# Functional analysis of lactic acid bacteria for efficient γ-aminobutyric acid production from processed tomato products

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## CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AOAA | aminooxyacetic acid |
| BLAST | Basic Local Alignment Search Tool |
| CTE | concentrated tomato extracts |
| GABA | γ-aminobutyric acid |
| GAD | glutamate decarboxylase |
| GRAS | generally recognized as safe |
| $g_{wcw}$/L | g (wet cell weight)/L |
| L-Glu | L-glutamic acid |
| HA | hydroxylamine |
| IPTG | isopropyl β-D-thiogalactopyranoside |
| LAB | lactic acid bacteria |
| LB | Luria-Bertani |
| MRS | de Man-Rogosa-Sharpe |
| MSG | monosodium glutamate |
| OD | optical density |
| PLP | pyridoxal 5′-phosphate |
| qPCR | quantitative polymerase chain reaction |
| SD | standard deviation |
| UV | ultraviolet |

# INTRODUCTRION

γ-Aminobutyric acid (GABA) is a four-carbon nonprotein amino acid widely distributed in nature and is known to function as a major inhibitory neurotransmitter in the mammalian central nervous system. GABA possesses various important physiological functions, including antihypertensive, anti-anxiety, tranquilizing, diuretic, and antidiabetic effects (1–4). In recent years, GABA has been used in supplements, beverages, and other healthcare foods (5,6).

GABA is synthesized from L-glutamic acid (L-Glu) by irreversible α-decarboxylation mediated by glutamate decarboxylase (GAD, E.C.: 4.1.1.15). GAD is a pyridoxal 5′-phosphate (PLP)-dependent enzyme (7–9), which is widely conserved among animals, plants, and microorganisms (9). Several microorganisms, including fungi, yeast, and lactic acid bacteria (LAB), have been reported to convert L-Glu into GABA (10–13). LAB have been applied in food industry, since LAB is well-known GRAS (generally recognized as safe) microorganisms and have been used for various fermented foods, including cheese, yogurt, sauerkraut, and other pickles (10,14–16). Many LAB can produce GABA and are distributed in species, including *Levilactobacillus brevis* (14,17–19), *Lactiplantibacillus plantarum* (14,20–24), *Lentilactobacillus buchneri* (25,26), *Lacticaseibacillus paracasei* (11,14,23), *Limosilactobacillus reuteri* (27,28), *Lacticaseibacillus rhamnosus* (23), and *Lactobacillus delbrueckii* (14).

To survive acidic environments, LAB have developed multiple acid-resistant systems, including $F_oF_1$-ATPase proton pump, sodium/proton antiporter, amino acid decarboxylation, alkali production, and biofilm formation (29–31). Regarding acid tolerance by decarboxylation of amino acids, proton consumption via GABA synthesis in *L. brevis* contributes significantly to maintaining cytoplasmic pH homeostasis (32). Regarding acid tolerance by decarboxylation of amino acids, proton consumption via GABA synthesis in *L. brevis* contributes significantly to maintaining cytoplasmic pH homeostasis (32). LAB strains have two isoforms of GADs. The gene encoding one isoform ($GadB_2$) is located adjacent to the *gad* operon, including the transcriptional regulatory gene (*gadR*), glutamic acid/GABA antiporter gene (*gadC*), and glutamyl-

tRNA synthetase gene (*gts*), whereas the gene encoding the other isoform ($GadB_1$) is separate and located far from the *gad* operon (24,33–35). *L. brevis* is well known as LAB with both *gadB1* and *gadB2* (24,33,35). In contrast, there are several reports that *L. plantarum* contains only *gadB1* (22,24), but *L. plantarum* KB1253 was reported by the authors as a first *L. plantarum* strain with both *gadB1* and *gadB2* (36). *L. reuteri* and *L. brevis* do not contain *gadB1* but *gadB2* (28,34). The expression of both genes is upregulated in response to low ambient pH when cells enter the late exponential growth phase (24,35).

In this study, the author focused on LAB as GABA-producers and carried out screening for GABA-producing LAB isolated from Japanese pickles, optimization of GABA production in tomato juice, and characterization of two GAD isomers of a selected LAB strain as GABA-producer.

Chapter I describes the screening for GABA producing LAB and optimization of GABA fermentation condition from tomato juice by screened LAB. In this chapter, the author found high GABA producing *L. plantarum* KB1253 by screening for 74 LAB strains.

Chapter II describes characterization of $GadB_1$ and $GadB_2$ involved in GABA production in *L. plantarum* KB1253.

Chapter III describes GABA production by whole cells of *L. plantarum* KB1253 using high concentrated tomato juice which is hard to use as a starting material for GABA production because it contains some GAD inhibitors. The stability of whole cells of *L. plantarum* KB1253 as a biocatalyst for industrial production of GABA from tomato juice was also evaluated for optimization the GABA production conditions.

# CHAPTER I

## Production of GABA-enriched tomato juice by *Lactiplantibacillus plantarum* KB1253

In previous studies, LAB has been mainly cultivated in a synthetic or semisynthetic medium for GABA production. However, it is of concern to increase GABA production costs, as the purification of fermented medium is required for use in foods (37). Tomato contains high L-Glu levels among agricultural products (38), and tomato juice has also been used as a medium for LAB cultivation. Previous study suggested that substances produced by heating tomatoes inhibit the production of GABA by LAB (39,40). In addition, 2,3-butanedione (diacetyl), which is produced when tomatoes are fermented with LAB, is known as an off-flavor and is an unfavorable volatile compound for food (41). Efficient production of GABA from processed tomato products while reducing the production of diacetyl is a problem for industrialization.

In this chapter, LAB that can produce high GABA and maintain desirable flavor as food after fermentation of tomato juice were selected from LAB derived from Japanese pickles. The optimal fermentation conditions for GABA production from tomato juice were also investigated.

# MATERIALS and METHODS

**Screening for GABA-producing LAB and growth conditions**

Ten kinds of pickles from different regions in Japan were suspended in sterile saline using Stomacher 80 (Seward, Worthing, UK), and the supernatant of the suspension was incubated on a de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Cambridge, UK) plate at 30°C for 24 h in a static incubator for the anaerobic condition. Each single colony was cultured in MRS broth at 30°C for 24 h without aeration and stored at -80°C. Each genomic DNA of LAB was purified using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured by NanoDrop one microvolume ultraviolet (UV) spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The isolates were identified by 16S rRNA gene sequencing. Upstream regions of 16S rRNA gene (about 500 bp) were amplified by PCR using following primers: 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and r1L (5′-GTATTACCGCGGCTGCTGG-3′). The PCR was done using a MyCycler (Bio-Rad, Hercules, CA, USA) as follows: denaturation at 94°C for 5 min, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. PCR products were examined by agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen). Nucleotide sequences were determined at an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA), and homology search was done using the Basic Local Alignment Search Tool (BLAST) and GenBank database (http://blast.ncbi.nlm.nih.gov). To screen GABA-producing strains, 74 isolates were cultured into MRS broth supplemented with 14 mM monosodium glutamate (MSG) at 30°C for 24 h without aeration, and GABA in cultured medium was measured. In addition, to evaluate production GABA and diacetyl from tomato juice by LAB fermentation, screened GABA-producing LAB were precultured in MRS broth at 30°C for 24 h without aeration. Subsequently, tomato juice (4.5°Bx; Kagome, Nagoya, Japan) was inoculated with 1% (v/v) MRS culture and incubated at 30°C for 48 h without aeration. GABA and diacetyl in fermented tomato juice by LAB were measured by high-performance liquid chromatography (HPLC) analysis and previously reported

method (42), respectively. To investigate the optimal pH for growth, finally selected LAB was cultivated in MRS broth (pH 3.5–5.5) at 30°C for 48 h. In addition, to investigate the optimal growth temperature, LAB was cultivated in MRS (optimal pH) at 25°C–45°C for 24 h.

**Gene expression analysis**

*L. plantarum* KB1253 cells cultivated at 35°C for 12 h without aeration were harvested by centrifugation at 8,000 × *g* at 4°C for 3 min, resuspended in an equal volume of MRS broth, and incubated for 3 h under different growth conditions (temperature of 30°C–50°C, pH of 3.0–4.5, and MSG concentration of 0–100 mM). *L. plantarum* KB1253 cells were harvested while inhibiting RNA degradation using RNA protect Bacteria Reagent (Qiagen), and total RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA concentration was measured by NanoDrop one microvolume UV spectrophotometer (Thermo Fisher Scientific). Genomic DNA removement and reverse transcription to cDNA were carried out using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The primers for quantitative polymerase chain reaction (qPCR) listed in Table 1-1 were designed using the program Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on *L. plantarum* KB1253 genomic DNA. The nucleotide sequence data reported in this study are shown in the DDBJ/EMBL/GenBank databases under accession numbers BIFE01000005 and BIFE01000033. The relative expression of $gadB_1$ and $gadB_2$ was calculated according to the comparative $2^{-\Delta\Delta Ct}$ method first described by K. Livak *et al*. (43). Expression levels were normalized to 16S rRNA gene expression, which was used as an internal housekeeping control. $C_t$ values were normalized to the samples cultured in MRS broth (pH 4.0) without MSG at 35°C (control). The following equations were used to determine the relative expression:

$\Delta C_t$ = $C_t$ of housekeeping gene (16S rRNA gene) - $C_t$ of target gene ($gadB_1$ or $gadB_2$)

$\Delta\Delta C_t$ = $\Delta C_t$ of control condition ($gadB_1$) - $\Delta C_t$ of treated condition ($gadB_1$ or $gadB_2$)

Relative expression = $2^{-\Delta\Delta Ct}$

Gene expression was measured by qPCR in triplicate with a QuantStudio® 3

(Applied Biosystems) using KOD SYBR qPCR/RT (Toyobo), and the mean was used for final analysis.

**Table 1-1** Primer sequences used for qPCR during the study for *L. plantarum* KB1253.

| Gene | Enzyme | Primer | Primer sequences (5′-3′) | Amplicon size (bp) |
|---|---|---|---|---|
| *gadB1* | Glutamate decarboxylase | $gadB_1$-F | CCTCGAGAAGCCGATCGCTTAGTTCG | 542 |
| | | $gadB_1$-R | TCATATTGACCGGTATAAGTGATGCCC | |
| *gadB2* | Glutamate decarboxylase | $gadB_2$-F | GGTCAACCCTCGACAGATT | 592 |
| | | $gadB_2$-R | GGAAATAACACCAGAACCCTTG | |
| 16S rRNA | Small subunit of prokaryotic ribosome | 16S-F | TCATGATTTACATTTGAGTG | 121 |
| | | 16S-R | GACCATGCGGTCCAAGTTGTT | |

**GABA production by LAB resting cells**

*L. plantarum* KB1253 cells cultivated in MRS broth at 30°C for 12 h without aeration were harvested by centrifugation at 8,000 × *g* at 4°C for 3 min, washed twice, and resuspended in the appropriate buffer. To investigate optimal GABA conversion conditions, the prepared resting cells were resuspended in 200 mM glycine-HCl buffer (pH 2.0–2.5), 200 mM sodium citrate buffer (pH 3.0–3.5), or 200 mM sodium acetate buffer (pH 4.0–5.5) supplemented with 50 mM MSG and 50 g wet cell/L and incubated for 1 h with different reaction conditions (temperature of 25°C–45°C and PLP concentration of 0–1.0 mM). In addition, to enhance GABA production, resting cells were resuspended in 200 mM sodium citrate buffer (pH 3.5) supplemented with 50 to 250 mM MSG and 150 g wet cell/L and incubated with 0.2 mM PLP at 45°C for 1 h.

**Tomato fermentation**

Tomato paste TP-4 Turkey (Kagome) was diluted to a concentration of 5 to 30°Bx with distilled water, heated at 105°C for 5 min, and centrifuged at 12,000 × *g* at 4°C for 15 min to remove lees. Each supernatant, tomato medium, was used for GABA fermentation without the addition of any compounds (e.g., glucose, L-Glu, or PLP). *L. plantarum* KB1253 was precultured in MRS broth at 35°C for 12 h without aeration.

Subsequently, to determine the maximum concentration of tomatoes capable of growth, tomato medium (5–30°Bx) was inoculated with 1% (v/v) MRS culture and incubated at 35°C for 48 h without aeration.

**Determination of biomass amount, GABA, L-Glu, lactic acid, and glucose**

Biomass cultured in MRS broth was determined by measuring the optical density (OD) at 600 nm using a spectrophotometer U-3900 (Hitachi High-Tech Science, Tokyo, Japan). Biomass cultured in tomato medium was determined by viable cell count. GABA and L-Glu were determined after ninhydrin derivatization through HPLC analysis using a Hitachi L-8900 system (Hitachi High-Tech Science) equipped with No. 2622 PF column (φ4.6 × 60 mm; Hitachi High-Tech Science) and a UV detector operating at 570 nm (44). Lactic acid was determined after bromothymol blue derivatization (45) through HPLC analysis using a Hitachi L-7000 system (Hitachi High-Tech Science) equipped with a GL-C610H-S column (φ7.8 × 300 mm; Hitachi High-Tech Science) and a UV detector operating at 440 nm (45). Glucose was determined through HPLC analysis using a Hitachi L-7000 system equipped with a Shodex Asahipak NH2P-50 4E column (φ4.6 × 250 mm; Hitachi High-Tech Science) and a refractive index detector (46).

**Statistical analysis**

All data in the bar charts and tables correspond to the mean ± standard deviation (SD). One-way analysis of variance and subsequent Tukey-Kramer's multiple-comparison tests were conducted to evaluate the significance of differences ($p<0.05$).

# RESULTS

## Isolation and screening of GABA-producing LAB

Three hundred twenty-five strains of LAB were isolated from pickles. Seventy-four isolates were randomly selected and screened through selection based on GABA productivity (Table 1-2). Strain identification by 16S rRNA sequencing revealed that all strains shared 98% to 100% identity with previously reported nucleotide sequences of each corresponding species. The obtained isolates were mainly constituted by *Latilactobacillus sakei* (24 isolates) and *L. plantarum* (23 isolates), and the others were *Companilactobacillus alimentarius* (14 isolates), *Pediococcus ethanolidurans* (5 isolates), *Lactiplantibacillus pentosus* (2 isolates), *P. parvulus* (2 isolates), *Enterococcus avium* (1 isolate), *Loigolactobacillus coryniformis* (1 isolate), *Latilactobacillus curvatus* (1 isolate), and *Lactiplantibacillus paraplantarum* (1 isolate). Two *L. plantarum* strains (KB1253 and KB1231) converted L-Glu into GABA at a rate of >90% (Table 1-3). In addition, both strains could also convert L-Glu in tomato juice into GABA at a rate of >90% (Table 1-4), although it was reported that substances produced by heating tomatoes may inhibit GABA production by LAB (40). Furthermore, *L. plantarum* KB1253 was finally selected as the most suitable GABA-producer for application in the food industry because it produced less diacetyl, known as an off-flavor in some foods, than *L. plantarum* KB1231 (Table 1-4). The genomic information of *L. plantarum* KB1253 has already published by the authors (36).

**Table 1-2** LAB isolated from pickles.

| Pickles | Raw materials | No. pickles | Identified strain (no. isolates) |
|---|---|---|---|
| *Pesorazuke* | Eggplant | 7 | *E. avium* (1),<br>*L. coryniformis* (1),<br>*L. plantarum* (5),<br>*P. ethanolidurans* (2) |
| *Kukizuke* | Taro and shiso leaf | 1 | *P. ethanolidurans* (1) |
| *Shibazuke* | Eggplant, cucumber, and shiso leaf | 6 | *C. alimentarius* (1),<br>*L. paraplantarum* (1),<br>*L. pentosus* (2),<br>*L. plantarum* (9) |
| *Suguki* | Radish | 4 | *C. alimentarius* (4),<br>*L. plantarum* (2),<br>*P. ethanolidurans* (1) |
| *Aso-takanazuke* | Mustard leaf | 6 | *C. alimentarius* (1),<br>*L. plantarum* (3),<br>*L. sakei* (2) |
| *Inekokina* | Radish | 3 | *L. curvatus* (1),<br>*L. sakei* (4) |
| *Takanazuke* | Mustard leaf | 1 | *P. parvulus* (1) |
| *Nanohanazuke* | Canola blossom | 4 | *C. alimentarius* (8) |
| *Akakabuzuke* | Radish | 4 | *L. plantarum* (4),<br>*L. sakei* (1),<br>*P. ethanolidurans* (1),<br>*P. parvulus* (1) |
| *Tsudakabuzuke* | Radish | 6 | *L. sakei* (17) |

**Table 1-3** High GABA-producing LAB

| Strain | LAB species identification based on the 16S rRNA sequence | Sequence homology with type strain (%) | GABA (mM) | GABA/L-Glu molar conversion rate (%) |
|---|---|---|---|---|
| KB1253 | *L. plantarum* | 99 | 13.4 | 95.7 |
| KB1231 | *L. plantarum* | 99 | 12.6 | 90.2 |
| KB1256 | *E. avium* | 99 | 2.9 | 20.4 |
| KB620 | *L. plantarum* | 99 | 1.3 | 9.6 |
| KB1146 | *L. plantarum* | 99 | 1.3 | 9.5 |
| KB660 | *L. plantarum* | 99 | 1.2 | 8.9 |
| KB619 | *L. plantarum* | 98 | 1.2 | 8.4 |
| KB1274 | *L. plantarum* | 99 | 1.1 | 7.8 |
| KB623 | *L. plantarum* | 99 | 1.0 | 7.3 |
| KB621 | *L. plantarum* | 98 | 0.9 | 6.4 |
| KB622 | *L. plantarum* | 99 | 0.8 | 5.8 |
| KB657 | *L. plantarum* | 98 | 0.7 | 5.1 |
| KB656 | *L. plantarum* | 98 | 0.7 | 5.1 |
| KB655 | *L. plantarum* | 98 | 0.7 | 4.9 |
| KB1086 | *C. alimentarius* | 99 | 0.7 | 4.7 |
| KB1502 | *L. sakei* | 99 | 0.6 | 4.6 |
| KB1090 | *C. alimentarius* | 98 | 0.6 | 4.2 |
| KB1039 | *L. plantarum* | 98 | 0.5 | 3.8 |
| KB1046 | *P. ethanolidurans* | 99 | 0.5 | 3.4 |
| KB950 | *L. plantarum* | 99 | 0.5 | 3.0 |
| KB1238 | *L. plantarum* | 99 | 0.4 | 2.9 |
| KB1280 | *L. plantarum* | 99 | 0.4 | 2.8 |
| KB1201 | *L. plantarum* | 98 | 0.4 | 2.7 |
| KB926 | *P. parvulus* | 99 | 0.4 | 2.6 |
| KB956 | *P. ethanolidurans* | 99 | 0.3 | 2.5 |
| KB1048 | *C. alimentarius* | 99 | 0.3 | 2.1 |
| KB1022 | *L. plantarum* | 99 | 0.2 | 1.7 |
| KB1034 | *L. plantarum* | 98 | 0.2 | 1.5 |
| KB1013 | *L. plantarum* | 98 | 0.2 | 1.5 |
| KB1166 | *L. plantarum* | 98 | 0.2 | 1.4 |
| KB642 | *L. paraplantarum* | 98 | 0.2 | 1.1 |
| KB1208 | *L. plantarum* | 99 | 0.1 | 1.1 |
| KB627 | *L. pentosus* | 99 | trace | 0.0 |
| KB629 | *L. pentosus* | 99 | trace | 0.0 |

**Table 1-3** *Cont.*

| Strain | LAB species identification based on the 16S rRNA sequence | Sequence homology with type strain (%) | GABA (mM) | GABA/L-Glu molar conversion rate (%) |
|---|---|---|---|---|
| KB694 | *C. alimentarius* | 98 | trace | 0.0 |
| KB951 | *L. sakei* | 98 | trace | 0.0 |
| KB1023 | *P. parvulus* | 98 | trace | 0.0 |
| KB1094 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1163 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1175 | *L. sakei* | 99 | trace | 0.0 |
| KB1194 | *L. sakei* | 99 | trace | 0.0 |
| KB1219 | *P. ethanolidurans* | 98 | trace | 0.0 |
| KB1236 | *P. ethanolidurans* | 99 | trace | 0.0 |
| KB1242 | *L. coryniformis* | 99 | trace | 0.0 |
| KB1286 | *L. sakei* | 100 | trace | 0.0 |
| KB1287 | *L. sakei* | 100 | trace | 0.0 |
| KB1288 | *L. sakei* | 98 | trace | 0.0 |
| KB1290 | *L. sakei* | 99 | trace | 0.0 |
| KB1323 | *L. curvatus* | 98 | trace | 0.0 |
| KB1434 | *P. ethanolidurans* | 99 | trace | 0.0 |
| KB1458 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1460 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1461 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1462 | *C. alimentarius* | 98 | trace | 0.0 |
| KB1463 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1468 | *C. alimentarius* | 98 | trace | 0.0 |
| KB1484 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1491 | *C. alimentarius* | 99 | trace | 0.0 |
| KB1495 | *L. sakei* | 99 | trace | 0.0 |
| KB1500 | *L. sakei* | 99 | trace | 0.0 |
| KB1503 | *L. sakei* | 99 | trace | 0.0 |
| KB1504 | *L. sakei* | 99 | trace | 0.0 |
| KB1542 | *L. sakei* | 98 | trace | 0.0 |
| KB1546 | *L. sakei* | 98 | trace | 0.0 |
| KB1548 | *L. sakei* | 98 | trace | 0.0 |
| KB1551 | *L. sakei* | 98 | trace | 0.0 |
| KB1553 | *L. sakei* | 98 | trace | 0.0 |
| KB1554 | *L. sakei* | 99 | trace | 0.0 |

**Table 1-3** *Cont.*

| Strain | LAB species identification based on the 16S rRNA sequence | Sequence homology with type strain (%) | GABA (mM) | GABA/L-Glu molar conversion rate (%) |
|---|---|---|---|---|
| KB1556 | *L. sakei* | 99 | trace | 0.0 |
| KB1571 | *L. sakei* | 98 | trace | 0.0 |
| KB1572 | *L. sakei* | 98 | trace | 0.0 |
| KB1573 | *L. sakei* | 98 | trace | 0.0 |
| KB1576 | *L. sakei* | 98 | trace | 0.0 |
| KB1579 | *L. sakei* | 98 | trace | 0.0 |

**Table 1-4** Tomato juice fermentation with GABA-producing LAB.

| Strain | L-Glu (mM) | GABA (mM) | GABA/L-Glu molar conversion rate (%) | Diacetyl (mg/L) |
|---|---|---|---|---|
| *L. plantarum* KB1253 | 0.1 | 16.8 | 93.2 | 0.36 |
| *L. plantarum* KB1231 | 1.6 | 15.5 | 83.4 | 1.04 |
| Tomato juice | 13.9 | 3.9 | – | 0.08 |

## Growth properties of *L. plantarum* KB1253

This strain grew well at an initial pH of 4.0 to 6.0 and reached the stationary phase in 18 to 24 h, with an $OD_{600}$ of 1.6 to 1.9 (Fig. 1-1A). However, the growth was significantly inhibited at an initial pH of 3.5, and the OD was ~1.4 even after 48 h cultivation. An optimal incubation temperature for the growth of this strain was also investigated. Under various temperature conditions, the $OD_{600}$ at 13 h after the start of incubation, corresponding to the late logarithmic growth phase, was investigated (Fig. 1-1B). This strain grew well from 30°C to 36°C, but growth decreased at 38°C.

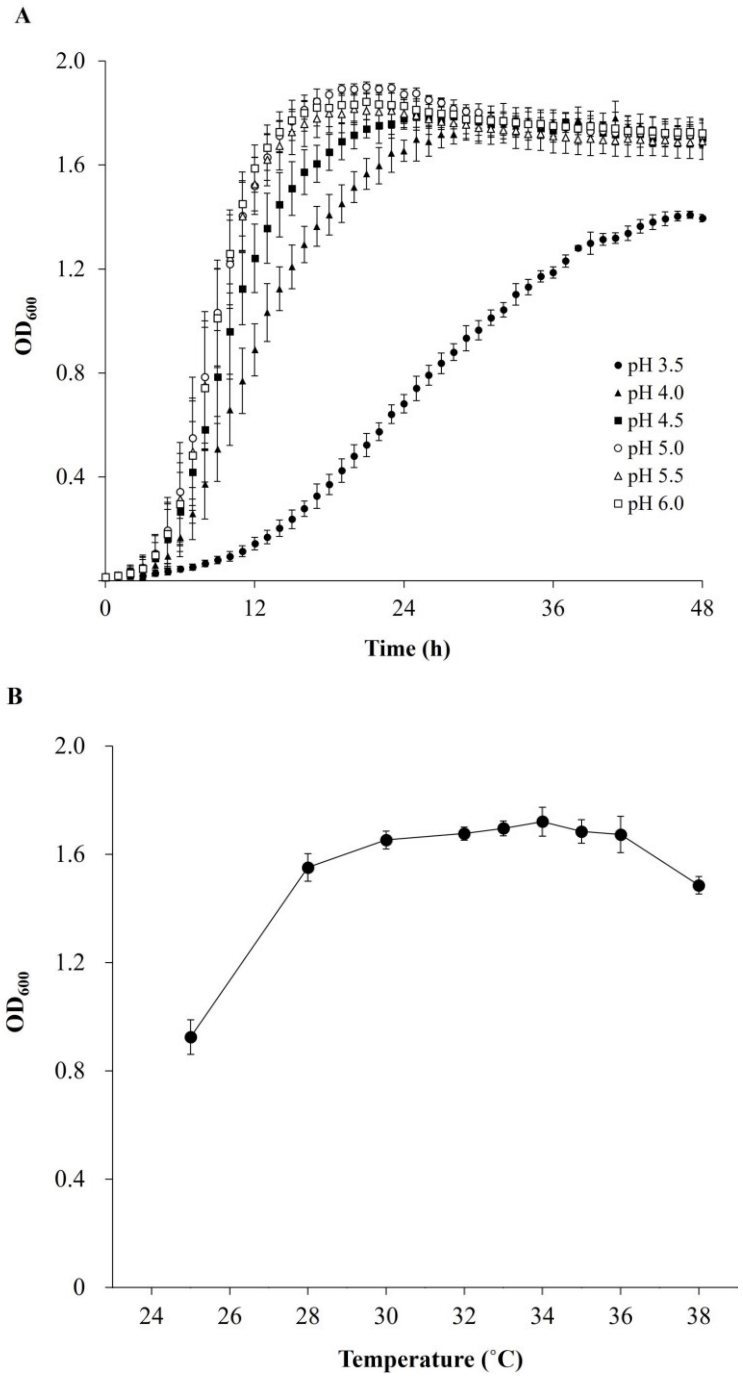



**Fig. 1-1.** Effects of initial pH of MRS medium and temperature on the growth of *L. plantarum* KB1253. To investigate the optimal pH for growth, *L. plantarum* KB1253 was cultivated in MRS broth (pH 3.5–6.0) at 30°C for 48h. In addition, to investigate the optimal growth temperature, *L. plantarum* KB1253 was cultivated in MRS (pH 5.0) at 25°C–38°C for 24h. Data are the mean ± SD of three independent experiments. A, initial pH; B, temperature.

**Gene expression level of two isoforms of the *GAD* gene**

After 13 h cultivation, corresponding to the late logarithmic growth phase of *L. plantarum* KB1253, cells were harvested by centrifugation, resuspended in different buffer solutions, and further incubated for 3 h, and gene expression profiles of cells were investigated. $gadB_1$ expression at pH 3.0 was highest (2.8-fold higher than at pH 4.0; Fig. 1-2). The lower the pH, the higher the expression (between pH 3.0 and 5.0), and almost no expression at pH 5.0 ($2.5 \times 10^{-1}$-fold). With $gadB_2$, the expression level increased with decreasing pH; however, at pH 5.0, almost no expression was observed ($3.5 \times 10^{-5}$-fold). The effects of incubation temperature on the amount of *gad* expression were also investigated. In common with both genes, little expression was observed at low temperatures (30°C). $gadB_1$ showed high expression at 40°C to 45°C, with the highest expression at 40°C (2.9-fold). In contrast, $gadB_2$ was hardly expressed at temperatures above 45°C. As a factor other than pH, there are few reports on the effects of temperature on *gad* gene expression. Furthermore, the effects of MSG concentration on the expression level were investigated. *gad* genes are inducible (33,35), and it is thought that MSG induces *gad* expression in this strain as well. The expression levels of $gadB_1$ and $gadB_2$ were investigated with 0.1 to 100 mM MSG in the buffer solution. Results showed that the expression levels of both genes increased as the MSG concentration increased from 0.1 to 50 mM MSG, up to ~6.0-fold. Moreover, under optimal conditions, $gadB_2$ was 35- to 90-fold more expressed than $gadB_1$.

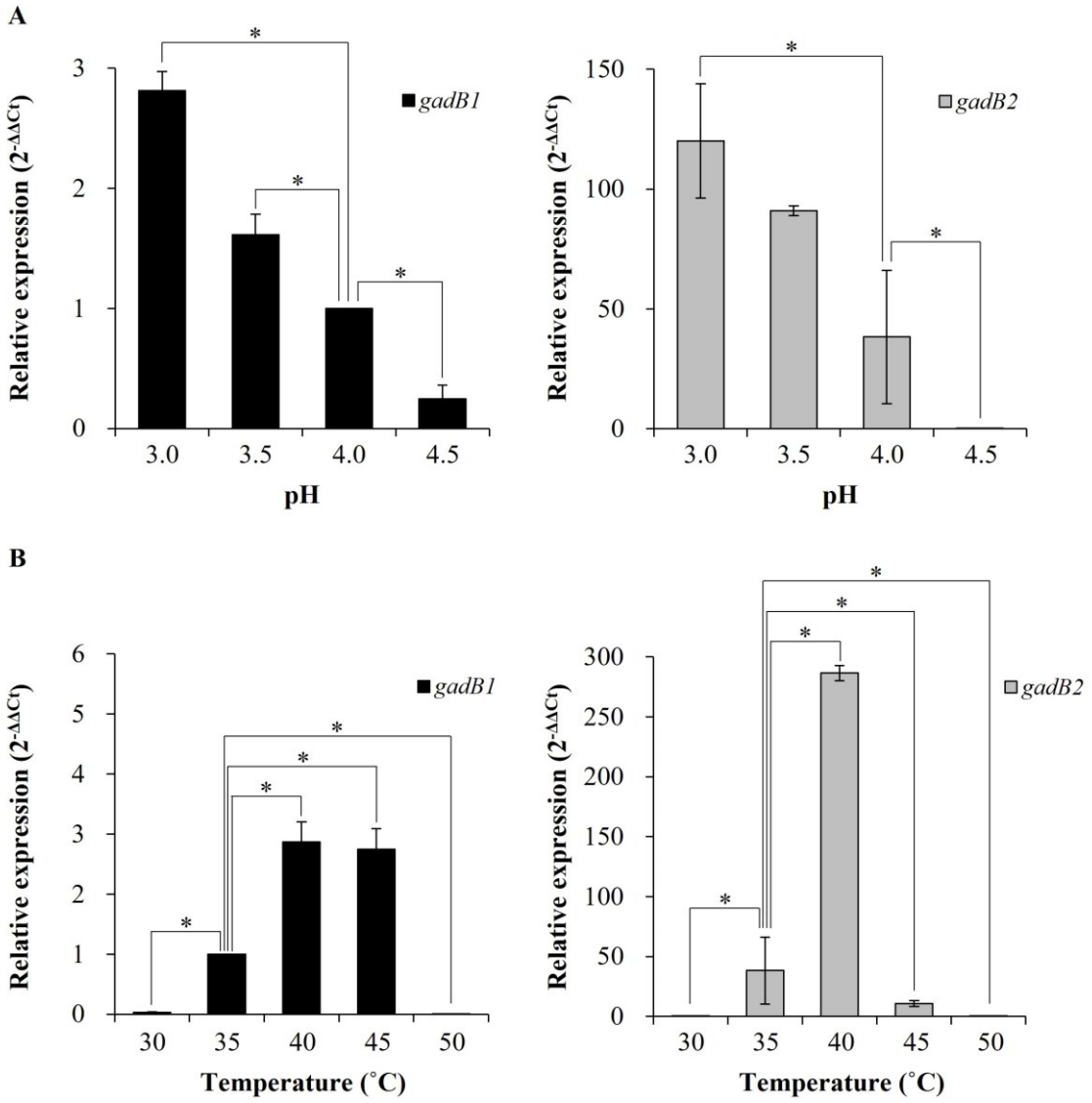



**Fig. 1-2.** *gadB1* and *gadB2* expression in *L. plantarum* KB1253 at different culture conditions for 3 h. Total RNA extracted from *L. plantarum* KB1253 cells incubated under various conditions (pH of 3.0–4.5, temperature of 30°C–50°C, and MSG concentration of 0–100 mM) were evaluated for *gadB1* and *gadB2* expression. Expression levels were normalized to 16S rRNA gene expression, which was used as an internal housekeeping control. $C_t$ values were normalized to *gadB1* of the samples cultured in MRS broth (pH 4.0) without MSG at 35°C (control). The following equations were used to determine the relative expression:

$\Delta C_t$ = $C_t$ of housekeeping gene (16S rRNA gene)- $C_t$ of target gene (*gadB1* or *gadB2*)

$\Delta\Delta C_t$ = $\Delta C_t$ of control condition (*gadB1*) - $\Delta C_t$ of treated condition (*gadB1* or *gadB2*)

Relative expression = $2^{-\Delta\Delta Ct}$

Data are the mean ± SD of three independent experiments. *p<0.05 compared to the control condition (pH 4.0, 35°C, without MSG). A, pH; B, temperature; C, MSG concentration.

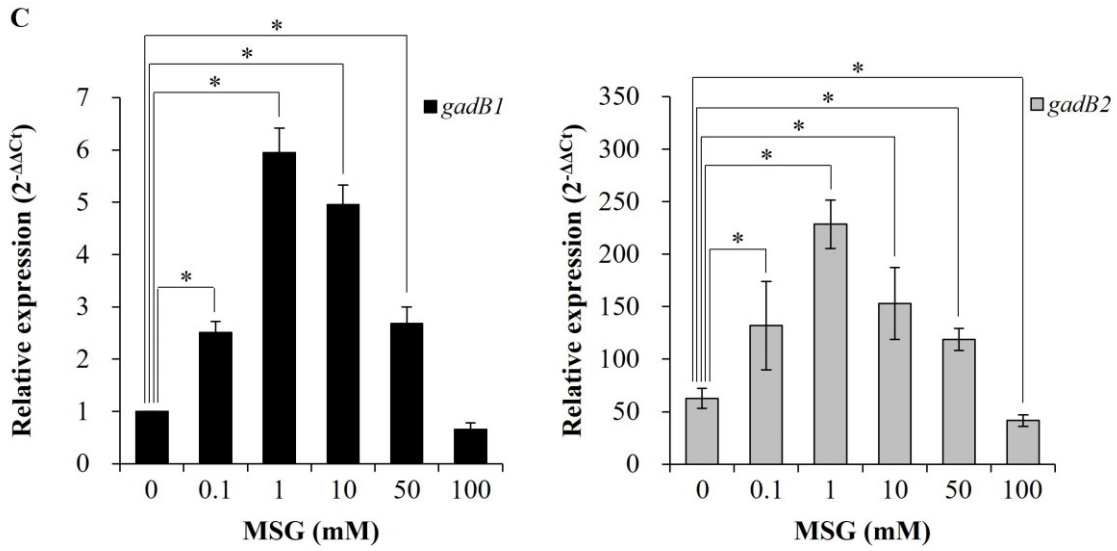



**Fig. 1-2.** *Cont. $gadB_1$* and *$gadB_2$* expression in *L. plantarum* KB1253 at different culture conditions for 3 h. Total RNA extracted from *L. plantarum* KB1253 cells incubated under various conditions (pH of 3.0–4.5, temperature of 30°C–50°C, and MSG concentration of 0–100 mM) were evaluated for *$gadB_1$* and *$gadB_2$* expression. Expression levels were normalized to 16S rRNA gene expression, which was used as an internal housekeeping control. $C_t$ values were normalized to *$gadB_1$* of the samples cultured in MRS broth (pH 4.0) without MSG at 35°C (control). The following equations were used to determine the relative expression:

$\Delta C_t$ = $C_t$ of housekeeping gene (16S rRNA gene)- $C_t$ of target gene (*$gadB_1$* or *$gadB_2$*)

$\Delta\Delta C_t$ = $\Delta C_t$ of control condition (*$gadB_1$*) - $\Delta C_t$ of treated condition (*$gadB_1$* or *$gadB_2$*)

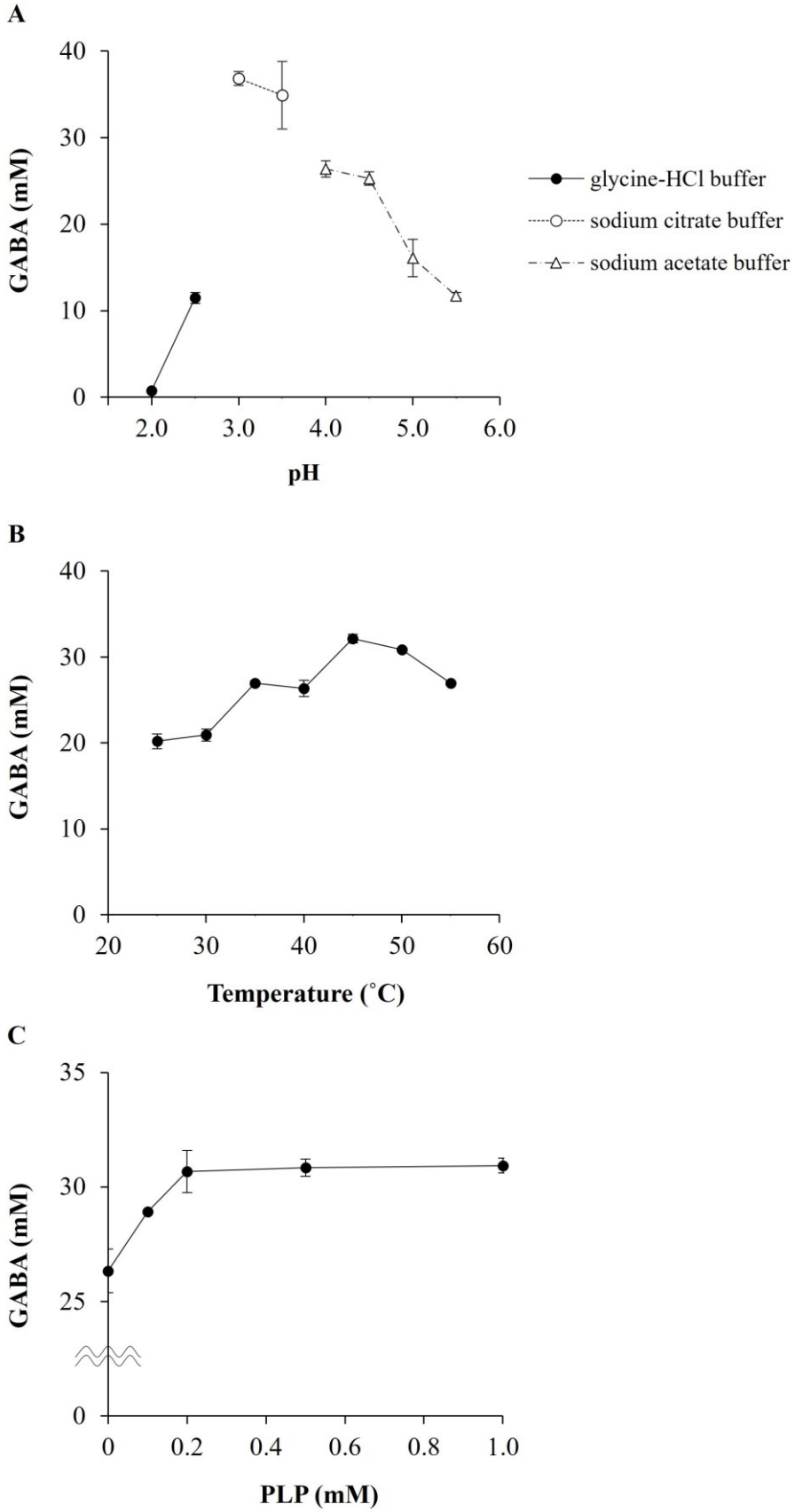
Relative expression = $2^{-\Delta\Delta Ct}$

Data are the mean ± SD of three independent experiments. *p<0.05 compared to the control condition (pH 4.0, 35°C, without MSG). A, pH; B, temperature; C, MSG concentration.

## Characterization of GAD activity of *L. plantarum* KB1253 in resting cell reactions

To understand the GAD activity of *L. plantarum* KB1253 without the effects of changing environments during cultivation, the GABA conversion rate under various conditions was investigated using resting cells. *L. plantarum* KB1253 produced GABA most efficiently at pH 3.0 (200 mM citrate buffer), producing 36.8 ± 0.8 mM GABA from 50 mM MSG in 1 h (Fig. 1-3A). GABA production at pH 3.0 to 4.5 was 26.4 ± 0.9 to 36.8 ± 0.8 mM. In contrast, when the pH was <2.5 and >5.0, GABA production decreased significantly (<16.1 ± 2.2 mM). Compared to the previously reported optimal

pH (4.2–5.0) (32,47–51), the optimal pH for GABA production in *L. plantarum* KB1253 was suggested to be much lower. As the reaction temperature increased, GABA production increased, reaching a maximum of 32.1 ± 0.5 mM at 45°C (Fig. 1-3B). However, at >50°C, the GABA conversion rate decreased. When the reaction was carried out at 55°C, GABA production decreased to the same level (27.0 ± 0.2 mM) as that at 40°C (26.3 ± 0.9 mM). GAD is a PLP-dependent enzyme and requires PLP for its activity (24,47,52). Therefore, the effects of PLP addition on GABA conversion by resting cells of *L. plantarum* KB1253 were investigated. The decarboxylation activity of *L. plantarum* KB1253 depended on the PLP concentration (up to ~0.2 mM; Fig. 1-3C). However, the activity did not change when more PLP was added. Additionally, to improve GABA production, a resting cell reaction was performed in a buffer solution with higher MSG concentrations. Thirty minutes after the start of the resting cell reaction, GABA conversion rates of 98.5 ± 1.8%, 92.9 ± 1.3%, 86.6 ± 3.8%, and 80.2 ± 4.0% were shown in the reaction system with initial MSG concentrations of 50, 100, 200, and 250 mM, respectively (Fig. 1-3D). One hour after the start of the reaction, the conversion rates were >90% with all MSG concentrations (48.5 ± 0.9, 84.3 ± 0.3, 185.5 ± 5.0, and 245.8 ± 3.4 mM GABA, respectively; Fig. 1-3E).


A
GABA (mM)
pH
glycine-HCl buffer
sodium citrate buffer
sodium acetate buffer
B
GABA (mM)
Temperature (˚C)
C
GABA (mM)
PLP (mM)

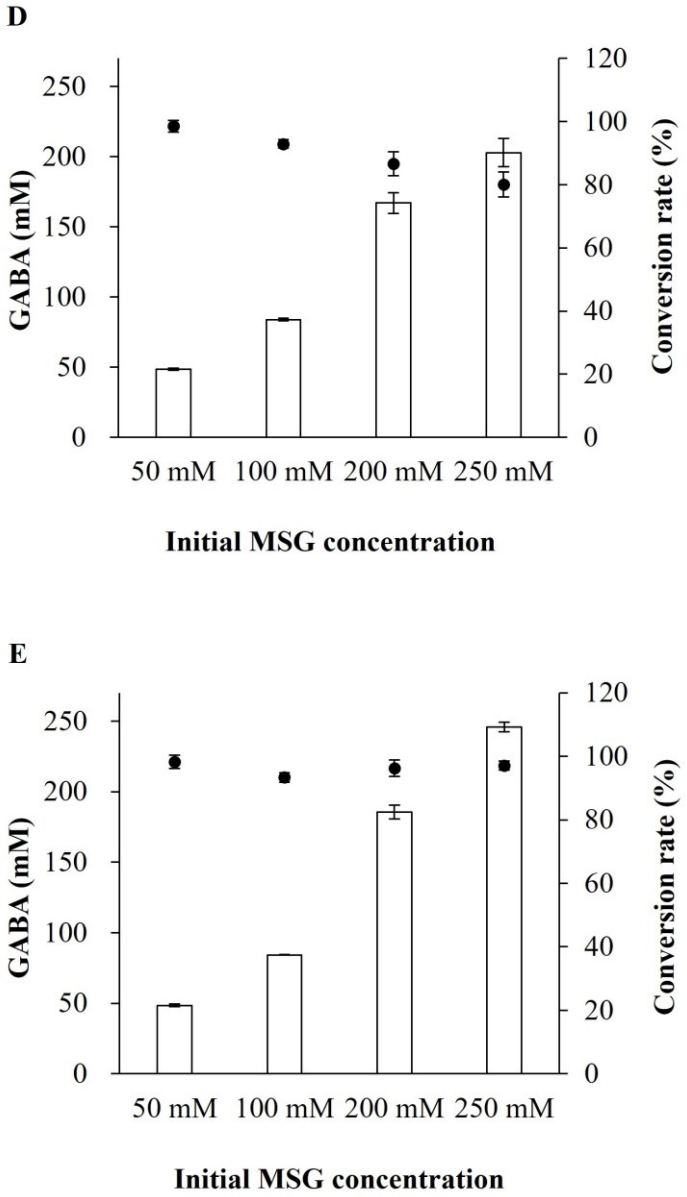



**Fig. 1-3.** Effects of pH, temperature, PLP concentration, and initial MSG concentration on GABA production by resting cells of *L. plantarum* KB1253. To investigate optimal GABA conversion conditions, the prepared resting cells were resuspended in 200 mM glycine-HCl buffer (pH 2.0–2.5), 200 mM sodium citrate buffer (pH 3.0–3.5), or 200 mM sodium acetate buffer (pH 4.0–5.5) supplemented with 50 mM MSG and 50 g wet cell/L and incubated for 1 h with different reaction conditions (temperature of 25°C–45°C and PLP concentration of 0–1.0 mM). In addition, to improve GABA production, resting cells were resuspended in 200 mM sodium citrate buffer (pH 3.5) supplemented with 50 to 250 mM MSG and 150 g wet cell/L and incubated with 0.2 mM PLP at 45°C for 1 h. Data are the mean ± SD of three independent experiments. A, pH (40°C, without PLP); B, temperature (pH 4.5, without PLP); C, PLP concentration (40°C, pH 4.5); D and E, initial MSG concentration [GABA conversion rate at 0.5 h (D) and 1.0 h (E) after the start of the reaction].

**GABA production during tomato fermentation by *L. plantarum* KB1253**

To produce more GABA-enriched tomato juice, higher concentrations of tomato medium with higher glutamate concentrations might be better. However, there is concern about growth inhibition due to osmotic pressure and/or acid stress. To determine the maximum concentration of tomato medium for GABA fermentation, *L. plantarum* KB1253 was incubated for 24 h in tomato medium (5–30°Bx, pH 4.0) at 35°C. This strain grew well in tomato medium (5–20°Bx) and reached the stationary phase in 12 to 24 h (Fig. 1-4). However, the growth was significantly inhibited in tomato medium (25–30°Bx).

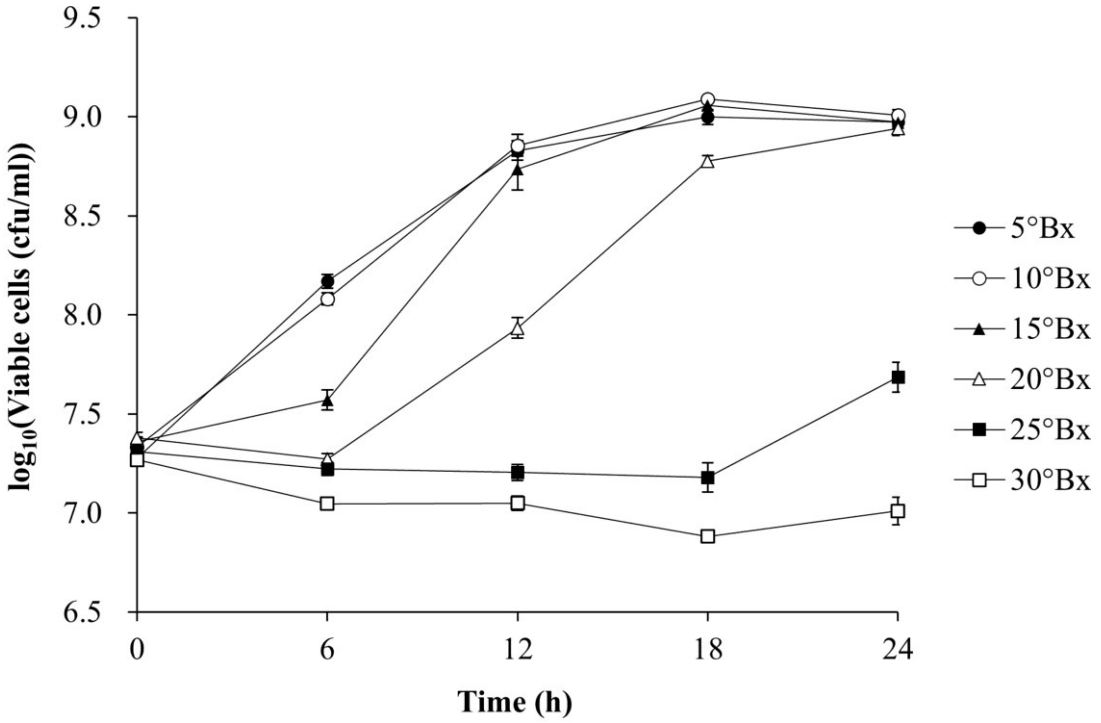



**Fig. 1-4.** Effects of tomato concentration on the growth of *L. plantarum* KB1253. *L. plantarum* KB1253 was precultured in MRS broth at 35°C for 12 h without aeration. Subsequently, to determine the maximum concentration of tomatoes capable of growth, tomato medium (5–30°Bx) was inoculated with 1% (v/v) MRS culture and incubated at 35°C for 48 h without aeration. Data are the mean ± SD of three independent experiments.

Therefore, to investigate the optimal pH for GABA production in tomato medium, *L. plantarum* KB1253 was incubated in tomato medium (20°Bx, pH 4.0–5.0).

The higher the initial pH, the faster the growth. In the tomato medium with an initial pH of 5.0, the growth almost entered the stationary phase 12 h after the start of culture (Fig. 1-5A). With this, glucose utilization and lactic acid production also differed depending on the initial pH (Fig. 1-5B and C). The higher the pH, the more glucose was utilized for growth, and the more lactic acid was produced, resulting in a lower pH of the culture medium (Fig. 1-5D). In the tomato medium with an initial pH of 4.5, the first 12 h of culture were the logarithmic growth phase, and the pH was ~4.0 (Fig. 1-5D). In contrast, in the tomato medium with an initial pH of 5.0, the pH became ~4.0 at 12 h (Fig. 1-5D). GABA production in tomato medium with an initial pH of 5.0, which grew well, was significantly inhibited, and insufficient GABA conversion was observed from 24 to 48 h cultivation even at pH 4.0, the optimal pH for GAD activity and *gad* expression (Fig. 1-5E). In contrast, when a tomato medium with initial pH of 4.0 was used, the GABA conversion rate already reached >85% at 24 h after the start of incubation (40.2 ± 1.4 mM GABA; Fig. 1-5E). Eventually, after 24 h incubation at pH 4.0, this strain produced 41.0 ± 1.1 mM GABA (Fig. 1-5F) despite inferior growth compared to growth in a tomato medium with higher initial pH of 5.0 (Fig. 1-5E). Although there was a difference in growth at the beginning of the culture (0–24 h), the number of viable cells after 24 h, corresponding to the stationary phase, was almost the same, even with different initial pH values. Therefore, it is suggested that the pH profile in the tomato medium up to 12 h incubation may significantly affect GABA production.

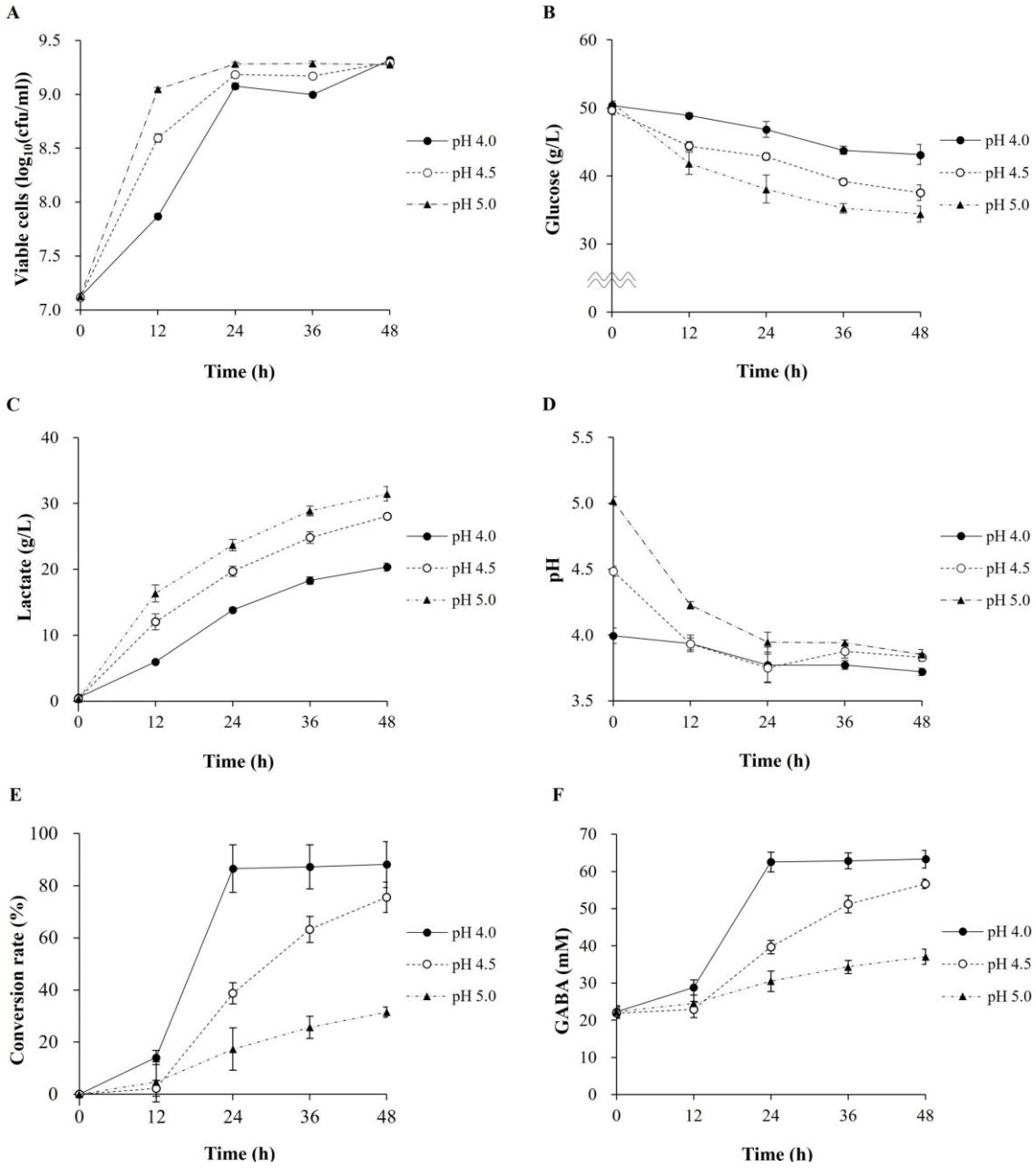



**Fig. 1-5.** Time profiles of growth (A–D) and GABA production (E and F) by *L. plantarum* KB1253. To investigate the optimal pH for GABA production in tomato medium, *L. plantarum* KB1253 was incubated in tomato medium (20°Bx, pH 4.0–5.0) at 35°C for 48 h without aeration. Data are the mean ± SD of three independent experiments. A, viable cell count; B, glucose; C, lactic acid; D, pH; E, GABA conversion rate; F, GABA.

# DISCUSSION

The lactobacilli most reported as a high GABA producer is *L. brevis* (0.1–591.5 mM GABA) (14,17–19,53). *L. plantarum* is reported to produce only 0.2 to 38.8 mM GABA at most in batch reaction systems (14,20–23,53). Nevertheless, *L. plantarum* KB1253 produced GABA in a shorter time and at a higher concentration than the previously reported GABA-producing *L. plantarum*. Previous studies revealed that *L. plantarum* KB1253 has two glutamate decarboxylase genes ($gadB_1$ and $gadB_2$) (36). The putative loci of GAD-related proteins in *L. plantarum* 90sk (24), *L. plantarum* Taj-Apis362 (22), *L. brevis* 15f (24), *L. brevis* NBRC12005 (49), *L. sakei* A156 (54), and *L. zymae* GU240 (47) were analyzed against the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the protein BLAST program and shown in Fig. 1-6 with the amino acid sequence identity scores along with GAD-related proteins of *L. plantarum* KB1253. GABA-producing *L. plantarum* strains have been reported to possess $gadB_1$ (22,24) but have never been reported to possess $gadB_2$ included in the *gad* operon. However, unlike other GABA-producing *L. plantarum* strains, *L. plantarum* KB1253 possesses $gadB_1$ and *gad* operon genes, including $gadB_2$ and *gadC* (24,49). It has been suggested that the *gad* operon, including $gadB_2$ in *L. brevis* strains, leads to high GABA production (35). Under optimal expression conditions, $gadB_2$ was 35- to 90-fold more expressed than $gadB_1$, suggesting that $gadB_2$ plays a key role in GABA production in *L. plantarum* KB1253. In addition, unlike other *L. plantarum* strains, *L. plantarum* KB1253 possesses *gadC* involved in L-Glu uptake and GABA export, it can affect the direct GABA production from L-Glu. Thus, the ability of *L. plantarum* KB1253 to produce high GABA levels may be attributed to its unique genetic constitution.

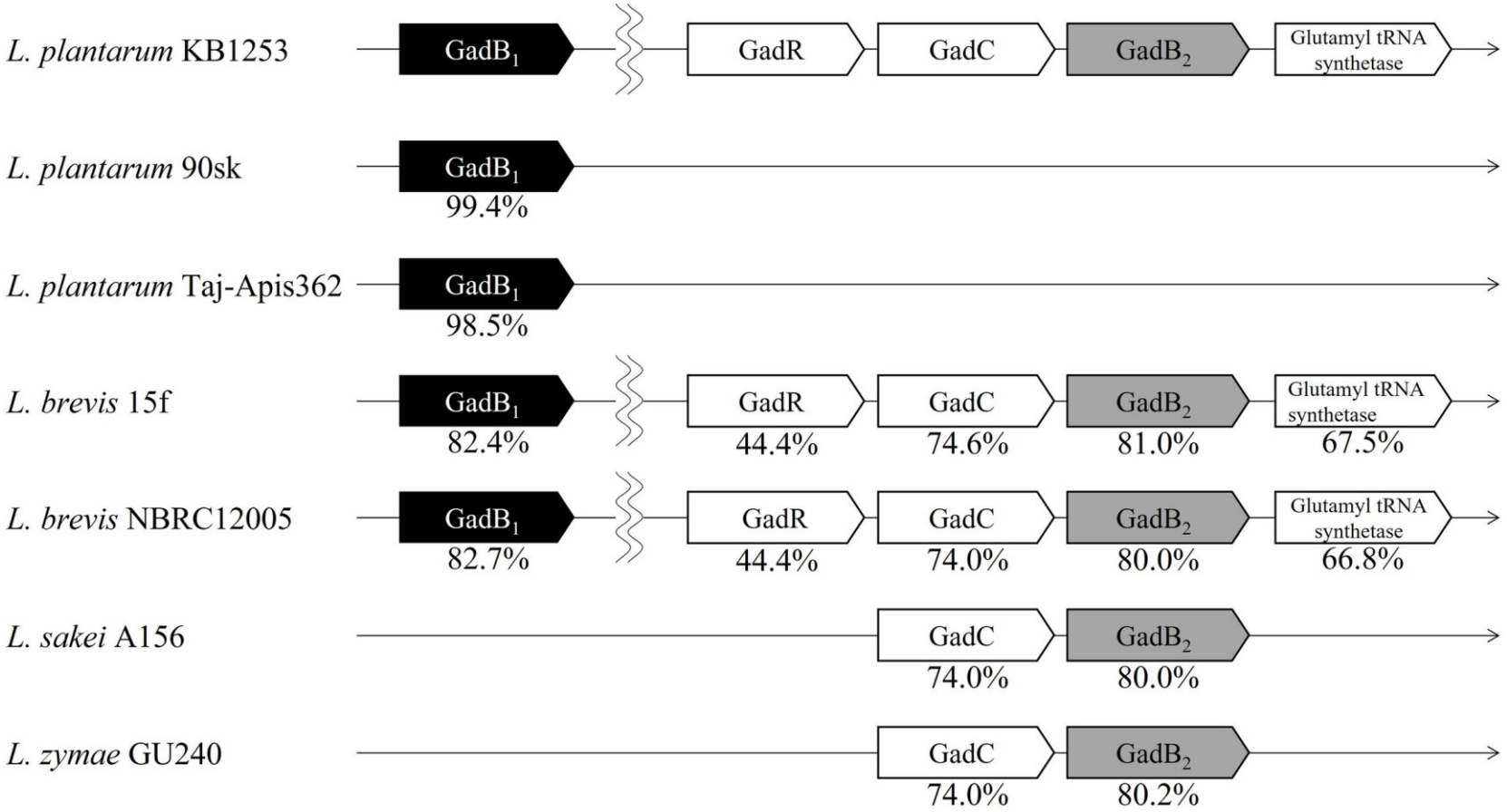



**Fig. 1-6.** Representation of gene loci encoding the proteins $GadB_1$, GadR, GadC, $GadB_2$, and Glutamyl tRNA synthetase in the 7 strains. Numbers indicate the protein identity with *gad*-related proteins of *L. plantarum* KB1253.

This strain grew well up to 20°Bx concentration of tomato medium (initial pH of 4.0); however, growth was significantly inhibited when the tomato medium concentration was >25°Bx. Osmotic stress and organic acids, including acetic acid, propionic acid, lactic acid, and citric acid, affect LAB growth (55,56). The growth inhibition of *L. plantarum* KB1253 in tomato medium with >25°Bx is thought to be due to sugar and the organic acids, such as citric acid, contained in tomato at high concentrations. Therefore, this study investigated the fermentation condition under which GABA can be efficiently produced in 20°Bx concentration of tomato medium by *L. plantarum* KB1253. As mentioned above, at 20°Bx, glutamate did not suppress $gadB_1$ and $gadB_2$ expression. Furthermore, the optimal pH for growth was 4.0 to 6.0, the optimal pH for gene expression of $gadB_1$ and $gadB_2$ was 3.0 to 4.0, and the optimal pH for resting cell activities for GABA production was 3.0 to 4.5. Therefore, it is appropriate to use a tomato medium with an initial pH of 4.0 for GABA production. In addition, a tomato medium with an initial pH of 4.5 or 5.0 may also be in the optimal pH range for $gadB_1$ and $gadB_2$ expression and $GadB_1$ and $GadB_2$ activities by the time it reaches the stationary phase, as it is assumed that the pH drops with time due to lactic acid production during growth. At an initial pH of 4.0 to 4.5, growth was poorer and entered the stationary phase at 24 h; at pH 5.0, growth was good and entered the stationary phase at 12 to 24 h. At the initial pH of 4.0, the pH dropped slowly and reached pH 3.72 ± 0.03 at 48 h. In contrast, at the initial pH of 4.5 to 5.0, it took 12 and 24 h, respectively, to reach pH <4.0, the range of high $gadB_1$ and $gadB_2$ expression and $GadB_1$ and $GadB_2$ activities. The growth was pH-dependent, and the higher the pH, the better the growth. However, in actual GABA production, the conversion rate was considerably higher at pH 4.0, where growth was poor. At the initial pH of 4.0, growth was not very high, but GAD was expressed in the early stages of growth, and the pH was optimal for GAD reaction, suggesting that GABA production was observed at an early stage of growth.

This study evaluated GABA production in tomato medium by *L. plantarum* KB1253, a newly found GABA-producing lactic acid bacterium. By adjusting tomato Bx to 20°Bx and initial pH to 4.0 and incubating at 35°C for 24 h, it was possible to produce 41.0 ± 1.1 mM GABA from 46.8 ± 3.4 mM glutamate in tomato medium.

During the fermentation process by *L. plantarum* KB1253, L-Glu, which contributes to umami, is reduced and acidity is increased by lactic acid production. This makes it possible to give fermented tomato juice a refreshing taste. Therefore, blending this concentrated fermented tomato juice with various vegetable juices enables a wide range of flavor designs and can be useful in the production of various health-promoting vegetable beverages. There are no reported cases of enhancing GABA concentration in tomato juice, and it is believed that this will provide useful knowledge to produce functional vegetable beverages and seasonings with health functions in the future. This study could not investigate the details of the two GAD isoforms in the newly reported *L. plantarum* KB1253 and the precise biochemical characteristics of each enzyme. In the next chapter, evaluation of each enzyme was carried out to establish more efficient GABA production.

# SUMMARY

To produce tomato juice with health-promoting functions, LAB capable of converting L-glutamic acid in tomatoes into GABA was screened from LAB stocks isolated from Japanese pickles. *L. plantarum* KB1253 was selected as the highest GABA producer among 74 strains of LAB stocks. *gad* gene expression and glutamic acid decarboxylation activity increased at low pH (3.0–3.5), whereas the growth decreased. Under optimal reaction conditions using resting cells as catalysts, this strain produced 245.8 ± 3.4 mM GABA. Furthermore, this strain produced 41.0 ± 1.1 mM GABA from L-glutamic acid in tomato juice under optimal fermentation conditions (pH 4.0, 20°Bx). This study may provide the basis for developing health-promoting functional foods rich in GABA from tomatoes and other agricultural products.

# CHAPTER II

## Characterization of two glutamate decarboxylases from *Lactiplantibacillus plantarum* KB1253

In chapter I, the author has isolated GABA-producing LAB, *L. plantarum* KB1253, from Japanese pickle. This strain possessed two GAD isoforms $GadB_1$ and $GadB_2$ (57). Whole cells of *L. plantarum* KB1253 could convert L-Glu into GABA in buffer without the addition of PLP, and this strain could produce 41.0 ± 1.1 mM of GABA from L-Glu derived from tomato juice (pH 4.0, 20°Bx) (57). These results suggested that one or both GAD isoforms of this strain may exist as PLP-containing apo-enzymes (apoGADs) not requiring exogenous PLP for glutamate decarboxylation reaction such as GADs in *E. coli* (58). However, to date, there is no information on that this strain biosynthesizes PLP and utilizes it in the reaction of PLP-dependent enzymes. In addition, GABA production by each purified GAD of *L. plantarum* KB1253 has not yet been characterized in detail. The biochemical characterization of each GAD of *L. plantarum* KB1253 is expected to contribute to industrial biosynthesis of GABA by fermentative methods or enzymatic methods from tomatoes and other agricultural products.

In this chapter, the author obtained his-tagged fusion proteins ($GadB_1$-$(His)_6$ and $GadB_2$-$(His)_6$) and evaluated the biochemical characteristics. The biochemical characteristics of two GAD isoforms from *L. plantarum* KB1253 were also compared with those of GADs from other LAB species.

# MATERIALS and METHODS

## Bacterial strains, plasmids, and media

*L. plantarum* KB1253 with high γ-aminobutyric acid (GABA)-producing capacity was isolated from the eggplant pickles in Japan (36) and used as DNA source. *L. plantarum* KB1253 was cultured at 30°C without aeration in a de Man-Rogosa-Sharpe medium (Oxoid, Cambridge, UK). *Escherichia coli* JM109 (Takara, Shiga, Japan) and *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) were used as the cloning and expression host cells, respectively. All *E. coli* strains were cultivated with aeration in Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) medium at 37°C. Ampicillin was added, when necessary, at a final concentration of 100 μg/mL.

## Cloning of *gadB*$_1$ and *gadB*$_2$ genes from *L. plantarum* KB1253

Genomic DNA was isolated from *L. plantarum* KB1253 and purified using a DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A *gadB*$_1$ gene was amplified from the *L. plantarum* KB1253 genome by using a primer set based on the *gadB*$_1$ sequence from *L. plantarum* KB1253 (36) : *gadB*$_1$-F (5′-GGGCATATGGCAATGTTATACGGTA-3′, *Nde*I site underlined) and *gadB*$_1$-R (5′-ACTGTCGACGTGTGTGAATC-3′, *Sal*I site underlined). A *gadB*$_2$ gene was amplified by using a primer set based on *gadB*$_2$ from *L. plantarum* KB1253 (36): *gadB*$_2$-F (5′-GGGCATATGACAAATAACGATGAAC-3′, *Nde*I site underlined) and *gadB*$_2$-R (5′-GGGCTCGAGTTTTGTCACCCTAT-3′, *Xho*I site underlined). PCR was performed in 2×PCR Buffer for KOD FX Neo (Toyobo, Osaka, Japan), 20 ng of genomic DNA, 10 pmol of each primer, 0.4 mM dNTPs (Toyobo), and 1.0 U of KOD FX Neo DNA polymerase (Toyobo) in a total volume of 50 μl. After initial denaturation for 2 min at 94°C, DNA was amplified over 30 cycles (10 sec of denaturation at 98°C, 30 sec of annealing at 65°C, and 1 min of extension at 68°C). Amplified PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The *gad* genes and expression vector pET-21a (+) (Novagen) were digested with restriction enzymes and purified with a QIAquick Gel Extraction Kit. The purified *gad* genes were ligated into the pET-21a (+) using T4

DNA ligase (Toyobo), and the ligated vectors were introduced into *E. coli* JM109. Colonies were selected on LB-Ampicillin plates and positive clones were identified by colony PCR. The recombinant expression vectors were purified using a Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions. The recombinant expression vectors were transformed into *E. coli* BL21 (DE3). Colonies were selected on LB-Ampicillin plates, and positive clones were identified by colony PCR. For expression of the recombinant enzymes, *E. coli* BL21 (DE3) cells transformed with recombinant expression vectors were grown in an LB medium with 100 μg/ml ampicillin at 37°C induced at midexponential phase ($A_{600}$ = 0.6) with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), incubated at 16°C for an additional 16 h, and harvested by centrifugation at 8,000 ×*g* for 20 min.

**Purification of the GAD-$(His)_6$ fusion proteins**

The harvested cells were washed twice with saline and resuspended in 20 mM potassium phosphate buffer (pH 7.4). The cells were disrupted by using an ultrasonic oscillator (Insinator 201 M, Kubota, Tokyo, Japan). The cell debris were removed by centrifugation at 20,000 ×*g* for 10 min. The expressed target proteins were purified using a Capturem™ His-Tagged Purification Kit (Takara). SDS-PAGE was carried out using a 12% (w/v) acrylamide gel. The amount of protein was determined spectrophotometrically using a protein assay (BCA Protein Assay, Pierce) (59) with bovine serum albumin as the standard.

**Relative molecular mass estimation**

The relative molecular mass of the purified enzymes was estimated both by gel filtration on a Superdex™ 200 Increase 10/300 GL column (GE Healthcare Life Sciences, Piscataway, NJ, USA) (20 mM potassium phosphate buffer, containing 0.15 M NaCl (pH 7.4)) and by SDS-PAGE as described above. Conalbumin (75,000), ovalbumin (44,000), carbonic anhydrase (29,000), ribonuclease A (13,700), and cytochrome c (12,400) were used as molecular weight standards for gel filtration. All protein standards were purchased from GE Healthcare Life Sciences.

**Properties of recombinant GADs in *L. plantarum* KB1253**

Enzyme activity was determined by measuring the amount of L-Glu consumed by using the L-glutamic acid assay F-kit (Roche Diagnostics, Basel, Switzerland). The reaction mixture (25 μL) contained monosodium glutamate (MSG), PLP, and recombinant GAD (10 μg) in appropriate buffer. After incubation, the enzyme reactions were stopped by adding 99.5% ethanol. The amount of residual L-Glu was analyzed as described above. One unit of GAD activity was defined as the amount of enzyme consuming 1 μmol L-Glu for 1 min under the experimental conditions. The optimal pH of GAD activity were determined by incubating the reaction mixture (25 μL) contained 50 mM MSG, 0.2 mM PLP, and recombinant GAD (10 μg) at different pH range (pH 2.5–6.5) for 15 min at 50°C. Buffers of 200 mM concentration were used: glycine-HCl buffer (pH 2.5), sodium citrate buffer (pH 3.0–3.5), sodium acetate buffer (pH 4.0–5.5), and potassium phosphate buffer (pH 6.0–6.5). The optimal temperatures of GAD activity were determined by incubating the reaction mixture (25 μL) contained 50 mM MSG, 0.2 mM PLP, and recombinant GAD (10 μg) in 200 mM sodium citrate buffer (pH 4.0–4.5) for 15 min at temperature ranging from 35°C to 75°C. The effects of PLP concentration (0–1 mM) metal ions (1 mM), and PLP inhibitors (aminooxyacetic acid (AOAA) and hydroxylamine (HA)) (1 mM) on GAD activity were determined by incubating the reaction mixture (25 μL) contained 50 mM MSG and recombinant GAD (10 μg) in 200 mM sodium acetate buffer (pH 4.0–4.5) for 15 min at 50°C–60°C. The effect of substrate MSG concentration on GADs activity was determined in a volume 25 μl substrate solution (200 mM sodium acetate buffer (pH 4.0–4.5), 0.2 mM PLP) and 10 μg of GADs, ranging in MSG concentration from 1 to 10 mM, with incubation at 50°C–60°C for 5 min. $K_m$, $V_{max}$, $k_{cat}$, and $k_{cat}/K_m$ were calculated using Lineweaver-Burk plots. All experiments were performed in triplicate.

**Statistical analysis**

All data in the bar charts and tables correspond to the mean ± standard deviation (SD). One-way analysis of variance and subsequent Tukey-Kramer's multiple-comparison tests were conducted to evaluate the significance of differences (*$p<0.05$, **$p<0.01$).

# RESULTS and DISCUSSION

## Cloning, and overexpression of two gad genes from *L. plantarum* KB1253 and purification of the gene products

The *gadB₁* and *gadB₂* genes were amplified by PCR from genomic DNA of *L. plantarum* KB1253 and were ligated into expression vector pET-21a (+). These recombinant vectors were transformed into expression host cell *E. coli* BL21 (DE3). Expression of the gad genes was driven by the T7 promoter and induced by addition of IPTG. The *gadB₁* and *gadB₂* from *L. plantarum* KB1253 were overexpressed in *E. coli* BL21(DE3). Proteins with relative molecular masses of 52,100 and 55,400 were obtained from IPTG-induced cells, respectively (Fig. 2-1A). Gel filtration on a Superdex™ 200 Increase 10/300 GL column eluted $GadB_1$ and $GadB_2$ as a single peak at a relative molecular mass of 104,700 and 219,600, respectively (Fig. 2-1B). These results suggested that $GadB_1$ is a dimer and $GadB_2$ is a tetramer (Table 2-1). To the best of my knowledge, GADs of lactic acid bacteria (LAB) strains were dimer (51,60,61), and this is the first report of tetrameric GAD of LAB strain.

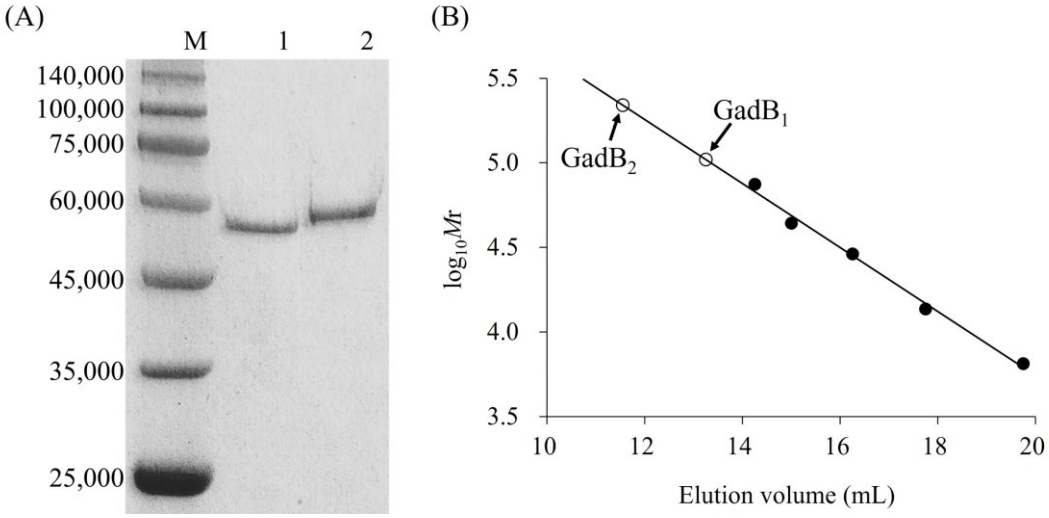



**Fig. 2-1.** The relative molecular mass estimation. (A) SDS-PAGE analysis of purified recombinant glutamate decarboxylases (GADs). The figure shows a 12% SDS-PAGE gel stained with Coomassie Blue. *Lanes: M* low-weight protein marker, *1* purified recombinant $GadB_1$, *2* purified recombinant $GadB_2$. (B) gel filtration analysis of purified recombinant GADs. The figure shows standard curve from native molecular mass determination by gel filtration chromatography. For calibration, a premixed protein molecular mass marker containing the following proteins was used: Conalbumin (75,000), ovalbumin (44,000), carbonic anhydrase (29,000), ribonuclease A (13,700), and cytochrome c (12,400).

**Table 2-1** Comparison of biochemical and kinetic properties of GADs from LAB.

| GAD | Origin | Relative molecular weight | | Number of subunits | $pH_{opt}$ | $Temp._{opt}$ (°C) | $K_m$ (mM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SDS-PAGE | Gel filtration | | | | | | | |
| $GadB_1$ | *L. plantarum* KB1253 | 52,100 | 104,700 | 2 | 4.0 | 60 | 28.3 | 381.6 | 13.5 | This study |
| $GadB_1$ | *L. plantarum* ATCC 14917 | 53,000 | 110,000 | 2 | 4.5 | 40 | 22.8 | 1304.8 | 57.2 | (61) |
| $GadB_1$ | *L. brevis* 877G | 50,000 | – | – | 5.2 | 45 | 3.6 | 17.1 | 4.8 | (48) |
| $GadB_1$ | *L. brevis* CGMCC 1306 | 53,470 | – | – | 4.8 | 48 | 10.3 | – | – | (50) |
| $GadB_2$ | *L. plantarum* KB1253 | 55,400 | 219,600 | 4 | 4.5 | 50 | 31.1 | 599.1 | 19.3 | This study |
| $GadB_2$ | *L. paracasei* NFRI 7415 | 57,000 | 110,000 | 2 | 5.0 | 50 | – | – | – | (51) |
| $GadB_2$ | *L. brevis* IFO 12005 | 60,000 | 120,000 | 2 | 4.2 | 30 | 9.3 | 390.0 | 41.9 | (49) |
| $GadB_2$ | *L. sakei* A156 | 54,400 | – | – | 5.0 | 55 | 16.0 | – | – | (54) |
| $GadB_2$ | *L. zymae* GU240 | 55,000 | – | – | 4.5 | 41 | 1.7 | 540.0 | 324.0 | (47) |

**Properties of two recombinant GADs from *L. plantarum* KB1253**

The recombinant GADs activities were measured using 200 mM buffer solution systems with pH varying from 2.5 to 6.5. The optimal pH for recombinant $GadB_1$ and $GadB_2$ were 4.0 and 4.5, respectively (Fig. 2-2), which are like those of recombinant GADs from other LAB (Table 2-1). The optimal temperature of the recombinant $GadB_1$ and $GadB_2$ were 60°C and 50°C, respectively (Fig. 2-3). Those temperatures were higher than those of other GADs of LAB (30°C–50°C, Table 2-1). Without addition of PLP to the reaction mixture, $GadB_1$ did not convert glutamate to GABA; however, the enzyme showed the activity with the addition of PLP (Fig. 2-4). Previous reports (24,51,61) supported these results. In contrast, $GadB_2$ showed the activity even without addition of PLP to the reaction mixture and showed a GABA conversion yield of 41.2 ± 3.4% without exogenous PLP (Fig. 2-4). The activities of both GADs increased with increasing PLP concentration, with maximum activity at around 0.2 mM PLP. The maximum GABA conversion yield of $GadB_1$ and $GadB_2$ were 61.4 ± 4.8% and 77.9 ± 3.9%, respectively. Within the two GAD isomers, $GadB_2$ was reported to mainly contribute to the decarboxylation of glutamate (35,62), supporting the results of this study. $GadB_1$ of LAB strains were reported to require exogenous PLP for those activities. In contrast, it was suggested that $GadB_2$ of *Bifidobacterium* strains does not require exogenous PLP; nevertheless, there are no reports of detailed research on the PLP dependency of $GadB_2$ in LAB strains. This is the first report suggesting that one of the two GAD isoforms possessed by *L. Plantarum* needs exogenous PLP for its activity while the other dose not.

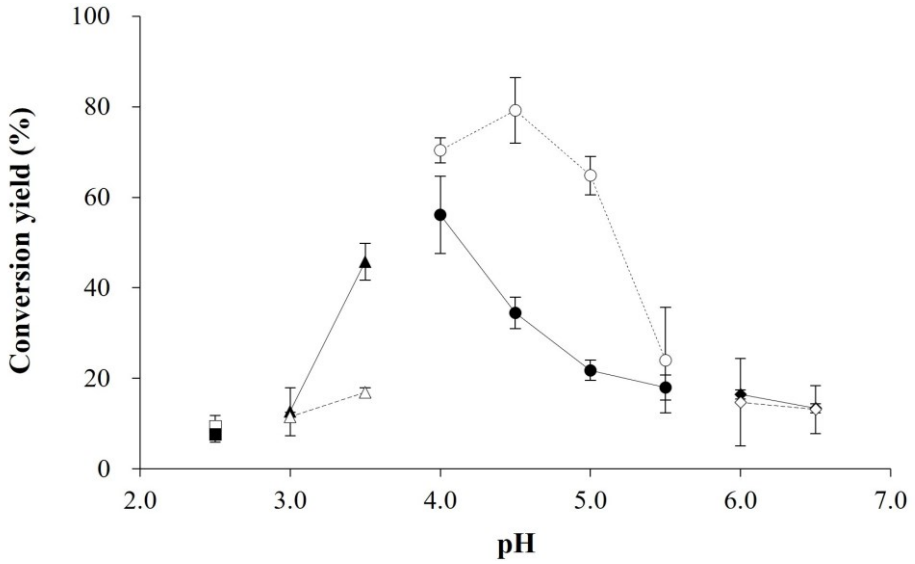



**Fig. 2-2.** Effects of pH on GADs activities. $GadB_1$ activity (closed) and $GadB_2$ activity (open) were determined at 50°C in 200 mM buffer containing 50 mM monosodium glutamate (MSG) and 0.2 mM pyridoxal 5′-phosphate (PLP) that ranged in pH from 2.5 to 6.5. Glycine-HCl buffer, pH 2.5 (squares), sodium citrate buffer, pH 3.0–3.5 (triangles), sodium acetate buffer, pH 4.0–5.5 (circles), and potassium phosphate buffer, pH 6.0–6.5 (diamonds) were used. Data are expressed as the mean ± SD of three independent experiments.

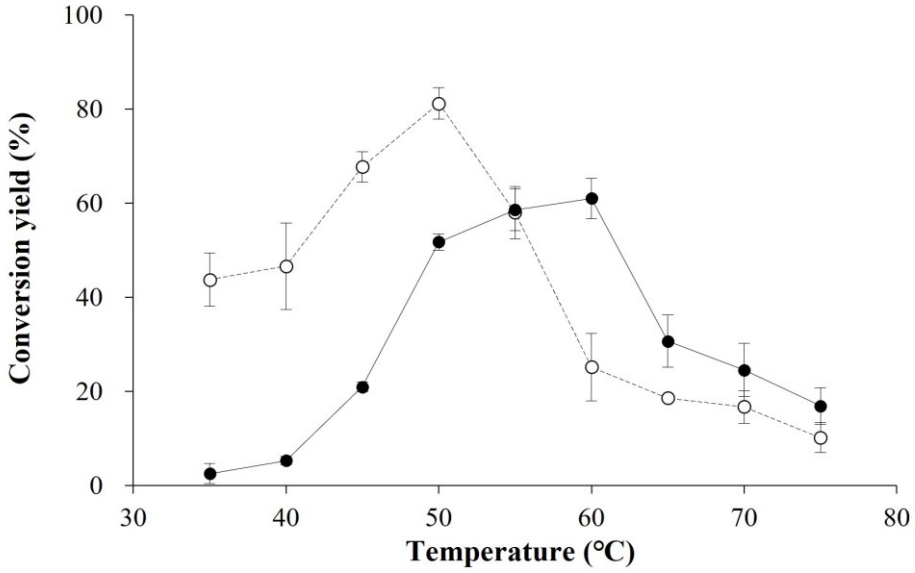



**Fig. 2-3.** Effects of temperatures on GADs activities. $GadB_1$ activity (closed circles) was determined at temperature ranging from 35°C to 75°C in 200 mM sodium acetate buffer (pH 4.0) containing 50 mM MSG and 0.2 mM PLP; $GadB_2$ activity (open circles) was determined at temperature ranging from 35°C to 75°C in 200 mM sodium acetate buffer (pH 4.5) containing 50 mM MSG and 0.2 mM PLP. Data are expressed as the mean ± SD of three independent experiments.

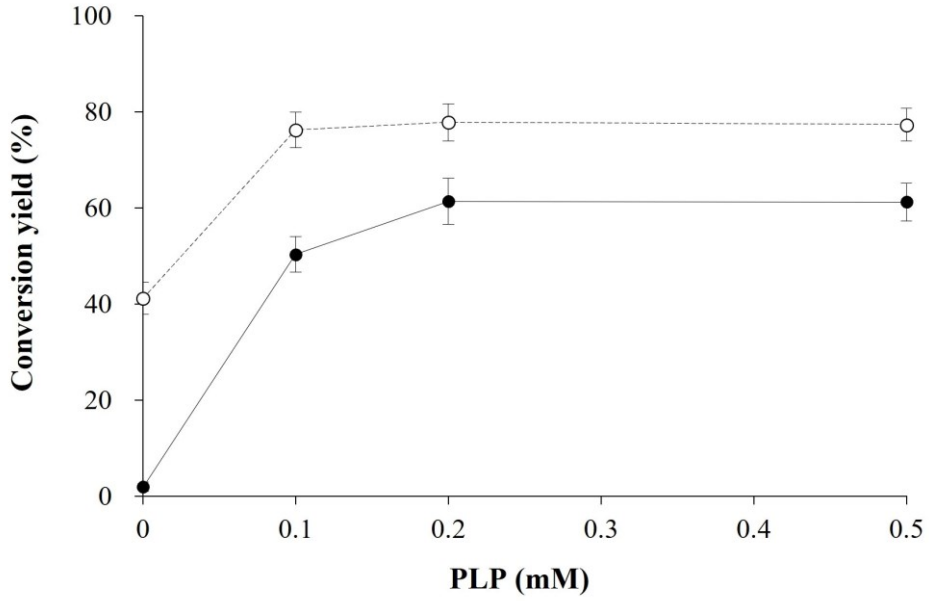
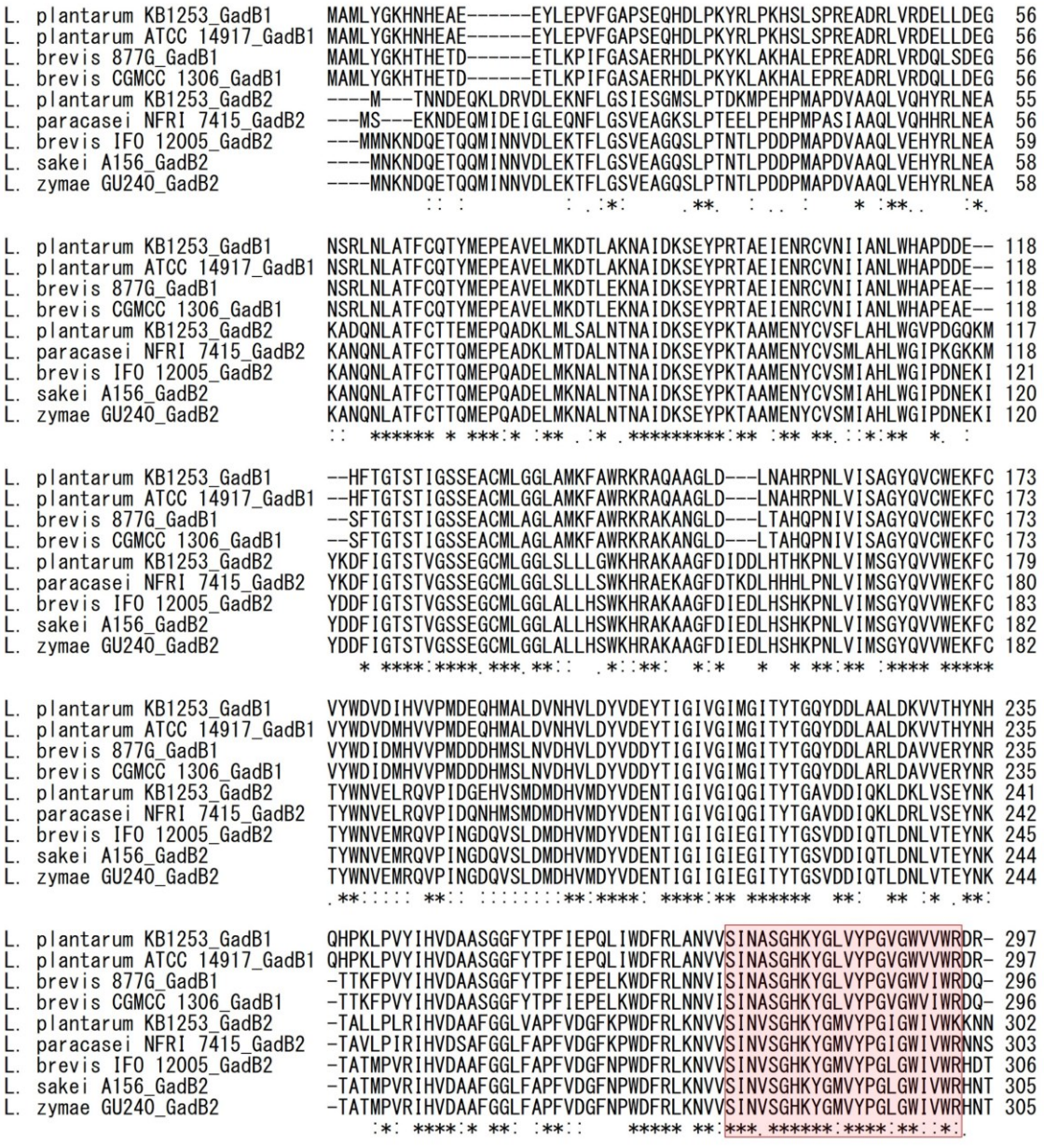



**Fig. 2-4.** Effects of PLP concentrations on GADs activities. $GadB_1$ activity (closed circles) was determined at PLP concentrations ranging from 0 to 0.5 mM in 200 mM sodium acetate buffer (pH 4.0) containing 50 mM MSG at 60°C; $GadB_2$ activity (open circles) was determined at PLP concentrations ranging from 0 to 0.5 mM in 200 mM sodium acetate buffer (pH 4.5) containing 50 mM MSG at 50°C. Data are expressed as the mean ± SD of three independent experiments.

The amino acid sequences of $GadB_1$ and $GadB_2$ were multiply aligned with other LAB strains GAD sequences from GenBank, respectively (Fig. 2-5). Both GAD domains of *L. plantarum* KB1253 also possess conserved lysine residues which are reported to be essential for glutamate decarboxylation (63). PLP-bound lysine of amino acid decarboxylases is known to play a key role in catalysis by the decarboxylases through acceleration of the Schiff base formation, decarboxylation, and product release steps (64). The sequences of SIN(A/V)SGHKYG(L/M)VYPG(V/I/L)GW(V/I)(V/I)W(R/K) are known as part of the PLP-binding domain and are thought to contribute GAD activities (65). The sequences of PLP-binding domains of $GadB_1$ in 4 strains are highly conserved, and 293rd amino acid is suggested to be species-specific (293rd: V, *L. plantarum*, I: *Levilactobacillus brevis*). Those of $GadB_2$ in 5 strains are also highly conserved, and 293rd and 299th amino acids are suggested to be also species-specific (293rd: I, *L. plantarum* and *Lacticaseibacillus paracasei*; L, *L. brevis*, *Latilactobacillus sake*i, and *Levilactobacillus*

*zymae*; 299th: K, *L. plantarum*; R, *L. paracasei*, *L. brevis*, *L. sakei*, and *L. zymae*). Comparison of the PLP binding sites of $GadB_1$ and $GadB_2$ in *L. plantarum* KB1253 revealed different amino acid sequences at three residues, and the same was observed in other LAB GADs. These differences in amino acid sequences may affect the interaction between PLP and GAD and resulted in the different PLP requirements of GADs.


L. plantarum KB1253_GadB1 MAMLYGKHNHEAE------EYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEG 56
L. plantarum ATCC 14917_GadB1 MAMLYGKHNHEAE------EYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEG 56
L. brevis 877G_GadB1 MAMLYGKHTHETD------ETLKPIFGASAERHDLPKYKLAKHALEPREADRLVRDQLSDEG 56
L. brevis CGMCC 1306_GadB1 MAMLYGKHTHETD------ETLKPIFGASAERHDLPKYKLAKHALEPREADRLVRDQLLDEG 56
L. plantarum KB1253_GadB2 ----M---TNNDEQKLDRVDLEKNFLGSIESGMSLPTDKMPEHPMAPDVAAQLVQHYRLNEA 55
L. paracasei NFRI 7415_GadB2 ---MS---EKNDEQMIDEIGLEQNFLGSVEAGKSLPTEELPEHPMPASIAAQLVQHHRLNEA 56
L. brevis IFO 12005_GadB2 ---MMNKNDQETQQMINNVDLEKTFLGSVEAGQSLPTNTLPDDPMAPDVAAQLVEHYRLNEA 59
L. sakei A156_GadB2 ----MNKNDQETQQMINNVDLEKTFLGSVEAGQSLPTNTLPDDPMAPDVAAQLVEHYRLNEA 58
L. zymae GU240_GadB2 ----MNKNDQETQQMINNVDLEKTFLGSVEAGQSLPTNTLPDDPMAPDVAAQLVEHYRLNEA 58
L. plantarum KB1253_GadB1 NSRLNLATFCQTYMEPEAVELMKDTLAKNAIDKSEYPRTAEIENRCVNIIANLWHAPDDE-- 118
L. plantarum ATCC 14917_GadB1 NSRLNLATFCQTYMEPEAVELMKDTLAKNAIDKSEYPRTAEIENRCVNIIANLWHAPDDE-- 118
L. brevis 877G_GadB1 NSRLNLATFCQTYMEPEAVELMKDTLEKNAIDKSEYPRTAEIENRCVNIIANLWHAPEAE-- 118
L. brevis CGMCC 1306_GadB1 NSRLNLATFCQTYMEPEAVELMKDTLEKNAIDKSEYPRTAEIENRCVNIIANLWHAPEAE-- 118
L. plantarum KB1253_GadB2 KADQNLATFCTTEMEPQADKLMLSALNTNAIDKSEYPKTAAMENYCVSFLAHLWGVPDGQKM 117
L. paracasei NFRI 7415_GadB2 KANQNLATFCTTQMEPEADKLMTDALNTNAIDKSEYPKTAAMENYCVSMLAHLWGIPKGKKM 118
L. brevis IFO 12005_GadB2 KANQNLATFCTTQMEPQADELMKNALNTNAIDKSEYPKTAAMENYCVSMIAHLWGIPDNEKI 121
L. sakei A156_GadB2 KANQNLATFCTTQMEPQADELMKNALNTNAIDKSEYPKTAAMENYCVSMIAHLWGIPDNEKI 120
L. zymae GU240_GadB2 KANQNLATFCTTQMEPQADELMKNALNTNAIDKSEYPKTAAMENYCVSMIAHLWGIPDNEKI 120
L. plantarum KB1253_GadB1 --HFTGTSTIGSSEACMLGGLAMKFAWRKRAQAAGLD---LNAHRPNLVISAGYQVCWEKFC 173
L. plantarum ATCC 14917_GadB1 --HFTGTSTIGSSEACMLGGLAMKFAWRKRAQAAGLD---LNAHRPNLVISAGYQVCWEKFC 173
L. brevis 877G_GadB1 --SFTGTSTIGSSEACMLAGLAMKFAWRKRAKANGLD---LTAHQPNIVISAGYQVCWEKFC 173
L. brevis CGMCC 1306_GadB1 --SFTGTSTIGSSEACMLAGLAMKFAWRKRAKANGLD---LTAHQPNIVISAGYQVCWEKFC 173
L. plantarum KB1253_GadB2 YKDFIGTSTVGSSEGCMLGGLSLLLGWKHRAKAAGFDIDDLHTHKPNLVIMSGYQVVWEKFC 179
L. paracasei NFRI 7415_GadB2 YKDFIGTSTVGSSEGCMLGGLSLLLSWKHRAEKAGFDTKDLHHHLPNLVIMSGYQVVWEKFC 180
L. brevis IFO 12005_GadB2 YDDFIGTSTVGSSEGCMLGGLALLHSWKHRAKAAGFDIEDLHSHKPNLVIMSGYQVVWEKFC 183
L. sakei A156_GadB2 YDDFIGTSTVGSSEGCMLGGLALLHSWKHRAKAAGFDIEDLHSHKPNLVIMSGYQVVWEKFC 182
L. zymae GU240_GadB2 YDDFIGTSTVGSSEGCMLGGLALLHSWKHRAKAAGFDIEDLHSHKPNLVIMSGYQVVWEKFC 182
L. plantarum KB1253_GadB1 VYWDVDIHVVPMDEQHMALDVNHVLDYVDEYTIGIVGIMGITYTGQYDDLAALDKVVTHYNH 235
L. plantarum ATCC 14917_GadB1 VYWDVDMHVVPMDEQHMALDVNHVLDYVDEYTIGIVGIMGITYTGQYDDLAALDKVVTHYNH 235
L. brevis 877G_GadB1 VYWDIDMHVVPMDDDHMSLNVDHVLDYVDDYTIGIVGIMGITYTGQYDDLARLDAVVERYNR 235
L. brevis CGMCC 1306_GadB1 VYWDIDMHVVPMDDDHMSLNVDHVLDYVDDYTIGIVGIMGITYTGQYDDLARLDAVVERYNR 235
L. plantarum KB1253_GadB2 TYWNVELRQVPIDGEHVSMDMDHVMDYVDENTIGIVGIQGITYTGAVDDIQKLDKLVSEYNK 241
L. paracasei NFRI 7415_GadB2 TYWNVELRQVPIDQNHMSMDMDHVMDYVDENTIGIVGIQGITYTGAVDDIQKLDRLVSEYNK 242
L. brevis IFO 12005_GadB2 TYWNVEMRQVPINGDQVSLDMDHVMDYVDENTIGIIGIEGITYTGSVDDIQTLDNLVTEYNK 245
L. sakei A156_GadB2 TYWNVEMRQVPINGDQVSLDMDHVMDYVDENTIGIIGIEGITYTGSVDDIQTLDNLVTEYNK 244
L. zymae GU240_GadB2 TYWNVEMRQVPINGDQVSLDMDHVMDYVDENTIGIIGIEGITYTGSVDDIQTLDNLVTEYNK 244
L. plantarum KB1253_GadB1 QHPKLPVYIHVDAASGGFYTPFIEPQLIWDFRLANVVSINASGHKYGLVYPGVGWVVWRDR- 297
L. plantarum ATCC 14917_GadB1 QHPKLPVYIHVDAASGGFYTPFIEPQLIWDFRLANVVSINASGHKYGLVYPGVGWVVWRDR- 297
L. brevis 877G_GadB1 -TTKFPVYIHVDAASGGFYTPFIEPELKWDFRLNNVISINASGHKYGLVYPGVGWVIWRDQ- 296
L. brevis CGMCC 1306_GadB1 -TTKFPVYIHVDAASGGFYTPFIEPELKWDFRLNNVISINASGHKYGLVYPGVGWVIWRDQ- 296
L. plantarum KB1253_GadB2 -TALLPLRIHVDAAFGGLVAPFVDGFKPWDFRLKNVVSINVSGHKYGMVYPGIGWIVWKKNN 302
L. paracasei NFRI 7415_GadB2 -TAVLPIRIHVDSAFGGLFAPFVDGFKPWDFRLKNVVSINVSGHKYGMVYPGIGWIVWRNNS 303
L. brevis IFO 12005_GadB2 -TATMPVRIHVDAAFGGLFAPFVDGFNPWDFRLKNVVSINVSGHKYGMVYPGLGWIVWRHDT 306
L. sakei A156_GadB2 -TATMPVRIHVDAAFGGLFAPFVDGFNPWDFRLKNVVSINVSGHKYGMVYPGLGWIVWRHNT 305
L. zymae GU240_GadB2 -TATMPVRIHVDAAFGGLFAPFVDGFNPWDFRLKNVVSINVSGHKYGMVYPGLGWIVWRHNT 305

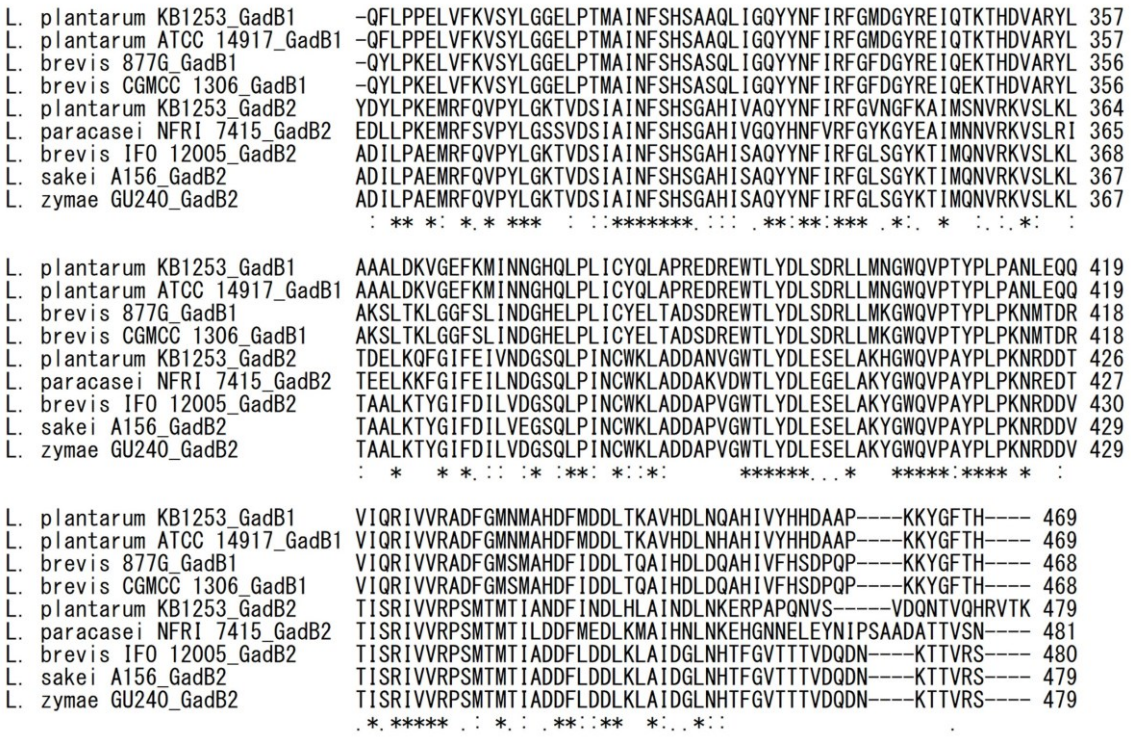



**Fig. 2-5.** Comparison of the amino acid sequences of $GadB_1$ and $GadB_2$ from *L. plantarum* KB1253 ($GadB_1$, GCD85323.1; $GadB_2$, GCD87347.1), GADs from *L. plantarum* ATCC 14917 ($GadB_1$, EFK28268.1), *L. brevis* 877G ($GadB_1$, AFU61547.1), *L. brevis* CGMCC 1306 ($GadB_1$, ADG02973.1), *L. paracasei* NFRI 7415 ($GadB_2$, AB295641.1), *L. brevis* IFO 12005 ($GadB_2$, GEB06935.1), *L. sakei* A156 ($GadB_2$, KM982734.1), and *L. zymae* GU240 ($GadB_2$, KF690143.1). The sequences in box are PLP binding domain. Consensus key: * (asterisk) -positions have a single, fully conserved residue, : (colon) -conservation between groups of strongly similar properties, . (period) -conservation between groups of weakly similar properties, and blank spaces mean no consensus.

The effects of metal ions and PLP inhibitors (1 mM) on GAD activity were evaluated. The addition of any metal ion did not increase the activities of both GADs. In contrast, both $GadB_1$ and $GadB_2$ were significantly decreased by $CuSO_4$, with residual activity of 35.2 ± 8.1% and 57.8 ± 7.7%, respectively (Fig. 2-6). Previous reports on GADs of LAB strains also showed inhibition of GAD activity by $Cu^{2+}$ (47,48), supporting the results of this study. Inactivation by EDTA (51) and activation by $Ca^{2+}$ (47,48,51) were also reported, suggesting that divalent cations contribute to the GADs activity. However, no significant inactivation by EDTA and activation by divalent cations including $Ca^{2+}$ was observed in this study. Furthermore, the addition of PLP

inhibitors (AOAA and HA) significantly decreased both GADs, suggesting that these GADs are PLP-dependent enzymes.

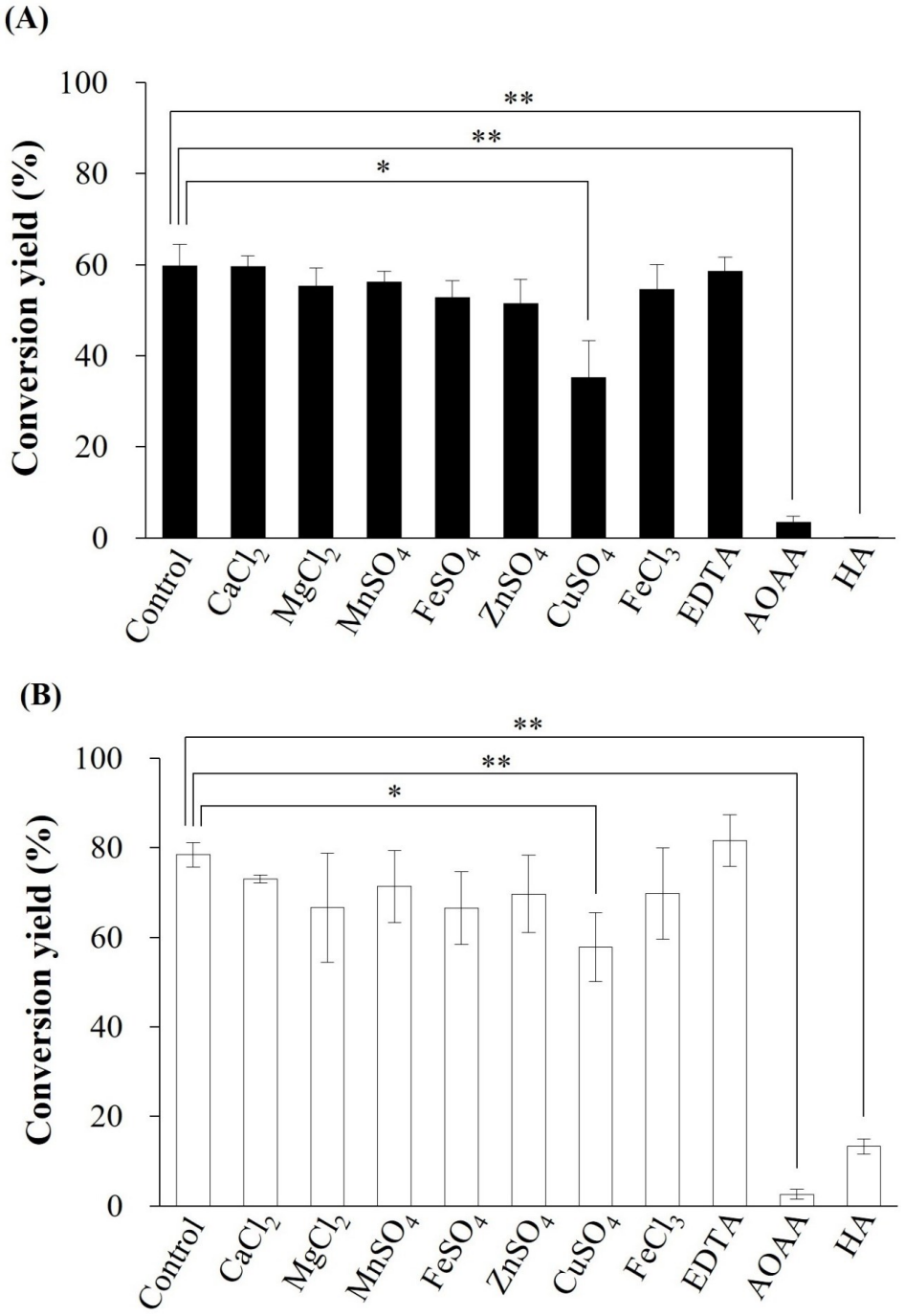



**Fig. 2-6.** Effects of metal ions and PLP inhibitors on GADs activities. $GadB_1$ activity (black bar) was determined in 200 mM sodium acetate buffer (pH 4.0) containing 50 mM MSG, 0.2 mM PLP, and 1 mM metal ions or PLP inhibitors at 60°C; $GadB_2$ activity (white bar) was determined in 200 mM sodium acetate buffer (pH 4.5) containing 50 mM MSG, 0.2 mM PLP, and 1 mM metal ions or PLP inhibitors at 50°C. AOAA and HA indicate aminooxyacetic acid and hydroxylamine, respectively. Data are expressed as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$ as compared to the control condition.

Kinetic parameters of two recombinant GADs were determined for the reaction using glutamate as a substrate and calculated from a Lineweaver-Burk plot. The $K_m$ of $GadB_1$ for glutamate was 28.3 mM and the $V_{max}$ was 2.93 mM $min^{-1}$ (Table 2-1). The $k_{cat}$ of $GadB_1$ was 381.6 $min^{-1}$ and $k_{cat}/K_m$ was 13.5 $mM^{-1}$ $min^{-1}$. In contrast, the $K_m$ of $GadB_2$ for glutamate was 31.1 mM and the $V_{max}$ was 4.33 mM $min^{-1}$. The $k_{cat}$ of $GadB_2$ was 599.1 $min^{-1}$ and $k_{cat}/K_m$ was 19.3 $mM^{-1}$ $min^{-1}$. As expected, two GADs of *L. plantarum* KB1253 also showed very similar kinetic properties in comparison with other microbial GADs (Table 2-1). These results suggested that $GadB_2$ has higher affinity for glutamate than $GadB_1$, supporting the results of high GABA production by $GadB_2$ (Fig. 2-4).

In this study, the biochemical characteristics of the two GAD isomers of *L. plantarum* KB1253 were compared. It was suggested that $GadB_2$ is the superior enzyme in terms of GABA production, as it shows decarboxylative activity without exogenous PLP in the reaction mixture. In contrast, $GadB_1$ was suggested to be active even under severe conditions (low pH, high temperature) than the previously reported GAD. Two GAD isomers have been reported in several *L. brevis* strains (24,62), but their presence has not been previously reported in *L. plantarum* strains. *L. plantarum* KB1253 isolated from pickles may have acquired this unique GAD system as a survival strategy in severe environments.

## SUMMARY

*Lactiplantibacillus plantarum* KB1253 is the first γ-aminobutyric acid (GABA) producer reported to possess two glutamate decarboxylase (GAD) isomers in the same lactic acid bacteria species. To reveal the GABA production capacity of this strain, the two isomers, $GadB_1$ and $GadB_2$, were characterized. Molecular weight estimation by SDS-PAGE and gel filtration suggested that $GadB_1$ and $GadB_2$ form dimer and tetramer, respectively. Maximum activity of $GadB_1$ was observed at pH 4.0 and 60°C and the activity was dependent on exogenous pyridoxal 5′-phosphate (PLP). In contrast, that of $GadB_2$ was observed at pH 4.5 and 55°C and $GadB_2$ could produce GABA without exogenous PLP. The $k_{cat}$ of $GadB_1$ was 381.6 $min^{-1}$ and $k_{cat}/K_m$ was 13.5 $mM^{-1}$ $min^{-1}$ when glutamate was used as a substrate. In contrast, the $k_{cat}$ of $GadB_2$ was 599.1 $min^{-1}$ and $k_{cat}/K_m$ was 19.3 $mM^{-1}$ $min^{-1}$. *L. plantarum* KB1253 isolated from pickles may have acquired this unique GAD system as a survival strategy in severe environments.

# CHAPTER III

## Gamma-aminobutyric acid production from highly concentrated tomato extracts using *Lactiplantibacillus plantarum* KB1253 whole cells

Lactic acid bacteria (LAB) have been applied in the food industry because it is known as generally recognized as safe microorganisms and has been used for various fermented foods, such as cheese, yogurt, sauerkraut, and other pickles (10,14–18,20–27,36). Tomato is one of the most glutamate-abundant agricultural products and may be a suitable raw material for GABA fermentation by LAB. *Lactiplantibacillus plantarum* KB1253 was shown to produce 41.0 ± 1.1 mM gamma-aminobutyric acid (GABA) from glutamate in tomato juice for 24 h (57). However, LAB growth was significantly inhibited when the tomato medium concentration exceeded 25°Bx, making it difficult to produce GABA from processed tomato products with higher concentrations. GABA production from tomatoes by the fermentation method is less efficient because it takes a long time to produce GABA and the concentration of substrate available for the reaction is limited.

A whole cell reaction is a method of enzymatic conversion of substrates to target products using whole cells as biocatalysts. The enzymatic GABA conversion method using the whole cell reaction can enable the use of a high glutamate concentration, unlike the fermentation method where microbial growth must be considered. *L. plantarum* KB1253 can produce 245.8 ± 3.4 mM GABA from 250 mM glutamate in a buffer solution for 1 h.

In this chapter, the author investigated the optimal conditions for preparing *L. plantarum* KB1253 whole cells to produce high GABA concentrations and the reaction conditions for producing high GABA concentrations from tomato without glutamate addition.

# MATERIALS and METHODS

## Bacterial strain

*L. plantarum* KB1253 with high GABA-producing activity was isolated from eggplant pickles in Japan (36).

## Preparation of *L. plantarum* KB1253 whole cells

Tomato paste TP-4 Turkey (Kagome, Nagoya, Japan) was diluted from 30°Bx to 10°Bx with distilled water and centrifuged at 12,000 × *g* at 4°C for 15 min. The supernatant (10°Bx) was concentrated to 40°Bx in a rotary evaporator at 50°C. Obtained concentrated tomato extract (CTE) was diluted to 5°Bx–40°Bx with distilled water, and the pH was adjusted to 3.0–5.0 using 5 M HCl or 5 M NaOH. *L. plantarum* KB1253 was cultivated in a de Man–Rogosa–Sharpe (MRS) medium (pH 6.2) (Oxoid, Cambridge, UK) at 30°C for 12 h without aeration. The seed culture was transferred to a 100-fold volume of CTE (5°Bx, pH 4.0–5.5) and incubated at 25°C–40°C for 9–22 h. After cultivation, the cells were harvested by centrifugation at 8,000 × *g* at 4°C for 3 min, washed twice with saline, and resuspended in CTE, as described below. The whole cells were stored in 50-mL plastic tubes frozen (−20°C) or refrigerated (4°C) until use.

## GABA production from CTE by the whole cell reaction

CTE with 30–150 g (wet cell weight)/L ($g_{wcw}$/L) whole cells were incubated at 35°C–60°C for 0.5–3 h. The effects of a monosodium glutamate (MSG) or a pyridoxal 5′-phosphate (PLP) addition to CTE on GABA production was also evaluated.

## Stability of LAB whole cells

To evaluate the effect of cell reuse on GABA production, cells were harvested by centrifugation at 8,000 × *g* at 4°C for 3 min from reaction solutions at the end of the whole cell reaction, washed twice with saline, and resuspended in CTE reaction solutions. CTE with 150 $g_{wcw}$/L whole cells was incubated at 45°C for 30 min. This procedure was performed 0–6 times to evaluate its effect on GABA production. In addition, to evaluate the effect of cold storage on GABA production, whole cells were

stored at 4°C for 0–7 days. CTE with 150 $g_{wcw}$/L whole cells was incubated at 45°C for 30 min. Furthermore, to evaluate the freeze–thaw stress of whole cells, cells frozen at −20°C and thawed at 4°C were used for whole cell reactions in CTE. Freeze–thaw was performed 0–5 times. CTE with 150 $g_{wcw}$/L whole cells was incubated at 45°C for 30 min.

**Determination of GABA and glutamate**

GABA and glutamate were determined after ninhydrin derivatization (Friedman, 2004) by high-performance liquid chromatography (HPLC) analysis using a Hitachi L-8900 system (Hitachi High-Tech Science, Tokyo, Japan) equipped with No. 2622 PF column (φ4.6 × 60 mm; Hitachi High-Tech Science) and an ultraviolet detector operating at 570 nm (44).

**Statistical analysis**

All data in the bar charts and tables correspond to the mean ± standard deviation (SD). One-way analysis of variance and subsequent Tukey–Kramer's multiple-comparison tests were conducted to evaluate the significance of differences ($p<0.05$).

# RESULTS and DISCUSSION

**Preparation of *L. plantarum* KB1253 whole cells**

The effects of whole cells, including preparation conditions, initial pH of MRS medium, cultivation temperature, and cultivation time, on GABA production were investigated. To evaluate the effect of the initial pH of MRS medium, whole cells were obtained by incubation in MRS medium (initial pH 4.0–5.5) at 30°C for 12 h, and CTE (25°Bx) with 30 $g_{wcw}$/L whole cells was incubated at 40°C for 3 h. MRS medium with the highest GABA production had an initial pH of 4.5 and achieved a GABA conversion yield of 85.9% ± 0.8% (Fig. 3-1A). To evaluate the effect of cultivation temperature, whole cells were obtained by incubation in MRS medium (initial pH 4.5) at 25°C–40°C for 12 h, and CTE (25°Bx) with 30 $g_{wcw}$/L whole cells was incubated at 40°C for 3 h. The cultivation temperature at which GABA production was highest was 30°C with a GABA conversion yield of 81.0% ± 2.0% (Fig. 3-1B). To evaluate the effect of cultivation time, whole cells were obtained by incubation in MRS medium (initial pH 4.5) at 30°C for 9–22 h, and CTE (25°Bx) with 30 $g_{wcw}$/L whole cells was incubated at 40°C for 3 h. The highest GABA production was observed at 12 h of cultivation, which is the phase corresponding to the late logarithmic growth phase and resulted in a GABA conversion yield of 83.8% ± 2.3% (Fig. 3-1C). Therefore, in the subsequent experiments, *L. plantarum* KB1253 whole cells with high GABA-producing activity, which enable high GABA production obtained by cultivation in MRS medium adjusted to pH 4.5 at 30°C for 12 h, were used.

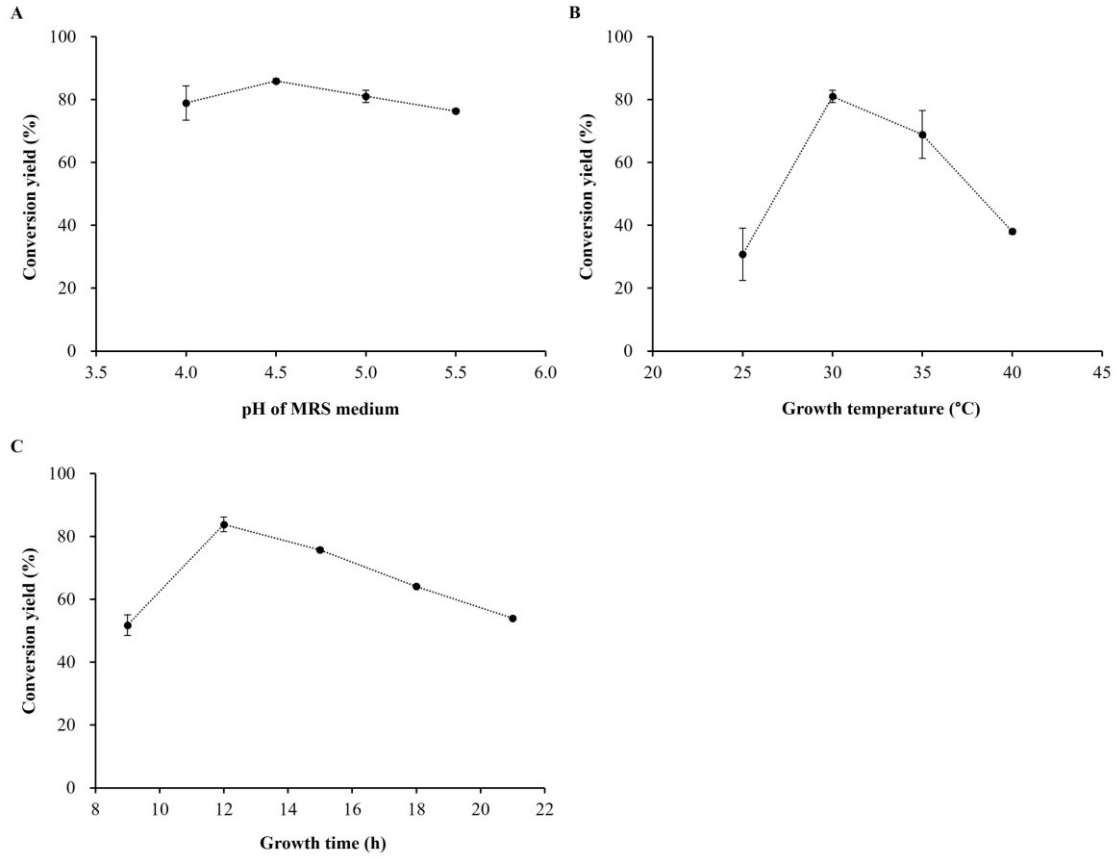



**Fig. 3-1.** Effects of whole cell-preparation conditions on gamma-aminobutyric acid (GABA) production. To evaluate the effect of the initial pH, cultivation temperature, and cultivation time, whole cells were obtained by incubation in concentrated tomato extract (CTE, initial pH 4.0–5.5) at 25°C–40°C for 9–22 h, and CTE (25°Bx) with 30 g (wet cell weight)/L ($g_{WCW}$/L) whole cells was incubated at 40°C for 3 h. Data are expressed as the mean ± SD of three independent experiments. (A) Initial pH of MRS medium, (B) growth temperature, and (C) growth time.

## GABA production from CTE using LAB whole cells

The optimal conditions for producing GABA from CTE were investigated using whole cells prepared under optimal conditions for GABA production. To investigate the effect of CTE pH on GABA production, CTE (pH 3.0–5.0, 25°Bx) was incubated at 40°C for 30 min with 30 $g_{wcw}$/L of the whole cells. The optimal pH was 4.0 with a GABA conversion yield of 78.0% ± 2.8% (Fig. 3-2A). In chapter I, GABA was produced without adding PLP, cofactor of GAD, to the whole cell reaction solution. Since PLP biosynthesis pathway has been reported for *Lactiplantibacillus pentosus* (66) genotypically related to *L. plantarum*, suggesting that the whole cells of *L. plantarum*

KB1253 contained sufficient PLP for GABA production. To investigate the effect of reaction temperatures, CTE (pH 4.0, 25°Bx) was incubated at 35°C–60°C for 30 min with 30 $g_{wcw}$/L of the whole cells. As the reaction temperature increased up to 45°C, the GABA conversion yield increased to 84.9% ± 3.0% (Fig. 3-2B). In contrast, the GABA conversion yield gradually decreased as the reaction temperature increased above 45°C and dropped to <25% at 60°C. The decrease in GABA conversion yield is thought to be caused by heat induced by the inactivation of the enzyme GAD. Notably, GAD activities are inhibited around 40°C–60°C (49,67,68). Two GADs possessed by *L. plantarum* KB1253 may be heat sensitive. The effects of CTE concentration on GABA production were investigated.

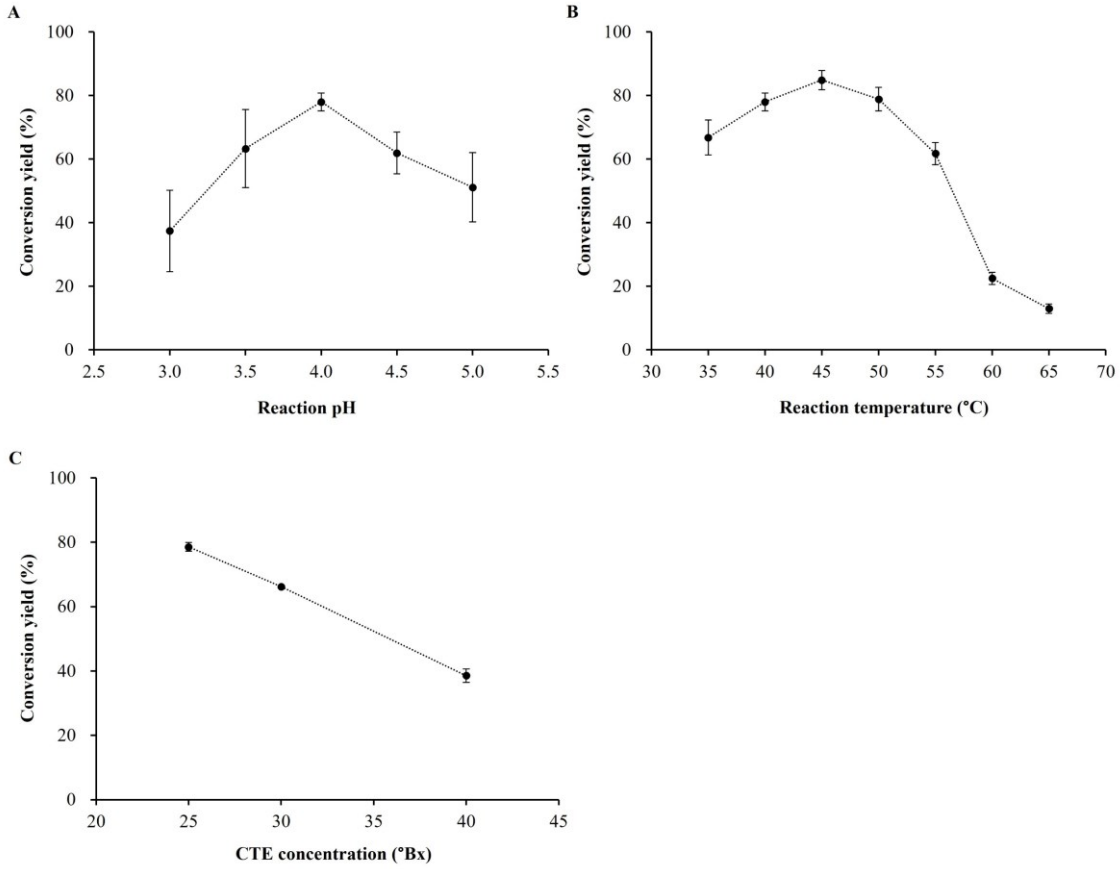



**Fig. 3-2.** Effects of pH, temperature, and tomato concentration on GABA production by whole cells. To investigate optimal GABA production conditions, CTE (25°Bx–40°Bx, pH 3.0–5.0) with 30 $g_{WCW}$/L whole cells was incubated at 35°C–65°C for 0.5 h. Data are expressed as the mean ± SD of three independent experiments. (A) Reaction pH, (B) reaction temperature, and (C) CTE concentration.

**Table 3-1.** Effects of CTE concentrations on GABA conversion yield

| CTE conc. (°Bx) | Before reaction | | After reaction[a] | | GABA conversion yield[b] (%) |
|---|---|---|---|---|---|
| | Glutamate (mM) | GABA (mM) | Glutamate (mM) | GABA (mM) | |
| 25 | 46.0 ± 0.7[c] | 37.4 ± 0.5 | 12.0 ± 0.6 | 73.6 ± 0.6 | 78.6 ± 1.4 |
| 30 | 54.1 ± 0.1 | 41.8 ± 0.2 | 16.7 ± 0.8 | 77.6 ± 0.1 | 66.2 ± 0.1 |
| 40 | 81.9 ± 0.9 | 53.8 ± 1.1 | 48.1 ± 2.3 | 85.3 ± 2.4 | 38.6 ± 2.1 |

[a] The washed cells of *L. plantarum* KB1253 were suspended in CTE (pH 4.0, 25°Bx–40°Bx) at 30 $g_{WCW}$/L and incubated at 40°C for 30 min.

[b] The GABA conversion yield was defined by the following equation:

$$\text{GABA conversion yield} = ([\text{GABA}]_t - [\text{GABA}]_0)\,[\text{Glutamate}]_0^{-1}$$

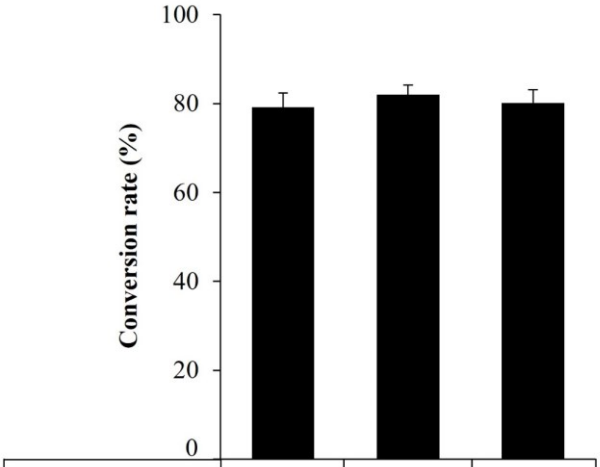
$[GABA]_t$: GABA concentration after t-hour reaction (mM)

$[GABA]_0$: GABA concentration before reaction (mM)

$[Glutamate]_0$: Glutamate concentration before reaction (mM)

[c] Data are expressed as the mean ± SD of three independent experiments.

Table 3-1 lists the concentrations of glutamate and GABA at different CTE concentrations. As CTE concentration increased, the GABA conversion yield significantly decreased. The GABA conversion yield using CTE (40°Bx) was approximately 50% lower than that using CTE (25°Bx). Since substrate inhibition of GAD by glutamate may have caused the decrease in GABA conversion yield, the effect of MSG addition to CTE (25°Bx) on the GABA conversion yield was evaluated. However, the GABA conversion yield was more than 80.2% from 76.3 mM MSG, including CTE (25°Bx) derived glutamate (Fig. 3-3). This result suggested that GAD of this strain did not show substrate inhibition by glutamate in this assay when the glutamate concentration was ≤76.3 mM. A previous report showed that approximately 250 mM glutamate could be converted to GABA for a 30 min reaction with a high conversion yield when MSG was used as the substrate in a buffer solution instead of CTE (57), supporting the results of the current study.




| Additional MSG conc. (mM) | 0 | 10 | 30 |
|---|---|---|---|
| Total MSG conc. (mM) before whole cell reaction | 46.3 | 56.3 | 76.3 |

**Fig. 3-3.** Effects of MSG addition to CTE (25°Bx) on GABA production by whole cells. To investigate the effect of MSG concentration on the GABA conversion yield, CTE (25°Bx, pH 4.0) with 30 $g_{WCW}$/L whole cells and MSG (0–30 mM) was incubated at 45°C for 0.5 h. Data are expressed as the mean ± SD of three independent experiments.

Furthermore, to improve the GABA production, the whole cell reaction was performed by adding PLP (0–0.2 mM), coenzyme of GAD, to CTE (25°Bx–40°Bx). PLP concentration-dependently enhanced the GABA conversion yield, and the addition of 0.2 mM PLP to CTE (40°Bx) led to a conversion yield of more than 74.4% (Fig. 3-4). This result suggested that CTE contains a compound that has some interaction with PLP, causing inhibition of the GAD reaction.

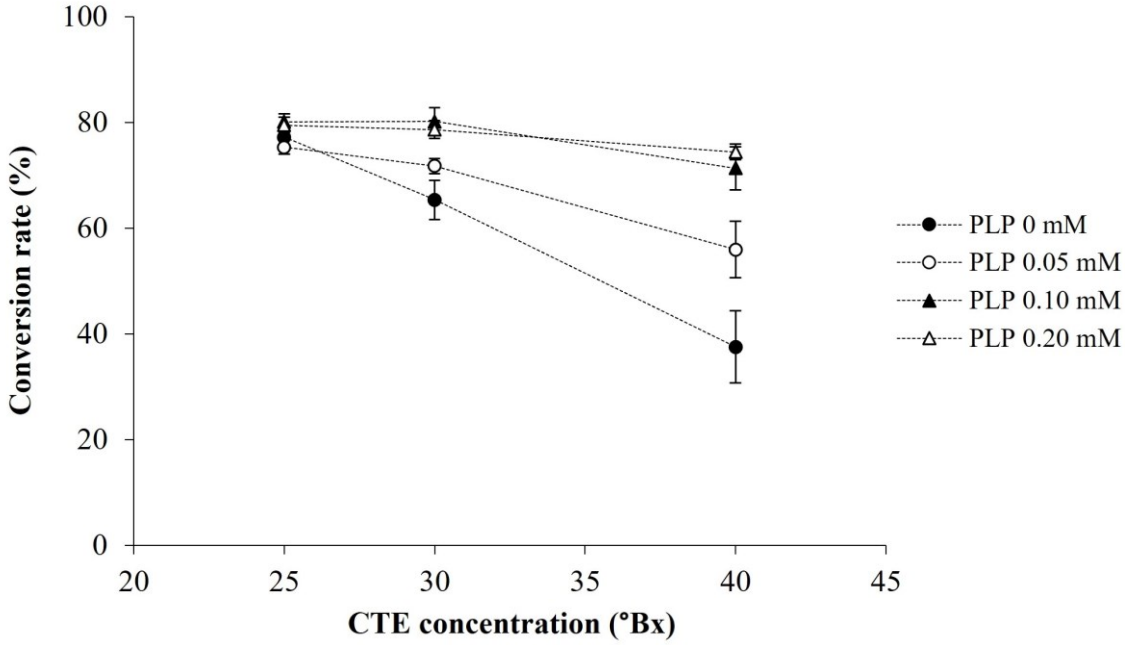



**Fig. 3-4.** Effects of a pyridoxal 5′-phosphate (PLP) addition to CTE on GABA production by whole cells. To investigate the effect of PLP addition on the GABA conversion yield, CTE (25°Bx–40°Bx, pH 4.0) with 30 $g_{WCW}$/L whole cells and PLP (0–0.2 mM) was incubated at 45°C for 0.5 h. Data are expressed as the mean ± SD of three independent experiments.

To achieve a high GABA conversion yield using CTE (40°Bx), the optimal cell concentrations were investigated. When the whole cell concentrations were 30 and 60 $g_{wcw}$/L, the maximum GABA conversion yields observed 2 h after initiating the reaction were 57.8% and 85.7%, respectively (Fig. 3-5A). The conversion yield of glutamate to GABA decreased about 1 to 2 h after initiating the reaction, although glutamate in CTE was not completely consumed. This may be attributed to the heat-induced inactivation of GAD in the reaction at 45°C. In contrast, when the whole cell concentration was 150 $g_{wcw}$/L (33 times higher than that of the culture medium), GABA could be produced at a conversion yield of more than 90% for a 30 min reaction. Apart from GABA in CTE before the reaction, 82.3 ± 1.1 mM GABA was eventually produced from glutamate in CTE (Fig. 3-5B). By increasing the concentration of whole cells, sufficient PLP was thought to be supplied for the whole cell reaction at CTE. In chapter I, 40 mM GABA was produced from CTE (25°Bx) in fermentation over 24 h (57). The whole cell reaction in CTE is a means of GABA production that is about twice as productive in a

shorter time than the fermentation method. These results suggest the efficient industrial production of GABA from high concentrations of processed tomatoes at lower costs than the fermentation method, but the costs of preparing whole cells require future evaluation. In addition, more efficient GABA production would be expected if PLP accumulation in the cells could be increased.

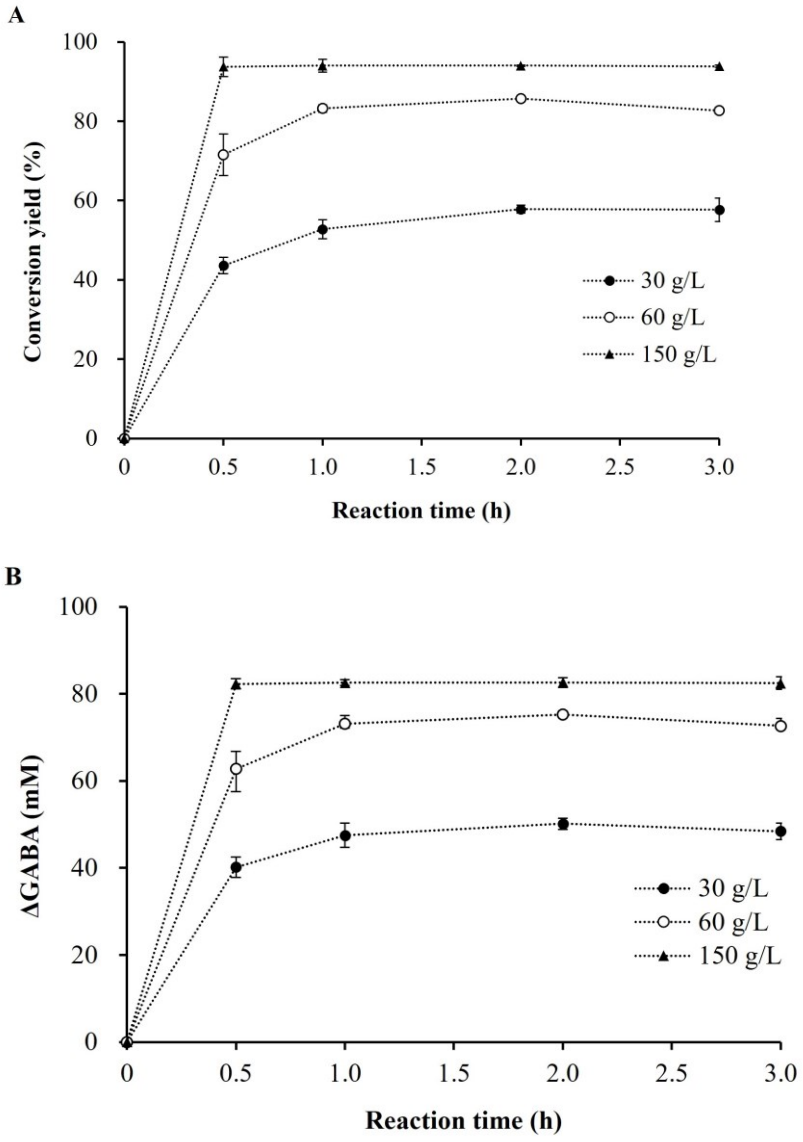



**Fig. 3-5.** Effect of the cell concentration used in the whole cell reaction on GABA production. To investigate the optimal GABA production conditions, CTE (40°Bx, pH 4.0) with 30–150 $g_{WCW}$/L whole cells was incubated at 45°C for 3 h. Data are expressed as the mean ± SD of three independent experiments. (A) Conversion yield and (B) ΔGABA (GABA generated by the whole cell reaction).

**Characterization of LAB whole cells for GABA production**

As the stable GABA production activity of whole cells is also important for industrial applications, especially for catalyst recycling, the reuse of whole cells for GABA production was evaluated. When the cells were reused for less than two cycles, the GABA conversion yield was maintained >79.6% (Fig. 3-6A). In contrast, when the cells were reused three cycles, the GABA conversion yield rapidly decreased (47.5%). Furthermore, when the whole cells were reused for more than five cycles, the GABA conversion yield decreased to <4.9%. All reactions were performed at 45°C. Notably, the reuse of the whole cells for more than three cycles decreased GABA-producing activities of the whole cells because the whole cells were exposed to 45°C for more than 90 min (49,67,68). In addition, GABA production may have been decreased by accumulation of compounds predicted to be included in CTE as PLP inhibitors in the cells during repeated multiple reactions. The effect of cold storage of whole cells on GABA production was also investigated. The GABA conversion yield did not change after 1 day of storage at 4°C (0 day, 98.5%; 1 day, 96.4%), and the GABA conversion yield was maintained at 82.5% for 2 days of storage (Fig. 3-6B). When the storage days were ≥5, the GABA conversion yield dropped to <18.9%, revealing whole cell unsuitability for long-term cold storage (≥5 days) for GABA production. Furthermore, the effect of the freeze–thaw stress of the whole cells on GABA production was investigated. One freeze–thaw cycle was defined as the cycle from the storage of the whole cells at −20°C until complete rethawing at 4°C. The GABA conversion yield gradually decreased after repeated freeze–thaw cycles (Fig. 3-6C). Nevertheless, the GABA conversion yield was maintained >80% after at least five freeze–thaw cycles.

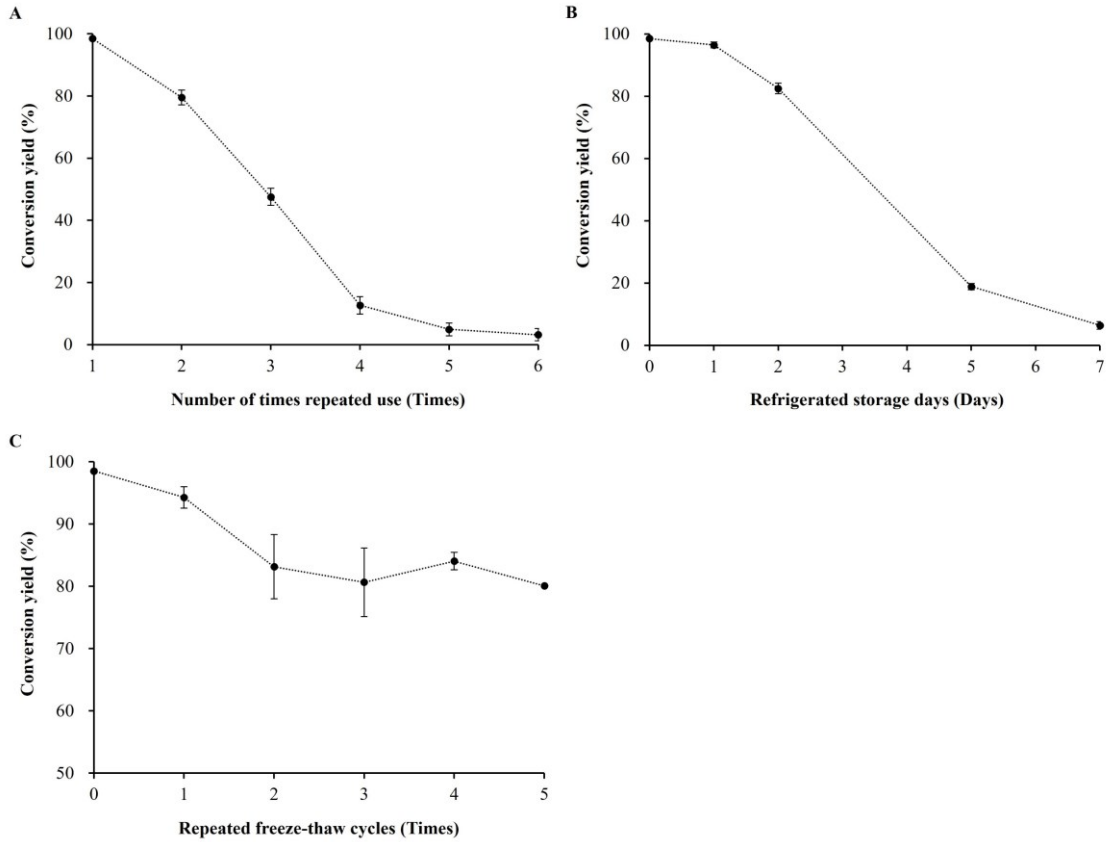



**Fig. 3-6.** Stability of *L. plantarum* KB1253 whole cells. To evaluate whole cell stability, CTE (40°Bx, pH 4.0) with 150 $g_{WCW}$/L whole cells was incubated at 45°C for 30 min. This procedure was performed 0–6 times to evaluate its effect on GABA production. In addition, to evaluate the effect of cold storage on GABA production, whole cells were stored at 4°C for 0–7 days. CTE (40°Bx, pH 4.0) with 150 $g_{WCW}$/L whole cells was incubated at 45°C for 30 min. Furthermore, to evaluate the freeze–thaw stress of whole cells, cells frozen at −20°C and thawed at 4°C were used for whole cell reactions in CTE. Freeze–thaw was performed 0–5 times. CTE (40°Bx, pH 4.0) with 150 $g_{WCW}$/L whole cells was incubated at 45°C for 30 min. Data are expressed as the mean ± SD of three independent experiments. (A) Reuse cycles; (B) refrigerated storage days, and (C) freeze–thaw cycles.

GABA fermentation from agricultural products such as tomatoes by LAB is difficult for industrialization due to the limited concentrations of substrates available for the reaction and the prolonged production time. In this study, GABA could be produced at a conversion yield of more than 90% in a 30 min LAB whole cell reaction. Apart from GABA in CTE before the reaction, 82.5 ± 1.4 mM GABA was eventually produced from glutamate in CTE. In addition, this study suggested that CTE contains a

compound that has some interaction with PLP, causing inhibition of the GAD reaction. To produce GABA from highly concentrated CTE (40°Bx), the concentration of the cells which contain PLP should be increased to 150 $g_{wcw}$/L. Improvements in whole cell stabilities and development of PLP accumulation method in the cells are expected to a more stable industrial GABA production.

# SUMMARY

An efficient process for γ-aminobutyric acid (GABA) production from concentrated tomato extract (CTE) was investigated using *Lactiplantibacillus plantarum* KB1253 whole cells. GABA production proceeded well when the whole cells were suspended in CTE (pH 4.0, 25°Bx) at 40°C for 30 min, resulting in a GABA conversion yield of 84.9% ± 3.0% with a whole cell concentration of 30 $g_{wcw}$/L. However, as CTE concentration increased, the GABA conversion yield significantly decreased. Notably, CTE (25°Bx) GABA production exceeded that of CTE (40°Bx) by approximately 50%. This study also suggested that CTE contains a compound that has some interaction with PLP, causing inhibition of the glutamate decarboxylase reaction. GABA production was improved by adding 0.2 mM PLP to CTE (40°Bx). Since this strain is thought to have endogenous PLP, efficient GABA production from CTE (40°Bx) was achieved by increasing cell concentration to 150 $g_{wcw}$/L (GABA: 82.5 ± 1.4 mM, conversion yield: 93.7 ± 2.4 mM). This study provides the basis for the process development of GABA-enriched, health-promoting functional food production from tomatoes and other agricultural products.

# CONCLUSION

## CHAPTER I

To produce tomato juice with health-promoting functions, LAB capable of converting L-glutamic acid in tomatoes into GABA was screened from LAB stocks isolated from Japanese pickles. *L. plantarum* KB1253 was selected as the highest GABA producer among 74 strains of LAB stocks. *gad* gene expression and glutamic acid decarboxylation activity increased at low pH (3.0–3.5), whereas the growth decreased. Under optimal reaction conditions using resting cells as catalysts, this strain produced 245.8 ± 3.4 mM GABA. Furthermore, this strain produced 41.0 ± 1.1 mM GABA from L-glutamic acid in tomato juice under optimal fermentation conditions (pH 4.0, 20°Bx). This study may provide the basis for developing health-promoting functional foods rich in GABA from tomatoes and other agricultural products.

## CHAPTER II

*Lactiplantibacillus plantarum* KB1253 is the first GABA producer reported to possess two GAD isomers in the same lactic acid bacteria species. To reveal the GABA production capacity of this strain, the two isomers, $GadB_1$ and $GadB_2$, were characterized. Molecular weight estimation by SDS-PAGE and gel filtration suggested that $GadB_1$ and $GadB_2$ form dimer and tetramer, respectively. Maximum activity of $GadB_1$ was observed at pH 4.0 and 60°C and the activity was dependent on exogenous pyridoxal 5′-phosphate (PLP). In contrast, that of $GadB_2$ was observed at pH 4.5 and 55°C and $GadB_2$ could produce GABA without exogenous PLP. The $k_{cat}$ of $GadB_1$ was 381.6 $min^{-1}$ and $k_{cat}/K_m$ was 13.5 $mM^{-1}$ $min^{-1}$ when glutamate was used as a substrate. In contrast, the $k_{cat}$ of $GadB_2$ was 599.1 $min^{-1}$ and $k_{cat}/K_m$ was 19.3 $mM^{-1}$ $min^{-1}$. *L. plantarum* KB1253 isolated from pickles may have acquired this unique GAD system as a survival strategy in severe environments.

# CHAPTER III

An efficient process for GABA production from concentrated tomato extract (CTE) was investigated using *Lactiplantibacillus plantarum* KB1253 whole cells. GABA production proceeded well when the whole cells were suspended in CTE (pH 4.0, 25°Bx) at 40°C for 30 min, resulting in a GABA conversion yield of 84.9% ± 3.0% with a whole cell concentration of 30 $g_{wcw}$/L. However, as CTE concentration increased, the GABA conversion yield significantly decreased. Notably, CTE (25°Bx) GABA production exceeded that of CTE (40°Bx) by approximately 50%. This study also suggested that CTE contains a compound that has some interaction with PLP, causing inhibition of the GAD reaction. GABA production was improved by adding 0.2 mM PLP to CTE (40°Bx). Since this strain is thought to have endogenous PLP, efficient GABA production from CTE (40°Bx) was achieved by increasing cell concentration to 150 $g_{wcw}$/L (GABA: 82.5 ± 1.4 mM, conversion yield: 93.7 ± 2.4 mM). This study provides the basis for the process development of GABA-enriched, health-promoting functional food production from tomatoes and other agricultural products.

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# PUBLICATIONS

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