temperature ranged from 20°C to 40°C. In contrast, when the temperature was raised to 50°C, there was an obvious decrease in the activity of the immobilized and free cells after incubation. After incubation at 60°C for 30 min, the activity of the free cells decreased to 42.7% of the initial value, whilst the immobilized biocatalyst still retained 79.3% of its initial activity. It is obvious that the cells immobilized in gellan gum beads displayed a broader temperature profile and provided a distinct advantage in thermal stability than suspended cells. Accordingly, the greater stability of immobilized cells can be ascribed to the stabilizing effects of immobilization. Overall, these observations were generally reported during the biotransformation by immobilized biocatalysts (23,31).

The effects of initial substrate concentration ranging from 40 to 200 mmol/L on bioconversion to GABA were also determined at 40°C in the 0.1 mol/L Na_2HPO_4-citric acid buffer with pH controlled at 4.4. Results are shown in Fig. 4D. The GABA levels progressively increase in response to increasing concentration of MSG, with the maximum values being observed at 140 mmol/L and 160 mmol/L for the free and immobilized cells, respectively, above which the increase finally leveled off. After 5 h of incubation at the optimal substrate concentration, the corresponding GABA level and conversion rate of immobilized cells were 10.15 g/L and 61.5%, respectively. In the case of suspended cells, those values were found to be 11.97 g/L and 82.9%, respectively. Furthermore, the immobilized cells showed a similar trend in GABA production with that of free cells during the course of incubation on the optimum conditions, although the reaction rate (V) was relative lower during the first 2 h of the reaction (Fig. 4E). However, the reaction rates of immobilized biocatalyst and free cells were retained less than 20.9% and 15.4% of their initial values after incubation for up to 3 h, respectively, and then diminished almost after another hour. Take these factors into consideration, a 4-h cycle was suitable for the production of GABA by using the immobilized *L. brevis* $GadA_{\Delta C14}$ cells in the above reaction system.

Repeated batch process of free and immobilized cells of *L. brevis* The reuse stability of biocatalysts is one of the most important factors affecting the utilization of an immobilized cells system in industrial process (23). Here, the operational stability of immobilized *L. brevis* cells was ascertained by reusing the biocatalysts for a total of 10 times within 2 days, each of 4 h duration. As shown in Fig. 5, the yield of entrapped *L. brevis* $GadA_{\Delta C14}$ cells remained unchanged during the first 4 cycles, while diminished progressively with the increase of number of reaction cycles. In contrast, the reusability of free cells was inferior to that of immobilized biocatalyst during successive cycles of the decarboxylation reaction. The yield of GABA dropped to about 43.6% after seven 4-h cycles of reaction with the free-cell biocatalyst, however, when the immobilized cells was used, the yield maintained at 61.9% even after 10 cycles of reaction.

Developing a repeatedly available immobilized *L. brevis* $GadA_{\Delta C14}$ cells provides a promising technique for improving the biocatalyst used in the biotransformation of MSG to GABA. As described previously (34), the gellan gel is a polymer free of toxicity

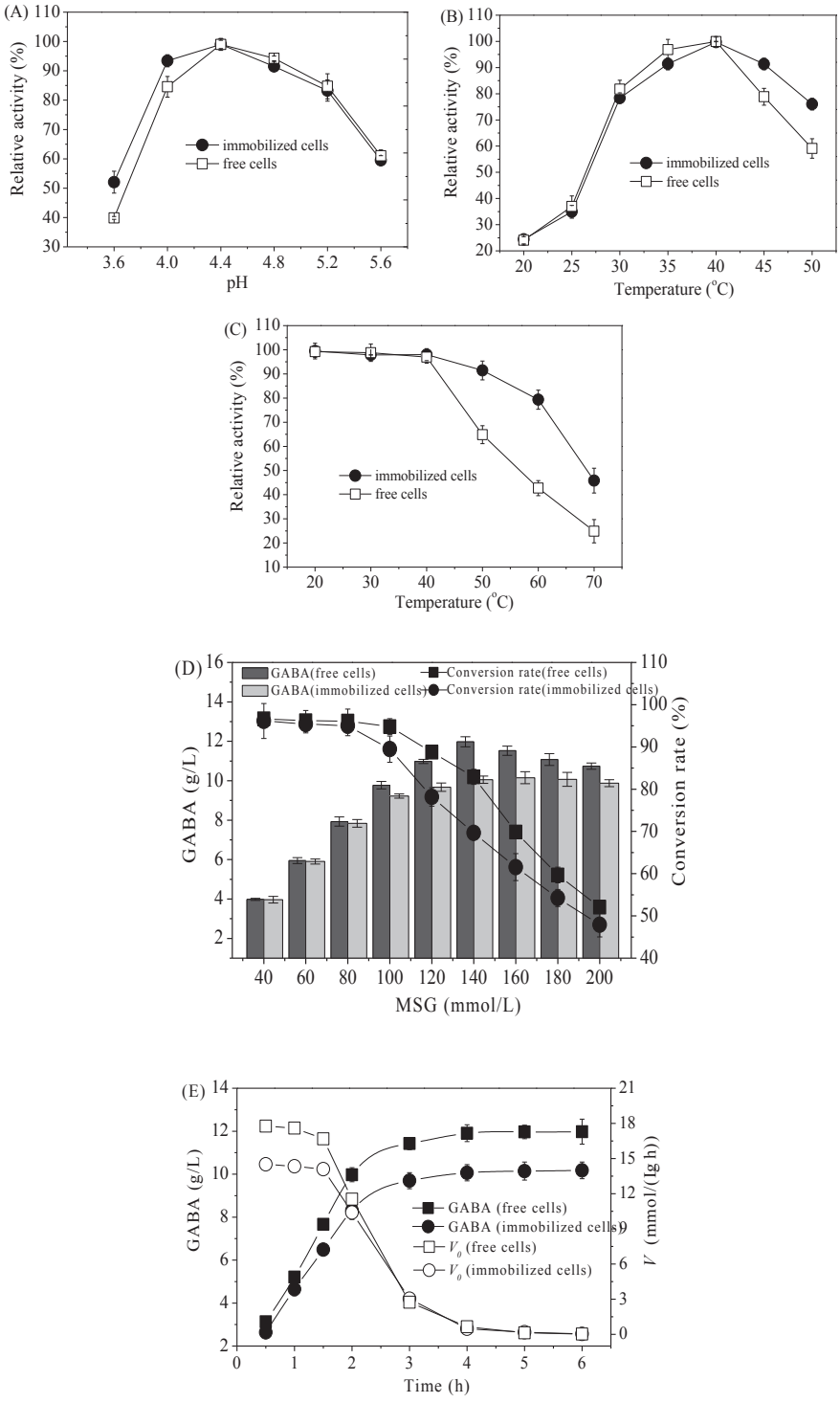

FIG. 4. (A) Effects of pH value on the GAD activity of free and immobilized *L. brevis* $GadA_{\Delta C14}$ cells. The activity obtained at pH 4.4 was taken as 100%. (B) Effect of temperature on the GAD activity of free and immobilized cells. The activity obtained at 40°C was taken as 100%. (C) Effect of temperature on the stability of free and immobilized cells. Samples were incubated for 30 min at different temperatures, and then put into the 0.1 mol/L Na_2HPO_4-citric acid buffer, respectively, containing 120 mmol/L MSG at pH 4.4 and 40°C to determine the GAD activity. (D) Effects of MSG concentration on the GABA production and conversion rate. The initial substrate concentrations were 140 mmol/L and 160 mmol/L for free and immobilized cells, respectively. (E) Time-dependent changes in GABA concentrations by free and immobilized cells of *L. brevis* $GadA_{\Delta C14}$. Squares, free cells; circles, immobilized cells.

and has a stable pH value ranging from 2 to 10. Our results also displayed that no broken pieces were found during the catalytic process, and the *L. brevis* $GadA_{\Delta C14}$ cells could be clearly observed on the surfaces and insides of gellan gel beads (Fig. S2). However, it should be noted that a disadvantage of immobilization was the decrease in reaction kinetics due to diffusion effects across the gel, partly offsetting the objective of process intensification through the use of higher cell concentrations (35). Nevertheless, the repeated use of immobilized cells can compensate it. As expected, the encouraging alteration in GABA level has occurred at the third cycle of the biotransformation experiments. After seven consecutive reaction cycles, the total amounts of GABA for free and immobilized

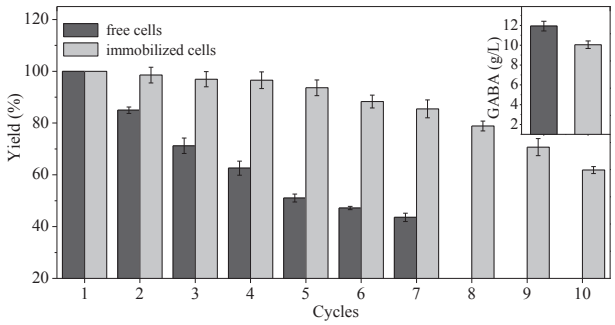

FIG. 5. Effects of reuse number on the GABA yield of free (dark gray columns) and immobilized *L. brevis* $GadA_{\Delta C14}$ cells (light gray columns). Biotransformation of each batch was carried out in the 0.1 mol/L Na_2HPO_4-citric acid buffer (pH 4.4) at 40°C for 4 h. The initial MSG concentrations were 140 mmol/L and 160 mmol/L for free and immobilized cells, respectively.

cells were 54.97 g/L and 66.31 g/L, respectively. Consequently, the immobilization of *L. brevis* $GadA_{\Delta C14}$ cells offered significant advantages in terms of increasing the process stability of the biocatalyst, which was likely to make the immobilized cells more valuable for industrial applications. In further studies, the commonly used three-phase fluidized bed and airlift bioreactor could be applied to evaluate the ability of continuous operation for GABA production by immobilized gellan gel beads.

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References

1. **Mccormick, D. A.:** Gaba as an inhibitory neurotransmitter in human cerebral-cortex, J. Neurophysiol., **62**, 1018–1027 (1989).
2. **Wong, C. G. T., Bottiglieri, T., and Snead, O. C.:** GABA, γ-hydroxybutyric acid, and neurological disease, Ann. Neurol., **54**, S3–S12 (2003).
3. **Ge, S. Y., Goh, E. L. K., Sailor, K. A., Kitabatake, Y., Ming, G. L., and Song, H. J.:** GABA regulates synaptic integration of newly generated neurons in the adult brain, Nature, **439**, 589–593 (2006).
4. **Dhakal, R., Bajpai, V. K., and Baek, K. H.:** Production of gaba (gamma-aminobutyric acid) by microorganisms: a review, Braz. J. Microbiol., **43**, 1230–1241 (2012).
5. **Li, H. X. and Cao, Y. S.:** Lactic acid bacterial cell factories for gamma-aminobutyric acid, Amino Acids, **39**, 1107–1116 (2010).
6. **Higuchi, T., Hayashi, H., and Abe, K.:** Exchange of glutamate and gamma-aminobutyrate in a *Lactobacillus* strain, J. Bacteriol., **179**, 3362–3364 (1997).
7. **Gaspar, P., Carvalho, A. L., Vinga, S., Santos, H., and Neves, A. R.:** From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria, Biotechnol. Adv., **31**, 764–788 (2013).
8. **Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S., and Hwang, K. T.:** Production of gamma-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100, Int. J. Food Microbiol., **130**, 12–16 (2009).
9. **Wu, Q. L. and Shah, N. P.:** High γ-aminobutyric acid production from lactic acid bacteria: emphasis on *Lactobacillus brevis* as a functional dairy starter, Crit. Rev. Food Sci. Nutr., **57**, 3661–3672 (2017).
10. **Huang, J., Mei, L. H., and Xia, J.:** Application of artificial neural network coupling particle swarm optimization algorithm to biocatalytic production of GABA, Biotechnol. Bioeng., **96**, 924–931 (2007).
11. **Lyu, C. J., Hu, S., Huang, J., Luo, M. Q., Lu, T., Mei, L. H., and Yao, S. J.:** Contribution of the activated catalase to oxidative stress resistance and gamma-aminobutyric acid production in *Lactobacillus brevis*, Int. J. Food Microbiol., **238**, 302–310 (2016).
12. **Huang, J., Mei, L. H., Sheng, Q., Yao, S. J., and Lin, D. Q.:** Purification and characterization of glutamate decarboxylase of *Lactobacillus brevis* CGMCC 1306 isolated from fresh milk, Chin. J. Chem. Eng., **15**, 157–161 (2007).
13. **Fan, E. Y., Huang, J., Hu, S., Mei, L. H., and Yu, K.:** Cloning, sequencing and expression of a glutamate decarboxylase gene from the GABA-producing strain *Lactobacillus brevis* CGMCC 1306, Ann. Microbiol., **62**, 689–698 (2012).
14. **Yu, K., Lin, L., Hu, S., Huang, J., and Mei, L. H.:** C-terminal truncation of glutamate decarboxylase from *Lactobacillus brevis* CGMCC 1306 extends its activity toward near-neutral pH, Enzyme Microb. Technol., **50**, 263–269 (2012).
15. **Lyu, C. J., Zhao, W. R., Hu, S., Huang, J., Lu, T., Jin, Z. H., Mei, L. H., and Yao, S. J.:** Physiology-oriented engineering strategy to improve gamma-aminobutyrate production in *Lactobacillus brevis*, J. Agric. Food Chem., **65**, 858–866 (2017).
16. **Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., and Grutter, M. G.:** Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase, EMBO J., **22**, 4027–4037 (2003).
17. **Dutyshev, D. I., Darii, E. L., Fornenkova, N. P., Pechik, I. V., Polyakov, K. M., Nikonov, S. V., Andreeva, N. S., and Sukhareva, B. S.:** Structure of *Escherichia coli* glutamate decarboxylase (GADα) in complex with glutarate at 2.05 Å resolution, Acta Crystallogr. D, **61**, 230–235 (2005).
18. **Gut, H., Pennacchietti, E., John, R. A., Bossa, F., Capitani, G., De Biase, D., and Grutter, M. G.:** *Escherichia coli* acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB, EMBO J., **25**, 2643–2651 (2006).
19. **Ho, N. A. T., Hou, C. Y., Kim, W. H., and Kang, T. J.:** Expanding the active pH range of *Escherichia coli* glutamate decarboxylase by breaking the cooperativeness, J. Biosci. Bioeng., **115**, 154–158 (2013).
20. **Shin, S. M., Kim, H., Joo, Y., Lee, S. J., Lee, Y. J., Lee, S. J., and Lee, D. W.:** Characterization of glutamate decarboxylase from *Lactobacillus plantarum* and its C-terminal function for the pH dependence of activity, J. Agric. Food Chem., **62**, 12186–12193 (2014).
21. **Choi, J. W., Yim, S. S., Lee, S. H., Kang, T. J., Park, S. J., and Jeong, K. J.:** Enhanced production of gamma-aminobutyrate (GABA) in recombinant *Corynebacterium glutamicum* by expressing glutamate decarboxylase active in expanded pH range, Microb. Cell Fact., **14**, 21 (2015).
22. **Li, H. X., Qiu, T., Huang, G. D., and Cao, Y. S.:** Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation, Microb. Cell Fact., **9**, 85 (2010).
23. **Huang, J., Le-He, M., Wu, H., and Lin, D. Q.:** Biosynthesis of gamma-aminobutyric acid (GABA) using immobilized whole cells of *Lactobacillus brevis*, World J. Microbiol. Biotechnol., **23**, 865–871 (2007).
24. **Mierau, I. and Kleerebezem, M.:** 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*, Appl. Microbiol. Biotechnol., **68**, 705–717 (2005).
25. **Pavan, S., Hols, P., Delcour, J., Geoffroy, M. C., Grangette, C., Kleerebezem, M., and Mercenier, A.:** Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study in vivo biological effects, Appl. Environ. Microbiol., **66**, 4427–4432 (2000).
26. **Marquez, F. J., Quesada, A. R., Sanchezjimenez, F., and Decastro, I. N.:** Determination of 27 dansyl amino acid derivatives in biological fluids by reversed phase high performance liquid chromatography, J. Chromatogr., **380**, 275–283 (1986).
27. **Lopez, A., Lazaro, N., and Marques, A. M.:** The interphase technique: a simple method of cell immobilization in gel-beads, J. Microbiol. Methods, **30**, 231–234 (1997).
28. **Woodward, J.:** Methods of immobilization of microbial cells, J. Microbiol. Methods, **8**, 91–102 (1988).
29. **Sanders, J. W., Leenhouts, K., Burghoorn, J., Brands, J. R., Venema, G., and Kok, J.:** A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation, Mol. Microbiol., **27**, 299–310 (1998).
30. **Li, H. X., Li, W. M., Liu, X. H., and Cao, Y. S.:** *gadA* gene locus in *Lactobacillus brevis* NCL912 and its expression during fed-batch fermentation, FEMS Microbiol. Lett., **349**, 108–116 (2013).
31. **Datta, S., Christena, L. R., and Rajaram, Y. R. S.:** Enzyme immobilization: an overview on techniques and support materials, 3 Biotech, **3**, 1–9 (2013).
32. **Wang, X., Gai, Z., Yu, B., Feng, J., Xu, C., Yuan, Y., Lin, Z., and Xu, P.:** Degradation of carbazole by microbial cells immobilized in magnetic gellan gum gel beads, Appl. Environ. Microbiol., **73**, 6421–6428 (2007).
33. **Shi, S., Qu, Y., Ma, F., and Zhou, J.:** Bioremediation of coking wastewater containing carbazole, dibenzofuran and dibenzothiophene by immobilized naphthalene-cultivated *Arthrobacter* sp. W1 in magnetic gellan gum, Bioresour. Technol., **166**, 79–86 (2014).
34. **Giavasis, I., Harvey, L. M., and McNeil, B.:** Gellan gum, Crit. Rev. Biotechnol., **20**, 177–211 (2000).
35. **Chung, T. P., Tseng, H. Y., and Juang, R. S.:** Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems, Process Biochem., **38**, 1497–1507 (2003).
36. **Chen, X. H., Wang, X. T., Lou, W. Y., Li, Y., Wu, H., Zong, M. H., Smith, T. J., and Chen, X. D.:** Immobilization of *Acetobacter* sp. CCTCC M209061 for efficient asymmetric reduction of ketones and biocatalyst recycling, Microb. Cell Fact., **11**, 119 (2012).