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Robust production of gamma-amino butyric acid using recombinant *Corynebacterium glutamicum* expressing glutamate decarboxylase from *Escherichia coli*

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ABSTRACT

Gamma-amino butyric acid (GABA) is a component of pharmaceuticals, functional foods, and the biodegradable plastic polyamide 4. Here, we report a simple and robust system to produce GABA from glucose using the recombinant *Corynebacterium glutamicum* strain GAD, which expresses GadB, a glutamate decarboxylase encoded by the *gadB* gene of *Escherichia coli* W3110. As confirmed by HPLC analysis, GABA fermentation by *C. glutamicum* GAD cultured at 30 °C in GABA Production 1 (GP1) medium containing 50 g/L glucose without the addition of glutamate yielded 8.07 ± 1.53 g/L extracellular GABA after 96 h. Addition of 0.1 mM pyridoxal 5′-phosphate (PLP) was found to enhance the production of GABA, whereas Tween 40 was unnecessary for GABA fermentation. Using the optimized GABA Production 2 (GP2) medium, which contained 50 g/L glucose and 0.1 mM PLP, fermentation was performed in a flask at 30 °C with 10% (v/v) seed culture of *C. glutamicum* GAD. GABA was produced in the culture supernatant with a yield of 12.37 ± 0.88 g/L after 72 h with a space–time yield of 0.172 g/L/h, which is the highest yield obtained to date for GABA from fermentation with glucose as a main carbon source.

1. Introduction

Gamma-amino butyric acid (GABA) is a non-protein amino acid widely found in microorganisms, animals, and plants. GABA functions as a neurotransmitter signal in humans, has blood pressure lowering activity [1], and has been used as a component of pharmaceuticals and functional foods [2]. Recently, it was reported that GABA also represents a new building block of bio-plastics. For example, polyamide 4 (PA4) is a linear polymer of GABA which can be chemically synthesized from 2-pyrrolidone, a lactam of GABA [3]. PA4 has excellent physical properties based on its high melting point of 260 °C and biodegradability in the soil [4] and in activated sludge [5]. In contrast, polyamide 6, which is commonly used in plastics and nylon materials, is not biodegradable in soil [6]. It is anticipated that new bio-plastic materials can be synthesized at low cost from abundantly available biomass resources when GABA is produced by recombinant microorganisms.

GABA was originally produced in traditional fermented foods such as Korean kimchi, Chun-gu-chan, yoghurt and cheese by lactic acid bacteria, including *Lactobacillus brevis*, *Lactobacillus lactis*, and *Streptococcus salivarius* [7–9]. These bacteria possess intracellular glutamate decarboxylase (GAD, EC 4.1.1.15) activities and can be used for GABA fermentation. GAD catalyzes the alpha-decarboxylation reaction of L-glutamate to GABA [10]. Bacterial *gad* genes have been identified in *E. coli* [11], *L. brevis* [12], *L. paracasei* [13], and several other *Lactobacillus* and *Enterobacteria* species. Using lactic acid bacteria, GABA is produced by adding glutamate to the fermentation medium as a precursor. As this method is not cost-effective for producing chemicals, a new approach for GABA fermentation from biomass is required for the sustainable industrial production of monomers for bio-plastics.

The aim of our study was to express the *gad* gene in a glutamate-producing microorganism to create an efficient GABA production process. *Corynebacterium glutamicum* is a non-pathogenic, non-sporulating, non-motile, Gram-positive soil bacterium belonging to the order *Actinomycetales*, which includes species of

Abbreviations: BHI, brain heart infusion; GABA, gamma-amino butyric acid; GAD, glutamate decarboxylase; LB, Luria–Bertani; MSG, monosodium glutamate; PA4, polyamide 4; PLP, pyridoxal 5′-phosphate; Tween 40, polyoxyethylene sorbitan monopalmitate.

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Corynebacteria, *Nocardia*, *Rhodococci*, and other related microorganisms [14]. *C. glutamicum* is an important industrial microorganism due to its high productivity of glutamate and amino acids, which are widely used in medicine, animal feed, and as food supplements [15,16]. Genetically engineered strains of *C. glutamicum* are also superior for producing various kinds of organic compounds including bio-ethanol [17], cadaverine as a component of bio-based nylons [18], and succinic acid as a polymer building block under oxygen-deprivation conditions [19,20]. Recently, it was reported that recombinant *C. glutamicum* expressing GAD from *L. brevis* was able to produce extracellular GABA [21].

In this study, we developed an efficient system for the production of GABA using a recombinant *C. glutamicum* strain expressing GAD from *E. coli*. The results showed that addition of glutamate was not necessary for GABA production by the *C. glutamicum* GAD strain and fermentation conditions were optimized to enhance GABA production from glucose as the main carbon source. As a result of medium optimization, we found that the addition of pyridoxal 5′-phosphate (PLP), which is a cofactor of GAD, in the fermentation medium greatly increased the productivity of GABA to more than 12 g/L after 72 h of fermentation.

2. Materials and methods

2.1. Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) containing 50 μg/mL kanamycin at 37 °C. *C. glutamicum* ATCC 13032 and all recombinant strains were grown in BY medium (10 g/L peptone, 10 g/L meat extract, 5 g/L yeast extract, and 5 g/L sodium chloride) containing 25 μg/mL kanamycin at 30 °C [22]. For the selection of *C. glutamicum* transformants, Brain Heart Infusion (BHI) medium (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) supplemented with 25 μg/mL kanamycin and 1.5% agar was used. The transformants were first precultivated in 5 mL BHI medium containing 25 μg/mL kanamycin in a test tube at 30 °C for 24 h. The culture was then inoculated into 20 mL GP medium containing 25 μg/mL kanamycin in a 200-mL flask for fermentation.

2.2. Construction of plasmids

All genetic manipulations were performed using *E. coli* SCS110 to avoid DNA methylation, and polymerase chain reactions (PCRs) were conducted using KOD-plus2 DNA polymerase (Toyobo, Osaka, Japan). *C. glutamicum*–*E. coli* shuttle vectors with the High-Constitutive Expression (HCE) promoter, pCH, were constructed as reported in our previous study [23].

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Reference or source
Escherichia coli		
SCS110	*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44Δ* (*lac-proAB*) [*F'traD36 proAB laclqZΔM15*]	STRATAGENE
W3110	Wild-type	NBRC
Corynebacterium glutamicum		
ATCC 13032	Wild-type	ATCC
GAD	*C. glutamicum* ATCC13032 harboring pCH-gadB	This study
GAD2	*C. glutamicum* ATCC13032 harboring pCH-gadA	This study
W	*C. glutamicum* ATCC13032 harboring pCH	This study
Plasmids		
pCH	*E. coli*–*C. glutamicum* shuttle vector, Km^r	[Tateno]
pCH-gadB	pCH containing *gadB* from *E. coli* W3110, Km^r	This study
pCH-gadA	pCH containing *gadA* from *E. coli* W3110, Km^r	This study

Genomic DNA from *E. coli* W3110 grown in LB medium was purified using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The GAD gene, *gadB* (GeneBank accession no. BAA15163.1), from *E. coli* was amplified by PCR from the genomic DNA of *E. coli* W3110 using the following primer pairs: W3110-gadB_F (5′-GGC GAG CTC ATG TTT AAA GCT GTT CTG TTG GGC A-3′, *Sac*I restriction site is underlined) and W3110-gadB_R (5′-CCG CTC GAG TTA *CTT GTC ATC GTC ATC CTT GTA GTC* AGG TCG GAA CTA CTC GAT TCA CG-3′, *Xho*I restriction site is underlined, and the FLAG-tagged sequence is italicized). The amplified DNA fragment was purified from a 1.0% agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega) after gel electrophoresis. The purified 1.37-kbp *gadB* fragment was digested with *Sac*I and *Xho*I (New England Biolabs, MA, USA) and cloned into pCH to yield pCH-gadB. The sequence of the constructed plasmid was confirmed by DNA sequencing analysis using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). A second GAD gene, *gadA*, was cloned and used for expression in *C. glutamicum*. *gadA* (GeneBank accession no. M84024.1) was amplified from *E. coli* W3110 genomic DNA using the following primer pairs: W3110-gadA_F (5′-GGC GAG CTC ATG GAC CAG AAG CTG TTA ACG GAT TT-3′, *Sac*I sites are underlined) and W3110-gadA_R (5′-CCG CTC GAG TCA *CTT GTC ATC GTC ATC CTT GTA GTC* GGT GTG TTT AAA GCT GTT CTG CTG-3′, *Xho*I sites are underlined, and FLAG-tagged sequence is italicized). The amplified 1.40-kbp fragment was cloned into pCH to yield pCH-gadA.

The constructed plasmid pCH-gadB, pCH-gadA or pCH as a control, was individually introduced into *C. glutamicum* ATCC 13032. The transformation was conducted by electroporation with a 2.5-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette using a Gene Pulser Xcell (Bio-Rad, Richmond, CA, USA), followed by a heat shock of 46 °C for 6 min. The cells were then incubated in 1 mL BHI medium at 30 °C for 1.5 h. Transformants were selected on BHI agar plates containing 25 μg/mL kanamycin, and the presence of the *gadB* was confirmed by cell-directed PCR using KOD FX (Toyobo). The resulting strains, *C. glutamicum* ATCC13032 (pCH-gadB), *C. glutamicum* ATCC13032 (pCH-gadA), and *C. glutamicum* ATCC13032 (pCH), were named *C. glutamicum* GAD, *C. glutamicum* GAD2, and *C. glutamicum* W, respectively.

2.3. Western blotting analysis

C. glutamicum GAD, *C. glutamicum* GAD2, and *C. glutamicum* W were precultivated in 5 mL BHI medium containing 25 μg/mL kanamycin in a test tube at 30 °C for 24 h. A small volume (0.2 mL) of the preculture solution was transferred to 20 mL BY medium containing 25 μg/mL kanamycin in a 200 mL shaker flask. After 24 h of fermentation, the cells from 1 mL culture were centrifuged at 8000 × *g* for 5 min, washed once in 50 mM Tris–HCl (pH 6.8) buffer, suspended in 1 mL buffer, and then 0.7 g of 0.1-mm glass beads YGB01 (Yasui Kikai, Japan) was added to the tube. The cells were disrupted using a Shake Master Neo (Bio Medical Science) by shaking the tube three times at 1500 rpm for 1 min with 1-min intervals. After centrifugation at 9000 × *g* for 5 min, the supernatants were subjected to SDS-PAGE analysis. The separated proteins were electroblotted onto a PVDF membrane (Millipore, Boston, MA, USA) and then reacted sequentially with a mouse monoclonal anti-FLAG M2 (Sigma, St. Louis, MO, USA) and goat anti-mouse IgG alkaline phosphate conjugate (Promega). The membrane was stained with 4-nitro-blue tetrazolium chloride (NBT; Promega) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Promega) according to the manufacturer's instructions.

2.4. Culture conditions for GABA fermentation from glucose

C. glutamicum GAD and *C. glutamicum* W were precultivated in 5 mL BHI medium containing 25 μg/mL kanamycin in a test tube at 30 °C for 22 h. A preculture solution (0.2 mL) was transferred to 20 mL GABA Production 1 (GP1) medium containing 25 μg/mL of kanamycin in a 200 mL shaker flask. The composition of the GP1 medium was 50 g glucose, 50 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 3 g urea, 0.4 g $MgSO_4{\cdot}7H_2O$, 50 g soy peptone, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}5H_2O$, 200 μg thiamine, 0.5 mg biotin, 0.265 g PLP, and 5 g Tween 40 per liter. Stock solutions of thiamine, biotin, and PLP were filtrated using a 0.22 μm filter membrane and added to the medium prior to the addition of cells. The pH of the GP1 medium in the flask was not adjusted. The fermentation was carried out at 30 °C with an agitation speed of 120 rpm in a BR-13FR BioShaker (Taitec, Japan). Throughout the 120 h cultivation, 1 mL of the culture was collected every 24 h, centrifuged at 8000 × *g* for 5 min at 4 °C, and filtrated through a 0.45 μm DISMIC Mixed Cellulose Ester (Advantec, Tokyo, Japan). GABA, glutamate, and glucose concentrations of culture supernatants were analyzed as described below. The optical density at 600 nm (OD_{600}) was monitored at the same time.

2.5. Determination of the effects of PLP, Tween 40, and cell culture volume on GABA fermentation

For the determination of the effects of Tween 40, 0 (control) and 5 g/L Tween 40 (polyoxyethylene sorbitan monopalmitate; Nacalai Tesque, Kyoto, Japan) was added to the GP1 medium. After 22 h of preculture of *C. glutamicum* GAD, 0.2 mL of the preculture solution was transferred to 20 mL of GP1 medium containing 25 μg/mL kanamycin in a 200 mL shaker flask. For evaluation of the effects of Tween 40, the concentration of PLP in the medium was fixed at 1 mM.

For determination of the effects of the coenzyme PLP on GABA fermentation, 0 (control), 0.1 mM, and 1 mM PLP was added to the GP1 medium. After 22 h of