

Production of γ -Aminobutyrate (GABA) in Recombinant *Corynebacterium glutamicum* by Expression of Glutamate Decarboxylase Active at Neutral pH

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Cite This: *ACS Omega* 2022, 7, 29106–29115



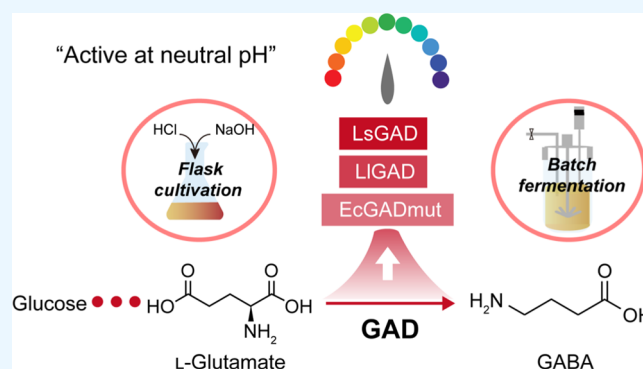
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ABSTRACT: γ -Aminobutyrate (GABA) is an important chemical by itself and can be further used for the production of monomer used for the synthesis of biodegradable polyamides. Until now, GABA production using *Corynebacterium glutamicum* harboring glutamate decarboxylases (GADs) has been limited due to the discrepancy between optimal pH for GAD activity (pH 4.0) and cell growth (pH 7.0). In this study, we developed recombinant *C. glutamicum* strains expressing mutated GAD from *Escherichia coli* (EcGADmut) and GADs from *Lactococcus lactis* CICC20209 (LIGAD) and *Lactobacillus senmaizukei* (LsGAD), all of which showed enhanced pH stability and adaptability at a pH of approximately 7.0. In shake flask cultivations, the GABA productions of *C. glutamicum* H36EcGADmut, *C. glutamicum* H36LsGAD, and *C. glutamicum* H36LIGAD were examined at pH 5.0, 6.0, and 7.0, respectively. Finally, *C. glutamicum* H36EcGADmut (40.3 and 39.3 g L⁻¹), H36LIGAD (42.5 and 41.1 g L⁻¹), and H36LsGAD (41.6 and 40.2 g L⁻¹) produced improved GABA titers and yields in batch fermentation at pH 6.0 and pH 7.0, respectively, from 100 g L⁻¹ glucose. The recombinant strains developed in this study could be used for the establishment of sustainable direct fermentative GABA production from renewable resources under mild culture conditions, thus increasing the availability of various GADs.



INTRODUCTION

γ -Aminobutyrate (GABA) is a non-proteinogenic amino acid that functions as an inhibitory neurotransmitter in animals and mediates the acid stress response in plants.¹ Additionally, GABA has widely been used in the pharmaceutical industry as the active compound in tranquilizers, analgesics, diuretics, and antianxiety medicines.^{2–4} Particularly, the production of GABA using bacteria has been assessed as an ideal strategy for functional food industrial applications.^{5–7} Furthermore, current environmental crises such as global warming and plastic waste accumulation have led to the exploration of new applications of GABA, a carbon-neutral chemical that can be used for further synthesis of 2-pyrrolidone, an important C4 platform chemical and monomer of bio-based polyamides such as polyamide 4.^{8,9} GABA biosynthesis is initiated from L-glutamate by glutamate decarboxylases (GADs; EC: 4.1.1.15), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, via α -decarboxylation. Numerous studies have assessed the synthesis of GABA with GADs through enzymatic reactions or whole-cell reactions using *Escherichia coli*^{9–13} and lactic acid bacteria (LAB)^{14–16} as biocatalysts (Table 1). In enzymatic GABA production, immobilization of *E. coli* GAD could produce

223.8 g L⁻¹ of GABA in a 1 L reactor within 100 min, retaining more than half of its activity after 10 sequential uses.¹² Although enzymatic reactions are among the most powerful strategies for GABA production, whole-cell reactions are considered a more cost-efficient alternative because they can lower the total production cost related to the removal of GAD purification and immobilization steps. Whole-cell reaction, in which an endogenous *gadA* is overexpressed in *E. coli* BL21(DE3), resulted in the production of 280–300 g L⁻¹ of GABA with an 89.5% yield via direct L-glutamate biotransformation.¹⁰ It was also reported that whole-cell conversion using the engineered *E. coli* XL1-blue strain expressing *gadB* from *Lactococcus lactis* subsp. *lactis* Il1403 could produce 94.8 g L⁻¹ of GABA from 200 g L⁻¹ monosodium glutamate (MSG).⁹

Received: May 12, 2022

Accepted: July 27, 2022

Published: August 4, 2022



Table 1. Summary of Recently Reported GABA Production in Recombinant Microbial Hosts^a

strain	features	scale	carbon source	titer (g L ⁻¹)	productivity (g L ⁻¹ h ⁻¹)	yield (g g ⁻¹)	ref
<i>E. coli</i> BL21(DE3)	P _{T7} : GAD from <i>S. cerevisiae</i>	whole-cell	L-glutamate	245	5.83*	0.99 (mol mol ⁻¹)	52
<i>E. coli</i> BL21(DE3)	P _{T7} : GAD from <i>L. lactis</i> FJNUGA01	whole-cell	L-glutamate	204.1	34	0.99 (mol mol ⁻¹)	53
<i>E. coli</i> BW25113	Δ <i>gadC</i> Δ <i>gadAB</i> , P _{araBAD} : <i>gadB</i> (M4)- <i>groES-groEL</i> , <i>gadB</i> mutant from <i>L. lactis</i> IL1403	whole-cell	L-glutamate	308.26	44.04	0.996 (mol mol ⁻¹)	54
<i>E. coli</i> EDK11	P _{LetO} : <i>gadB</i> , <i>gadC</i> , <i>gabT</i> , and <i>gltA</i>	shake flask	glucose	1.2	0.05*		55
<i>E. coli</i> Nissle 1917 pMT1-G/pMT2-R/EcNP	P _{trc} : <i>gadB</i>	whole-cell	MSG	17.9			56
<i>E. coli</i> BL21 (DE3)/GADZ ₁₁	P _{T7} : GAD from <i>Bacillus</i> spp.	whole-cell	L-glutamate	1640	96.4*	0.985 (mol mol ⁻¹)	44
<i>Corynebacterium glutamicum</i> ATCC 13032	Δ <i>gabT</i> , Δ <i>gabP</i> ; P _{tac} : <i>gadB2</i> from <i>L. brevis</i> ATCC 367	shake flask	glucose	28.7	0.3		57
<i>C. glutamicum</i> SH	P _{tac} : <i>gadB1-gadB2-ppc</i>	fed-batch	glucose	26.3	0.365		58
<i>C. glutamicum</i> H36GD1852	P _{H36} : <i>gadB_{mut}xyLAB</i> from <i>E. coli</i>	fed-batch	empty fruit bunch (EFB) solution	35.47	0.68		22
<i>C. glutamicum</i> SH	P _{tacM} : R4a- <i>gabB2B1^{mut}</i> from <i>L. brevis</i>	shake flask	glucose	26.5	0.442*	0.269*	59
<i>C. glutamicum</i> H36EcGADmut	P _{H36} : GAD from <i>E. coli</i>	batch	glucose	40.3	1.12	0.40	this study
<i>C. glutamicum</i> H36LIGAD	P _{H36} : GAD from <i>L. lactis</i> CICC20209	batch	glucose	42.5	1.18	0.43	this study
<i>C. glutamicum</i> H36LsGAD	P _{H36} : GAD from <i>L. senmaizukei</i>	batch	glucose	41.6	1.16	0.42	this study

^a* indicates calculated data based on reported studies.

Although high titers of GABA could be obtained from the conversion of L-glutamate or MSG through enzymatic or whole-cell biocatalysis reactions, there is a growing need for the establishment of more sustainable and cost-effective alternative microbial processes, the direct fermentative production of GABA from renewable carbon sources such as glucose.^{17,18}

To establish an efficient system for one-step fermentative GABA production from glucose, a sufficient amount of intracellular L-glutamate should be ensured because it is a key precursor for GABA synthesis. Among various host strains, *C. glutamicum* is considered the ideal host since it is already known as a robust industrial commercial L-glutamate-producing strain. Moreover, *C. glutamicum* is a Gram-positive and nonpathogenic bacterium that is generally recognized as safe (GRAS) serving benefits on target chemical production.^{19,20} In a previous study, an engineered *C. glutamicum* 13 032 strain expressing the EcGADmut produced 38.6 g L⁻¹ of GABA from glucose during fed-batch fermentation at pH 6.0.²¹ Recently, the heterologous expression of EcGADmut along with *E. coli* xylose isomerase (*xyIA*) and xylulose kinase (*xyIB*) in an L-glutamate overproducing *C. glutamicum* KCTC 1852 strain resulted in the production of 35.5 g L⁻¹ of GABA from an empty fruit bunch biosugar solution (a mixture of glucose and xylose) during batch fermentation at pH 6.0.²²

One of the main challenges of direct GABA synthesis from glucose is that there is a trade-off between GAD activity and cell growth. During fermentation, GABA production is limited due to the significant difference between the optimal pH for GAD activity (pH 4.0) and the optimal pH for cell growth (pH 7.0). Mostly, GADs used for enzymatic conversion or whole-cell conversion of MSG into GABA have shown specific activity under acidic conditions. Particularly, GADs from *Lactobacillus zymae*,²³ *E. coli*,²⁴ *Lactobacillus sakei* A156,²⁵ and *L. brevis* HYE1²⁶ were reported to be only active at pH 4.0–

5.0. Given that the optimal pH for cell growth is higher than that for optimal GAD activity, these GADs cannot be used in direct fermentative GABA production from renewable resources, as they are unable to support the efficient production of GABA during the exponential growth phase of the host strain. Moreover, GAD is active during the stationary phase when both pH and cell growth have begun to significantly decrease, which ultimately results in retarded productivity of GABA requiring long cultivation times of over 120 h.^{24,27}

Therefore, the employment of various GADs exhibiting enhanced activity within a wider pH range (pH 5.0–7.0) has been attempted to produce high GABA yields in a growth-associated manner. To this end, many studies have contributed to the screening of GADs active in a broad pH range using two primary strategies: (i) site-directed mutagenesis on previously reported GADs and (ii) discovery of novel GADs. For example, (i) a site-specific mutant of *E. coli* GAD (EcGADmut; E89Q/Δ452–466) was developed based on the structural changes observed during pH shifts, which led to enhanced activity within a wider pH range^{21,22,28} and (ii) novel GADs active at pH 7.0 were elucidated in LAB via genome mining.²⁹

In this study, recombinant *C. glutamicum* strains capable of producing high amounts of GABA under near-neutral pH conditions were developed by employing several GADs from *E. coli* (EcGADmut), *L. lactis* (LIGAD), and *L. senmaizukei* (LsGAD), which had been reported to be active at a broad pH range, as demonstrated by in vitro assays.^{21,29} Therefore, our study sought to extend the applicability of GADs active at a pH close to 7.0 and to secure a diverse gene pool of GADs with a broad pH range during in vivo experiments. First, the GABA biosynthesis pathway was established in recombinant *C. glutamicum* KCTC 1852 strains via the construction of plasmids for heterologous expression of three GADs under the control of two different strong synthetic promoters, H30

Table 2. Strains and Plasmids Used in This Study

strains and plasmids	relevant characteristics	reference or source
Strains		
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [FA1 <i>proAB lacI^qZΔM15 Tn10</i> (Tet ^R)]	stratagene
<i>C. glutamicum</i> KCTC 1852	L-glutamate producers	KCTC
<i>C. glutamicum</i> 1852 H36EcGADmut	<i>C. glutamicum</i> KCTC 1852 harboring pCES208H36EcGADmut	22
<i>C. glutamicum</i> 1852 H30LsGAD	<i>C. glutamicum</i> KCTC 1852 harboring pCES208H30LsGAD	this study
<i>C. glutamicum</i> 1852 H36LsGAD	<i>C. glutamicum</i> KCTC 1852 harboring pCES208H36LsGAD	this study
<i>C. glutamicum</i> 1852 H30LIGAD	<i>C. glutamicum</i> KCTC 1852 harboring pCES208H30LIGAD	this study
<i>C. glutamicum</i> 1852 H36LIGAD	<i>C. glutamicum</i> KCTC 1852 harboring pCES208H36LIGAD	this study
Plasmids		
pCES208H30GFP	pCES208 derivative; H30 promoter, eGFP, Km ^r	30
pCES208H36GFP	pCES208 derivative; H36 promoter, eGFP, Km ^r	30
pCES208H36EcGADmut	pCES208H36GFP derivative; H36 promoter, GAD mutant from <i>E. coli</i> (Glu89Gln/Δ452–466), Km ^r	21
pCES208H30LsGAD	pCES208H30GFP derivative; H30 promoter, GAD from <i>L. senmaizukei</i> , Km ^r	this study
pCES208H36LsGAD	pCES208H36GFP derivative; H36 promoter, GAD from <i>L. senmaizukei</i> , Km ^r	this study
pCES208H30LIGAD	pCES208H30GFP derivative; H30 promoter, GAD from <i>L. lactis</i> CICC20209, Km ^r	this study
pCES208H36LIGAD	pCES208H36GFP derivative; H36 promoter, GAD from <i>L. lactis</i> CICC20209, Km ^r	this study

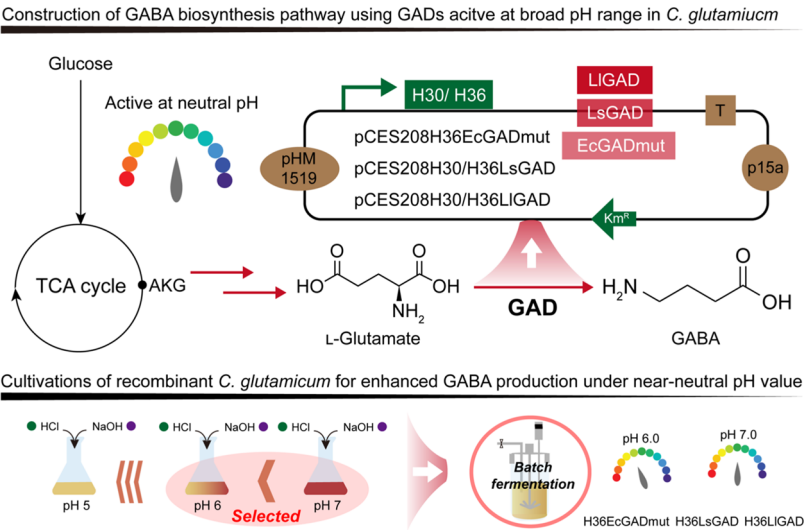


Figure 1. Diagram of the GABA biosynthetic pathway in *C. glutamicum* and proposed production strategies for GABA using various GADs through flask and batch fermentation. The abbreviation shown is AKG, α -ketoglutarate.

Table 3. List of Primers Used in PCR Experiments

glutamate decarboxylase	primer
EcGADmut-F	GGATCCATGGATAAGAAGCAAGTAACGG
EcGADmut-R	GCGGCCGCTTAGGTATGTTTAAAGCTGTTCT
LsGAD-F	GGATCCATGAGTAAAAACGATCAGGAAACGC
LsGAD-R	GCGGCCGCTAACGAACAGTCGTCTTGTCTTTC
LIGAD-F	GGATCCATGTTATACGAAAAAGAAATCGCG
LIGAD-R	GCGGCCGCTTAGTGAGTAAATCCATATGTTTTATTTTC

and H36 (Table 2).^{29,30} Then, further control of pH in flask cultivation by the intermittent addition of an acid or a base was implemented to investigate the optimal pH for GABA production. Finally, batch fermentation of the engineered strains with heterologous expression of EcGADmut, LsGAD, and LIGAD under the H36 promoter was conducted by maintaining the culture pH at 6.0 and 7.0 (Figure 1). Taken together, we have, here, demonstrated the extended applicability of GADs in neutral pH conditions.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Table 2 summarizes all of the bacterial strains and plasmids used in this study. *E. coli* XL1-Blue (Stratagene, La Jolla, CA) was used as the main host strain for gene cloning and plasmid maintenance. The L-glutamate overproducing *C. glutamicum* KCTC 1852 strain was purchased from the Korean Collection for Type Cultures (KCTC, Korea). The pCES208H30GFP, pCES208H36GFP, and pCES208H36EcGADmut plasmids containing mutated GAD from *E. coli* (EcGADmut) were constructed, as previously described.^{21,30}

Plasmid Construction. All DNA manipulations were conducted following standard procedures.³¹ A C1000 Thermal Cycler (Bio-Rad, Hercules, CA) was used for polymerase chain reactions (PCRs). The primers used in this study were synthesized by Bioneer (Daejeon, Korea) (Table 3). For the expression of GAD from *L. lactis* CICC20209 and *L. senmaizukei*, each gene was codon-optimized and synthesized by Cure bio (Seoul, Korea) based on sequence data from a previous report.³⁰ The DNA fragments were digested with *Bam*HI/*Not*I and ligated into the *Bam*HI/*Not*I sites of pCES208H30GFP or pCES208H36GFP to make pCES208H30LsGAD and pCES208H30LIGAD or pCES208H36LsGAD and pCES208H36LIGAD, respectively (Table 2), in which the *gfp* gene was replaced with the corresponding GAD gene.

Culture Conditions. *E. coli* was grown in the Luria–Bertani (LB) medium containing 10 g L^{−1} tryptone, 5 g L^{−1} yeast extract, and 5 g L^{−1} NaCl at 37 °C, 200 rpm. Shake flask culture for GABA production using the recombinant *C. glutamicum* 1852 strains was conducted using 250 mL baffled flasks with 50 mL of the GP1 medium. These flasks were incubated at 30 °C and 250 rpm. The GP1 medium contained 50 g L^{−1} glucose, 50 g L^{−1} (NH₄)₂SO₄, 1 g L^{−1} K₂HPO₄, 3 g L^{−1} urea, 0.4 g L^{−1} MgSO₄·7H₂O, 50 g L^{−1} peptone, 0.01 g L^{−1} FeSO₄, and 0.01 g L^{−1} MnSO₄·5H₂O. Glucose was separately autoclaved and added to the culture medium. Each flask culture was supplemented with 25 μg mL^{−1} kanamycin (Km), 0.1 mM pyridoxal 5-phosphate hydrate (PLP), 50 μg L^{−1} biotin, and 200 μg L^{−1} thiamine. These supplements were prepared as stock solutions and sterilized using 0.22 μm filter membranes prior to addition to the culture medium. To minimize pH fluctuations during cultivation, 10 g L^{−1} of calcium carbonate (CaCO₃) was added to the culture medium. For pH-controlled flask culture, the pH of flask cultures was checked every 24 h prior to dropwise addition of 1 M HCl or 1 M NaOH to adjust pH 5.0, 6.0, or 7.0.

For batch fermentation, 50 mL of seed culture was prepared by making 4 mL overnight cultures of recombinant *C. glutamicum* 1852 strains for inoculation of 50 mL of the GP1 medium in a 500 mL baffled flask. These were incubated at 30 °C and 250 rpm. Batch fermentations were carried out at 30 °C and 600 rpm using a 2.5 L jar fermenter (BioCNS, Korea), which contains a total volume of 500 mL of the CG100 medium. The CG100 medium is composed of 100 g L^{−1} of glucose, 30 g L^{−1} of yeast extract, 30 g L^{−1} of (NH₄)₂SO₄·7H₂O, 0.5 g L^{−1} of KH₂PO₄, 0.5 g L^{−1} of MgSO₄·7H₂O, 0.01 g L^{−1} of MnSO₄·H₂O, 0.01 g L^{−1} of FeSO₄·7H₂O, 0.5 mg of biotin, and 0.3 mg of thiamine-HCl. Kanamycin and PLP were added to the culture medium at 25 μg mL^{−1} and 0.1 mM, respectively. Two different pH values (6.0 and 7.0) were examined for GABA production and maintained via automatic addition of 28% (v/v) NH₄OH. Foam formation was suppressed by adding Antifoam 204 (Sigma-Aldrich, St. Louis, MO).

Analytical Procedures. Cell growth was monitored by measuring absorbance at 600 nm (OD₆₀₀) using a UV-1700 spectrophotometer (Shimadzu, Japan). Glucose concentration was analyzed by high-performance liquid chromatography [HPLC, Infinity 1260 (Agilent Technologies, Santa Clara, CA)] equipped with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87H column. Elution was performed with 5 mM of H₂SO₄ at a 0.8 mL min^{−1} flow rate at 50 °C. The concentrations of L-glutamate and GABA were analyzed by

HPLC equipped with a variable wavelength detector (VWD) and a C18 column (RStech, Daejeon, Korea) according to the DEEMM method.³² Elution was performed with mobile phase A (acetonitrile) and mobile phase B (25 mM sodium acetate buffer, pH 4.8).

RESULTS AND DISCUSSION

Construction of the GABA Biosynthesis Pathway in *C. glutamicum* KCTC 1852. Most bacterial GADs are only active under acidic conditions, especially at 4.0, and GAD activity is strongly diminished as the pH of the culture medium increases.^{23,25,26} In contrast, *C. glutamicum* is widely regarded as a moderately alkali-tolerant bacterium with an optimal pH range between 7.0 and 8.5.^{33,34} Therefore, the efficient conversion of L-glutamate into GABA largely relies on maintaining an optimal balance between maximum bacterial growth and GAD activity.

To identify GADs that efficiently produce GABA under a wider pH range, several studies have reported the production of GABA with engineered or novel GADs that possess a broad active pH range from 5.0 to 7.0.^{21,28,29,35} Recently, there has been an attempt to search for novel GADs from GRAS probiotic bacteria including LAB based on genome mining and sequence alignment analysis, and consequently, 16 putative GADs were screened.²⁹ Among them, LsGAD and LIGAD were found to possess a wider optimal pH range and a more durable enzymatic stability than GAD from *L. brevis*, which was used as a probe.²⁹ Therefore, in this study, we hereby examined three different GADs (EcGADmut, LsGAD, and LIGAD) that were previously reported to exhibit superior activity under a wide pH range (pH 5.0–7.0) for GABA production from glucose at near-neutral pH in L-glutamate-overproducing *C. glutamicum* KCTC 1852 strains. To this end, we evaluated five different recombinant *C. glutamicum* KCTC 1852 strains expressing EcGADmut, LsGAD, or LIGAD under the control of a synthetic H30 or H36 promoter: (i) H36EcGADmut strain (*C. glutamicum* KCTC 1852 harboring pCES208H36EcGADmut), (ii) H30LsGAD strain (*C. glutamicum* KCTC 1852 harboring pCES208H30LsGAD), (iii) H36LsGAD strain (*C. glutamicum* KCTC 1852 harboring pCES208H36LsGAD), (iv) H30LIGAD strain (*C. glutamicum* KCTC 1852 harboring pCES208H30LIGAD), and (v) H36LIGAD strain (*C. glutamicum* KCTC 1852 harboring pCES208H36LIGAD) (Figure 1). Strong synthetic H30 and H36 promoters have been successfully implemented for the construction of recombinant *C. glutamicum* strains that can produce various chemicals such as cadaverine, glutaric acid, GABA, and 5-HV.^{22,36–38} Only the H36 promoter was examined for the expression of EcGADmut in *C. glutamicum* KCTC 1852, as we had previously reported that EcGADmut expression under the H36 promoter in *C. glutamicum* KCTC 1852 supported higher GABA production from an empty fruit bunch biosugar solution.²²

Comparison of GADs in Recombinant *C. glutamicum* during pH-Controlled Flask Cultivation. Flask cultivation using each recombinant *C. glutamicum* strain was conducted under three different pH conditions (pH 5.0, 6.0, and 7.0) to determine which GAD and promoter combinations produced the highest titers of GABA at near-neutral pH. Even though the initial pH values of the flask cultures were established according to previous studies,^{21,22} cell growth and production of L-glutamate could be adversely affected as the pH in the culture medium decreased over time. Therefore, the pH in the

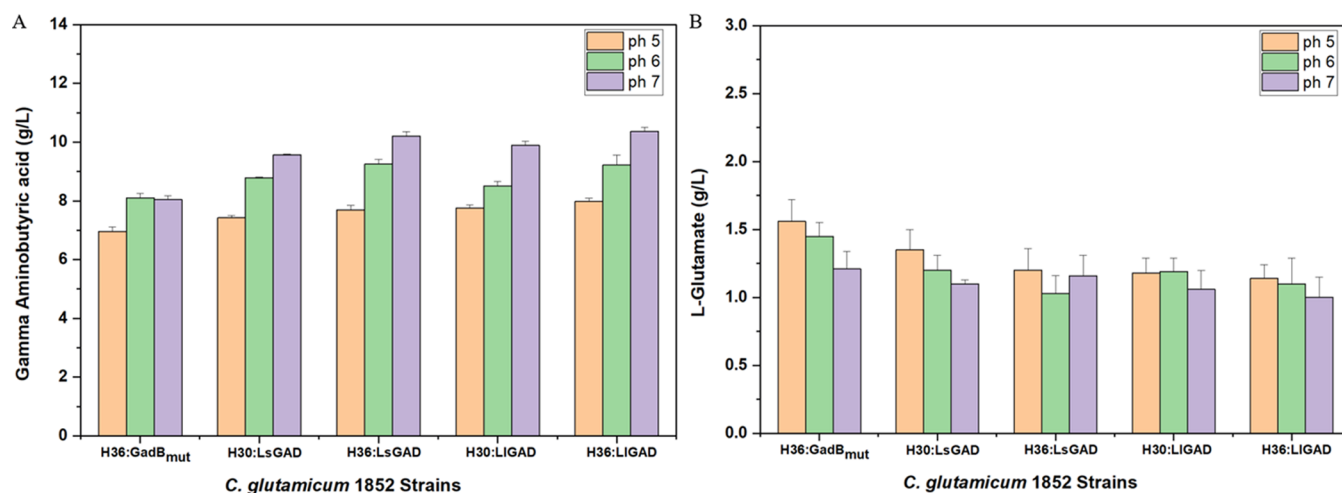


Figure 2. Comparison of GABA production (A) and L-glutamate accumulation (B) during shake flask culture by different recombinant *C. glutamicum* 1852 strains with different combinations of GADs under the control of the strong synthetic promoters H30 or H36: *C. glutamicum* H30LsGAD, *C. glutamicum* H30LIGAD, *C. glutamicum* H36EcGADmut, *C. glutamicum* H36LsGAD, and *C. glutamicum* H36LIGAD. All flask cultures were conducted in triplicate. All measurements are presented as mean \pm standard deviation.

flask was manually controlled during cultivation to maintain a target pH range. To this end, flask cultivation was performed to produce GABA from 50 g L⁻¹ of glucose, and proper amounts of 1 M HCl or 1 M NaOH were added every 24 h to adjust the pH to 5.0, 6.0, and 7.0 (Figure 2).

At pH 5.0, the final titers of GABA achieved by the five different engineered strains were 7.42 \pm 0.08 g L⁻¹ (H30LsGAD), 7.75 \pm 0.11 g L⁻¹ (H30LIGAD), 6.95 \pm 0.16 g L⁻¹ (H36EcGADmut), 7.69 \pm 0.15 g L⁻¹ (H36LsGAD), and 7.99 \pm 0.10 g L⁻¹ (H36LIGAD) (Figure 2A). Consistent with previous studies,^{21,22} the recombinant *C. glutamicum* strains with the H36 promoter exhibited higher GABA production than those with the H30 promoter. However, compared with the recombinant *C. glutamicum* H36EcGADmut strain, *C. glutamicum* expressing novel GADs (LsGAD and LIGAD) achieved higher GABA production with lower L-glutamate accumulation.

At pH 6.0, *C. glutamicum* H30LsGAD and H30LIGAD yielded 8.79 \pm 0.02 and 8.50 \pm 0.16 g L⁻¹ of GABA, respectively. Similarly, *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD strains yielded 8.10 \pm 0.15, 9.25 \pm 0.16, and 9.22 \pm 0.34 g L⁻¹ of GABA, respectively (Figure 2A). Therefore, in addition to the higher GABA concentrations obtained by the novel GADs compared to EcGADmut, the recombinant strains with the H36 promoter also yielded higher GABA titers than those with the H30 promoter.

In the case of cultivation at pH 7.0, *C. glutamicum* H30LsGAD and H30LIGAD produced 9.56 \pm 0.03 and 9.89 \pm 0.14 g L⁻¹ of GABA, respectively, whereas *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD could produce 8.05 \pm 0.13, 10.20 \pm 0.15, and 10.36 \pm 0.18 g L⁻¹ of GABA, respectively (Figure 2A). Similar to the other flask culture conditions, the H36 promoter induced a higher GABA production. Additionally, higher pH conditions resulted in higher GABA concentrations, and therefore, the largest differences in GABA concentration between EcGADmut and novel GADs were observed at pH 7.0.

In accordance with previous studies,^{21,22,29} expressing GADs from LAB and engineered GADs from *E. coli* in host strains that are reported to maintain their activities even at near-

neutral pH could efficiently produce GABA at pH 7.0. The produced GABA concentrations at various pH conditions obtained by *C. glutamicum* H36LsGAD (pH 5.0, 7.69 \pm 0.15 g L⁻¹; pH 6.0, 9.25 \pm 0.16 g L⁻¹; pH 7.0, 10.20 \pm 0.15 g L⁻¹) and *C. glutamicum* H36LIGAD (pH 5.0, 7.99 \pm 0.10 g L⁻¹; pH 6.0, 9.22 \pm 0.34 g L⁻¹; pH 7.0, 10.36 \pm 0.18 g L⁻¹) were marginally improved compared to those obtained from *C. glutamicum* H36EcGADmut (pH 5.0, 6.95 \pm 0.16 g L⁻¹; pH 6.0, 8.10 \pm 0.15 g L⁻¹; pH 7.0, 8.05 \pm 0.13 g L⁻¹). This demonstrated that LsGAD and LIGAD have a slightly stronger in vivo catalytic activity at pH 5.0–7.0. In addition, recombinant strains with the H36 promoter produced consistently higher concentrations of GABA than those with the H30 promoter. The strains with the H36 promoter harboring LsGAD produced 3% (pH 5.0), 5% (pH 6.0), and 6% (pH 7.0) higher GABA yields. Moreover, the strains harboring LIGAD under the control of the H36 promoter resulted in 3% (pH 5.0), 8% (pH 6.0), and 5% (pH 7.0) enhanced production of GABA than those of the H30 strains (Figure 2A). This may be because a stronger promoter could support the high-level expression of related enzymes along with the overproduction of target compounds.^{21,30,39,40} Furthermore, the *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD strains exhibited lower accumulations of unconverted L-glutamate at pH 7.0, with final concentrations of 1.21 \pm 0.09, 1.16 \pm 0.10, and 1.00 \pm 0.19 g L⁻¹ of L-glutamate, respectively, while *C. glutamicum* H30LsGAD and H30LIGAD strains have also accumulated less L-glutamate at pH 7.0 (1.10 \pm 0.16 and 1.06 \pm 0.15 g L⁻¹, respectively) than at pH 5.0 (Figure 2B).

Batch Fermentations of Recombinant *C. glutamicum* for the Enhanced Production of GABA under Near-Neutral pH. Even though pH adjustment was conducted during flask cultivation, strict regulation of fermentation environments by tightly adjusting pH is essential to achieve high-level production of the desired products. Previously, GABA was produced using GAD that was only active at low pH, thus, the following fermentation strategies were carried out. For example, two-stage cultivation is employed for the production of target compounds in low pH conditions, which is divided into two parts: (i) the early cell growth phase, where

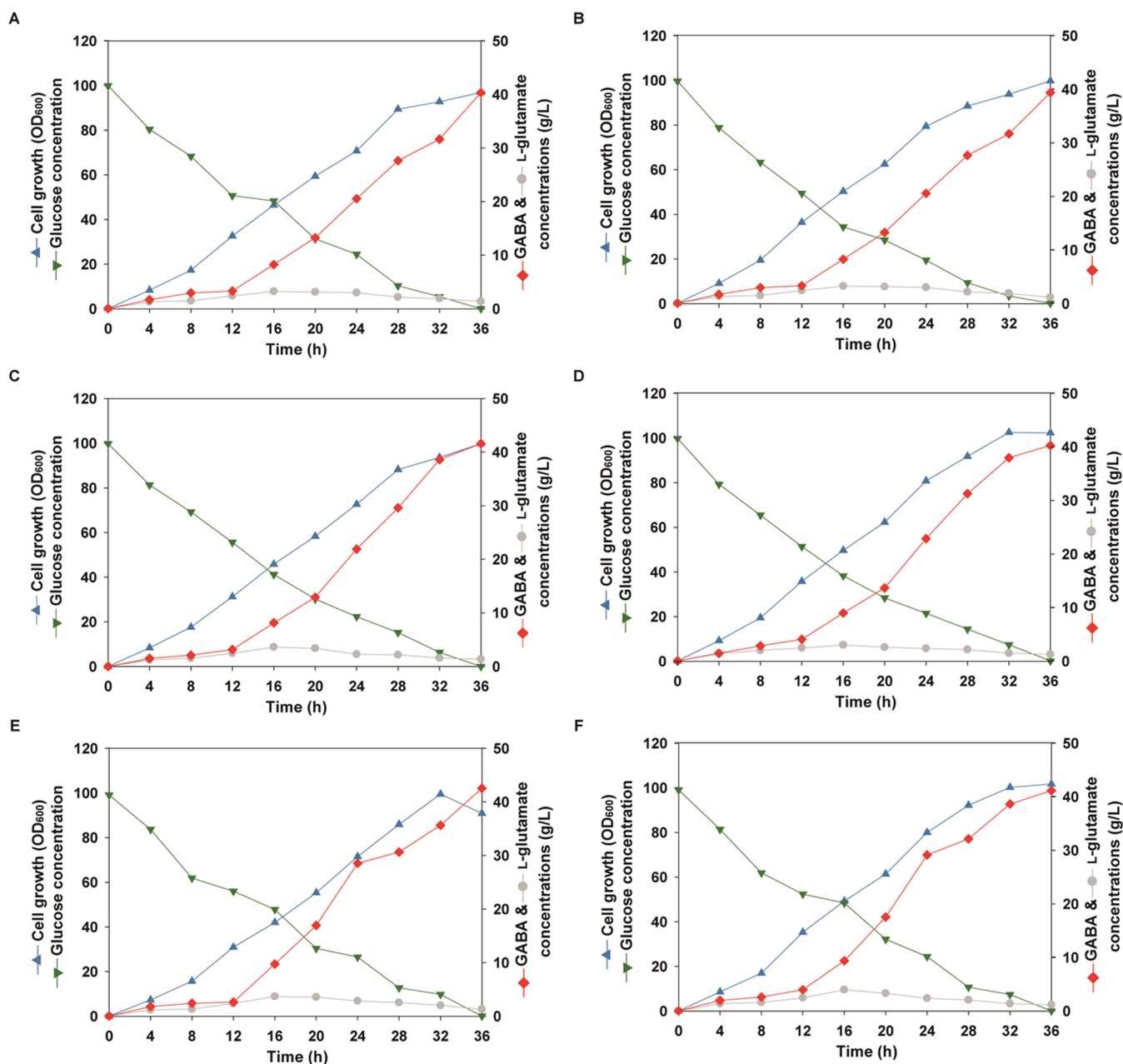


Figure 3. Time profiles of GABA, L-glutamate, glucose concentration, and cell density during batch fermentation in recombinant *C. glutamicum* H36EcGADmut at pH 6.0 (A), recombinant *C. glutamicum* H36EcGADmut at pH 7.0 (B), recombinant *C. glutamicum* H36LsGAD at pH 6.0 (C), recombinant *C. glutamicum* H36LsGAD at pH 7.0 (D), recombinant *C. glutamicum* H36LIgAD at pH 6.0 (E), and recombinant *C. glutamicum* H36LIgAD at pH 7.0 (F). All batch fermentations were done in duplicates and the figure shows a representative fermentation profile.

cells grow well using a carbon source but without much GABA production and (ii) the GABA production phase, where the pH decreases to approximately 4.0, after which GAD becomes active and substantial amounts of GABA production are initiated.^{41,42} Nevertheless, it is more beneficial to proceed with the fermentation with GADs, which show activity in almost neutral pH, as they can render high GABA yields in short periods. Therefore, we conducted batch fermentation of recombinant *C. glutamicum* strains to obtain high GABA yields in a 2.5 L lab-scale bioreactor maintained at pH 6.0 and 7.0 without compromising production efficiency. This was achieved by utilizing three GADs exhibiting comparable activities at near-neutral pH conditions. Since recombinant strains expressing EcGADmut, LsGAD, and LIgAD under the

control of the H36 promoter have supported higher productions of GABA in flask cultures, batch fermentation of *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIgAD was conducted herein. Figure 3 illustrates the time profiles of each batch fermentation of the final *C. glutamicum* strains at different pH values.

The final GABA titers obtained from batch fermentation at pH 6.0 were 40.3 g L⁻¹ (H36EcGADmut), 41.6 g L⁻¹ (H36LsGAD), and 42.5 g L⁻¹ (H36LIgAD), with a final OD₆₀₀ of 96.8 (H36EcGADmut), 90.8 (H36LIgAD), and 99.8 (H36LsGAD) (Figure 3A,C,E). After 36 h of cultivation, a GABA yield of 0.40 g g⁻¹ glucose, 0.42 g g⁻¹ glucose, and 0.43 g g⁻¹ glucose and GABA productivities of 1.12, 1.16, and 1.18 g L⁻¹ h⁻¹ were achieved using the *C. glutamicum*

H36EcGADmut, H36LsGAD, and H36LIGAD strains, respectively. L-Glutamate began to accumulate in the early stage of fermentation, and the final concentrations of L-glutamate were 1.34 g L⁻¹ (H36EcGADmut), 1.26 g L⁻¹ (H36LsGAD), and 1.35 g L⁻¹ (H36LIGAD) after 36 h of cultivation.

During batch fermentation at pH 7.0, the GABA titers and final cell concentrations achieved by the engineered *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD strains were 39.3 g L⁻¹ with an OD₆₀₀ of 99.6, 40.2 g L⁻¹ with an OD₆₀₀ of 102.1 and 41.1 g L⁻¹ with an OD₆₀₀ of 101.6, respectively (Figure 3B,D,F). Additionally, the *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD strains could produce GABA with a yield of 0.39 g g⁻¹ glucose, 0.39 g g⁻¹ glucose, and 0.41 g g⁻¹ glucose and a productivity of 1.09, 1.12, and 1.14 g L⁻¹ h⁻¹, respectively. The accumulation of L-glutamate was observed at the end of the cultivation with final concentrations of 1.11 g L⁻¹ (H36EcGADmut), 1.19 g L⁻¹ (H36LsGAD), and 1.12 g L⁻¹ (H36LIGAD).

The highest GABA production was achieved in the batch fermentation of the *C. glutamicum* H36LIGAD strain at pH 6.0. It was first expected to achieve the enhanced production of GABA at pH 7.0 as with the flask cultivation results; however, a slightly higher concentration of GABA with improved productivity was observed at pH 6.0. It was reasoned that slightly acidic environments are still preferred in fermentative GABA production even with the employment of GADs active in a wide pH range, because the conversion of L-glutamate to GABA is a proton-consuming reaction.⁴³ Furthermore, LAB GADs were reported to favor lower pH conditions (approximately pH 5.0–6.0) in vitro analyses. For example, both GADs retained more than 80% of their relative activity at pH 5.0–6.0 but substantially lost their stability (up to 50%) if they deviate from their optimum pH.²⁹ Nevertheless, comparable amounts of GABA could be obtained by the recombinant *C. glutamicum* strains at pH 7.0. Furthermore, approximately 1 g L⁻¹ of unconverted L-glutamate accumulation was observed during the batch fermentations (Figure 3). Given that the unconverted L-glutamate concentrations during flask cultivation were over 10% of the final GABA concentrations (Figure 2), we concluded that batch fermentation where pH was tightly regulated was effective for GABA production with substantially reduced L-glutamate accumulation. Compared to our previous study,²² the employment of three different GADs demonstrated a higher production of GABA with a much lower L-glutamate accumulation. In our previous study,²² batch fermentation of *C. glutamicum* H36EcGADmut at pH 6.0 and 7.0 rendered 30.8 and 33.8 g L⁻¹ of GABA production, respectively, from 90 g L⁻¹ of glucose and 10 g L⁻¹ of xylose, with over 8 g L⁻¹ of L-glutamate accumulation. These high amounts of L-glutamate accumulated in the culture medium led to lower GABA productivity. In contrast, the present study achieved L-glutamate accumulation as low as 1.0 g L⁻¹, with GABA concentrations over 40 g L⁻¹ during the batch fermentation of the *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD strains under both pH 6.0 and pH 7.0. These findings indicated that all three GADs used in this study could efficiently produce GABA from glucose at a near-neutral pH.

More recently, novel GADs from *Bacillus* spp. were identified, and engineered *E. coli* expressing GADZ₁₁ could convert L-glutamate into GABA via whole-cell reaction at pH 5.0 with a 98.6% average conversion yield.⁴⁴ However, these GADs were not considered feasible for the industrial

production of GABA from glucose via direct fermentation, as they were only active under low pH conditions. In another study, the authors found that the PucR-type transcriptional regulator (*gadR*), GABA permease, GABA transaminase, and succinate semialdehyde dehydrogenase (*gadTDP*) are involved in GABA degradation pathways,⁴⁵ suggesting that the deletion of related enzymes could improve GABA production. Therefore, further enzyme engineering (crystal structure analysis and enzymatic characterization, de novo design of enzymes, and alteration of enzyme–substrate specificity) and evolutionary engineering (adaptive laboratory evolution (ALE)) of LsGAD and LIGAD could provide important insights into the exact mechanisms that drive the structural and kinetic changes of key enzymes in response to pH variations. Moreover, a combination of metabolic engineering and synthetic biology tools has enabled the development of engineered GABA-producing *C. glutamicum* strains with broader metabolic capacities to enhance the biosynthesis of target products.^{46–49}

CONCLUSIONS

Herein, we examined the *E. coli* GAD mutant and novel LAB-derived GADs, which were reported to be active in expanded pH range in vivo analysis for growth-coupled fermentative GABA production in L-glutamate-overproducing *C. glutamicum*, thus widening the usefulness of GADs active at near-neutral culture conditions. First, we compared the activities of two novel GADs from *L. senmaizukei* and *L. lactis*²⁹ and engineered GAD from *E. coli*^{21,22} under the control of two strong synthetic promoters (H30 and H36) having different strengths in *C. glutamicum*. During the flask cultivation, the concentration of GABA increased proportionally to the pH in the culture medium up to 7.0, indicating that the recombinant strains with engineered GADs could result in enhanced GABA production from glucose even at an almost neutral pH value. Further batch fermentation of *C. glutamicum* H36EcGADmut, H36LsGAD, and H36LIGAD rendered slightly higher final concentrations of GABA from 100 g L⁻¹ of glucose at pH 6.0, reaching yields of up to 40.3, 41.6, and 42.5 g L⁻¹, respectively. Since comparable amounts of GABA can be produced by the engineered strains even at a neutral pH value, the recombinant *C. glutamicum* strains developed in this study could be used for direct fermentative production of GABA in a growth-coupled manner, thus increasing the applicability of GADs that can produce GABA from glucose at an almost neutral pH. In addition, developed competitive bioprocesses with high amounts of GABA titer, productivity, and yield using glucose as a sole carbon source could contribute to the establishment of the economically feasible and efficient one-step fermentative GABA production system. However, the remaining issues must be addressed to ensure the widespread adoption of the approaches proposed herein, and therefore, the following strategies will be explored in our future studies: (i) characterizing the crystal structure of the enzymes and their functions, (ii) additional expression of pyridoxal kinase without the addition of external PLP for enhanced GAD activity,^{50,51} and (iii) blocking competitive pathways related to GABA degradation.⁴⁵

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) & funded by the Korean government (MSIT) (NRF-2018M3A9H3020459). Y.J.S. appreciates the support from Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A6A3A13045939).

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