

Acceleration of Aglycone Isoflavone and γ -Aminobutyric Acid Production from Doenjang Using Whole-Cell Biocatalysis Accompanied by Protease Treatment

Yincong Li^{1,2†}, Seockmo Ku^{3†}, Myeong Soo Park⁴, Zhipeng Li¹, and Geun Eog Ji^{1,2*}

¹Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Seoul 08826, Republic of Korea

²Research Center, BIFIDO Co. Ltd., Hongcheon 25117, Republic of Korea

³Fermentation Science Program, School of Agribusiness and Agriscience, College of Basic and Applied Sciences, Middle Tennessee State University, Murfreesboro, TN 37132, USA

⁴Department of Hotel Culinary Arts, Yeonsung University, Anyang 14011, Republic of Korea

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*Corresponding author
Phone: +82-2-880-6282;
Fax: +82-2-884-0305;
E-mail: geji@snu.ac.kr

†These authors contributed
equally to this work.

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Recently, soybean isoflavone aglycones (*i.e.*, daidzein and genistein) and γ -aminobutyric acid (GABA) have begun to receive considerable consumer attention owing to their potential as nutraceuticals. To produce these ingredients, multiple microorganisms and their enzymes are commonly used for catalysis in the nutraceutical industry. In this work, we introduce a novel fermentation process that uses whole-cell biocatalysis to accelerate GABA and isoflavone aglycone production in doenjang (a traditional Korean soybean paste). Microbial enzymes transform soybean isoflavone glycosides (*i.e.*, daidzin and genistin) and monosodium glutamate into soybean isoflavone aglycones and GABA. *Lactobacillus brevis* GABA 100 and *Aspergillus oryzae* KACC 40250 significantly reduced the production time with the aid of a protease. The resulting levels of GABA and daidzein were higher, and genistein production resembled the levels in traditional doenjang fermented for over a year. Concentrations of GABA, daidzein, and genistein were measured as 7,162, 60, and 59 $\mu\text{g/g}$, respectively on the seventh day of fermentation. Our results demonstrate that the administration of whole-cell *L. brevis* GABA 100 and *A. oryzae* KACC 40250 paired with a protease treatment is an effective method to accelerate GABA, daidzein, and genistein production in doenjang.

Keywords: Whole-cell catalysis, probiotics, doenjang, γ -aminobutyric acid, soybean isoflavone, bioconversion

Introduction

A traditional condiment in the Korean diet, doenjang (fermented soybean paste, an age-old ethnic staple), is commonly added as a basal material to multiple Korean foods, including sauces (jang, yangnyum) and soup (kuk) [1]. Major Korean food conglomerates mass-produce doenjang using koji (a filamentous mold), which is artificially spiked with *Aspergillus oryzae* [2]. The koji is mixed with heat-treated soybeans to produce doenjang.

There are active discussions among food scientists regarding the functional effects and bioavailability of

soybean phytochemicals. Among the various soybean molecules, soybean isoflavone aglycones (*i.e.*, daidzein and genistein) are widely used as nutraceuticals to protect against osteoporosis, malignant neoplastic (*e.g.*, prostate and breast cancer), and cardiovascular diseases [3, 4]. Soybean isoflavone aglycones are assumed to be more bioactive than soybean glycosides (*e.g.*, daidzin and genistin), due to their effective absorption and administration in intestinal tissues and serum [5]. Multiple microbial glycosylhydrolases (E.C. 3.2.1) catalyze and hydrolyze glucosyl residues of phytochemical glycoconjugates. Among them, the β -glucosidase (E.C. 3.2.1.21) produced by food micro-

organisms has been widely utilized to detach the sugar moieties of soybean daidzin and genistin to increase the level of daidzein and genistein in soybean products [6].

Another biofunctional molecule, γ -aminobutyric acid (GABA), is an inhibitory neurotransmitter that regulates cortical functions and blocks nerve impulses. GABA continues to receive attention in academia and functional food markets [7]. Multiple studies have proven GABA's role in the treatment of illnesses and its utility in dietary supplements. Recent clinical studies provide significant evidence of GABA's bioavailability; the GABA-treated group showed statistically significant improved prioritized planned action and inhibitory control [8]. Recently, multiple groups have attempted to produce soybean isoflavone aglycones and GABA using upstream and/or downstream bioprocessing (e.g., genetic engineering, biotransformation, chemical synthesis, and bioseparation) to develop a product that meets consumers' needs [9, 10].

Microbial biocatalysis for producing biofunctional and bioactive molecules is of a growing importance in the pharmaceutical and nutraceutical industries. Recent progress in genetic engineering (e.g., the design and use of expression vectors and the production of recombinant proteins) makes biocatalysis a practical tool for producing value-added ingredients (i.e., GABA and isoflavone aglycones) inexpensively [6, 10, 11]. Given that the characteristics, trends, and prospects for the nutraceutical market differ from the pharmaceutical market, the use of genetically modified organisms (GMOs) in processed foods, especially functional foods, can pose potential marketing strategy issues [12]. Despite a number of studies that report the safety of GMOs and the endorsement of GMO safety by over 110 Nobel Laureates, consumers' negative opinions of GMOs are still reflected in their food choices [13]. Without a doubt, many consumers are willing to pay more to purchase non-GMO food products [12, 14].

Enzymes and microorganisms have a significant impact on capital cost distributions in complex downstream processing [15, 16]. Therefore, simple techniques are necessary in the process development of isoflavone aglycones and GABA. Moreover, the application of whole-cell biocatalysis linked to mixed fermentation could be an advantage to simplify the manufacturing process. As de Carvalho [17] and Ku *et al.* [18] summarized, the use of whole-cell biocatalysis offers multiple benefits. Specifically, simple microbial processes decrease manufacturing costs and constitute a crucial best practice for the food industry.

Although some catalytic properties of microorganisms have been investigated, no previous studies have linked

mixed fermentation to the simultaneous production of isoflavone aglycones and GABA. Therefore, a comparison of the characteristics of microorganisms that express varied catalytic properties to isoflavone glycosides and glutamic acid from soybean paste has not yet been published. In the present study, we report the co-cultivation of *Lactobacillus brevis* GABA 100 and *A. oryzae* strains to doenjang and the resulting rapid production of GABA and isoflavone aglycones.

Materials and Methods

Microorganisms

All strains of *A. oryzae* (i.e. KACC 40234, KACC 40250, KACC 41736, KACC 44967, KACC 45001, KACC 45002, KACC 45006, KACC 45007, KACC 46471, and KFRI 995) were grown on potato dextrose agar: BAM Media M127(BD, USA) at 30°C under aerobic conditions for 7 days before being inoculated on the soybean medium. *L. brevis* GABA 100 was cultured in *Lactobacillus* de Man, Rogosa, and Sharpe broth (BD, USA) with 0.05% (w/v) L-cysteine-hydrochloride anhydrous ($\geq 98\%$; Sigma-Aldrich, USA) at 37°C under anaerobic conditions for 18 h before inoculation onto the soybean medium [19]. To determine the best *A. oryzae* strain for the soybean medium fermentation, 5 ml of various strains of *A. oryzae* (7 log spores/ml) and 2.5 ml of *L. brevis* GABA 100 (9 log CFU/ml) were inoculated in 50 ml of soybean medium and incubated aerobically at 30°C.

Media and Starter Preparation

To select the best type of fungi from the 10 different *A. oryzae* strains, their isoflavone aglycone and GABA productivities were evaluated using soybean medium. Organic soybeans were purchased from a local grocery store (Korea). Kim and Ji [19] and Dhakal *et al.* [20] developed the methods used to produce the soybean medium. In brief, 5 g of the milled soybean and 1 g of monosodium glutamate (MSG) were added to 50 ml of distilled water followed by a pH adjustment of 5.0 ± 0.2 with 1 M citric acid based on the data we reported. The naturally occurring microbiota in the soybean medium was autoclaved at 121°C for 15 min. To accelerate the production of isoflavone aglycones, two different types of commercially available enzymes were treated. A protease cocktail (i.e., 0.3% (w/w) of Prozyme 2000P (Bision Biochem Corporation, Korea) and 0.6% (w/w) of Multifect PR 7L (Bision Biochem Corporation, Korea)) was used to treat the autoclaved soybean medium. Prozyme 2000P and Multifect PR 7L are proteolytic enzymes extracted from *Aspergillus* and *Bacillus* spp., respectively. The microbial contaminants in the enzyme cocktail were removed by 0.2 μ m cut-off microfiltration. The enzyme activities of Prozyme 2000P and Multifect PR 7L were 1,400 and 1,600 AU/g, respectively. For the starter preparation, milled soybean and distilled water (in the ratio of 1:2 (w/w) and MSG (2% (w/w))) were added to the medium and the pH was

adjusted to 6.75 using 1 N NaOH. After the enzyme treatment described above, the pH of the medium was adjusted to 5.0 ± 0.2 using 1 M citric acid and steam-sterilized at 121°C for 15 min. Five milliliters of 8 log spores/ml of the optimum *A. oryzae* was co-inoculated with 2.5 ml of 10 log CFU/ml of *L. brevis* GABA 100 in 50 g of soybean medium and fermented in a shaking incubator at 30°C and 150 rpm for 1 day.

Doenjang Fermentation

The soybeans were washed and soaked for 15 h. Before being steamed at 121°C for 1 h using a steam sterilizer, 100 g of soybean was mixed with 2 g of MSG. Once the sterilized soybeans were cooled to room temperature, they were homogenized. The pH was adjusted to 5.0 ± 0.2 using 1 M citric acid. For the enzyme stock, Prozyme 2000P and Multifect PR 7L were mixed with distilled water to produce 10% (w/v) of Prozyme 2000P and 50% (w/v) of Multifect PR 7L aqueous solution. Then, 3 ml of Prozyme 2000P and 1 ml of Multifect PR 7L stock solution were added to 100 g of the homogenized soybean after microfiltration (0.2 µm cut-off) to separate the naturally occurring microbiota. Fifty grams of the starter was added to the crocks and mixed evenly. The crocks were placed into a 30°C incubator for fermentation. After 3 days of fermentation, 100 ml of a 20% sterilized NaCl solution was added and mixed thoroughly. Afterward, the crocks were placed back into the 30°C incubator for another 10 days of fermentation.

Extraction of Isoflavones from Fermented Soybean

Methods adapted from Kao and Chen [21] were used to extract isoflavone from fermented soybean as follows. One gram of the sample was mixed with 3 ml of hexane (Sigma-Aldrich) and vortexed for 30 min. The mixture was then centrifuged at 2,900 ×g and 25°C for 30 min (2236R; LaboGene, Denmark). After the supernatant was discarded, 300 µl of hexane (Sigma-Aldrich Co.) was added and centrifugation was carried out at 2,900 ×g and 25°C for 30 min. After repeating this protocol three times, the sediment was dried in a Speed Vacuum Concentrator (ScanSpeed 40, SCANVAC; LaboGene) at 1,500 rpm and 27°C for 10 h. Then, 28 ml of 70% ethanol was added and the sediment was extracted

in a 60°C water bath for 2 h, followed by 1 h of sonication at 60°C. Then, the sample was centrifuged at 11,000 ×g and 25°C for 15 min. The supernatant was collected and volatilized in the speed vacuum concentrator at 1,500 rpm and 27°C for 14.5 h. Finally, 1 ml of 70% ethanol was added to dissolve the solid.

Detection of Isoflavones by High-Performance Liquid Chromatography

The extracted solution was filtered out using a syringe filter (0.22 µm cut-off; PALL Corporation, USA) prior to high-performance liquid chromatography (HPLC) analysis. Daidzin, genistin, daidzein, and genistein were separated from 20 µl samples using an Eclipse XDB-C18 column (150 mm column length: 150 mm; inner diameter: 3 mm; particle size: 5 µm; Agilent Technologies, USA). Standard daidzin, genistin, daidzein, and genistein were purchased from Nanjing Dilger Medical Technology Co., Ltd, China. These four standards were dissolved in 80% methanol. The concentration of the analyzed isoflavone standards were 10, 15, 20, and 25 µg/ml. The mobile phase and ratio changes for each standard are shown in Table 1. The column temperature was 35°C, and the flow rate was 0.5 ml/min. The UV detection wavelength was 254 nm. The system used for HPLC analysis was the 1090 Series-II Model HPLC System (Hewlett-Packard, USA).

Quantitative Analysis of GABA and Free Amino Acids by HPLC

One gram of the sample was mixed with 10 ml of 70% ethanol. After 1 h of sonication, the mixture was placed on a rocker (NB-104; N-Biotek, Korea) and extracted at 20 rpm at room temperature for 24 h. The mixture was then centrifuged at 2,900 ×g for 1.5 min at 25°C. The supernatant was collected and filtered out through a 0.22-µm cut-off microfiber syringe filter membrane (PALL Corporation) before HPLC analysis. HPLC analysis of GABA was carried out at the National Instrumentation Center for Environmental Management (Korea). The column used for separation was the VDSpher 100 C18-E (150 mm long, 4.6 mm internal diameter, 3.5 µm particle size; VDS Optilab, Germany). Standard samples were made by dissolving 17 amino acids into 0.1 N HCl. The mobile phase and ratio changes are shown in Table 2. The column temperature was 40°C, with a flow rate of 1.5 ml/min. The level

Table 1. HPLC analysis conditions for soybean isoflavones.

Time (min)	Solution A ^a (%)	Solution B ^b (%)
0	95	5
4	95	5
48	65	35
54	15	85
56	0	100
66	0	100
75	95	5
80	95	5

^aSolution A was 0.1% (v/v) acetic acid in water.

^bSolution B was 0.1% (v/v) acetic acid in acetonitrile.

Table 2. HPLC analysis conditions for free amino acids.

Time (min)	Solution C ^a (%)	Solution D ^b (%)
0	95	5
3	95	5
24	45	55
25	0	100
31	0	100
34.5	95	5
35	95	5

^aSolution C was 40 mM sodium phosphate dibasic, pH 7.0.

^bSolution D was water-acetonitrile-methanol (10:45:45 (v)).

of free amino acids was quantified by a fluorescence detector (338 nm, Dionex Ultimate 3000 HPLC System; Thermo Fisher Scientific, USA).

Statistical Analysis

Significant differences were evaluated using t-tests to analyze changes in isoflavone levels during doenjang fermentation, using Microsoft Excel 2016. To analyze the effect of *L. brevis* GABA 100 and 10 varieties of *A. oryzae* strains, analysis of variance followed by Tukey's post hoc test was applied to make group comparisons, using Minitab 16.

Results and Discussion

Screening of *A. oryzae* for the Enhancement of Isoflavone Aglycones during Fermentation

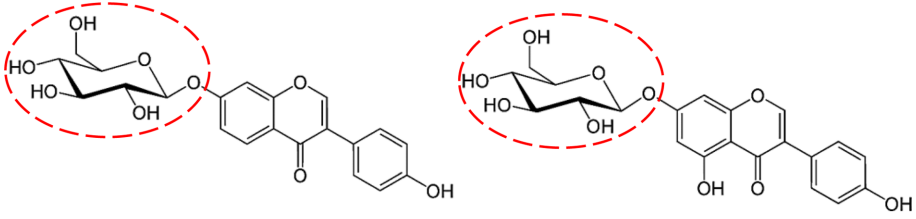
During soybean fermentation, several health-promoting compounds, such as GABA and isoflavone aglycones, are produced. Glutamic acid is produced via the hydrolyzation of soybean protein and can be transformed into GABA under the catalysis of glutamic acid decarboxylase [22]. Some lactic acid bacteria have been proven to synthesize glutamic acid decarboxylase, which produces GABA from glutamate. Recently, multiple *Lactobacillus* spp. have been used in GABA production [23]. Specifically, *L. brevis* GABA 100, which was originally isolated from kimchi (a traditional Korean fermented vegetable), is known to produce a large amount of GABA under the presence of MSG using glutamic acid decarboxylase [24]. *Aspergillus* spp. are regarded as owing of the most extensively and naturally occurring mold species that exhibit strong β -glucosidase activity. For example, *A. oryzae* and its β -glucosidase are traditionally used in the following fermented soybean and rice products of Asia: doubanjiang in China, doenjang and soy sauce in Korea, and miso, natto, and sake in Japan [2]. Specifically, *A. oryzae* is a major fungus growing in meju, which is a dried soybean paste (major raw ingredient in doenjang) prepared by traditional fermentation methods [25]. Recently, these microorganisms and enzymes have been applied in various nutraceutical and biological engineering applications; moreover, they are linked to the bioconversion of soybean isoflavones, due to their significant hydrolytic and transferase properties.

To achieve the simultaneous production of GABA and isoflavone aglycones from doenjang, whole-cell *L. brevis* GABA 100 was co-inoculated with 10 strains of *A. oryzae* spp. and fermented for 2 days without interruption under aerobic conditions. Samples were taken every day and the concentration of the isoflavones and GABA was determined by HPLC and thin-layer chromatography (TLC), respectively.

As shown in Table 3, within 24 h of incubation, the levels of daidzin and genistin in all 10 groups had decreased significantly ($p < 0.05$, $n = 3$). The final concentrations of daidzin and genistin were almost constant once the incubation time approached 48 h. Both daidzin and genistin were successfully converted into daidzein and genistein within 1 day. Among the 10 groups, four (Table 3, Exp.# 1, 2, 6, and 7) were inoculated with *A. oryzae*; KACC 40234, KACC 40250, KACC 45002, and KACC 45006 showed the highest daidzein and genistein productivity compared with the other groups ($p < 0.05$, $n = 3$). Although the five other groups (spiked *A. oryzae* KACC 44967, KACC 45001, KACC 45007, KACC 46471, and KFRI 995, respectively, with *L. brevis* GABA 100) (Table 3, Exp.# 4, 5, 8, 9, and 10) catalyzed daidzin and genistin into daidzein and genistein within 24 h, the levels of daidzein and genistein were significantly decreased at 48 h fermentation ($p < 0.05$, $n = 3$). In terms of the catalytic properties of the group treated with *A. oryzae* KACC 41736 (Table 3, Exp. #3), the conversion rate of genistin was somewhat lower than that of the *A. oryzae* KACC 45006-treated group (Table 3, Exp. #7) and significantly lower than the conversion rate of the aforementioned three groups (Table 3, Exp.# 1, 2, and 6) ($p < 0.05$, $n = 3$).

Application of Mixed Fermentation to Produce GABA

Although *L. brevis* GABA 100 represented strong MSG catalytic properties and produced significant levels of GABA in certain condition [24], these catalytic properties and GABA productivity can be altered by the medium composition, the type of microbial flora, and the condition of mixed fermentation [12, 14, 26]. Consequently, the condition that best facilitates GABA and isoflavone aglycone production should be determined. After our initial screening of 10 strains of *A. oryzae* and isoflavone aglycone productivity, four candidate strains (*i.e.*, *A. oryzae* KACC 40234, KACC 4025, KACC 45002, and KACC 45006) were selected and utilized for further GABA production analysis. When assessing GABA productivity using TLC, the treatment of whole-cell *A. oryzae* KACC 40234, KACC 45006, and KACC 40250 with whole-cell *L. brevis* GABA 100 showed effective catalytic activity after 3 days of incubation (Fig. 1). Although, *L. brevis* GABA 100, the key source of glutamic acid catalysis, was treated to all samples, significant synergistic and/or antagonistic activities in the glutamic acid conversion were observed. GABA's catalytic patterns varied in each sample by type of spiked microorganisms. Specifically, *A. oryzae* KACC 40250 showed the best glutamic acid catalytic properties, whereas the *A. oryzae* KACC 45002-treated group

Table 3. Changes of soybean isoflavones during fermentation via co-cultivation of *L. brevis* GABA 100 and 10 varieties of *A. oryzae* strains ($n = 3$).


Exp.#	Spiked cell (<i>Aspergillus</i>)	Daidzin			Genistin		
		0 h	24 h	48 h	0 h	24 h	48 h
1	KACC 40234	14.0 ± 0.3	3.9 ± 0.2	3.9 ± 0.8	12.5 ± 0.7	3.9 ± 0.2	3.9 ± 0.2
2	KACC 40250	14.0 ± 0.3	3.6 ± 0.4	3.3 ± 0.4	12.5 ± 0.7	2.8 ± 0.5	3.4 ± 0.2
3	KACC 41736	14.0 ± 0.3	3.7 ± 0.1	3.5 ± 0.4	12.5 ± 0.7	4.0 ± 0.7	3.9 ± 0.2
4	KACC 44967	14.0 ± 0.3	3.9 ± 0.3	3.7	12.5 ± 0.7	2.9 ± 0.2	3.3 ± 0.6
5	KACC 45001	14.0 ± 0.3	5.6 ± 0.3	3.3	12.5 ± 0.7	5.5	3.6 ± 0.1
6	KACC 45002	14.0 ± 0.3	5.0 ± 0.1	4.6 ± 1.1	12.5 ± 0.7	3.6 ± 0.6	4.3 ± 0.5
7	KACC 45006	14.0 ± 0.3	3.4 ± 1.8	2.9 ± 0.2	12.5 ± 0.7	4.1 ± 0.5	3.6 ± 0.4
8	KACC 45007	14.0 ± 0.3	3.0 ± 0.4	3.0 ± 1.1	12.5 ± 0.7	2.9 ± 0.1	3.2 ± 0.7
9	KACC 46471	14.0 ± 0.3	5.1 ± 1.4	3.4 ± 0.1	12.5 ± 0.7	5.0 ± 0.5	3.7 ± 0.2
10	KFRI 995	14.0 ± 0.3	5.6 ± 0.2	3.4 ± 0.2	12.5 ± 0.7	5.2 ± 0.5	3.7 ± 0.2



Exp.#	Spiked cell (<i>Aspergillus</i>)	Daidzein			Genistein		
		0 h	24 h	48 h	0 h	24 h	48 h
1	KACC 40234	4.6 ± 0.3	11.3 ± 0.4	11.7 ± 0.4b ^a	3.6	10.7 ± 0.1	11.6 ± 1.6a
2	KACC 40250	4.6 ± 0.3	12.9 ± 1.6	12.9 ± 0.4a	3.6	13.1 ± 3.0	13.1 ± 0.5a
3	KACC 41736	4.6 ± 0.3	12.0 ± 0.6	12.9 ± 0.4a	3.6	11.0 ± 0.1	8.3 ± 0.2b
4	KACC 44967	4.6 ± 0.3	12.1 ± 0.2	5.1 ± 0.7de	3.6	13.0 ± 0.2	6.0c
5	KACC 45001	4.6 ± 0.3	10.1 ± 0.1	4.2 ± 0.1e	3.6	8.8 ± 0.2	4.0 ± 0.1d
6	KACC 45002	4.6 ± 0.3	14.0	13.0 ± 0.4a	3.6	12.8 ± 3.7	12.1a
7	KACC 45006	4.6 ± 0.3	13.7 ± 0.4	13.5 ± 0.1a	3.6	11.0 ± 0.1	9.3 ± 0.1b
8	KACC 45007	4.6 ± 0.3	12.3 ± 2.4	7.1 ± 0.4c	3.6	13.5 ± 0.6	5.6 ± 0.4cd
9	KACC 46471	4.6 ± 0.3	12.9 ± 0.4	6.1 ± 0.4cd	3.6	11.9 ± 0.3	5.0 ± 0.2cd
10	KFRI 995	4.6 ± 0.3	13.2 ± 0.1	4.7 ± 0.6e	3.6	7.8 ± 0.1	4.0 ± 0.1d

^aCorresponding letters within a column are significantly different at $p < 0.05$.

showed limited catalytic properties. Recently, *A. oryzae* KACC 40250 was reported not to produce aflatoxin and cyclopiazonic acid, which are hazardous to consumers' health [20, 27]. Therefore, *A. oryzae* KACC 40250 was applied in subsequent research on GABA, daidzein, and genistein

with the use of *L. brevis* GABA 100.

Protease Treatment to Accelerate GABA, Daidzein, and Genistein Production

The quantities of daidzein and genistein in the fermented

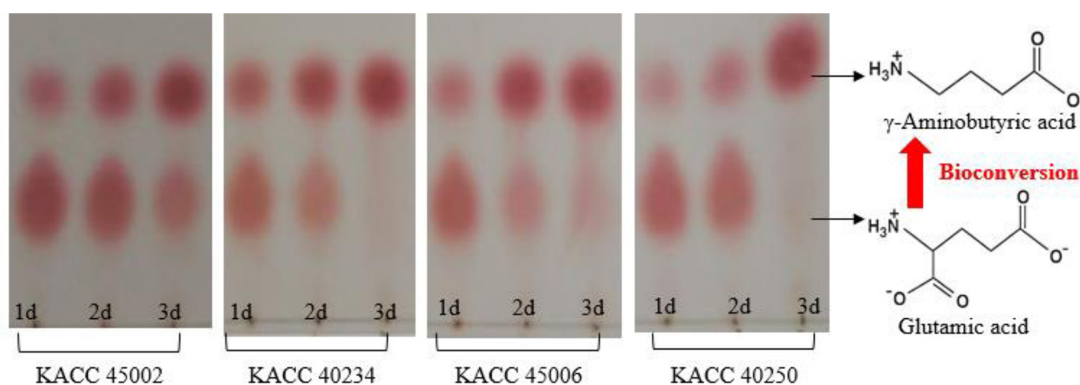


Fig. 1. Production of GABA in fermented soybean media using strains of *L. brevis* GABA 100 and *A. oryzae*.

products tended to plateau after 24 h of incubation (Table 3), while the GABA contents tended to increase until the third day of fermentation (Fig. 1). According to Komatsuzaki *et al.* [28], GABA productivity is significantly reduced when high levels of MSG are added due to growth inhibition. This result was also confirmed by Zhang *et al.* [29], whose study reported that GABA productivity was not significantly changed when 0.5–0.7% (w/v) of MSG was added to the media. However, GABA productivity was reduced dose-dependently when the culture medium was treated with more than 0.8% (w/v) of MSG, due to microbial growth inhibition. Accordingly, simple treatments of additional MSG are not a solution worth troubleshooting in this case.

According to Taylor [30], the addition of protease is another option for improving GABA productivity. This method was previously used to produce jiuqu, a traditional mold starter for the production of Chinese rice wine. The level of amino acid is increased owing to soybean degradation by protease treatment. Generation of free amino acids positively affects the physiological activity of microorganisms, such as enhanced microbial growth and increased glutamic acid decarboxylase activity. When microbial growth is activated, the production of organic acids is increased, which leads to promoted GABA production at low pH [19, 24]. Because one goal of this work was to simultaneously produce GABA, daidzein, and genistein, we treated the soybean medium with artificial-cell spiking combined with a protease treatment in order to accelerate GABA production. GABA productivity is highly related to levels of glutamic acid, which can be generated by the hydrolysis of soybean protein.

When the samples were analyzed at 6, 12, 18, 24, and 48 h, free amino acids were detected in all samples via protease treatment. Moreover, GABA production reached its peak level within 24 h of incubation (Fig. 2). The use of

protease significantly reduced the GABA production time from 3 days to 1 day when the mixed fermentation of whole-cell *L. brevis* GABA 100 and *A. oryzae* KACC 40250 was applied.

Doenjang Fermentation

Based on our data from the aforementioned experiments, the best conditions for the rapid production of GABA, daidzein, and genistein was practically administered to doenjang production using a mixed microbial fermentation accompanied by protease treatment. The samples were analyzed on the seventh day of doenjang fermentation. As

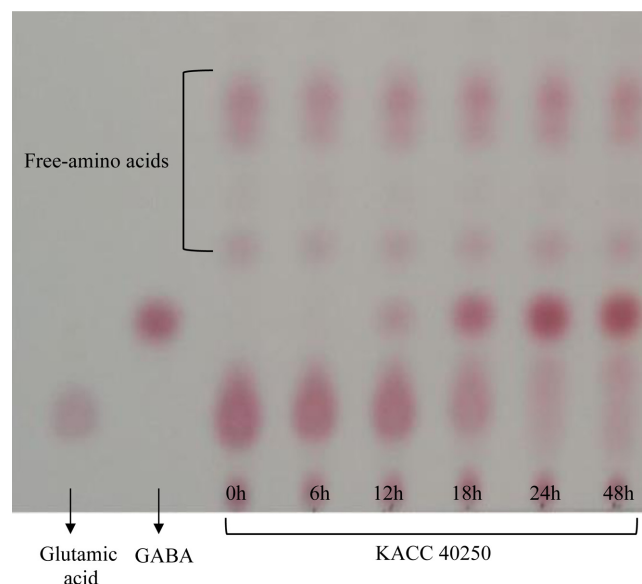


Fig. 2. Time course TLC analysis of metabolites of glutamic acid bioconverted by mixed fermentation using *A. oryzae* KACC 40250 and *L. brevis* GABA 100.

The soybean medium was treated with protease cocktail to accelerate GABA production.

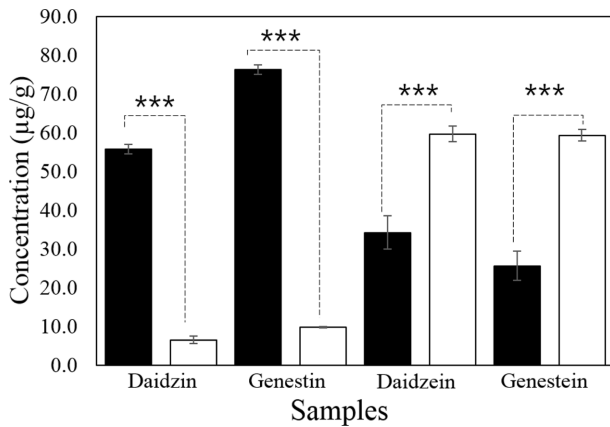


Fig. 3. Changes in isoflavone levels (before (■) and after 7 days fermentation (□)) in doenjang fermented by co-cultivation of *L. brevis* GABA 100 and *A. oryzae* KACC 40250. Error bars denote ± standard deviation. Asterisks show a significant difference between the two groups (***p* < 0.001).

shown in Fig. 3, the quantity of aglycone isoflavones and GABA in the seventh day doenjang were significantly higher than the initial doenjang samples. The levels of daidzein and genistein were 60 and 59 µg/g, respectively. The GABA concentration of the doenjang on day 7 of fermentation was determined to be 7,162 µg/g, which is notably higher than that of conventional doenjang (Fig. 4). According to Jo *et al.* [31], traditional Korean doenjang fermented for 1 year contained 44 µg/g GABA, while a 10-year-old doenjang contained 1,939 µg/g GABA.

It is assumed that MSG supplementation is not an issue in this study because MSG has already been administered

to commercialized doenjang for flavor purposes. As far as we know, no studies have analyzed the GABA concentrations in commercialized doenjang. However, the GABA concentration of commercialized doenjang is expected to be lower than this study because it does not use specific GABA-producing microorganisms. In addition, it is also expected that the concentration of GABA will be lower or similar to that of the traditional method because the case of aging doenjang for 1 year is rare. The daidzein contents in the 1-year-old and 10-year-old doenjang were 55 and 101 µg/g, respectively. As for genistein, 72 and 95 µg/g were contained in the 1-year-old and 10-year-old doenjang, respectively [31]. Therefore, there are significantly higher levels of daidzein and GABA in the doenjang fermented with our proposed method compared with the concentrations in 1-year-old and 10-year-old traditional doenjang.

In summary, the GABA and aglycone isoflavone contents in doenjang were significantly increased by mixed fermentation paired with protease treatments. *L. brevis* GABA 100 (*i.e.*, glutamic acid decarboxylase producing cell) and *A. oryzae* 40250 (*i.e.*, β-glucosidase-producing cell) were co-inoculated in the protease-treated soybean starter for doenjang fermentation. The starter was then mixed with steamed soybean for the GABA- and aglycone isoflavone-enriched doenjang production. GABA and soybean isoflavone quantities were evaluated after 7 days of fermentation. The GABA, daidzein, and genistein levels were 7162, 60, and 59 µg/g, respectively, which were comparable to that in long-term fermented traditional doenjang. GABA and aglycone isoflavone production in doenjang was significantly accelerated by our proposed method.

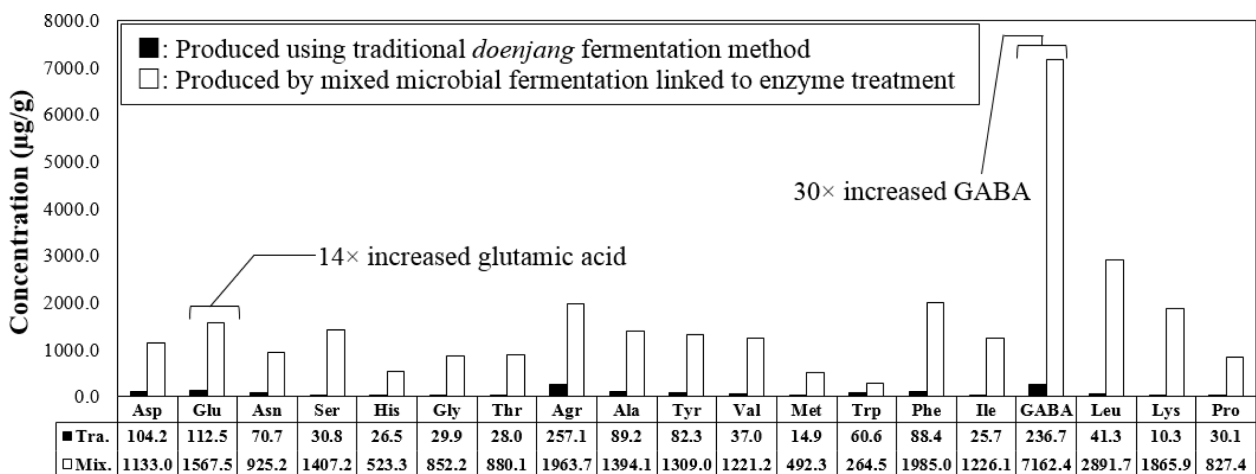


Fig. 4. Increased GABA and free amino acids concentration in deonjang after mixed fermentation using *A. oryzae* KACC 40250 and *L. brevis* GABA 100 accompanied by protease cocktail treatments compared with the conventional deonjang production method.

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