THERMOTOLERANT α-GALACTOSIDASE PRODUCING BACTERIA APPLIED FOR FERMENTATION OF SOYBEAN MEAL IN ANIMAL FEED

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Received: July 25, 2019; Revised: December 10, 2019; Accepted: December 11, 2019

Abstract

Raffinose family oligosaccharides (RFOs) are one of anti-nutritional factors (ANFs) found in soybean meal (SBM) and unable to hydrolyze by enzymes in intestinal tract of monogastric animals resulted in flatulence which reduced their diet performance. Removal these oligosaccharides from SBM by α-galactosidase is therefore an effective approach for monogastric animal feeds. The aim of this research was to isolate and screen thermotolerant bacteria capable to digest RFOs in SBM. Only 11 out of 28 thermotolerant isolates appeared light blue colony on selective medium. The isolates showed the α-galactosidase activity in range of 0.100-0.600 U/ml. By 16S rDNA sequence analysis, the isolate S2 having enzyme activity at 0.417 U/ml was identified as *Bacillus megaterium* which is one of bacteria in the list of animal feed supplement. The α-galactosidase production of that S2 isolate was maximal reached at 32 h after cultivating in LB supplemented 1% raffinose. Increasing of *B. megaterium* S2 growth on SBM from 7.47 to 9.46 log CFU/g indicated its ability to use SBM as sole nutrient source. Moreover, it was also able to degrade 87.21% raffinose and 97.46% stachyose in autoclaved SBM within 72 h of fermentation which indirectly suggested the removal of flatulence-causing oligosaccharides by its α-galactosidase activity. Therefore, *B. megaterium* S2 could be potentially applied for production of fermented SBM containing low RFOs further used as animal feed supplement.

Keywords: α-Galactosidase, animal feed, raffinose family oligosaccharides, soybean meal

Introduction

Soybean meal (SBM) is a main plant protein source widely used in animal feed industry. Apart from about 48% protein mainly found in SBM, carbohydrate is also a dominant (35%) composition in SBM. Those carbohydrates composed of non-starch polysaccharides (NSP) and free sugars including mono-, di- and oligosaccharides. Among free sugars content in SBM, oligosaccharides especially raffinose family oligosaccharides (RFOs) has reported as an anti-nutritional factors effected to diet performance of monogastric animals. Without α-galactosidase in intestinal tract of these animals, therefore raffinose and stachyose that are main RFOs in SBM are unable to hydrolyze and generate the flatulence of those animals (Choct *et al*., 2010). α-Galactosidase (EC 3.2.1.22) is a galactohydrolase

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enzyme. It functions by hydrolysis α-1,6-galactosidic bonds existed in these oligosaccharides resulted in α-D-galactose releasing (Scussiato De Andrade Taborda *et al*., 2016). To date, α-galactosidase from many microorganisms including bacteria, yeast, and filamentous fungi were produced and characterized (Mukesh Kumar *et al*., 2012; Lee *et al*., 2012; Park *et al*., 2012; Hema and Helen, 2012; Lee *et al*., 2013a, 2013b; Carevic *et al*., 2016; Scussiato De Andrade Taborda *et al*., 2016). In case of bacteria, there has been reported the α-galactosidase producing bacteria in many species such as *Bacillus* sp. SPE10 and *Bacillus* sp. SPE15 (Mukesh Kumar *et al*., 2012), *Bacillus* sp. LX-1 (Lee *et al*., 2012; Lee *et al*., 2013a, 2013b), *Paenibacillus* sp. LX-20 (Park *et al*., 2012), *Pseudomonas* sp. MCCMB3 (Hema and Helen, 2012), *B. licheniformis*, *B. megaterium*, *Lactobacillus plantarum*, *L. reuteri*, *L. helveticus*, *L. fermentum*, *L. acidophilus*, *L. lactis*, *Leuconostoc mesenteriodes*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptomyces erythrus*, *S. griseoloalbus* and *Sulfolobus solfataricus* (Carevic *et al*., 2016). However, isolation of microorganisms producing thermotolerant α-galactosidase is rare. Thermophilic microorganisms, which are capable to grow at optimum temperature 50°C or above is more interesting than other extremophiles (Mohammad *et al*., 2017). It dues to their potential to produce thermostable enzymes which is beneficial for many industries which are getting involved in high temperature processes. The fermentation temperature to produce fermented SBM in feed industry is in range of 30-45°C depended on the operation system of each producer. Considering to the advantages of thermostable enzymes, it is certainly that thermotolerant microorganisms are more attractive. Additionally, in order to produce fermented SBM for applying as animal feed supplement in Thailand, microorganisms in the list of qualified presumption of safety (QPS) by European Food Safety Authority (EFSA) must be taken consideration.

The aim of this research was focused on the isolation and screening of thermotolerant bacteria to reduce RFOs especially raffinose and stachyose in SBM. Moreover, bacteria permitted to use in animal feed production in Thailand is also emphasized. The fermented SBM with low RFOs obtained from this study can further apply as feed supplement for improving the diet performance of monogastric animals.

Materials and Methods

Bacterial Isolation

Soya milk (SM), fermented SBM (F), yoghurt (Y), fermented vegetable (FV), and soybean growing soil (S) were used for bacterial isolation. The SM, Y and FV were purchased from grocery shops in local market of Nakhon Pathom Province, Thailand. While the S and F was collected from agricultural field in Nakhon Pathom Province and Feed Techno Focus Co., Ltd in Chachoengsao, respectively. To isolate bacteria, suspended those of the materials in sterile distilled water with ratio of 1:4 (w/v) and incubated at 37ºC, overnight. The sample suspensions with dilution of 10^{-1}-10^{-4} were then plated on nutrient agar medium (NA) and incubated at 37ºC for 24 h. Picked up the colonies growing on medium and re-streaked until pure colonies were obtained. The pure isolates were sub-cultured on NA slants and preserved at 4ºC for further analysis.

Thermotolerant α-Galactosidase Producing Bacterial Screening

To obtain thermotolerant bacteria, inoculated single colony of the previous isolates in 5 ml of nutrient broth (NB) and incubated at 37, 45, and 50ºC for 24 h. Then streaked the culture broths on NA and incubated at those of each temperature for additional 24 h. The isolates growing at the temperature up to 50ºC were selected for screening of α-galactosidase production.

The secondary screening was conducted by streaking the thermotolerant isolates on selective agar medium (LB supplementing 0.2% lactose and 32 μg/ml of 5-bromo-4-chloro-3-indolyl-α-D-gal (X-α-Gal) and incubated at 37°C for overnight (Lee *et al*., 2012). The isolates showing blue colonies were further determined for α-galactosidase activity.

α-Galactosidase Activity Determination

Inoculated single colony of the selected thermotolerant isolates in 5 ml nutrient broth which consisted of 1% raffinose, 3% peptone, 0.05% yeast extract, and 10% salt solution (1% K_2HPO_4, 0.9% $MgCl_2.6H_2O$, 0.1% $CaCl_2$, 1% NaCl) and incubated at 37ºC on rotary shaker with 150 rpm for 24 h. Separated the cell pellets by centrifugation at 10,000 rpm, 4ºC for 8 min. Then, washed cell pellets with 0.1 M sodium citrate buffer pH 5.78. The cell pellets were re-suspended with 5 ml of the same buffer and disrupted with ultrasonication at 20 kHz for 4 min (2 min pause on/off). To remove cell debris, the suspensions were centrifuged at 10,000 rpm at 4ºC for 8 min. The supernatant was collected for α-galactosidase activity determination.

The α-galactosidase activity was measured by using *p*-nitro phenyl-α-D-galactopyranoside (*p*NPG) as substrate (Du *et al*., 2012). The reaction mixture contained 50 μl of properly diluted supernatant, 50 μl of 10 mM *p*NPG solution in 0.1 M sodium

citrate buffer pH 5.78. The mixture was incubated at 37ºC for 10 min. The reaction was then stopped by adding 400 µl of 0.5 M Na_2CO_3. The enzyme activity was determined from the released amount of *p*-nitrophenol (*p*NP) which was spectrophotometrically measured at 405 nm. One unit (U) of enzyme activity was defined as the amount of released 1.0 µmole of *p*NP from substrate per ml per min under the assay condition.

Bacterial Identification

The bacteria were identified by morphological observation, gram staining and 16s rDNA gene analysis. For 16s rDNA gene analysis, the nucleotides were amplified by PCR with primer pair of 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') or 785F (5'GGA TTA GAT ACC CTG GTA3') and 907R (5'CCG TCA ATT CMT TTR AGT TT3'). The PCR condition for gene amplification was performed by the Humanizing Genomics macrogen Inc. Briefly, *Taq* polymerase was activated at 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 55°C, and 72°C for 1 min each. Ended up with a 10 min at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. Homology sequence analysis was performed by Blastn program via GenBank database (NCBI).

Growth Kinetics and α-Galactosidase Production

Inoculated single colony of *B. megaterium* S2 to 10 ml of LB medium and incubated at 37ºC for 24 h on rotary shaker with 150 rpm. Then, transferred 5% inoculum (OD600 = 0.6) to the 50 ml of LB and incubated at the same condition for another 24 h. The broth was consequently transferred to 1 L flask containing 500 ml of LB supplemented with 1% raffinose and cultivated under the same condition. Sample (8 ml) was withdrawn for measurement of cell growth and α-galactosidase activity. Cell growth was monitored by measuring the absorbance at 600 nm while the determining of enzyme activity was followed by the previously mentioned method.

Growth of The Isolated Bacteria on Soybean Meal Determination

SBM obtained from an animal feed company (Feed Techno Focus Co., Ltd) was moisten to 40% by mixing with distilled water. The SBM was then autoclaved at 121ºC for 15 min. Inoculated single colony of bacterial strain in 5 ml of LB and incubated on rotary shaker with 150 rpm, at 37ºC for 24 h. Then, transferred 10% inoculum (OD600 = 0.6) to the autoclaved SBM. The fermentation was conducted at 37ºC for 72 h. The fermented SBM was dried at 60°C for 24 h by hot air oven. Grinded the fermented SBM to powder before sugar contents analysis.

Degradation of Sugars (Glucose, Raffinose, and Stachyose)

Inoculated single colony of bacterial strain in 5 ml of LB and incubated on rotary shaker with 150 rpm, at 37ºC for 24 h. Then, transferred 1% inoculum (OD600 = 0.4) to LB supplemented 1% sugars (glucose, raffinose, or stachyose) as carbon source. Cultivated on rotary shaker with 150 rpm at 37ºC for 24 h. Bacterial growth was determined by spectrophotometry at optical density (OD) 600 nm. The supernatant after removal of cell pellet was taken to sugar analysis.

Sugar Analysis

Sugar analysis in terms of total sugar, reducing sugar and sugar type was conducted by phenol sulfuric method (Dubois *et al*., 1956), DNS method (Miller, 1959) and HPLC (Bampensin *et al*., 2019), in respectively.

The mono-, di- and oligosaccharides (stachyose and raffinose) were qualified and quantified by HPLC (Shimadzu LC-20A, Japan) equipped with refractive index detector (Shimadzu RID, Japan) and Rezek RNM carbohydrate column of Phenomenex (7.8×300 ml). Briefly, the extract or supernatant was filtered through 0.22 µm filter before injection to HPLC system. A volume of 10 µl of each sample was injected to the HPLC system with 100% deionized water as mobile phase. The temperature of system was set at 45°C and flow rate of mobile phase was 0.4 ml/min. The sugar type and concentration were analyzed by comparison retention time of each sugar and area peak of HPLC chromatogram of extract or supernatant with standard sugar.

Results and Discussion

Isolation and Screening of Thermotolerant α-Galactosidase Producing Bacteria

A total of 37 bacterial colonies were isolated from various sources as aforementioned. Primary screening of the isolated bacteria at different temperatures (37, 45, and 50ºC) revealed that only 28 isolates were capable to grow at temperature up to 50ºC as shown in Table 1. According to the optimum growth temperature of thermophilic microorganisms is 50ºC or above (Mohammad *et al*., 2017) therefore the 28 primary screened isolates are in a group of thermotolerant bacteria.

Table 1. Growth of the 37 isolated bacteria at 37, 45, and 50°C

Isolation source	Isolate	Thermotolerant test		
		37°C	45°C	50°C
Soya milk	SM1	+	+	+
	SM2	+	+	+
	SM3	+	+	+
	SM4	+	+	-
	SM5	+	+	+
	SM6	+	+	+
Soybean growing soil	S1	+	+	+
	S2	+	+	+
	S3	+	+	-
	S4	+	+	-
	S5	+	+	-
	S6	+	+	-
	S7	+	+	-
Fermented soybean meal	F1	+	+	+
	F2	+	+	+
	F3	+	+	+
Yoghurt	Y1	+	+	+
	Y2	+	+	-
	Y3	+	+	-
	Y4	+	+	+
	Y5	+	+	-
Fermented vegetable	FV1	+	+	+
	FV2	+	+	+
	FV3	+	+	+
	FV4	+	+	+
	FV5	+	+	+
	FV6	+	+	+
	FV7	+	+	+
	FV8	+	+	+
	FV9	+	+	+
	FV10	+	+	+
	FV11	+	+	+
	FV12	+	+	+
	FV13	+	+	+
	FV14	+	+	+
	FV15	+	+	+
	FV16	+	+	+
Total		37	37	28

+ and - indicates growth and non-growth of bacteria on NA medium for 24 h.

For screening of α-galactosidase producing bacteria, the light blue colony of 11 out of 28 isolates appeared on selective medium indicates their ability to produce that enzyme. As shown in Figure 1, the α-galactosidase activity in range of 0.100-0.600 U/ml was observed from all of those isolated thermotolerant bacteria. The isolate FV9 showed the highest enzyme activity at 0.600 U/ml followed by FV4 at 0.419 U/ml and S2 at 0.417 U/ml, respectively.

Bacterial Identification

Gram staining suggested isolate FV9 and FV4 is negative gram while S2 is a rod shape and positive gram bacterium. By amplification of 16s rDNA gene with primer pair of 785F and 907R, the nucleotide sequence of the isolate FV9 and FV4 derived from fermented vegetable were 99% identical to *Cronobacter malonaticus* and *C. sakazakii*, respectively. In case of strain S2, it shows 99% identical of 16s rDNA sequence to *Bacillus megaterium* in Genbank database. *C. sakazakii*, *C. malonaticus* are two of seven species which belonged to *Cronobacter* spp. It is rod shape and negative gram bacterium. Additionally, it is considered as pathogenic bacteria in food that effected to children and the elderly illness (Parra-Flores *et al*., 2018). While *Bacillus megaterium* is one of probiotic *Bacillus* species (Elshaghabee *et al*., 2017) and has also reported in probiotic formulas used in nutrition of pig (Markowiak and Śliżewska, 2018). Besides that, it is in the list of qualified presumption of safety as previously mentioned, therefore it can use for SBM processing in animal feed industry. Moreover, the capable of its growth at the temperature up to 50°C leaded to take its advantage in fermentation process of animal feed ingredients production which involved rather high temperature. Its growth temperature is consistent with the one previously isolated from sugarcane industrial waste, *Bacillus megaterium* VHM1 (Patil *et al*., 2010). However, the higher α-galactosidase activity of strain VHM1 than that determined in S2 may due to the different carbon source in production medium. *B. megaterium* VHM1 grew in production medium supplemented guar gum while *B. megaterium* S2 used raffinose as carbon source. In order to increase enzyme activity of *B. megaterium* S2, optimization of carbon source in enzyme production medium should be further investigated.

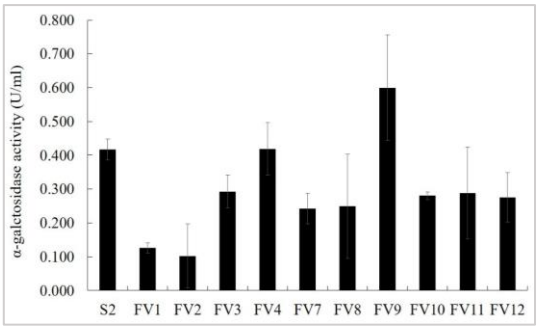

Figure 1. α- Galactosidase activity of 11 screened thermotolerant bacteria. S, soybean growing soil; FV, fermented vegetable

Growth Kinetic and α-Galactosidase Production

The growth profile and α-galactosidase production of *B. megaterium* S2 showing in Figure 2 indicated lag phase is in the first 2 h of cultivation; thereafter the bacterial grew very rapidly and reached maximum biomass within 48 h of fermentation. Meanwhile, α-galactosidase production started at the exponential phase (18 h) and increased quickly up to 0.470 U/ml at 32 h of fermentation. After that, the enzyme production slowly decreased. It seems to be growth-associated enzyme. In

B. megaterium VHM1, α-galactosidase production maximum achieved after 20 h of fermentation (Patil *et al*., 2010) while the highest α-galactosidase production by *Bacillus* sp. LX-1 was observed at a 72 h of fermentation. (Lee *et al*., 2012). It indicated that the growth and enzyme production of each microorganism is different depended on strain, medium, and the cultivation condition. Moreover, the α -galactosidase produced by *B. megaterium* S2 is an intracellular enzyme while enzyme from an alkalophilic bacterium, *B. megaterium* VHM1 reported as an extracellular (Patil *et al*., 2010). There has reported that α-galactosidase of *B. amyloliquefaciens* strain H102, *Bacillus* sp. and *B. thuringiensis* strain 2PR5618 isolated from legumes was also an intracellular enzyme (Kumar *et al*., 2014). Different physical culture conditions for enzyme production may effect to enzyme secretion of each strain even in the same genus. Higher activity of the enzyme in culture broth was detected when *B. megaterium* VHM1 was grown at 50-55°C while lower activity of that enzyme observed at 35-45°C and 60-65°C. For *B. megaterium* S2, the same temperature at 37°C as those cultures of *B. amyloliquefaciens strain* H102, *Bacillus* sp. and *B. thuringiensis* strain 2PR5618 (Kumar *et al*., 2014) was conducted to produce enzyme. To address this assumption, various temperatures for enzyme production should be investigated further. However, in order to increase nutritional value of SBM, directly applied cell biomass of *B. megaterium* S2 for fermentation is feasible.

Growth of *B. megaterium* S2 on Soybean Meal

The aim of this study was isolation and screening of thermotolerant bacteria to reduce RFOs especially raffinose and stachyose which are flatulence-causing sugars in SBM, therefore the growth of *B. megaterium* S2 on SBM without other nutrients adding was performed. The composition of SBM obtained from the animal feed company used in this study was shown in Table 2. It contained 29.1% carbohydrate (include dietary fiber), 3% fat and 50.2% protein which indicated the richness of carbon and nitrogen source. As shown in Figure 3, increasing of *B. megaterium* S2 growth on autoclaved SBM with moisture content of 40% for 72 h from 7.47 to 9.46 log CFU/g indicated its ability to use soybean meal as sole nutrient source.

The growth of *B. megaterium* S2 is consistent with the decreasing of total sugar content upon the time course of cultivation as shown in Figure 4. It indicates the sugar consumption of *B. megaterium*

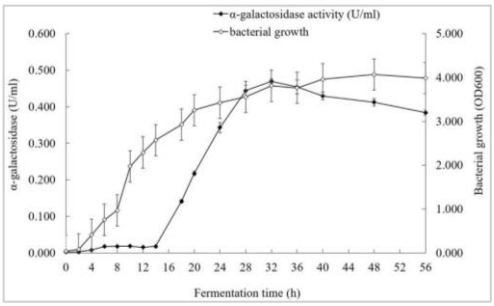

Figure 2. Growth kinetic and α-galactosidase production of *B. megaterium* S2. Cells were grown in LB medium supplemented with 1% raffinose at 37°C on rotary shaker at 150 rpm

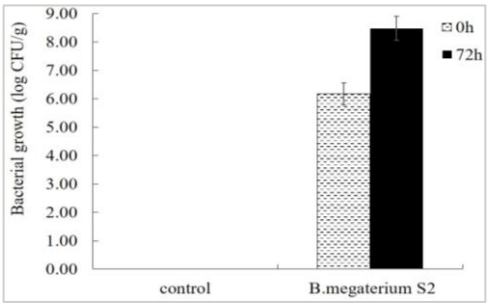

Figure 3. Growth of *B. megaterium* S2 on soybean meal with 40% moisture content for 72 h. Control, autoclaved SBM with 40% moisture content without bacterial inoculation

Table 2. The composition of SBM

Compounds	unit	The amount of compound	Method
Ash	g/100g	6.2	AOAC (2012), 942.05
Total carbohydrate (Include Dietary Fiber)	g/100g	29.1	Method of Analysis for nutrition labelling (1993) p.106
Calories (Include dietary fiber)	kcals/100g	344	Method of Analysis for nutrition labelling (1993) p.106
Fat	g/100g	3.0	Based on AOAC (2012), 954.02
Fiber (crude)	g/100g	3.0	Based on AOAC (2012), 978.10
Moisture	g/100g	11.5	AOAC (2012), 930.15
Protein (N×6.25)3.0	g/100g	50.2	Based on AOAC (2012), 981.10
Stachyose	%	3.80	HPLC
Raffinose	%	1.84	HPLC

S2 for increasing biomass. Increasing of reducing sugar in autoclaving SBM (control 0 h) from initial to the end of cultivation (*B. megaterium* S2, 72 h) suggested the production of enzymes involved in sugar cleavage by *B. megaterium* S2. The released reducing sugars were then consumed by bacteria for biomass accumulation. It is generally known that microorganisms required preferred sugars for their growth.

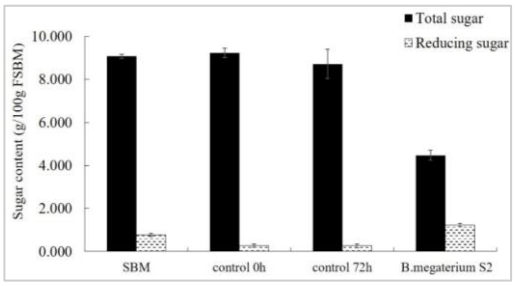

Figure 4. Total sugar and reducing sugar profile when cultivating *B. megaterium* S2 on soybean meal with 40% moisture content for 72 h. SBM, unautoclaved SBM; control oh, autoclaved SBM with 40% moisture content without bacterial inoculation; control 72 h, autoclaved SBM with 40% moisture content without bacterial inoculation for 72 h; *B. megaterium* S2, autoclaved SBM with 40% moisture content with bacterial inoculation for 72 h

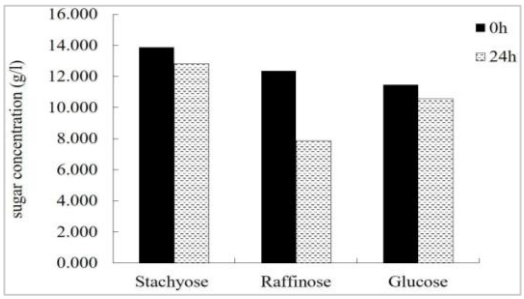

Figure 5. Stachyose, raffinose and glucose concentration in LB when *B. megaterium* S2 was cultured for 0 h and 24 h

Degradation of Sugars (Glucose, Raffinose, and Stachyose) by *B. megterium* S2

According to α-galactosidase plays major role in cleavage of terminal non-reducing α-1,6-linked galactosyl residues from diverse range of galacto-oligosaccharides and polysaccharides (Naumoff, 2004), the capable of *B. megaterium* S2 to digest oligosaccharides was then conducted both in cultivation of bacterium in LB containing each sugar type as sole carbon source and SBM as nutrient source. As shown in Table 3 and Figure 5, degradation of stachyose, raffinose, and glucose in LB was 7.58, 36.40 and 7.88%, respectively. Higher degradation of raffinose than the rate that detected in glucose suggested the production of α-galactosidase by *B. megaterium S2*.

Table 3. Degradation of stachyose, raffinose and glucose supplemented in LB by *B. megaterium* S2

Sugar (g/l)	Control	*B.megaterium* S2	%Degradation
Stachyose	13.883	12.831±0.557	7.58
Raffinose	12.362	7.862±0.369	36.40
Glucose	11.459	10.556±0.239	7.88

Control, LB containing 1% stachyose, raffinose, or glucose without bacterial inoculation *B. megaterium* S2; LB containing 1% stachyose, raffinose, or glucose with bacterial inoculation; % Degradation, sugar content in control- sugar content in medium with bacterial inoculation/sugar content in control ×100

The ability of *B. megaterium* S2 to reduce oligosaccharides in SBM was also determined by cultivating bacterium in unautoclaved and autoclaved SBM. As shown in Table 4, it was found that 92.63% and 97.46% of stachyose as well as 94.31% and 87.21% of raffinose from unautoclaved and autoclaved SBM, respectively were degraded by *B. megaterium* S2. It indirectly suggested the removal of these galacto-oligosaccharides by its α-galactosidase activity. It is in agreement with the ability to hydrolyze stachyose and raffinose in soy milk of *Bacillus megaterium* VHM1 (Patil *et al*., 2010). Therefore, *B. megaterium* S2 reported as a probiotic, nonpathogenic organism, and is in the list of qualified presumption of safety by European Food Safety Authority can apply to remove RFOs from SBM for improving the nutritional value of animal feed.

Table 4. Degradation of stachyose and raffinose in soybean meal by *B. megaterium* S2

Item	Stachyose		Raffinose	
	Content, g/100g	%Degradation	Content, g/100g	%Degradation
Unautoclaved SBM	3.796	-	1.840	-
Uninoculated unautoclaved SBM, 0h	4.640	-	2.302	-
Inoculated unautoclaved SBM, 72h	0.342	92.63	0.131	94.31
Uninoculated autoclaved SBM, 0h	3.620	-	1.736	-
Inoculated autoclaved SBM, 72h	0.092	97.46	0.222	87.21

%Degradation, sugar content in control-sugar content in SBM with bacterial inoculation/sugar content in control ×100

Conclusions

Bacillus megaterium S2 isolated from soybean growing soil is thermotolerant α-galactosidase produced bacteria. It can reduce stachyose and raffinose in soybean meal. Moreover, it has reported as a probiotic and nonpathogenic organism, therefore it could be used to produce fermented soybean meal with low oligosaccharides causing the flatulence of monogastric animals in feed industry.

Acknowledgments

This research was financially supported by Research and Researchers for Industries (RRI) from the Thailand Research Fund (TRF), contract no. PHD59I0073 and Silpakorn University Research, Innovation and Creativity (Fiscal Year 2560). Author would like to thank Feed Techno Focus, Co, Ltd., Thailand for supporting raw material and reagents.

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