Journal of Nutrition & Food Sciences
ISSN: 2155-9600

Ping et al., J Nutr Food Sci 2012, 2:7
DOI: 10.4172/2155-9600.1000153

Research Article Open Access

Effect of Isoflavone Aglycone Content and Antioxidation Activity in Natto by Various Cultures of Bacillus Subtilis During the Fermentation Period

Shieh Ping Ping[1], Shu Cheng Shih[2], Chen Tong Rong[2] and Wei Que King[1]*

[1]*Department of Food Science and Technology, National Taitung Junior College, No.889, Jhengci North Road, Taitung, 95045 Taiwan*
[2]*Department of Food Science, National Chiayi University, 300 Syuefu Road, Chiayi, 60004, Taiwan, ROC*

Abstract

This study investigated the isoflavone aglycones content and antioxidant activities (total phenol, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging ability) of cooked soybeans fermented by various cultures of *Bacillus subtilis*, including *B. subtilis* N205 (BS205), *B. subtilis* BCRC14718 (BS14718) and a co-culture of *B. subtilis* N205 with *B. subtilis* BCRC 14718 (BSCO) at 42˚C for 48 h. Fermentation by the various *B. subtilis* strains produced with varying contents of isoflavone aglycones, arranged in increasing concentration as follows: BS14781 (172.94 mg/100 g dry wt.) >BSCO (117.20 mg/100 g dry wt.) >BS205 (34.20 mg/100 g dry wt.). However, the total phenolic content, DPPH free radical scavenging activity and ABTS free radical scavenging ability in Natto fermented by BSCO was higher than those produced by BS205 and BS14718 after fermentation for 48 h.

Keywords: Isoflavone aglycones; Antioxidant activity; *Bacillus subtilis;* Natto

Introduction

Isoflavones are diphenolic secondary metabolites of plants, and are frequently found to be rich in soybeans. Isoflavones possess both antioxidant activities and metal ion-chelating properties [1,2]. They also exhibit strong antioxidant potency in liposomes challenged with UV exposure, peroxyl, and hydroxyl free radicals *in vitro* [3].

The main isoflavones found in soybeans consist of aglycones (daidzein, glycitein and genistein) their glycosides (daidzin, glycitin and genistein) [4]. Several researchers have reported that cooked soybeans fermented with *B. subtilis* enhance the antioxidant activities in chungkookjang (in Korea), natto (in Taiwan and Japan), kinema (in India) [5-7] and tempeh (in Indonesian) [8]. Specific of the isoflavone aglycones of genistein and daidzein have been suggested to be responsible for these observed benefits [9].

Most researchers have been interested in the fermentation of soybeans with *B. subtilis* because it increases the hydrolysis of isoflavone glucosides, resulting in higher concentrations of aglycones [10-13]. We have previously reported that *B. subtilis* BCRC 14718 is more effective in converting isoflavone glucosides to aglycones in prepared natto [13]. The fermentation process changes the isoflavone content, and its composition causes hydrolysis by β-glucosidase derived from microorganisms, thereby increasing the concentration of isoflavone aglycones in fermented cooked soybean [14,15].

Traditionally natto is a popular fermented soybean produced by *Bacillus subtilis* that provided a potent nattokinase activity. Nattokinase activity also plays an indirect part in promoting fibrinolysis [16]. In this study, we used the *B. subtilis* N205 (BS205) isolated from commercial natto, which exhibited the high nattokinase activity, and the *B. subtilis* BCRC14718 (BS14718) produced high isoflavone aglycone content in fermentation processing [13]. We try to develop a high antioxidant activity natto production which simultaneously provided the high nattokinase activity and isoflavone aglycone content. Thus, this study was to compare of isoflavone aglycones content and their antioxidant activities after cooked soybeans fermented by a single culture of BS205 or BS14718, or a co-culture of BS205 with BS14718 (BSCO).

Materials and Methods

Materials

The isoflavone standards, daidzein and genistein, were purchased from Sigma Chemical (St. Louis, MO, USA). Glycitein standards were purchased from LC Laboratories (Woburn, MA, USA). Trifluoroacetic acid, LC grade acetonitrile, methanol, and n-hexane were purchased from Merck (Darmstadt, Germany).

Bacteria

The *B. subtilis* N205 strain possesses high nattokinase activity and was isolated from commercial natto products. Commercial natto products were purchased from supermarkets in Taiwan. The nattokinase activities of the strains identified by the API 50 CHB computerized identification system (bioMérieux, Nürtingen, Germany), and analyzed by the APILAB PLUS 3.3.2 software. A pure culture of *B. subtilis* BCRC 14718 was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). The pure culture and isolated culture were stored at 80˚C. Two cultures were activated using two consecutive transfers into nutrient agar (Oxoid CM3) at 37˚C for 24 h, and the activated cultures were again inoculated under the same conditions to serve as the pre-cultures to the inoculum for the preparation of fermented cooked soybean.

Preparation of fermented natto

Soybeans were prepared according to previously described

***Corresponding author:** Wei Que King , Department of Food Science and Technology, National Taitung Junior College, No.889, Jhengci North Road, Taitung, 95045 Taiwan, Tel: 886-89-226389; Fax: 886-89-236941; E-mail: weiqueking@yahoo.com.tw

Received May 10, 2012; **Accepted** July 23, 2012; **Published** July 25, 2012

Citation: Ping SP, Shih SC, Rong CT, King WQ (2012) Effect of Isoflavone Aglycone Content and Antioxidation Activity in Natto by Various Cultures of *Bacillus Subtilis* During the Fermentation Period. J Nutr Food Sci 2:153. doi:10.4172/2155-9600.1000153

procedures [13]. First, 10 g of soybeans was soaked in water for 12 h at room temperature. After decanting the water, the soaked soybeans were placed into a glass jar and autoclaved at 121°C for 45 min. The surface of the sterilized soybeans was sprayed with 1% suspensions inoculum (approximately 10^5 cfu/g) described above by microtips and the mixture was then fermented at 42°C for 48 h. The inocula included BS205, BS14718, or BSCO with the same proportions of *B. subtilis* N205 with *B. subtilis* BCRC 14718 inoculated in the fermented steamed soybeans. The prepared natto samples were stored at 20°C for isoflavone extraction and analysis.

Extraction and analysis of isoflavones

The isoflavone extraction and analysis methods used have been previously described [17]. 1 g of the freeze-dried samples was extracted with 20 ml of 80% methanol in a flask and was stirred for 1 h at 60°C. The extracted solutions were centrifuged at 5000× *g* for 10 min, and the supernatants were dried by evaporation. The dried extracts were dissolved in 5 ml of 50% methanol and treated with 20 ml of n-hexane four times to remove the lipids. Following the evaporation of aqueous methanol, the insoluble residue was dissolved in 10 ml of 80% methanol and filtered through a 0.45 μm filter membrane prior to HPLC analysis.

Reversed phase HPLC analysis was carried out with a Hitachi Model L-6200 (Hitachi Ltd., Tokyo, Japan) equipped with an ODS-AM-303 column (250 mm×4.6 mm i.d.; 5 μm; YMC Inc.) and an ultraviolet spectrophotometer at 254 nm. The mobile phase was composed of 0.1% (v/v) glacial acetic acid in acetonitrile (A) and 0.1% (v/v) glacial acetic acid in water (B). The following steps occurred for the HPLC analysis: 10 μl of the sample was injected onto the column starting at 15% (A) and held for 20 min; the mobile phase was then increased to 24% (A) within 10 min and was held for 10 min; the gradient consisted of an increase to 35% (A) within 4 min and was held for 8 min; finally, it was decreased back to 15% (A) for another 5 min. The flow rate was 1.0 ml/min. Quantitative data for each isoflavone was obtained by comparison to known standards.

Determination of total polyphenol content

The total polyphenol concentrations in the fermented natto were determined as previously described [18]. The methanolic extract (0.5 μl) was mixed with 0.5 μl of Folin-Ciocalteau phenol reagents, and 200 μl of 5% Na_2CO_3 was added and allowed to react for 1 h at room temperature. The absorbance was measured at 750 nm using an automated microplate reader and compared to a gallic acid standard.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay

The DPPH radical scavenging effect was estimated as previously described [19]. A 0.5 mM DPPH solution in methanol was prepared and 200 μl of the DPPH solution was then added to 25 μl of each test sample. After a 90-min incubation period at ambient temperature, the absorbance at 517 nm was measured. The inhibitory percentage of DPPH was calculated according to the following equation: Scavenging effect (%)=[1-absorbance of sample/absorbance of control]×100%.

2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical-scavenging assay

The total antioxidant activity of the seed extracts was measured by the $ABTS^+$ radical cation decolorization assay involving preformed $ABTS^+$ radical cations [20]. ABTS was dissolved in 0.01 M sodium phosphate buffer to a 7 mM concentration. The $ABTS^+$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. The stock solutions of the soybean extracts in methanol were diluted so that a 2 μl aliquot of each dilution was used in the assay. After the addition of 200 μl of the diluted $ABTS^+$ solution to the antioxidant compounds or Trolox standards in methanol, samples were taken at 37°C exactly 7 min after initial mixing. The absorbance of the resulting solution was measured at 734 nm.

The inhibitory percentage of the $ABTS^+$ was calculated according to the following equation:

Scavenging effect (%)=[1- absorbance of sample/absorbance of control]×100%.

Statistical analysis

Each test was performed in triplicate. Data were analyzed using an

Time (h)	Isoflavones (mg 100 g^{-1})					
	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
***B. subtilis* N205**						
0	132.67 ± 0.66^a	51.24 ± 0.79^a	157.00 ± 0.35^a	11.82 ± 0.16^d	1.49 ± 0.19^a	9.26 ± 0.35^d
12	113.61 ± 2.34^b	53.61 ± 1.02^a	116.57 ± 2.04^b	14.96 ± 0.26^c	1.38 ± 0.02^a	11.15 ± 1.58^c
24	99.10 ± 1.32^d	56.18 ± 1.36^a	100.45 ± 1.69^d	17.16 ± 0.09^c	1.07 ± 0.56^a	15.97 ± 0.58^b
36	107.08 ± 0.47^c	56.06 ± 0.52^d	98.21 ± 1.18^d	27.55 ± 0.86^b	1.37 ± 0.19^a	21.59 ± 0.50^a
48	98.82 ±1.06^d	55.66 ± 0.57^b	104.00 ± 0.74^c	37.20 ± 0.97^a	1.48 ± 0.12^b	21.15 ± 0.88^a
***B. subtilis* BCRC14718**						
0	132.67 ± 0.66^a	51.24 ± 0.79^a	157.00 ± 0.35^a	11.82 ± 0.16^c	1.49 ± 0.19^a	9.26 ± 0.35^d
12	56.71 ± 1.30^b	59.58 ± 4.34^a	78.88 ± 2.21^b	103.62 ± 0.01^b	1.53 ± 0.04^a	38.03 ± 1.84^b
24	40.83 ± 1.06^c	30.18 ± 0.08^c	60.76 ± 0.33^c	133.91 ± 2.74^a	1.19 ± 0.06^a	37.85 ± 1.55^b
36	48.49 ± 2.95^c	27.89 ± 0.45^d	43.24 ± 2.68^e	127.96 ± 3.48^a	1.34 ± 2.06^a	45.59 ± 0.83^a
48	42.42 ± 0.77^c	37.17 ± 0.01^b	54.6 ± 1.50^d	106.63 ± 1.41^b	1.92 ± 0.26^a	23.06 ± 2.41^c
Co-culture *B. subtilis N205* with *B. subtilis* BCRC14718						
0	132.67 ± 0.66^a	51.24 ± 0.79^a	157.00 ± 0.35^a	11.82 ± 0.16^e	1.49 ± 0.19^b	9.26 ± 0.35^e
12	113.61 ± 8.86^b	53.61 ± 1.14^a	106.57 ± 4.19^b	33.76 ± 0.09^d	1.44 ± 0.05^b	12.45 ± 2.16^d
24	89.10 ± 5.42^c	46.18 ± 4.39^b	90.45 ± 0.49^c	88.29 ± 2.77^b	1.07 ± 0.14^c	27.84 ± 0.53^b
36	55.57 ± 0.87^d	49.84 ± 0.42^{ab}	59.72 ± 2.18^e	108.26 ± 1.31^a	1.46 ± 0.01^b	36.23 ± 0.25^a
48	58.82 ± 1.06^d	45.66 ± 1.03^b	84.28 ± 1.63^d	77.14 ± 1.78^c	1.82 ± 0.22^a	22.88 ± 1.93^c

Mean± standard deviation; n=3. Means in the same column with different letters are significantly different (P<0.05).

Table 1: Isoflavones content in cooked soybean fermented with various cultures of *Bacillus subtilis* at 42°C for 48 hrs.

ANOVA and the SAS software (version 6.03, SAS Institute Inc., Cary, NC). Duncan's multiple range tests were utilized to analyze differences between treatments (the significance level was P<0.05).

Results

Isoflavone aglycone content in fermented natto

The total isoflavone aglycone content in cooked soybeans fermented by BS14718 was significantly increased (P<0.05) compared to those fermented by BS205 and BSCO (Table 1). The total isoflavone glucoside concentrations in those fermented product were significantly exhibited the opposite results (Table 1). After fermentation for 24 h, the total isoflavone aglycone concentration was significantly increased in cooked soybeans fermented by BS14178 (172.94 mg/100 g), followed by BSCO (117.20 mg/100 g) and BS205 (34.20 mg/100 g) (P<0.05) (Table 1). After fermenting for 48 h, the isoflavone aglycone content was observed to be 131.61 mg/100 g for BS14718, 101.84 mg/100g for BSCO and 59.83 mg/100 g for BS205. The major composition of the isoflavone aglycones daidzein and genistein exhibited a significant increase in content after fermentation for 48 h (Table 1). The concentration of daidzein was significantly increased from 11.82 mg/100 g to 106.63 mg/100 g, 77.14 mg/100 g and 37.20 mg/100 g in cooked soybeans fermented by BS14718, BSCO and BS205, respectively (P<0.05) (Table 1). However, a similar increase in concentration was observed for genistein from 9.26 mg/100 g to 23.06 mg/100 g, 22.88 mg/100 g and 21.15 mg/100 g, respectively (P<0.05), when the cooked soybeans were fermented with BS14718, BSCO and BS205 (Table 1).

Total phenolic content in fermented natto

The total phenolic content in the methanolic extract of cooked soybeans fermented by the three cultures of *B. subtilis* at 42°C for 48 h was significantly higher than non-fermented soybeans (Figure 1). The total phenolic content in the different extractions of non-fermented soybeans showed a significant increase from 13.29 μg/g-80.91 μg/g (for the control). The fermented cooked soybeans also exhibited increased total phenolic content when incubated with various cultures of *B. subtilis* for 24 h (Figure 1). However, the total phenolic content in various extractions of natto fermented by BSCO (64.73 μg/g-1.32 μg/g) and BS205 (65.71 μg/g-30.56 μg/g) was slightly higher than in those fermented by BS14718 (68.03 μg/g-121.06 μg/g) (Figure 1). Furthermore, the total phenolic content in the three types of fermented natto was similar in the range of 82 μg/g-136 μg/g after fermentation for 48 h (Figure 1).

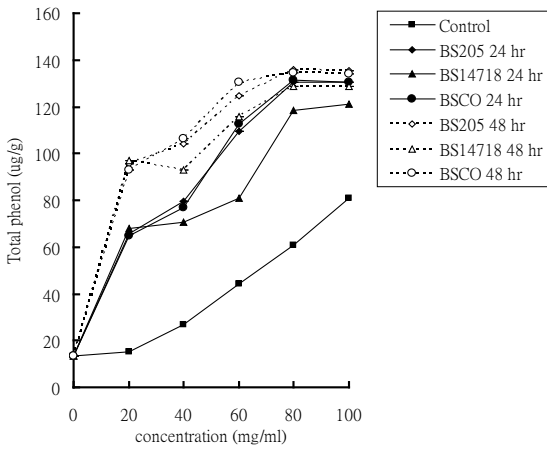

Figure 1: The total phenol content in the methanolic extracts from cooked soybeans fermented by various cultures of *Bacillus subtilis* at 42°C for 48 h.

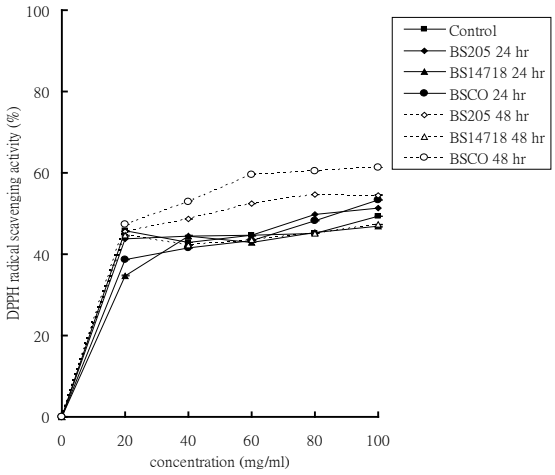

Figure 2: The DPPH free radical-scavenging activity in the methanolic extracted from cooked soybeans fermented by various cultures of *Bacillus subtilis* at 42°C for 48 h.

DPPH free radical activity in fermented natto

The DPPH free radical activity of the extraction of cooked soybeans fermented by the three cultures of *B. subtilis* at 24 h was in the range of 43%-53%, and that for the non-fermented soybeans were in the range of 43%-49% (Figure 2). The inhibition of $DPPH^+$ absorption for the methanolic extracts of fermented soybeans by BSCO (43%-61%) and BS205 (43%-54%) was more effective than that for cooked soybeans fermented by BS14718 (43%-48%) and the non-fermented cooked soybeans (Figure 2). After 48 h of fermentation, antioxidant activity of DPPH showed BSCO (IC_{50}=32.39 mg/ml) higher than BS205 (IC_{50}=51.46 mg/ml). However, the methanolic extract from the cooked soybeans fermented by BS 14718 did not have an obvious change in its scavenging effect compared with the non-fermented soybeans (Figure 2). Therefore, the cooked soybeans fermented by the various strains may affect the DPPH radical scavenging activity differently.

ABTS free radical activity in fermented natto

The ABTS free radical-scavenging effect was significantly increased in the methanolic extracts from the three types of natto after 48 h of fermentation (Figure 3). After the initial fermentation period of 24 h, the ABTS radical scavenging ability in the extraction of cooked soybeans fermented by *B. subtilis* fermented BSCO was higher than non-fermented soybeans (Figure 3). The ABTS radical-scavenging ability in the extraction was significantly increased after 48 h of fermentation (P<0.05). The BS205-Natto (58%-89%) and BSCO-Natto (41%-88%) had greater ABTS free radical-scavenging abilities than the BS14718-Natto (25%-87%) (Figure 3). Following fermentation for 48 h, the ABTS free radical-scavenging abilities increased to 87%-89% for BS205 (IC_{50}=11.44 mg/ml), 72%-89% for BS14718 (IC_{50}=13.80 mg/ml) and 83%-89% for BSCO (IC_{50}=12.08 mg/ml) (Figure 3).

Discussion

In this study, cooked soybeans fermented by a single culture of BS14718 exhibited a greater increase in isoflavone aglycone content, as previously reported [13], compared to those fermented by a single culture of BS 205 or a co-culture of BSCO. This is consistent with the

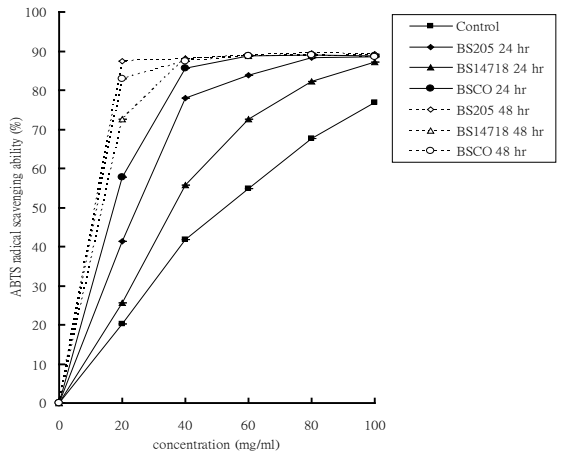

Figure 3: The ABTS^{+} free radical-scavenging activity in the methanolic extracts from cooked soybean fermented by various cultures of *Bacillus subtilis* at 42°C for 48 h.

results reported by Dajanta et al. [21], which stated that the isoflavone aglycone content in fermented soybeans varies with the starter culture. Therefore, a promising use of the *Bacillus* starter culture incubated with soybean is to improve the isoflavone aglycone content in fermented processing [21]. Non-fermented soybeans contain an abundance of glycosides and a smaller amount of aglycones. The glucoside conjugates of the isoflavones were converted to isoflavone aglycones by β-glucosidase during soybean processing [22]. Setlow et al. [23] reported that *B. subtilis* contains β-glucosidase, which catalyzes the hydrolysis of the isoflavones from the glycoside to the aglycone. Kuo et al. [12] reported that the incubation of *B. subtilis* natto NTU-18 in soymilk from black soybean catalyzes the hydrolysis of isoflavone glycosides in 3% to 5% black soymilk.

The increased total phenolic content in fermented soybeans caused by β-glucosidase derived from microorganisms catalyzes the release of aglycones from the bean substrate and thereby increases their phenolic content [24]. The total polyphenol content in fermented natto was higher than that of non-fermented soybeans in this study, and this is consistent with the findings of most investigations [6,7,24]. Similar reports revealed that total phenolic contents in kinema [7] and chungkookjang [6] were higher than in non-fermented cooked soybeans. Moreover, the incubation with a single culture of BS 205 or co-culture of BSCO in cooked soybeans produced higher total phenolic content than did incubation with a single culture of BS14718. The cooked soybeans fermented for 48 h by either BSCO or BS205 also exhibited enhanced DPPH radical-scavenging effect compared with BS 14718. Similar results have been reported in other fermented soybean products, i.e., *Aspergillus*-soybean koji [25] and *Bacillus subtilis*-soybean kinema [7]. Thus, this suggests that the different fermented strain in the soy fermented product could affect the DPPH radical scavenging activity.

Fermented cooked soybean by BSCO exhibited higher ABTS scavenging activity than those produced by either BS205 or BS14718 during fermentation periods in this study. The ABTS scavenging activity in soybean meal fermented by *B.* spp. (70%-99%) and *R oligosporus* (49%) was observed to be higher than that of soybean meal [16]. Moreover, Fan et al. [26] also indicated that the soybeans fermented by *B. subtilis* B1 had strong antioxidant activities against the ABTS radicals in douche. In this study, the three nattos fermented for 48 h displayed greater ABTS scavenging activities than the non-fermented cooked soybeans. Pyo et al. [27] reported a good linear correlation between the isoflavone aglycone concentration and the scavenging activity of ABTS in ethanolic extracts from soybeans fermented by lactic acid bacteria.

According to the previous showed cooked soybeans fermented by BSCO exhibited higher antioxidant ability than those produced by either BS205 or BS14718 during the fermentation period; however, a large increase in isoflavone aglycone content in BS14718-Natto did not produce the most improved antioxidant activities, including total phenolic content, DPPH free radical scavenging ability and ABTS free scavenging ability. The BSCO-Natto showed higher antioxidant activity, specific for ABTS free radical scavenging ability during the fermentation periods. Furthermore, another mechanism that may have higher antioxidant activity caused the increase in peptides or free amino acids by microbial protease activity during soybean fermentation [28]. Fan et al. [26] demonstrated that the douche antioxidant activity was dependent on the increase in peptides with fermentation rather than on the increase in isoflavone aglycones. This interesting result suggests that the effect of antioxidant activity in fermented natto was not only due to the isoflavone aglycone content but also due to the content of another metabolism component, i.e., peptides in soybeans fermented by a different microorganism [29]. Thus, total phenolic content, DPPH free radical scavenging ability and ABTS free scavenging ability of antioxidant activities in methanolic extractions were observed in natto fermented by various *B. subtilis* strains.

Conclusion

The isoflavone aglycone concentration in fermented natto was affected by various *B. subtilis* strains after fermentation processing, which caused different DPPH free radical-scavenging activities and ABTS radical-scavenging abilities. The antioxidant activities in methanolic extracts from cooked soybeans fermented by BS205 and BSCO were significantly higher ($P<0.05$) than those fermented by BS14718, suggesting a role for fermentation in enhancing antioxidant activities at appropriate concentrations of isoflavone aglycone during fermentation periods.

Acknowledgments

This study was financially supported by the National Science Council of the Republic of China in Taiwan under Contract No. NSC 93-2313-B-273-002.

References

1. Kao TH, Chen BH (2006) Functional components in soybean cake and their effects on antioxidant activity. J Agric Food Chem 54: 7544-7555.
2. Wang D, Wang L, Zhu F, Zhu J, Chen XD, et al. (2008) *In vitro* and *in vivo* studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). Food Chem 107: 1421-1428.
3. Record IR, Dreosti IE, Mcinerney JK (1995) The antioxidant activity of genistein *in vitro*. J Nutr Biochem 6: 481-485.
4. Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, et al. (1991) Malonyl isoflavone glucosides in soybean seeds (Glycine max Merrill)(Food & Nutrition). Agric Biol Chem 55: 2227-2233.
5. Juan MY, Chou CC (2010) Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with Bacillus subtilis BCRC 14715. Food Microbiol 27: 586-591.
6. Kwak CS, Lee MS, Park SC (2007) Higher antioxidant properties of Chungkookjang, a fermented soybean paste, may be due to increased aglycone and malonylglycoside isoflavone during fermentation. Nutr Res 27: 719-727.
7. Moktan B, Saha J, Sarkar PK (2008) Antioxidant activities of soybean as affected by *Bacillus*-fermentation to kinema. Food Res Int 41: 586-593.
8. Babu PD, Bhakyaraj R, Vidhyalakshmi RA (2009) Low cost nutritious food "Tempeh"- A review. World Journal of Dairy and Food Sciences 4: 22-27.

9. Markham KR, Ternai B, Stanley R, Geiger H, Mabry TJ (1978) Carbon-13 NMR studies of flavonoids-III: Naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron.

10. Chien HL, Huang HY, Chou CC (2006) Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. Food Microbiol 23: 772-778.

11. Choi YM, Kim YS, Ra KS, Suh HJ (2007) Characteristics of fermentation and bioavailability of isoflavones in Korean soybean paste (doenjang) with application of sp. KH-15. International Journal of Food Science and Technology 42: 1497-1503.

12. Kuo LC, Cheng WY, Wu RY, Huang CJ, Lee KT (2006) Hydrolysis of black soybean isoflavone glycosides by Bacillus subtilis natto. Appl Microbiol Biotechnol 73: 314-320.

13. Wei QK, Chen TR, Chen JT (2008) Use of Bacillus subtilis to enrich isoflavone aglycones in fermented natto. J Sci Food Agric 88: 1007-1011.

14. Kwon DY, Jang JS, Lee JE, Kim YS, Shin DH, et al. (2006) The isoflavonoid aglycone-rich fractions of Chungkookjang, fermented unsalted soybeans, enhance insulin signaling and peroxisome proliferator-activated receptor-gamma activity in vitro. Biofactors 26: 245-258.

15. Nakajima N, Nozaki N, Ishihara K, Ishikawa A, Tsuji H (2005) Analysis of isoflavone content in tempeh, a fermented soybean, and preparation of a new isoflavone-enriched tempeh. J Biosci Bioeng 100: 685-687.

16. Sumi H, Hamada H, Nakanishi K, Hiratani H (1990) Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. Acta Haematol 84: 139-143.

17. Wei QK, Jone WW, Fang TJ (2004) Study on isoflavones isomers contents in Taiwan's soybean and GM soybean. Journal of Food and Drug Analysis 12: 324-331.

18. Waterhouse Al L (2000) Determination of Total Phenolics. In "Curr Protoc Food Anal Chem."

19. Katsube T, Tabata H, Ohta Y, Yamasaki Y, Anuurad E, et al. (2004) Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. J Agric Food Chem 52: 2391-2396.

20. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, et al. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26: 1231-1237.

21. Dajanta K, Chukeatirote E, Apichartsrangkoon A, Frazier RA (2009) Enhanced aglycone production of fermented soybean products by *Bacillus* species. Acta Biologica Szegediensis 53: 93-98.

22. Toda T, Sakamoto A, Takayanagi T, Yokotsuka K (2000) Changes in isoflavone compositions of soybean foods during cooking process. Food Science and Technology Research 6: 314-319.

23. Setlow B, Cabrera-Hernandez A, Cabrera-Martinez RM, Setlow P (2004) Identification of aryl-phospho-beta-D-glucosidases in Bacillus subtilis. Arch Microbiol 181: 60-67.

24. Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, et al. (1991) Malonyl isoflavone glucosides in soybean seeds (Glycine max Merrill). Agriculture Biology Chemistry Journal 55: 2227-2233.

25. Vatterm AD, Shety K (2002) Solid-state production of phenolic antioxidants from cranberry Pomace by Rhizopus Oligosporus. Food Biotechnol 16: 189-210.

26. Lin CH, Wei YT, Chou CC (2006) Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. Food Microbiol 23: 628-633.

27. Fan J, Zhang Y, Chang X, Saito M, Li Z (2009) Changes in the radical scavenging activity of bacterial-type douchi, a traditional fermented soybean product, during the primary fermentation process. Biosci Biotechnol Biochem 73: 2749-2753.

28. Pyo YH, Lee TC, Lee YC (2005) Effect of lactic acid fermentation on enrichment of antioxidant properties and bioactive isoflavones in soybean. J Food Sci 70: S215-S220.

29. Wang W, Mejia EGD (2005) A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. Comp Rev Food Sci Food Saf 4: 63-78.

30. Wu J, Muir AD (2010) Isoflavone during protease hydrolysis of defatted soybean meal. Food Chem 118: 328-332.