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Bacillus subtilis natto fermentation to improve aglycone isoflavones content of black soybean varieties detam 2

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Abstract

The utilization of *Bacillus subtilis natto (B.natto)* in fermentation was expected to increase the content of aglycone isoflavones which is beneficial for health. This study aimed to measure the isoflavone aglycone daidzein and genistein content in fermented black soybean varieties detam 2 using *B. natto* strain IFO 3335. HPLC analysis on aglycone showed that 100 g dry weight of defatted raw soybean sample contained 1.29 mg and 1.16 mg of genistein and daidzein respectively, while undefatted raw soybean sample contained 1.19 mg and 1.07 mg. After fermentation, both genistein and daidzein concentration increased up to 8 times as much as those in the raw soybean. After fermentation, genistein concentration was measured 10.43 mg (defatted) and 9.43 mg (undefatted) one while daidzein concentration was 9.60 mg (deffated) and 8.68 mg (undefatted) one. Thus, fermentation using *Bacillus subtilis natto* was proven to improve the content of genistein and daidzein in black soybean varieties detam 2.

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Introduction

Soybean and its products have been gaining the world interest in recent years, especially for its benefits for human health. Black soybean (*Glycine max* L.merr) has been consumed since hundreds years ago and has been processed into variety of food products. Soy contains many nutrients and various functional components such as isoflavones which are very useful to protect the body from metabolic diseases, such as obesity and type 2 diabetes (Nanri *et al*., 2010). Similar to soybean, black soybean and its isoflavones have been reported to reduce the DNA injury that cause cyclophosphamide (Ribeiro *et al*., 2003), suppress lipoprotein oxidation (Takahashi *et al*., 2005), and reduce risk cardiovascular disease (Rimbach *et al*., 2008)

Soybean contains 12 isoflavones, three of them are aglycones and the others are glucosides (glycosides, malonyl glucosides, acetyl glycosides). Among those twelve isoflavones, glycoside is the most form found in soybean (Izumi *et al*., 2000). However, glycosides are poorly ingested by human (low bioavailability) while aglycone is a type with a higher bioavailability (Shao *et al*., 2009; Ferreira *et al*., 2011). That's why increasing the amount of aglycone becomes the aim of many soybean modifications and processing.

Several modifications have been reported to be able to improve the amount of aglycone in soybean, one of them is fermentation. During fermentation, isoflavone glycoside was hydrolyzed by β-glucosidase enzyme produced by microorganisms (Haron *et al*., 2009). *Bacillus subtilis natto* is a gram-positive bacteria commonly used in the manufacture of natto, a Japanese fermented food product made from soybean. Through fermentation process, low bioavailability glycosides can be hydrolyzed into aglycone.

Fermented soybean products such as chuungkookjang, kochujang, and meju (Korean traditional food) are reported to have better anti diabetic effects than the unfermented ones in animals and human with diabetes (Taniguchi *et al*., 2008; Kwon *et al*., 2009, 2011). Fermented soybean is better for diabetes because it contains more aglycone form than the non-fermented soybean does. Aglycone is a very important isoflavone, because it is more efficiently absorbed by the body compared to isoflavone glycoside forms found in non-fermented soy (Ferreira *et al*., 2011).

The problem of this research was whether the *Bacillus subtilis natto* fermentation could improve

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the isoflavone aglycone content of black soybean varieties detam 2. This research aimed to measure the aglycone isoflavone content, particularly daidzein and genistein in fermented black soybean varieties detam 2 by *B. natto* strain IFO 3335. This study was expected to provide quantitative data of aglycone isoflavones content produced from fermentation of black soybean using *B. natto.* Therefore it would be possibly used as the basis of further research, like anti-diabetic effect of fermented black soybean varieties detam 2.

Materials and Methods

Preparation of the inoculum

The preparation of inoculum was carried out using the method reported by Wei *et al*. (2001) with slight modification. *B. natto* culture was obtained from PAU Microbiology Laboratory, University of Gajah Mada, Indonesia, which had been stored previously in 30% glycerol stock was used as a source of inoculum. A total of 100 µL of glycerol stock was grown in 10 mL Nutrient Broth (NB) sterile (80 mg NB powder in 10 mL of distilled water) and incubated at 40°C for 24 hours (200 rpm). A total of 2% of the NB medium that had been overgrown with *B. natto* (seen from media opacities) was transferred into a 150 mL new NB sterile and incubated at 40°C, 200 rpm for 16 hours. Culture with OD_{660} value was measured with spectrophotometer. After a 16 hour incubation, OD_{660} value was 1.5 with a bacterial population ranged from 10^7 to 10^8 CFU/mL. Bacterial culture was ready to use for the preparation of inoculums.

Preparation B. natto *for fermentation process*

Inoculum was made also based on Wei *et al.* (2001) method. A total of 7.5 gram of black soybean (Seed Resources Management Unit (UPBS), Research Institute for Legumes and Tuber Crops (Balitkabi), Malang, East Java, Indonesia) was added into 150 mL inoculum of *B. natto* that has been ready to harvest, then was left for ± 30 minutes. The culture was centrifuged at 12.000 rpm for 25 min at 4°C. The pellet obtained was diluted with 15 mL sterile butterfield phosphate buffer. *B. natto* inoculum in buffer was ready to be used for fermentation.

Black soybean fermentation using B. natto

The black soybean fermentation using *B. natto* was carried out based on the method of Wei *et al.* (2001) with slight modification. The modification was applied during the incubation stage in wich it was carried out in the waterbath instead of a fermentor, using 600 mL beaker glass. In addition, there was an extra step for maturation process of fermentation product by storing them in the refrigerator for 8 hours. First, soybean was washed and soaked in distilled water (the distilled water was 3 times as much as the soybean weight) at room temperature (21-23°C) for 16 hours until the weight ratio reached 2.1-2.3. Soybean was drained and steamed in an autoclave at a temperature of 121°C for 40 minutes, then cooled down until the temperature reached 50°C, 60 g of soybean with temperature of 50°C was put into a 600 mL beaker glass, immediately inoculated with 5 mL of inoculum *B. natto*, and mixed well. The surface was sealed and covered with plastic film. The beaker glass was then covered with sterile aluminum foil, with some holes on it for air circulation. Samples were incubated in the waterbath for 24 hours at 42°C. After fermentation, samples were stored in a refrigerator at a temperature of 3-10°C for 8 hours. The sample was dried at 42°C for 24 hours until completely dried.

Sample preparation and extraction of isoflavones

Soybean isoflavones were extracted based on the method of Shao *et al.* (2009). Dried soybean was crushed using a blender for 3-4 minutes. A total of 5 g of soy powder was wrapped in a filter paper and defatted in a soxhlet containing 75 mL of n-hexane for 3-4 hours until all the fat gone. The powder was placed in fume hood overnight to remove the residual solvent. A total of 1 g of defatted soybean powder was extracted with 10 mL of 80% methanol for 2 hours at room temperature. Soybean extract was then centrifuged at a speed of 5000 rpm for 10 min at 4°C. Supernatant was filtered through Whatmann filter paper no. 40 to obtain a clear yellow filtrate. Similar treatment was carried out on non-fermented soy and crude soybean.

Isoflavones analysis using HPLC

Isoflavone aglycone content was determined based on Shao *et al.* (2009) procedure. HPLC Shimadzu LC SOLUTION 1.2 type-2 Column ODS (150 x 4.6 mm, 5 µL) was used for analysis of soy isoflavone content. Binary mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid (solvent B), while the elution gradients were as follow: 0-40 min, 100-50% B; 40-42 minutes, 50-20% B; 42-45 min, 20-100% B. Volume injection was 10 µL with absorbance wavelength of 260 nm. The isoflavone content of crude soybean, non-fermented, and the fermentation one were measured based on external standards with concentrations of daidzein and genistein (Sigma-Aldrich, Castle Hill, NSW, Australia) were 2.5, 5, 10, 20, 50, and 100 µg/mL. Each standard was injected twice (in duplicate)

and the six concentrations obtained were used to determine the concentration of daidzein and genistein in samples.

Results

Soybean sample chromatograms

The chromatograms of aglycone content of the two samples were determined using HPLC were presented in Figure 1 and 2. All chromatograms showed clear and sharp peaks, separated from each other.

The aglycone content of soybean

The analysis on chromatogram of the three samples were done by linear regression which was formed by the external standard daidzein and genistein curves. Each standard, daidzein and genistein, was injected in duplicate at several concentrations: 2.5, 5, 10, 20, 50, and 100 µg/mL. Based on linear regression analysis, linear regression equation for daidzein was $y = 102167x - 6731$ with $R^2 = 0.999$ and $p<0.05$. While the linear regression equation for genistein was $y = 150817x + 32198$ with $R^2 = 0.999$ and $p<0.05$. Based on those two regression equations, daidzein and genistein concentrations of samples were as shown in Table 1.

Quantitative analysis of aglycone

Comparison of daidzein and genistein concentrations in all three samples in the form of mg/100 g dry weight sample was shown in Figure 3, each for defatted and undefatted one. The concentrations of daidzein and genistein of the defatted samples were obtained from chromatogram analysis, while those of the undefatted samples were obtained from calculation.

Table 1. The analysis result of HPLC chromatograms of raw, non-fermented, and fermented soybean

Sample	Isoflavone	concentration (µg/mL)
Raw Soybean	Daidzein	3.60
	Genistein	3.99
Non-fermented Soybean	Daidzein	3.28
	Genistein	3.22
Fermented Soybean	Daidzein	26.49
	Genistein	28.79

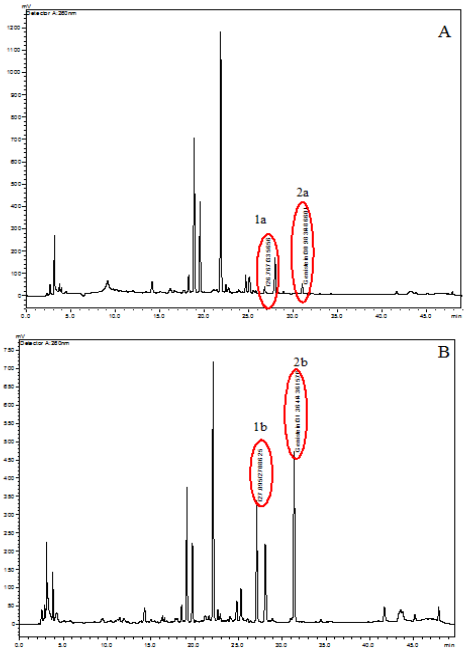

Figure 1. Chromatogram of isoflavones daidzein (1a) and genistein (2a) of non-fermented soybean samples (A); isoflavones daidzein (1b) and genistein (2b) of fermented soybean samples (B)

Discussion

Fermented black soybean detam 2 with B. natto

In this study, black soybean detam 2 that was fermented by *B. natto* strain IFO 3335 into natto (traditional Japanese food) showed main features of natto; soft texture, natto flavor, and covered by white sticky mucus. These were key features that appear when the fermentation was successful. White mucus produced by *B. natto* is generally a polyglutamate acid or γ-PGA and polysaccharides. γ-PGA is a non-toxic environmentally friendly biopolymer and beneficial for health, cosmetics, agriculture, and industry. The unique characteristic of the strain IFO 3335 is that it will only produce γ-PGA in large quantity without any by-products such as polysaccharides (Goto and Kunioka, 1992).

B. natto strain IFO 3335, which was used to ferment the black soybean, was first isolated by M. Yamazaki in 1954. Not only effective to produce γ-PGA, IFO 3335 also produce β-glucosidase like other type of *B. natto.* Meanwhile the black soybean used in this study was a local soybean which consist 45.58% protein in dry weight sample, higher than other soybean varieties that only contain 35% protein (Balitkabi, 2012).

Fermentation of soybean into natto, according to Wei *et al.* (2001), was affected by boiling time, the strain of bacterium, and the soybean. Natto fermentation which had been successfully done by

Table 2. Daidzein and genistein concentration of 100 g sample of soybean and its derived products

Sample	Isoflavone concentration (mg)		Microorganism	References
	Daidzein	Genistein		
Whole soybean flour (Brazil)	-	1.23[a]	-	da Silva *et al.*, 2011
Autoclaved whole soybean flour	-	1.89[a]	-	da Silva *et al.*, 2011
Fermented autoclaved whole soybean flour-24 h	7.45[b]	14.30[b]	*A oryzae*	da Silva *et al.*, 2011
Grade A Soymilk powder (GASP)	3.13[b]	0.84[b]	-	Tyug *et al.*, 2010
Grade B Soymilk powder (GBSP)	2.7-2.9[b]	0.4-0.6[b]	-	Tyug *et al.*, 2010
Soybean husk powder (SHP)	1.17[b]	-	-	Tyug *et al.*, 2010
Acid hydrolyzed Soybean husk powder	19.02[b]	-	-	Tyug *et al.*, 2010
Raw soybean (*defatted*)	-	0.46[a]	-	Fukutake *et al.*, 1996
Soybean flour (*defatted*)	-	1.82[a]	-	Fukutake *et al.*, 1996
Tofu (*defatted*)	-	1.39[a]	-	Fukutake *et al.*, 1996
Miso brand A (*defatted*)	-	22.9[a]	-	Fukutake *et al.*, 1996
Miso brand B (*defatted*)	-	5.6[a]	-	Fukutake *et al.*, 1996
Natto brand A (*defatted*)	-	3.85[a]	-	Fukutake *et al.*, 1996
Natto brand B (*defatted*)	-	6.42[a]	-	Fukutake *et al.*, 1996
Soy sauce brand A (*defatted*)	-	0.28[a]	-	Fukutake *et al.*, 1996
Soy sauce brand B (*defatted*)	-	0.25[a]	-	Fukutake *et al.*, 1996
Black soybean Sakushuu-kuro (tempe)	6.80[b]	9.9[b]	*Rhizopus sp*	Nakajima *et al.*, 2005
Yellow soybean Tamahomare (tempe)	8.0[b]	7.2[b]	*Rhizopus sp*	Nakajima *et al.*, 2005
Defatted yellow soybean (*germ-tempeh*)	206.1[b]	32[b]	*Rhizopus sp*	Nakajima *et al.*, 2005
Rich isoflavones tempeh	65.9[b]	24.6[b]	*Rhizopus sp*	Nakajima *et al.*, 2005
Defatted raw soybean varieties USA	1.66[a]	2.67[a]	-	Iskandar and Priatni, 2006
Tempeh	8.37[b]	10.45[b]	*R. oligosporus* C	Iskandar and Priatni, 2006
Tempeh	9.05[b]	11.40[b]	*R oryzae* L16	Iskandar and Priatni, 2006
Tempeh	3.36[b]	6.99[b]	Commercial cultur	Iskandar and Priatni, 2006
Raw black soybean varieties detam 2 (undefatted)	1.07[a]	1.19[a]	-	Hasim *et al.*, 2014*
Raw black soybean varieties detam 2 (defatted)	1.16[a]	1.29[a]	-	Hasim *et al.*, 2014*
Fermented black soybean varieties detam 2 (undefatted)	8.68[a]	9.43[a]	*B. natto* IFO 3335	Hasim *et al.*, 2014*
Fermented black soybean varieties detam 2 (defatted)	9.60[a]	10.43[a]	*B. natto* IFO 3335	Hasim *et al.*, 2014*

Table 2. Daidzein and genistein concentration of 100 g sample of soybean and its derived products
a isoflavone concentration in 100 g dry weight sample
bisoflavone concentration in 100 g wet sample
*Results on this research

Wei *et al*. (2001) had number of bacterial inoculum ranged from 10^7 to 10^8 CFU/mL with the absorbance value of 1.5. In this study, the number of inoculum of bacteria obtained through Total Plate Count (TPC) method was 1.2 x 10^7 CFU/mL with OD660 value of 1.6. Thus, the inoculum used in this study was qualified to optimize the fermentation process. Inoculum *B.natto* IFO 3335 which had been successfully cultured and then was centrifuged, the pellet was dissolved in butterfield phosphate buffer. Butterfield phosphate buffer is a common solvent for microorganisms used by the American Public Health Association (APHA). It is cheap and easy to prepare and has a fixed value of pH 7.2 therefore it is more specific and scalable than the non-solvent one that has a wide variety of pH. In the process of making natto in laboratory scale, fermentation process generally occured in a styrofoam container, placed inside a fermentor with high humidity, reaching 85-90% RH (Relative Humidity). However, due to the limited equipment, this study modified the fermentation process in which the fermentation was carried out in a waterbath instead of in a fermentor.

The fermentation methods described by Ueda (1989); Kiuchi and Watanabe (2004) were failed to be applied in this study because the fermentation temperature was too high (50°C). Sample became dry and had hard texture, different from a good natto.

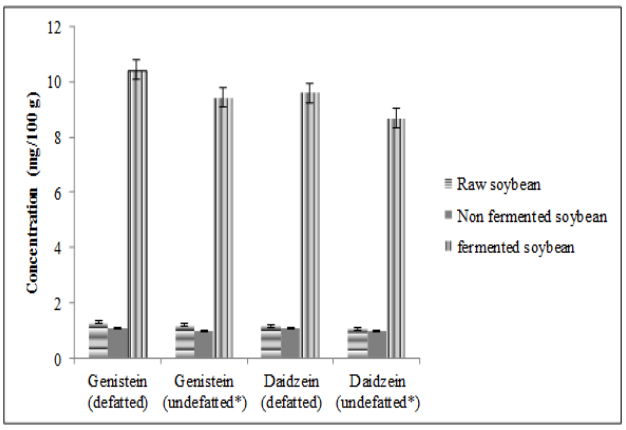

Figure 2. Comparison of isoflavones genistein and daidzein concentrations in 100 grams dry weight of raw, non-fermented and fermented soybean

At first, the fermentation method described by Wei *et al.* (2001) was also failed, although it was done in the right temperature (40-42°C). This happened because the fermentation was done in a regular incubator that had no moisture (RH) regulation mechanism, resulting in the dry soybean. That is the reason to modify the fermentation process by using a waterbath filled with water with a stable temperature, so that the high humidity needed for fermentation process can be obtained.

Analysis of chromatograms and the content of aglycone in soybean

The aglycone content in soybean was measured by using HPLC. The chromatogram resulted from the three sample showed only the area of daidzein and genistein, without showing their actual concentration. The concentration of daidzein and genistein of the samples were calculated from the linear regression curve formed by daidzein and genistein external standards using LC SOLUTION 1.2 program. As shown in Table 1, the concentration of daidzein and genistein of raw soybean were 3.59 mg/mL and 3.99 mg/mL respectively. The concentration of daidzein and genistein of non-fermented soybean were not much different from 3.28 mg/mL and 3.22 mg/mL. However, the concentrations of both aglycones increased sharply up to 8 times as much as those in fermented soybean, which was 26.49 mg/mL for daidzein and 28.80 mg/mL for genistein. In addition, based on the two equations, both p values was less than 5%. Thus, it was concluded that the fermentation affects the aglycones content.

Until now, it was reported that there are 12 kinds of isoflavones in soy, consist of three types of aglycone isoflavones (genistein, daidzein, and glisitein), isoflavone 7-O-β-D-glucoside, known as glycosides (genistin, daidzin, and glisitin), isoflavones 6 "-O-malonil-7-O-β-D-glucoside (malonilgenistin, malonildaidzin, and malonilglisitin), and isoflavones 6"-O-acetyl-7-O-β-D-glucoside (asetilgenistin , asetildaidzin, and asetilglisitin). Among all types of isoflavones, isoflavone 7-O-β-D-glucosides or glycosides is the most commonly found in raw soybean. However, a variety of soybean processing such as heating, cooking, enzymatic hydrolysis, and fermentation can turn glycoside into an aglycone form. Food products that contain large amounts of aglycone will be more beneficial for human because aglycone is easier to absorb in human intestine than other forms (Shao *et al.*, 2009; Ferreira *et al.*, 2011). In this study, glycosides (genistin and daidzin) were converted into aglycone (genistein and daidzein) due to the activity of β-glucosidase enzyme produced by *Bacillus subtilis natto* while Prasad and Shah (2011) revealed that *B. animalis* Bb12 hydrolysed isoflavone glucosides into aglycones.

Quantitative analysis of aglycone

Before the concentration of the aglycone was determined by HPLC analysis, samples must first be defatted. This fat removal process is very important because the high fat content in the sample will not produce a clean chromatogram, leading to some peaks to coincide. In addition, the fat removal with soxhlet will not cause a significant loss of isoflavones (Taniguchi *et al*., 2008). In defatted samples, 8-10% of the fat would be removed. Black soybean detam 2 fat content was 14.83%, lower than that of other soybeans that reach 18-20%. The highest concentration of genistein and daidzein was produced by fermented soybean, increasing up to 8 times (10.43 mg genistein and 9.6 mg daidzein in 100 g dry weight of soybean) as much as those in raw soybean samples. The lowest concentration was produced by non-fermented soybean with 1.07 mg genistein and 1.09 mg daidzein, followed by raw soybean with slightly higher concentration, 1.29 mg and 1.16 mg for genistein and daidzein respectively.

However, soybean and its derived products are largely consumed in the form of fat-containing soy (undefatted). Therefore, a conversion was needed to know the concentration of daidzein and genistein in undefatted samples. Overall, undefatted samples had lower concentration of daidzein and genistein than the fat-free (defatted) samples. Figure 3 showed that fermentation could improve genistein and daidzein content up to 8 times as much as those of the raw sample. Genistein and daidzein concentration in each fermented sample reached 9.43 mg and 8.68 mg per 100 g dry sample weight respectively, much higher than those in the raw soybean, which only 1.19 mg

for genistein and 1.07 mg for daidzein. Meanwhile, non-fermented soybean only contained 0.97 mg genistein and 0.98 mg of daidzein.

Based on previous studies about fermented soybean, it appears that fermentation and acid hydrolysis in soy will increase the daidzein and genistein concentration (Nakajima *et al*., 2005; Tyug *et al*., 2010; da Silva *et al.*, 2011). Fermentation with *A. oryzae* successfully increased the genistein concentration from 1.23 to 14.30 mg (da Silva *et al.*, 2011). In addition, yellow fat-free soybean fermented with *Rhizopus* sp. into germ tempeh on research conducted by Nakajima *et al.* (2005) also succeeded in increasing the concentration up to 206.1 mg for daidzein and 32 mg for genistein. This is the highest concentration of all kinds of soy and dairy products (Table 2).

Compared to other varieties of soybean, black soybean detam 2 contained lower concentration of daidzein and genistein. It was 1.162 mg for daidzein and 1.29 mg for genistein in 100 g dry sample, much lower than those of American varieties which contain 1.66 mg daidzein and 2.67 mg genistein (Nakajima *et al.*, 2005). But after fermentation, the concentration of daidzein and genistein were quite higher than those of other black soybean product (natto, soy sauce, tempeh) as shown on Table 2. Black soybean derived products such as natto and soy sauce contain so little daidzein that it cannot be detected by HPLC (not measurable). Black soy products such as tempeh also contain less concentration of daidzein (6.80 mg) compared to that in this study, 8.68 mg daidzein (the highest among the samples of other types of black soybean and its derived products). Meanwhile, genistein content of black soybean and its derived products in Table 2 quite varied with the lowest concentration of 0.25 mg in soy sauce brand B and the highest one 9.9 mg in black soybean tempe-kuro Sakushuu. While the result of this study showed that the highest genistein content of the samples was 9.43 mg, which is the second highest level for black soybeans. Meanwhile the daidzein and genistein concent of non-fermented soybean were lower than those of raw soybean. It was because most of the isoflavones were dissolved into water during the process of soaking (± 16 hours immersion). In addition, the steaming process at 121^{o}C for 40 minutes could be causing the loss of most of the aglycone isoflavones as well (Grun *et al.*, 2001).

The increase of daidzein and genistein concentration in fermented soybean was due to the activity of *B. natto*. During fermentation, the bacteria will produce β-glucosidase, an enzyme that hydrolyzes isoflavone glycosides into aglycones (Ribeiro *et al*., 2003). In hydrolysis process, the glucose group attached to the oxygen atom (glycosides) would be disconnected and the position of the glucose would be replaced by a hydrogen atom to form aglycone isoflavones. Naturally, soybeans that are consumed will be hydrolized by β-glucosidase as the activity of microflora in human intestine (Behloul and Wu, 2013) or as a result of activity of the lactase enzyme (acid) in small intestine intestine (Raimondi *et al*., 2009).

β-glucosidase enzyme activity in hydrolizing soy isoflavones was first reported by Matsuura *et al.* (1989). Matsuura reported that β-glucosidase plays a role in increasing daidzein and genistein concentration during the soaking process of soybean in the making of soy milk. In addition, β-glucosidase also plays a role in hydrolizing glycosides into aglycones in fermented soy flour (da Silva *et al*., 2011).

A study conducted by Kwon *et al.* (2011) showed that meju, Korean traditional food that was fermented by *Bacillus* and *Aspergillus* without salt for 20-60 days, and chungkookjang (fermented soybeans with *B. subtilis* without salt for 2-3 days) proved to be able to work as an anti-diabetes by improving insulin sensitivity. Genistein itself is beneficial in improving glucose and lipid metabolism as well as protecting the β-cells of the pancreas (Choi *et al*., 2008).

Conclusion

Fermented black soybeans detam 2 with *B. subtilis natto* strain IFO 3335 proved to increase the content of daidzein and genistein. Black soybean detam 2 (raw soybean samples) naturally contains 1.29 mg genistein and 1.16 mg daidzein in 100 grams dried samples (defatted) and 1.19 mg of genistein and daidzein at 1.07 mg free fat sample (undefatted). This amount would decrease as a result of washing and cooking process that occured in non-fermented soybean containing 1.07 mg genistein and daidzein 1.09 mg (0.97 mg and 0.98 mg of the sample undefatted). The fermentation process was able to improve the content of aglycone isoflavones as much as 8 times those of raw samples up to 10.43 mg (9.43 mg undefatted) for genistein and 9.60 mg (8.68 mg undefatted) for daidzein, quite high compared to fermentation using other microorganisms.

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