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PROTEIN QUALITY IMPROVEMENT AND ANTI-NUTRITIONAL FACTORS REDUCTION IN SOYBEAN MEAL BY *Bacillus velezensis* K1

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Abstract: The solid-state fermentation (SSF) of soybean meal (SBM) by *Bacillus velezensis* K1 to improve protein quality and reduce allergenic proteins as well as raffinose family oligosaccharides was investigated in this research paper. The fermentation parameters affected protein hydrolysis, namely soybean meal moisture, size of inoculums, time and temperature of fermentation were evaluated in the soybean meal. The best solid-state fermentation conditions with the highest degree of hydrolysis (16.65 ± 4.08%) were 50% (w/w) moisture content, 10% (v/w) inoculum size, 40°C and 72 hours fermentation. Under these optimal conditions, large proteins in the soybean meal were hydrolysed. Proteins with molecular weight <10 kDa increased significantly from 34.05 to 48.22% ($P<0.05$). Additionally, allergens (glycinin and β-conglycinin) and raffinose family oligosaccharides decreased. The bands representing glycinin and β-conglycinin on SDS-PAGE were considerably reduced and their degradations were 96.47% and 49.60%, respectively. The raffinose family oligosaccharides, primarily stachyose and raffinose also decreased by 73.57% and 22.76%, respectively. With the capability of producing protease and other carbohydrate hydrolytic enzymes (cellulase, pectinase, amylase and invertase), *B. velezensis* K1 presents great potential for increasing the nutritional quality of the soybean meal as an ingredient in the feed industry.

Keywords: Allergenic proteins, anti-nutritional factors, raffinose oligosaccharides, solid-state fermentation, soybean meal.

Introduction

Soybean meal, a by-product of soybean oil extraction is one of important plant-based protein sources. It is a high protein substrate widely supplied in the feed industry. However, the nutritional values of soybean meal are limited by some anti-nutritional factors (ANFs) such as trypsin inhibitor, allergenic proteins, raffinose family oligosaccharides and phytic acid.

These anti-nutritional factors have a negative effect on animal growth performance, particularly in young animals with immature gut structures (Chen *et al*., 2013a). As a result, it is desirable to remove these components.

Thermal processing could partially eliminate heat labile factors such as trypsin inhibitor and lectins (Anderson & Wolf, 1995; Fasina *et al.,* 2003). However, the heat stable ANFs require other approaches for complete removal. Many studies have revealed that microbial fermentation of soybean meal could not only improve protein quality but also reduce some ANFs. For example, fungal strains *Neurospora crassa* (Li *et al*., 2019b) and *Aspergillus oryzae* (Hong *et al*., 2004; You-ling *et al*., 2013) have been proven to have a profound effect on enhancing nutrient quality by lowering soybean meal allergenic proteins during solid-state fermentation (SSF). Apart from fungal fermentation, yeast and bacteria have also been used to increase protein quality, nutritional bioavailability, and reduce some ANFs in soybean meal (Chi & Cho, 2016; Shi *et al*., 2017; Yuan *et al*., 2017; Medeiros *et al*., 2018; Cui *et al*., 2020). The dominant bacteria used for enhancing soybean meal nutritional values and reducing some ANFs is a species of *Bacillus* such as *B. subtilis* (Shi *et al*., 2017; Medeiros *et al*., 2018), *B. cereus* and *B. amyloliquefaciens*

(Medeiros *et al*., 2018). The *Bacillus* genus is categorized as GRAS (Generally Recognised as Safe) microorganisms.

Various fermented soy products are produced by the generally recognised as safe. These strains are able to secrete proteolytic enzymes which degrade complex proteins into small molecular mass proteins, resulting in easier digestion and absorption by young animals (Wongputtisin *et al*., 2012; Kook *et al*., 2014; Sanjukta *et al*., 2015; Chi & Cho, 2016; Yuan *et al*., 2017; Zhang *et al*., 2017; Medeiros *et al*., 2018; Cheng *et al*., 2019; Cui *et al*., 2020; Li *et al*., 2020).

Glycinin and β-conglycinin are major allergenic proteins, which account for approximately 30% of SBM. It has been reported that the potential allergens of sensitized piglets are α', α and β-subunits of β-conglycinin as well as acidic and basic subunits of glycinin (Sadeghi *et al*., 2006; Zheng *et al*., 2014).

The elimination of allergens in soybean meal by fermentation, carbohydrate based anti-nutritional factors such as raffinose family oligosaccharides (mainly raffinose and stachyose) which generate gastrointestinal gases resulting in flatulence and discomfort in monogastric animals (Liying *et al*., 2003) could also be eliminated by *B. subtilis* TP6 (Kook *et al*., 2014). Thus, bacteria in the genus of *Bacillus* which are able to produce protease together with the ability to reduce glycinin and β-conglycinin as well as raffinose family oligosaccharides in soybean meal are of particular interest.

In the present study, *B. velenzensis* K1 was isolated from Kimchi, traditional Korean fermented vegetables. Previously, *B. velezensis* was widely used for agricultural biocontrol (Palazzini *et al.*, 2016; Myo *et al.*, 2019; Balderas-Ruíz *et al.,* 2020; Chen *et al.*, 2020; Ben Gharsa *et al*., 2021; Kim *et al.*, 2021; Medhioub *et al*., 2022). Although *B. velezensis* has been reported as a probiotic applied in feed (Yi *et al.*, 2018; Li *et al.*, 2019a; Thurlow *et al.*, 2019; Ye *et al*., 2020), it has received less attention for its use in increasing the nutritional value of soybean meal by fermentation (Liu *et al.*, 2020; Chen *et al.*, 2021; Tsai *et al.*, 2021).

Additionally, most studies have emphasized on reducing anti-nutritional factors through microbial fermentations at 37°C as a controlled temperature (Liu *et al.*, 2020; Chen *et al.*, 2021). However, in practice, metabolic heat is generated during SSF, resulting in an increment in temperature which could affect the microbial growth and corresponding cellular functions.

Therefore, it is of interest in the current work to obtain a microorganism that can not only grow at a moderately elevated temperature (~37-45°C) but also possess proteolytic and non-starch polysaccharides (NSP) hydrolytic activities. The main focus was to increase the soybean meal nutritional value by *B. velezensis* K1 fermentation.

The fermented soybean meal (FSBM) with high content of low molecular mass proteins as well as less content of anti-nutritional factors emphasized on allergenic proteins and RFOs were optimised. The FSBM with the desired characteristics from this research can be used in the feed industry.

Materials and Methods

Microorganisms and Enzyme Production

The strain K1 isolated from Kimchi was primary screened on skim milk agar medium at 37°C, 24 hours for protease production. The strain K1 was cultured in skim milk broth at different temperatures (37, 40, 45 and 50°C) and enzyme activity was determined following the protocol laid down by Sarath *et al.* (1989).

Briefly, a diluted supernatant was reacted with 0.5% (w/v) azocasein in 50 mM Tris-HCl buffer (pH 8.0). The mixture was incubated at 37°C for 30 minutes. After that, 10% TCA solution was added and set at room temperature for 30 minutes. The reaction was centrifuged at 12,000 rpm for 7 minutes. The supernatant was pipetted and mixed with 2.0 M NaOH and left for 10 minutes. The resulting solution absorbance was then determined at 440 nm.

One unit of protease activity is the amount of the enzyme which increased 0.01 of the absorbance in 30 minutes under the assay condition. Apart from protease, the productions of cellulase, α-galactosidase, pectinase, amylase and invertase (carbohydrate hydrolytic enzymes) were also examined.

Amylase production was performed according to Mageswari *et al.* (2012) method by spotting bacterial culture on starch agar (NA + 1% starch (w/v)) while pectinase production was determined on screening agar plate following the method of Takcı and Turkmen (2016). The clear zone after flooding with iodine-potassium iodide solution indicated the ability to produce amylase or pectinase. Similarly, cellulase production was determined on cellulose Congo-Red agar according to the work of Gupta *et al.* (2012).

A colony with a clear zone indicated positive cellulose-degrading bacteria. For the α-galactosidase production, it was evaluated by the presence of blue colonies on selective medium (Lee *et al.*, 2012). Meanwhile, the production of invertase enzyme was analysed based on bacteria's ability to grow on a sucrose containing medium (Ghasemi *et al.*, 2014).

For the strain K1 identification by 16s rDNA gene analysis, the amplified PCR product from a genomic DNA template primed with specific primer pairs was purified and sequenced. The homology of sequences was determined by the blastn tool on the website of the National Centre for Biotechnology Information (NCBI).

Optimisation of Soybean Meal Fermentation

For the culture inoculum preparation, the *B. velezensis* K1 colony was transferred into fresh nutrient broth and incubated (37°C) with shaking at 150 rpm for 24 hours. Then, 5% inoculum was transferred to 50 mL and 100 mL of the medium and cultured for 24 hours and 8 hours, respectively. The absorbance at 600 nm of the bacterial cultures was adjusted to 0.5 (10^8 CFU/mL). The strain was used as the starter for soybean meal fermentation.

To optimise the soybean meal fermentation parameters, initial soybean meal moisture, fermentation temperature, size of inoculum and time were investigated. To find the optimum content of moisture, soybean meal was mixed with distilled water to obtain the required moisture content (35, 40, 50, 60% w/w) and steamed at 121°C for 15 minutes. Then, 10% (v/w) (10^8 CFU/mL) of *B. velezensis* K1 was added into the cooled soybean meal and thoroughly mixed.

The inoculated soybean meal was performed at 40°C for 48 hours. The optimum fermentation temperature (37, 40, 45 and 50°C) was evaluated by keeping the other parameters constant at the optimal moisture content, 10% (v/w) inoculum size and 48 hours fermentation. Then, the optimal inoculum size (5, 10, 15 and 20% v/w) and fermentation time (0, 24, 48, 72 and 96 hours) for FSBM production was consequently investigated. After fermentation, the FSBM was dried at 60°C for 24 hours. The un-inoculated soybean meal contained the same components as the inoculated one except that sterile medium was added instead of the inoculum and served as a control.

Determination of Bacterial Growth

The FSBM was mixed with 0.85% NaCl solution and serially diluted to proper dilutions. The diluted sample (0.1 mL) was plated on nutrient agar. Bacterial growth was counted and calculated as colony forming units per gram sample (CFU/g) after a 24 hours incubation at 37°C.

Determination of Degree of Protein Hydrolysis (DH)

To measure the DH, the FSBM samples were extracted with distilled water and stirred at 160 rpm for 30 minutes at 37°C. Then, the mixture was centrifuged at 7,000 rpm for 10 minutes at 4°C to harvest the supernatant (Fang *et al.*, 2015). The TNBS method based on the work of Nalinanon *et al.* (2011) was used to determine the DH of FSBM.

Protein Analysis by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Extraction of protein from fermented soybean meal was performed according to the protocol of Li *et al.* (2019b). The FSBM powder was mixed with 120 mM Tris-HCl (0.05% Tween 20, 20% SDS and 2% β-mercaptoethanol, pH 7.4) and shaken at 2-4°C overnight. Then, the supernatant was collected by centrifugation at 7,000 rpm at 4°C for 20 minutes**.** The soluble protein in the FSBM extract was measured using the Bradford method. For protein pattern study by SDS-PAGE, proteins (20 µg) were loaded on a 12% polyacrylamide separation gel and electrophoresed at 100 V for 120 minutes. After Coomassie brilliant blue R-250 staining and acetic acid de-staining, protein bands were appeared.

Molecular Weight (MW) Distribution of Proteins

The FSBM protein was extracted according to the protocol of Sanjukta *et al.* (2015). 1 g of FSBM was mixed with 10 mL of distilled water and shaken for 4 hours at 28 ± 2°C. Then, the extracts were collected by centrifugation at 7,000 rpm for 20 minutes. The extracts were fractionated into >30 kDa, 10-30 kDa and <10 kDa by 10 and 30 kDa molecular weight cut offs (MWCO) membranes (Sartorius Stedim Biotech GmbH). The peptide concentrations of each fraction were determined by the TNBS method as mentioned above (Nalinanon *et al.*, 2011).

Determination of Allergenic Protein Content

The glycinin and β-conglycinin contents in soybean meal and fermented soybean meal samples were determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit (Beijing Longkefangzhou Bio-Engineering Technology Co., Ltd.).

Determination of Sugar Content

The extraction of sugar from fermented soybean meal was performed according to the protocol of Chen *et al.* (2013b) and analysed by HPLC. In brief, 1 g of FSBM sample was mixed with 10 mL of distilled water and shaken at 200 rpm, 50°C for 30 minutes. Then, the supernatant was collected by centrifugation at 3,000 rpm for 15 minutes.

For HPLC analysis, the 10 µL of properly diluted filtrates were injected into a HPLC system (Shimadzu LC-20A, Japan) in an isocratic mode**.** The samples were separated on a Rezex RNM carbohydrate column (7.8x300 mL; Phenomenex, USA**)** using deionised water with a flow rate of 0.4 mL/min as the mobile phase. The temperature of column was set at 45°C. The separation peaks were detected by a refractive index detector (Shimadzu RID, Japan). Stachyose, raffinose and sucrose were used as sugar standards.

Statistical Analysis

Statistical analysis of data was performed using a one-way analysis of variance by SPSS. Significant differences were assessed at a 5% level of probability ($P<0.05$). Data were expressed as means ± standard deviations.

Results and Discussion

Protease and Carbohydrate Hydrolytic Enzyme Production

To improve protein quality and reduce allergenic proteins as well as raffinose family oligosaccharides, mainly stachyose and raffinose in soybean meal via solid-state fermentation, the bacterial strain K1 was isolated from Kimchi. Primary screening on skim milk as a selective medium indicated the ability of K1 to produce protease. Apart from proteolytic enzymes, the K1 could produce all tested carbohydrate hydrolytic enzymes except α-galactosidase (Figure 1).

For protease production, the growth temperature effect on the protease activity was investigated. There were no significant differences ($P<0.05$) in protease activity at 37°C, 40°C and 45°C with the highest activity (5536.0 ± 256.6 U/mL) was observed at 40°C.

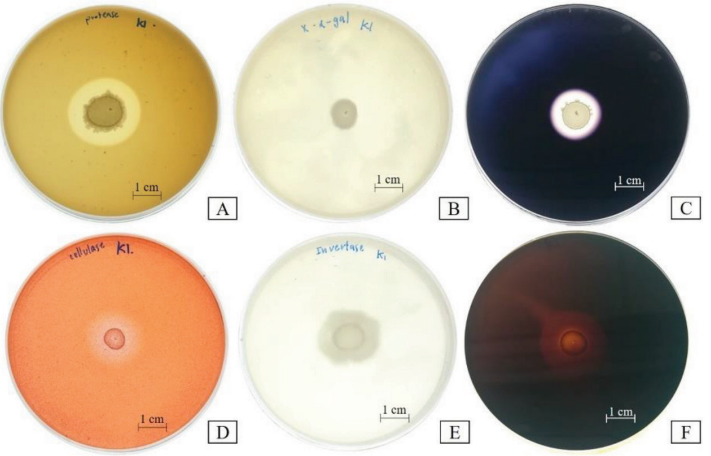

Figure 1: The ability of enzyme production by *B. velezensis* K1 on the selective medium: Protease (A), α-galactosidase (B), amylase (C), cellulase (D), invertase (E) and pectinase (F)

However, a sharp decrease in enzyme activity was found when the temperature was increased to 50°C (Figure 2). The ability to produce proteolytic enzyme by K1 implied that it can possibly be used to improve protein quality in soybean meal by degrading complex proteins into small molecules. Moreover, the presence of cellulase, amylase, pectinase and invertase activities in K1 suggested that it can certainly consume carbohydrates (starch and non-starch components) in soybean meal as a nutrient source (Shi *et al.*, 2017).

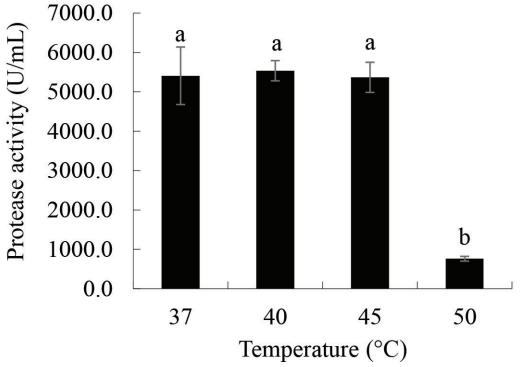

Figure 2: Protease activity of *B. velezensis* K1 at different temperatures (37, 40, 45 and 50°C). Data were expressed as means ± SD. Different letters indicate significant differences at P<0.05

For the strain identification, the results suggested that K1 is a gram-positive bacteria. The 16s rDNA gene sequence of K1 was 99% identical to that of *Bacillus velezensis* in the GenBank database. *Bacillus* spp. is generally a type of GRAS microorganism that has been used to make a variety of fermented soy products. The secretion of proteolytic enzymes by *Bacillus* species to digest complex proteins in soybean meal into small molecular mass proteins resulting in easier digestion and absorption by young animal has been reported in many studies (Wongputtisin *et al.*, 2012; Kook *et al.*, 2014; Sanjukta *et al.*, 2015; Chi & Cho, 2016; Yuan *et al.*, 2017; Zhang *et al.*, 2017; Medeiros *et al.*, 2018; Cheng *et al.*, 2019; Cui *et al.*, 2020; Li *et al.*, 2020).

It was reported that *B. velezensis* is used widely as an agricultural biocontrol (Palazzini *et al.*, 2016; Myo *et al.*, 2019; Balderas-Ruíz *et al.,* 2020; Chen *et al.*, 2020; Ben Gharsa *et al.*, 2021; Kim *et al.*, 2021; Medhioub *et al.*, 2022) and probiotic in aquaculture feed (Yi *et al.*, 2018; Li *et al.*, 2019a; Thurlow *et al.*, 2019).

However, there are a few reports involved in the application of *B. velezensis* in soybean meal fermentation. Chen *et al.* (2018) isolated *B. velezensis* 157 from the bark of *Eucommia ulmoides*. The strain 157 showed various lignocellulolytic activities that indicated its

ability to use agro-industrial waste including soybean meal under solid-state fermentation.

Additionally, the single-stage fermentation by *B. velezensis* (Liu *et al*., 2021) and two-stage fermentation by *Lactobacillus* spp. (Chen *et al.*, 2020; Tsai *et al.*, 2021) in order to reduce the content of soybean meal was recently reported.

The European Food Safety Authority introduced the Qualified Presumption of Safety (QPS) which indicated that *B. velezensis* would be safely applied in feed and food (Na *et al*., 2022). Hence, it is possible to use *B. velezensis* K1 obtained from this study to increase the soybean meal nutritional value for further serving as an ingredient in animal feed.

The Optimal Fermentation Conditions

This study focused on finding the optimal conditions to produce fermented soybean meal with a high content of low molecular mass proteins as well as less content of anti-nutritional factors, emphasised on allergenic proteins and RFOs. Protein hydrolysis could be evaluated by different parameters, including the determination of trichloroacetic acid (TCA) soluble nitrogen, amino acid content, degree of protein hydrolysis and SDS-PAGE profile. The protein hydrolysis degree (DH) based on released free amino acids determination after the hydrolysis is a preferred parameter for analysis of the fermented soybean meal products. Many studies revealed that an increase in DH during soybean meal fermentation resulted from protease hydrolysis (Chen *et al*., 2013a). The relation of soybean meal initial moisture content and bacterial growth in Figure 3 (A) suggested that the population of *B. velezensis* K1 in all tested moisture content levels were increased after 48 hours of fermentation and showed no significant differences (P<0.05).

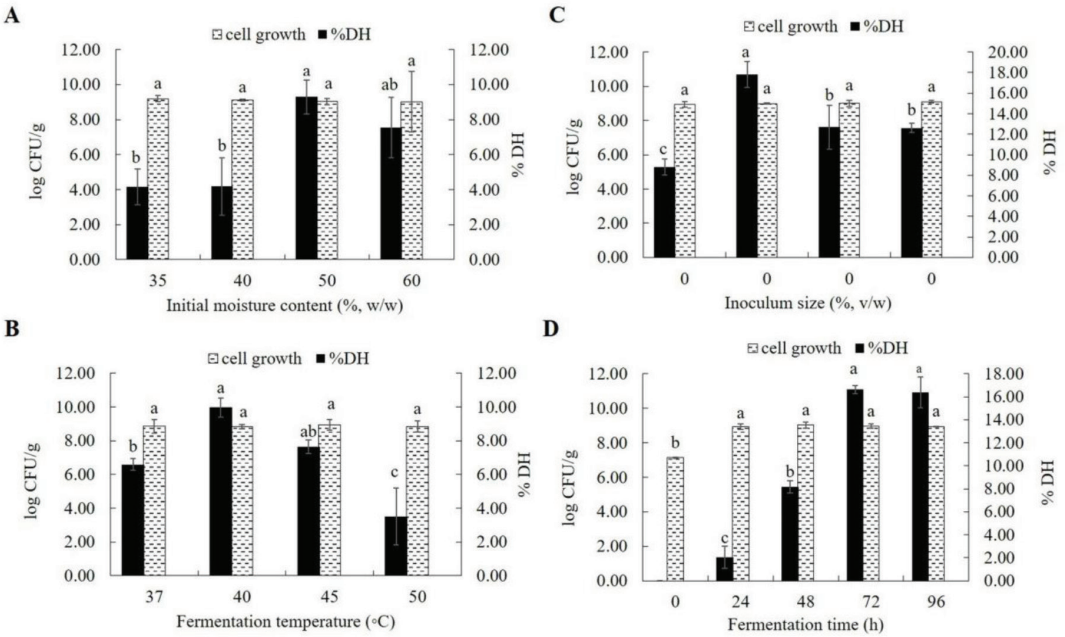

Figure 3: Effects of moisture content (A), temperature (B), inoculum size (C) and time (D) on bacterial growth (log CFU/g) and degree of protein hydrolysis (DH) in soybean meal fermentation. Data were expressed as means ± SD. Different letters within the same data set (cell growth or % DH) indicate significant differences at P<0.05

Considering the DH, a significant effect of moisture content on protein hydrolysis was observed. The highest DH (9.30 ± 0.97%) was obtained in fermented soybean meal at 50% moisture content. Similar to previous results, increasing the moisture content in SSF can improve the bacterial growth and hydrolytic enzyme production (Kook *et al.*, 2014; Wang *et al.*, 2014b). The DH of soybean meal effected by fermentation temperatures was also examined.

One of the important factors in SSF is temperature. It has been reported that the optimal temperature for the growth of different microorganisms is different (Zhang *et al.*, 2017). Figure 3 (B) shows that fermentation temperature affected DH significantly. The highest DH (9.97 ± 0.58%) was achieved when soybean meal was fermented at 40°C, whereas the DH decreased sharply when increased the temperature to 50°C. However, the DH of soybean meal fermented at 40 and 45°C did not differ significantly ($P<0.05$). This result is consistent with the protease production of *B. velezensis* K1 in liquid medium that was previously presented.

Growth and proteolytic activity of this strain at moderately elevated temperatures in the range of 37 - 45°C suggested more advantages for practical application in SSF than those of the previously reported strains which showed optimal fermentation of soybean meal at 37°C (Liu *et al.*, 2020; Chen *et al.*, 2021). For SSF processing, the metabolic heat from microbial growth is generated and accumulated in the substrate, resulting in a rapid increase in fermenter temperature (Pandey, 2003; Manpreet *et al*., 2005, Wang *et al*., 2014b). Additionally, large-scale fermentation in industry is conducted in a closed system, which generates the heat increment (Matsushita *et al*., 2016). The temperature in some areas of the fermenter may be 20°C higher than the incubation temperature (Pandey, 2003).

Apart from moisture content and temperature, the effects of bacterial inoculum and fermentation time on bacterial growth and DH of soybean meal were also investigated. As can be seen in Figure 3 (C), the inoculum size had a significant influence on the DH but not on cell growth. The highest DH was obtained when using an inoculum size of 10% (v/w).

Fermentation time also showed a remarkable effect on DH (Figure 3 (D)), revealing that DH was increased with the increment of time and remained constant after 72 hours of fermentation. It has been reported that the optimum conditions for soybean meal fermentation are different depending on microorganism. Jia *et al.* (2013) reported the optimization conditions of SSF to improve the DH of soybean meal by *B. subtilis* BS-GA15 at 30°C, soybean meal and water with ratio of 1:1 (w/w) and 10% inoculum.

Meanwhile, the optimal fermented soybean meal production conditions with the minimum trypsin inhibitor content by *Lactobacillus brevis* were 47.2% of moisture, pH 5.1, 10% inoculum and 72 hours (You-ling *et al.*, 2013). In this study, the optimal fermentation conditions for fermented soybean meal production by *B. velezensis* K1 with the highest DH of soybean meal (16.65 ± 4.08%) via SSF were delineated as follows: 50% (w/w) moisture content, 10% (v/w) inoculum size, 40°C for 72 hours.

Protein Quality Improvement and Allergenic Protein Degradation

Although soybean meal contains a high protein content, its nutritive value is low due to the complex and allergenic proteins (glycinin and β-conglycinin). There have been reported that these proteins are one of the anti-nutritional factors, which cause reduced growth performance and diarrhoea in young animals (Hotz & Gibson, 2007; Medeiros *et al.*, 2018).

Therefore, it is necessary to degrade these proteins into oligopeptide and free amino acids which are more readily utilized by animals before applying them in animal feed (Zhang *et al.*, 2017). The protein profiles of SBM, control (un-inoculated SBM) and fermented soybean meal with K1 at optimal fermentation conditions are shown in Figure 4. SBM, control and fermented soybean meal at 0 hour had the same protein profile in the range of between 20 and 100 kDa

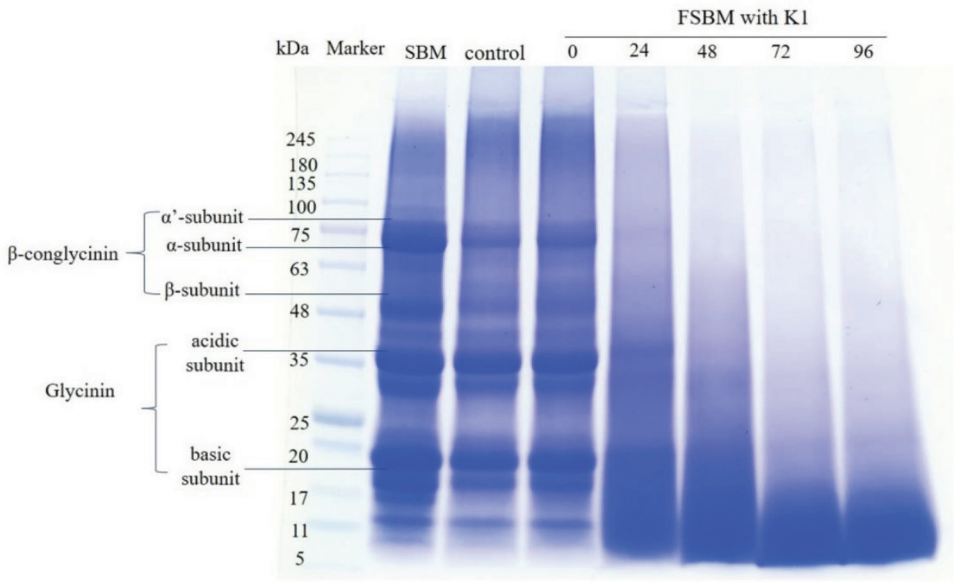

Figure 4: SDS-PAGE protein patterns of soybean meal and fermented soybean meal at different fermentation times (0, 24, 48, 72 and 96 hours). Marker: Protein molecular weight marker (3.5-245 kDa), SBM: Raw soybean meal, control: Un-inoculated autoclaved soybean meal. FBSM with K1: Autoclaved soybean meal fermented with *B. velezensis* K1 at optimal fermentation conditions (50% moisture content, 10% inoculum size (v/w), 40°C)

with dense protein bands of β-conglycinin and glycinin at 75, 48, 35 and 20 kDa.

The approximate molecular mass of α', α and β-subunits of β-conglycinin were reported as 90.5, 71.5 and 55.2 kDa, respectively, whereas the acidic and basic subunits of glycinin were 37.6 and 19.8 kDa, respectively (Sadeghi *et al.*, 2006).

After fermentation, these bands were almost completely degraded and the small molecular weight proteins less than 17 kDa were observed on SDS-PAGE. The hydrolytic activity of protease enzyme produced by *B. velezensis* K1 during the fermentation caused decreasing of complex proteins and allergens.

The result was in accordance with the previous studies on solid-state fermentation by *B. subtilis* and *Enterococcus faecium* (Shi *et al.*, 2017), *Saccharomyces cerevisiae*, *B. amyloliquefaciens* and *Lactobacillus* spp. (Chi & Cho, 2016) can remove the anti-nutritional factors as well as allergens from the soybean meal.

Apart from the protein profile on SDS-PAGE, the amounts of glycinin and β-conglycinin in Table 1 were also determined. The glycinin and β-conglycinin contents in raw soybean meal was 86.97 and 51.71 mg/g sample, respectively. After sterilization by steaming at 121°C for 15 minutes, these compounds decreased to 6.51 and 5.08 mg/g sample, respectively. This can be explained by the fact that glycinin and β-conglycinin could be denatured at temperatures of around 92 and 72°C, respectively (Liu *et al.,* 2004).

Also, it was reported that fermentation by microorganisms is an effective way to degrade allergenic proteins in soybean meal. For instance, Zheng *et al.* (2017) presented that *in vitro* digestibility and absorbability of soybean meal after fermentation by *B. siamensis* isolate JL8 were enhanced by 86.0% and 70.3% due to the reduction of protein allergens content, respectively.

Shi *et al.* (2017) have reported that the amount of soybean allergenic proteins in the

Table 1: Effects of fermentation on glycinin and β-conglycinin content and degradation

Samples	Glycinin		β-conglycinin	
	Amount (mg/g sample)	Degradation (%)	Amount (mg/g sample)	Degradation (%)
SBM	86.97 ± 6.10	-	51.71 ± 1.65	-
Control	6.51 ± 0.71	-	5.08 ± 0.23	-
FSBM	0.23 ± 0.13	96.47	2.56 ± 0.57	49.60

Degradation rate = (allergenic soy protein content of control – allergenic soy protein content in FSBM)/allergenic soy protein content of control) x 100% SBM: Raw soybean meal, Control: Un-inoculated autoclaved SBM, FSBM: Autoclaved SBM fermented with K1 at optimal fermentation conditions for 72 hours

mixed feed (corn and SBM) decreased after the fermentation by *B. subtilis* and *E. faecium*. Moreover, it has been recently presented that the solid-state fermentation of soybean meal with *B. subtilis* and *E. faecium* (Wang *et al.*, 2020) as well as *B. velezensis* and *L. plantarum* (Chen *et al.*, 2021) could decrease glycinin and β-conglycinin in soybean meal.

Similarly, when compared with the control (un-inoculated autoclaved SBM), both allergenic proteins in FSBM, glycinin and β-conglycinin were declined by 96.47 and 49.60%, respectively. This result indicated the ability of *B. velezensis* K1 to hydrolyze the high molecular mass proteins and allergens in soybean meal.

Molecular Weight (MW) Distribution of Proteins

An important parameter reflecting protein hydrolysis is MW of protein distribution (Wang *et al.*, 2014a). As presented in Table 2, after 72 hours of fermentation, the proportion of large MW proteins (> 30 kDa) decreased significantly from 63.48% to 49.01%.

Simultaneously, the smaller ones (<10 kDa) increased from 34.05% to 48.22%. These results agree well with those of Chi and Cho (2016) as well as Yang *et al.* (2019) on the increment of small proteins/peptide fraction (<3 kDa) after microbial fermentation.

The increase of the small MW proteins was also consistent with the protein pattern of fermented soybean meal on SDS-PAGE (Figure 4). At this point, it could be confirmed that the secretion of proteolytic enzymes during the fermentation of *B. velezensis* K1 not only resulted in soybean meal protein quality improvement but also allergenic protein elimination.

The increased small MW proteins and low level of glycinin and β-conglycinin in fermented soybean meal products by *B. velezensis* K1 would be beneficial as animal feed due to its facilitated digestion, more readily absorbed and utilized by animals, especially young animals (Zhang *et al.*, 2017).

Table 2: Distribution of protein molecular weight in FSBM

Molecular Weight (kDa)	FSBM with K1	
	0 hour	72 hours
>30	63.48 ± 6.06%^{a}	49.01 ± 4.06%^{b}
10-30	2.54 ± 0.27%^{a}	2.78 ± 0.07%^{a}
<10	34.05 ± 6.28%^{b}	48.22 ± 4.00%^{a}

Data were expressed as means ± SD. Means in the same row with different letters indicate significant differences at P<0.05

Sugar Profile and Raffinose Family Oligosaccharides Degradation in FSBM

Figure 5 shows the sugar profile of FSBM at 0 and 72 hours under the optimal fermentation conditions. As can be seen, there were no differences in the sugar profile (number of sugar types, i.e., stachyose, raffinose, sucrose and fructose) of fermented soybean meal at 0 and 72 hours. Considering the peak areas of each sugar that corresponded to the sugar amount, they were decreased after fermentation. This indicated that *B. velezensis* K1 could utilize these sugars in soybean meal as a source of carbon.

To obtain degradation (consumption) rates of these sugars, the amounts of raffinose family oligosaccharides mainly stachyose and raffinose as well as sucrose in fermented soybean meal were also quantified as presented in Table 3.

The amounts of stachyose, raffinose and sucrose in raw soybean meal and fermented soybean meal at zero hours were not significantly different. Sucrose and stachyose, on the other hand, decreased remarkably after fermentation (96.8 and 73.6%, respectively). Several studies have shown that α-galactosidase, invertase or both can completely hydrolyse raffinose family oligosaccharides into monosaccharides.

Galactose and sucrose are released as the end products by α-galactosidase activity while invertase cleaves the α-1,2 linkage between fructose and glucose in sucrose, raffinose or stachyose, producing melibiose and fructose (Rehms & Barz, 1995; Rezende *et al.*, 2005; Singh & Vij, 2017).

However, this research paper found that *B. velezensis* K1 cannot produce the α-galactosidase enzyme because there was no blue colony formed in the selective medium. On the other hand, as presented earlier in Figure 1, *B. velezensis* K1 can grow on the selective medium with only sucrose. This indicated the ability to produce invertase enzyme of this strain.

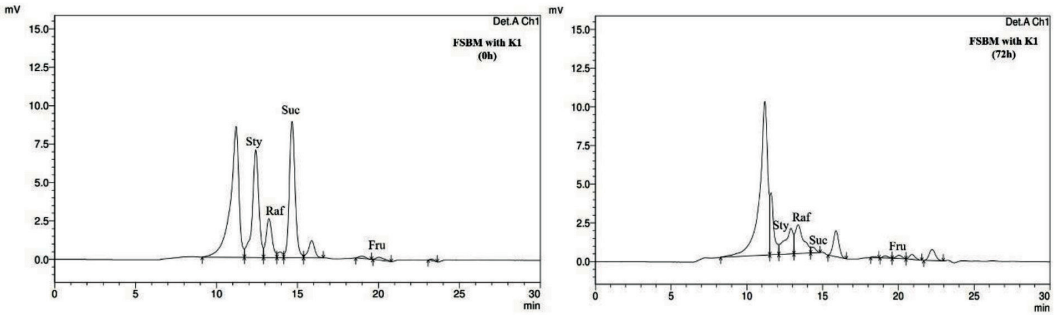

Figure 5: HPLC chromatograms of sugars in fermented soybean meal (FSBM) samples at optimal fermentation conditions at 0 hour (left) and 72 hours (right). Fru: Fructose, Glu: Glucose, Suc: Sucrose, Raf: Raffinose and Sty: Stachyose

Table 3: The sugar content in soybean meal and fermented soybean meal with *B. velezensis* K1 sample at the optimal fermentation conditions

Samples	Sugar Content (g/100 g of Sample)		
	Stachyose	Raffinose	Sucrose
SBM	3.69 ± 0.17^{a}	1.25 ± 0.06^{a}	4.01 ± 0.15^{a}
FSBM at 0 hour	3.67 ± 0.88^{a}	1.23 ± 0.29^{a}	3.70 ± 0.69^{ab}
FSBM at 72 hours	0.97 ± 0.13^{b}	0.95 ± 0.02^{a}	0.12 ± 0.03^{c}

Data were expressed as means ± SD. Different letters indicate significant differences at $P<0.05$. SBM: Raw soybean meal, FSBM: Autoclaved soybean meal fermented with *B. velezensis* K1 at the optimal fermentation condition (50% (w/w) moisture content, 10% (v/w) inoculum size at 40°C)

Additionally, the sucrose content in fermented soybean meal was decreased after the fermentation (Table 3). Based on these two findings, the capability of *B. velezensis* K1 to produce invertase enzyme during fermentation is proven. Similar to previous results of Chi and Cho (2016), *B. amyloliquefaciens* U304 can secrete proteolytic and glycolytic enzymes to degrade protein anti-nutritional factors and carbohydrate anti-nutritional factors (stachyose and raffinose) during soybean meal fermentation.

With the capacity to produce both proteolytic and carbohydrate hydrolytic enzymes, *B. velezensis* K1 could be a good candidate to increase the nutritional value of soybean meal by degrading allergenic proteins and raffinose family oligosaccharides, primarily stachyose and raffinose.

Although soybean meal is rich in protein, it also contains a high carbohydrate content. Non-starch polysaccharides (NSP) (cellulose, hemicellulose and pectin) and free sugars (mono-, di- and oligosaccharides) are the main carbohydrates found in soybean meal (Choct *et al.*, 2010).

Both NSP and some oligosaccharides (raffinose and stachyose) have been reported as anti-nutritional factors in pigs and poultry. It is because of the lack of endogenous enzymes in these animals to digest those of anti-nutritional factors (Opazo *et al.*, 2012; Wongputtisin *et al.*, 2012; Tian *et al.*, 2019). Thus, the bacteria used in fermented soybean meal production should be able to produce carbohydrate hydrolytic enzymes to hydrolyze carbohydrate-based anti-nutritional factors in soybean meal as mentioned before.

In this study, *B. velezensis* K1 can produce some carbohydrate hydrolytic enzymes such as amylase, cellulase, invertase and pectinase. However, no α-galactosidase activity was observed. These findings suggest that *B. velzensis* K1 has the feasibility of being used in the soybean meal fermentation process to eliminate anti-nutritional factors like raffinose family oligosaccharides.

To broaden the applicability of this work, further investigations should be conducted to determine whether fermented soybean meal produced by *B. velezensis* K1 could benefit the health and growth performance of young animals.

Conclusion

The optimal conditions for fermented soybean meal production by *B. velezensis* K1 via SSF were obtained as follows: 50% (w/w) moisture content, 10% (v/w) inoculum size at 40°C, 72 hours. Under these conditions, high mass proteins (20-100 kDa) were significantly hydrolysed into the small molecules (<10 kDa) as well as the soybean allergens (glycinin and β-conglycinin) were removed.

Furthermore, raffinose family oligosaccharides especially stachyose and raffinose were also hydrolysed. It suggested that *B. velezensis* K1 can increase the nutritional values of soybean meal and that fermented soybean meal products can be excellent protein sources that could be applied to animal feed.

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