Functional beverage from fermented soymilk with improved amino nitrogen, β -glucosidase activity and aglycone content using *Bacillus subtilis* starter

Kyung Ha Lee^{1,2}, Sae Hun Kim¹, Koan Sik Woo², Hyun Joo Kim², Hye Sun Choi², Young Hoon Kim⁴, and Jin Song^{3,*}

¹Division of Food Bioscience and Technology, Korea University, Seoul 02841, Korea

²Department of Central Area Crop Science, National Institute of Crop Science, Rural Development Administration, Suwon, Gyeonggi 16613, Korea

³National Academy of Agricultural Science, Rural Development Administration, Jeonju, Jeonbuk 55365, Korea

⁴BK21 Plus Graduate Program, Department of Animal Science and Institute Agricultural Science & Technology, Chonbuk National University, Jeonju, Chonbuk 54896, Korea

Received March 19, 2016
Revised July 16, 2016
Accepted July 17, 2016
Published online October 31, 2016

*Corresponding Author
Tel: +82-63-238-3671
Fax: +82-63-238-3662
E-mail: songjin@korea.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract The bioactivity of soymilk was enhanced by fermentation with three strains of β -glucosidase-producing *Bacillus subtilis* for 36 h at 37°C. The results indicated that protease, cellulase, and β -glucosidase activities were significantly ($p < 0.05$) increased with increasing fermentation time. In addition, the amino-type nitrogen content in *B. subtilis*-fermented soymilk was increased to 121.1–140.7 mg% after 36 h of fermentation. Among the isoflavones in soymilk, the contents of β -glucosides or acetyl-glucosides were decreased, while aglycone content was increased by fermentation. In particular, the soymilk fermented with *B. subtilis* HJ18-9 had highest β -glucosidase activity and the largest increase in aglycone content. The total aerobic and anaerobic cell counts were increased with increasing fermentation time. Therefore, this study suggests that soy beverages fermented with β -glucosidase-producing *B. subtilis* have the potential to enhance the health and nutritional status of consumers.

Keywords: *Bacillus subtilis*, soymilk, fermentation, isoflavone, enzyme activity

Introduction

Soybean is an abundant and inexpensive source of nutrients and calories (1). Soymilk, a beverage made from soybeans, is one of the traditional soy foods consumed by Asian people. Recently, it attracted attention because of its ability to reduce the risk of coronary heart disease, cancer, osteoporosis, and alleviate hot flashes in menopausal women (2). In particular, soymilk contains high amounts of isoflavones, which have a structure homologous to human oestrogens (3,4).

The main isoflavones in soymilk, soy bread, or *douchi* comprise 12 chemical compounds, which include three aglycones (daidzein, genistein, and glycitein) and their β -, malonyl-, and acetyl-glucosides (5,6). The bioactivities of isoflavones are strongly influenced by their chemical structures (7). The parent isoflavones have the aglycone structure, which are conjugated to form β -, malonyl-, and acetyl-glucoside configurations. Aglycones, the biologically active oestrogen-like isoflavones, are absorbed faster and in higher amounts than their glucoside derivatives in humans (8). Most of the soybean isoflavones

exist in the β -glycoside form, which include daidzein, glycitein, and genistin, constituting 50–90% of the isoflavones. Isoflavone aglycones exhibit biological activity such as oestrogenic action and antioxidant capacity (9) whereas the native forms of β -glucosides are not readily bioavailable in humans because of the difficulties in their absorption through the intestinal tract (10).

The biological activity and composition of isoflavones in soy products, including soymilk, depend on the type and condition of processing like enzyme hydrolysis, heat treatment, and fermentation (11). Hydrolysis of β -glucosides by bacterial β -glucosidases produces active and more readily metabolized aglycone forms (12). In particular, β -glucosidase produced by fermentation has numerous advantages over several current processing methods because it is cost-efficient and easily applicable for large-scale production.

Bacteria with β -glucosidase activity, such as lactic acid bacteria, bifidobacteria, and *Saccharopolyspora erythraea*, have been examined for their ability to convert glucosides to aglycones. *Lactobacillus rhamnosus* CRL981 completely hydrolysed the glucosides into their aglycone forms after 12 h of fermentation (13). *Bacillus subtilis* is an

important starter culture used in the processing of fermented soybean foods such as Korean *doenjang*, Japanese *natto*, Thai *thua nao*, and Chinese *douche* (14). Studies have been reported that soymilk can be improved functionality and quality characteristics by fermentation with *B. subtilis* (15,16). However, soymilk has not been examined yet for application of β -glucosidase-producing *B. subtilis* isolated from *chungkukjang*. Only fermented soymilk by *B. subtilis* natto with high β -glucosidase was reported (17). Fermentation of soymilk may not only enhance its beneficial health properties but it also has the potential to lead to new types of soy products (18).

Therefore, this study was aimed to investigate the potential for application of β -glucosidase-producing *B. subtilis* as a functional starter culture to soymilk for production of fermented soymilk. In addition, the three *B. subtilis* strains were compared for their activity to hydrolyze isoflavone glycosides and for their increasing ability amino type nitrogen in soymilk.

Materials and Methods

Microorganisms *B. subtilis* HJ18-9 and HJ25-8 were obtained from the Fermentation Food Science Division at NAAS (National Academy of Agricultural Science, Jeonju, Korea). *B. subtilis* KACC 15935 was purchased from KACC (Korean Agricultural Culture Collection, Jeonju, Korea). *B. subtilis* HJ18-9 and HJ25-8 were isolated from buckwheat *sokseongjang*, a traditional Korean fermented soybean food, showed broad-spectrum antimicrobial activity against foodborne pathogens, including *B. cereus*. Also *B. subtilis* HJ18-9 and HJ25-8 have amylase, cellulase and β -glucosidase activities. *B. subtilis* KACC was isolated from commercial *deonjang*, a traditional Korean fermented soybean food (19).

These strains were cultured on Luria-Bertani broth (LB, Pronadisa, Conda, ID, USA). A single colony of *B. subtilis* HJ18-9, HJ25-8, and KACC 15935 were chosen for further use. This was then added to 100 mL of LB and cultured at 30°C for 24 h under aerobic conditions. After incubation, the cultures were centrifuged at 6,000× *g* for 10 min. Cell pellets were washed twice in buffered peptone water (0.1% sterile peptone, w/v). The cell concentration of these individual cultures was approximately 6 log CFU/mL.

Preparation of soymilk and fermentation Soybean ‘Dae-Won’ (*Glycine max*) harvested in Kyungpook province in South Korea in 2012 were used for soymilk preparation. Whole beans were washed, soaked in distilled water overnight, and allowed to drain for 1 h. The swollen soybeans were dehulled and ground with warm water at a ratio of 1:10 (w/v) using a blender (Waring Commercial, Torrington, CT, USA) for 3 min. The soybeans were steam-heated at 80°C for 15 min and filtered to separate insoluble residue to extract the soymilk. The soymilk was sterilized by autoclaving at 121°C for 15 min. For fermentation of the soymilk with *B. subtilis*, the sterilized soymilk was inoculated with the *B. subtilis* HJ18-9, HJ25-8, and KACC 15935

at a 1:100 (v/v) ratio. The soymilk was fermented at 37°C for 36 h.

Assay of enzyme activity To measure protease, cellulase, and β -glucosidase activities, 10 mL of fermented soy milk sample was mixed with 40 mL distilled water and placed on a shaker for 30 min at room temperature and each sample centrifuged at 6,000× *g* for 10 min (Beckman Instruments, Inc., Palo Alto, CA, USA).

The β -glucosidase activity was assayed as described by Choi *et al.* (20) with some modifications. One millilitre of each sample was centrifuged at 6,000× *g* for 5 min, and then 0.2 mL of the solution was added to 0.6 mL of sodium phosphate buffer (pH 7.0) containing 5 mM *p*-nitrophenyl- β -D-glucoside (*p*-NPG) and incubated at 37°C for 20 min. The reaction was stopped by addition of 0.5 mL 2 M Na₂CO₃ and centrifuged at 15,000× *g* for 5 min. The absorption of the supernatant was measured at 405 nm. One unit of β -glucosidase was equivalent to 1 μ mol of *p*-nitrophenol liberated by 1 mL of the sample under assay conditions.

Protease activity was assayed as described by Kim *et al.* (21). A 0.5 mL aliquot of supernatant was added to 1.5 mL of 0.6% casein (pH 7.0) and reacted at 37°C for 10 min. Subsequently, the reaction was stopped by adding 2 mL of 0.4 M trichloroacetic acid (TCA), and the mixture was filtered using Whatman No. 2 filter paper. Next, 1 mL of supernatant was reacted with 5 mL of 0.4 M Na₂CO₃ and 1 mL of Folin’s reagent at 37°C for 20 min, and the absorbance of the solution was measured at 660 nm. One unit of protease was equivalent to 1 μ g of tyrosine liberated by 1 mL of the sample under assay conditions.

To determine cellulase activity, 0.5 mL aliquot of filtrate was added to 0.25 mL of 1% (w/v) carboxymethyl cellulose (CMC) in 0.2 M sodium phosphate buffer (pH 7.0) and 1 mL of 0.2 M sodium phosphate buffer and incubated at 50°C for 15 min. The reaction was stopped by adding 3 mL of dinitrosalicylic acid and heated in a boiling water bath for 5 min. After cooling, the absorbance was read at 540 nm. The enzyme activity was expressed as U/mL of soymilk sample. One unit of cellulase activity was equivalent to 1 μ g of glucose liberated by 1 mL of the sample under assay conditions.

Isoflavone analysis by HPLC The extraction of isoflavones, including glucosides and aglycones from fermented soymilk, was carried out according to previous reports with some modifications (22). Each sample was extracted with 24 mL of acetonitrile and 6 mL of 1 M HCl for 1 h with shaking at room temperature. The supernatant was filtered with a syringe filter (0.2 μ m PVDF membrane filter, Millipore, Billerica, MA, USA) for a HPLC analysis. Isoflavones separation was performed using an ultra-performance liquid chromatography (UPLC) (Waters Co., Milford, MA, USA) equipped with binary pump, auto sampler, and photodiode array detector (PDA). Separations were performed with a reversed phase column (BEH Shield RP18, 1.7 μ m particle size, 2.1×100 mm, Waters Co.). The mobile phase was composed of 0.1% acetic acid in 10% methanol (A) and 0.1% acetic acid in methanol (B). Following the injection of 0.8 μ L of each

sample, solvent B increased from 26 to 50% over 17 min, and then held at 26% for 8 min for re-equilibration of the column before injection of the next sample. The solution was pumped at a flow rate of 0.3 mL/min and the eluted isoflavones were detected at 254 nm. The amount of isoflavone in the soymilk samples was calculated using calibration curves constructed with 0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL of isoflavone standards.

Amino-type and ammonia-type nitrogen content assay For amino-type nitrogen content analysis, 5 mL aliquot of filtrate was added to 10 mL of neutral formalin and 10 mL of distilled water. Two to three drops of 0.5% phenolphthalein solution was added and then adjusted to pale pink colour with 100 mM NaOH. Distilled water was used as a blank. The amino-type nitrogen content was calculated as follows:

$$\text{Amino-type nitrogen (mg\%)} = (V_1 V_0) \times F \times 0.0014 \times D \times 100 / S$$

where V_1 was the titration value (mL) of sample, V_0 was the titration value (mL) of blank, F is the factor of 0.1 mM NaOH, D is the dilution ratio, S is the weight (g) of sample, and 0.0014 represents the nitrogen weight in 1 mL of 0.1 mM NaOH.

Ammonia-type nitrogen content in the fermenting substrates was determined using the phenol-hypochlorite reaction. A 0.1 mL aliquot of diluted extract was added to 2 mL of solution A (1 L distilled water including 10 g phenol and 0.05 g sodium nitroprusside dihydrate) and solution B (1 L distilled water including 9 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6 g NaOH, and 10 mL NaOCl). The reaction was carried out at 37°C for 20 min, and the absorbance was measured at 630 nm using a UV spectrophotometer (V560; Jasco, Tokyo, Japan). The total concentration of ammonia-type nitrogen was determined from a standard curve prepared with known concentrations of ammonium sulphate.

Microbiological analysis During fermentation, total aerobic cell count was determined by the plate dilution method using plate count agar (PCA; Difco, Livonia, MI, USA). Serial dilutions of each fermented soymilk sample were plated onto PCA agar plates in triplicate and the plates were incubated at 37°C for 24 h. The results

were expressed as colony forming units per milliliter (CFU/g).

Statistical analysis The mean value and standard deviation were calculated from the data obtained from three trials. Analysis of variance and Duncan's multiple range tests were performed on all the results obtained in this study by using the SPSS package (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Enzyme activity Enzymes such as protease, β -glucosidase, and cellulase in soymilk play an important role in changes of composition of nutrients in soymilk, imparting improved flavor and taste to it (23). In addition, the activities of protease, cellulase, and β -glucosidase in soymilk fermented with *B. subtilis* would greatly increase during fermentation. Therefore, the activities of the three enzymes were measured during fermentation of soymilk with *B. subtilis* (Table 1). Soybean protease is one of the critical factors that determine the unique taste of soy products because soy protein is hydrolysed generating amino acids and peptides (24).

The initial protease activity of soymilk was 52.3–56.1 unit/mL, indicating there were negligible differences between the strains. After 24 h of fermentation, the protease activity of soymilk fermented with *B. subtilis* KACC 15935, HJ18-9 and HJ25-8 significantly increased to 87.4, 96.5, and 109.0 unit/mL, respectively. In particular, the protease activity of soymilk fermented with *B. subtilis* HJ18-9 increased to 140.7 unit/mL at 36 h fermentation until the end of fermentation, resulting in 87.5 unit/mL increase, compared to the beginning of fermentation.

In *chungkookjang* fermentation with *B. natto* and *B. licheniformis*, Youn *et al.* (7) reported that the protease activity consistently increased during 40 h of fermentation. Odunfa (25) also showed an increase in the protease activity of African locust bean dawadawa during the first 36 h of fermentation, after which a decline was observed until 60 h when the fermentation was terminated. Therefore, these results indicated that there are significant differences in the

Table 1. Changes in enzyme activities (U/mL) during soymilk fermentation by the three strains of *B. subtilis*

Enzymes	Strains	Fermentation time (h)			
		0	12	24	36
Protease	KACC15935	52.3±1.4 ^A ^d	78.2±1.4 ^B ^c	87.4±2.6 ^C ^b	121.1±1.4 ^B ^a
	HJ18-9	53.2±1.4 ^A ^d	80.7±1.4 ^B ^c	96.5±2.1 ^B ^b	140.7±0.7 ^A ^a
	HJ25-8	56.1±11.3 ^A ^c	99.9±3.2 ^A ^b	109.0±2.5 ^A ^b	137.8±4.3 ^A ^a
Cellulase	KACC15935	0.0±0.0 ^C ^c	0.0±0.0 ^B ^c	11.8±4.4 ^B ^b	16.6±1.9 ^C ^a
	HJ18-9	6.3±4.7 ^A ^B ^b	20.2±13.5 ^A ^b	22.7±9.9 ^A ^B ^b	50.2±19.8 ^A ^a
	HJ25-8	8.1±2.8 ^A ^b	14.0±9.3 ^A ^B ^b	35.1±0.8 ^A ^a	43.7±16.3 ^A ^B ^a
β -Glucosidase	KACC15935	146.7±29.6 ^A ^c	234.4±13.9 ^A ^b	231.1±44.4 ^A ^b	331.1±15.8 ^B ^a
	HJ18-9	156.7±5.8 ^A ^c	223.3±29.6 ^A ^b	234.4±18.4 ^A ^b	1255.6±48.3 ^A ^a
	HJ25-8	121.1±22.2 ^A ^c	198.9±9.6 ^A ^b	220.0±18.6 ^A ^b	323.3±35.3 ^B ^a

Means in the same row^{a-c} and column^{A-E} followed by different letters are significantly different ($p < 0.05$).

changes in protease activity during fermentation depending on the type of substrates, starters, fermentation time, and temperature, which are in agreement with our current data.

The cellulase activity in soymilks was observed relatively lower at the beginning of fermentation (Table 1). After 36 h of fermentation, cellulase activities in soy milk fermented with *B. subtilis* HJ18-9 and HJ25-8 were 50.2 and 43.7 unit/mL, respectively, whereas cellulase activity in soy milk fermented with *B. subtilis* KACC 15935 was 16.6 unit/mL.

The β -glucosidase activities in soymilk fermented with *B. subtilis* KACC 15935, HJ18-9 and HJ25-8 is shown in Table 1. β -Glucosidase plays a key role in breaking down the β -glucosidic bond in isoflavone glucosides to release the biologically active aglycones (26,27). The β -glucosidase activities in the soymilk fermented by the three strains of *B. subtilis* were in the range of 121.1–156.7 unit/mL at the beginning of fermentation with no significant ($p < 0.05$) differences. On the other hand, β -glucosidase activity in soymilk fermented with *B. subtilis* HJ18-9 significantly increased to 1255.6 unit/mL at the end of fermentation, compared to that of the other strains. This result may be attributed to the differences in the bacterial strains and growth media used. In addition, a sharp increase in the activity of β -glucosidase in soymilk was associated with the increase of the content of isoflavone aglycones such as daidzein, genistein, and glycitein in the soymilk by fermentation with *B. subtilis* HJ18-9. Some researchers reported that β -glucosidase produced hydrolysed glycosidic bonds of isoflavones in soybean flour during its fermentation (28,29).

Yang et al. (30) reported that β -glucosidase from *Paecilomyces thermophila* J18 converted glycosides in soybean flour extract and

soybean embryo extract into aglycones, resulting in more than 93% hydrolysis of three glycosides composed of daidzein, genistin, and glycitin. Therefore, the strains of β -glucosidase-producing *B. subtilis* could be used to increase the amounts of isoflavone aglycones in soy products. Overall, our results indicated that the type of *B. subtilis* strain is directly related to the differences in the production of enzymes including protease, cellulase, and β -glucosidase, even when the same medium was used.

Changes in isoflavone content and composition The content and composition of isoflavones in soymilk fermented with *B. subtilis* KACC 15935, HJ18-9 and HJ25-8 are presented in Table 2. The total isoflavone content ranged from 3,742.5 to 4,061.7 $\mu\text{g/g}$ dry weight (DW) during fermentation, indicating that there were no significant differences between the *B. subtilis* strains and fermentation time. The malonyl-glucoside content of soymilk fermented with the three strains of *B. subtilis* was in the range of 782.3–867.2 $\mu\text{g/g}$ DW, indicating that there were negligible differences among the samples during fermentation. Therefore, *B. subtilis* KACC 15935, HJ18-9 and HJ25-8 did not affect the content of malonyl-glucosides in soymilk during fermentation. On the other hand, there were distinct changes in the acetyl-glucoside content in soymilk during fermentation by *B. subtilis* KACC 15935, HJ18-9 and HJ25-8, depending on fermentation time. The acetyl-glucoside content in soymilk samples was initially 230.1–242.2 $\mu\text{g/g}$ DW, whereas in fermented soymilks, it decreased to 68.3–89.3 $\mu\text{g/g}$ DW after 36 h of fermentation, corresponding to a reduction of 152.3–169.9 $\mu\text{g/g}$ DW. In particular, after 36 h of fermentation, the soymilk fermented with *B. subtilis* HJ18-9 exhibited

Table 2. Changes in isoflavone contents ($\mu\text{g/g}$ DW) during soymilk fermentation by the three strains of *B. subtilis*

Isoflavones	Strains	Fermentation time (h)			
		0	12	24	36
β -Glucoside ¹⁾	KACC 15935	2,343.7 $\pm$ 187.6 ^{Ab}	2,725.8 $\pm$ 149.6 ^{Aa}	2,797.8 $\pm$ 189.0 ^{Aa}	2,578.0 $\pm$ 202.5 ^{Ab}
	HJ18-9	2,413.8 $\pm$ 190.1 ^{Ab}	2,749.3 $\pm$ 218.3 ^{Ab}	2,855.3 $\pm$ 263.0 ^{Aa}	2,035.0 $\pm$ 168.8 ^{Bc}
	HJ25-8	2,503.7 $\pm$ 178.9 ^{Aa}	2,650.3 $\pm$ 241.0 ^{Aa}	2,702.2 $\pm$ 200.4 ^{Aa}	2,638.1 $\pm$ 180.5 ^{Aa}
Malonyl-glucoside ²⁾	KACC15935	804.0 $\pm$ 31.3 ^{Aa}	867.2 $\pm$ 41.1 ^{Aa}	810.3 $\pm$ 57.4 ^{Aa}	782.3 $\pm$ 41.9 ^{Aa}
	HJ 18-9	795.2 $\pm$ 44.2 ^{Aa}	842.1 $\pm$ 26.4 ^{Aa}	862.6 $\pm$ 35.4 ^{Aa}	828.1 $\pm$ 53.0 ^{Aa}
	HJ 25-8	845.3 $\pm$ 58.0 ^{Aa}	847.3 $\pm$ 45.1 ^{Aa}	819.4 $\pm$ 51.2 ^{Aa}	820.6 $\pm$ 52.5 ^{Aa}
Acetyl-glucoside ³⁾	KACC15935	230.1 $\pm$ 44.8 ^{Aa}	202.7 $\pm$ 38.5 ^{Aa}	140.3 $\pm$ 22.6 ^{Ab}	77.8 $\pm$ 11.6 ^{Ac}
	HJ 18-9	238.2 $\pm$ 44.3 ^{Aa}	182.1 $\pm$ 38.2 ^{Aa}	115.1 $\pm$ 22.2 ^{Ab}	68.3 $\pm$ 10.5 ^{Ab}
	HJ 25-8	242.2 $\pm$ 44.6 ^{Aa}	192.1 $\pm$ 36.6 ^{Aa}	131.7 $\pm$ 20.3 ^{Ab}	89.3 $\pm$ 17.7 ^{Ab}
Aglycone ⁴⁾	KACC15935	364.5 $\pm$ 19.5 ^{Ab}	216.3 $\pm$ 6.3 ^{Bc}	202.5 $\pm$ 7.6 ^{Bc}	460.3 $\pm$ 29.1 ^{Ba}
	HJ 18-9	374.4 $\pm$ 17.4 ^{Ab}	207.1 $\pm$ 9.3 ^{Bc}	184.3 $\pm$ 12.6 ^{Bc}	977.4 $\pm$ 49.1 ^{Aa}
	HJ 25-8	395.5 $\pm$ 12.0 ^{Ab}	321.6 $\pm$ 19.4 ^{Ac}	297.9 $\pm$ 11.4 ^{Ac}	513.5 $\pm$ 31.2 ^{Ba}
Total ⁵⁾	KACC15935	3,742.5 $\pm$ 220.7 ^{Aa}	4,012.2 $\pm$ 153.3 ^{Aa}	3,951.1 $\pm$ 157.1 ^{Aa}	3,898.6 $\pm$ 177.5 ^{Aa}
	HJ 18-9	3,821.7 $\pm$ 208.0 ^{Aa}	3,980.7 $\pm$ 239.4 ^{Aa}	4,017.4 $\pm$ 262.6 ^{Aa}	3,908.9 $\pm$ 172.0 ^{Aa}
	HJ 25-8	3,986.7 $\pm$ 177.5 ^{Aa}	4,011.4 $\pm$ 252.0 ^{Aa}	3,951.4 $\pm$ 181.0 ^{Aa}	4,061.7 $\pm$ 176.8 ^{Aa}

Means in the same row^{a-c} and column^{A-B} followed by different letters are significantly different ($p < 0.05$).

¹⁾ β -glucoside: β -daidzin, β -glycitin, and β -genistin.

²⁾Malonyl-glucoside: M-daidzin, M-glycitin, and M-genistin.

³⁾Acetyl-glucoside: A-daidzin, A-glycitin, and A-genistin.

⁴⁾Aglycone: daidzein, glycitein, and genistein.

⁵⁾Total: β -glucoside+malonyl-glucoside+acetyl-glucoside+aglycone.

lower β -glucoside content than did soymilks fermented with KACC 15935 and HJ25-8. In addition, soymilk fermented with *B. subtilis* HJ18-9 showed the highest aglycone content of 977.4 $\mu\text{g/g}$ DW. The aglycone content in soymilk fermented with *B. subtilis* KACC 15935 and or HJ25-8 was slightly increased without the reduction of β -glucosides at the end of fermentation. This difference could be attributed to the fermentation characteristics of the different strains. Overall, the decrease in β -glucoside or acetyl-glucoside content in soymilk during fermentation is due to the bioconversion of glycosidic form into aglycone form (31). Setchell *et al.* (32) reported that isoflavone glucosides were not efficiently absorbed across the intestinal epithelium in human adults because of their higher molecular weight and higher hydrophilicity of the glucosides.

Chen *et al.* (33) also reported that β -glucosidase immobilized on coffee grounds was capable of catalysing hydrolysis of isoflavone glycosides in black soymilk in a highly efficient manner. Along with other reports, the results indicated that an enzymatic method by β -glucosidase-producing *B. subtilis* can be used to deglycosylate isoflavone glycosides in soymilk. Lee *et al.* (34) showed that soy milks fermented with *L. paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU 102 effectively inhibited adipocyte differentiation, up-regulated lipolysis activity, and suppressed HR-LPL (heparin-releasable lipoprotein lipase) activity to decrease free acid accumulation by increasing daidzein and genistein levels.

Isoflavones aglycone forms are absorbed more easily than glycosylated conjugates because their low molecular weight improves diffusion (35). The aglycones in soymilk are more quickly absorbed than the glycosylated forms (36). Isoflavone content and conversion during processing is directly affected by the chemical structures and other parameters, such as the soybean cultivar, pH, temperature and time of soaking and thermal treatment, soybean: water proportion, filtration, and factors affecting the β -glucosidase activity (37,38). Few investigators have proposed modifications in the process in order to increase aglycone content by increasing endogenous β -glucosidase activity (39). Therefore, soymilk with high aglycone contents can be a source of functional beverage by modifying some processing stage.

Amino-type and ammonia-type nitrogen contents Amino-type and ammonia-type nitrogen are the standards for determining

Fig. 1. Changes of total aerobic counts during soymilk fermentation by the three strains of *B. subtilis*.

fermentation efficiency of fermented food. They include palatable free amino acids formed by reaction of enzymes such as protease secreted from microorganisms during fermentation of soybean products including *doenjang* and the free amino acids are further broken down into ammonia during later stage of fermentation (40). Thus, the changes in contents of amino-type and ammonia-type nitrogen during soymilk fermentation were investigated (Table 3). The amino-type nitrogen content in soymilk samples fermented with all three strains increased from 52.3–56.1 mg% at the initial stage of fermentation to 121.1–140.7 mg% at 36 h of fermentation. In particular, soymilk fermented with *B. subtilis* HJ18-9 and HJ25-8 showed the higher amino-type nitrogen content (140.7 mg%) compared to that fermented with KACC 15935 (121.1 mg%). The ammonia-type nitrogen content in soymilk also increased markedly from 15.9–29.6 mg% at the beginning of fermentation to 49.8–52.0 mg% at 36 h of fermentation. Regardless of the strains, amino-type nitrogen in soymilk showed higher content throughout fermentation, compared to the ammonia-type nitrogen content.

Microbiological changes during fermentation Changes in the counts of total number of aerobic cells in soymilk during fermentation are

Table 3. Changes in amino-type nitrogen (mg%) and ammonia-type nitrogen (mg%) contents during soymilk fermentation by different *B. subtilis* strains ($n=\bar{X}\pm\text{SD}$)

Type	Strains	Fermentation time (h)			
		0	12	24	36
Amino-type nitrogen	KACC15935	52.3 $\pm$ 1.4 ^{Ab}	78.2 $\pm$ 1.4 ^{Bb}	87.4 $\pm$ 2.6 ^{Cb}	121.1 $\pm$ 1.4 ^{Ba}
	HJ18-9	53.2 $\pm$ 1.4 ^{Ab}	80.7 $\pm$ 1.4 ^{Bc}	96.5 $\pm$ 2.1 ^{Bbc}	140.7 $\pm$ 0.7 ^{Aa}
	HJ25-8	56.1 $\pm$ 1.3 ^{Aa}	99.9 $\pm$ 3.2 ^{Aa}	109.0 $\pm$ 2.5 ^{Aa}	137.8 $\pm$ 4.3 ^{Aa}
Ammonia-type nitrogen	KACC15935	16.43 $\pm$ 1.12 ^{Cc}	17.96 $\pm$ 0.60 ^{Bc}	24.67 $\pm$ 3.67 ^{BCb}	50.46 $\pm$ 0.47 ^{ABa}
	HJ18-9	15.90 $\pm$ 0.59 ^{Cb}	16.07 $\pm$ 0.46 ^{Bb}	19.98 $\pm$ 4.67 ^{Cb}	52.04 $\pm$ 3.10 ^{ABa}
	HJ25-8	29.58 $\pm$ 0.92 ^{Bbc}	24.32 $\pm$ 6.37 ^{Ac}	33.18 $\pm$ 1.82 ^{Ab}	49.76 $\pm$ 2.22 ^{Ba}

Means in the same row^{a-c} and column^{A-E} followed by different letters are significantly different ($p<0.05$).

shown in Fig. 1. Counts of total aerobic cells in soymilk were 4.32 log CFU/mL for KACC 15935, 5.61 log CFU/mL for HJ18-9, and 5.69 log CFU/mL for HJ 25-8, respectively. The counts of total aerobic cells in soymilk fermented with *B. subtilis* KACC 15935, HJ18-9 and/or HJ25-8 rapidly increased up to 12 h of fermentation, reaching more than 7 log CFU/mL after 36 h of fermentation.

Our results were similar to those of Lee et al. (41) who reported that viable cell counts of soymilk fermented with *Lactobacillus curvatus* Gk36 and/or KCTC 3168 increased from 6.29-6.46 log CFU/mL at the initial stage to 8.15-8.41 log CFU/mL at 24 h of fermentation.

In conclusion, this study demonstrated that fermentation of soymilk with *B. subtilis* is an effective method for manufacturing an improved health beverage by increasing enzyme activities in soymilk compared to non-fermented soymilk. Our results also indicate that the production of isoflavone aglycones in soymilk fermented with *B. subtilis* was correlated with the increase in β -glucosidase activity during fermentation. In particular, the β -glucosidase from *B. subtilis* HJ18-9 was able to more effectively hydrolyze the glycoside form of soymilk isoflavones to their aglycones than the β -glucosidase from *B. subtilis* KACC 15935 and/or HJ25-8. Amino-type and ammonia-type nitrogen content in the fermented soymilk increased by fermentation with three strains of *B. subtilis*. Therefore, although any of the three strains of *B. subtilis* KACC 15935, HJ18-9 and HJ25-8 can be used for fermentation of soymilk, *B. subtilis* HJ18-9 strain would be more optimal as a starter culture in the development of functional soy beverages rich in biological activities.

Acknowledgments This work carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011695012016)” Rural Development Administration, Republic of Korea.

Disclosure The authors declare no conflict of interest.

References

- Tikkanen MJ, Adlercreutz H. Dietary soy-derived isoflavone phytoestrogens: Could they have a role in coronary heart disease prevention? *Biochem. Pharmacol.* 60: 1-5 (2000)
- Jassi HK, Jain A, Arora S, Chitra R. Effect of soy proteins vs soy isoflavones on lipid profile in postmenopausal women. *Indian J. Clin. Biochem.* 25: 201-207 (2010)
- Reiter E, Gerster P, Jungbauer A. Red clover and soy isoflavone-an *in vitro* safety assessment. *Gynecol. Endocrinol.* 27: 1037-1042 (2011)
- Molla MDJ, Hidalgo-Mora JJ, Soteras MG. Phytotherapy as alternative to hormone replacement therapy. *Front. Biosci. (Schol Ed)* 1: 191-204 (2011)
- Hsu C, Ho HW, Chang CF, Wang ST, Fang TF, Lee MH, Su NW. Soy isoflavone-phosphate conjugates derived by cultivating *Bacillus subtilis* var. *natto* BCRC 80517 with isoflavone. *Food Res. Int.* 53: 487-495 (2013)
- Islam MA, Punt A, Spenkelink B, Murk AJ, Leeuwen FXR, Rietjens IMCM. Conversion of major soy isoflavone glucosides and aglycones in *in vitro* intestinal models. *Mol. Nutr. Food Res.* 58: 503-515 (2014)
- Youn KC, Kim DH, Kim JO, Park BJ, Yook HS, Cho JM, Byun MW. Quality characteristics of the *chungkookjang* fermented by the mixed culture of *Bacillus natto* and *B. licheniformis*. *J. Korean Soc. Food Sci. Nutr.* 31: 204-210 (2002)
- Brouns F. Soya isoflavones: A new and promising ingredient for the health foods sector. *Food Res. Int.* 35: 187-193 (2002)
- Marazza JA, Nazareno MA, Giori GS, Garro MS. Bioactive action of β -glucosidase enzyme of *Bifidobacterium longum* upon isoflavone glucosides present in soymilk. *Int. J. Food Sci. Tech.* 48: 2480-2489 (2013)
- Donkor ON, Shah NP. Production of β -glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus casei* in soymilk. *J. Food Sci.* 73: 15-20 (2008)
- Yeo KE, Kim WJ. Effects of acid hydrolysis on isoflavone of defatted soybean flour. *Korean J. Food Sci. Technol.* 34: 916-918 (2002)
- Villares A, Rostagno MA, García-Lafuente A, Guzmán E, Martínez JA. Content and profile of isoflavones in soy-based foods as a function of the production process. *Food Bioprocess Tech.* 4: 27-38 (2011)
- Marazza JA, Garro MS, Savoy DG. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. *Food Microbiol.* 26: 333-339 (2009)
- Wang LJ, Yin LJ, Li D, Zou L, Saito M, Tatsumi E, Li LT. Influences of processing and NaCl supplementation on isoflavone contents and composition during douchi manufacturing. *Food Chem.* 101: 1247-1253 (2007)
- Choi JM, Kim JH, Cho EJ. Protective activity of purple sweet potato extract-added soymilk fermented by *Bacillus subtilis* against oxidative stress. *Food Sci. Biotechnol.* 19: 457-462 (2010)
- Seo KC, Kim MJ, Hong SH, Cha SY, Noh JS, Kim MJ, Song YO. The hypocholesterolemic effects of soymilk fermented with *Bacillus subtilis* compared to soymilk with *cheonggukjang* powder in apolipoprotein E knockout mice. *Prev. Nutr. Food Sci.* 15: 83-87 (2010)
- Kuo LC, Cheng WY, Wu RY, Huang CJ, Lee KT. Hydrolysis of black soybean isoflavone glycosides by *Bacillus subtilis* natto. *Appl. Microbiol. Biot.* 73: 314-320 (2006)
- Wang J, Wu R, Zhang W, Sun Z, Zhao W, Zhang H. Proteomic comparison of the probiotic bacterium *Lactobacillus casei* Zhang cultivated in milk and soy milk. *J. Dairy Sci.* 96: 5603-5624 (2013)
- National Academy of Agricultural Science. Study about improving antidiabetic and liver function of fermented soy-food and technology development of ingredient. Available from: <http://report.ndsl.kr/repDetail.do?cn=TRKO201500010551>. Accessed Feb. 27, 2015
- Choi YB, Kim KS, Rhee JS. Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria. *Biotechnol. Lett.* 24: 2113-2116 (2002)
- Kim JY, Lee SY, Park NY, Choi HS. Quality characteristics of black soybean paste (*Daemaekjang*) prepared with *Bacillus subtilis* HJ18-4. *Korean J. Food Sci. Technol.* 44: 743-749 (2012)
- Wang G, Kuan SS, Francis OJ, Ware GM, Carman AS. Simplified HPLC method for determination of phytoestrogens in soybean and its processed product. *J. Agr. Food Chem.* 38: 185-190 (1990)
- Park JS. Histological changes of *doenjang* during the fermentation with different strains. *Korean J. Food Sci. Technol.* 24: 477-481 (1992)
- Nora NT, Esther SD, Wisdom Kofi AA. The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into dawadawa. *Int. J. Food Microbiol.* 106: 145-152 (2006)
- Odunfa SA. Biochemical changes in fermenting African locust bean (*Parkia biglobosa*) during 'iru' fermentation. *Int. J. Food Sci. Tech.* 20: 295-303 (1985)
- Marazza JA, Nazareno MA, Giori GS, Garro MS. Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *J. Funct. Foods* 4: 594-601 (2012)
- Ewe JA, Wan-Abdullah WN, Alias AK, Liong MT. Ultraviolet radiation enhanced growth of lactobacilli and their bioconversion of isoflavones in biotin-supplemented soymilk. *LWT-Food Sci. Technol.* 50: 25-31 (2013)
- King RA, Bignell CM. Concentrations of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. *Aust. J. Nutr. Diet.* 57: 70-78 (2000)
- Handa CL, Couto UR, Vicensoti AH, Georgetti SR, Ida EI. Optimisation of soy flour fermentation parameters to produce α -glucosidase for bioconversion into aglycones. *Food Chem.* 152: 56-65 (2014)
- Yang S, Wang L, Yan Q, Jiang Z, Li L. Hydrolysis of soybean isoflavone glycosides by a thermostable β -glucosidase from *Paecilomyces thermophila*. *Food Chem.* 115: 1247-1252 (2002)
- Katekan D, Ekachai C, Arunee A, Richard AF. Enhanced aglycone production of fermented soybean products by *Bacillus* species. *Acta Biologica. Szegediensis* 53: 93-98 (2009)
- Setchell KDR, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AS, Heubi JE. Evidence of lack of absorption of soy isoflavones in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* 76: 447-453 (2002)
- Chen KI, Lo YC, Liu CW, Yu RC, Chou CC, Cheng KC. Enrichment of two isoflavone aglycones in black soymilk by using spent coffee grounds as an immobiliser for α -glucosidase. *Food Chem.* 139: 79-85 (2013)
- Lee BH, Lo YH, Pan TM. Anti-obesity activity of *Lactobacillus* fermented soy milk products. *J. Funct. Foods* 5: 905-913 (2013)
- Xu X, Wang HJ, Murphy PA, Hendrich S. Neither background diet nor type of soy food affects short-term isoflavone bioavailability in women. *J. Nutr.* 130:

- 798-801 (2000)
36. Kano M, Takayanagi T, Harada K, Sawada S, Ishikawa F. Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. *J. Nutr.* 136: 2291-2296 (2006)
37. Chun J, Kim JS, Kim JH. Enrichment of isoflavone aglycones in soymilk by fermentation with single and mixed cultures of *Streptococcus infantarius* 12 and *Weissella* sp. 4. *Food Chem.* 109: 278-284 (2008)
38. Jung S, Murphy P, Sala I. Isoflavone profiles of soymilk as affected by high-pressure treatments of soymilk and soybeans. *Food Chem.* 111: 592-598 (2008)
39. Baú TR, Ida EI. Soymilk processing with higher isoflavone aglycone content. *Food Chem.* 183: 161-168 (2015)
40. Mann SY, Kim EA, Lee GY, Kim DY. Characteristics of *chungkookjang* produced by *Bacillus subtilis* MC31. *Life Sci.* 23: 560-568 (2013)
41. Lee LS, Jung KH, Choi UK, Cho CW, Kim KI, Kim YC. Isolation and identification of lactic acid producing bacteria from *Kimchi* and their fermentation properties of soymilk. *J. Korean Soc. Food Sci. Nutr.* 42: 1872-1877 (2013)