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Enhancement of Aglycone, Vitamin K_2 and Superoxide Dismutase Activity of Black Soybean through Fermentation with *Bacillus subtilis* BCRC 14715 at Different Temperatures

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In the present study, the change in the content and activity of some functional constituents including aglycone, the bioactive form of isoflavone, vitamin K_2, and superoxide dismutase (SOD) of black soybeans during their solid fermentation with *Bacillus subtilis* BCRC 14715 at different tempaeratures (35, 40, 45, and 50 °C) for 18 h was investigated. It was generally found that fermentation resulted in an enhancement of these constituents, regardless of fermentation temperature, while varying the fermentation temperature of black soybeans produced variations in the enhancement. The 50 °C -fermented black soybean showed the most marked increase in the content of daidzein and genistein aglycone. On the other hand, the highest SOD activity and vitamin K_2 content were found in the black soybeans fermented at 45 and 40−45 °C, respectively. Thus functional properties of black soybeans can be further improved through fermentation with *B. subtilis* BCRC 14715.

KEYWORDS: Black soybeans; fermentation; isoflavone; superoxide dismutase activity; vitamin K_2

INTRODUCTION

Black soybean [*Glycine max* (L.) Merr.] is a soybean cultivar containing a considerable amount of anthocyanin located primarily in its seed coat (*1*). Anthocyanin has been reported to show free radical scavenging effect (*2*) and exhibit potential physiologic effects as antineoplastic agents (*3*). Similar to soybean, black soybean is a nutritionally rich food with a plentiful supply of protein and calories. Black soybean also contains constituents such as isoflavones, vitamin E, saponins, and carotenoids in addition to anthocyanin, which has have been shown to exert biological functions (*1*, *4*, *5*). In China, it has been widely utilized as a tonic food and of medicinal value. Recently research has further demonstrated that black soybean suppresses low-density lipoprotein oxidation (*6*), reduces the DNA injury caused by cyclophosphamide (*7*) and exerts antioxidative as well as antimutagenic effects (*7*−*9*). Additionally, *Rhizopus oligosporus*-fermented black soybean with rice has been proposed as a nutritious weaning food, (*10*) and *Aspergillus awamori*-fermented black soybean, possessing enhanced antioxidant and antimutagenic activities, has also been suggested as an useful ingredient in the formulation of healthy food (*8*, *9*).

Natto, a popular Japanese traditional fermented product of soybean prepared with *Bacillus subtilis* (natto) as the starter organism, is gaining wide acceptance as a health food. Nattokinase, a clot-dissolving agent that has been used in the treatment of cardiovascular disease, is produced by *B. subtilis* (*11*). In addition to nattokinase, *B. subtilis* has also been reported to produce compounds with various forms of biological function such as aglycone, vitamin K_2, and superoxide dismutase (*12*−*14*). In an attempt to develop a useful health food or health food ingredient possessing enhanced and broadened biological functionality, we have previously found that black soybean possessed increased content of anthocyanin, antioxidant activity and angiotensin coverting enzyme inhibitory activity after fermentation with *B. subtilis* 14715 (*15*). This study was further performed to investigate the change of some other functional constituents including aglycone, the bioactive form of isoflavone, vitamin K_2, and superoxide dismutase (SOD) during the fermentation of black soybean with *B. subtilis* BCRC 14715 at various temperatures.

MATERIALS AND METHODS

Black Soybeans and *B. subtilis* BCRC 14715. In the present study, black soybeans [*Glycine max* (L.) Merrill] Tainan #3, harvested in spring of 2007 at Taiwan county, Taiwan were obtained from the local market (Taiepi, Taiwan) and used as the fermentation substrate. *B. subtilis* BCRC 14715, which served as the starter organism for the fermentation of black soybeans, was obtained from the Bioresources Collection and Research Center (BCRC), Hsinchu, Taiwan. To prepare the inoculum, the test organism was first activated twice by growing in Nutrient broth (NB, Acumedia Manufactures, Inc. Lansing, Michigan, USA) at 40 °C and 120 rpm for 24 h. The activated culture was inoculated into NB and incubated at 40 °C for 16 h. After centrifugation of the culture, cell pellets were suspended in phosphate buffer solution (PBS, pH 7.2) at a viable population of ca. 10^{7-8} CFU/mL. This was then used as the inoculum for the fermentation of black soybeans.

Fermentation of Black Soybeans. Black soybeans were fermented with *B. subtilis* BCRC 14715 according to the procedures described by Wei et al. (*16*). First, the whole black beans were washed and soaked in distilled water at room temperature (21−23 °C) and weighed three times the weight of the beans for ca. 16 h. After decanting the water, the black soybeans were steam-cooked in an autoclave at 121 °C for 110 min. After cooling, the steamed black soybeans (100 g) were inoculated with the test organism

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by evenly spraying 5 mL inoculum of *B. subtilis* BCRC 14715. After a thorough mixing, the inoculated black bean substrate was placed on a round screen with 30-mesh and then incubated for 18 h at 35, 40, 45, or 50 °C and 95% RH for various periods of the time (0−18 h) as specified in the Results and Discussion. After fermentation, the steamed black soybeans were first dried by a freeze-dryer and homogenized to pass 30-mesh screen. The ground dried powder of the samples were then stored at −20 °C until further analyses were performed.

The Measurement of Isoflavones. The HPLC analysis procedures described by Lee and Chou (*17*) were followed to determine isoflavones in the samples. Briefly, the dried powder of the samples was first extracted with 80% methanol. The extract was then combined with fluorescein (Sigma-Aldrich Co., St. Louis, Missouri, U.S.A.) as an internal standard and subjected to HPLC analysis for isoflavones. The HPLC equipment used was a chromatograph (Model 7200, Jasco Co., Tokyo, Japan) equipped with a YMC-Pack ODS-AM-303 column (250 × 4.6 mm, 5 μm, YMC Co., Ltd., Kyoto, Japan), a UV−vis detector (Model UV-970, Jasco), and a SISC Chromatography data processor (SISC Co., Davis, CA, U.S.A.). A linear HPLC gradient was composed of (A) 0.1% glacial acetic acid in H_2O and (B) 0.1% glacial acetic acid in acetonitrile. After injecting the sample onto the column (25 °C), solvent B increased from 15 to 20% in 20 min, then increased to 24% in 30 min and held at 24% for 4 min, then 10 min later further increased to 35% at which time it held at 35% for 8 min, and then finally reduced to 15% after a further 13 min. The solvent flow rate was 1.0 mL/min. The content of the isoflavones, expressed as micrograms per grams of dried sample, was calculated from the standard curves of the area responses for authentic isoflavone standards (LC Laboratories, Woburn, MA, U.S.A.) normalized to the constant amount of fluorescein added to each sample.

Measurement of Vitamin K_2 (Menaquinone-7). Vitamin K_2 content was measured according to the method described by Tsukamoto et al. (*18*) with minor modification. One gram of the dried powder of samples was first mixed with 5.0 mL each of distilled water and isopropanol, shaken for 30 min, then centrifuged (12 000 × g, 10 min). The supernatant was combined with 8.0 mL hexane and the upper phase was collected after shaking for 1 h. It was then properly diluted with hexane and filtered through a PVDF filter system (0.45 μm, Schleicher and Schuell GmbH, Dassel, Germany). The filtrate was injected into HPLC system equipped with a fluorescence detector. The sample was separated on an YMC-pack ODS-AM-303 column (YMC co., Ltd., Kyoto, Japan). Detection was carried out at an excitation wavelength of 280 nm. Acetonitrile (Merck, Darmstadt, Germany) was used as the mobile phase with a flow rate of 1.0 mL/min and the column temperature was controlled at 30 °C .

Determination of SOD Activity. To determine the SOD activity, extraction of samples was first performed by mixing one gram of the dried powder of samples with 10.0 mL of distilled water and shaken for 30 min then centrifuged (12,000 × g, 10 min). The supernatant was then filtered through a PVDF filter system (0.45 um, Schleicher and Sehuell GmbH) and properly diluted.

The SOD-like activity of sample was measured with the SOD Ransod kit (Ransod, Cat. No. SD 125, Randox Laboratories Ltd., Antrim, U.K.). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. The assays were performed using an ELISA reader adjusted to 37 °C inside the chamber. The 200 μL of reaction mixture, which contained 5 μL of sample, 170 μL of mixed substrate, and 25 μL of xanthine oxidase with a read time of 3 min. The superoxide dismutase activity was measured by the degree of inhibition of this reaction at 505 nm. Results are expressed in units of SOD/g dry fermented black soybean. One unit (U) of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Determination of β-Glucosidase Activity and Dry Weight. The procedures described by Lee and Chou (*17*) were followed to measure the activity of β-glucosidase activity and dry weight of the sample. One unit of enzyme was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min under the assay condition.

Enumeration of Viable *B. subtilis* BCRC 14715. Method described by Wei et al. (*12*) was followed to determine the viable population of *B. subtilis* BCRC 14715, samples (10 g) were first homogenized with 90 mL of PBS (pH 7.2). Serial decimal dilutions were then made and 1.0 mL of each dilution was pour-plated onto nutrient agar. Colonies were counted after 16 h of incubation at 40 °C.

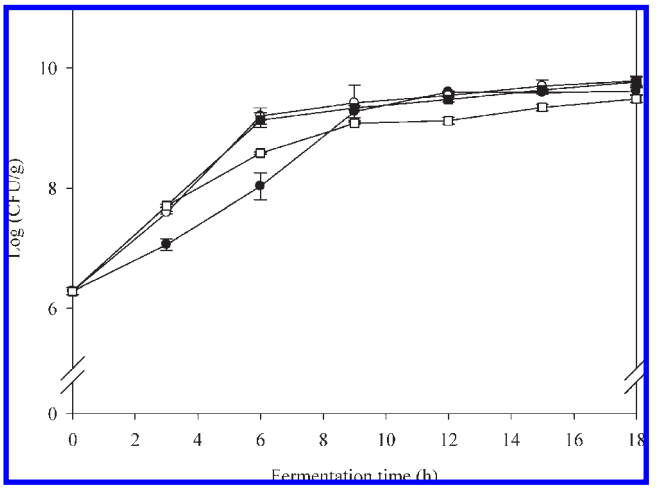

Figure 1. Change of viable population during the fermentation of steamed black soybean with *B. subtilis* BCRC 14715 at various temperatures. ●, 35 °C; ○, 40 °C; ■, 45 °C; □, 50 °C.

Statistical Analysis. The mean value and standard deviation were calculated from the data obtained from the three separate experiments. Means were compared using Duncan's multiple range test method in SAS, version 9.1 (SAS Institute, Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Growth at Different Temperatures. Figure 1 shows the growth of *B. subtilis* BCRC 14715 in black soybeans incubated at 30−50 °C for a period of 18 h. It was found that *B. subtilis* BCRC 14715 grew quickly and entered its stationary phase with a viable population of 9.5−9.8 log CFU/mL after 6−9 h of cultivation, regardless of cultivation temperature. As shown in **Figure 1**, a relatively lower growth rate of test organism was noted in black soybeans incubated at 35 or 50 °C during the first 6 h of cultivation when compared to that observed at 40 or 45 °C. However, at the end of fermentation, the viable population detected in all the black soybeans showed no significant difference ($P > 0.05$).

The Transformation of Isoflavones during Fermentation at Different Temperatures. Generally, isoflavones are present in four chemical forms with daidzein, glycitein, and genestein serving as the three basic chemical structures for aglycones. There are three other forms, namely, β-glucoside, acetyl glucoside, and malonyl glucoside derivatives from each aglycones (*19*). Chemical structural differences of soy isoflavones may result in variable bioavailabilities in the biological system. It is generally suggested that aglycones, the bioactive isoflavones, are more bioavailable than their respective glucosides (*20*). However conflicting opinions have been proposed. For example, Zubik and Meydani (*21*) reported that the apparent bioavailability of genistein is not different when it consumed as either glycoside or aglycone. Setchell et al. (*22*) indicated that the bioavailability of isoflavones is greater for the ingestion of glycosides than for aglycones.

Previously, Lee and Chou (*17*) reported that culture temperature affected the transformation of isoflavone isomers in fermented products. They observed that black soybeans fermented at 30 °C with *Aspergillus awamori* contained a higher content of aglycone than that fermented at 25 or 35 °C.

Table 1 shows the content of total and individual isoflavone isomers of unfermented steamed black soybean and *B. subtilis* BCRC 14715-fermented black soybeans prepared at 25, 30, 45,

Table 1. Isoflavone Contents of Steamed Black Soybean after 18 h of Fermentation with *B. subtilis* BCRC 14715 at Various Temperatures

	content (μg/g)				
isoflavone	unfermented	35 °C	40 °C	45 °C	50 °C
β-Glucoside					
daidzin	768.8 ± 33.4a	673.5 ± 30.1c	715.6 ± 7.8b	732.7 ± 1.2ab	746.1 ± 21.9ab
glycitin	118.6 ± 4.1ab	108.7 ± 2.1b	131.6 ± 5.7a	123.2 ± 1.3a	132.3 ± 14.2a
genistin	983.3 ± 30.4a	834.4 ± 28.3c	891.4 ± 16.1b	924.3 ± 1.9b	908.4 ± 27.3b
Malonylglucoside					
daidzin	1.51 ± 0.0a	1.1 ± 0.2c	1.2 ± 0.1bc	1.2 ± 0.1bc	1.4 ± 0.2ab
glycitin	5.80 ± 0.2a	2.1 ± 0.4b	1.2 ± 0.2c	1.1 ± 0.1c	1.4 ± 0.4c
genistin	2.57 ± 0.3ab	2.4 ± 0.2bc	2.8 ± 0.2ab	2.1 ± 0.3c	3.0 ± 0.2a
Acetylglucoside					
daidzin	92.4 ± 1.0a	81.8 ± 1.7b	74.6 ± 2.1c	67.9 ± 2.8d	63.2 ± 3.4e
glycitin	13.8 ± 0.1ab	15.2 ± 0.8a	8.5 ± 0.8d	10.5 ± 0.7c	12.4 ± 1.3b
genistin	112.3 ± 0.7a	105.8 ± 2.2a	72.8 ± 0.9c	79.4 ± 6.0b	85.5 ± 4.9b
Aglycone					
daidzein	17.5 ± 0.2e	22.0 ± 0.5d	33.7 ± 0.7c	42.1 ± 1.6b	47.1 ± 2.5a
glycitein	14.3 ± 0.1a	11.8 ± 0.2b	11.9 ± 0.4b	11.6 ± 0.1b	11.7 ± 1.7b
genistein	18.3 ± 0.2e	33.5 ± 0.4d	37.5 ± 0.4c	40.9 ± 0.9b	52.0 ± 1.8a
total	2149.1 ± 68.9b	1892.3 ± 35.4a	1982.2 ± 11.5a	2037.0 ± 18.8a	2064.4 ± 72.1a

*1 Values are presented as means ± SD (n = 3). Means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

Table 2. Contents and Distribution Profile of Isoflavone in Steamed Black Soybean after 18 h of Fermentation with *B. subtilis* BCRC 14715 at Various Temperatures

	Content of isoflavone							
	β-glucoside		malonylglucoside		acetylglucoside		aglycone	
fermentation temperature (°C)	content (μg/g)	distribution[a] (%)	content (μg/g)	distribution (%)	content (μg/g)	distribution (%)	content (μg/g)	distribution (%)
control	1870.7 ± 67.7a[b]	87.0 ± 3.2a	9.9 ± 0.5a	0.5 ± 0.0a	218.5 ± 1.7a	10.2 ± 0.1a	50.1 ± 0.4e	2.3 ± 0.0e
35 °C	1616.6 ± 39.7c	85.4 ± 2.1a	5.6 ± 0.8b	0.3 ± 0.0b	202.8 ± 4.5b	10.7 ± 0.2a	67.4 ± 0.8d	3.6 ± 0.0d
40 °C	1738.5 ± 13.1b	87.7 ± 0.7a	4.7 ± 0.3c	0.2 ± 0.0b	155.9 ± 1.8c	7.9 ± 0.1b	83.1 ± 0.9c	4.2 ± 0.0c
45 °C	1780.2 ± 11.9b	87.4 ± 0.6a	4.3 ± 0.2c	0.2 ± 0.0c	157.8 ± 9.5c	7.8 ± 0.5b	94.7 ± 2.6b	4.7 ± 0.1b
50 °C	1786.8 ± 58.8b	86.6 ± 2.9a	5.8 ± 0.7b	0.3 ± 0.0b	161.1 ± 9.4c	7.8 ± 0.5b	110.75 ± 5.8a	5.4 ± 0.3a

[a] Distribution (%) was obtained by dividing the content of isoflavone with the sum of total isoflavone content of the sample. [b] Values are presented as means ± SD (n = 3). Means in the same column with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

and 50 °C for 18 h. Regardless of fermentation temperature, the prepared fermented black soybeans, like fermented soybeans (*19*), contained a lower total isoflavone content of 1892.3−2064.4 μg/g of dry fermented black soybean than that (2149.1 μg/g of dry black soybean) of the unfermented steamed black soybeans. This may be attributed to the hydrolytic cleavage of glucose moiety from isomers, which contribute to the mass of glucoside isoflavone (*19*). Additionally, hydroxylation of daidzein and genistein with the formation of 8-hydroxydaidzein and 8-hydroxygenistein, respectively, (*23*) and the transformation of β-glucoside to other isoflavone isomer such as succinylglucose isoflavone (*24*, *25*) may occur during fermentation. These isoflavone derivatives were not examined in the present study and this may have led to the lower total isoflavone content observed in the fermented black soybean.

In comparing the distribution patterns of various isoflavone isomers in the unfermented black soybeans, it was generally noted that fermentation with *B. subtilis* BCRC 14715, regardless of fermentation temperature, caused a major increase in the content of daidzein and genistein along with a reduced content of daidzin, genistin, malonyldaidzin, malonylglycitin, and all the acetylglucoside isoflavone isomers. The extent of increased aglycone content and the reduction in the content of the glucoside isoflavone isomers varied with fermentation temperature. Malonyl glucoside might convert to acetyl glucoside and β-glucosides isoflavone through decarbosylation and hydrolysis, respectively (*20*). Additionally, degradation and transformation of isoflavones to other derivatives may also occur (*23*−*26*). These reactions may vary depending on the fermentation temperatures examined and this variation may thus contribute to the change as well as difference in the content of isoflavone observed.

Table 2 summarizes the total content and the distribution profile of β-glucoside, acetylglycoside, malonylglucoside, and aglycone isoflavone in the steamed black soybeans before and after fermentation at different temperatures. Compared with the respective isoflavone content in the unfermented black soybeans (control), the fermented black soybean, regardless of fermentation temperature, shows a reduced content of β-glucoside, acetylglucoside, and malonylglucoside isoflavone but an increased aglycone content. For example, the unfermented steamed black soybeans contains 1870.7, 9.9, and 218.5 μg/g of dry black soybean, respectively, of β-glucoside, acetylglucoside, and malonylglucoside compared to a lower level of 1786.8, 5.8, and 161.1 μg/g of dry fermented black soybean noted in the 50 °C-fermented black soybean. On the other hand, the aglycone content increased from 50.1 μg/g of dry black soybean noted in the unfermented black soybean to 110.7 μg/g of dry fermented black soybeans after fermentation at 50 °C.

Despite the reduced content of β-glucoside observed, its distribution in fermented black soybeans showed no significant ($P > 0.05$) difference from that of the control. Moreover, all

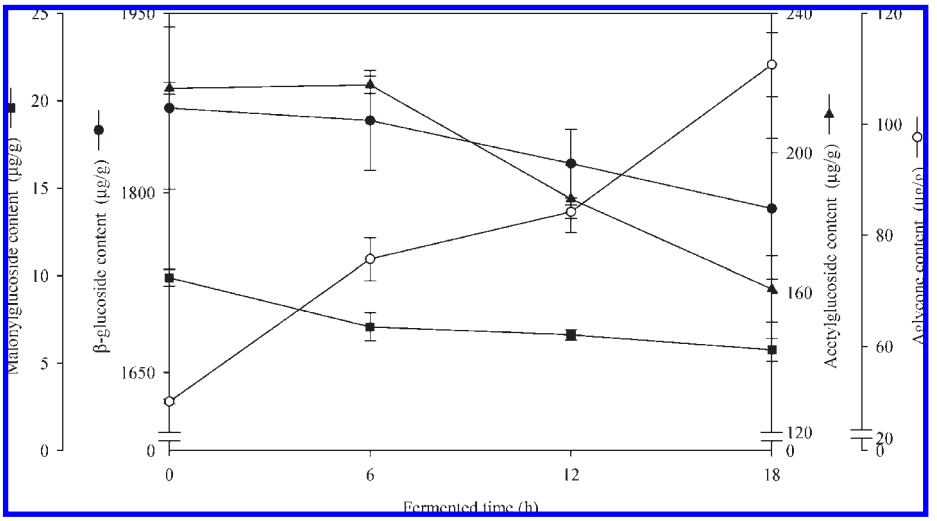

Figure 2. Change of β-glucoside, malonylglucoside, acetylglucoside isoflavone, and aglycone during the fermentation of steamed black soybean with *B. subtilis* BCRC 14715 at 50 °C. ●, β-glucoside; ■, malonylglucoside; ▲, acetylglucoside; ○, aglycone.

the fermented black soybeans exhibit a significant ($P < 0.05$) reduction in the distribution of acetylglucoside and malonylglucoside isoflavone and an increased distribution of aglycone isoflavone. Most importantly, a regular trend in the increased aglycone content and distribution with increasing fermentation temperature was noted in the fermented black soybean. The extent of the increase in aglycone content and distribution was the highest with black soybeans fermented at 50 °C followed by that fermented at 45, 40, and 35 °C in descending order. The 50 °C-fermented black soybean showed a 60.6 μg/g black soybeans increase in aglycone content with a distribution changed from 2.3 to 5.4%. **Figure 2** shows the changes of total β-glucoside, acetylglucoside, malonylglucoside, and aglycone content in black soybeans during the fermentation at 50 °C. It was generally found that contents of β-glucoside, acetyl glucoside, and malonyl glucoside reduced as the fermentation proceeded. On the other hand, content of aglycone increased from the start of fermentation and through out the entire fermentation period examined.

Changes of β-glucosidase activity during fermentation. Consistent with report of Ibe et al. (*25*), the activity of β-glucosidase was also detected in the black soybean substrate during fermentation at various temperatures by *B. subtilis* BCRC 14715. As shown in **Figure 3**, β-glucosidase activity in the fermented black soybeans exhibited the highest level after 6 h of fermentation with 50 °C fermented black soybean exhibiting the highest activity level. However, activity reduced as fermentation further proceeded. At the end of fermentation, no significant ($P > 0.05$) difference in the activity of β-glucosidase was detected in all the fermented black soybeans examined. β-glucosidase has been reported to catalyze the hydrolysis of glycoside isoflavones to aglycone and other isoflavones (*20*). Thus the catalytic action of β-glucosidase along with other reactions such as decarbolylation and de-esterfication play an important role in the observed conversion of isoflavone isomers (**Tables 1**, **2**, and **Figure 2**).

Changes of vitamin K_2 content during fermentation. Vitamin K_2 is an essential cofactor for the posttranslational conversion of glutamic acid residues of specific proteins in the blood and bone into r-carbosyglutamic acid (*27*). Thus, vitamin K_2 plays an important role in the regulation of bone metabolism. It has been suggested that supplementation with vitamin K_2 may be an important therapeutic tool for osteoporosis (*28*). Previously, Sato et al. (*13*) observed that the production of vitamin K_2 by *B. subtilis* MH-1 was lower in agitated cultures than static cultures. In addition, different optimal temperatures for vitamin K_2 production

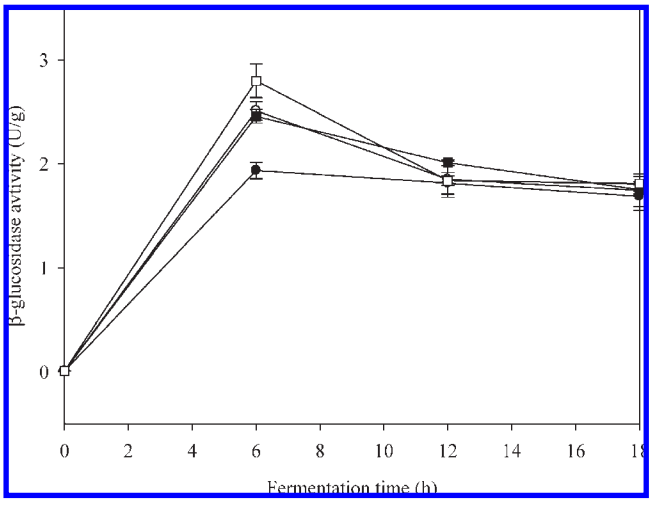

Figure 3. Changes of β-glucosidase activity during the fermentation of steamed black soybean with *B. subtilis* BCRC 14715 at various temperatures. ●, 35 °C; ○, 40 °C; ■, 45 °C; □, 50 °C.

were reported depending on culture conditions with 45 °C optimal for agitated cultures and 37 °C optimal for static cultures. As shown in **Figure 4**, we also found the amount of vitamin K_2 present in the black soybeans substrate during solid fermentation with *B. subtilis* BCRC 14715 varied with culture temperature and fermentation period. It was found that the content of Vitamin K_2 increased from the start of fermentation. Further, the production of vitamin K_2 by *B. subtilis* BCRC 14715 was higher at 45 °C than at other cultivation temperatures. The vitamin K_2 content increased from 1.79 μg/g of dry black soybean at the beginning to 8.25 μg/g of fermented black soybean, which accounts for a ca. 4.6 folds increase, after 18-h of fermentation at 45 °C. Fermentation at other temperatures also resulted in an increase in the vitamin K_2 content of black soybeans, however this increase was less than that observed in the 45 °C-fermented black soybeans.

Changes in SOD activity during fermentation. SOD is a metalloenzyme that possesses the ability to detoxify superoxide radicals by conversion to hydrogen peroxide and oxygen. SOD has also shown a therapeutic application in the treatment of inflammatory disease, the prevention of tumor promotion (*29*), and in the protection of tissue following both infective and ischemic complications and traumatic or burn injuries (*30*, *31*). Enhanced SOD

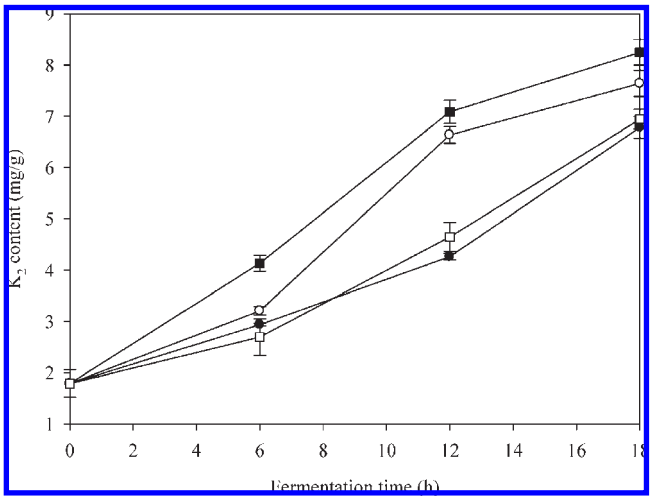

Figure 4. Changes of vitamin K_2 contents during the fermentation of steamed black soybean with *B. subtilis* BCRC 14715 at various temperatures. ●, 35 °C; ○, 40 °C; ■, 45 °C; □, 50 °C.

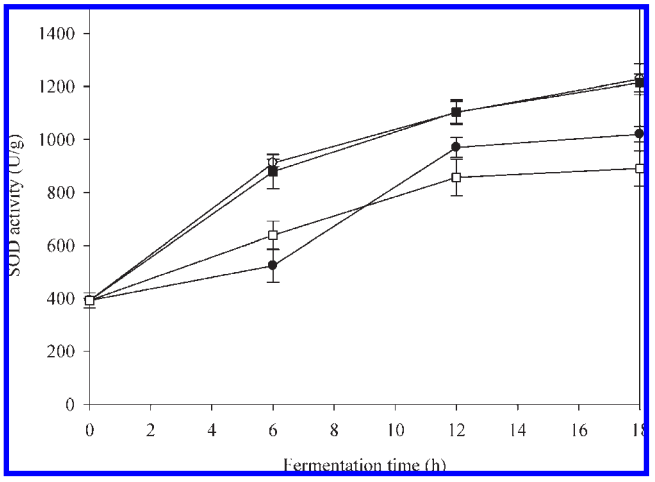

Figure 5. Changes of superoxide dismutase activity during the fermentation of steamed black soybean with *B. subtilis* BCRC 14715 at various temperatures. ●, 35 °C; ○, 40 °C; ■, 45 °C; □, 50 °C.

activity has been detected in natto prepared with a fermentation of soybeans by *B. subtilis* (*15*). A similar observation was made in the present study with an increase of SOD activity detected in black soybeans during fermentation with *B. subtilis* BCRC 14715 as shown in **Figure 5**. Cultivation temperature was also found to affect the SOD activity detected in the fermented black soybeans. Among the various culture temperatures examined, fermentation at 40−45 °C resulted in the highest SOD activity of 1213.5−1227.0 U/g of dry fermented black soybean. In contrast, the lowest SOD activity of 890.9 U/g of dry fermented black soybean was noted in the black soybeans fermented at 50 °C. This temperature-based variation in SOD production has also been reported by Chuang et al. (*32*). They found that the production of SOD in the submerged culture of *B. subtilis* B-12 was highest at 37 °C. Raising the cultivation temperature to 40 or 50 °C resulted in a reduced production of SOD. This discrepancy in findings may be attributed to differences in test organisms and the culture conditions utilized in each study.

In addition to enhancing the antioxidant activity of black soybeans, as observed by Juan (*15*), results obtained from the present study further demonstrated that fermentation with *B. subtilis* BCRC 14715 may enhance their level of aglycone, the bioactive form of isoflavone, their SOD activity, and their vitamin K_2 content. These findings provide a means to further improve the functional properties of black soybeans. Thus a healthy food with multifunctional properties can be developed through the fermentation of black soybeans with *B. subtilis* BCRC 14715. Furthermore, this study also showed that fermentation temperature and length of fermentation affected the enhancement of the functional constituents examined in the *B. subtilis*-fermented black soybean. This information should be taken into account when functional food or functional food ingredients are prepared through the fermentation of black soybean with *B. subtilis* BCRC 14715.

LITERATURE CITED

(1) Choung, M. G.; Baek, I. Y.; Kang, S. T.; Han, W. Y.; Shin, D. C.; Moon, H. P.; Kang, K. H. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *J. Agric. Food Chem.* **2001**, 49, 5848–5851.

(2) Kathkonen, M. P.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycones. *J. Agric. Food Chem.* **2003**, 51, 628–633.

(3) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, 13, 590–594.

(4) Rao, A. V.; Sung, M. K. Saponins as anticarcinogens. *J. Nutr.* **1995**, 125, S717–S724.

(5) Miyazawa, M.; Sakano, K.; Nakamura, S.; Kosaka, H. Antimutagenic activity of isoflavones from soybean seeds (*Glycin max* Merrill). *J. Agric. Food Chem.* **1999**, 47, 1346–1349.

(6) Takahashi, R.; Ohmori, R.; Kiyose, C.; Momiyama, Y.; Ohsuzu, F.; Kondo, K. Antioxidant activities of black and yellow soybeans against low density lipoprotein oxidation. *J. Agric. Food Chem.* **2005**, 53, 4578–4582.

(7) Ribeiro, L. R.; Saloadori, D. M. F. Dietary components may prevent mutation-related diseases in humans. *Mutat. Res.* **2003**, 544, 195–201.

(8) Hung, Y. H.; Huang, H. Y.; Chou, C. C. Mutagenic and antimutagenic effects of methanol extracts of unfermented and fermented black soybeans. *Int. J. Food Microbiol.* **2007**, 118, 62–68.

(9) Lee, I. H.; Hung, Y. H.; Chou, C. C. Total phenolic and anthocyanin contents as well as antioxidant activity of black bean koji fermented by *Aspergillus awamori* under different culture conditions. *Food Chem.* **2007**, 104, 936–942.

(10) Rodriguez-Bürger, A. P.; Mason, A.; Nielsen, S. S. Use of fermented black beans combined with rice to develop a nutritious weaning food. *J. Agric. Food Chem.* **1998**, 46, 4806–4813.

(11) Sumi, H.; Hamada, H.; Nakanish, K.; Hiratani, H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* **1990**, 84, 130–143.

(12) Wei, Q. K.; Chen, T. R.; Chen, J. T. Use of *Bacillus subtilis* to enrich isoflavone aglycones in fermented natto. *J. Sci. Food Agric.* **2008**, 88, 1007–1011.

(13) Sato, T.; Yamada, Y.; Ohtani, Y.; Mitsui, N.; Murasawa, H.; Araki, S. Production of menaquinone (Vitamin K_2)-7 by *Bacillus subtilis*. *J. Biosci. Bioeng.* **2001**, 91, 16–20.

(14) Sumi, H.; Yatagai, C.; Sumi, A. Superoxide radical scavenging enzymes detected in the fermented soybean Natto. *J. Brew. Soc. Jap.* **1999**, 94, 1016–1018.

(15) Juan, M. Y. The antioxidant activities and the inhibitory effect on angiotensin I converting enzyme exerted by the extract of Bacillus subtilis BCRC 14715-fermented black soybean. MS Thesis. National Taiwan University, Taipei, Taiwan, **2008**.

(16) Wei, Q.; Hall-wolf, C.; Chang, K. C. Natto characteristics as affected by steaming time, *Bacillus* strain, and fermentation time. *J. Food Sci.* **2001**, 66, 167–173.

(17) Lee, I. H.; Chou, C. C. Distribution profiles of isoflavone isomers in black bean kojis prepared with various filamentous fungi. *J. Agric. Food Chem.* **2006**, 54, 1309–1314.

(18) Tsukamoto, Y.; Kasai, M.; Kakuda, H. Construction of *Bacillus subtilis* (natto) with high productivity of Vitamin K_2 (menaquinone-7)

by analog resistance. *Biosci. Biotechnol. Biochem.* **2001**, 65, 2007–2015.
(19) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, 42, 1666–1673.
(20) Ismail, B.; Hayes, K. beta-Glycosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, 53, 4918–4924.
(21) Zubik, L.; Meydani, M. Bioavailability of soybean isoflavones from aglycone and glucoside forms in American women. *Am. J. Clin. Nutr.* **2003**, 77, 1459–1465.
(22) Setchell, K. D. R.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, 131, 1362S–1375S.
(23) Esaki, H.; Watanabe, R.; Onozaki, H.; Kawakishi, S.; Osawa, T. Formation mechanism for potent antioxidative o-dihydroxyisoflavones in soybeans fermented with *Aspergillus saitoi*. *Biosci. Biotechnol. Biochem.* **1999**, 63, 851–858.
(24) Toda, T.; Uesugi, T.; Hirai, K.; Nukaya, H.; Tsuji, K.; Ishida, H. New 6-*O*-acyl isoflavone glycosides from soybeans fermented with *Bacillus subtilis* (*natto*). I. 6-*O*-succinylated isoflavone glycosides and their preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet. *Biol. Pharm. Bull.* **1999**, 11, 1193–1201.
(25) Ibe, S.; Kumada, K.; Yoshiba, M.; Onga, T. Production of natto which contains a high level of isoflavone aglycones. *Nippon Shokuhin Kagaku Kogaku Kaishi* **2001**, 48, 27–34.
(26) Esaki, H.; Onozaki, H.; Morimitsu, Y.; Kawakishi, S.; Osawa, T. Potent antioxidative isoflavones isolated from soybeans fermented with *Aspergillus saitoi*. *Biosci. Biotechnol. Biochem.* **1998**, 62, 740–746.
(27) Shearer, M. J. Vitamin K and vitamin K-dependent proteins. *Br. J. Hamaetol.* **1990**, 75, 156–162.
(28) Yamaguchi, M.; Kakuda, H.; Gao, Y. H.; Tsukamoto, Y. Prolonged intake of fermented soybean (natto) diets containing vitamin K_2 (menaquinone-7) prevents bone loss in ovariectomized rats. *J. Bone Miner. Metab.* **2000**, 18, 71–76.
(29) Nishikawa, M.; Nagatomi, H.; Nishijima, M.; Ohira, G.; Chang, B. J.; Sato, E.; Inoue, M. Targeting superoxide dismutase to renal proximal tubule cells inhibits nephrotoxicity of cisplatin and increases the survival of cancer-bearing mice. *Cancer Lett.* **2001**, 171, 133–138.
(30) Yunoki, M.; Kawauchi, M.; Ukita, N.; Sugiura, T.; Ohmoto, T. Effects of lecithinized superoxide dismutase on neuronal cell loss in CA3 hippocampus after traumatic brain injury in rats. *Surg. Neurol.* **2003**, 59, 156–601.
(31) Emerit, J.; Samuel, D.; Pavio, N. Cu-Zn super oxide dismutase as a potential antifibrotic drug for hepatitis C related fibrosis. *Biomed. Pharmacother.* **2006**, 60, 1–4.
(32) Chuang, C. Y.; Lin, C. S.; Sieh, P. C. Study of submerge fermentation of *Bacillus subtilis* B-12 for optimal production of superoxide dismutase. *Taiwanese J. Agric. Chem. Food Sci.* **2006**, 44, 345–350.

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