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

1Hasim, 1Astuti, P., 1Falah, S. and 2,3Faridah, D.N.

1Department of Biochemistry, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Darmaga Campus IPB, Bogor 16680, Indonesia
2Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, Darmaga Campus IPB, Bogor 16002, Indonesia
3Southeast Asia Food and Agricultural Science and Technology Center, Jl. Paspa Lingkar, Bogor Agricultural University, Darmaga Campus IPB, Bogor 16680, Indonesia

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 (defatted) 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.

Keywords
Daidzein
Fermentation
Genistein
Black soybean

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 chungkookjang, 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...
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<sub>660</sub> value was measured with spectrophotometer. After a 16 hour incubation, OD<sub>660</sub> value was 1.5 with a bacterial population ranged from 10<sup>7</sup> to 10<sup>8</sup> 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 which 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isoflavone</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Soybean</td>
<td>Daidzein</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>3.99</td>
</tr>
<tr>
<td>Non-fermented</td>
<td>Daidzein</td>
<td>3.28</td>
</tr>
<tr>
<td>Soybean</td>
<td>Genistein</td>
<td>3.22</td>
</tr>
<tr>
<td>Fermented</td>
<td>Daidzein</td>
<td>26.49</td>
</tr>
<tr>
<td>Soybean</td>
<td>Genistein</td>
<td>28.79</td>
</tr>
</tbody>
</table>

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 \( \gamma \)-PGA and polysaccharides. \( \gamma \)-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 \( \gamma \)-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
\( \gamma \)-PGA, IFO 3335 also produce \( \beta \)-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
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 occurred 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isoflavone concentration (mg)</th>
<th>Microorganism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole soybean flour (Brazil)</td>
<td>-</td>
<td>-</td>
<td>da Silva et al., 2011</td>
</tr>
<tr>
<td>Autoclaved whole soybean flour</td>
<td>-</td>
<td>-</td>
<td>da Silva et al., 2011</td>
</tr>
<tr>
<td>Fermented autoclaved whole soybean flour-24 h</td>
<td>7.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.30&lt;sup&gt;o&lt;/sup&gt;</td>
<td><em>A. oryzae</em></td>
</tr>
<tr>
<td>Grade A Soymilk powder (GASP)</td>
<td>3.13&lt;sup&gt;=&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Grade B Soymilk powder (GBSP)</td>
<td>2.7-2.9&lt;sup&gt;p&lt;/sup&gt;</td>
<td>0.4-0.6&lt;sup&gt;o&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Soybean husk powder (SHP)</td>
<td>1.17&lt;sup&gt;=&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid hydrolyzed Soybean husk powder</td>
<td>19.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raw soybean (defatted)</td>
<td>-</td>
<td>0.46&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Soybean flour (defatted)</td>
<td>-</td>
<td>1.82&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Tofu (defatted)</td>
<td>-</td>
<td>1.39&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Miso brand A (defatted)</td>
<td>-</td>
<td>22.9&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Miso brand B (defatted)</td>
<td>-</td>
<td>5.6&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Natto brand A (defatted)</td>
<td>-</td>
<td>3.85&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Natto brand B (defatted)</td>
<td>-</td>
<td>6.42&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Soy sauce brand A (defatted)</td>
<td>-</td>
<td>0.28&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Soy sauce brand B (defatted)</td>
<td>-</td>
<td>0.25&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Black soybean Sakushu-kuro (tempe)</td>
<td>6.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.9&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>Rhizopus sp</em></td>
</tr>
<tr>
<td>Yellow soybean Tamahore (tempe)</td>
<td>8.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.2&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>Rhizopus sp</em></td>
</tr>
<tr>
<td>Defatted yellow soybean (germ-tempe)</td>
<td>206.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>Rhizopus sp</em></td>
</tr>
<tr>
<td>Rich isoflavones tempeh</td>
<td>65.9&lt;sup&gt;h&lt;/sup&gt;</td>
<td>24.6&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>Rhizopus sp</em></td>
</tr>
<tr>
<td>Defatted raw soybean varieties USA</td>
<td>1.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.67&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Tempeh</td>
<td>8.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.45&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>R. oligosporus C</em></td>
</tr>
<tr>
<td>Tempeh</td>
<td>9.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>R. oryzae L16</em></td>
</tr>
<tr>
<td>Raw black soybean varieties det 2 (undeft)</td>
<td>3.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.99&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Commercial cultur</td>
</tr>
<tr>
<td>Raw black soybean varieties det 2 (defatt)</td>
<td>1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.19&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Fermented black soybean varieties det 2 (undeft)</td>
<td>1.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.29&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Fermented black soybean varieties det 2 (defatt)</td>
<td>8.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.43&lt;sup&gt;h&lt;/sup&gt;</td>
<td><em>B. natto IFO 3335</em></td>
</tr>
</tbody>
</table>

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
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

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 content 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°C for 40 minutes could be causing the loss of most of the aglycone isoflavones as well (Grun 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 hydrolyzed 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 occurred 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.

### References


by elevating insulin level and altering hepatic gluco-
neogenic and lipogenic enzyme activities in non-
Effect of the fermentation of whole soybeans flour on the
Ferreira, M. P., da Silva, M. P., de Oliveira, M. C. B.,
Mandarino, J. M., da Silva J. B., Ida E. I. and Panizzi,
M. C. C. 2011. Changes in the isoflavone profile and in
the chemical composition of tempeh during processing
Fukutake, M., Takahashi, M., Ishida, K., Kawamura,
Quantification of genistein and genistin in soybean
and soybean products. Food Chemical Toxicology 34:
457-461.
hydrolysis of polyglutamic acid from Bacillus subtilis
Grun, I. U., Adhikari, K., Li, C., Li, Y., Lin, B., Zhang,
J. and Fernando, L. N. 2001. Changes in the profile
of genistin, daidzein, and their conjugates during
thermal processing of tofu. Journal of Agricultural and
S. 2009. Daidzein and genistein contents in tempeh
and selected soy products. Food Chemistry 115: 1350
1356.
[IFO] Institute for Fermentation Osaka. 2000. “List of
 Cultures: Microorganism”, 11th Edn, IFO Pr., Osaka.
Internet: Kedelai hitam detam 2. 2012 . [Balitkabi]
Balai Penelitian Kacang-kacangan dan Umbi-umbian
Download from http://balitkabi.litbang.deptan.go.id/
varietas-unggul/va-kedelai/108-varietas-unggul-
kedelai-detam-2.html on 19/01/2014
Iskandar, Y. M. and Priatni, S. 2006. Isoflavone aglycones
produce from fermented soybean. Proceeding
Seminar Nasional Iptek Solusi Kemandirian Bangsa.
Yogyakarta, Indonesia.
Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe,
K. and Saito, M. 2000. Soy isoflavone aglycones
are absorbed faster and in higher amounts than their
glycosides in humans. Journal of Nutrition 130: 1695–
1699.
Kiuchi, K. and Watanabe, S., 2004. Industrialization of
Japanese natto, In: Steinkraus, K., (Ed.).
Kwon, D. Y., Hong, S. M., Ahn, S., Kim, M. J., Yang, H. J.
Y. and Park, S. 2009. Kochujang, a korean fermented
red pepper plus soybeans paste, improves glucose
homeostasis in 90% pancreatectomized diabetic rats.
Kwon, D. Y., Hong, S. M., Ahn, S., Kim, M. J., Yang, H.
J. Y. and Park, S. 2011. Isoflavonoids and peptides
from meju, long-term fermented soybeans, increase
insulin sensivity and exert insulinitropic effect in
Objectionable flavor of soy milk developed during the
soaking of soybeans and its control. Journal of Food
Science 54: 602–605.
Nakajima, N., Nozaki, N., Ishihara, K., Ishikawa, A. and
Tsui, H. 2005. Analysis of isoflavone content in
tempeh, a fermented soybean and preparation of a new
isoflavone enriched tempeh. Journal of Bioscience
and Bioengineering 100(6): 685-687.
Nanri, A., Mizoue, T., Takahashi, Y., Kirii, K., Inoue, M.,
Noda, M. and Tsugane, S. 2010. Soy product and
isoflavone intakes are associated with a lower risk of
type 2 diabetes in overweight Japanese woman. The
Prasad, L. N. and Shah, N. P. 2011. Conversion of
isoflavone glycoside to aglycones in soy protein isolate
(SPI) using crude enzyme extracted from
Bifidobacterium animalis Bb12 and Lactobacillus
delbrueckii sp. bulgaricus ATCC 11842. International
Raimondi, S., Roncaglia, L., de Lucia, M., Amaretti, A.,
Bioconversion of soy isoflavones daidzin and daidzein
by Bifidobacterium strains. Applied Microbiology and
Biotechnology 81: 943–950.
Rimbach, G., Boesch-Sa datmandi, C., Frank, J., Fuchs,
D., Wenzel, U., Daniel, H., Hall, W. L. and Weinberg,
P. D. 2008. Dietary isoflavones in the prevention of
cardiovascular disease – A molecular perspective.
Food and Chemical Toxicology 46: 1308–1319.
components may prevent mutation-related diseases in
Shao, S., Duncan, A. M., Yang, R., Marcone, M. F.,
Rajcan, I. and Tsao, R. 2009. Tracking isoflavones:
from soybeans to soy flour, soy protein isolates to
functional soy bread. Journal of Functional Food 1:
119-127.
Takahashi, R., Ohmori, R., Kiyose, C., Momiyama, Y.,
Ohsuzu, F. and Kondo, K. 2005. Antioxidant activities
of black and yellow soybeans against low density lipo-
protein oxidation. Journal of Agricultural and Food
Chemistry 53: 4578-4582.
Taniuchi, A., Yamanaka-Oakumura, H., Nishida, Y.,
Natto and viscous vegetables in a Japanese style meal
suppress postprandial glucose and insulin responses.
Tyug, T.S., Prasad, K. N. and Ismail, A. 2010. Antioxidant
capacity, phenolics and isoflavones in soybean by
Ueda, S. 1989. Industrial application of B. subtilis, In:
Maruo, B. and Yoshikawa, H. (Eds.). Bacillus subtilis :
Molecular Biology and Industrial Application, p.
characteristics as affected by steaming time, Bacillus
strain, and fermentation time. Journal of Food and