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Preparation of γ-Aminobutyric Acid Using *E. coli* Cells with High Activity of Glutamate Decarboxylase

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Abstract

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter synthesized in the central nervous system from glutamate by glutamate decarboxylase (GAD). It has applications in the production of many drugs. The technology of GABA synthesis by treating L-glutamic acid with the cells of the gene-engineered GAD superproducer strain of *Escherichia coli* GADK10 was developed. Cell growing in the presence of 0.02 m*M* pyridoxal phosphate (PLP) causes the 2- to 2.5-fold increase of total productivity of the cells. The best way to prepare the cells for the reaction was their thermal activation by pretreatment for 1 h at 53°C. The optimal conditions for this reaction were 37°C and pH 4.6. The rate of the enzymatic reaction is the function of acetate concentration with the maximum at 0.5 *M* acetate. The total amount of GABA synthesized using 1 g of wet cells reached 23–25 g. The final concentration of GABA in the reaction medium was 280–300 g/L. The yield of the product was about 99%.

Index Entries: γ-Aminobutyric acid; glutamate decarboxylase; *Escherichia coli*; pyridoxal 5-phosphate.

Introduction

γ-Aminobutyric acid is an efficient neurotransmitter of inhibition in the central nervous system (CNS) *(1)*.

GABA is widely used in medicine in a number of drugs, such as aminalone, gammalone, pycamilone, pantogam, for the treatment of

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disturbances of brain activity, including those of memory and speech, hemiplegia, and hypertension *(2,3)*.

There are several chemical methods of GABA synthesis *(1)*, the pitfalls of which are severe process conditions and expensive raw materials.

The enzymatic method for GABA preparation from L-glutamic acid using glutamate decarboxylase of *Escherichia coli* (EC 4.1.1.15) was first described in refs. *4* and *5*. The reaction is expressed by the following scheme:

$$HOOC—CH_2—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH \Rightarrow HOOC—CH_2—CH_2—CH_2—NH_2 + CO_2\uparrow$$

Almost complete conversion of the substrate into the target product is achieved in this reaction.

Low activity of cell preparations was the main restriction for commercial enzymatic synthesis until now. We have developed a highly productive *E. coli* strain GADK10 (available from the All-Russian Collection of Industrial Microorganisms) providing the GABA-saving technology.

This work aimed to optimize the enzymatic synthesis of GABA using the described superproducer.

Materials and Methods

Construction of GAD Overproducing Strain

The cloning of *gad*A *E.coli* gene and the construction of the expression vector pGAD1 are described in ref. *6*. The plasmid pGADK10 conferring resistance to two antibiotics—ampicillin and canamicin—was assembled from *Bam*HI restriction fragments of pUC4K and pGAD1. It consists of the tandem of two consecutive pGAD1 fragments, containing GAD expression cassette, and a single fragment of pUC4K, containing *neo* gene. The overproducing strain GADK10 that was used in this work was prepared by transformation of the *E. coli* strain BL21(DE3) with pGADK10 plasmid.

Growing Conditions

The producer strain was grown at 37°C in a fermenter (Model MSJ-301, Marubishi Laboratory Equipment, Japan) in 20 L of nutrient medium of the following composition: 10.0 g/L of glucose; 0.2 g/L of monopotassium phosphate; 15.0 g/L of protein hydrolysate; 0.5 g/L of magnesium sulfate; 1.0 g/L of ammonium chloride; 5.0 g/L of peptone; 0.05 g/L of kanamycin; up to 20 L of tap water (pH 7.0). The mixer rotation speed was 300 min^{-1}, the flow rate of sterile air was of 1:1 [v/v] per min The automatic pH-stating control at pH 7.0 ± 0.1 was carried out by subtitration with glucose–ammonia mixture with glucose and ammonia concentrations of 450.0 and 62.5 g/L, respectively. Optical density (D) of culture medium was measured with a spectrophotometer (Model SP-46, LOM Company, Russia) at 540 nm. When the process was over, the culture medium was cooled to 15°C and the cells were separated in a separator (Model ASG-3M, Plava, Russia) at 4000*g*. The obtained biomass was frozen at –20°C.

Cell Preparation for Synthesis

Cell treatment with organic solvents and thermal activation were carried out as follows. Sixty milligrams of frozen biomass were introduced into 15 mL of 0.9% aqueous solution of NaCl and stirred with a magnetic stirrer for 15 min. The suspension was divided into three equal portions. Twenty-five microliters of ethyl acetate, 12.5 μL toluene + 12.5 μL NaCl solution, and 25 μL NaCl solution were added to the first, second, and third portion, respectively, then mixtures 1 and 2 were incubated in a shaker for 45 min at 37°C, and mixture 3 was incubated at 53°C.

Sonication of the cells was carried out with a disintegrator (Model MSE 200, Scientific Instruments, UK) for 1 min at an amplitude of 10 μm.

After the treatment, the suspensions were separated by centrifugation for 10 min at 5000*g* in a centrifuge (Model RC-5B, Sorvall, France). The pellet was used for GABA synthesis.

Biotransformation of L-Glutamic Acid

Concentrations of L-glutamic acid and GABA assayed by paper chromatography on FN-11 paper (German) in butanol:acetic acid:water mixture 4:1:2. GAD activity was determined after 15 min incubation of the cells at the biomass concentration of 0.25 mg wet wt/mL of 1% solution of L-glutamic acid at 37°C and pH 4.6. The reaction was terminated by the addition of 30 μL 30% NaOH. The enzyme activity was expressed in units (1 U—1 μ*M* GABA in 1 min at 37°C). GAD specific activity was expressed in U/mg cells, wet wt.

Protein was determined according to Bradford *(7)*.

Total cell productivity (TCP) was determined as the product amount formed by 1 g of wet biomass during transformation. The synthesis was carried out in fermenters (Model BioFlo C30, New Brunswick, NJ) with the working volume of 500 mL at the initial concentration of glutamic acid 100 g/L, cell concentration 2 g/L, the mixer rotation rate 100 min^{-1} with controlled temperature and pH. pH adjustment was achieved by the addition of 35% hydrochloric acid to reaction mixture.

L-Glutamic acid, γ-aminobutyric acid, and pyridoxal phosphate were from Serva, Germany. Other reagents produced in Russia were of the highest quality available.

Results and Discussion

It is known from the literature *(4,5)* that in the result of decarboxylation L-glutamic acid is almost completely converted into GABA without by-product formation. According to the classification proposed in ref. *8*, GABA biosynthesis is a process with a labile biocatalyst and stable substrate and product. For this reason, the biotransformation rate and TCP were taken as main criteria for the process optimization. The rate of biotransformation was dependent on the enzyme activity and its

intracellular concentration. In addition, TPC depends on the enzyme stability.

Growing the GAD Producer

Effect of PLP on GAD Biosynthesis

One of the factors responsible for enzyme inactivation during L-glutamic acid decarboxylation is the side transamination reaction resulting in PLP (GAD coenzyme) conversion to pyridoxamine 5-phosphate *(9)*. To estimate the PLP effect on GAD biosynthesis, the culture was grown on the above-described nutrient medium with the different PLP content, and the GAD and TCP dependence on the coenzyme concentration was determined. The addition of 0.02 PLP caused a double increase in GAD specific activity (Fig. 1A, curve 1).

A similar coenzyme effect was observed with TCP (Fig. 1A, curve 2). Optimal PLP concentration was equal to 0.04 m*M* and it was used in further studies.

Intracellular Content of the Enzyme and Its Molecular Mass

The gene engineering techniques and optimization of GAD biosynthesis allowed us to obtain an ultra-high level of the target enzyme production. This is illustrated by the electropherogram of protein preparations (Fig. 1B) isolated after sonication of the initial plasmid-free *E. coli* strain BL-21 (DE3) and developed recombinant *E. coli* strain GADK10.

The molecular mass of the enzyme subunit is 50 kD; this value is in agreement with the previous data *(10)*.

Analysis of Productivity of GAD Biosynthesis by *E. coli* GADK10

Figure 2 shows comparative characteristics of two GAD-producing strains. Analysis of these data indicates that *E. coli* strain GADK10 exhibits the best growth as compared with the wild-type GAD producer *E.coli* 600 *(9)*. In this case, the total enzymatic activity at the end of fermentation is 10- to 20-fold higher than that for other cultures. The use of our strain makes possible a marked decrease in fermentation expenses.

Preparation of the Cells for Biotransformation

Because GAD is an intracellular enzyme, the increase of the cell wall permeability controls the substrate access to the enzyme. To increase the permeability, the cells underwent a special treatment including sonication, treatment with ethyl acetate, treatment with toluene, and thermal activation.

Analysis of the GAD activity dependence on the cell treatment method (Fig. 3A) shows that thermal activation is the best, since this procedure doubles the enzyme activity and avoids toxic solvents. For these reasons, only thermally activated biomass was used in our further experiments.

The dependence of GAD-specific activity on thermal activation duration is shown in Fig. 3B. The activity maximum was achieved in 1 h of cell incubation at 53°C, the activity drop to the end of the incubation was 40%

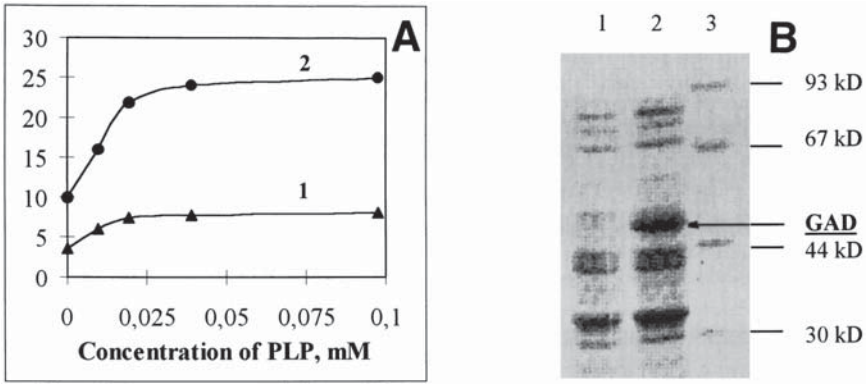

Fig. 1. **(A)** PLP effect on GAD biosynthesis; 1, GAD activity, U/mg; 2, TCP, g GABA/g cells. (B) 10% SDS-PAGE: 1, initial plasmid-free strain *E. coli* BL21(DE3); 2, gene-engineering strain *E. coli* GADK10; 3, markers.

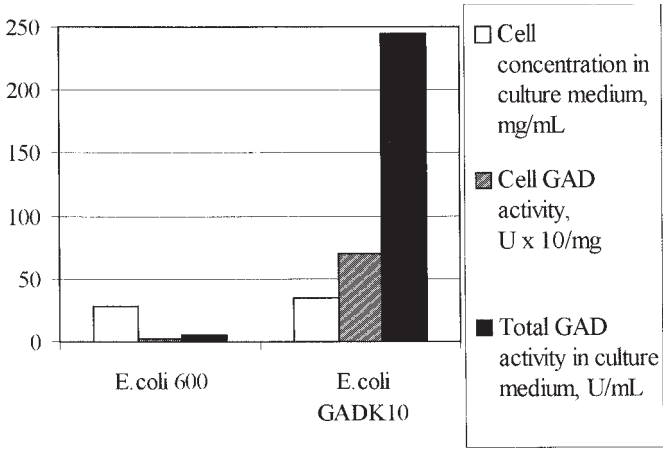

Fig. 2. Comparative characteristics of GAD-producing strains.

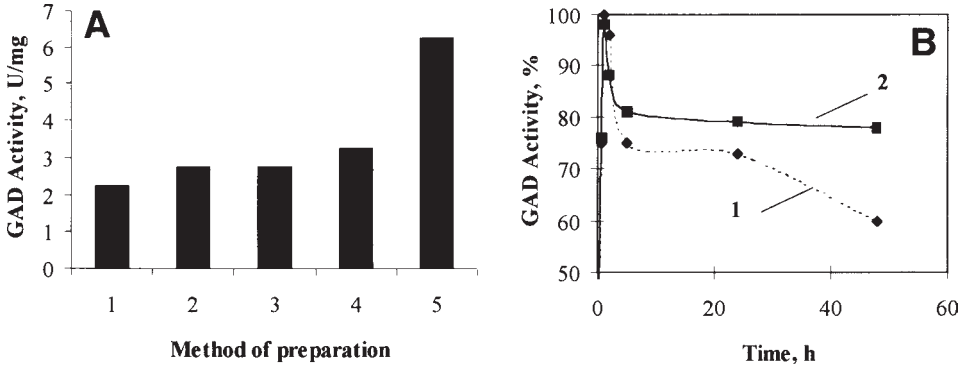

Fig. 3. **(A)** Comparison of different mehtods of the cell wall treatment: 1, without treatment (control); 2, ultrasonication; 3, ethylacetate; 4, toluene; 5, thermal activation. **(B)** Thermal stability of GAD at 53°C (1) and 37°C (2).

(curve 1). When the temperature of the cell suspension was lowered to 37°C after 1 h incubation, this drop was lower—20% (curve 2).

Based on the obtained results, the following regime of biomass thermal activation was proposed:

1. biomass freezing at –20°C and maintaining this temperature for 24 h;
2. biomass thawing and incubation at 53°C for 1 h;
3. incubation of cell suspension during L-glutamic acid biotransformation at 37°C.

Biotransformation of L-Glutamic Acid

Effect of Temperature and pH

To optimize biotransformation conditions, the effects of temperature and pH on the reaction rate and TCP were studied. The temperature increase from 34 to 70°C shifted the optimal pH value from 4.0 to 4.5 (Fig. 4). The maximum reaction rate was equal to 25 g GABA/h per 1 L at 37°C and pH 4.0.

Maximum TCP value (Fig. 4) was equal to 25 g GABA/g wet biomass at pH 4.6, whereas at pH 4.0 it was only 12 g/g. Thus, the pH shift from 4.0 to 4.6 caused a certain decrease in the biotransformation rate and double increase of TCP due to the increasing of enzyme stability. That is why 37°C and pH 4.6 were considered as optimal for GABA synthesis.

Effect of Substrate and Product Concentrations

A peculiar feature of this process is the absence of substrate and product inhibition. The effect of different concentrations of L-glutamic acid and GABA added to the medium in the beginning of the process was studied. The results showed that the rate of biotransformation and GABA yield were unchanged within the interval of L-glutamic acid concentrations from 10 to 300 g/L and those of GABA from 0 to 300 g/L.

The reaction rate reaches maximum value at 100 m*M* glutamic acid concentration (Fig. 5A). The K_m value determined for this substrate is equal to 21 m*M*.

Effect of Acetate

It is known from a number of publications *(11,12)* that acetate inhibits purified GAD. However, other authors have disproved these results *(13)*.

Because acetic acid is used as a titrant during biotransformation, an experiment aimed to elucidate the acetate effect was carried out; see Fig. 5B. For intracellular GAD, the optimum concentration of acetate in reaction mixture is 0.5 *M*. The decarboxylation rate is practically constant until the acetate concentration is 0.8 *M*. At the concentration of 1.5 *M*, the rate falls to the value corresponding to the acetate absence. Further increase in the acetate concentration causes a significant inhibition of enzyme activity. For this reason it is necessary to terminate the biotransformation process when acetate concentration reaches 1.5 *M*.

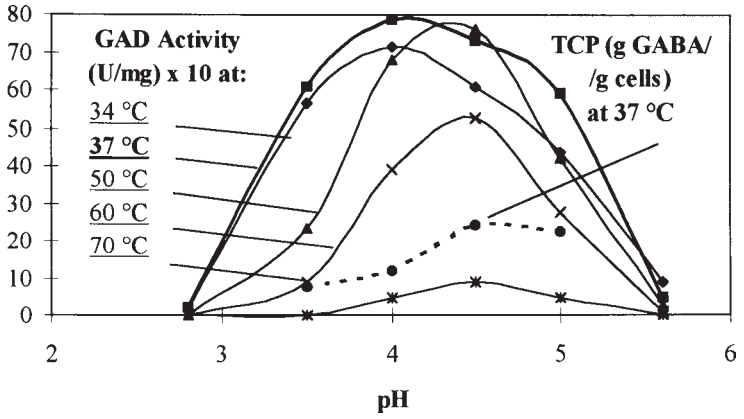

Fig. 4. Dependence of GAD activity on pH at differenct temperatures and TCP dependence on pH at 37°C.

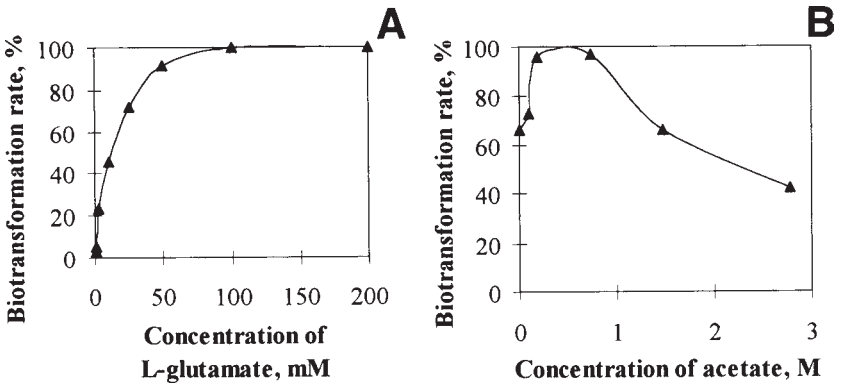

Fig. 5. **(A)** Dependence of biotransformation rate on substrate concentration. **(B)** Dependence of biotransformation rate on acetate concentration.

Effect of PLP

Comparative analysis of GABA synthesis kinetics after introduction of 0.1 m*M* PLP directly into the reaction mixture and in its absence was carried out with the biomass grown without PLP. No increases in TCP and biotransformation rate occur in the presence of PLP (Fig. 6A, curve 2). However, the addition of PLP at the fourth hour of decarboxylation to the control variant (arrow) showed insignificant activation of the enzyme (Fig. 6A, curve 1). Thus, the most efficient application of PLP within the interval of 0.02–0.05 m*M* concentrations is possible only at the step of producer growing.

Biocatalyst Regeneration

Marked foaming is the main limitation for the GABA synthesis rate. Foaming is caused by active formation of carbon dioxide and the presence of surfactants in reaction medium and their release from the cells.

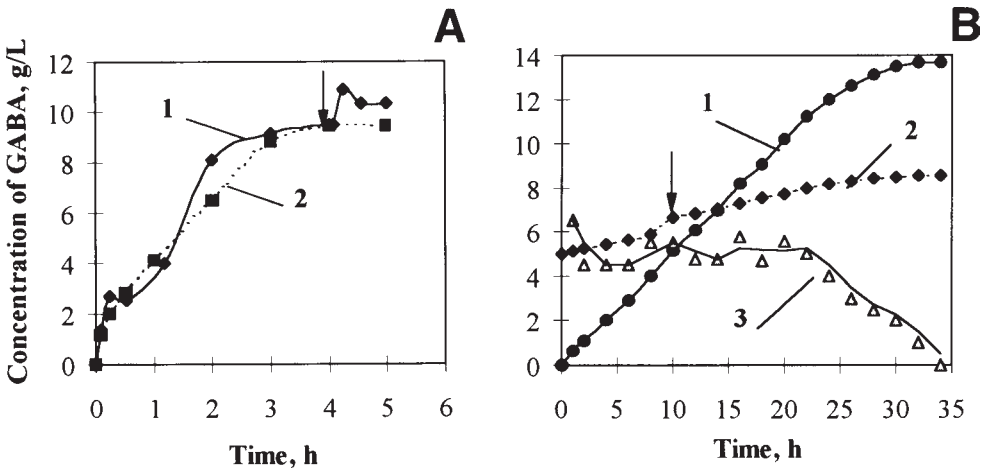

Fig. 6. **(A)** Kinetics of GABA synthesis without PLP (1) and in the presence of 0.1 m*M* PLP (2) in reaction medium. **(B)** Kinetics of synthesis of GABA at optimum conditions: 1, quantity GABA in reactor, g × 0.1; 2, reaction volume, mL × 0.01; 3, current productivity, g/h.

The foaming rate decreases as the surfactants are utilized. The maximally permissible concentration of active biomass in reaction mixture at specific activity of 5–6 U/mg should not exceed 2 mg/mL, otherwise the foam volume will reach 2–3 working volumes of the fermenter. Application of an organic foam suppressor is also inefficient, since its presence makes it difficult for the further purification of GABA. In connection with this, a gradual supply of biomass into the reaction mixture is proposed.

Our data show that with the cell concentration of 2 mg wet biomass/1 mL the biocatalyst can be efficiently used for 4 h. To determine the process optimal productivity at a fixed amount of active cells (approx 1 mg/mL), it is necessary to take into account the enzyme inactivation and to compensate the activity loss with a continuous supply of fresh biomass.

The kinetics of GABA synthesis in the process with additional supply of fresh biomass is shown in Fig. 6B, curve 1. Twice 100 g portions of dry glutamic acid, before biocatalyst addition and at the tenth hour of the reaction (arrow), introduced the substrate. The initial reaction volume was 500 mL. Cell suspension with the wet biomass concentration of 200 mg/mL was introduced in the beginning of the process in 5 mL, and then added at a constant rate of 0.7 mL/h. The synthesis conditions were 37°C and pH 4.6. During the first 24 h, the average productivity was about 5 g/h GABA (curve 3). In this case, a moderate foam volume was maintained 15–20% of the total mixture volume. A gradual decrease in the productivity after 24 h was caused by cessation of the biocatalyst supply. Final volume was 1.5 times larger than the initial one (curve 2), owing to which it was necessary to use a reactor with twice the volume. Period of decarboxylation was equal to 35 h; 138 g GABA were obtained from 200 g L-glutamic acid.

The final substrate concentration was no higher than 0.5 g/L. TCP reached 23 g/g. The extent of L-glutamic acid conversion reached 99% of the theoretical one, and the GABA yield 0.69 g/g of substrate.

Thus, the optimal conditions for L-glutamic acid biotransformation into GABA using the GAD superproducing strain were determined. Almost complete substrate conversion into the product is achieved in the course of the process developed.

GAD overproducing strains were used in biochemical and molecular biological studies *(6,14,15).* Here we developed their use in biotechnology.

The proposed method of GABA synthesis is applied for a large scale production of this amino acid by Mosagrogen company.

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References

1. Sytinsky, I. A. (1972), *γ-Aminobutyric Acid and Activity of the Central Nervous System*, Leningrad, Nauka.
2. Krnjevic, K. (1974), *Physiol. Rev*. **54,** 418–540.
3. Roberts, E. (1974), *Biochem. Pharm.* **23,** 2637–2649.
4. Camien, M. N., Mc Clure, L. E., Lepp, A., and Dunn, M. S. (1953), *Arch. Biochem. Biophys.* **43,** 378–380.
5. Gubarev, E. M. and Galaev, Yu. V. (1960), *Biokhimiya (Moscow)* **25,** 261–263.
6. Schulga, A. A., Kurbanov, F. T., Khristoforov, R. R., Darii, E. L., and Sukhareva, B. S. (1999), *Mol. Biol. (Moscow)* **33,** 560–566.
7. Bradford, M. (1976), *Anal. Biochem.* **72,** 248.
8. Erdman, I. E., Nys, P. S., and Biryukov, V. V. (1986), *Antibiotics Med. Biotechnol.* **4,** 249–254.
9. Sukhareva, B. S. and Braunstein, A. E. (1971), *Mol. Biol.* **5,** 302–317.
10. Strausbauch, P. N. and Fisher, E. (1970), *Biochemistry* **9,** 226–233.
11. Shukuya, R. and Schwert, G. W. (1960), *J. Biol. Chem.* **235,** 123–126.
12. Fonda, M. L. (1972), *Arch. Biochem. Biophys.* **153,** 763–768.
13. Lambrecht, R. H. D., Slegers, G., Mannens, G., amd Claes A. (1987), *Enzyme Microb. Technol.* **9,** 221–224.
14. Khristoforov, R. R., Sukhareva, B. S., Dixon, H. B. F., Sparkes, M. J., Krasnov, V. P., and Bukrina, I. M. (1995), *Biochem. Mol. Biol. Intern.* **36,** 77–85.
15. De Biase, D., Tramonti, A., John, A. R., and Bossa, F. (1996), *Prot. Expr. Purif.* **8,** 430–438.