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Chemical, enzymatic, and antioxidant enrichments of full-fat soybean and sunflower meal by *Bacillus subtilis* (ATCC® 6633™) fermentation using a solid-state bioreactor

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Abstract: This study was conducted to fortify nutritional and chemical composition of sunflower meal (SFM) and full-fat soybean (FFSB) used as a protein source in farm animal nutrition by *Bacillus subtilis* ATCC 6633 fermentation. An optimized set of fermentation parameters (60% moisture, 6.5–7.0 pH, 30–35 °C temperature, continuous aeration of 0.5–1.0 L/min and agitation of 20–100 rpm) were used to ferment SFM and FFSB samples with or without using bacterial inoculant for 0, 24, 48, and 72 h. The results indicated that the total organic acids (TOA) contents and crude ash (CA) of SFM and FFSB increased significantly, but there were significant reductions in dietary fiber (DF) components (acid detergent fiber (ADF) and neutral detergent fiber (NDF)) in both feed samples. Moreover, urease activity, trypsin inhibitors (TI), and phytic acid (PA) contents of both feed samples reduced by 50%, 35%, and 79%, respectively. Total flavonoid level reduced by 30% in FFSB samples, but remained unchanged in SFM samples. The levels of tannin in FFSB and SFM decreased significantly. Although there were no significant changes in the activities of amylase, celulase, and beta-glucanase; the protease and phytase activities increased significantly. The total phenolic compounds content and the antioxidant activities of FFSB and SFM samples increased significantly. In conclusion, the fermented FFSB (F-FFSB) and fermented SFM (FSFM) had lowered the levels of ADF, NDF, tannin, TI, urease activity, and PA but they were remarkably enriched with organic acids, enzymes, and antioxidants. These feed materials could be used as functional feed additives or feed materials in farm animal nutrition.

Key words: *Bacillus subtilis*, nutrient and chemical enrichment, fermentation, full-fat soybean, sunflower meal

1. Introduction

In 2016, the world's soybean meal production reached 217 million tones and nearly half of it was used as protein supplement in farm animal nutrition (1). Sunflower meal (SFM), the fourth largest oilseed meal produced in the world, also serves as a protein source, mostly in ruminant diets, while its use in poultry and pig diets is limited (2). Chemical composition of these plant meals determines their levels in complete feeds fed to farm animals. For instance, soybean and its by-products may contain appreciable levels of phytic acid (PA) up to 0.6%, trypsin inhibitors (TI) up to 21.0–30.3 mg/g, protease inhibitors up to 45–60 mg/g protein, oligosaccharides up to 15%, lectins up to 50–200 mg/g, glycinin up to 150–200 mg/g, and beta-conglycin up to 50–100 mg/g (27.74 mg/g); these are known as antinutritional factors (ANFs) in young monogastric animals and reduce the rates of nutrient assimilation and absorption at the sites of digestive tract (3,4). SFM has a proportionally less crude protein (CP) in comparison to soybean meal, and its dietary inclusion level is low in poultry diets since it contains high level of crude fiber (CF) up to 18%–29% and polyphenolic compounds, mainly chlorogenic acid, up to 2.70% (2). In this study, possible improvements in nutritional qualities of SFM and full-fat soybean (FFSB) for farm animal nutrition were targeted by a fermentation process.

Improved nutritional qualities of fermented FFSB (F-FFSB) and fermented SFM (FSFM) by solid-state fermentation (SSF) using GRAS (generally regarded as safe) microorganisms were reported earlier (5–7) and recently well documented by Mukherjee et al. (8). In addition, fermented feeds may contain biologically active compounds (biosurfactants, phenolic compounds, organic acids, enzymes) and less ANFs (9–14). The species of *Lactobacillus* and *Bacillus* are mostly used to ferment the feed materials (8,15,16). Recently, fermentation using *Bacillus subtilis* was found to be superior to fungal fermentation in terms of the increased soluble protein

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and the rate of degraded TI contents (10). Therefore, a GRAS *Bacillus subtilis* strain was chosen to ferment FFSB and SFM in this study based on its GRAS property and its high probiotic activities and capable of producing extracellular polysaccharides, antibiotics like compounds and biosurfactant (17–19). Herein we proposed an SSF process, specifically optimized for the strain of *Bacillus subtilis* subsp. *spizizenii* Nakamura et al. (ATCC 6633), that would yield high nutritional qualities in FFSB and SFM.

On the other hand, inducing a high degree of nutritional enhancement greatly depends on the optimization of fermentation conditions at set-values for this specific strain of ATCC 6633. Most of the fermentation studies were conducted on laboratory flask, where the control and optimization of fermentation conditions is difficult. Modern lab-scale bioreactors have been designed to offer an efficiently optimized SSF for this particular purpose.

FFSB and SFM are two important dietary protein sources in poultry nutrition, but consumption of both feed materials could negatively affect poultry species due to their ANF contents. The purpose of this study was to minimize ANFs and possibly to achieve chemical, enzymatic, and antioxidant enrichments of FFSB and SFM by means of fermentation. The feed materials in this study were therefore fermented by ATCC 6663 microorganism using a solid-state bioreactor under the following fermentation conditions: a moisture level of not less than 600 g/kg, a pH ranging from 6.5 to 7.0, a temperature ranging from 30 to 38 °C, a continuous aeration ranging from 0.5 to 1.0 L/min and a continuous agitation ranging from 20 to 100 rpm.

2. Materials and methods

2.1. Cultivation of active bacteria inoculants

The strain used in this study was ATCC 6633, which was cultivated on Nutrient Broth (NB) for 24 h at 37 °C to collect bacterial suspension for fermentation experiments. The pellets of bacteria after centrifugation of NB at 14,000 × *g* for 10 min at 4 °C were suspended in 8% (w/v) saline buffer solutions. The number of spores was counted on Neubauer chamber (Blaubrand®, Brand Gmbh+Co Kg, Wertheim, Germany) as well as reading its absorbance at 600 nm (Genesys™ 10S UV-VIS, Thermo Scientific, USA). The counts and absorbance readings were then confirmed by the determination of colony forming unit (cfu) per mL of bacterial suspension using a spread plate method (ISO 15784:2009) on nutrient agar (NA) at 37°C for 24 h. The bacterial suspension was then added to the medium in a quantity which can provide a 1×10^8 cfu per gram of solid substrate.

2.2. Fermentation of feed materials

Feeds of FFSB and SFM including shell obtained from a local feed mill were ground to pass a 3-mm sieve before autoclaving (Nüve, OT 40 L, Turkey) at 121 °C for 15 min (holding time). The bioreactor and its glassware accessories were sterilized at 175 °C for 2 h. A total of 3.5 L capacity of Labfors 5™ bioreactor (Infors AG, Bottmingen, Switzerland) was loaded with 500 g of autoclaved FFSB and SFM under a biosafety flow cabinet, followed by mixing with a sterilized distilled water to yield a fermenting substrate not less than 600 g/kg moisture content (w/w) in order to induce a porridge-like consistency. Substrate pH was adjusted to 6.50 by addition of sterilized acid buffer using 0.01 M sulfuric acid. Initial moisture contents for FFSB and SFM were 680 and 820 g/kg, respectively, to reach a porridge-like consistency, allowing a homogenous stirring at a level of torque permitted by the bioreactor. These moisture levels have allowed not only a homogenous substrate agitation, but also a perfect maintenance of a constant substrate temperature. A bacterial suspension of 1×10^8 ATCC 6633 cfu per gram of solid material was added. Then the bioreactor was set at the following values to operate for 72 h: a pH of 6.5, aeration at 0.75 L/min, temperature of 35 °C, and agitation of 50 rpm. The temperature of 35 °C was excellently managed by the control of agitation rate, which was observed to increase from 20 rpm to 100 rpm from 24 h to 48 h and thereby reduced to 50 rpm until 72 h of fermentation. The same fermentation parameters and processes were applied to the control feed fermentations, where no microbial inoculant was added. Three independent sterile samples were taken at 24, 48, and 72 h for analytical and microbiological measurements. Microbial growth at 24, 48, and 72 h of fermentation was determined as cfu/g of fresh sterile sample using a spread plate method (ISO 15784:2009) on NA at 37 °C for 24 h.

Air inlet and outlet were equipped with sterilized filters of 0.25-µm pore size (Infors AG, Bottmingen, Switzerland). A bio-gas detector (SA2Q from Beijing Shi-An Technology Instrument C0., Ltd) was attached to the air outlet to monitor the amount of released CO_2 (0% to 100%), CH_4 (0% to 100%) and H_2S (1 to 1000 ppm). In addition, the outlet air passed through a bottle with 10 mL of 2% boric acid solution was analyzed for nitrogen determination at a 24 h interval up to 72 h.

2.3. Chemical analysis

Samples taken at 0, 24, 48, and 72 h of fermentations were oven-dried at 50 °C and ground using a 1-mm sieve prior to chemical analysis. Analysis was carried out in duplicates for three independent samples taken at each incubation time, providing a total of 6 measurements per sample per incubation time. AOAC methods (20) were used to determine dry matter (DM) (method 930.15), crude ash (CA) (method 942.05), CP (method 984.13), crude lipid (CL) (method 920.39), and CF (method 14.020). Official methods reported by Karabulut and Canbolat (21) were employed to determine neutral detergent fiber (NDF), acid detergent fiber (ADF), total starch content and total

reducing sugar content. The method of analysis described by Chemesova and Chizhikov (22) was used to determine the content of tannin. The PA was analyzed according to the method of De Boland et al. (23). Analysis of TI in FFSB samples was carried out using an official method of analysis (EN ISO 14902:2001). Urease activity of FFSB samples was analyzed according to the method EN ISO 5506:1988. All analytical results were expressed as percentage of DM.

2.4. Total organic acid (TOA) determination

A method suitable to silage and fermented products (21) was modified to quantify individual organic acids. Ten grams of homogenous FFSB and SFM 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 (21).

2.5. Total phenolics and flavonoid compounds

Folin and Ciocalteu's method (24) was used to determine the total phenolic content of the samples. The results were expressed as mg of gallic acid equivalent. Total flavonoid content was determined according to the method described in (25), and the results were expressed as mg of catechin equivalents per gram of sample DM.

2.6. Antioxidant capacity (AOC)

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals scavenging activity (26,27) were determined as 1mol Trolox equivalent (TE) of FFSB and SFM samples for total AOC.

2.7. Enzyme activities

Protease activity was determined as the ability to hydrolyze 0.8% (w/v) azo-casein protein by the method of Cotta and Hespell (28). The sample was suspended in 10 mM citrate–20 mM sodium phosphate buffer (pH 6.0) for an hour at room temperature (25 °C) for enzyme extraction. The insoluble residue was removed by filtration through butter muslin and after centrifugation the supernatant was used as the crude enzyme (29). The reaction mixture containing 0.5 mL of 0.8% (w/v) azo-casein in 100 mM potassium phosphate buffer (pH 7) and 0.5 mL of the enzyme source was incubated in 1.8 mL microcentrifuge tubes for 3 h at 25 °C. The reaction was stopped by the addition of 0.5 mL cold 1.5 M $HClO_4$ to each tube, which was then held on ice for 30 min. Precipitated protein was removed by centrifugation (Allegra™ 64R, Beckman Coulter, USA) at 13000 × *g* for 5 min, and a 1.0 mL aliquot of supernatant was mixed with an equal volume of 1N NaOH. The concentration of acid-soluble azo-peptides was determined optically at 440 nm against a blank with 1.00 optical density unit equal to 320 µg/mL. One unit of protease activity was defined as microgram of azo-casein digested per hour at pH 7 and 25 °C. Amylase enzyme extraction was carried out according to a method described by Tsegaye and Gessesse (30) and final enzyme extract was used as amylase activity analyses. Amylase activity was determined by incubating the mixture of 0.5 mL aliquot of enzyme source and 1 mL 1% soluble starch dissolved in 0.1 M sodium acetate buffer. DNS (3,5-Dinitrosalicylic acid) reagent and Rochelle's Salt solution was added and released reducing sugar liberated was measured according to the absorbance read at 510 nm by dinitrosalicylic acid method (31). One unit of amylase activity was defined as the amount of enzyme liberating 1 µg of glucose per minute under the standard assay conditions. Phytase activities of all samples were determined using an official method (32). One unit of phytase activity was considered to be equal to 1 µmol of released inorganic phosphorus under the assay conditions. A colorimetric method described by König et al. (33) was used for the quantification of cellulase and beta-glucanase activities. One unit of enzyme activity was considered the amount of enzyme that released 1 µmol of reducing sugar (glucose or xylose) per minute under the assay conditions.

2.8. Statistical analysis

A general linear model (GLM) of 2 feed samples by 4 incubation periods by 6 independent replicates was used to analyze the data (SPSS 15 for Windows, Inc. 2016). Group means were separated at 5% level of significance. The results of GLM analysis indicated that the differences in all parameters between the control fermentations of SFM and FFSB samples, considered noninoculant feed fermentations were insignificant at 24, 48, and 72 h; therefore, all the control measurements were pooled for each samples and presented as an overall mean of "Control values" in order to improve the readability of the data.

3. Results

SSF process was well optimized by the bioreactor used in this study, particularly for the parameters of pH and temperature throughout the fermentation periods. A constant temperature of 35 °C recorded remained unchanged up to 72 h throughout the fermentation processes, with minor fluctuations. Throughout the fermentation periods, there were statistically insignificant ($P > 0.05$) changes in pH values of SFM ranging from 6.3 to 6.7 and in FFSB samples ranging from 6.46 to 6.53 (Figures 1 and 2). In comparison to the control fermentations, there were significant increases ($P < 0.05$) in CO_2 levels in both SFM and FFSB fermentations (Figures 1 and 2). The

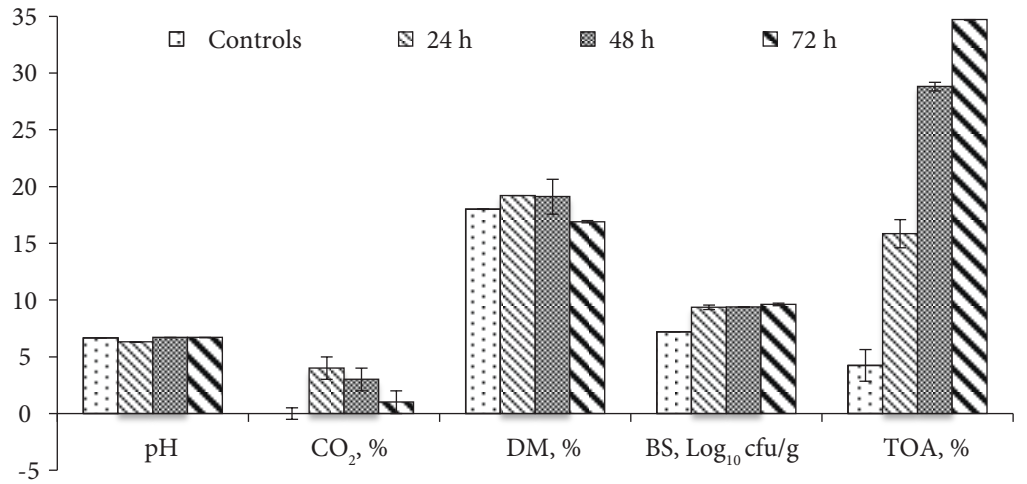

Figure 1. Changes in pH, CO_2 (%), DM (%), microbial growth (Log_{10} cfu/g), and TOA (%) during ATCC 6633 fermentation of SFM.

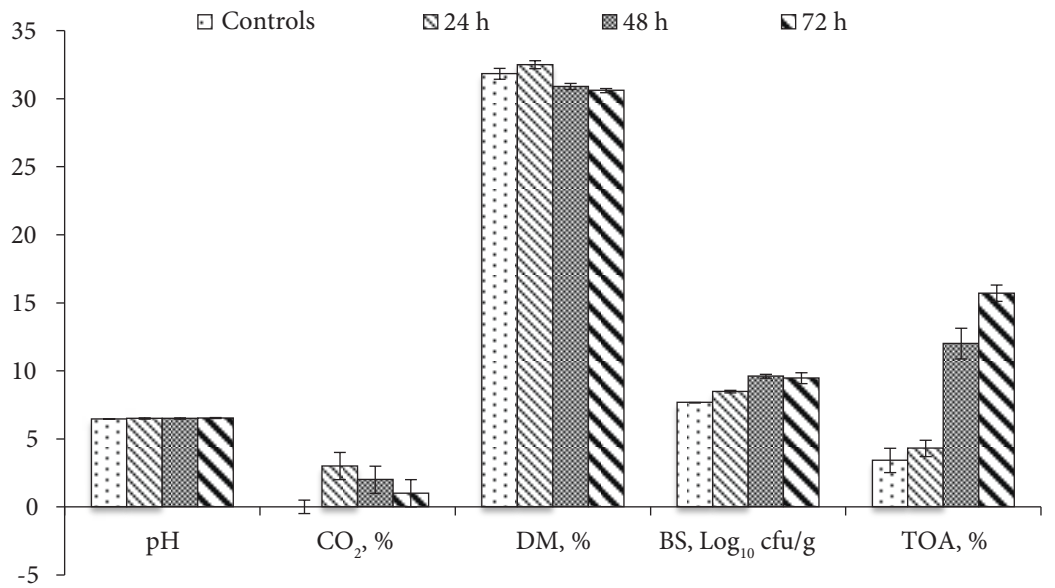

Figure 2. Changes in pH, CO_2 (%), DM (%), microbial growth (Log_{10} cfu/g), and TOA (%) during ATCC 6633 fermentation of FFSB.

maximum levels of CO_2 (4% in FSFM and 3% in F-FFSB) were reached in 24 h of fermentations. These values were then reduced to 1% at the end of 72 h both for FSFM and F-FFSB samples. A 7.18 and 7.6 log cfu of bacterial inoculant per gram of total mass of SFM and FFSB samples added respectively was increased approximately by 3 log. However, the maximum growth of 9.6 log_{10} cfu/g was reached at 24 h of SFM fermentation, and thereafter remained unchanged. In the case of FFSB fermentation, the same growth rate was reached at 48 h. Similar differences in TOA levels of SFM and FFSB fermentations were also observed (Figure 2). An 8-fold increase in TOA level, from 4.2% at 24 h to 34.7% at 72 h was observed with FSFM, whereas the increase in TOA level from 3.41% at 24 h to 15.7% at 72 h was remained 3-fold with F-FFSB. Furthermore, the production levels of the released N and

H_2S gases in FSFM were greater than the levels in F-FFSB (Figure 3). Overall, the released amount of nitrogen was 70 ppm in SFM fermentation and 66 ppm in FFSB fermentation, respectively. The amounts of H_2S obtained from 24, 48, and 72 h of fermentations of SFM were 11, 36, and 216 ppm, respectively, while no H_2S was detected with FFSB fermentation. During the fermentation there were reductions in DM content of fermenting substrates (Figures 1 and 2). Reduction in DM from 18% control to 16.9% at 72 h of SFM fermentation was significant ($P < 0.05$). Similarly, the reduced DM content from 31.8% control to 30.9% at 48 h and to 30.6% at 72 h of FFSB fermentation was also significant ($P < 0.05$).

ATCC 6633 fermentation significantly ($P < 0.05$) increased total CA contents of both SFM and FFSB samples (Tables 1 and 2). In comparison to the CA content of control samples, the CA contents in FSFM and F-FFSB increased by 1.93 and 1.27 times, respectively. In this study, the ATCC 6633 fermentation significantly ($P < 0.05$) reduced the levels of CP in FSFM and F-FFSB samples compared to the control samples. The CP was reduced from 35.25% in the control SFM samples to 32.28% in the 72-h FSFM samples. Similarly, the CP was reduced from 44.72% in the control FFSB samples to 40.41% in the 72-h F-FFSB sample. Similar reduction rates in CL contents of SFM and FFSB were observed by ATCC 6633 fermentations.

ATCC 6633 fermentation caused significantly ($P < 0.05$) reduced total CF content in FSFM (Table 1), only at 48 h of fermentation. The effect of ATCC 6633 fermentation on the CF content of F-FFSB was insignificant. However, ADF, NDF, and lignin fractions were significantly ($P < 0.05$) reduced in both FSFM and F-FFSB samples. The effect of ATCC 6633 fermentation on reduced fiber fractions was more pronounced for F-FFSB than that for FSFM samples. The percentages of reduced ADF and NDF in FSFM samples were 40% and 43%, respectively, while the same corresponding values in F-FFSB were 49% and 67%, respectively. NFE (nitrogen-free extract) content (calculated value) in FSFM sample was reduced from 32.65% to 26.98% at 72 h fermentation, while there was no change in NFE content of F-FFSB sample (Tables 1 and 2).

In this study, significant ($P < 0.05$) amounts of TOA (Table 3) were produced by ATCC 6633 fermentation, particularly when the SFM (7.3 times more than the control FSFM samples) was used as fermenting substrate compared to FFSB (3.6 times more than the control F-FFSB samples). In all feed fermentations, the greater proportion, more than 60%–80%, of organic acids was acetic + butyric acids. The percentage of lactic acid was 20% in FSFM samples and 40% in F-FFSB samples.

Tannin contents of SFM and FFSB samples were significantly ($P < 0.05$) reduced at 24 and 48 h of ATCC 6633 fermentation, but the effect of 72-h fermentation was insignificant on the tannin contents (Table 4). On the other hand, the PA of both feed samples was significantly ($P < 0.05$) degraded by increasing the time of fermentation. The rate of reduced PA by the end of fermentation was 79% in both FSFM and F-FFSB samples. In F-FFSB samples, the level of TI and urease activity was significantly ($P < 0.05$) reduced by 25% and 50%, respectively.

ATCC 6633 fermentation produced significantly high ($P < 0.05$) activities of protease around 3- to 4-fold in the case of SFM and around 3.5- to 5-fold in the case of FFSB used as substrates, in comparison to their control fermented samples with no inoculants of ATCC 6633 used in this study (Table 5). Although ATCC 6633

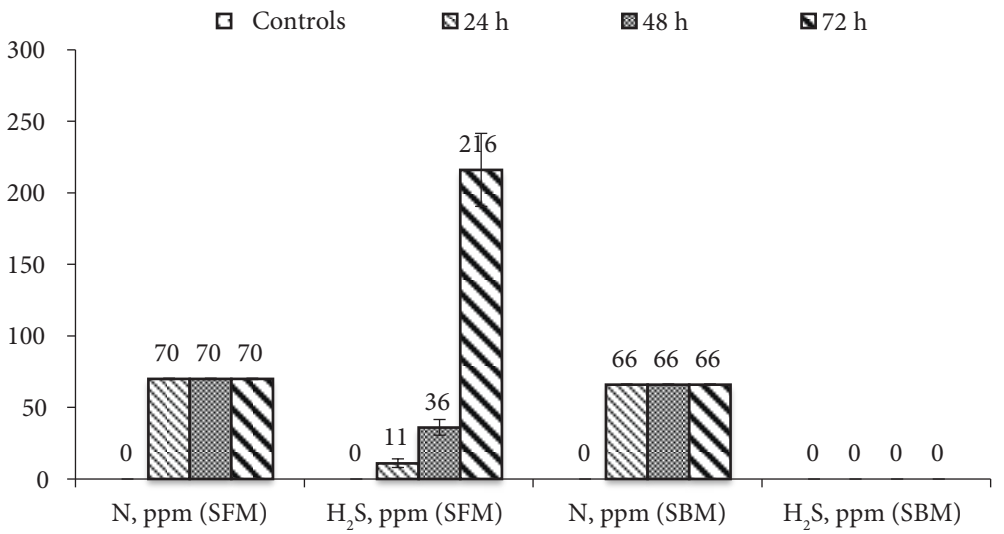

Figure 3. Release of N (ppm) and H_2S (ppm) gases during ATCC 6633 fermentation of SFM and FFSB.

Table 1. Changes in nutrient composition (% of DM) of SFM by ATCC 6633 fermentation.

Nutrients(%)	Controls*	24 h	48 h	72 h
CA	6.55 ± 0.08^{c}	14.2 ± 1.5^{b}	16.2 ± 1.0^{b}	19.2 ± 0.68^{a}
Organic matter**	84.29	74.87	76.41	75.44
CP	35.25 ± 0.14^{a}	33.76 ± 0.55^{b}	33.72 ± 0.14^{b}	32.28 ± 0.01^{c}
CL	0.28 ± 0.01^{b}	0.33 ± 0.01^{a}	0.22 ± 0.01^{c}	0.21 ± 0.01^{c}
CF	25.26 ± 1.80^{a}	20.33 ± 1.97^{ab}	18.57 ± 1.14^{b}	21.30 ± 1.66^{ab}
ADF	34.05 ± 0.97^{a}	25.59 ± 0.20^{b}	22.68 ± 0.93^{c}	20.25 ± 4.26^{bc}
NDF	52.12 ± 1.03^{a}	41.63 ± 1.44^{b}	35.67 ± 1.02^{c}	29.43 ± 4.86^{d}
Lignin**	8.79	5.26	4.11	0.00
Starch	0.00^{b}	0.34 ± 0.09^{a}	1.16 ± 0.97^{a}	0.00^{b}
Reducing sugar	13.75 ± 1.81	12.34 ± 0.79	11.55 ± 0.42	12.09 ± 0.37
NFE**	32.65	31.38	31.27	26.98

^{a,b,c}Different superscripts indicate significant differences ($P < 0.05$) between the means in the same row. *The data given as controls indicate the mean of all data pooled during 24-, 48-, and 72-h noninoculant feed fermentations. **The values were calculated but not subjected to statistical analysis. ADF: acid detergent fiber, CA: crude ash, CF: crude fiber, CL: crude lipid, CP: crude protein, DM: dry matter, NDF: neutral detergent fiber, NFE: nitrogen-free extract, SFM: sunflower meal.

Table 2. Changes in nutrient composition (% of DM) of FFSB by ATCC 6633 fermentation.

Nutrients(%)	Controls*	24 h	48 h	72 h
CA	6.49 ± 0.01^{a}	10.64 ± 0.25^{b}	13.57 ± 0.59^{c}	14.72 ± 0.43^{c}
Organic matter**	86.75	82.22	77.32	77.19
CP	44.72 ± 0.66^{a}	41.47 ± 0.23^{b}	41.04 ± 0.50^{b}	40.41 ± 0.41^{b}
CL	19.03 ± 0.02^{a}	17.0 ± 0.05^{b}	15.75 ± 0.01^{b}	14.80 ± 0.01^{c}
CF	6.33 ± 0.08	6.94 ± 0.50	6.23 ± 0.13	6.60 ± 0.93
ADF	10.70 ± 0.01^{a}	5.45 ± 0.98^{b}	5.45 ± 0.06^{b}	6.40 ± 1.04^{b}
NDF	45.17 ± 1.93^{a}	14.48 ± 1.08^{b}	14.73 ± 1.04^{b}	14.93 ± 0.95^{b}
Lignin**	3.37	0.00	0.00	0.00
Starch	0.00	2.12 ± 0.09	0.00	0.00
Reducing Sugar	10.76 ± 0.33	11.09 ± 0.12	11.56 ± 0.38	11.44 ± 0.40
NFE**	23.45	23.95	23.41	23.47

^{a,b,c}Different superscripts indicate significant differences ($P < 0.05$) between the means in the same row. *The data given as controls indicate the mean of all data pooled during 24-, 48-, and 72-h noninoculant feed fermentations. **The values were calculated but not subjected to statistical analysis. ADF: acid detergent fiber, CA: crude ash, CF: crude fiber, CL: crude lipid, CP: crude protein, DM: dry matter, FFSB: full-fat soybean, NDF: neutral detergent fiber, NFE: nitrogen-free extract.

Table 3. Changes in the levels of organic acid in SFM and FFSB samples by ATCC 6633 fermentation.

Fermentation of SFM by ATCC 6633				
Organic acids(%)	**Controls***	**24 h**	**48 h**	**72 h**
Acetate	2.10 ± 0.59^{a}	8.85 ± 0.23^{b}	12.67 ± 0.20^{c}	17.95 ± 0.44^{d}
Butyrate	1.17 ± 0.66^{a}	1.27 ± 0.07^{a}	7.28 ± 1.60^{b}	9.80 ± 0.23^{c}
Lactate	0.95 ± 1.30^{a}	5.70 ± 0.90^{b}	6.90 ± 1.02^{b}	6.92 ± 0.22^{b}
Total	4.23 ± 1.40^{a}	15.84 ± 1.24^{b}	26.80 ± 0.38^{c}	34.7 ± 0.01^{d}
Fermentation of FFSB by ATCC 6633				
Organic acids(%)	**Controls***	**24 h**	**48 h**	**72 h**
Acetate	1.33 ± 0.02^{a}	2.10 ± 0.35^{b}	4.90 ± 1.21^{c}	6.20 ± 0.13^{d}
Butyrate	0.79 ± 0.05^{a}	0.70 ± 0.50^{a}	2.54 ± 0.3^{b}	2.95 ± 0.4^{b}
Lactate	1.28 ± 0.90^{a}	1.48 ± 0.40^{a}	4.56 ± 0.30^{b}	6.54 ± 0.02^{c}
Total	3.41 ± 0.90^{a}	4.30 ± 0.60^{a}	12.00 ± 1.12^{b}	15.70 ± 0.20^{c}

^{a,b,c}Different superscripts indicate significant differences ($P < 0.05$) between the means in the same row. *The data given as controls indicate the mean of all data pooled during 24-, 48-, and 72-h noninoculant feed fermentations.

Table 4. The levels of tannin, urease activity, TI, total phenolic compounds, Total flavonoids, and PA in FSFM and F-FFSB samples affected by ATCC 6633 fermentation.

Fermentation of SFM by ATCC 6633				
Parameters(%)	**Controls***	**24 h**	**48 h**	**72 h**
Tannin	3.18 ± 0.52^{a}	0.63 ± 0.10^{b}	1.35 ± 0.13^{c}	3.2 ± 0.41^{a}
Phytic acid	0.54 ± 0.02^{a}	0.26 ± 0.01^{b}	0.19 ± 0.02^{c}	0.11 ± 0.01^{d}
Total phenolic compound	3.12 ± 0.09^{a}	4.34 ± 0.14^{b}	5.32 ± 0.14^{c}	6.90 ± 0.12^{d}
Total flavonoids	29.1 ± 2.3^{a}	16.5 ± 1.5^{b}	17.4 ± 1.20^{b}	19.60 ± 1.6^{b}
Fermentation of FFSB by ATCC 6633				
Parameters(%)	**Controls***	**24 h**	**48 h**	**72 h**
Tannin	3.75 ± 0.06^{a}	1.32 ± 0.11^{b}	1.15 ± 0.10^{b}	3.60 ± 0.49^{a}
Phytic acid	1.22 ± 0.05^{a}	1.09 ± 0.05^{b}	0.69 ± 0.01^{c}	0.26 ± 0.01^{d}
Tyripsin inhibitor	27.74 ± 0.01^{a}	23.11 ± 0.01^{b}	21.20 ± 0.01^{c}	20.57 ± 0.01^{d}
Urease activity	3.36 ± 0.05^{a}	2.64 ± 0.17^{b}	1.94 ± 0.07^{c}	1.65 ± 0.13^{d}
Total phenolic compound	0.75 ± 0.01^{a}	1.29 ± 0.01^{b}	2.31 ± 0.03^{c}	3.67 ± 0.08^{d}
Total flavonoids	3.0 ± 0.2	3.1 ± 0.1	3.3 ± 0.2	3.3 ± 0.3

^{a,b,c}Different superscripts indicate significant differences ($P < 0.05$) between the means in the same row. *The data given as controls indicate the mean of all data pooled during 24-, 48-, and 72-h noninoculant feed fermentations. Tannin: % of DM, urease activity: mg N/100 g of DM, TI: mg/g of DM, total phenolic compounds: % of DM as gallic acid equivalent, total flavonoids: % of DM as quercetin equivalent, PA: % of DM.

Table 5. The levels of enzyme activities and AOC of FSFM and F-FFSB samples affected by ATCC 6633 fermentation.

Fermentation of SFM by ATCC 6633				
Parameters (IU/g)	**Controls***	24 h	48 h	72 h
Protease	2.5 ± 0.4[a]	7.01 ± 0.3[b]	7.3 ± 0.5[c]	9.9 ± 0.7[d]
Alpha-amlyase	5.19 ± 0.82	5.80 ± 0.69	5.64 ± 0.54	5.66 ± 0.82
Cellulase	43.1 ± 2.2	42.8 ± 2.5	46.5 ± 2.1	46.5 ± 1.8
Phytase	0.9 ± 0.03[a]	1.4 ± 0.07[b]	2.8 ± 0.11[c]	1.6 ± 0.08[b]
Beta-glucanase	40.3 ± 2.1	42.4 ± 1.9	44.1 ± 2.2	44.3 ± 1.8
Antioxidant capacity				
ABTS	33.1 ± 0.66[d]	42.8 ± 0.52[c]	54.3 ± 0.51[a]	51.2 ± 0.29[b]
DPPH	3.1 ± 0.7	4.2 ± 0.6	4.8 ± 0.7	5.2 ± 0.9
Fermentation of FFSB by ATCC 6633				
Parameters(IU/g)	**Controls***	24 h	48 h	72 h
Protease	0.9 ± 0.02[a]	3.5 ± 0.3[b]	3.6 ± 0.3[b]	4.9 ± 0.2[c]
Alpha-amylase	5.21 ± 0.52[a]	5.35 ± 0.38[a]	6.20 ± 0.32[b]	7.38 ± 0.41[b]
Cellulase	45.3 ± 1.9[a]	45.3 ± 1.7[a]	46.4 ± 2.1[ab]	50.4 ± 2.3[b]
Phytase	1.3 ± 0.11[a]	2.4 ± 0.12[b]	3.6 ± 0.15[c]	2.1 ± 0.09[b]
Antioxidant capacity				
ABTS	9.6 ± 0.82[c]	13.8 ± 0.64[b]	29.2 ± 0.74[a]	30.6 ± 0.35[a]
DPPH	0.9 ± 0.1[c]	1.3 ± 0.11[b]	1.5 ± 0.13[ab]	1.6 ± 0.11[a]

[a,b,c]Different superscripts indicate significant differences ($P < 0.05$) between the means in the same row. *The data given as controls indicate the mean of all data pooled during 24-, 48-, and 72-h noninoculant feed fermentations.

fermentation did not have any amylolitic and cellulotic enzyme producing capability when SFM used, the increase in these enzyme activities of the same inoculant growing on the FFSB substrate was small but significant ($P < 0.05$). However, ATCC 6633 fermentation had a significant influence ($P < 0.05$) on increasing the phytase enzyme activities (1- to 2-fold) in both SFM and FFSB samples. The AOC measured as DPPH and ABTS activity was increased by ATCC 6633 fermentation. The ABTS binding ability was significantly increased by 1.5- and 3.0-fold in SFM and FFSB, respectively, while the increases in DPPH binding ability by ATCC 6633 fermentation by 1.6-fold was found significant ($P < 0.05$) in FFSB samples, but the same increase was found insignificant ($P > 0.05$) in SFM samples.

4. Discussion

The use of a modern bioreactor to optimize fermentation condition for ATCC 6633 was found very successful. Particularly, the fermentation temperature was optimized at the desired level throughout the fermentation process by an efficient rate of agitation and aeration. This was managed by an excellent agitation process of the bioreactor to distribute the heat homogenously within the fermenting substrate with the help of an averaged aeration rate of 0.75 L per minute. A porridge-like consistency of 3 mm size of substrate particles have partially accounted for these successful fermentations. The differences in bacterial growth between the tested feed materials of SFM and FFSB were parallel to the differences in released CO_2 levels and TOA between SFM and FFSB fermentations, indicating the ATCC 6633 fermentations were successful.

In our study, ATCC 6633 fermentation caused significantly increased CA; however, it significantly reduced CP, CL, ADF, NDF, and lignin contents. Previous studies using ATCC 6633, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus* sp. AR-009 and *Bacillus subtilis natto* fermentations to ferment various feed materials including SFM have reported a 3% to 104% increase of CP, a 7.7% increase of CL, a 17% reduction of CF, an 80% increase of CA, and an 11% to 59% increase of NFE (34). Except that increased CA and decreased fiber fraction by ATCC 6633 fermentation in our study, the reduced CP and CL contents as a consequence of reduced CP and CL were exceptionally contradictory to these previously reported results (5,7,8,10,15,16,34). In the case of reduced

CP, ATCC 6633 fermentation caused significantly increased protease enzyme activities (about 2.96–4.44-fold increase) in both feed materials, which could account for the reduced levels of CP in our study. Furthermore, fermentation conditions in our study were quiet similar to those in previous studies, except that there was continuous agitation in our fermentations (5,7,8,10,15,16,34). Silva and Yang (35) reported earlier that increasing the frequency of agitation in SSF processes has a reducing impact on protein production. Under these permanent agitation conditions, a constant release of nitrogen in gas state, 70 ppm in FSFM at every 24 h intervals and 66 ppm in the case of F-FFSB were inevitably produced. Therefore, it is more likely that continuous agitation and increased protease activity in our study was responsible for reduced CP and CL contents. In fact, microbial fermentation needs a nitrogen source for bacterial growth to produce bioactive molecules, enzymes and antioxidants. In the past, soybean was reported to be a good nitrogen source for the strain of *Bacillus subtilis* (12). Therefore, the CP and CL of feed samples may have been partially used-up by the strain of ATCC 6633 during fermentation. Both effects of agitation and the nitrogen requirements of ATCC 6633 may be responsible for lowered CP and CL contents in FSFM and F-FFSB samples in our study. On the other hand, in comparison to the above studies, the level of increased CA contents up to 3- to 8-fold and reduced CF fractions up to 50% to 100% in both feed samples by ATCC 6633 fermentation was greater than the levels reported by these studies (5,7,8,10,15,16,34). These levels of improvements by ATCC 6633 were even better than those obtained from yeast fermentations, except the increased protein content of the final product (13,14). Thus, these well-optimized fermentation conditions were suitably well fit for the purpose of producing other biological molecules rather than fortification of CP and CL contents of the fermenting substrates in this study. The lignin in FSFM and F-FFSB samples was completely degraded by ATCC 6633 fermentation. There were sporadic and inconsistent changes in total starch and sugar contents in FSFM and F-FFSB samples by ATCC 6633 fermentation. Nevertheless, ATCC 6633 fermentation caused significant levels of degradations in CF, ADF, and NDF in SFM and ADF and NDF in FFSB. In fact, unfermented FFSB contained less amount of CF and high levels of CP and CL compared to unfermented SFM, indicating that FFSB is an ideal substrate for microorganisms. Thus, ATCC 6633 grew on FFSB has produced more enzymes of amylase, cellulase and protease than those grew on SFM. The increase in amylase, cellulase, and protease activities in F-FFSB and FSFM were 41% versus 9%, 11% versus 7%, and 444% versus 296%, respectively. Similarly, the degradation percentages of ADF and NDF in F-FFSB and FSFM were 50% versus 42% and 68% versus 42%, respectively. The differences in enzymatic activities between the F-FFSB and FSBM were well reflected on the differences in ADF and NDF during the fermentation periods.

Fermentation with several strains of *Bacillus* spp. caused significant increases in the levels of low molecular weight proteins, lactic acid, total phenolic compounds, AOC, and increased activities of xylanase and cellulase (34). Similarly, we found enhanced production of these compounds with ATCC 6633 fermentation of SFM and FFSB samples. Zhang et al. (34) reported significantly increased levels of lactic acids with FFSB, cereal grains, and tomato pomace fermented with various *Bacillus* subspecies. The level of lactic acid produced was lower compared to the lactate production obtained from FFSB and SFM by ATCC 6633 fermentation in our study. In our study, it was shown that SFM can be a good source of acetate + butyrate and FFSB a good source of lactate since the production levels of these organic acids were significantly high by ATCC 6633 fermentation. However, there was a significantly increasing level of H_2S production from 24 to 72 h of fermentation of SFM sample. The level of H_2S was found to be too high, which limits the use of FSFM as animal feed, and its possible residues may remain in its organic acid content, which may not be safe. Nevertheless, some purification processes can be applied to cleanup organic acids from the residues of H_2S when these high levels of organic acids are of significant interest for commercial-scale production.

In our study, the effect of ATCC 6633 on the degradation of tannin known as ANFs was not consistent since there were insignificant changes in the level of tannin in SFM and FFSB samples at the end of 72 h although the tannin content at 24 and 48 h was significantly reduced. On the other hand, the ATCC 6633 fermentation has successfully degraded some of ANFs, PA, TI, and urease by 79%, 25%, and 50%, respectively. With an exception of tannin, these results were similar to those of previous studies where the fungal fermentations, mainly *Aspergillus* spp., degraded the tannin by around 30% to 66%, PA by 35%–74% and urease 58% (36) compared to a limited degradation of these ANFs by bacterial fermentations (34,36,37). In general, Soybean and molded soymilk was traditionally fermented by microbial intervention of *Bacillus* spp., (15). Moreover, fermentation with *Bacillus subtilis* was found to be superior to fungal fermentations in terms of the increased soluble protein and the rate of degraded TI contents (10). The fermentation of FFSB by yeast and bacteria did not change the contents of essential amino acids, while a decreased content of cysteine was found during bacterial fermentation (5). At the end, ATCC 6633 was successful to reduce both TI and urease activity of FFSB in our study.

Phytochemicals such as phenolic and flavonoid compounds in plant foods could be modified and made

readily available during microbial fermentation. An appreciable amount of total phenolic compounds and some other flavonoids, around 50% was reported by the *Bacillus subtilis* fermentations (34). These results were similar to our findings but the increase rate in total phenolic compounds was greater in our study. The rate of increase in phenolic compounds ranged from 2.0 to 3.9 times in FSFM and F-FFSB samples, respectively, although the ATCC 6633 fermentation had no effect on total flavonoid contents of F-FFSB samples, except a 43% increase in total flavonoid contents in FSFM. All these indicated that the ATCC 6633 fermentation was superior to any other bacterial fermentation for the production of bioavailable phytochemicals with its antioxidant effects in both human and animal nutrition (11).

The rate of increase in CP was 16% and the inhibition of TI was 90% after the fermentation of SFM with 60% moisture content fermented with *Bacillus subtilis* TP6 (11). Increased small-size peptides along with increased free amino acids (i.e. arginine, serine, threonine, aspartic acid, and glycine) were also reported by the researchers due to the degradation of long-chained proteins (10,38). In such cases, it is evidentially true to speculate that different proteinase enzymes (protease activity increased 3- to 5-fold in our case) may have been microbiologically activated during the fermentation. Having considered possible activations of other complex and noncomplex carbohydrate degrading enzymes (numerically insignificant increases in protease, amylase, betaglucanase, and cellulase activity in this study) and those degrading PA, fermenting FFSB (1- to 2-fold increase in our study) could have produced readily available forms of nutrients (39,40). It can be speculated that during the fermentation, bacteria needed readily available nutrients, particularly nitrogen and energy, and these were obtained from the substrates. As fermentation progressed, the dietary fiber of the substrate was attracted by bacterial degradation, and the reduced crude fiber in this study was caused by an efficient optimization of fermentation and increased enzymatic activity. The magnitude of changes in nutrient contents of feeds by fermenting with the same bacterial inoculant was different in comparison to their raw counterpart feeds. This was simply due to the fact that the SFM and FFSB remarkably differ in nutrient content.

Bacterial fermentation leads to significantly improved AOC of SFM (9) due to the increased phenolic compounds with increased metal chelating activities (41). The increased AOC, measured as ABTS binding ability in this study could be due to the increased total phenolic compounds in FSFM and F-FFSB.

In conclusion, a well-optimized fermentation process employed in this study has caused significant reductions in ANFs of the SFM and FFSB samples with no enrichments of CP and CL whose contents were lowered by the fermentation of ATCC 6633. Thus, this fermentation procedure fits for the purpose of reducing ANFs contents and increasing the contents of biologically active compounds (organic acids, enzymes, and antioxidants). F-FFSB and FSFM enriched with beneficial bioactive molecules can be used as "functional feed additive or feed materials" in the nutrition and feeding of farm animals, however, a further purification step is needed to clean-up the bad odor of H_2S left in FSFB during the fermentation.

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