

Degradation of Phytic Acid and Soy Protein in Soy Meal via Co-fermentation of *Aspergillus oryzae* and *Aspergillus ficuum*

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Abstract The usage of soy meal (SM) as a protein source for young monogastric animals is limited by the presence of antinutritional factors. To address this problem, fermentation was applied to simultaneously degrade phytic acid and soy protein in SM. *Aspergillus oryzae* (ATCC 9362) and *Aspergillus ficuum* (ATCC 66876), sources of protease and thermostable phytase, respectively, were co-fermented using a two-stage temperature protocol: 36.5 °C (0–28 h), then 50 °C (28–40 h). The two-stage co-fermentation approach achieved a 17 % increase of phytic acid degradation compared to *A. oryzae* fermentation and 72 % increase in protein degree of hydrolysis (DH) compared to *A. ficuum* fermentation. The two-stage temperature fermentation produced a 27 % increase in phytic acid degradation and 90 % increase in DH compared to a single-stage fermentation. The fermented SM with reduced levels of antinutritional factors would serve as high quality feedstuff.

Keywords Solid-state fermentation · Soy allergens · Protease · Phytase · Phytic acid · Degree of hydrolysis

Abbreviations

AF	Two-stage incubation of <i>A. ficuum</i>
AO	Two-stage incubation of <i>A. oryzae</i>
AOFC	Two-stage co-fermentation of <i>A. oryzae</i> and <i>A. ficuum</i>
ATCC	American Type Culture Collection

DH	Degree of hydrolysis
FSM	Fermented soy meal
SM	Soy meal
AOFC	Single-stage co-fermentation of <i>A. oryzae</i> and <i>A. ficuum</i>

Introduction

Soy meal (SM) is mainly used as a protein source for monogastric animals [1]. However, antinutritional factors contained in SM, such as trypsin inhibitors, oligosaccharides, and phytic acid, lower its nutritional value. Trypsin inhibitors and oligosaccharides are easily removed via fermentation [2]. But, degradation of phytic acid and soy protein cannot be obtained at a high level simultaneously.

Phosphorous groups in phytic acid cannot be absorbed by monogastric animals because they do not produce phytase. Phytic acid combines with other nutrients, such as protein and minerals, to form phytic acid–protein or phytic acid–protein–phytic acid complexes in SM [3, 4]. The nutrients in the complexes are difficult for animals to digest. Further, the unabsorbed phytate may produce excess phosphorous in monogastric animals' manure, leading to eutrophication. Only two to three peptides and amino acids can be directly absorbed by monogastric animal intestine cells, thereby hindering the digestion of SM protein. Although soy protein has been investigated as a low-cost protein source for chick broilers and newly weaned piglets [5, 6], young animals with limited stomach acid and enzymatic secretions in the small intestine will have difficulty in digesting soy proteins because of the complex structures and large molecular weight of soy proteins [7].

Aspergillus ficuum (American Type Culture Collection [ATCC] 66876) and *Aspergillus oryzae* (ATCC 9362)

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have been used to ferment SM to decrease antinutritional factors [2, 8]. Phytic acid degradation by these two cultures was 89 and 57 %, respectively [8, 9]. Although *A. ficuum* produces a more stable phytase than that from *A. oryzae*, the former does not secrete protease. Similarly, although *A. oryzae* has high protease activity, its phytase stability is low [8]. In order to achieve a high level degradation for both phytic acid and soy protein, the co-fermentation of *A. ficuum* and *A. oryzae* was investigated in this study.

To enhance the co-fermentation, a two-stage temperature protocol was employed. The objective of the first stage, or fermentation stage, is to achieve high activities of both phytase and protease. The goal for the second stage, or enzyme hydrolysis stage, is to achieve the highest extent of enzymatic hydrolysis for protein and phytic acid *via* operating at an optimal temperature for enzyme activity and stability. The advantage of a two-stage fermentation protocol has been described previously.

Materials and Methods

Materials

Microorganisms were purchased from the ATCC (Manassa, VA, USA). SM was procured from Archer Daniels Midland Alliance Nutrition (Abilene, KS, USA) in 2009 and contained 11 % moisture and 51 % protein on a dry matter basis (dmb). All the other chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), except for those specifically indicated in the text.

Microbial Cultures

Aspergillus oryzae (ATCC 9362) and *A. ficuum* (ATCC 66876) were preserved in 15 % glycerol and stored at -80°C in 1-ml aliquots in the Bioprocessing and Renewable Energy Lab in Kansas State University. The *A. oryzae* strain used in this research did not produce mycotoxins [10]. The *A. ficuum* strain is commonly used for phytase production [11]. Before experimentation, the frozen cultures were revived in potato dextrose broth twice. After revival, the culture was inoculated on a potato dextrose agar (PDA) slant and incubated at 30°C for 7 days. Spores were collected from the slants by gently washing with 0.1 % Tween[®] 80 (polyoxyethylene (20) sorbitan monooleate) to obtain a spore suspension at a concentration near 10^7 spores/ml.

Substrate Preparation and Fermentation

Solid-state fermentation (SSF) was carried out in 125-ml Erlenmeyer flasks. The moisture content of SM was

adjusted to 50 % by adding distilled water before autoclaving. Erlenmeyer flasks containing 10 g substrates (dmb) were autoclaved at 121°C for 15 min. Sterilized samples were separated into four groups of two replicates according to their different inoculums: (1) AO (*A. oryzae* 3.2 ml); (2) AOF and (3) AOFC (*A. oryzae*, 1.6 ml plus *A. ficuum*, 1.6 ml); and (4) AF (*A. ficuum*, 3.2 ml). AOFC served as a single-stage control for AOF.

The two-stage process for AO, AOF, and AF consisted of first fermenting SM isothermally at 36.5°C from 0 h to 28 h, followed by isothermal fermentation at 50°C from 28 h to 40 h. A single-stage fermentation at 36.5°C was employed for AOFC.

Protease Activity

To extract protease from fermented soy meal (FSM), samples were milled, then mixed with distilled water at a ratio of 1:20 (w/v). The mixture was then placed in a 40°C water bath and shaken at a speed of 200 rpm for 1 h. The mixture was centrifuged at 16,800g for 15 min at 4°C . The supernatant was diluted twice with pH 7.2 sodium phosphate buffer and then tested for protease activity using an assay described previously [12] with modification. Casein solution (2 %, w/v) was prepared in pH 7.2 sodium phosphate buffer. A mixture of 0.25 ml of casein solution and 0.25 ml of enzyme extract was incubated at 40°C for 10 min. The proteolysis of casein was terminated by adding 0.5 ml of 0.4 M trichloroacetic acid (TCA). The mixture was then centrifuged at 16,800g for 15 min at 4°C . The supernatant (0.25 ml) was added to 1.25 ml of 0.4 M sodium bicarbonate followed by 0.25 ml of 0.4 M Folin–Ciocalteu phenol reagent. The solution was incubated at 40°C for 20 min; absorbance was read at 660 nm using 1.0-cm-pathlength cuvettes. The blank sample was prepared by adding 2 % casein solution after adding 0.4 M TCA to mixtures. Experiments were performed in triplicate. One unit of protease (U) was defined as the amount of enzyme that yields the color equivalent of 1.0 μg of tyrosine per minute per gram FSM (dmb) at 40°C .

Effect of Temperature on Activity and Stability of *A. oryzae* Protease

The protease activity of *A. oryzae* was analyzed at 40, 45, 50, 55, 60, and 70°C using the assay described above. To study protease stability at different temperatures, the supernatant from fermentation was incubated at 36.5, 45, 50, 55, 60, and 65°C for 1 h; the protease activity was then measured using the assay described above. Protease stability is defined as the percentage of residual protease activity remaining after incubation relative to the protease activity before incubation.

Phytase and Phytic Acid Assay

Phytase was extracted by mixing FSM with 1:10 w/v of 2 % CaCl_2 (w/v) via shaking for 1 h. The mixture was then centrifuged at 16,800g for 20 min at 4 °C and the phytase activity of the supernatant was analyzed following the method of Gunashree and Venkateswaran [13]. Phytase activity is defined as the amount of enzyme that liberates 1.0 μmol of inorganic phosphate in 45 min. Phytic acid was detected with a phytic acid assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland).

Degree of Hydrolysis for Proteins

The degree of hydrolysis (DH) for soy protein contained in FSM was analyzed using the trinitrobenzenesulfonic acid (TNBS) method, following the procedure of Adler-Nissen [14] with modifications. DH analysis was applied to the protein solutions used for protease analysis. One milliliter of sample solution was mixed with 2 ml of 1 % (v/v) sodium dodecyl sulfate (SDS) solution. The protease was first inactivated by incubation at 75 °C for 15 min. Then, a 0.25-ml aliquot of the mixture was transferred to test tubes containing 2.0 ml of sodium phosphate buffer (0.2 M, pH 8.2). TNBS reagent (0.1 %) was then added, followed by mixing and incubation in the dark at 50 °C for 60 min. The reaction was stopped by adding 4 ml of 0.1 N HCl. Samples were then allowed to cool at room temperature for 30 min, before absorbance values were measured at 340 nm. L-Leucine (0–2.0 mM) was used to generate a standard curve by diluting 2.5 mM L-leucine with sodium phosphate buffer. TNBS was added to the sample controls after the addition of 0.1 N HCl. DH was calculated on the basis of L-leucine equivalents.

Statistical Analysis

All analyses were conducted in duplicate except where indicated. Data were analyzed using the least-significant difference (LSD) method at the 0.05 level in accordance with procedures in the SAS statistical software package (SAS Institute 2009, Cary, NC, USA).

Results and Discussion

To optimize the two-stage temperature protocol for fermentation, the duration of the first stage needs to be decided first. Our preliminary research showed that after 28 h, visible spores appeared for both *A. oryzae* and *A. ficuum*. Too many spores might cause digestive irritation of monogastric animals. Figure 1 shows the phytase activity of *A. ficuum* and protease activity of *A. oryzae* at different fermentation

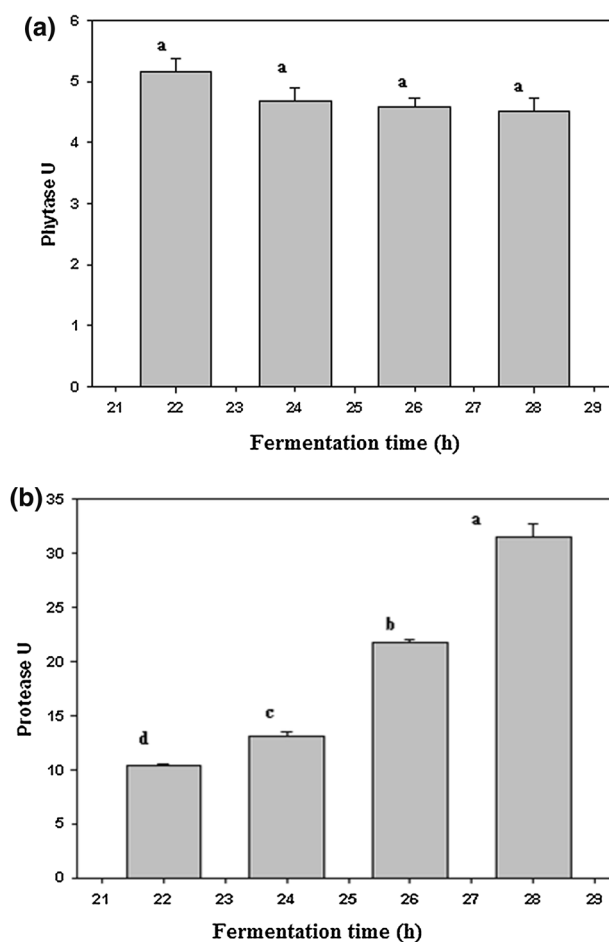


Fig. 1 Phytase activity of *Aspergillus ficuum* (a) and protease activity of *Aspergillus oryzae* (b) achieved for fermented soy meal (FSM) according to fermentation time. The incubation temperature was 36.5 °C for both cultures. Data with different letters (a–d) are statistically significantly different ($p < 0.05$)

times. From 22 to 28 h, phytase activity for *A. ficuum* did not change significantly; but, protease activity increased continuously with fermentation time up to 28 h. Therefore, 28 h was chosen as the fermentation time for the first stage.

To choose the optimal temperature for the second stage, enzymatic hydrolysis, the effect of temperature on the protease activity expressed by *A. oryzae* was investigated. The optimal temperature for protease activity is 55 °C, while protease activity at 50 °C (922 U) and 60 °C (884 U) were a little lower than that of 55 °C (1094 U, Fig. 2). But, as the temperature was increased, the protease stability decreased. For example, at 50 °C for 1 h, 33 % protease activity was lost; but if the incubation temperature was increased slightly, to 55 °C, 68 % protease activity was lost in 1 h. Therefore, 50 °C was chosen as the operating temperature for the second stage, to obtain both high *A. oryzae* protease activity and stability. We discovered that the optimal temperature for *A. ficuum* phytase was also at 50 °C, at which, *A. ficuum*

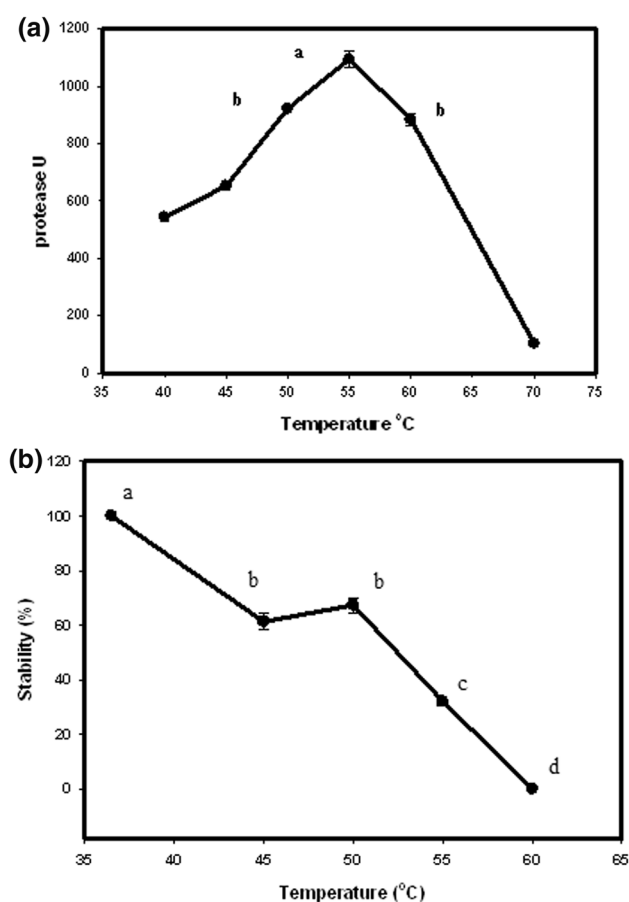


Fig. 2 Effect of temperature on the activity (a) and stability (b) of protease produced by *Aspergillus oryzae*. Data with different letters (a–d) are statistically significantly different ($p < 0.05$)

phytase possessed 93 % of its original activity after 3 h of incubation (data not shown). The phytase activity values of FSM at different incubation times for each of the four fermentation methods are shown in Fig. 3. The phytase activity for *A. oryzae* under monoculture conditions (AO) decreased when the temperature was increased from 36.5 to 50 °C during the second stage (after 28 h); in contrast, the phytase activity for *A. ficuum* under monoculture conditions (AF) increased during the second stage. Expression of phytase by *A. ficuum* is possibly spore-related. Phytase activity for co-fermentation samples (AOF) was higher than that of *A. oryzae* and lower than that of *A. ficuum* (Fig. 3).

Figure 4 displays the change of phytic acid concentration versus incubation time. The extent of phytic acid degradation was significantly different for four different fermentation methods. The single stage co-fermentation (AOFC) produced the lowest degree of phytic acid degradation (48 %), while *A. ficuum* two-stage incubation (AF) yielded the highest (72 %). Phytic acid degradation for two-stage co-fermentation (AOF) and for *A. oryzae* two-stage incubation (AO) were 61 and 52 %, respectively.

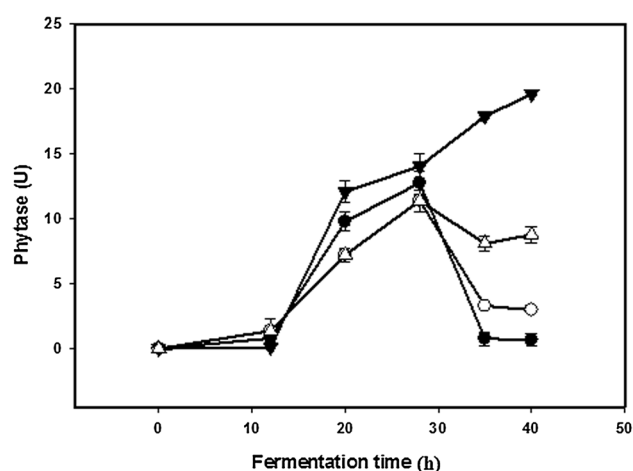


Fig. 3 Phytase activity of FSM versus incubation time and fermentation approach (Black circle AO, white circle AOF, inverted black triangle AF, white triangle AOFC). For two-stage fermentation (AO, AF, and AOF, referring to fermentation of *A. oryzae*, *A. ficuum*, and co-fermentation of *A. oryzae* and *A. ficuum*, respectively), the temperature was held at 36.5 °C for 0–28 h and 50 °C for 28–40 h. AOFC refers to the one-stage co-fermentation at 36.5 °C

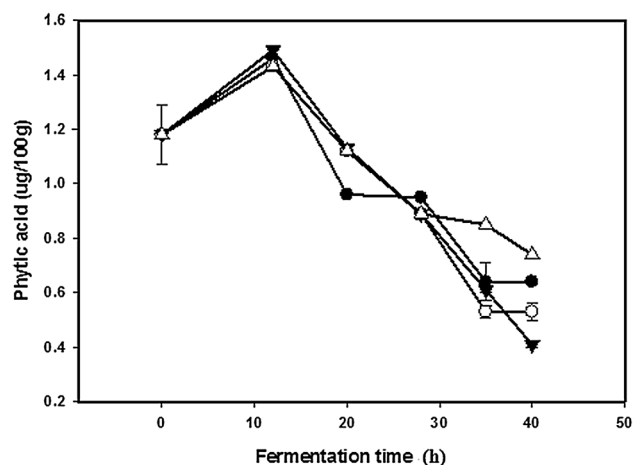


Fig. 4 Phytic acid concentration in FSM versus incubation time and fermentation approach. Fermentation conditions and abbreviations are given in Fig. 3

Although AOFC yielded a higher phytase activity than AO and AF during the second stage (Fig. 3), it led to a lower degree of phytate degradation (Fig. 4). That is because phytase had a much higher enzymatic activity at 50 °C (stage 2) than at 36.5 °C (stage 1 and the temperature employed for AOFC). With better phytase stability, two-stage co-fermentation led to a higher degradation degree than that for AO. Comparing AOF and AOFC, the two-stage fermentation afforded a 27 % increase in phytic acid degradation. Comparing AOF and AO, the co-fermentation of *A. ficuum* and *A. oryzae* had 17 % increase in phytic acid degradation.

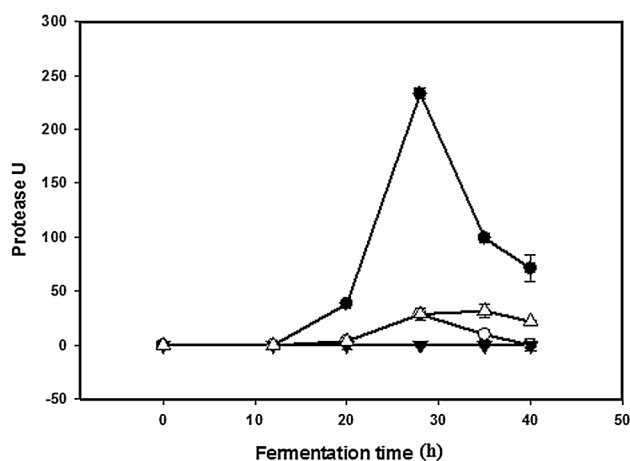


Fig. 5 Protease activity of FSM versus incubation time and fermentation approach. Fermentation conditions and abbreviations are given in Fig. 3

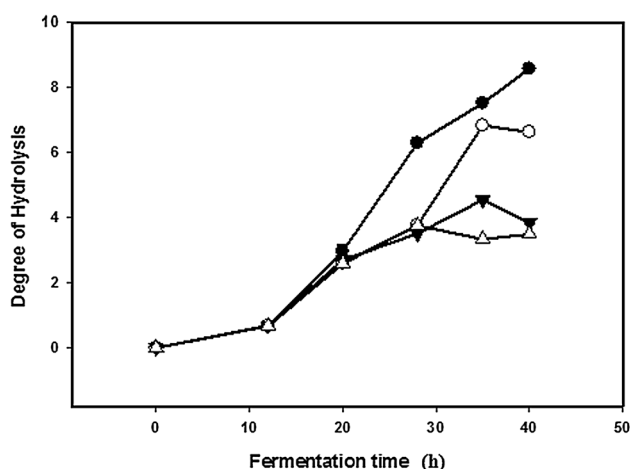


Fig. 6 Degree of hydrolysis for FSM versus incubation time and fermentation approach. Fermentation conditions and abbreviations are given in Fig. 3

Protease activities of FSM with different fermentation conditions are shown in Fig. 5. No protease was secreted by *A. ficuum*. Co-fermentation of *A. oryzae* and *A. ficuum* resulted in lower levels of protease activity than achieved for AO. *A. oryzae* protease activity decreased with time during the second stage for both AO and AOF, presumably because of the higher temperature.

Figure 6 plots the degree of protein hydrolysis for SM versus fermentation time. AO promoted the largest DH, followed by AOF. Comparing the two co-fermentations, the two-stage temperature fermentation produced a 90 % higher DH level than the single-stage control (Fig. 6). Although *A. ficuum* did not secrete protease to any significant extent (Fig. 5), the decrease of DH achieved for AF was higher than for AOFC. The underlying cause may be the release

of phytic acid from protein bodies catalyzed by *A. ficuum*, noting the latter's high phytase activity (Fig. 3), leading to release of protein and peptides from protein–cation–phytate complexes contained in the protein bodies [15].

Degradation of phytic acid and soy protein contained in SM will help alleviate their negative effect on animal growth and the release of phytic acid to the environment through manure, leading to reduced levels of eutrophication [3, 16]. Hirabayashi et al. [17, 18] investigated the effect of *Aspergillus usamii* fermentation on SM to allow the latter's use as feed for chicks, and they found that FSM improved the bioavailability of phosphorus, zinc, and iron in SM, thereby leading to body weight gain for the chicks. Fermentation and protease hydrolysis depolymerize soy protein, leading to its enhanced solubility in water and perhaps lowering soy protein immunoreactivity by altering the structure of antigen epitopes, thereby reducing the IgE binding potential [19]. Partial replacement of SM with protease-hydrolyzed soy protein for broilers used for 1- to 21-day-old chicks increased feed intake, body weight gain, and nutrient digestibility of crude protein, Ca, and P [20]. The FSM obtained in this research had both low phytic acid content and high protein DH; therefore, its nutritional value is much higher than SM fermented by other methods.

Conclusions

Both *A. oryzae* and *A. ficuum* grew well when cultivated together using SM as the sole carbon source. Co-fermentation of *A. oryzae* and *A. ficuum* produced higher phytase activity and better phytase stability than achieved by *A. oryzae* fermentation, and it also had higher protease activity than by *A. ficuum* fermentation. Two-stage temperature co-fermentation yielded a 27 % increase in phytic acid degradation and 90 % increase in protein DH compared to single stage co-fermentation, a 17 % increase in phytic acid degradation compared to *A. oryzae* two-stage fermentation, and a 72 % increase in DH compared to *A. ficuum* two-stage fermentation.

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