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ORIGINAL PAPER

Biosynthesis of γ-aminobutyric acid (GABA) using immobilized whole cells of *Lactobacillus brevis*

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Abstract On an industrial scale, the production of γ-aminobutyric acid (GABA) from the cheaper sodium L-glutamate (L-MSG) is a valuable process. By entrapping *Lactobacillus brevis* cells with higher glutamate decarboxylase (GAD) activity into Ca-alginate gel beads, the biotransformation conditions of L-MSG to GABA were optimized with the immobilized cells. The cells obtained from a 60-h culture broth showed the highest biotransformation efficiency from L-MSG to GABA. The optimal cell density in gel beads, reaction pH and temperature were 11.2 g dry cell weight (DCW) l^{-1}, 4.4 and 40°C respectively. The thermal stability of immobilized cells was significantly higher than free cells. Under the optimized reaction conditions, the yield of GABA reached above 90% during the initial five batches and the yield still remained 56% in the tenth batch. Continuous production of GABA was realized with a higher yield by incorporating cell re-cultivation using the packed bed reactor.

Keywords γ-Aminobutyric acid · Glutamate decarboxylase · Immobilization · Optimization · Packed bed reactor

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Introduction

γ-Aminobutyric acid (GABA), a four-carbon non-protein amino acid, acts as a major inhibitory neurotransmitter in the central nervous system (Krnjevic 1974). GABA has several physiological functions such as neurotransmission, induction of hypotensive effects, diuretic effects, treatment of epilepsy and tranquilizer effects (Jakobs et al. 1993; Cohen et al. 2002; Komatsuzaki et al. 2005). Some recent studies showed that GABA was also a strong secretagogue of insulin from the pancreas effectively preventing diabetes (Adeghate and Ponery 2002; Hagiwara et al. 2004). To date, GABA has been used extensively in pharmaceuticals and functional foods such as gammalone, cheese, gabaron tea, and shochu (Nomura et al. 1998; Sawai et al. 2001; Yokoyama et al. 2002).

Due to the increasing commercial demand for GABA, various chemical and biological synthesis methods for GABA have been studied (Plokhov et al. 2000; Komatsuzaki et al. 2005; Choi et al. 2006). Biosynthesis of GABA may be a much more promising method due to simple reaction procedure, high catalytic efficiency, mild reaction conditions and environmental compatibility. Glutamate decarboxylase (GAD) is the unique enzyme to catalyse the conversion of L-glutamate or its salts to GABA through the single-step α-decarboxylation (Ueno et al. 2000; Battaglioli et al. 2003). Immobilized cell technology is an interesting method for preparation of metabolic products in the pharmaceutical, food, and other industries. It can be advantageous to use immobilized microbial cells with GAD activity, thus avoiding separation and purification of the enzyme, simplifying the product purification process, and enhancing long-term operation stability.

The production of GABA by batch fermentation has been reported using various microorganisms, but the recovery of GABA from such complex fermentation broth is generally difficult and expensive to perform (Kono et al. 2000; Plokhov et al. 2000; Komatsuzaki et al. 2005; Choi et al. 2006). In this work, the *Lactobacillus brevis* cells were entrapped into Ca-alginate gel beads and the biotransformation of sodium L-glutamate (L-MSG) to GABA was investigated with the immobilized whole cells.

Materials and methods

Material and strain

All chemicals were of analytical reagent grade and used without further purification. GABA standard was purchased from Acros Organics (Geel, Belgium). Dansyl chloride (Dns-cl) was obtained from Sigma-Aldrich (St. Louis, USA). 5′-Pyridoxal phosphate (PLP) was provided by Shanghai Sangon Bioengineering Co. Ltd (Shanghai, China). L-MSG and other chemicals were purchased from China Medicine Co. Ltd (Shanghai, China). The strain used in the present work was isolated from fresh milk without pasteurization and then mutagenized with ultraviolet treatment and ^{60}Co radiation (Xia 2006). It is kept in the China General Microbiological Culture Collection Center as *Lactobacillus brevis* CGMCC 1306.

Cultivation and preparation of immobilized *Lactobacillus brevis* cells

The glucose-yeast extract-peptone (GYP) medium for seed preparation contained (g l^{-1}): glucose, 10; yeast extract, 10; peptone, 5; sodium acetate, 2; $MgSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; NaCl, 0.001; pH 6.8. The fermentation medium consisted of the following (g l^{-1}): glucose, 17.6; yeast extract, 15; peptone, 5; sodium acetate, 3; $FeSO_4 \cdot 7H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.001; $MnSO_4 \cdot 4H_2O$, 0.019; L-MSG, 10; pH 6.8. One loop of slant culture was inoculated into 50 ml seed medium in a 250-ml Erlenmeyer flask and incubated without agitation at 30°C for 24 h. The seed culture was then inoculated at 1% (v/v) into 250 ml fermentation medium in a 500-ml Erlenmeyer flask and cultivated without agitation at 30°C. After 60 h cultivation, the cells were harvested by centrifugation at 6,000 × *g* for 8 min at 4°C. Wet cells were added to sodium alginate solution (15 g l^{-1}) and the mixed solution was added drop by drop into calcium chloride solution (20 g l^{-1}). The gel beads thus formed were continuously stirred in calcium chloride solution for 30 min. Then the beads were collected by filtration, rinsed several times and stored in the distilled water at 4°C. The beads with a mean diameter (Dm) of about 2.2 mm were prepared without tails.

Diffusion coefficient of L-MSG during various cell densities

Ten-milliliter gel beads with different cell density were added rapidly into the well-stirred 20 ml sodium acetate buffer (50 mM, pH 4.4) containing 30 mM L-MSG at 35°C. Since the solute could diffuse into these gel beads, the solute concentration in bulk solution would decrease. 50 μl of sample was withdrawn from the bulk solution periodically and analyzed by HPLC. A diffusion equation (Crank 1975) for a solid sphere in solution of limited volume was used to calculate the diffusion coefficient:

$$C_L = C_{r=R} = \frac{\alpha C_0}{1+\alpha}\left[1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)}{9+9\alpha+q_n^2\alpha^2} \exp\left(-\frac{Dq_n^2 t}{R^2}\right)\right] \quad (1)$$

where R is the external radius of the sphere, C_0 the initial concentration of the bulk solution without capsules, t the time, α the ratio of volume of liquid to the capsules, and D the diffusion coefficient in a solid sphere. The q_n terms are the nonzero positive roots of the following equation:

$$\tan q_n = \frac{3q_n}{3 + \alpha q_n^2} \quad (2)$$

The cells were heated to 80°C in order to inactivate GAD prior to the immobilization.

Optimization of batch reaction conditions

0.066 g (dry cell weight, DCW) of the immobilized cells was added to 25 ml reaction mixture (50 ml flask) including 50 mM sodium acetate buffer, 10 μM PLP and 0.75 mmol L-MSG. The mixture was shaken at 150 rev min^{-1}. Temperature was 20–50°C and pH was 3.6–5.6. The supernatant (0.1 ml) was sampled at 0.5 h intervals and used for the analysis.

Comparison of packed bed and batch reactors

Continuous GABA production in a packed bed reactor was carried out in a column reactor (∅32 × 500 mm) equipped with a jacket for temperature control. The conversion obtained at a variety of feed flow rates in

the packed bed reactor is compared with that obtained at various times observed in a batch stirred tank reactor according the literature described by Hasegawa et al. (1998).

Analysis methods

The concentrations of L-MSG and GABA were determined by HPLC (Márquez et al. 1986; Chen et al. 1997). Agilent 1100 series HPLC system equipped with a Hypersil ODS C_{18} reverse-phase column (250 × 4.6 mm I.D., 5 μm particle) was used. The HPLC conditions were flow rate of 1.0 ml min^{-1}, u.v. detection at 254 nm and ambient column temperature. A gradient elution program with A (methanol)/B (tetrahydrofuran/methanol/50 mM pH 6.2 sodium acetate, 5:75:420, by vol.) as mobile phase was described as Chen et al. (1997). The retention time of L-MSG and GABA was 9.8 and 19.5 min, respectively. 100 μl of sample was mixed with 900 μl sodium bicarbonate solution (0.2 M, pH 9.8), and then the derivatization was carried out by adding 100 μl of the above mixture into 100 μl Dns-cl (8 g l^{-1}) and incubating at 30°C for 1 h. After derivatization, the sample was diluted and subjected for HPLC analysis. The initial reaction rate (V_0) was defined as the amount that produced GABA per gram DCW in the initial reaction time. The conversion (x_s) of L-MSG and yield (x_p) of GABA can be defined as follows:

$$x_s(\%) = \frac{M_{S0} - M_S}{M_{S0}} \times 100 \quad (3)$$

$$x_p(\%) = \frac{M_p}{M_{S0}} \times 100 \quad (4)$$

where M_{s0} is the mole of L-MSG added, M_s and M_p are the mole of L-MSG and GABA in the product.

Results and discussion

Effect of fermentation time on the biotransformation ability (BTA)

GAD enzyme activity differs during different growth phases of cells because it is an induced enzyme present in cytoplasm to achieve resistance to acid (Fonda 1985; Small and Waterman 1998). As shown in Table 1, BTA could not be detected during the first 24 h and then BTA increased with the extension of fermentation time, reaching a maximum at 60 h and then decreasing.

Table 1 Effect of fermentation time on the biotransformation ability (BTA) and biomass[a]

Fermentation time (h)	Biomass (g DCW/l)	BTA/Biomass (U/g DCW)
12	0.42	0
24	1.10	1.61
36	1.44	20.48
48	1.55	60.97
60	1.68	98.37
66	1.69	92.01
84	1.59	77.39
91	1.51	44.07

[a] The reaction mixture (25 ml) comprised of Na_2HPO_4-citric acid buffer (100 mM, pH 4.8), 60 mM L-MSG and the wet cells (0.066 g DCW) harvested from different fermentation time. The reaction mixture was incubated at 30 °C for 1 h, and centrifuged at 6,000 rev min^{-1} for 8 min, and the supernatant was analyzed for GABA. One unit (U) of the biotransformation ability (BTA) from L-MSG was defined as the amount of the formation of 1 μmol of GABA per min under the test conditions

In the following experiments, the resting cells obtained from a 60-h culture broth were used.

Optimization of cell density in *Ca-alginate gel beads*

In order to obtain higher reaction rate of GABA production, the effects of *Lactobacillus brevis* cells incorporated into the gel beads on the diffusion coefficient of L-MSG and catalytic activity were studied.

As shown in Fig. 1a, L-MSG concentration decreased rapidly after the gel beads were added into the solution and the diffusivity of L-MSG decreased with increasing cell density. The diffusion coefficient (D) of L-MSG under different cell density could be calculated based on the results of Fig. 1a. As shown in Fig. 1b, D decreased linearly from 9.74×10^{-10} m^2 s^{-1} to 2.92×10^{-10} m^2 s^{-1} when the cell density in Ca-alginate gel beads was increased from 0 to 22.2 g DCW l^{-1}. The possible explanation was that the cells (single or in aggregates) occupied the originally empty pores in the gel beads, leading to the additional obstruction effect by increasing the tortuosity of gel beads (Øyaas et al. 1995; Chen et al. 2003). Fig. 1b also showed that the optimal cell density in Ca-alginate gel beads was about 11.2 g DCW l^{-1}. When the cell density was less than 11.2 g DCW l^{-1}, the initial reaction rate (V_0) was lower than substrate diffusion rate and the system was known as the reaction-limited regime. If cell density exceeded 11.2 g DCW l^{-1}, the rate of substrate diffusion decreased and was lower than the substrate consumption rate and the reaction rate observed could be considered as diffusion-limited.

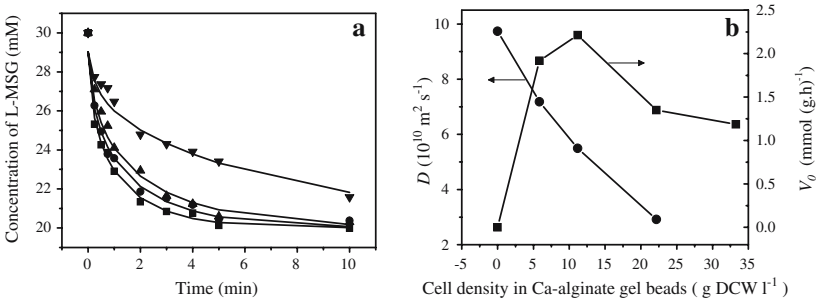

Fig. 1 (**a**) Diffusion of L-MSG from solution into Ca-alginate beads during various cell density. Cell density in gel beads: (■) 0 g DCW l^{-1}; (●) 5.85 g DCW l^{-1}; (▲) 11.17 g DCW l^{-1}; (▼) 22.17 g DCW l^{-1}. (**b**) Effect of cell density on the diffusion coefficient (D) of L-MSG and initial reaction rate (V_0). Biotransformation was carried out with sodium acetate buffer (50 mM, pH 4.4), 10 μM PLP, and a 150 rev min^{-1} shaking speed at 35 °C

Effects of substrate and product concentrations on the biotransformation of L-MSG to GABA

When the initial substrate concentration of L-MSG was within the range of 0–40 mM, V_0 increased with increasing substrate concentration and finally leveled off (Fig. 2a). When fixing the initial substrate L-MSG concentration at 30 mM and increasing the initial GABA concentration from 0 to 80 mM, Fig. 2b shows that V_0 decreased from 2.21 mmol $(g\ h)^{-1}$ to 0.96 mmol $(g\ h)^{-1}$. The result showed that feedback inhibition by the product GABA existed.

Effects of pH and temperature on the biotransformation of L-MSG to GABA

One pathway for GABA decomposition exists in a large variety of plants and microorganisms: GABA transaminase catalyses the reversible conversion of GABA to succinic semialdehyde using either pyruvate or α-ketoglutarate as amino acceptor and succinic semialdehyde dehydrogenase catalyses the reversible conversion of succinic semialdehyde to succinate (Shelp et al. 1999; Kumar et al. 2000). Therefore, it was necessary to block the two enzymes which have the alkaline pH optimum by adjusting the pH of the buffer (Shelp et al. 1999; Kumar et al. 2000). Figure 3a showed that the optimum pH for reaction with immobilized cells was at 4.4 and the corresponding yield of GABA reached about 82.3%.

Temperature has not only an effect on biocatalyst activity and stability, but the thermodynamic equilibrium of a reaction. As shown in Fig. 3b, higher temperature resulted in a higher reaction rate and GABA yield within the range from 25 to 40°C. However, V_0 and GABA yield decreased with further increase of temperature. The optimum reaction temperature was at 40°C and the corresponding yield of GABA after 8 h reached about 92%.

Comparison of immobilized and free *Lactobacillus brevis* cells thermal stability

Immobilized and free cells were incubated for 30 min at different temperature between 30°C and 70°C, and then put into sodium acetate and Na_2HPO_4-citric acid buffer (50 mM, pH 4.4) respectively, containing 0.75 mmol L-MSG at 40°C. Figure 4 shows the relative

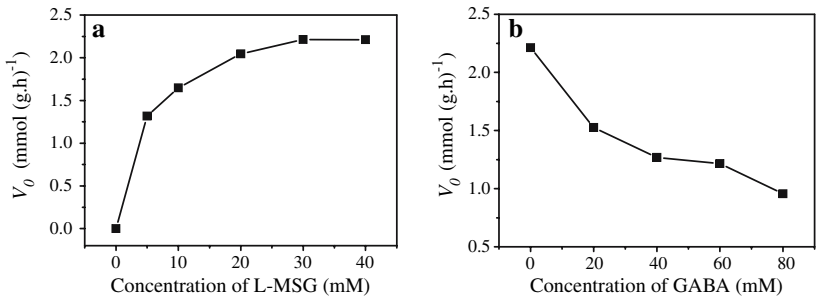

Fig. 2 (**a**) Effect of L-MSG concentration on the biotransformation of L-MSG to GABA. (**b**) Effect of GABA concentration on the biotransformation of L-MSG to GABA. Biotransformation was carried out with sodium acetate buffer (50 mM, pH 4.4), 10 μM PLP, 0.066 g DCW, and a 150 rev min^{-1} shaking speed at 35°C

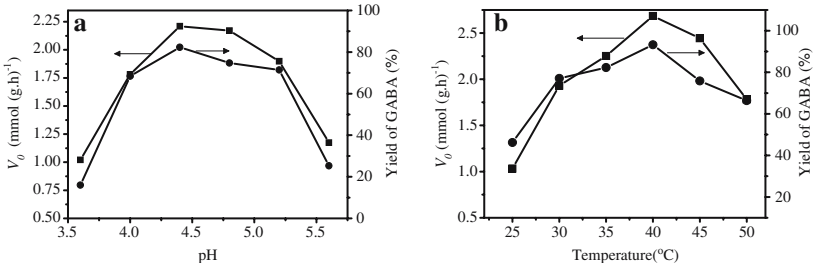

Fig. 3 **(a)** Effect of pH on the biotransformation of L-MSG to GABA. Biotransformation was carried out with sodium acetate buffer (50 mM, pH 3.6–5.6), 0.066 g DCW, 10 μM PLP, 35 °C and a 150 rpm shaking speed for 8 h. **(b)** Effect of reaction temperature on the biotransformation of L-MSG to GABA. Biotransformation was carried out with sodium acetate buffer (50 mM, pH 4.4), 0.066 g DCW, 10 μM PLP, 20–50 °C and a 150 rev min^{-1} shaking speed for 8 h

V_0 of immobilized cells still remained 70% after incubation 30 min at 70°C, while 36.6% of the free cells relative V_0 could be observed after being kept at the same temperature for 30 min. The result indicated that the thermal stability of immobilized cells was significantly enhanced. The possible reason was that the Ca-alginate gel beads would provide a favorable microenvironment for immobilized cells which might minimize the deactivation of GAD due to the thermal transport resistance effect.

Repeated batch process of immobilized *Lactobacillus brevis* cells

The usefulness of immobilized *Lactobacillus brevis* cells was examined by its reusability in the biotransformation from L-MSG to GABA. From the results presented in Fig. 5 can be seen that GABA yield reached above 90% during five cycles under the optimized reaction conditions. After that, GABA yield declined and still reached more than 74% after eight-times recycling of the immobilized cells, while it dropped to 56% at the tenth repeated run. The result showed that the immobilized cells were stable and efficient for GABA production in multi-batch processes.

Performance comparison of batch and packed bed reactors and continuous production of GABA in packed bed reactor

Figure 6 illustrates the relationship between the conversion and the variables of batch stirred tank reactor and packed bed reactor. When the substrate concentration was 30 mM, the differences of conversion with the two types of reactors were not remarkable.

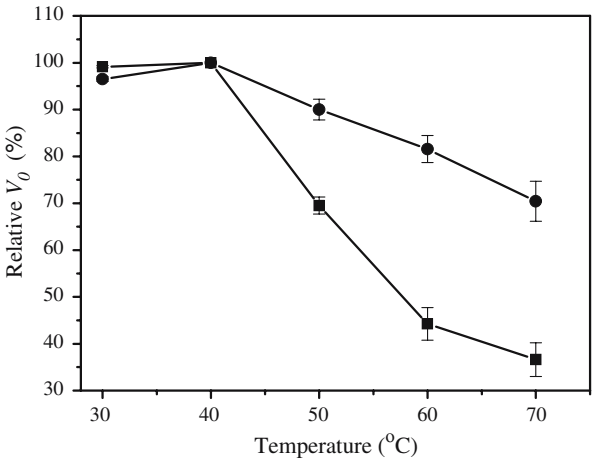

Fig. 4 Comparison of immobilized and free *Lactobacillus brevis* cells thermal stability (●) immobilized cells; (■) free cells. Each data point represents the mean ± SD from three independent samples. Biotransformation for the immobilized cells was carried out with sodium acetate buffer (50 mM, pH 4.4), 0.066 g DCW, 10 μM PLP and a 150 rpm shaking speed. Biotransformation for free cells was carried out with Na_2HPO_4–citric acid buffer (50 mM, pH 4.4), 0.066 g DCW and without agitation

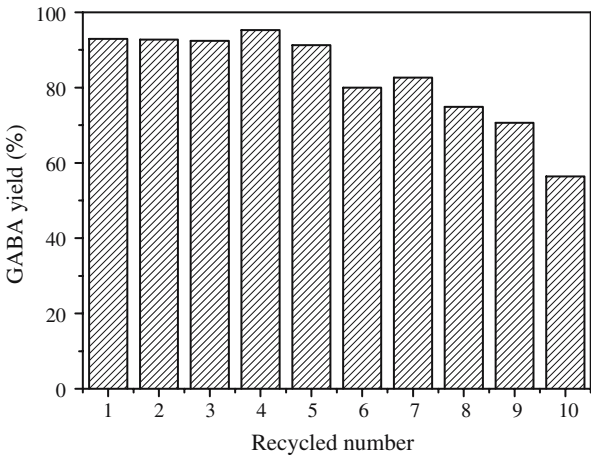

Fig. 5 GABA yield of sequential 10 batches operation with immobilized whole cells of *Lactobacillus brevis*. Biotransformation of each batch was carried out with sodium acetate buffer (50 mM, pH 4.4), 0.066 g DCW, 0.75 mmol L-MSG, 10 μM PLP and a 150 rev min^{-1} shaking speed at 40°C for 8 h

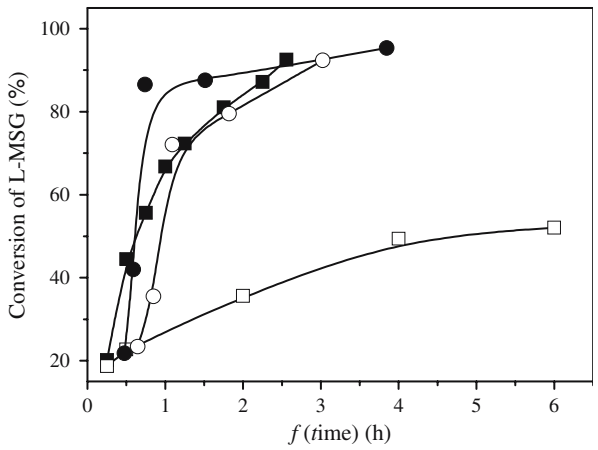

Fig. 6 Performance comparison of batch and packed bed reactors. f (time) represented the reaction time ($V_g t/V$) for batch stirred tank reactor and the retention time ($(1-\varepsilon_b)Z/u_0$) for packed bed reactor, respectively. V represents the volume of the reaction liquid, V_g the volume of the immobilized gel beads, and t time; Z is the packed bed height, u_0 the superficial velocity, ε_b the bed voidage. (■) 30 mM L-MSG for batch reactor; (□) 60 mM L-MSG for batch reactor; (●) 30 mM L-MSG for packed bed reactor; (○) 60 mM L-MSG for packed bed reactor. Biotransformation for the batch reactor was carried out with sodium acetate buffer (50 mM, pH 4.4), 0.066 g DCW, 10 μM PLP and a 150 rev min^{-1} shaking speed at 40 °C. The operation conditions of packed bed reactor were as follows: 95 mL immobilized gel beads containing 1.06 g DCW; 50 mM sodium acetate buffer (pH 4.4); 10 μM PLP; 1 mM $CaCl_2$; 40 °C

However, when the substrate concentration reached 60 mM, the conversion of the packed bed reactor outperformed significantly that of the batch reactor.

As shown in Fig. 7, GABA concentration of about 55 mM was obtained and maintained during the first 24 h of the operation. However, the GABA concentration decreased to 28 mM at 48 h. In order to regain GAD catalytic activity, the immobilized cells were then re-cultivated and the encapsulated beads were added to the fermentation medium with a volumetric ratio of 50% (v/v) and incubated at 30°C and 100 rev min^{-1} for 24 h. After the first re-cultivation, GABA concentration recovered to about 44.6 mM, but decreased to 19.9 mM at 80 h. After the second re-cultivation, GABA concentration recovered to about 33.7 mM and remained only about 12.4 mM at 96 h. The possible reasons could be the deactivation of GAD and the change of metabolic flux resulting from the lack of nutrients.

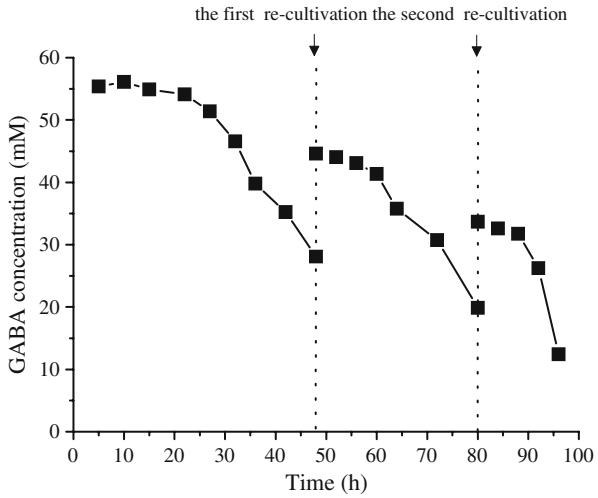

Fig. 7 Time course of GABA concentration in the packed bed reactor. The operation conditions of packed bed reactor were as follows: flow rate 2.4 ml/min; 220 ml immobilized gel beads containing 2.46 g DCW; sodium acetate buffer (50 mM, pH 4.4); 10 μM PLP; 1 mM $CaCl_2$; 40 °C

Conclusions

This paper reports biosynthesis of GABA by immobilized whole cells of *Lactobacillus brevis*. Our work interestingly indicated that the immobilized *Lactobacillus brevis* cells were stable and efficient for biosynthesis of GABA. By this bioprocess, the recovery of GABA from such a simple reaction liquid is generally easy and inexpensive to perform. Given the simplicity and the long-term stability of the GABA production, the use of whole-cell biocatalysts immobilized by Ca-alginate entrapment offers a promising means of GABA production for industrial application.

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