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Bacillus subtilis Fermentation for Enhancement of Feed Nutritive Value of Soybean Meal

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Abstract In order to increase the nutritional quality of soybean meal (SBM) as an animal feed, *Bacillus subtilis* TP6, a previously isolated strain from an Indonesian traditional fermented soybean food, Tempeh, was used as a starter organism for solid-state fermentation. In the pre-treated SBM with water content of 60% (v/w), *B. subtilis* TP6 was grown to a maximum viable cell number of 3.5×10^9 CFU/g. Compared to control, crude protein in *Bacillus* fermented SBM was increased by 16%, while raffinose, stachyose, and trypsin inhibitors were reduced by 31, 37, and 90%, respectively. The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that proteins in the fermented SBM were remarkably hydrolyzed into smaller molecular masses, resulting in a decrease in large sized proteins. Our data suggested that *B. subtilis* fermentation could increase the nutritive value of SBM through reduction of anti-nutritive factors and improvement of protein quality by hydrolysis of soy protein. In addition, *B. subtilis* TP6 produced a functional ingredient, poly-γ-glutamic acid which has various health benefits.

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Introduction

Soybean (*Glycine max*) is an oilseed crop belonging to the family *Leguminosae* and one of the world's largest and most efficient sources of plant protein. Soybean meal (SBM), which is a byproduct remaining after extraction of oil from soybean, has become the most important source of protein for poultry and other livestock, due to its high concentration of protein and excellent profile of essential amino acids in comparison to other plant protein sources (Park, 1987; Adeola, 1996; Emmert and Baker, 1997; Boonyaratpalin et al., 1998). SBM contains around 40–50% protein and is a rich source of the amino acids including lysine, tryptophan, threonine, isoleucine, and valine that are deficient in corn, grain sorghum and other cereal grains used for animal feed (Emmert and Baker, 1997; Kim et al., 2012).

Nutritive value of SBM is limited by several anti-nutritional factors such as protease inhibitor, oligosaccharide, and phytic acid that interfere with digestion, absorption, and utilization of nutrients in the diet (Liener, 1981; Grant, 1989). SBM possesses high content of protease inhibitors that adversely affect protein digestibility and amino acid availability. Especially, the presence of trypsin inhibitors in animal feed has been implicated in growth inhibition and pancreatic hypertrophy (Liener, 1981; Grant, 1989; Gumbmann et al., 1989; Friedman et al., 1991). Oligosaccharides such as raffinose and stachyose are also poorly utilized by some species of animals, and fermentation of these oligosaccharides in the lower gut causes the production of gastrointestinal gases (Krause et al., 1994). Most phosphorus present in SBM is in the form of phytic acid, which is generally indigestible by monogastric animals such as poultry and swine (Grant, 1989). The level of anti-nutritional factors can be reduced by heating during the processing of soybean (Ratner and Crawford, 1955; Friedman et al., 1991). However, an excess of heat may increase Maillard reactions between amino acids and reducing sugars, and consequently decrease the digestibility of amino acid (Parsons et al., 1992; Friedman and Brandon, 2001). Therefore, the conditions applied during heat processing must be carefully monitored to prevent the loss of essential available nutrients.

Treatment of SBM with either of protein hydrolysis (Caine et al., 1998; Dierick et al., 2004) or fermentation (Feng et al., 2007; Jones et al., 2010) increases the proportions of soluble proteins and small peptides, which may be potential to improve health status of animals. Hong et al. (2004) showed that fermentation of SBM with *Aspergillus oryzae* improved the nutritive value of soybeans, and suggested that fermented SBM can be used as a feed ingredient, especially for young animal diets. Several studies reported that during fermentation of SBM, the size of the peptides decreased, which could improve amino acid digestibility in young animals (Hong et al., 2004; Chen et al., 2010; Feng et al., 2010).

So far, there is little information available on the nutritional quality of SBM fermented by *Bacillus* strains. *Bacillus* fermentation technology has been widely applied in preparation of industrial proteinases as well as fermented soy foods such as Chungkookjang, a Korean traditional fermented food, and Natto, a Japanese traditional fermented food (Lee, 1998; Kim et al., 1999; Degering et al., 2010). Previously, *B. subtilis* TP6 was isolated from the Indonesian fermented soybean, Tempeh and found to possess strong protease activity (Kim et al., 2006). It is expected that fermentation of SBM by the *Bacillus* strain can reduce anti-nutritional factors, especially trypsin inhibitors and improve protein quality by the hydrolysis of soy protein. Thus, the purposes of this study were to investigate the applicability of a solid state fermentation of SBM by *B. subtilis* TP6 and to evaluate the nutritional quality of fermented SBM.

Materials and Methods

Preparation of strains. *B. subtilis* TP6 isolated from the Indonesian fermented soybean, Tempeh (Kim et al., 2006), was used as a starter organism. The stock cultures were maintained at −70^{o}C in a modified Nutrient broth (pH 7.0), which contains soytone (5 g/L), beef extract (5 g/L), and glucose (20 g/L). Before use in experiments, the culture was propagated twice in the modified Nutrient broth at 37^{o}C for 24 h.

Pre-treatment of SBM and solid state fermentation. In the present study, SBM, which served as the fermentation substrate, was obtained from CJ (Korea). SBM was washed and soaked in water by spraying adequate amount of water to achieve final water contents of 30−60% (v/w). The soaked SBM was heated at 60, 80, 105, and 121^{o}C for 10−30 min. After cooling, the heat-treated SBM was inoculated with *B. subtilis* TP6 to have a final viable cell number of 1.0×10^7 CFU/g. After a thorough mixing, the inoculated SBM was incubated at 37^{o}C for 24 h. Bacterial growth was determined by plating 0.1-mL portions of appropriately diluted culture on the modified Nutrient agar plates and incubating the plates at 37^{o}C for 48 h. The pH of fermented SBM was measured using a pH meter (MP 220 pH meter, Mettler Toledo, Switzerland).

Chemical analysis. Dry matter and crude protein were determined according to the method of AOAC (2006). The oligosaccharide composition was analyzed by high performance liquid chromatography according to the method of Delente and Ladenburg (1972). Determination of poly-γ-glutamic acid was carried out by the method of Choi et al. (2004). Amino acids were measured by the method of Hong et al. (2004).

Trypsin inhibitor assay. Trypsin inhibitor assay was carried out by the modified method of Hamerstrand et al. (1981). An aliquot (1 mL) of trypsin enzyme solution was mixed with 0.1 mL of crude extract of sample and pre-incubated in shaking water bath at 37^{o}C for 10 min. After pre-incubation, 0.6 mL of 0.1 M phosphate buffer (pH 7.5) and 0.3 mL of BApNA (α-Benzoyl-DL-arginine-*p*-nitroanilide) were added. The reaction mixture was incubated at 37^{o}C in shaking water bath for 10 min and reaction was stopped by adding 0.5 mL of 30% acetic acid. The optical density was measured at 410 nm using UV/VIS spectrophotometer to determine trypsin inhibitor activity.

Protease assay. Protease activity was measured using the modified method of Hagihara (Hagihara, 1958). The crude enzyme was obtained by fermenting soybean meal. The fermented soybean meal sample (10 g) and 90 mL of 0.85% (w/v) NaCl solution were mixed for 30 min at room temperature, and then cell-free extract was used as a crude enzyme solution. The enzymatic reaction mixture consisted of 1 mL of crude enzyme solution, 1 mL of 0.6% (w/v) azocasein (Sigma, USA), and 1 mL of 0.1 M phosphate buffered saline (pH 7.0). The enzymatic reaction was performed at 37^{o}C for 10 min. The reaction was stopped by addition of 3 mL of 5% (w/v) trichloroacetic acid (TCA; Sigma, USA) followed by centrifugation (12,000 rpm, 15 min). Hydrolyzed casein was measured by a folin-ciocalteu method. One unit of enzyme activity was defined as the amount of protease which produced 1 μg tyrosine per min at 37^{o}C.

KOH solubility. KOH solubility of protein was determined as described by Parsons et al. (1991). The protein sample mixture (1.5 g of sample and 75 mL of 0.2% KOH solution) was stirred for 20 min at 22^{o}C, and then centrifuged at 12,000 rpm for 10 min. The supernatant was collected and the nitrogen content was determined by the Kjeldahl method (AOAC, 2006). The protein solubility was calculated as a percentage of the total in the original SBM sample.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in 12.5% separating gel and 5% stacking gel according to the method of Hames and Rickwood (1990). Gels were run at 20^{o}C for 6 h at 80 mA. After electrophoresis, the gel was stained with Coomassie blue according to the method of Reed et al. (1998). Relative molecular weight of each protein was determined using a standard protein marker.

Results and Discussion

Pre-treatment of SBM. Solid state fermentation is performed on

Table 1 Effect of water content on growth of *B. subtilis* TP6 on soybean meal[a]

Water content (%)	Cell No. (CFU/g)	Final pH
30%	1.0×10^7	6.12
40%	7.0×10^8	5.90
50%	2.5×10^9	5.55
60%	3.5×10^9	5.26

[a]Values are the means of three replicated measurements.

Table 2 Effect of heat treatment on growth of *B. subtilis* TP6 on soybean meal[a]

Conditions	Cell No. (CFU/g)	Final pH
60°C, 10 min	NA[b]	NA
60°C, 30 min	NA	NA
80°C, 10 min	1.2×10^9	6.20
80°C, 15 min	1.5×10^9	6.19
80°C, 30 min	1.4×10^9	6.22
105°C, 15 min	2.0×10^9	5.47
105°C, 30 min	1.9×10^9	5.53
121°C, 15 min	3.4×10^9	5.32

[a]Values are the means of three replicated measurements.
[b]NA: not applicable due to microbial contamination.

Table 3 Physical and nutritional characterization of fermented soybean meal[a]

Items	Non-fermented SBM[b]	Fermented SBM
Dry matter (%)	88.04±1.21	89.68±0.64
Final pH	6.51±0.14	5.28±0.09
Crude protein (%)	48.53±0.52	56.42±2.08
KOH solubility	77.37±3.07	79.25±1.82
Total carbohydrate (%)	32.50±0.31	25.29±0.11
Raffinose (%)	1.26±0.04	0.87±0.06
Stachyose (%)	3.47±0.06	2.19±0.05
Poly-γ-glutamic acid (%)	ND[c]	0.60±0.03
Protease activity (U/mg)	ND	25.1±0.2

[a]Values are the means of three replicated measurements and standard deviations.
[b]SBM: soybean meal.
[c]ND: No detection.

a non-soluble material that acts both as physical support and source of nutrients under low moisture content. From the ancient, solid state fermentation has been used to prepare traditional fermented soy foods such as Chungkookjang, Natto, Tempeh, soybean paste, and soy sauces which are highly digestible and nutritious (Lee, 1998; Kim et al., 1999). Previously, *B. subtilis* TP6 was isolated from the Indonesian fermented soybean, Tempeh and found to produce a serine protease of subtilisin family consisting of 275 amino acid residues (Kim et al., 2006). In this study, *B. subtilis* TP6 was selected as a starter organism to ferment SBM because the strain has been shown to have a strong proteolytic activity.

A limited amount of usable water is present in SBM and thus the adjustment of water content by soaking is important for the growth of starter organism. Moisture content is also one of the most critical factors influencing protease production in solid state fermentation. To determine the influence of water content on cell growth of *B. subtilis* TP6, SBM was soaked in water to achieve water contents of 30–60% (v/w). The hydrated SBM was autoclaved at 121°C for 15 min. After cooling, the heat-treated SBM was inoculate with *B. subtilis* TP6 (1.0×10^7 CFU/g), mixed, and fermented for 37°C for 24 h. The effect of water content on growth of *B. subtilis* TP6 on SBM was present in Table 1. The cell growth of *B. subtilis* TP6 was not observed in SBM with 30% water, whereas the strain was able to grow in SBM with 40% water and its viable cell number reached 7.0×10^8 CFU/g. The maximum cell growth (3.5×10^9 CFU/g) was observed in SBM with 60% water. The growth profile of *B. subtilis* TP6 on SBM with 60% water was similar to that of the strain grown in modified Nutrient Broth (Data not shown). Our data suggest that SBM as a substrate for the growth of the bacterial strain is required to have 60% of water content for industrial application.

In order to remove undesirable microorganisms from SBM that could interfere with fermentation, SBM was soaked to have water content of 60% (v/w), and subsequently heated at 60, 80, 105, and 121°C for 10–30 min. After heat treatment at 60°C, undesirable microorganisms were detected in the SBM. In the SBM treated at 80°C for 10 min, microbial contamination was not observed during 72-h fermentation, and the viable cell number of *B. subtilis* TP6 reached 1.2×10^9 CFU/g. However, the maximum cell growth (3.4×10^9 CFU/g) was observed in the heat-treated SBM at 121°C for 15 min (Table 2). Therefore, the SBM, which was soaked to have water content of 60% and heated at 121°C for 15 min, was used as a substrate for solid-state fermentation in subsequent experiments.

Physical and nutritional characteristics of fermented SBM. Characteristics of solid-stated fermented SBM are shown in Table 3. The final pH was decreased from pH 6.5 to pH 5.3, probably due to the production of organic acids during the fermentation. Crude protein was increased from 48.5 to 56.4%, while the total carbohydrate dropped from 32.5 to 25.3%. The increase in crude protein appears to be resulted from the digestion of carbohydrate in SBM for microbial growth during fermentation. Similar findings were observed by other studies that showed an increase in crude protein content and a decrease in carbohydrate content after fermentation of SBM (Chen et al., 2010; Teng et al., 2012; Gao et al., 2013).

The KOH protein solubility of heat-treated plus non-fermented SBM and fermented SBM was 77 and 79%, respectively and was slightly lower than that of the untreated raw SBM (85%), due to the application of heat process. However, the solubility of fermented SBM is still above 75%, indicating that the fermented SBM maintains high quality of soluble protein. The KOH protein solubility is a good index of *in vivo* soybean quality for animal feeding. Parsons et al. (1991) suggested that feed efficiency could decrease significantly when the solubility was below 66%. Willis (2003) reported that 18 minutes of autoclaving raw soybeans were

Table 4 Amino acid composition in fermented soybean meal (mg/g) [a]

Amino acid	Non-fermented SBM[b]	Fermented SBM
Aspartic Acid	5.09±0.17	5.42±0.22
Threonine	2.06±0.06	2.25±0.11
Serine	2.11±0.03	2.19±0.05
Glutamic Acid	8.78±0.20	10.57±0.47
Proline	2.07±0.04	2.98±0.09
Glycine	1.92±0.42	2.08±0.16
Alanine	2.20±0.07	2.43±0.05
Valine	2.40±0.34	2.69±0.17
Isoleucine	2.33±0.13	2.60±0.06
Leucine	3.60±0.05	3.99±0.12
Tyrosine	1.97±0.12	2.16±0.06
Phenylalanine	2.54±0.10	2.80±0.21
Histidine	1.25±0.05	1.28±0.02
Lysine	2.74±0.08	3.28±0.19
Arginine	3.91±0.10	4.19±0.22
Cysteine	0.71±0.04	0.70±0.15
Methionine	0.69±0.07	0.87±0.13
Tryptophan	0.41±0.02	0.48±0.06

[a]Values are the means of three replicated measurements and standard deviations.
[b]SBM: soybean meal.

required to maximize the growth of chicks and there was very little change in KOH solubility value during the heat treatment.

SBM contains raffinose and stachyose, the anti-nutritive oligosaccharides that cause osmotic problems in animals (Krause et al., 1994). After fermentation, raffinose and stachyose were lowered by 31 and 37%, respectively. Possible explanation from the decrease in the oligosaccharides is probably due to the release of exogenous enzymes hydrolyzing the oligosaccharides from the *Bacillus* strain during the fermentation.

Fermentation resulted in the production of poly-γ-glutamic acid, an anionic polypeptide, in which D-and/or L-glutamic acid units are polymerized via γ-amide linkages (Kang et al., 2005). Poly-γ-glutamic acid is a major component of the extracellular mucilage produced by some strains of *B. subtilis* and is found in the Korean traditional soy product, Cheongkukjang (Shih and Van, 2001; Lee et al., 2010). Several studies have shown that poly-γ-glutamic acid increases calcium absorption in small intestine, intensifies femur strength, enhances immune-stimulating activity, and potentially promotes antitumor activity (Lee et al., 2006; Kim et al., 2007).

It is well established that *Bacillus* strains have the ability to secrete large amounts of extracellular proteases and are recognized as important sources of commercial proteases. In this study, *B. subtilis* TP6 grown in SBM exhibited protease activity of 25.1 U/mg.

Table 4 presents the amino acid profile of non-fermented and fermented SBM. The contents of most amino acids in SBM were increased by the fermentation with *B. subtilis* TP6. This consists with the results of the previous studies on the fermentation of SBM with fungi or bacteria (Hong et al., 2004; Chen et al., 2010; Gao et al., 2013), suggesting that the increase in amino acid

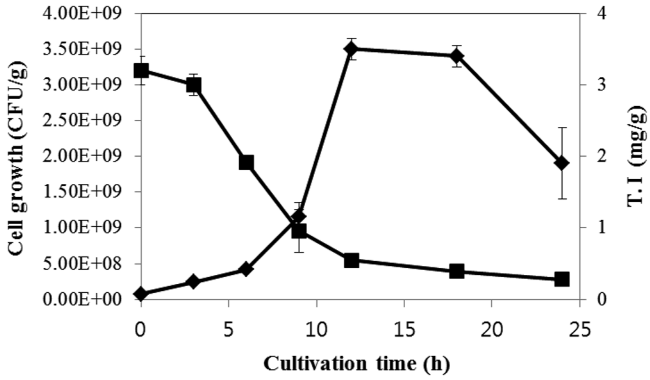

Fig. 1 Time profile of cell growth and trypsin inhibitor during soybean meal fermentation by *Bacillus subtilis* TP6. Filled diamond; cell growth, filled square; trypsin inhibitor. Data points are the means of three replicated measurements. Error bars represent standard deviations.

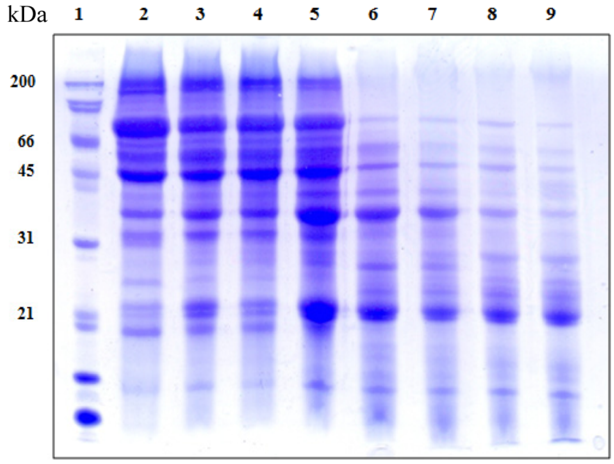

Fig. 2 Protein pattern of fermented soybean meal. Lane 1; size marker, lane 2; raw soybean meal, lane 3; non-fermented soybean meal, lane 4; fermentation for 3 h, lane 5; fermentation for 6 h, lane 6; fermentation for 9 h, lane 7; fermentation for 12 h, lane 8; fermentation for 18 h, lane 9; fermentation for 24 h.

content could be attributed to the simple protein constituents of microbial mass as well as microbial metabolism during fermentation.

Profiles of cell growth, trypsin inhibitor, protein distribution during fermentation. To determine the growth of *B. subtilis* TP6 during fermentation, SBM was soaked in water to achieve water contents of 60% (v/w), heated at 121°C for 15 min., inoculate with the *Bacillus* strain (1.0×10^7 CFU/g), and fermented at 37°C for 24 h. Growth profile of *B. subtilis* TP6 in SBM was presented in Fig. 1. The growth of *B. subtilis* TP6 was increased rapidly after 6-h fermentation, and the maximum viable cell number (3.5×10^9 CFU/g) was obtained at 12 h. On the other hand, trypsin inhibitor started to decrease after 3-h fermentation, and gradually decreased until 18 h (Fig. 1). The trypsin inhibitor content in fermented SBM was decreased from 3.2 to 0.33 mg/g after 18 h, resulting in almost 90% reduction.

Fig. 2 presents the SDS-PAGE analysis of raw SBM, non-fermented SBM and fermented SBM. The protein profile of heat-treated plus non-fermented SBM exhibited similar to that of raw SBM, although larger proteins with MW above 66 kDa were decreased slightly in the non-fermented SBM. The profile of raw SBM was in agreement with the study of Feng et al. (2007). After 9-h fermentation, the large proteins in SBM were remarkably hydrolyzed into smaller molecular masses, resulting in a decrease in large sized proteins. The proteins with MW above 31 kDa were almost hydrolyzed at 24-h fermentation. Our data suggested that *Bacillus* fermentation could increase the nutritive value of SBM and enhance the content of small-size peptides, due to hydrolysis of large size proteins by protease secreted from *B. subtilis* during fermentation. The result of SDS-PAGE was consistent with previous studies of SBM fermented with fungi or bacteria (Hong et al., 2004; Chen et al., 2010). It is expected that an increase in small size peptides by fermentation may improve health status of animals because soybean-derived peptides have been shown to have many health benefits such as antimicrobial, antioxidant, anticancer, antihypertensive, anti-inflammatory, hypocholesterolemic, and immunostimulatory activities (Wang and De Mejia, 2005).

In conclusion, *Bacillus subtilis* fermentation could increase the nutritive value of SBM in which fermentation increased in protein content, reduced trypsin inhibitor content, produced poly-γ-glutamic acid, and improved protein quality by the hydrolysis of soy protein. Therefore, SBM fermented with *B. subtilis* is a promising alternative protein source to improve health status of animals and potentially reduce the cost of animal production. The effectiveness of fermented SBM on piglet growth is still pending to be confirmed in the near future.

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