Increased organic acid production and decreased phytate phosphorus by high level of water content of *Bacillus subtilis* ATCC PTA-6737 fermentation of soybean meal

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Abstract

In order to enrich soybean meal (SBM) by microbial fermentation, SBM (3 mm particle size) with 78 or 85% moisture content were inoculated with 0.0 or 1×10^7 CFU/g (colony forming unit) *Bacillus subtilis* ATCC PTA-6737 and fermented at 37 °C for 0, 24, 48 and 72 h. There was a marked decrease in pH with 85% moisture content, associated at higher organic acid production, compared to increased pH at 24 h with 78% moisture content and a gradual decrease hereafter. Bacterial growth peaked at 48 h with both moisture contents. Fermenting SBM increased crude ash level by 65%, soluble amino acid nitrogen by 2.7 folds and degraded phytic acid by 65-75%. Moreover, there was a high level of organic acid production despite of decreased crude protein by 6-7%, but the effect of high moisture level was more pronounced in all case. In conclusion, fermentation SBM with water content of 85% at 35 °C by *B. subtilis* provided SBM an appreciable amount of bio-functionalities such reduced phytate content, increased soluble amino acid content and more importantly fortified with organic acids. The fermented SBM can therefore be used an antimicrobial agent due to its high organic acid level in animal nutrition.

Keywords: *Bacillus subtilis*, soybean meal, fermentation, nutrient composition

Introduction

Soybean meal (SBM) high in protein content is an important plant protein feed material in animal nutrition (Senkoylu *et al.*, 2005), and the use of SBM is limited by the presence of some antinutritional factors (ANFs). In addition, the phosphorous of SBM is in the form of phytate which may not be well utilised by monogastric animals (Balloun, 1980 and Liener, 1980).

There has been high interest in testing the effects of microbial fermentation of SBM for enrichment of nutritional contents and degradation of ANFs in animal nutrition (Roubosvan den Hil *et al.*, 2009; Roh *et al.*, 2015; Chi and Cho 2016). In many studies, the size of peptides

was reduced while digestibility of some amino acids was increased by microbial fermentation of SBM (Hong *et al.*, 2004; Feng *et al.*, 2007a). Fermentation of SBM with *B. subtilis* alone caused to increased protein and soluble proteins and decreased trypsin inhibitor (TI) (Dai *et al.*, 2017). Feeding the diets containing fermented soybean meal (SBM) with *B. subtilis* BS12 with the reduced contents of antinutritional factors (ANFs) remarkably improved the performance and intestinal immune system of the piglets (Feng *et al.*, 2007b and Zhang *et al.*, 2018).

B. subtilis and *Aspergillus oryzae* were tested to ferment SBM (Teng *et al.*, 2012). The increased contents of protein, small size proteins and

antioxidant capacity and decreased contents of trypsin inhibitors and antigenic proteins were superior with *B. subtilis* than *Aspergillus oryzae*. Fermenting SBM with a mixture of *B. subtilis*, *Hansenula anomala* and *Lactobacillus casei* (2:1:2) was found a best microbial combination for increased peptide contents and reduced ANFs (Yuan *et al.*, 2017). The replacement of SBM with fermented SBM with mixed microorganisms containing *B. subtilis* at a dietary level of 10% caused significantly increased animal performance and gut health in weaned piglets (Yuan *et al.*, 2017 and Zhu *et al.*, 2017).

All the above has clearly indicated that the use of *B. subtilis* is very promising microorganism for the improvement of SBM nutritional composition. *Bacillus subtilis* is a Gram-positive, found in soil and the gastrointestinal tract (Yonsel, 2010). *B. subtilis* is safe microorganism which has been widely used in many industrial microbial applications (Constantinescu and Petruta, 2015) as well as in food and feed industry (Yonsel, 2010). Solid state fermentation (SSF) using microorganisms is a process of immersed solid materials in absolute water (Özşölen, 2010 and Mukherjee *et al.*, 2016) under an optimal temperature required for the species of microorganisms, and the effect of moisture content has been found important on nutrient enrichment of SBM (Gervais and Molin, 2003; Amadou *et al.*, 2010; Rashid *et al.*, 2011). In these studies, it was demonstrated that the ratio of added water to solid materials in SSF is crucial for improved metabolic activity of microorganism, and directly interacts together with oxygen for the removal of heat generated during the fermentation. In SSF, the water content of fermenting substrate is preferable above 60%, and moreover the optimal level of water content in SBM fermentation studies ranged from 75 to 85% water content (Amadou *et al.*, 2010; Yasar *et al.*, 2018; Karakurt *et al.*, 2019).

Therefore, the objective of this study was to test the effect of two levels of moisture (78 and 85%) during the *B. subtilis* ATCC PTA-6737 fermentation on nutritional composition of SBM.

Materials and methods

B. subtilis ATCC PTA-6737 was purchased from ATCC (LGC Standards GmbH Mercatorstr. 51 46485 Wesel Germany) in the form of lyophilised pellet, which was cultured on Brain Heart Infusion Broth at 37 °C for 24 h. Active cultured

cells were suspended in saline solution after the centrifugation of 15000 rpm at 4 °C for 10 min. The suspension was collected in the required quantity used as inoculant in the fermentation process. A spread plate method using nutrient agar (Merck 1.05450) was used to determine the total number of CFU (colony forming unit) of *B. subtilis* according to the standard protocol of EN ISO 2009. The sterile wet sample taken under aseptic conditions was suspended (1:10 w/v) in a sterilised phosphate buffered saline. The suspended solution was serially diluted, and then spread on nutrient agar plates for incubation at 37°C for 24 h. The CFU was counted and calculated per g of wet mass (EN ISO 2009).

Soybean meal was bought in 20 kg from a local feed mill in air-dried form (90% dry matter) and ground to pass a 3-mm sieve. A 200 gram of ground homogenous SBM was autoclaved at 121°C for 20-25 min for SSF conducted in Pyrex Erlenmeyer flask of 1000 ml. The moisture content of SBM with or without bacteria inoculants were adjusted to 78 and 85% (w/w) by adding sterilised distilled water. Finally, a freshly prepared bacteria suspension was added at a rate of 1×10^7 CFU per g solid material, and homogeneously mixed under a flow-cabinet. The flasks covered with sterilised cotton and aluminium folio were left for fermentation at 37 °C for 0, 24, 48 and 72 h at which each flask was stirred with sterilised glass rods for 5 min under the flow cabinet. The experimental design was of 2 moisture levels (78 and 85%) x 3 replicates of flasks x 4 incubation periods (0, 24, 48 and 72 h). At the end of each incubation period, pH values of fermenting substrate were measured. The wet samples were immediately taken for the determination of bacteria count (ISO 2004). The remaining wet samples were dried at a constant temperature of 50 °C until a constant weight of sample was obtained for the determination of dry matter content. The crude protein and crude ash was determined by the methods of AOAC (1990). The content of soluble amino nitrogen and organic acids (mainly acetate, lactate and butyrate) were determined by the methods reported by Karabulut and Canbolat (2005). The method of Karabulut and Canbolat (2005) suitable to silage and fermented products was modified to quantify individual organic acids. Ten grams of homogenous SBM samples were diluted 10 times in distilled water prior to incubation at 50 °C for 30 min at 150

rpm. Then the supernatants were collected after a centrifugation at 4100 rpm and were immediately steam distilled. Three subsequent distillates were collected. The last distillate was obtained after an oxidation of the remaining supernatant from first two distillates in a potassium dichromate solution. All distillates were then titrated with 0.05 N NaOH. The amount of NaOH consumed was used to calculate individual organic acids of acetic, butyric, and lactic acids, and total organic acid (% of sample DM) were expressed as sum of acetic, butyric, and lactic acids of all samples (Karabulut and Canbolat 2005). Phytate content was determined spectrophotometrically by the method of De Boland *et al.* (1975). The fine samples were extracted for 18-24 h with a solution containing 1.2% HCL and 10% Na₂SO₄. Then 10 ml of extract was treated with 10 ml water and 5 ml of 0.4% FeCl₃ in 0.6% HCL containing 5% Na₂SO₄. The phosphorous content of insoluble ferric salts was determined colorimetrically after digestion in 3 ml H₂SO₄ and 5 ml nitric acid. Three independent samples (flasks) taken at the end of each fermentation period were analysed triplicate, resulting in 9 (3x3) independent measurements per treatment.

A general linear model (GLM) was used to test the effect of water levels on the studied parameters using SPSS statistical program (IBM SPSS Inc, version 23), where the differences between the treatments were separated by Duncan's Multiple Comparison Test at a 0.05 significance level.

Results and discussions

The effect of moisture content on pH of fermenting substrate was significant ($P < 0.05$). The pH values at 78% moisture condition significantly ($P < 0.05$) increased at 24 h, and then gradually decreased to the same pH of the 0 h of fermentation (Tab. 1). Initially, there was an increase in pH at 24 h of fermentation with low moisture level (78%) in which there were no changes in total organic acid production. However, the level of acetic acid decreased significantly ($P < 0.05$) whereas the production of lactic acid increased during the 24 h of fermentation. On the other hand, fermenting SBM with 85% moisture level gradually caused a significant ($P < 0.05$) decrease in pH. In parallel, the production of organic acids significantly ($P < 0.05$) changed with the changes in the pH of SBM at both levels of moisture contents. In both 78 and

85% moisture conditions, a maximum bacteria growth from 10^7 to 10^{10} CFU/g occurred at 48 h of fermentation, and then lowered gradually (Tab. 1). *B. subtilis* usually grows at a pH range from 5.5 to 8.5 (Sneath, 1986), and pH values in the current study were within these ranges. It was earlier shown that fermentation with *B. subtilis* of SBM caused increased alkaline protease production which may have a proteolytic effect on ammonia release, and thereby increased pH (Sarkar *et al.*, 1993 and Chantawannakul *et al.*, 2002). This indicated that even though the bacteria used in our study could have an alkaline protease activity during 24 h due to an initial increase in pH over 6.0 (Abusham *et al.*, 2009) the significant drops in pH after 24 h in both SFM samples differed in moisture content clearly indicated the suitability of fermentation for the organic acid production rather than an activity of protease which was not measured in this study. Thus, there is a high level of organic acid production which was parallel to decreased pH of the substrate in our study at both levels of moisture contents. Many species of *Bacillus* were reported to produce more lactic acids in anaerobic than aerobic fermentation (Ohara and Yahata, 1996), similar to our study conducted at anaerobic condition. Moreover, the production of organic acids in our study was high in the case of high moisture condition. This result was found contradictory to the results reported by Yasar *et al.* (2018), using the same strain of *B. subtilis* to ferment sunflower meal. However, Ohara and Yataha (1996) reported lactic acid production by *B. spp.*, fermentations, similar to our results. Furthermore, Karakurt *et al.*, (2019) demonstrated clearly that fixing pH at around 5.5 for *Bacillus subtilis* fermentation of both sunflower and soybean meal samples produced an appreciable amount of organic acids in the form of acetate, butyrate and lactate, similar to the results of pH and organic acids obtained from both moisture levels at 48 and 72 h of fermentation in our experiment.

Dry matter contents of SFM with 78% moisture content were significantly ($P < 0.05$) increased at 48 and 72 h of the fermentation. The increase in dry matter content of SFM with 85% moisture content was insignificant ($P > 0.05$). There were similarly significant ($P < 0.05$) increases in crude ash contents of SFM with both moisture contents. Phytic acid considered as ANF in monogastric

Table 1. Changes in pH, bacteria count (CFU/g) and organic acids (%) of fermented SBM

Time (h)	pH	Bacteria count CFU/g	Acetic acid %	Butyric acid %	Lactic acid %
78% moisture content					
0	6.28±0.01 ^a	1×10 ⁷ ±0.05 ^a	4.2±0.12 ^a	0.0±0.0 ^a	0.0±0.0 ^a
24	7.02±0.02 ^b	5.0×10 ¹⁰ ±0.02 ^b	1.43±0.032 ^b	0.00±0.00 ^a	2.75±0.03 ^b
48	6.60±0.01 ^c	6.4×10 ¹⁰ ±0.03 ^c	9.47±0.93 ^c	0.73±0.01 ^c	3.42±0.023 ^b
72	6.30±0.01 ^a	3.6×10 ¹⁰ ±0.10 ^d	4.54±0.03 ^d	0.96±0.01 ^d	7.58±0.05 ^c
85% moisture content					
0	6.28±0.01 ^a	1×10 ⁷ ±0.05 ^a	4.2±0.12 ^a	0±0.0 ^a	0.0±0.0 ^a
24	6.04±0.02 ^b	4.4×10 ¹⁰ ±0.1 ^b	6.97±0.08	0.0±0.0 ^b	1.77±0.027 ^b
48	5.74±0.01 ^c	7.3×10 ¹⁰ ±0.15 ^c	7.83±0.04	0.0±0.0 ^c	8.45±0.06 ^c
72	5.28±0.01 ^d	5.3×10 ⁷ ±0.02 ^d	7.97±0.05 ^c	2.08±1.07 ^d	7.48±4.55 ^c

Note: Different letters in the same column indicated significant (Duncan test, $P<0.05$) differences between the means ± standard deviation.

Table 2. Effect of fermenting SBM at two levels of moisture content on nutritional composition

Parameters %	Moisture %	0	24	48	72
Dry matter	78	21.35±0.25 ^{aX}	21.38±0.10 ^{aX}	23.40±0.26 ^{bX}	23.35±0.90 ^{bX}
	85	15.15±0.15 ^{aY}	15.05±0.03 ^{aY}	15.75±0.6 ^{aY}	15.55±0.5 ^{aY}
Crude ash	78	6.30±0.4 ^{aX}	8.9±0.6 ^{bX}	9.97±0.03 ^{cX}	10.57±0.2 ^{dX}
	85	6.3±0.4 ^{aX}	9.3±0.02 ^{bY}	9.9±0.44 ^{cX}	10.1±0.37 ^{cX}
Crude protein	78	51.3±0.3 ^{aX}	51.0±0.5 ^{aX}	51.5±0.2 ^{aX}	51.3±0.5 ^{aX}
	85	51.3±0.3 ^{aX}	47.8±0.4 ^{bX}	49.4±0.4 ^{cY}	47.1±0.2 ^{bY}
Soluble amino nitrogen	78	4.8±0.25 ^{aX}	12.53±0.35 ^{bX}	14.71±0.04 ^{cX}	13.90±0.15 ^{cX}
	85	4.8±0.25 ^{aX}	13.68±0.11 ^{aY}	18.60±0.39 ^{bY}	17.0±0.75 ^{cY}
Phytic acid	78	1.22±0.05 ^{aX}	1.18±0.04 ^{aX}	0.76±0.01 ^{bX}	0.33±0.01 ^{cX}
	85	1.22±0.05 ^{aX}	1.25±0.06 ^{aX}	0.82±0.04 ^{dX}	0.43±0.02 ^{eY}

Note: Different letters in the same column indicated significant (Duncan test $P<0.05$) differences between the means ± standard deviation. Different letters in the same row indicated significant (Duncan test $P<0.05$) differences between the means ± standard deviation.

animals were remarkably degraded up to 65 to 75% with fermentation of SBM at both moisture contents in the present study. The percentage increase in crude ash content in this study was about 65%. The changes in crude protein content of SBM with 78% moisture level was not significant, but the crude protein content was decreased by 6-7% after 24 h of fermentation with 85% moisture content. Fermentation with 85% water content caused more increases (2.7 folds) in soluble amino nitrogen content than the increase with fermentation with 78% water content (2.0 folds).

It was earlier reported that anaerobic fermentation of *Bacillus spp.*, grown on polypepton obtained from soybean produced appreciable amount of organic acids (Ohara and Yahata, 1996). And, *Bacillus spp.* can degrade macro nutrient to water-soluble low molecular weight components (Kiers *et al.*, 2000). These indicated that fermentation of SBM in high level of moisture

content in our study caused significantly increased soluble amino nitrogen with low molecular weight used by microorganisms to produce high levels of organic acids, thereby lead to a reduced level of crude protein up to 6-7%. This result was found contradictory to the increased level of crude protein with *Bacillus subtilis* fermentation of SBM (Teng *et al.*, 2012; Dai *et al.*, 2017; Yuan *et al.*, 2017). This difference was basically due to the high moisture content of fermenting substrate in the present study. The fermentation increased amount of soluble amino nitrogen in the SBM samples and the more production of soluble amino nitrogen the more digestibility of SBM in animal nutrition. In this study fermented SBM containing less phytate and enriched with organic acid may have a high potentiality used in animal nutrition as functional feed materials, similar to the cases reported by Yuan *et al.* (2017) and Zhu *et al.* (2017). Organic acids were used as antimicrobial agents in the diet of farm animals (Weber *et al.*, 2012; Meeusen and

Hagemans, 2010; Torallardona *et al.*, 2016). On the other hand, the fermentation of SBM at both moisture contents increased crude ash content and amount of small size protein molecules (soluble amino nitrogen) which are almost water soluble, and decreased amount of phytic acid, which were also supported by earlier results (Teng *et al.*, 2012; Dai *et al.*, 2017; Yuan *et al.*, 2017).

In conclusion, the results showed that the higher water content in SSF of SBM the higher decreased crude protein content, but the more production of organic acids and soluble amino nitrogen, and that the SSF of SBM with 78 to 85% water content lead to significant degradation of phytic acid. We recommend that the fermented SBM containing appreciable amount of organic acid could be used as functional feed material for a better animal performance and health.

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