DETOXIFICATION AND NUTRITIONAL ENHANCEMENT OF SOY MEAL *VIA* MICROBIAL BIOPROCESSING

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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College of Agriculture

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Manhattan, Kansas

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Abstract

Soy meal (SM) is the main protein source for monogastric animals. Anti-nutritional factors in SM limited its usage for young monogastric animals. Aspergillus was investigated to degrade these factors and to enhance its nutritional value *via* solid state fermentation. Galacto-oligosaccharides were totally degraded from the initial 9.48 mmol/100 g, and trypsin inhibitor decreased from 10.7 TIU/mg to a non-detectable level after 36 hr fermentation. Structural polysaccharides decreased by 59% (w/w) and the degree of hydrolysis of SM protein increased from 0.9% to 7% (w/w) through the 7 d fermentation. Fermentation also modified nutritional factors. Protein content increased from 50.47% (w/w) to 58.93% (w/w) after 36 hr fermentation. Amino acid profile was significantly enhanced.

Two - stage temperature-induced fermentation protocol was developed to increase the degradation rate of phytic acid by *A. oryzae* (ATCC 9362) and by *A. ficuum* (ATCC 66876). The first stage maximized phytase production with fermentation parameters obtained by central composite design. The second stage achieved maximum enzymatic degradation with parameters obtained by studying the phytase temperature characteristics. While using *A. oryzae*, 57% of phytic acid in SM was degraded by the two stage protocol compared to 39% degradation from single stage fermentation. For *A. ficuum*, the two-stage temperature fermentation protocol achieved a 98% degradation level of phytic acid degradation compared with the single stage process. Two-stage temperature-induced co-fermentation of *A. oryzae* and *A. ficuum* was investigated to simultaneously degrade phytic acid and soy protein with high efficiency. Co-fermentation of *A. oryzae* and *A. ficuum* resulted in higher phytic acid degradation than *A. oryzae* fermentation and superior protein hydrolysis compared to *A. ficuum* fermentation.

Sterilization distorted the results of fermentation effect on soy allergens and soy protein degradation. Virginiamycin is a kind of bacterisin. It was added to *A. oryzae* solid state fermentation, to exclude the necessity of SM sterilization. Nonsterile, solid state fermentation using *A. oryzae* and virginiamycin showed the complete degradation of α and α' subunits of β-conglycinin and decreased immunoreactivity of soy protein. The modified SM after microbial bioprocessing created an innovative product with enhanced characteristics with potential wider applications for feed industry.

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Chapter 1 - Anti-nutritional factors in soy meal and their detoxification[1]

Soy meal

Soybean is the second largest acreage crop in the United States (29%), right after corn (35%) according to the American Soybean Association (Chen et al 2013). Soybean contains around 40% protein, 20% oil, 35% carbohydrates and 5% ash (Liu 1997). In the United States, soybean is mainly used for biodiesel production. Soy oil is extracted either using solvent extraction or mechanical extraction (Chen et al 2010a). After oil extraction, the leftover material is soy meal (SM).

According to Grieshop et al (2003), SM contains protein (53.5-56.2%), crude fat (0.8-2.3%), total dietary fiber (17.0-20.7%), total sugar (13.6-17.9%). The sugar includes sucrose (6.8-7.72%), raffinose (0.98-1.43%), stachyose (4.1-5.72%), verbascose (0.16-0.24%). Soy meal is the main protein source for animal feed, because of its high protein content and its amino acids profile complementary to that of grains. The portions of different animal usages are poultry (48%), swine (26%), beef (12%), dairy (9%), pets (2%) and others (3%) (Chen et al 2013). Because of the large markets of poultry and swine in feed industry, our research will focus on the SM usage of monogastric animals.

Soy meal also contains several kinds of anti-nutritional factors, which lower down its nutritional value and limit its usage for broilers and newly-weaned piglets, whose digestive tract is not mature enough. Detrimental effects due to SM in young animals include reduction in growth rate, nutrient digestibility and intestinal villus height (Dreau et al 1994). Newly weaned piglets fed SM had worse performance and more diarrhea than pigs fed no protein diet (Song et al 2010).

Carbohydrates

Soy meal contains 35-40% carbohydrates (Liu, 1997). These carbohydrates could be separated into three categories: soluble sugar, storage polysaccharides, structural polysaccharides. Soluble sugar constitutes approximately 50% of total carbohydrates, mainly

[1] Chapter 1 is parts of two book chapters: Chen et al (2010) / Soybeans: Cultivation, Uses and Nutrition; Chen et al (2013) / Soybean: A Review

sucrose, raffinose, stachyose and verbascose. Free galactose, glucose, and fructose were not found in SM because they were removed or destroyed during processing. Sucrose in SM ranged from 42.4 to 73.4 mg/g DM. In SM produced at 10 commercial processing plants in the US, concentrations ranged from 41.0 to 57.2, 9.8 to 14.3, and 1.6 to 2.4 mg/g DM for stachyose, raffinose, and verbascose, respectively (Grieshop et al 2003). Storage polysaccharide in SM is starch, constituting less than 1% of DM. Soy cotyledon polysaccharides are made up of acidic components and a neutral polysaccharide. The complex of these polysaccharides contains structural features common with several pectinic acids and with tragacanthic acid. Therefore, it belongs to the pectinic acid group of polysaccharides that contain unusually high proportions of neutral sugar units (Karr-Lilienthal et al 2005).

Galacto-oligosaccharides (GOS)

Fermentation of GOS results in the production of short chain fatty acids and the gases, CO_2 and H_2, potentially resulting in increased flatulence. Carbon dioxide and H_2 can cause flatulence, nausea, and discomfort in swine.

In general, alpha-galactosidase acts upon gal-gal bonds in the tetrasaccharide stachyose, releasing galactose and raffinose, and also acts upon gal-glu bonds with the release of sucrose. Mono-gastric animals can't digest galacto-oligosaccharide themselves, due to the lack of alpha-galactosidase. The oligosaccharides will be utilized by microorganisms in their small intestine, resulting in the production of CO_2 and H_2 (Parsons et al 2000). In some cases, the accumulation of flatulent rectal gas provokes gastrointestinal distress such as abdominal pain, nausea, and diarrhea. Weanling pigs fed a GOS-free diet supplemented with 2% stachyose or fed a diet containing soy meal had increased incidence of diarrhea compared with pigs fed a control diet (Karr-Lilienthal et al 2005). Additionally, fermentation of GOS has been implicated to have negative effects on nutrient digestibilities and energy availability of soy meal. Roosters fed soy meal with low oligosaccharide concentrations had higher total net metabolizable energy values (2931 kcal/kg dm) than those fed conventional soy meal (2739 kcal/kg DM) (Nout and Rombouts 1990). The removal of polysaccharides from soy foods and feed is, therefore, a major factor in improving their nutritive value.

Structural polysaccharides

SM structural polysaccharides may be retained in the large intestine of swine for 20-38 hr (Karr-Lilienthal et al 2005). This allows for prolific microbial growth. But they may have an impact on digestibilities of other nutrients in the diet (Karr-Lilienthal et al 2005).Structural polysaccharides were also considered as anti-nutritional factors (Jezierny et al 2010). These structural polysaccharides reduced digesta passage rate, which may result in a lowered feed intake and decreased growth performance (Dunshea et al 2001).

Trypsin inhibitors

Protease inhibitors constitute around 6% of soybean protein (Hocine and Bove 2007). Two protein protease inhibitors have been isolated from soybeans. The Kunitz trypsin inhibitor has a specificity directed primarily toward trypsin and a molecular weight of about 21.5 kDa. The Bowman-Birk (BB) inhibitor is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites and has a molecular weight of 7.8 kDa (Isanga and Zhang 2008). Trypsin and chymotrypsin, the two major proteolytic enzymes produced in the pancreas, belong to the serine protease class.

Trypsin inhibitors present in soybeans are responsible for growth depression by reducing proteolysis and by an excessive fecal loss of pancreatic enzymes rich in sulfur-containing amino acid which can't be compensated by dietary soy protein (Kakade et al 1973). Trypsin inhibitors account for 30-50% of the growth inhibition effect (Denter et al 1998). Rats fed a raw soybean extract from which trypsin inhibitors had been inactivated showed improved growth performance when compared with control rats fed diets containing raw soybeans from which inhibitors had not been inactivated (Sato et al 2001). Diets with a trypsin inhibitor concentration of 0.77 mg/g and less did not reduce the growth of pigs according to reference (Hong et al 2004).

Phytic acid

Phytate is the calcium-magnesium-potassium salt of inositol hexaphosphoric acid commonly known as phytic acid [Figure1.2] (Oatway et al 2001). Phytate and phytic acid are also referred to as phytin in some literature.

Phytate is the main storage form of phosphorous in soybean. Its content in soybean ranged from 1.00 to 1.47% on a dry matter basis (Liu 1997). Phytate is known to be located in

the protein bodies, mainly within their globoid inclusions. Phosphorous in the phytate form could not be absorbed by monogastric animals, because they lack phytase, the digestive enzyme required to release phosphorus from the phytate molecule. Phytic acid could form protein-phytate or protein-phytate-protein complexes; these have more resistance to digestion by proteolytic enzymes; thus, utilization of dietary protein is reduced. Also, phytic acid has a strong binding affinity to important minerals such as calcium, magnesium, iron, and zinc. When a mineral binds to phytic acid, it becomes insoluble, precipitates, and is not absorbable in the intestines. In food industry, the presence of phytic acid in high concentration is undesirable. In feed industry, the unabsorbed phytate passes through the gastrointestinal tract of monogastric animals, elevating the amount of phosphorus in the manure. Excess phosphorus excretion can lead to environmental problems such as eutrophication. With the pressure on the swine industry to reduce the environmental impact of pork production, it is important to use feed ingredients that can minimize this influence.

Soy protein

The high protein content and comparatively low cost makes soy meal a major protein source for mono-gastric animals feed. Soy protein is high in lysine, but low in sulfur-containing amino acids, with methionine being the most limiting amino acid (Liu 1997). The special amino acid pattern of soy meal is complementary to corn, and makes it perfectly combine with corn as feedstuff.

Soy protein basic information

Based on solubility patterns, soy protein is divided into water soluble albumins and salt soluble globulins. Globulins are further divided into two distinct types: legumin and vicilin, which are commonly known as glycinin and conglycinin, respectively. Basing on sedimentation coefficients using ultracentrifugation, soy protein could be separated into 2, 7, 11, and 15S. 2S and 7S fractions are heterogeneous. The 2S fraction accounts for about 20% of the extractable protein and includes the Kunitz and Bowman-Birk trypsin inhibitors and cytochrome C. The 7S fraction accounts for one third of the extractable protein and consists of conglycinin, α-amylase, lipoxygenase, and hemagglutinin. 11S and 15S fractions are pure proteins. 11S fraction is the soybean glycinin and accounts for at least one third of extractable protein, whereas the 15S fraction is the polymer of glycinin and accounts for about 10% of extractable protein.

Conglycinin is made of α-, β-, γ-conglycinins. Because of its highest proportion in the 7S fraction, β-conglycinin has been mainly studied. The fraction β-conglycinin is a trimer with a molecular weight of about 180 kilo Daltons (kDa). Its three subunits are designated as α', α, and β, with molecular weight of 76, 72, and 53 kDa, respectively . Conglycinin has no disulphide bond. Glycinin is a hexamer and have molecular weight (MW) of about 300 – 450 kDa. It consists of six subunits of MW about 60 kDa associated by noncovalent forces. Each subunit is composed of acidic (MW about 40 kDa) and basic (MW about 20 kDa) chains, associated by a single disulphide bond. Glycinin and β-conglycinin account for 65-80% of total seed proteins (Burks et al 1991).

Soy protein as anti-nutritional factors

The anti-nutritional effects of soy protein include two aspects. One is the large protein molecular weight. According to Kim S.W. (Kim 2010), newly weaned pigs have immature digestive tract, with limited HCl in stomach and enzymatic secretion in small intestine. They have difficulties to digest proteins with complex structures and large molecular weights. The other aspect is the allergenic protein, which is discussed below.

Soy protein is one of the "big 8" food allergens designated by the Food Allergen Labeling and Consumer Protection Act of 2004 (Frias et al 2008). It cause Type I hypersensitivity reactions, which involve generation of an IgE antibody response (Mills et al 2004). To date, 34 soybean proteins have been identified as allergens (Wilson et al 2008). All three parts of the β-conglycinin (Krishnan et al 2009), both acidic (Zeece et al 1999) and basic subunits of glycinin (Helm et al 2000), P34, and 2S, have been identified as main allergens for young pigs (Hong et al 2004; Mills et al 2004; Frias et al 2008; Wilson et al 2008; Zhao et al 2008).

Glycinin, β-conglycin and 2S protein have been introduced above. Now we will give the introduction for P34. P34, also referred as Gly m Bd 30K or Gly M 1, has been identified as a predominant immunodominant allergen. P34 is an insoluble glycoprotein that consists of 258 amino acid residues contained by disulfide linkages in the 7S globulin fraction of soybean storage protein and may play a role in protein folding (Wilson et al 2008). P34 is a relatively minor seed constituent comprising less than 1% of total seed protein and has a tendency to bind to lipid and oil, which is likely significant in its role as an allergen.

The deduction of immunoreactive glycinin and beta-conglycinin in stomach, middle jejunum, caecum, colon were studied by Zhao et al (2008), when purified glycinin and beta-conglycinin were fed to newly weaned piglets. Dietary glycinin or beta-conglycinin reduced weight gain and feed efficiency and increased the incidence of diarrhea in piglets. The imunoreactivity of glycinin or beta-conglycinin decrease as the digesta descended down the digestive tract, which followed the general rules of the protein digestion.

Enzymes addition

In order to remove the negative effect caused the anti-nutritional factors in SM, enzymes have been added to the diet of monogastric animals. The common enzymes used are phytase and carbohydrase.

Phytase is probably the enzyme most familiar to monogastric nutritionists. The current pressure to reduce environmental phosphorus pollution in both Europe and the United States has created a market opportunity for exogenous phytases. Consequently, phytase enzyme preparations have became important feed additives with a wide range of applications in swine and poultry production. Phytase hydrolyzes phytic acid to inositol and phosphoric acid and thereby eliminates the metal-chelating properties of phytic acid. For a thorough review of the benefits of phytase, see Singh (2008). Adding microbial phytase to plant-derived feed ingredients fed to poultry improved growth performance, feed intake, and feed efficiency (Ravindran et al 2000) and retention of phosphorus, calcium, zinc, and copper (Chu et al 2006; Hill et al 2009). Phytase may also improve protein digestibility (Kies et al 2006). Higher retention of nutrients ultimately reduces environmental pollution (Chu et al 2006; Hill et al 2009).

Various kinds of carbohydrase have been used to solve problems caused by carbohydrates. Xylanase, glucanase, cellulase (Cowieson et al 2010), hemicellulase, pectinase, galactanase, mannanse, and polygalacturonase have been tried. Both positive and negative results have been obtained and published in the literature. Cowieson et al (2010) found that xylanase and glucanase could improve the feed conversion ratio and ileal nutrient digestibility. The combination of the two enzymes resulted in greater benefits than either enzyme alone. Galactanase had the ability to hydrolyze soy polysaccharides, but its residual activity in the intestine was poor. In vivo experiments showed that addition of multiple enzymes (galactanase and mannanse) did not enhance the body weight gain of broiler chickens and that enzyme

activity decreased gradually from stomach to caecum. There is still no research to find the proper carbohydrase for monogastric animals feed. One reason is the complex soy carbohydrates. The other is the low stability of in vivo enzyme activity.

Even phytase addition could be added to partially degrade phytic acid in SM. It could only solve the disadvantages cause by one anti-nutritional factor. Fermentation has been investigated to applly to detoxify several anti-nutritional factors in SM at one time. Animal test about fermented SM (FSM) involvement has been done and positive results have been obtained.

Animal test of FSM

Several research have been tried to apply FSM to mono-gastric animals and positive results have been obtained. Feeding piglets and broilers FSM significantly increased average daily gain and average daily feed intake and improved feed conversion efficiency compared with feeding piglets unfermented SM (Feng et al 2007a; Feng et al 2007d). With regard to the soybean allergy, Liu et al (2007) showed that fermentation of SM decreased the immune response to soybean protein in piglets; the level of serum IgG decreased 27.2%. Song et al (2010) found that plasma from pigs fed FSM exhibited reduced immunoreactivity toward α, α' subunits of β-conglycinin and acidic subunits of glycinin, due to the partial hydrolysis of those proteins during fermentation. Antigenic soybean proteins in the diet of early weaned pigs provoke a transient hypersensitivity associated with morphological changes including villi atrophy and crypt hyperplasia in the small intestine (Dreau and Lalles 1999). All of these morphological changes can cause a malabsorption syndrome (Gu and Li 2004; Sun, Li, and Dong 2008), growth depression, and diarrhea (Dreau et al 1994; Sun et al 2008). Feng et al (2007b) investigated differences of the villi condition in pigs fed SM and FSM by using scanning electron microscopy and found that piglets fed SM had shorter, disordered, and broader villi, whereas piglets fed FSM had long, round, regular, and tapering villi that could better digest and absorb nutrients. Also piglets and broilers fed FSM had higher activities of total protease and trypsin at the duodenum and jejunum of piglets than those fed with SM (Feng et al 2007a; Feng et al 2007c). Moreover, Hirabayashi et al (1998a) found that *Aspergillus usamii* fermentation improved phosphorous bioavailability in SM and supplemental inorganic phosphorous was not necessary for the FSM –based diet, which remarkably reduced phosphrous excretion in chicks.

Current research of FSM

But the current research did not provide enough information for technology improvement of the current product. The nutritional profile change of SM via fermentation, including the decrease of trypsin inhibitor content, GOS content, protein molecular weight, and increase of crude protein, crude fat, phosphorous, total amino acids, TCA soluble protein were studied by Hong et al (2004), Chen et al (2010b) and Teng et al (2012). All these three were used *A. oryzae* as the fermentation culture. Hong and Chen used SM as the sole substrate and Teng used the mixture of SM with wheat bran at a ratio of (3:1). As to the immunity, Song et al (2008) studied the immunoreactivity of FSM, fermented by natural organisms, *Lactobacillus plantarum*, *Bifidobacterium lactis*, *Saccharomyces cereviseae*. FSM showed reduced immunoreactivity to human plasma, particularly if proteins were <20 kDa. *S.cereviseae* and naturally FSM showed the highest reduction in IgE immunoreactivity, up to 89% and 88%, respectively, against human pooled plasma. Frias et al (2008) studied immunoreactivity of *A. oryzae*, *R.oryzae* or *B.subtilis* FSM. *R.oryzae* and *A. oryzae* FSM showed 66% and 68% reduction in IgE immunoreactivity, respectively, for human plasma 97.5 kUA/L. *B.subtilis* fermentation yielded a 81 and 86% reduction in immunoreactivity against both human plasma 97.5 IgE kUA/L and human pooled plasma samples, respectively.

Not only limited information was provided, but there are other limitations in these researches. Hong et al (2004) used 60-70 ^{o}C for 1 hr as sterilization before *A. oryzae* fermentation, which was not enough to kill the natural bacteria. Chen et al (2010b) and Song et al (2008) didn't use sterilization. The existing bacteria in SM would cause contamination to FSM and the quality of FSM is hard to control. Teng et al (2012) used the mixed substrate of SM with wheat bran. Considering the large particle gap caused by the wheat bran, the fermentation environment was totally different with SM as the sole substrate. Besides, the mixing of wheat bran would increase the content of structural polysaccharides and decrease the value of final product. Frias et al (2008) studied the fermentation on the immunoreactivity reduction of sterilized SM. Since sterilization at 121^{o}C could denature soy protein and reduce the immunoreactivity, there is no way to make sure whether the immunoreactivity reduction is caused by fermentation. In order to study how fermentation will influence the SM allergens, new method is needed.

Our research

Our research focuses on the reduction of anti-nutritional factors, including GOS, trypsin inhitor, large protein molecular size, protein allergy and phytic acid. We investigated the way to increase the processing efficiency and product quality and solid state fermentation was used with *A. oryzae* and *Aspergillus ficuum* as the microbial culture.

Solid state fermentation

Solid state fermentation (SSF) is defined as a fermentation process in which microorganisms grow on solid materials without the presence or near absent of free water. In SSF, the moisture necessary for microbial growth exists in an absorbed state or complexed within the solid matrix (Krishna et al 2005).

SSF has been used to produce various kinds of enzymes, chemicals, antibiotics etc., which has been reviewed by Krishna et al (2005). It also has been used to detoxify agro-industrial residues. Compared with submerged state fermentation, solid state fermentation has the following advantages: SSF usually use raw materials as substrates, and this lowers the capital cost. It uses low water content compared with SmF, which also lead to low wastewater output. It has high volumetric productivity. Like animal feed production, the whole fermented substrates are expected to be the product. Using SSF for this kind of product, less downstream processing is needed compared with SmF.

The hypha growth mode and their good tolerance to low water activity (*Aw*) give fungi advantages in the colonization of solid substrates and utilization of available nutrients (Krishna et al 2005). Fungi have aerial hyphae and penetrative hyphae. The role of penetrative and aerial hyphae in the growth process has not been fully elucidated. Aerial hyphae may make significant contributions to the overall oxygen uptake rate, while penetrative hyphae may bring the site of hydrolytic enzyme release closer to the polymers in the interior of the particles (Nopharatana et al 2003). Varzakas et al (1998) studied the mycelia penetration and enzyme diffusion in soya bean tempeh and defatted soy flour. Hyphae infiltrated to a depth of about 2 mm in 40 hr for soy bean tempeh, whereas for tempe-flour, the depth was about 5-7 mm over same time.

Aspergillus oryzae

Aspergillus oryzae is the fermentation culture for the production of soy sauce (shoyu) and soy paste (jiang). *A. oryzae* could secret various kinds of enzymes, which may include but is not

limited to: α-amylase, carboxymethyl cellulase, pectinlyase (Nakadai and Nasuno 1988), protease (Wang et al 2005), endo-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55) (Szendefy et al 2006), α-galactosidase (EC 3.2.1.22) (Shankar and Mulimani 2007), endo- and exdo-polygalacturonase (Fontana et al 2009), and α-L-arabinofuranosidase (Matsumura et al 2004) to degrade complex carbohydrates into available sugar for their growth needs. *A. oryzae* could secret neutral protease (Yasuhara et al 1994; Sandhya et al 2005), acid protease (Vishwanatha et al 2010) and alkaline protease (Ikegaya et al 1992). Characteristics of neutral and acid protease have been studied by Bombara et al (1994) and Vishwanatha et al (2009). During industrial koji making, *A. oryzae* produced both phytase (EC 3.1.3.8) and acid phosphatase (EC 3.1.3.2) (Fujita et al 2003).

Aspergillus ficuum

Aspegillus ficuum NRRL 3135 was designated to be the highest producer of active phytase (Ullah 1988). The optimum pH for extracelluar phytase activity is pH2.5 and pH5.0. Phytase and acid phosphatase sequentially hydrolyze myo-inositol hexakis-, pentakis-, tetrakis-, triphosphate, diphosphate and monophosphate (Ullah and Phillippy 1994). Fermentation has also been directly applied to degrade phytic acid. Nair and Duvnjak (1990) used *A. ficuum* NRRL3135 solid state fermentation to degrade phytic acid in canola meal, and a complete reduction of phytic acid content was achieved in 48hr. *A. ficuum* has been reported to secret alpha-galactosidase (Shankar and Mulimani 2007; Zapater et al 1990) and xylanase (Lu et al 2008).

Virginiamycin

Virginamycin is the bactericin to feed industry. Also, it is used in ethanol production to prevent contamination of materials used for fermentation. In our research, we applied the virginiamycin to inhibit bacteria growth during *A.orzyae* solid state fermentation in unsterilized SM. Our preliminary research found that 0.05% (dmb) of virginamycin in SM could inhibit bacteria growth while *A. oryzae* could still grow well. By applying virginamycin, the respective effect of heat and fermentation on the immunoreactivity reduction and soy protein degradation could be investigated.

Specific research objectives

First, we utilized *A. oryzae* (ATCC12896) for the SSF of SM, to see the physical and chemical changes of SM and the growth characteristics of *A. oryzae*.

Preliminary research indicate: (1) the optimal temperature for *A. oryzae* growth is 36.5 ^{o}C; (2) the optimal temperature for *A. oryzae* phytase was 45-50 ^{o}C; (3) from around 28 hr, visible spores emerge and cover the substrate surface at 28 hr of fermentation. As a result of the two temperature regime, two - stage temperature solid state fermentation was investigated. The first stage was for *A. oryzae* growth and enzyme production. The second stage was for enzymatic hydrolysis.

Considering that *A. oryzae* fermentation could not totally hydrolyze phytic acid in SM, and *A. ficuum* phytase has better stability, we investigated phytic acid degradation, using two - stage temperature protocol with *A. ficuum*.

Based on three facts: (1) *A. ficuum* better hydrolyzes SM phytic acid than *A. oryzae*. But *A. ficuum* didn't secret protease; (2) *A. oryzae* had both phytase and protease; (3) *A. oryzae* and *A. ficuum* belonged to the same genus and their growth rate on SM was similar, which suggested that growing them together will not lead to antagonism. We performed co-fermentation of SM using *A. oryzae* and *A. ficuum* to achieve better degradation of both phytic acid and protein.

To study the effect of fermentation on SM immunity, sterilization of SM would distort the results, since heat could also decrease protein immunity. However, without sterilization, the chance of contamination is enhanced. In our research, virginiamycin was added to SM to inhibit the growth of contaminant bacteria and the effects of fungal fermentation on SM protein degradation and immunoreactivity were investigated.

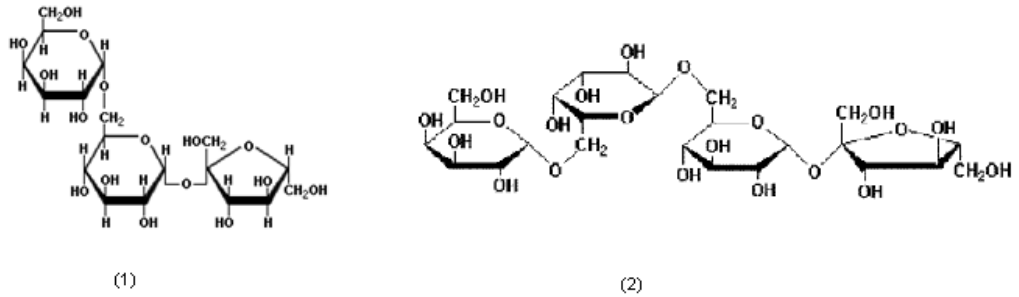


Figure 1.1 Structure of raffinose and stachyose (1) raffinose; (2) stachyose

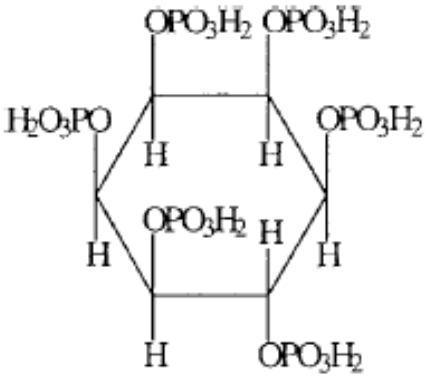


Figure 1.2 Basic structure of phytic acid

Chapter 2 - Nutritional enhancement of soy meal *via Aspergillus orzyae* solid – state fermentation[2]

Abstract

Anti-nutritional factors in soy meal (SM) include trypsin inhibitor, galacto-oligosaccharides (GOS), structural polysaccharides and large molecular weight protein. These anti-nutritional factors limit the usage of SM for young monogastric animals. *A. oryzae* solid state fermentation was applied to eliminate these factors. Changes in physical and chemical characteristics of SM during fermentation were investigated. *A. oryzae* was more nutrient- than oxygen-oriented, illustrated by scanning electron microscopy. GOS (raffinose, stachyose and verbascose) were totally degraded from the initial 9.48 mmol/100 g, and trypsin inhibitor decreased from 10.7 TIU/mg in original SM to a non-detectable level after 36 hr fermentation. Structural polysaccharides decreased by 59% (w/w) and the degree of hydrolysis of SM protein increased from initial value of 0.9% to 7% (w/w) through the 7 d fermentation. Fermentation also modified nutritional factors. Protein content increased from 50.47% (w/w) to 58.93% (w/w) after 36 hr fermentation. Amino acid profile was significantly enhanced. The research thoroughly studied the *A. oryzae* solid-state fermentation process on SM. Results showed that the process could provide a better protein source for monogastric animals and provides detailed information for future process optimization.

Introduction

Soy meal (SM), the residue from soy oil extraction, is mainly used for animal feed. Poultry and swine usage accounts for 74% of total animal usages (Chen et al 2010a). Anti-nutritional factors in SM, including galacto-oligosaccharides (GOS), trypsin inhibitors, structural polysaccharides, large molecular weight protein etc., limit its usage for young animals with immature gut structure. The concentration of GOS ranged from 41.0 to 57.2, 9.8 to 14.3, and 1.6 to 2.4 mg/g dry matter for stachyose, raffinose and verbascose, respectively (Grieshop et al 2003). Fermentation of GOS by intestinal bacteria in the lower intestinal tract results in the production of CO_2 and H_2, potentially resulting in increased flatulence. CO_2 and H_2 can cause

[2] Chapter 2 is included as a part of Chen L. et al. in Cereal Chemistry (minor revision)

flatulence, nausea, and discomfort in monogastric animals (Karr-Lilienthal et al 2005). Weanling pigs fed a GOS-free diet supplemented with 2% stachyose or fed a diet containing SM had increased incidence of diarrhea compared with pigs fed a GOS–free diet (Zhang et al 2003). Trypsin inhibitors constitute around 6% of soy protein (Friedman and Brandon 2001) in SM. They account for 30–50% of the growth inhibition effect (Isanga and Zhang 2008) by reducing proteolysis and by an excessive fecal loss of pancreatic enzymes rich in sulfur-containing amino acids. Loss of the S- containing amino acids cannot be compensated by dietary soy protein (Rackis and Gumbamann 1981). SM structural polysaccharides may be retained in the large intestine of swine for 20-38 hr (Karr-Lilienthal et al 2005). These polysaccharides were also considered as anti-nutritional factors (Jezierny et al 2010). They could reduce digesta passage rate, which may result in a lowered feed intake and decreased growth performance (Dunshea et al 2001). Newly weaned pigs have difficulties to digest original soy protein with complex structures and large molecular weights, due to limited HCl in stomach and enzymatic secretion in small intestine (Kim 2010).

Fermentation can effectively eliminate the adverse effect of many anti-nutritional factors in SM, which we have reviewed (Chen et al 2013). Fermented SM (FSM) has been applied for feeding young monogastric animals and positive results have been obtained. The FSM could increase average daily gain, improve intestinal morphology and digestive enzyme activities, digestibility of crude protein in weaned piglets and broilers (Feng et al 2007a; Feng et al 2007b; Feng et al 2007c; Feng et al 2007d; Liu et al 2007). Also, Song et al (2010) found that newly weaned pigs fed with FSM have less diarrhea than those fed with SM. FSM could also partially replace dried skim milk and plasma protein at 3 to 7 week of age nursery pigs (Kim et al 2010).

But the research about FSM producing is limited. Hong et al (2004), Chen et al (2010b) and Teng et al (2012) had studied the changes of protein content, amino acid, protein molecular weight, trypsin inhibitors, and GOS. For research of Hong et al (2004) and Chen et al (2010b), no sterilization was applied to fermentation. Contamination from the natural culture in SM might exist, so the product quality is hard to control. Teng et al (2012) used the mixture of SM with wheat bran at a ratio of 3:1 as fermentation substrate. The addition of wheat bran increased the content of structural polysaccharides and decreased the value of FSM. In total, the current research was not enough to understand the process and provide enough information for future process modification.

Our research further studied the solid state fermentation of SM. *Aspergillus oryzae*, which secretes enzymes such as alpha-galactosidase (Shankar and Mulimani 2007) and protease (Wang et al 2005), was used. We summarized the common characteristics of fungi which could grow on SM, the reason for the nutrition enhancement, the change of anti-nutritional factors. This research gave the clear nutritional profile of FSM, and also it provided sufficient information for our future research on improving fermentation process of FSM.

Materials and Methods

Microbial cultures

A. oryzae (American Type Culture Collection (ATCC 12892) was preserved in 15% glycerol solution at -80 ^{o}C in our lab. Each time before use, one 1 ml - Erlenmeyer tube of *A. oryzae* spore solution was placed into 50 ml sterilized potato dextrose broth (PDB) and incubated at 30 ^{o}C for 48 hr. The activation process was done twice. To prepare the spore suspension, fungal spores from the above medium were cultured on PDB agar tube slants at 30 ^{o}C for 72 hr. Spores were collected from the slants by gently washing them with 0.1% Tween 80 to obtain spore suspension of around 10^{7} spores/ml. Spore suspension was stored at 4 ^{o}C until used.

Substrate preparation and fermentation

SM was procured from ADM Alliance Nutrition (Abilenne, KS). Solid-state fermentation (SSF) was carried out in 125-ml Erlenmeyer flasks. Before autoclaving, moisture content of SM was adjusted to 50% with distilled water. Erlenmeyer flasks with 5 g substrate were autoclaved at 121 ^{o}C for 15 min and inoculated with 1 ml spore suspension containing 10^{7}spores/ml. The flasks were incubated at 30 ^{o}C. At different time intervals, flasks were harvested and fermented products were lyophilized and milled with mortar and pestle for chemical analyses. Samples were duplicates and chemical analyses were triplicates for each sample.

Chemical analysis

Total sugar and total reducing sugar analysis

To extract carbohydrate from samples, 1 g of milled FSM sample was diluted with 10 ml distilled water in 50-ml tubes. The tubes were put in a 50 ^{o}C water bath shaker for 30 min at a speed of 200 rpm. The mixture was then centrifuged at 3,000 rcf for 15 min. Supernatant was

used for total sugar and for reducing sugar analyses. Structural polysaccharides were defined by subtracting reducing sugar from total sugar.

Total sugar analysis followed the method of Dubois (1956). One milliliter of supernatant was pipetted into a colorimetric tube and mixed with 1 ml of 5% phenol. Five milliliters concentrated sulfuric acid was added directly to the liquid surface. After standing for 10 min, the mixture was then shaken and placed for 15 min in a water bath at 30 ^{o}C. The absorbance of the characteristically yellow-orange color was measured at 490 nm. Glucose was used as the standard.

Total reducing sugar analysis followed the method of Miller (1959) with modifications. The DNS reagent contained 0.63% dinitrosalicylic acid, 18.2% Rochelle salts, 0.5% phenol, 0.5% sodium bisulfate, and 2.14% sodium hydroxide. A 3-ml aliquot of reagent was added to 3-ml diluted samples in 14-mm tubes. The mixtures were then heated for 5 min in a boiling water bath and cooled in ice adjusted to ambient temperature. The color intensities were measured at 575 nm in a spectrophotometer. Glucose was used as the standard.

Protease activity

To extract protease, milled samples were diluted with distilled water at a ratio of 1:20 (w/v). The mixture was then placed in a 40 ^{o}C water bath and shaken at a speed of 200 rpm for 1 hr. After protease extraction, the mixture was centrifuged at 5000 rcf for 15 min at 4 ^{o}C. The supernatant was used as crude enzyme and diluted two times with pH 7.2 sodium phosphate buffer for protease analysis. Casein (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 ^{o}C for 10 min. The reaction was terminated by adding 0.5 ml of 0.4 M trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000 rcf for 15 min at 4 ^{o}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 reaction stood for 20 min at 40 ^{o}C; absorbance was read at 660 nm. Sample blank was prepared by adding 2% casein solution after adding 0.4 M TCA to mixtures. Experiments were performed in triplicates. One unit of protease (U) was defined as the amount of enzyme that yields the color equivalent to 1 μg of tyrosine per minute at 40 ^{o}C per gram FSM (dmb).

TCA soluble nitrogen content

A 0.625-g sample was diluted with 10 ml of distilled water and shaken at 200 rpm for 2 hr. The mixture was then fully mixed with 10 ml of 20% (w/v) TCA solution and held at room temperature. After standing for 1 hr, the mixture was then centrifuged at 3000 rcf for 20 min. Soluble nitrogen content was analyzed with the same method used for crude protein analysis.

Other chemical analysis methods

Raffinose and stachyose were analyzed using Raffinose/Sucrose/Glucose assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland) and followed its analysis instructions. Protein contents were analyzed using the combustion method (AOAC Official Method 990.03) by the Analytical Laboratory in the Department of Animal Sciences and Industry, Kansas State University (KSU, Manhattan, KS). The ratio of nitrogen to protein used 6.25. Trypsin inhibitor analysis followed the AACC official method 22-40.01. Trypsin inhibitor activity is expressed in terms of trypsin inhibitor units (TIU). Degree of hydrolysis (DH) of protein was based on the formal titration method. Total amino acid profile, total fatty acid profile, and crude fat analysis were conducted at the Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO) using the following methods: total amino acid profile (AOAC Official Method 982.30 E (a,b,c), chapter 45.3.05, 2006), total fatty acid profile (AOAC Official Method 996.06), crude fat (AOAC Official Method 920.39 (A) and 954.02, 2006)(Official methods of analysis of AOAC international. 2006)

Scanning Electron Microscopy (SEM)

The SEM was conducted in the Biological Analytical Laboratory in the Biology Department, KSU (Manhattan, KS). A Nova NanoSEM 430 (FEI Company) SEM was used to observe the surface morphology of fermented SM (FSM). FSM samples from different time intervals were affixed to an aluminum stub with two-sided adhesive tape. The SEM of the FSM was performed at an acceleration voltage of 15.0 kV with low vacuum mode.

Statistics analysis

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

Results

Figure 1 shows *A. oryzae* spreading in SM substrate at different fermentation time intervals, both from the surface and from the inside of the substrate, by SEM. High-density mycelia could be seen in all the pictures. In Fig. 1, the mycelia density was almost the same for 20-hr, 24-hr, 36-hr, and 48-hr samples, but the mycelia width was different. With longer fermentation time, the mycelia became stronger. Spores existed on the surface of the 36-hr and 48-hr samples (Fig. 1c and Fig. 1d), but their respective inside views were spore-free (Fig. 1g and Fig. 1h).

Figure 2 displays the consumption of sucrose and GOS by *A. oryzae* during solid-state fermentation. The GOS in SM includes raffinose, stachyose and verbascose. Sucrose was consumed within 20 hr from the initial 23.18 mmol/100g. When sucrose was deficient, GOS began to be degraded, and their content decreased from the initial 9.48 mmol/100g until totally degrading around 28 hr.

The activity of trypsin inhibitor in SM was 10.7 TIU/mg SM (dmb), which was also 5.65 mg TI/g SM (dmb), assuming that 1.9 TIU/mg is equivalent to 1.0 mg TI/g (Kakade et al 1974). This result is consistent with that of Jezierny et al (2010), with 5.8 mg TI/g crude protein (CP). After autoclave, the trypsin inhibitor for autoclaved SM was 2.1 TIU/mg. After 36 hr fermentation, the activity of trypsin inhibitor decreased to a non-detectable level.

Fig. 3 shows the SM protein content change at different fermentation time intervals. The protein content kept increasing from the original 50% (dmb) and attained approximately 63% (dmb) after 7 d fermentation. Fermentation is a way to enhance protein content of soybean products.

In Fig. 4, substrate dry matter content kept decreasing during fermentation, in parallel with the trend of protein increase. Total sugar decreased from 32.26% to 15.82% (w/w) and reducing sugar decreased from 10.43% to 2.79% (w/w) after 7 d fermentation, which was a 51% and 73% decrease, respectively. Structural polysaccharides decreased by 59% (w/w). The peak value for total sugar, reducing sugar appeared at 20 hr fermentation.

Protease activity during *A. oryzae* fermentation is shown in Fig. 5. The activity attained its peak value (552 U) at 36 hr fermentation. With extended fermentation time, the protease activity decreased to around 25 U because of the non-optimal environment. TCA protein content

was increased from 2.18% to 51.1% (w/w). The degree of hydrolysis of SM protein kept increasing from initial value of 0.9% to 7% (w/w) throughout the 7 d fermentation.

The amino acid profile is shown in Table 1. Amino acid levels in SM control (SMC) and autoclaved SM (ASM) were not significantly different. The total amino acid content in FSM was increased by 13.13% compared with the original sample. All but three of the 23 single amino acids were higher in FSM. The total content of 12 amino acids in the ideal protein pattern (arginine, histidine, isoleucine, leucine, lysine, methionine, methionine+cystine, phenylalanine, phenylalanine+tyrosine, threonine, tryptophan, and valine), suggested by National Research Council Committee on nutrient requirements of swine (National Research Concil (U.S.) Committee on Animal Nutrition 1998), increased by 5.6% of the original SM after fermentation. Available lysine content of FSM increased by 6.21%, although the autoclave procedure before fermentation decreased its content. The content of methionine, cysteine, threonine and tryptophan increased by 11.11%, 28.57%, 18.65% and 6.76%, respectively. Valine content also increased by 12%.

Fatty acid profile is shown in Table 2. There was no significant difference between SM fatty acid profile and that of ASM. Crude fat content increased about 12.29%. The unsaturated fatty acid increased approximately 0.58%, and saturated fatty acid decreased about 3.12% of the total fat after fermentation. Linoleic content increased by 2.54%.

Discussion

The particle gaps for moistened SM are small when compared with soybean and the bran materials. Growing fungi in such sticky materials, like SM, during solid-state fermentation is challenging due to oxygen transfer and evaporative moisture loss. From the high-density mycelia, we saw that *A. oryzae* could grow very well in SM alone. Ammonium nitrogen source was excluded from the experimental design to avoid producing high urea, which is not welcome in monogastric animals. The SEM graph clearly showed the formation of biofilm of *A. oryzae* hyphae. The biofilm formation could protect fungus from the environment, create resistance to physical and chemical removal of cells, and enable metabolic cooperation and a community-based regulation of gene expression (Ramage et al 2009).

Fungus spores were generated when nutrient limitations stopped fungus growth and metabolism. The substrate surface is rich in oxygen, whereas the inside substrate is nutrient-rich.

The fact that spores were generated in different sites illustrated that *A. oryzae* was more nutrient- than oxygen-limited. This explained why *A. oryzae* could grow well in sticky, moist SM. The common characteristics for such fungi were investigated by testing the growth of *Rhizopus oligosporus* (ATCC22959), but *R.oligosporus* could not grow well in SM, and spores came out first from inside SM rather than SM surface. One difference between *R.oligosporus* and *A. oryzae* is that the latter has short hyphae. This helps explain the conclusion that for solid-state medium with short hypha may find it easier to penetrate substrate to form biofilm and make full use of it.

A. oryzae has been reported to secret α-galactosidase (Shankar and Mulimani 2007), the specific enzyme to degrade GOS. In general, α-galactosidase acts on gal-gal bonds in the tetrasaccharide stachyose, releasing galactose and raffinose; it also acts on gal-glu bonds with the release of sucrose. Our result is consistent with Chen et al (Chen et al 2010b), who reported 91.5% oligosaccharide decrease in SM after 24 hr fermentation by *A. oryzae*. The removal of GOS from SM will improve its nutritive value for feed. Parsons et al (Parsons et al 2000) found that roosters fed SM with low oligosaccharide concentrations had higher total net metabolizable energy values (2931 kcal/kg dry matter) than those fed conventional SM (2739 kcal/kg dry matter).

According to Gabert et al (Gabert et al 1996), trypsin inhibitor activity up to 3.2 mg TI/g diet did not affect pancreatic secretion of nitrogen, protein, or pancreatic chymotrypsin activity in young pigs, but Huisman and Tolman (Huisman and Tolman 2001) recommended a lower dietary maximum tolerance level for fattening pigs of approximately 0.5 mg TI/g. Although the literature threshold of trypsin inhibitor for monogastric animals is inconsistent, our fermented SM with non-detectable trypsin inhibitor could meet all the requirements. Hong et al (Hong et al 2004) reported that after 48 hr fermentation by *A. oryzae*, trypsin inhibitor of SM decreased from 2.7 mg/g to 0.42 mg/g. Different fermentation process and fermentation parameters influence the results. As we mentioned above, protease was produced during fermentation. The trypsin inhibitor may be degraded or modified during fermentation and lose its active binding site to trypsin. Removing trypsin inhibitor will benefit animal growth. Rats fed a raw soybean extract from which trypsin inhibitors had been inactivated showed improved growth performance compared with control rats fed diets containing raw soybeans from which inhibitors had not been inactivated (Kakade et al 1973).

The protein content of FSM in the current research is higher than those from other research. Protein content of *A. oryzae* FSM, from research of (Chen et al 2010b) increased from 43% to 47.8%, and from research of (Hong et al 2004) increased from 44.30% to 46.23%. Autoclave was applied in current research but not in Chen and Hong's researches. Nutrition might be more available for fungi growth after sterilization. Two reasons contributed to protein increase. The consumption of polysaccharides resulted in the dry matter loss, which made the protein become concentrated. *A. oryzae* used polysaccharides for their own growth and the single cell protein contributed to the increase of the total potein.

A. oryzae could secret various kinds of enzymes, which may include but is not limited to: α-amylase, carboxymethyl cellulase, pectinlyase (Nakadai and Nasuno 1988), protease (Wang et al 2005), endo-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55) (Szendefy et al 2006), α-galactosidase (EC 3.2.1.22) (Shankar and Mulimani 2007), endo- and exdo-polygalacturonase (Fontana et al 2009), and α-L-arabinofuranosidase (Matsumura et al 2004) to degrade complex carbohydrates into available sugar for their growth needs. The excretion of enzymes during fermentation also could be reflected by the results that the peak value for total and reducing sugar appeared at 20 hr fermentation as shown in Fig. 4. Due to the degradation of the complex polysaccharides by enzymes, 20-hr samples extracted more sugar than 0-hr and 12-hr samples. The utilization of carbohydrates contributed to the dry matter loss. *A. oryzae* single cell protein and dry matter loss were the reasons for protein content increase. Further, the degradation of structural polysaccharides excluded its negative effect on feed intake and growth performance of monogastric animals.

Usually peptides with 10 or fewer amino acids will dissolve in TCA (Low 1980). The increase of TCA protein content demonstrated the degradation of large protein molecules; this is consistent with the results of protein degree of hydrolysis, which is also demonstrated in Fig. 5. Our result was also consistent with that of Chen et al. (2010b), who reported the increase of TCA protein content of SM from the original 65.26 μmol/g to110.33 μmol/g after *A. oryzae* fermentation. Soybean is defined as one of the "big 8" food allergens in the United States (Frias et al 2008). Protein hydrolysis could lower soy protein immunoreactivity by hydrolyzing soy protein into peptides and amino acids and altering the structure of antigen epitopes, therefore reducing the IgE binding potential (Song et al 2008). Studies have confirmed that degradation of soybean allergens occurred during fermentation by microbial proteolytic enzymes in fermented

soybean products, such as soy sauce, miso, and tempeh (Kobayashi 2005; Song et al 2008). Newly weaned pigs with limited stomach acid and enzymatic secretions in the small intestine can have difficulty in digesting proteins with complex structures and large molecular weights (Kim 2010). FSM with hydrolyzed protein by *A. oryzae* could be used for weaning pigs, which will be tested in further animal study.

The non-significant difference between SM and ASM means that autoclaving did not modify the amino acid profile. But with high heat, some amino acids are susceptible to react with other compounds, resulted in the amino acids loss. An example was the available lysine loss, which might be because of lysine reacting with reducing sugars to form Maillard compounds. Amino acid enhancement after fermentation was due to growth of *A. oryzae*. The percentage of amino acids content increase in our research was much higher than former research of Hong (2004) and Chen (2010b), who reported a 5.6% increase and a 4.9% increase of total amino acid in FSM after 48 hr fermentation by *A. oryzae,* respectively. The reason might be because of the different fermentation conditions used. Good amino acid balance is essential for animal feed efficiency. Millet et al (2011) found that restricting amino acid levels in pigs' diets during growing and early finishing led to decreased growth rates. According to Toride et al (2002), for growing pigs, the limiting amino acid sequence is lysine, threonine, and tryptophan, and for broilers, the sequence is methionine, lysine, and threonine. All these four amino acids content increased after fermentation. *A. oryzae* amino acid profile might complement that of SM. Ravinder et al (Ravinder et al 2003) analyzed the nutrition profile of *A. oryzae* grown on rice bran and found that methionine content of the fungus is higher than that of SM.

The main fatty acids for SM and its fermented products were linoleic, oleic, palmitic, and linolenic. Autoclaving did not significantly modify fatty acid profiles. Oil seeds, vegetable oils, marine oils, or animal fats are often used to supplement fat in animal feed (Ananda and Vadlani 2010). The increased fat content enhanced the FSM value. Linoleic acid and linolenic acid are recognized as metabolically essential fatty acids, which are converted to long-chain polyunsaturated fatty acids (PUFA) through a series of desaturation and elongation steps. In poultry, specific PUFA are biosynthesized into compounds called eicosanoids, which act as potent biological regulators (National Research Council (U.S.) Committee on Animal Nutrition 1994). Linoleic acid is the only essential fatty acid with a dietary requirement for poultry. The

inadequacies of linoleic acid would decrease resistance to disease, and the increased linoleic acid content in FSM would help decrease the extra linoleic acid addition and reduce the feed cost.

CONCLUSION

A. oryzae grew well in SM as the sole substrate. Anti-nutritional factors in SM, including trypsin inhibitors, GOS, structural polysaccharides and large molecular protein decreased significantly by *A. oryzae* fermentation. GOS and trypsin inhibitors were totally degraded within 36 hr fermentation. Non-reducing polysaccharidies decreased by 59% (w/w) and the degree of hydrolysis of SM protein kept increasing from initial value of 0.9% to 7% (w/w) throughout the 7 d fermentation. Nutritional factors, including protein, amino acid and fatty acid, were also modified. Protein content increased from 50.47% (w/w) to 58.93% (w/w) after 36 hr fermentation. Amino acid content was significantly enhanced. The final product from our research had lower anti-nutritional factors and higher nutritional ingredients than any former research, and it was expected to be higher value protein source for monogastric animals and have wider market than before.

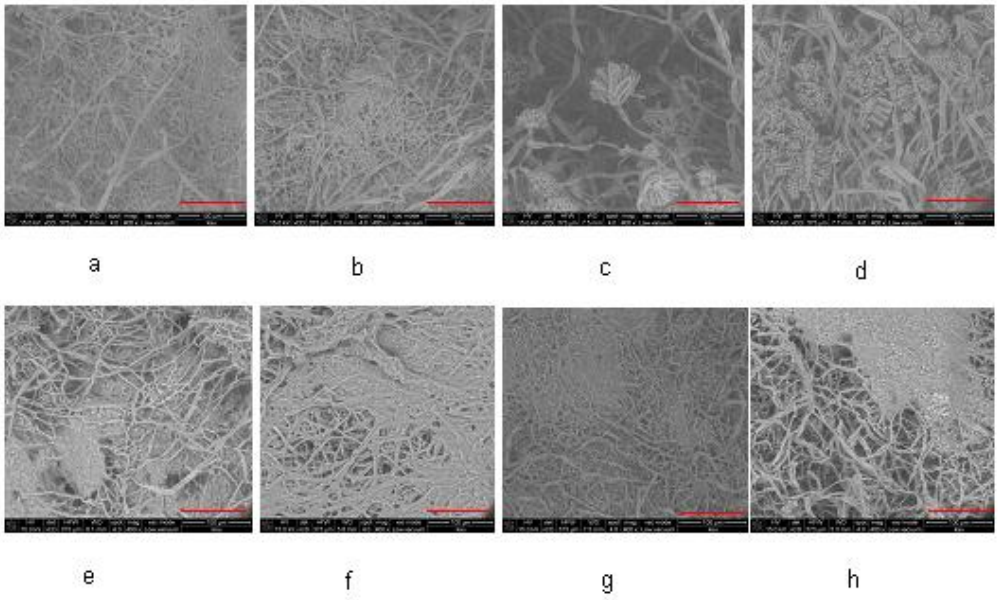


Figure 2.1 *Aspergillus oryzae* fermented SM with different fermentation times: (A) 20 hr surface, (B) 24 hr surface, (C) 36 hr surface, (D) 48 hr surface, (E) 20 hr inside, (F) 24 hr inside, (G) 36 hr inside, and (H) 48 hr inside; the scale stands for 100 um

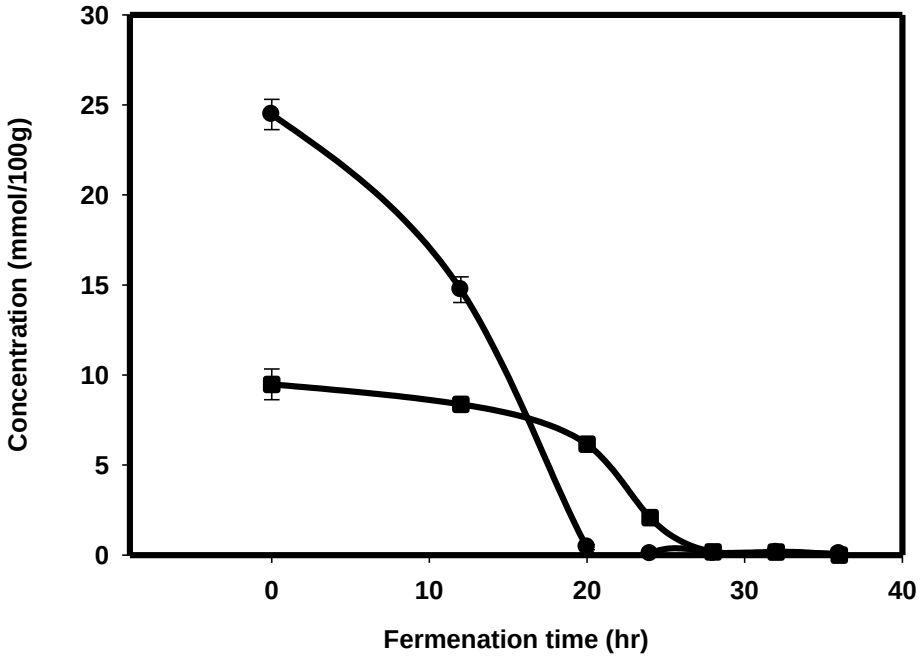


Figure 2.2 Sucrose and oligosaccharides content change during *Aspergillus oryzae* fermentation of SM. Circle: sucrose; square: oligosaccharides

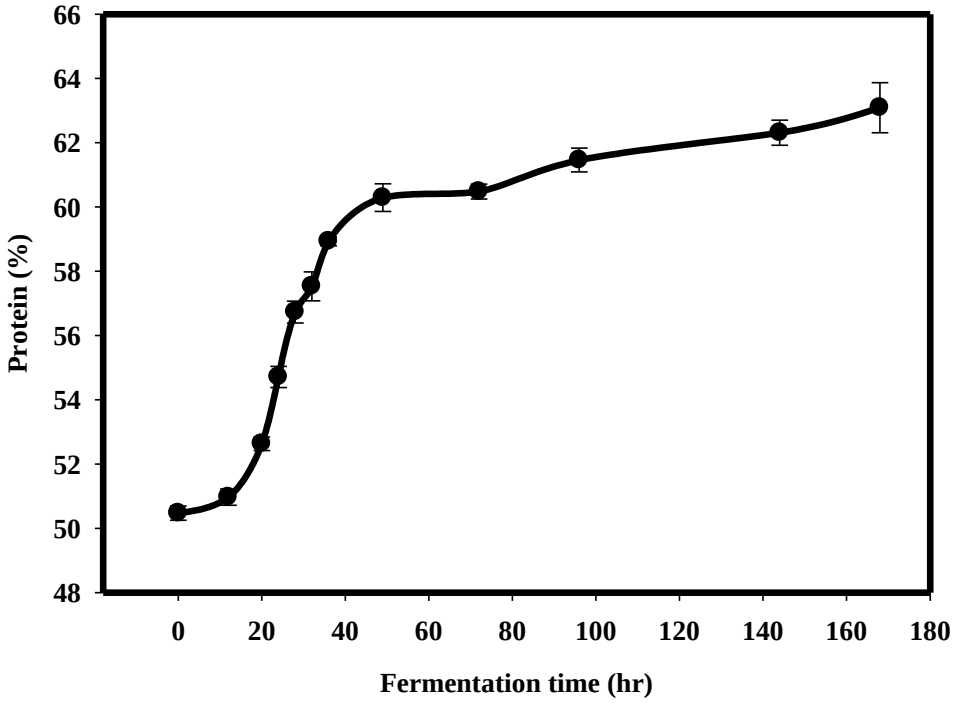


Figure 2.3 Protein content change during *Aspergillus oryzae* solid-state fermentation of SM

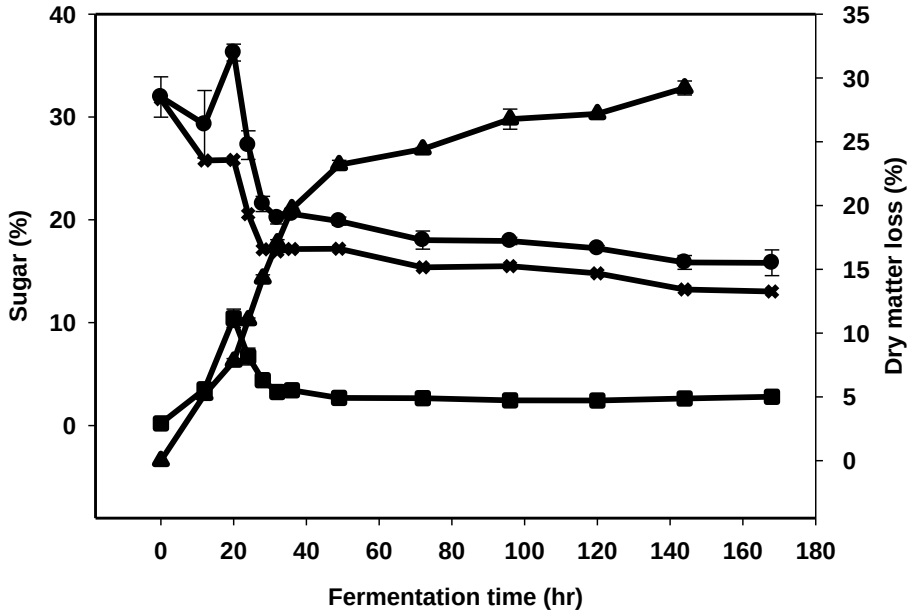


Figure 2.4 Total sugar, reducing sugar, and dry matter loss change during *Aspergillus oryzae* solid-state fermentation of SM. Circle: total sugar; square: reducing sugar; triangle: dry matter loss; thin x: structural polysaccharides

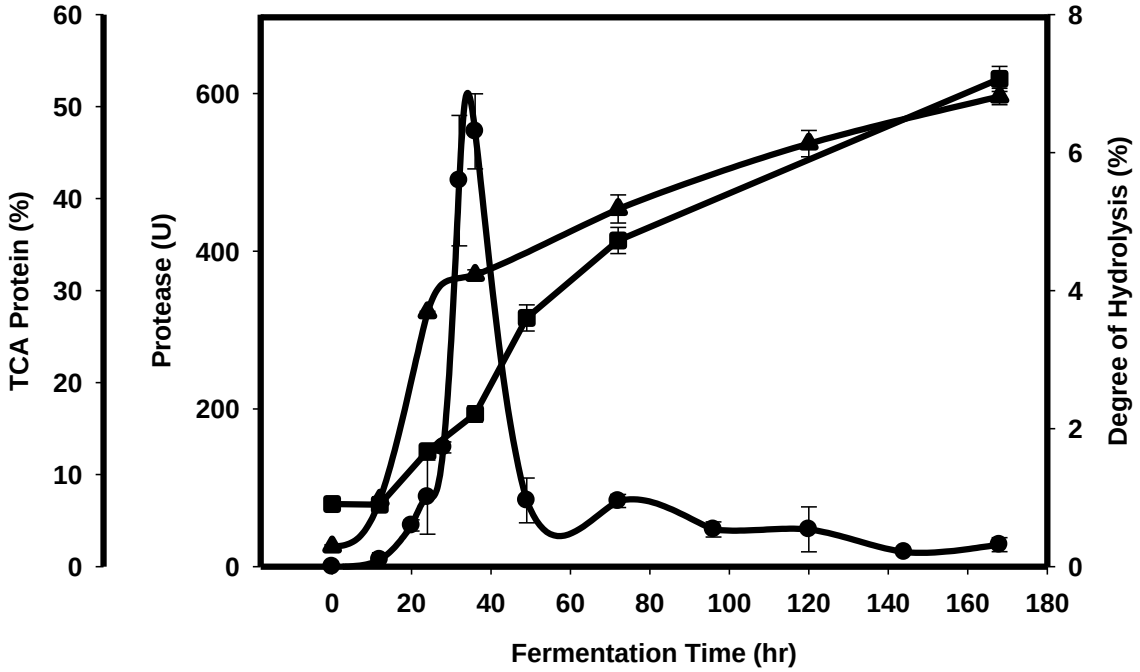


Figure 2.5 Protease, TCA protein content and degree of hydrolysis (DH) changes during *Aspergillus oryzae* fermentation. Circle: protease; triangle: TCA protein; square: degree of hydrolysis

Table 2.1 Amino acid profile of SM control, autoclaved SM and fermented SM

	SM (%)*	ASM (%)**	FSM (%)***
Taurine	0.01[a]	0.01[a]	0.03[a]
Aspartic acid	5.77±0.03[b]	5.66[b]	6.68±0.04[a]
Threonine	1.93±0.06[b]	1.85±0.02[b]	2.29±0.05[a]
Serine	2.14±0.08[b]	2.04±0.01[b]	2.85±0.12[a]
Glutamic acid	8.85±0.12[b]	8.66±0.07[b]	9.81±0.02[a]
Proline	2.53±0.05[b]	2.51±0.08[b]	3.24±0.01[a]
Glycine	2.14±0.03[b]	2.07[b]	3.06±0.02[a]
Alanine	2.19±0.02[b]	2.13[b]	2.71±0.04[a]
Cysteine	0.7±0.02[b]	0.67±0.01[b]	0.9±0.01[a]
Valine	2.52±0.02[b]	2.51±0.03[b]	2.83±0.05[a]
Methionine	0.72±0.01[b]	0.7[b]	0.8±0.01[a]
Isoleucine	2.39±0.01[b]	2.33±0.01[c]	2.64[a]
Leucine	3.92±0.03[b]	3.84[b]	4.14±0.03[a]
Tyrosine	1.84±0.04[b]	1.77±0.01[b]	1.94[a]
Phenylalanine	2.53±0.02[a]	2.48[a]	2.46[a]
Hydroxylysine	0.03[a]	0.02[b]	0.02[b]
Ornithine	0.03[b]	0.04[b]	0.11[a]
Lysine	3.24±0.02[b]	3.1[b]	3.54±0.05[a]
Histidine	1.32±0.01[a]	1.27[a]	1.39±0.04[a]
Arginine	3.64±0.02[a]	3.52±0.01[ab]	3.37±0.07[b]
Tryptophan	0.74±0.03[a]	0.71±0.01[a]	0.79±0.01[a]
Total	49.13±0.48[b]	47.84±0.02[b]	55.58±0.53[a]
Available lysine	3.06±0.03[b]	2.84[c]	3.25±0.05[a]
Crude protein (Kjeldahl)	50.46±0.04[b]	50.62±0.07[b]	58.99±0.35[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009)
Amino acids with a content of zero were deleted from the list
*SMC is the original soy meal sample
**ASM control is soy meal after 121 ^{o}C, 15 min autoclave
***FSM is soy meal after fermentation

Table 2.2 Fatty acid profile of SM control, autoclaved SM, and fermented SM

	SM (%)*	ASM (%)**	FSM (%)***
Crude fat	2.93±0.01[b]	2.24±0.03[c]	3.29±0.05[a]
Myristic (14:0)	0.5±0.01[a]	0.54±0.02[a]	0.12±0.01[b]
(C15:0)	0.00	0.00	0.12±0.01
Palmitic (16:0)	14.4±0.01[b]	14.49[a]	14.35±0.03[b]
Palmitoleic (16:1)	0.12[b]	0[c]	0.24±0.01[a]
(17:0)	0.15[b]	0.15[b]	0.25[a]
(17:1)	0[b]	0[b]	0.21±0.01[a]
Stearic (18:0)	4.41[a]	4.37±0.03[a]	3.97±0.01[b]
Oleic (18:1*n9*)	15.24[a]	15.2[a]	15.05±0.07[a]
Linoleic (18:2)	54.69[b]	54.85±0.02[b]	56.08±0.01[a]
Linolenic (ω18:3)	9.3[a]	9.25±0.02[a]	8.23±0.05[b]
Arachidic (20:0)	0.25±0.01[a]	0.25±0.01[a]	0.21±0.01[a]
Docosanoic (22:0)	0.39[a]	0.36±0.01[a]	0.37±0.01[a]
Lignoceric (24:0)	0.4±0.01[ab]	0.37±0.01[b]	0.47±0.03[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009)
Amino acids with a content of zero were deleted from the list
Fatty acids with a content of zero were deleted from the list
*SMC is the original soy meal sample
**ASM control is soy meal after 121 ^{o}C, 15 min autoclave
***FSM is soy meal after fermentation

Chapter 3 - High efficiency to degrade phytic acid in soy meal *via* two-stage temperature protocol during *Aspergillus oryzae* solid state fermentation[3]

Abstract

Aspergillus oryzae (ATCC 9362) solid state fermentation was applied to degrade phytate in soy meal. The experiment design: two - stage temperature fermentation protocol, was applied. Central Composite Design was used to obtain the optimal fermentation condition for phytase production in the first 25 hr fermentation. And a combination of moisture 41%, temperature of 37 ^{o}C, and inoculum size of 1.7 ml in 5 g substrate (dmb) would favor maximum phytase production, yielding phytase activity 58.7 U. Phytase properties study show that optimal temperature for phytase activity was 45 – 50 ^{o}C, at which, phytase also had good stability. After 25 hr fermentation, temperature was changed to 45 ^{o}C and 50 ^{o}C for another 11 hr fermentaiton. By the end of fermentation, 57% phytic acid was degraded from soy meal fermented at 50 ^{o}C, comparing with 39% of that fermented at 37 ^{o}C and 49% at 45 ^{o}C. Nutritional profile of fermented soy meal was also studied. Oligosaccharides were totally removed after fermentation and 67% of total non – reducing polysaccharides were decreased. Protein content increased by 9.5%. SM nutritional value was greatly enhanced.

Introduction

Soy meal (SM) is the residue after soy oil extraction. With around 48% protein content and high lysine content, it is mainly used as protein supplement for feed. Mono-gastric animals account for more than 74% of total usage (Chen et al 2010a). However, presence of phytic acid in SM diminishes its nutritional value.

Phytic acid is the main storage form of phosphorous in soybean, accounting for 61 – 70% of phosphorous in SM (Nelson et al 1968). Phosphorous in the phytate form could not be absorbed by mono-gastric animals, because they lack phytase, the digestive enzyme required to release phosphorous from the phytate molecule. Non-digestible phytic acid is, therefore, an anti-nutritional factor for mono – gastric animals. It could influence the digestion of protein and

[3] Chapter 3 is accepted as a part of Chen L. et al (2013) Journal of the Science of the food and agriculture

important minerals, such as zinc, iron, calcium etc. (Selle and Ravindran 2007). In the feed industry, unabsorbed phytate passes through the gastrointestinal tract of mono-gastric animals, elevating the amount of phosphorus in the manure. Excess phosphorus excretion can lead to environmental problems such as eutrophication.

The common way to alleviate the negative effect of phytic acid is to add phytase to feedstuffs, but the average degree of phytate degradation by adding phytase was only around 50% (Selle and Ravindran 2007). Phytase has been produced from various agricultural residues, like canola meal (Nair et al 1991), rapeseed meal (El-batal and Karem 2001), SM (Chantasartrasamee et al 2005). Fermentation has also been directly applied to degrade phytic acid. Nair (Nair and Duvnjak 1990a) used *A. ficuum* NRRL3135 solid state fermentation to degrade phytic acid in canola meal, and a complete reduction of phytic acid content was achieved in 48hr. Ilyas (1995) applied *Aspergillus usami* to degrade phytic acid in SM. By the end of 60 hr fermentation, phytic acid was totally degraded. Although for both Nair (Nair and Duvnjak 1990) and Ilyas' research, good degradation degrees have been obtained, the problem is the long fermentation period. Aspergillus would produce spores from 22 hr - 28 hr depending on the growth environment. Too many spores in feed ingredients may cause digestive stress for mono-gastric animals. Also, long lasting fermentation period lowers production efficiency.

A two-stage temperature solid state fermentation protocol was investigated in our research to shorten fermentation time for phytic acid degradation in SM. *Aspergillus oryzae* (ATCC 9362) was chosen as the fermentation culture. It could secrete phytase (Shimizu 1993), and it doesn't produce aflatoxin (Stoloff et al 1977). Besides, *A. oryzae* has been reported to secret alpha-galactosidase (Shankar and Mulimani 2007) and carbohyrases (Matsumura et al 2004). It is preferred for feed stuff production. The anti-nutritional factors, including oligosaccharides and non-reducing polysaccharides, together with protein content, were also studied.

Material and methods

Microbial culture

Lyophilized cultures of *Aspergillus sojae* (ATCC 9362) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived in potato dextrose broth twice. After revival, culture was inoculated on potato dextrose (PDA) slant, incubated at 30 ^{o}C for 7

days, and later stored at 4 ^{o}C for short term preservation. For routine experiments, spore solution was used. Spores were collected from the slants by gently washing with 0.1% Tween 80 to obtain spore suspension of around 10^7 spores/ml. Additionally, spores were suspended in 15% glycerol and stored at -80 ^{o}C in 1 ml aliquots for long term preservation.

Substrate preparation and fermentation

SM was procured from ADM Alliance Nutrition (Abilene, KS). Solid state fermentation was carried out in 125-ml Erlenmeyer flasks. Moisture content of SM was adjusted with distilled water before autoclaving. Erlenmeyer flasks containing 5 g substrates (dmb) were autoclaved at 121 ^{o}C for 15 min and inoculated with *Aspergillus sojae* spore suspension containing 10^7 spores/ml. The flasks were incubated at certain temperature for desired time. The activity of phytase was checked at different time intervals.

Optimization of factors affecting phytase production by response-surface methodology

Experimental design

The critical parameters that could affect the production of phytase were determined by initial experiments to be: temperature, moisture, and inoculum level. Response surface methodology (RSM) was used for studying effects of interaction among these variables. The optimized ranges for the selected variables were temperature (31 – 39 ^{o}C), moisture (35 - 55%), and inoculum amount (0.5 – 1.7 ml) in 5 g SM substrate. Design Expert. V8.0.6 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experiment designs, estimate the responses of dependent variables, and generate the contour and/or response surface plots. Central Composite Design (CCD) was adopted to optimize the levels of the three factors, with 6 center points yielding a set of 20 experiments. The factors at three different levels (-1, 0, +1) with minimum and maximum range of values were as presented in Table 1. The treatment schedule for the model is given in Table 2. The experiments were randomized to maximize the effects of unknown variability due to irrelevant factors in the observed responses.

Average maximum phytase activity was taken as the dependent variable (response) with duplicates. The response value (Y) in each trial was the average of duplicates. A second-order polynomial equation (1) was fitted to the data:

$$Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \varepsilon \quad (1)$$

Where Y is the predicted response for phytase produced; β_0 is the constant coefficient; β_1, β_2, and β_3 are the linear coefficients; β_{11}, β_{22}, and β_{33} are the quadratic coefficients; β_{12}, β_{13} and $\beta_{23}$are the interaction coefficients; while ε is the random error. The responses under different combinations as defined by the design (Table 2) were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters.

Effect of temperature on phytase activity and stability

Phytase activity was analyzed at 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C to investigate temperature effect. To study phytase stability at different temperatures, samples were stored at 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C. After 3 hr, residue phytase activities were analyzed. The percentage of residue phytase activity to phytase activity before storage was used to describe phytase stability.

Phytase and phytic acid assay

Phytase was extracted by mixing the fermented SM (FSM) with 1:10 w/v of 2% $CaCl_2$ (w/v), shaking for 1 hr with an orbital shaker. The mixture was then centrifuged at 5,000 rcf for 20 min at 4 ^{o}C and the supernatant was used as crude enzyme preparation for further investigation. Phytase analysis was following the method of Gunashree and Venkateswaran (2008). The method is as followings: the assay was initiated by mixing 1 ml of diluted (1:10) crude enzyme with 0.5 ml of sodium acetate (0.2 M) buffer of pH 5.0 and 0.5 ml of sodium phytate (15 mM) (Sigma Chemicals Co, USA). The reaction mixture was incubated at 40 ^{o}C in a water bath for 45 min. The reaction was terminated by adding 2 ml of 15% trichloroacetic acid. Assay mixture of 0.5 ml was then mixed with 4 ml of 2:1:1 v/v of acetone, 10 mM ammonium molybdate and 5 N sulfuric acid (AAM solution) and 0.4 ml of citric acid (1M). The amount of free phosphate released was determined spectrophotometrically at 355 nm. A starndard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 30 to 360 μM (Gunashree and Venkateswaran, 2008). Phytase activity was defined as the amount of enzyme that liberates 1 μmol of inorganic phosphate in 45 min. Phytic acid was analyzed with Phytic acid assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland).

Other chemical analysis

Oligosaccharides and sucrose content were analyzed using Raffinose/Sucrose/Glucose assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland). Protein contents were analyzed using the Combustion method (AOAC Official Method 990.03) by Soil Analytical Laboratory in Department of Agronomy, Kansas State University (KSU, Manhattan, KS). Total sugar analysis was followed the method of Dubois (Dubois et al 1956). Total reducing sugar analysis was followed that method of Miller (Miller 1959). Total sugar deducting total reducing sugar was defined as total structural polysaccharides.

Statistics analysis

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

Results

A. oryzae solid state fermentation to degrade phytate in SM was separated into two stages, based on the temperature difference. The first stage was from 0 hr to 25 hr. CCD design was applied to generate the optimal fermentation condition to maximize phytase production. The second stage was from 25 hr to 36 hr. By studying the phytase properties, temperature was adjusted to the optimal temperature for phytase activity.

Optimization of parameters influencing phytase activity by RSM using CCD design

Optimization of process parameters temperature, moisture and inoculums size to maximize phytase production was carried out by RSM using CCD design. The actual yield of phytase (response) obtained is presented in Table 2. Maximum phytase activity (U of dry substrate) was observed at moisture content of 45%, temperature of 35 ^{o}C, and inoculum size of 2.11 ml. The overall quadratic model for phytase activity in coded terms was given below:

$$Y= 52.93 – 0.89A + 5.77B + 7.77C – 8.61AB – 5.51AC + 0.31BC – 16.78A^2 – 9.53B^2 - 4.14C^2 \quad (2)$$

The ANOVA of the final quadratic model was shown in Table 3. The model was significant, with an F value of 28.87. The coefficient of regression (R^2) for this model was 0.9629. The suitable R^2 suggested that the model (Equ.2) could be sufficient to represent the actual relationship between the response and the significant variables.

Contour plots for phytase production as a function of two variables, with the other one kept at its central point, were generated in Fig. 1. The center point was indicated as a red spot, and the number of 6 indicates the number of repeats. The curves implied that the responses varied significantly from the single-variable optimized conditions. As moisture, temperature and inoculum size were changed from their optimum values, phytase activity decreased significantly.

Analysis of response – surface curves and contour plots indicated the necessity to optimize variable levels to achieve better results. The results predicted by CCD design showed that a combination of moisture 41%, temperature of 37 ^{o}C, and inoculum size of 1.7 ml in 5 g substrate (dmb) would favor maximum phytase production, yielding phytase activity 58.7 U. The confirmation experiment based on three replicates obtained the phytase activity 52 U, which was within the 95% confidence level of the predicted value (46 – 71 U).

Effect of temperature on phytase activity and stability

The optimum temperature for *A. oryzae* (ATCC9362) phytase activity was 45 ^{o}C (Fig. 2). At 35 ^{o}C, 40 ^{o}C and 55 ^{o}C, phytase activities were also high, just a little lower than the optimum temperature of 45 ^{o}C. Our result was close to that of Wang (Wang et al 2005), which studied the properties of the same strain cultivated in rice medium and optimal temperature for phytase activity was 50 ^{o}C. *Aspergilus oryzae* (ATCC9362) phytase stability was shown in Fig. 3. At 35 ^{o}C to 45 ^{o}C, phytase stability was 100%. At 50 ^{o}C, phytase was still relatively stable, with 62% activity remaining. Phytase from different strains of *A. oryzae* may have different temperature properties. Phytase of *A. oryzae* from Chantasartrasamee (Chantasartrasamee et al 2005) has the optimum temperature 70 – 75 ^{o}C and 66% activity remaining after storing at 80 ^{o}C for 2 hr.

Phytic acid degradation of SM via two-stage temperature protocol during *Aspergillus oryzae* solid state fermentation

Phytic acid degradation and phytase activity change during the temperature two-stage fermentation was shown in Fig. 4 and Fig. 5. In the first stage, from 0 hr to 25 hr, phytic acid degraded from 1.18 g/100g SM to 0.93 g/100g SM. Phytase activity increased rapidly from 12 hr to 24 hr and achieved its highest level at 25 hr.

In the second stage, from 25 hr to 36 hr, fermentation at 45 ^{o}C and 50 ^{o}C achieved larger phytic acid degradation than that of 37 ^{o}C fermentation. Especially at 50 ^{o}C, 57% the SM phytic acid was degraded, compared with 39% at 37 ^{o}C and 49% at 45 ^{o}C. For 50 ^{o}C, 24 hr to 28 hr had

the highest degradation rate, and after 28 hr, phytic acid degradation stopped. That was due to the loss of phytase activity when incubated at high temperature as illustrated by Fig. 5.

Nutrient profile change of SM *via* two-stage temperature protocol during *Aspergillus oryzae* solid state fermentation

Fig. 6 showed the sucrose and oligosaccahrides changes during *A. oryzae* fermentation. After 18 hr, when sucrose was depleted, oligosaccharides degradation rate was highly enhanced. At 25 hr, SM oligosaccharides were totally removed. Total sugar and total non - reducing sugar content (Fig. 7) also kept decreasing. Meanwhile total reducing sugar was increased and 67% of total structural polysaccharides was lost by the end of fermentation. Sugar content changed largely during 13 hr to 25 hr fermentation, when fungus growth and enzyme secretion took place. Same trend was observed for protein change (Fig. 8). Protein content increased 9.5% totally, of which, during first temperature stage, protein increase had already attained 8.2%.

Discussion

In the present study, we investigated phytic acid degradation of SM using *A. oryzae* solid state fermentation. *A. oryzae* mainly produces extracellular phytase (Wang et al 1980). Phytase production was growth related. Fujita (Jin et al 2003) found that phytase activity increased subsequent to the logarithmic growth of *A. oryzae* RIB-128 mycelia. Our research also found that the optimal temperature for phytase production by *A. oryzae* was 37 ^{o}C, given by CCD design. But the optimal temperature for phytase activity was 45 – 50 ^{o}C, which was obtained by both of our research and the former research (Wang et al 1980). Considering the optimal temperature difference between phytase production and phytase activity, two-stage temperature experimental design was applied to obtain the largest phytate degradation in SM by *A. oryzae* solid state fermentation.

In the first stage, CCD design was applied to maximize phytase production. Paramters, including temperature, moisture and inoculum size, were studied. Although pH may be also an influencing factor, it was excluded, since the final product would be directly used for feed and pH adjustment may influence flavor. Phytic acid in SM is located in the protein bodies and forms protein – phytate complex (Liu 1997). During fermentation, protease was secreted (Wang et al 2005). Hyrolysis of protein might ease phytic acid extraction. That was the reason that the highest amount of phytic acid was measured at 12 hr.

The second stage started when phytase activity reached plateau (25 hr). Temperature changed to 45 ^{o}C and 50 ^{o}C, respectively, compared with the 37 ^{o}C control. Highest phytate degradation degree was obtained at fermentation temperature 50 ^{o}C, but not at 45 ^{o}C, which was the optimal temperature for phytase activity. The variation may be because the phytase acitivity was analyzed in liquid, which was different from solid state fermentation conditions. At optimal temperature for phytase activity, the speed for phytic acid degradation was enhanced. A 46% increase of phytate degradation degree was attained by the two-stage temperature protocol fermentation.

Degradation of phytate in animal feed ingredients would alleviate the negative effect caused by phytate. The benefit of low phytate diet achieved by adding phytase has been reviewed (Chen et al 2010a). The enzymatic modified diet, which had around 50% phytate degradation (Selle and Ravindran 2007), could increase protein/amino acid availability, enhance phosphorous, zinc, calcium retention, and improve animals' growth performance. Hirabayashi (Hirabayashi et al 1998a; Hirabayashi et al 1998b) also investigated the effect of *Aspergillus usamii* FSM on chicks. The *Aspergillus usamii* FSM could improve phosphorus, zinc and iron bioavailability in SM and enhance body weight gain of chicks.

Nutritional profile was also studied. Anti-nutritional factor, the galacto-oligosaccharides and structural polysaccharides content were decreased. Protein content was increased due to the growth of *A. oryzae* and the consumption of SM dry matter during fermentation. Oligosaccharides in soybean were mainly stachyose and raffinose. They represent approximately 4 – 6% of soybean dry matter (Liu 1997). Galacto-oligosaccharides in SM could cause gastrointestinal disturbance and influence the nutrient digestibility and energy availability of SM (Zhang et al 2003). Around 20% of non-starch-polysaccharides (NSP) are present in SM (Hollung et al 2006). These NSP reduce digesta passage rate, which in turn may lower feed intake and decrease growth performance (Dunshea et al 2001). The two-stage temperature protocol solid state fermentation by *A. oryzae* could not only achieve a higher phytate degradation level, but also remove oligosaccharides, decrease non – reducing carbohydrates and increase protein content. This process greatly enhanced the nutritional value of SM.

Conclusion

As to the degradation of phytic acid in SM, two-stage temperature protocol solid state fermentation by *A. oryzae* worked well. In the first stage, a combination of moisture 41%, temperature of 37 ^{o}C and inoculum size of 1.7 ml in 5 g substrate (dmb) given by CCD experiment favored maximum phytase production, yielding phytase activity 58.7 U. The second state fermentation used temperature 45 ^{o}C and 50 ^{o}C, which were the optimal temperature for phytase activity, and 46% increase of phytate degradation was obtained at 50 ^{o}C compared with that from the fermentation at 37 ^{o}C. Besides phytate degradation, oligosaccharides were removed, and non – reducing polysaccharides content decreased by 67 %. Meanwhile, protein content increased by 9.2 %. The nutritional enhanced FSM would be better feed ingredients compared with original SM.

Table 3.1 Parameter levels for CCD design

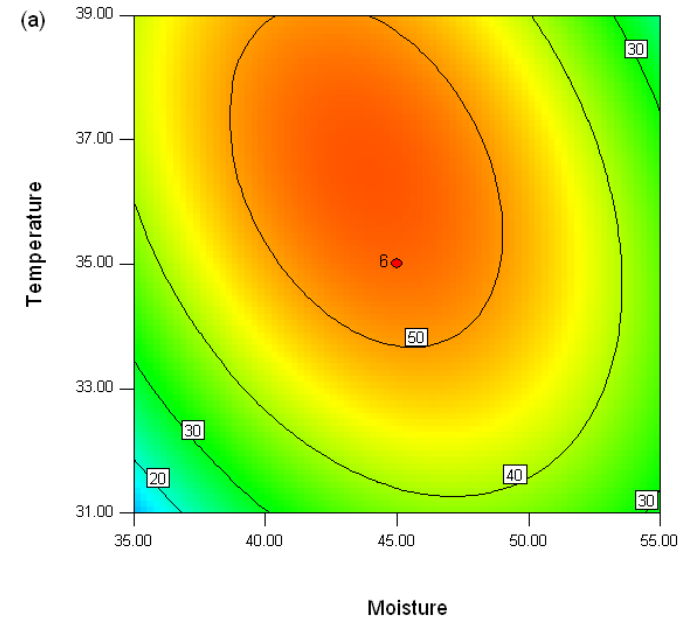
Variables	Symbol	Coded level				
		-1.682 (-α)	-1	0	1	1.682 (α)
Moisture (%)	A	28.18	35	45	55	61.82
Temperature (^{o}C)	B	28.27	31	35	39	41.73
Inoculum size (ml)	C	0.09	0.5	1.1	1.7	2.11

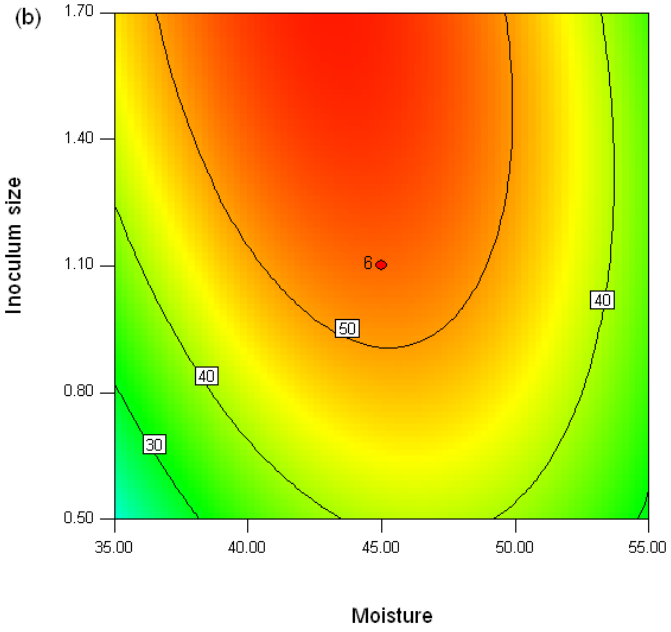
Table 3.2 Phytase activity under different fermentation conditions

Run Order	A	B	C	Phytase (U)
1	0	0	0	50.71
2	0	0	0	49.51
3	0	0	0	52.13
4	0	-1.682	0	15.73
5	0	0	-1.682	24.89
6	0	0	0	54.76
7	-1	-1	-1	3.38
8	1	1	-1	18.40
9	-1	1	1	54.67
10	0	0	0	55.24
11	1	1	1	13.24
12	1	-1	-1	18.44
13	1.682	0	0	9.56
14	-1	1	-1	23.51
15	0	1.682	0	37.22
16	0	0	1.682	58.53
17	1	-1	1	26.31
18	0	0	0	55.02
19	-1.682	0	0	2.40
20	-1	-1	1	19.02

Table 3.3 ANOVA for the fitted quadratic model for phytase production as per CCD design

Souce	Sum of Squares	df	Mean Square	F value	p-value
Model	7128.22	9.00	792.02	28.87	<0.0001
A-moisture	10.80	1.00	10.80	0.39	0.54
B-temperature	454.75	1.00	454.75	16.57	0.0022
C-inoculum size	824.20	1.00	824.20	30.04	0.0003
AB	593.21	1.00	593.21	21.62	0.0009
AC	242.98	1.00	242.98	8.86	0.0139
BC	0.77	1.00	0.77	0.03	0.8699
A^2	4055.93	1.00	4055.93	147.83	< 0.0001
B^2	1308.40	1.00	1308.40	47.69	< 0.0001
C^2	247.31	1.00	247.31	9.01	0.0133


(a)
39.00
37.00
35.00
33.00
31.00
Temperature
35.00
40.00
45.00
50.00
55.00
Moisture
30
6
50
30
20
40
30


(b)
1.70
1.40
1.10
0.80
0.50
Inoculum size
35.00
40.00
45.00
50.00
55.00
Moisture
6
50
40
40
30

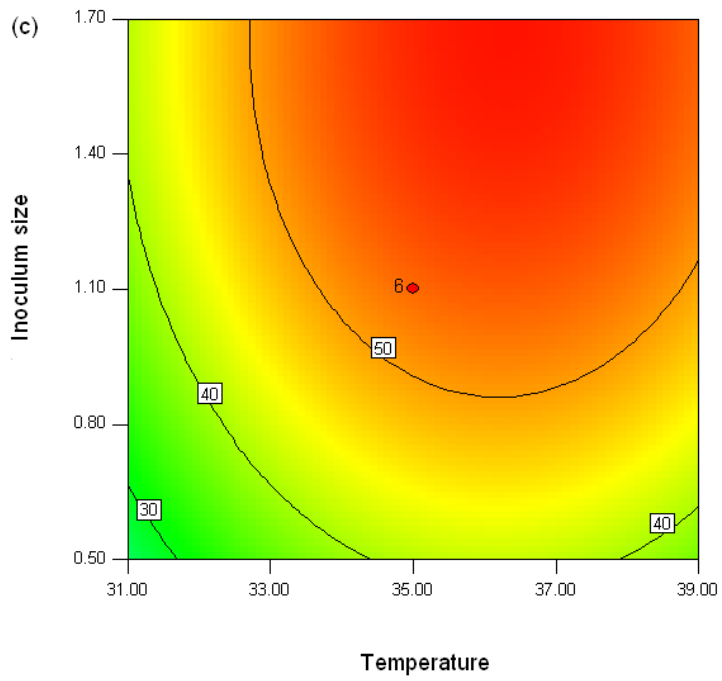


Figure 3.1 Contour plots illustrating the interaction of (a) moisture and temperature, (b) moisture and inoculums size (c) temperature and inoculums size on phytase production

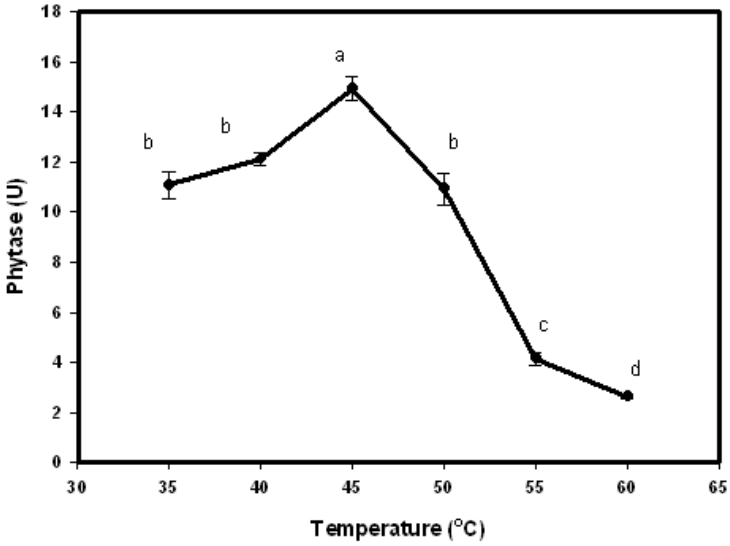


Figure 3.2 Effect of temperature on phytase activity (data with different letters were significantly different at the 0.05 level)

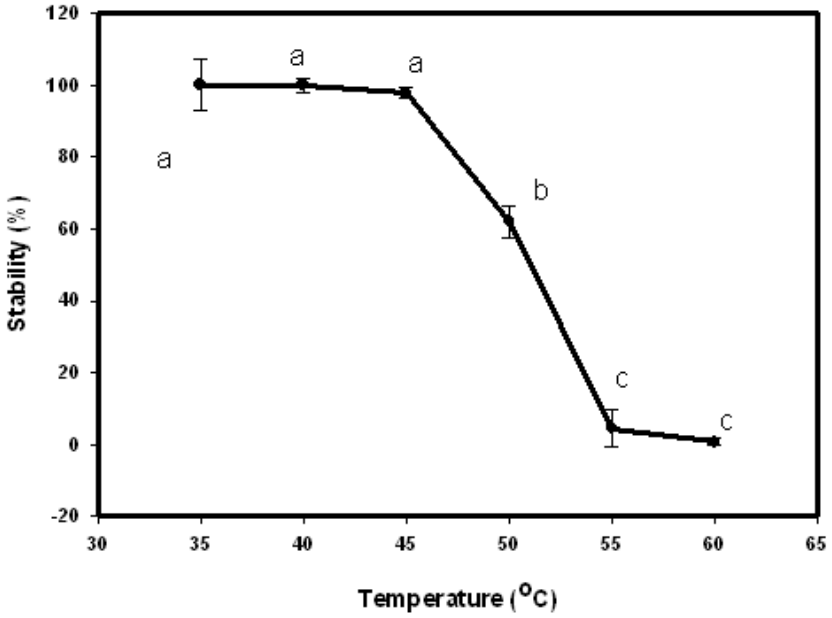


Figure 3.3 Effect of temperature on phytase stability (data with different letters were significantly different at the 0.05 level)

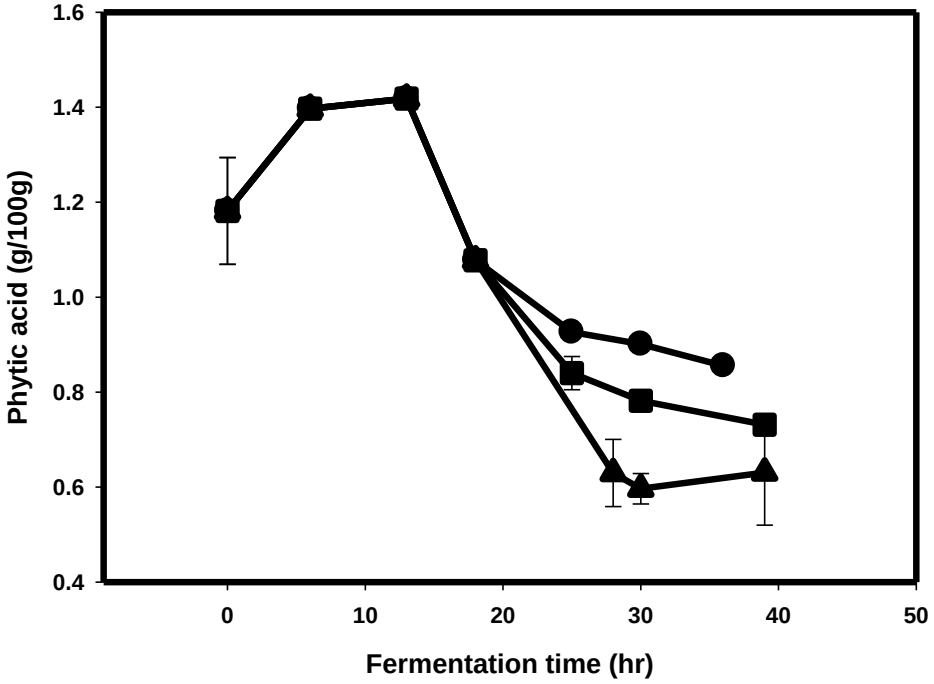


Figure 3.4 Phytic acid content of SM with different fermentation temperature at different fermentation time (circle: 37 ^{o}C; square: 45 ^{o}C; triangle: 50 ^{o}C)

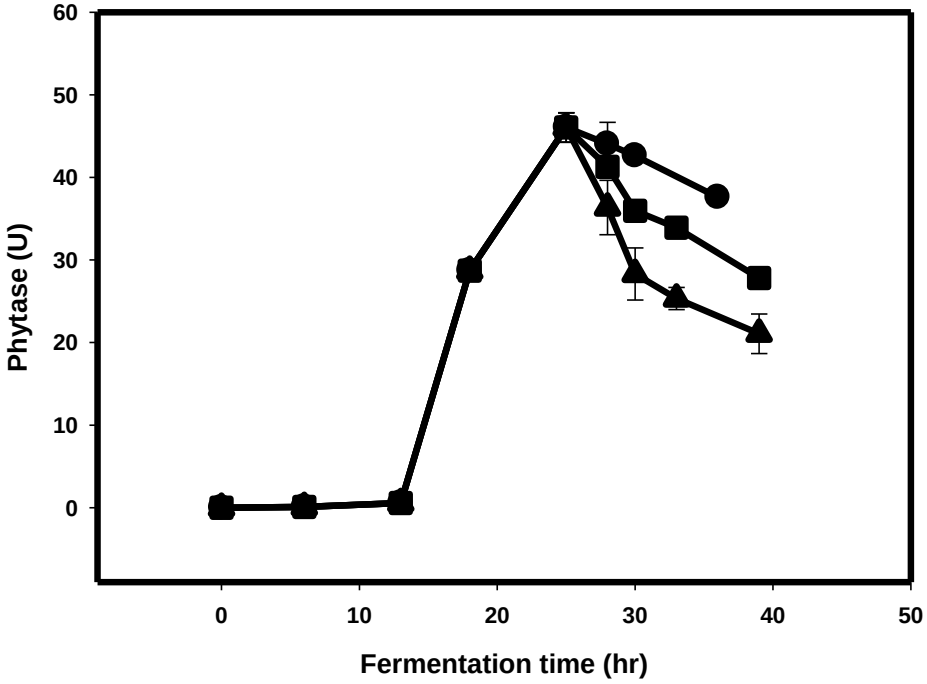


Figure 3.5 Phytase activity at different fermentation temperature with fermentation time (circle: 37 ^{o}C; square: 45 ^{o}C; triangle: 50 ^{o}C)

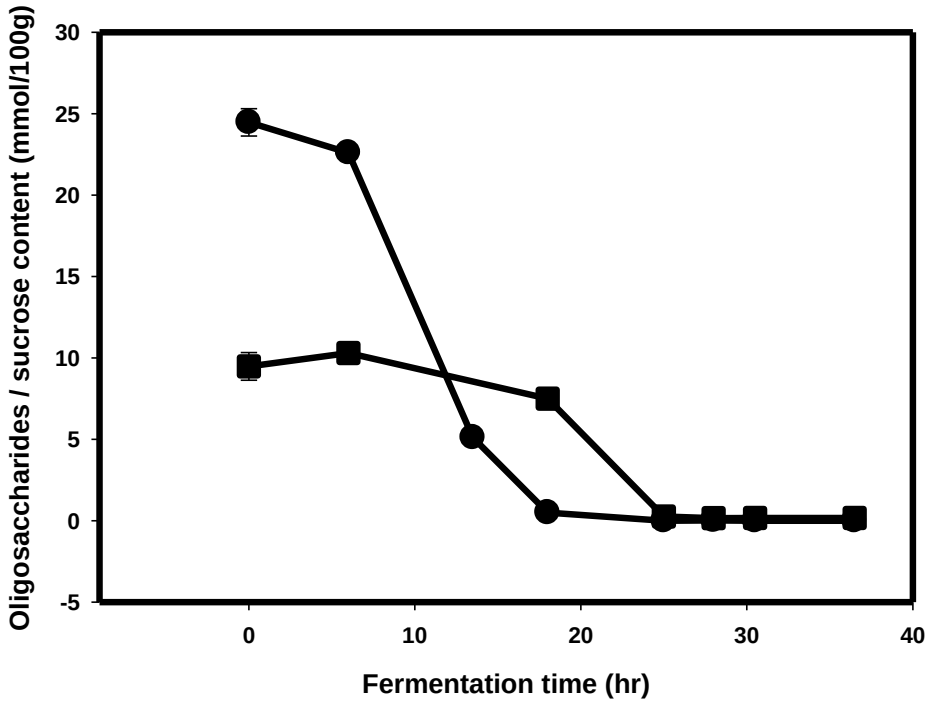


Figure 3.6 Oligosaccharides and sucrose content change of SM during *Aspergillus oryzae* solid state fermentation (circle: sucrose; square: oligosaccharides)

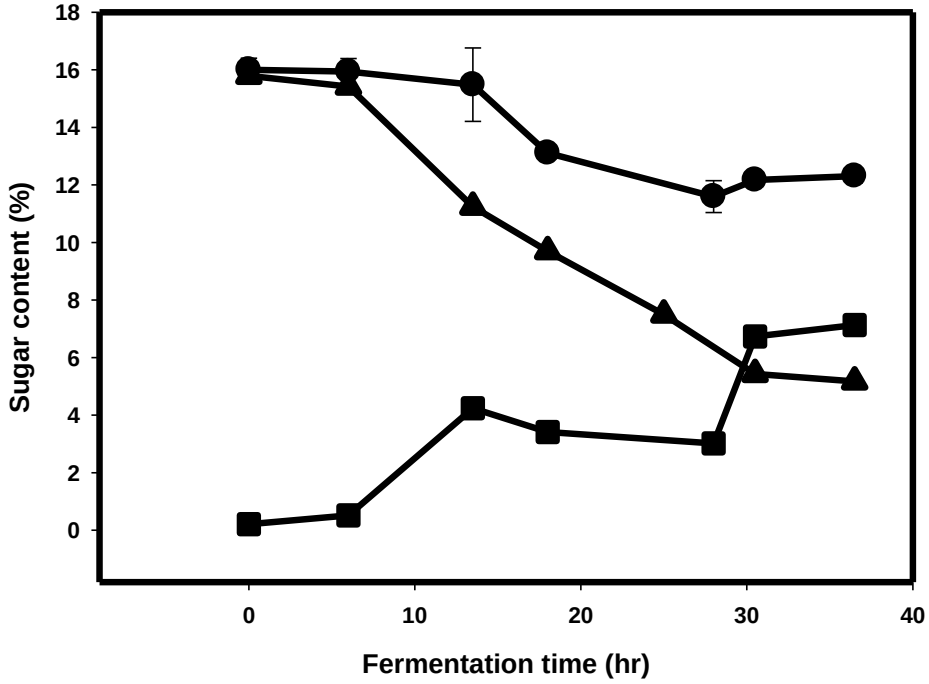


Figure 3.7 Total, reducing sugar and total structural polysaccharides content changes of SM during *Aspergillus oryzae* solid state fermentation (triangle: total structural polysaccharides; circle: total carbohydrate; square: reducing sugar)

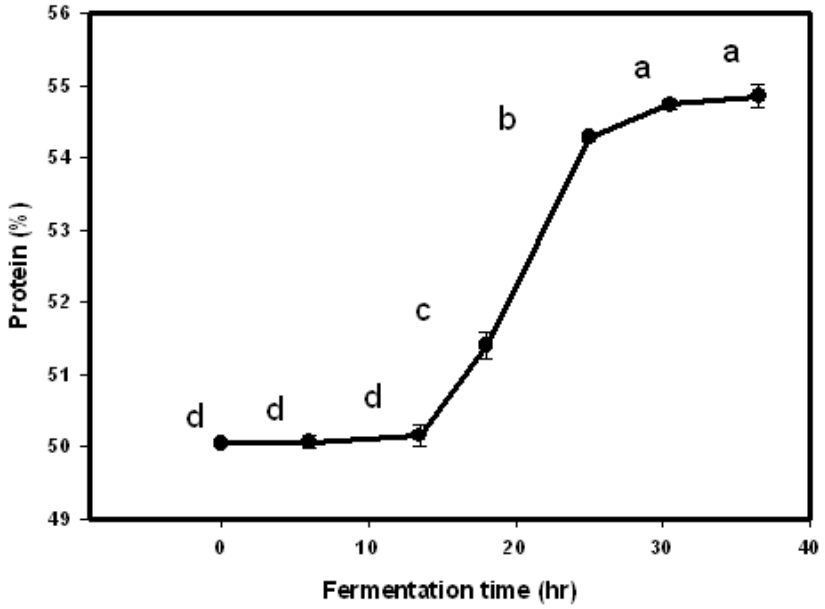


Figure 3.8 Protein content change of SM during *Aspergillus oryzae* solid state fermentation (data with different letters were significantly different at the 0.05 level)

Chapter 4 - High efficiency degradation of phytic acid in soy meal *via* two-stage temperature protocol during *Aspergillus ficuum* solid state fermentation

Abstract

Two - stage temperature protocol was applied to *Aspergillus ficuum* (ATCC 66876) solid state fermentation to degrade phytate in soy meal. Central Composite Design was used to obtain the optimal fermentation condition for phytase production in the first 24 hr fermentation. And a combination of moisture 51% and temperature of 36 ^{o}C would favor maximum phytase production, yielding phytase activity 13.5 U. Phytase properties study show that optimal temperature for phytase activity was 50 ^{o}C, at which, phytase also had good stability. Temperature was changed to 50 ^{o}C from 12 hr to 24 hr fermentaiton. The two-stage temperature fermentation protocol achieved a 98% increase of phytic acid degradation level. Nutritional profile of fermented soy meal was also studied. Oligosaccharides were totally removed after fermentation and 87% of total non-reducing polysaccharides were decreased. Protein content increased by 6.7%. SM nutritional value was greatly enhanced.

Introduction

Soy meal (SM) is the residue after soy oil extraction. With around 48% protein content and high lysine content, it is mainly used as protein supplement to feed. Mono-gastric animals account for more than 74% of total usage (Chen et al 2010a). However, presence of phytic acid in SM diminishes its nutritional value.

Phytic acid is the main storage form of phosphorous in soybean, accounting for 61 – 70% of phosphorous in SM (Nelson et al 1968). Phosphorous in the phytate form could not be absorbed by mono-gastric animals, because they lack phytase, the digestive enzyme required to release phosphorous from the phytate molecule. Non-digestible phytic acid is, therefore, an anti-nutritional factor for mono-gastric animals. It could influence the digestion of protein and important minerals, such as zinc, iron, calcium etc (Selle and Ravindran 2007). In the feed industry, unabsorbed phytate passes through the gastrointestinal tract of mono-gastric animals, elevating the amount of phosphorus in the manure. Excess phosphorus excretion can lead to environmental problems such as eutrophication.

The common way to alleviate the negative effect of phytic acid is to add phytase to feedstuffs, but the average degree of phytate degradation by adding phytase was only around 50% (Selle and Ravindran 2007). Phytase has been produced from various agricultural residues, like canola meal (Nair et al 1991), rapeseed meal (El-batal and Karem 2001), SM (Chantasartrasamee et al 2005). Fermentation has also been directly applied to degrade phytic acid. (Nair and Duvnjak 1990) used *Aspergillus ficuum* NRRL3135 solid state fermentation to degrade phytic acid in canola meal, and a complete reduction of phytic acid content was achieved in 48hr. Ilyas (1995) applied *Aspergillus usami* to degrade phytic acid in SM. By the end of 60 hr fermentation, phytic acid was totally degraded. Direct fermentation is prior to phytase addition, since mycelia penetration into solid matter could facilitate enzyme activity (Ito et al 1989; Matsunaga et al 2002). Although for both the research of Nair and Duvnjak (1990) and Ilyas (1995), good degradation degrees have been obtained, the problem is the long fermentation period. Aspergillus would produce spores from 22 hr - 28 hr depending on the growth environment. Too many spores in feed ingredients may cause digestive stress for mono-gastric animals. Also, long lasting fermentation period lowers production efficiency.

A two-stage temperature protocol for solid state fermentation was investigated in our research to shorten fermentation time for phytic acid degradation in SM. *A. ficuum* (ATCC 66876), which is preferred for producing phytase (Howson and Davis 1983; Shimizu 1993), was chosen as the fermentation culture. Besides, *A. ficuum* has been reported to secret alpha-galactosidase (Shankar and Mulimani 2007; Zapater et al 1990) and xylanase (Lu et al 2008). The anti-nutritional factors, including oligosaccharides and non-reducing polysaccharides, together with protein content, were also studied.

Materials and methods

Microbial cultures

Lyophilized cultures of *A. ficuum* (ATCC 66876) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived in potato dextrose broth twice. After revival, culture was inoculated on potato dextrose (PDA) slant, incubated at 30 ^{o}C for 7 days, and later stored at 4 ^{o}C for short term preservation. For routine experiment, spore solution was used. Spores were collected from the slants by gently washing with 0.1% Tween 80 to obtain

spore suspension of around 10^7 spores/ml. Additionally, spores were suspended in 15% glycerol and stored at -80 ^oC in 1 ml aliquots for long term preservation.

Substrate preparation and fermentation

Soy meal (SM) was procured from ADM Alliance Nutrition (Abilen, KS). Solid state fermentation (SSF) was carried out in 125-ml Erlenmeyer flasks. Moisture content of SM was adjusted with distilled water before autoclaving. Erlenmeyer flasks containing 5 g substrates (dmb) were autoclaved at 121 ^oC for 15 min and inoculated with *A. ficuum* spore suspension containing 10^7 spores/ml. The flasks were incubated at certain temperature for desired time. The activity of phytase was checked at different time intervals.

Optimization of factors affecting phytase production by response-surface methodology experimental design

The critical parameters that could affect the production of phytase were determined by initial experiments to be: temperature and moisture. Central Composite Design (CCD) was used for studying effects of interaction among these variables. The optimized ranges for the selected variables were temperature (33 – 39 ^oC) and moisture (40 - 60%). Design Expert. V8.0.6 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experiment designs, estimate the responses of dependent variables, and generate the contour and/or response surface plots. CCD design was adopted to optimize the levels of the two factors, with 3 center points yielding a set of 11 experiments. The factors at three different levels (-1, 0, +1) with minimum and maximum range of values were as presented in Table 1. The treatment schedule for the model is given in Table 2. The experiments were randomized to maximize the effects of unknown variability due to irrelevant factors in the observed responses.

Average maximum phytase activity was taken as the dependent variable (response) with duplicates. The response value (Y) in each trial was the average of duplicates. A second-order polynomial equation (1) was fitted to the data:

$$Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A_2 + \beta_{22} B_2 + \beta_{12} AB + \varepsilon \qquad (1)$$

Where Y is the predicted response for phytase produced; β_0 is the constant coefficient; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients; β_{12} is the interaction coefficients; while ε is the random error. The responses under different combinations as defined

by the design (Table 2) were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters.

Effect of temperature on phytase activity and stability

Phytase activity was analyzed at 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C, 60 ^{o}C and 65 ^{o}C to investigate temperature effect. To study phytase stability at different temperatures, samples were stored at 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C, 60 ^{o}C and 65 ^{o}C. After 3 hr, residue phytase activities were analyzed. The percentage of residue phytase activity to phytase activity before storage was used to describe phytase stability.

Phytase and phytic acid assay

Phytase was extracted by mixing the fermented SM (FSM) with 1:10 w/v of 2% $CaCl_2$ (w/v), shaking for 1 hr. The mixture was then centrifuged at 5,000 rcf for 20 min at 4 ^{o}C and the supernatant was used as crude enzyme preparation for further investigation. Phytase analysis was following the method of (Gunashree and Venkateswaran 2008, for details please see Chapter 3). Phytase activity was defined as the amount of enzyme that liberates 1 μmol of inorganic phosphate in 45 min. Phytic acid was analyzed with Phytic acid assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland).

Other chemical analysis

Oligosaccharides and sucrose content were analyzed using Raffinose/Sucrose/Glucose assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland). Protein contents were analyzed using the Combustion method (AOAC Official Method 990.03) by Soil Analytical Laboratory in Department of Agronomy, Kansas State University (KSU, Manhattan, KS). Total sugar analysis was followed the method of (Dubois et al 1956). Total reducing sugar analysis was followed that method of (Miller 1959). Total sugar deducting total reducing sugar was defined as total structural polysaccharides.

Statistics analysis

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

Results and discussion

A. ficuum solid state fermentation to degrade phytate in SM was separated into two stages, based on the temperature difference. The first stage was from 0 hr to 24 hr. CCD design was applied to generate the optimal fermentation condition to maximize phytase production. The second stage was from 24 hr to 36 hr. By studying the phytase properties, temperature was adjusted to the optimal temperature for phytase activity.

Optimization of parameters influencing phytase activity by RSM using CCD design

Optimization of process parameters temperature and moisture to maximize phytase production was carried out by RSM using CCD design. The actual yield of phytase (response) obtained is presented in Table 2. Maximum phytase activity (U of dry substrate) was observed at moisture content of 50% and temperature of 36 ^{o}C. The overall quadratic model for phytase activity in coded terms was given below:

$$Y = 13.44 + 0.26\ A + 0.90\ B - 0.60\ A\ B - 4.50\ A^2 - 4.60\ B^2 \quad (2)$$

The ANOVA of the final quadratic model was shown in Table 3. The model was significant, with an F value of 28.87. Lack of fit value was not significant, which mean the model fitted. The coefficient of regression (R^2) for this model was 0.9680. The suitable R^2 suggested that the model (Equ.2) could be sufficient to represent the actual relationship between the response and the significant variables.

Contour plot for phytase production as a function of the two variables was generated in Fig. 1. The center point was indicated as a red spot, and the number of 3 indicates the number of repeats. The curves implied that the responses varied significantly from the single-variable optimized conditions. As moisture and temperature were changed from their optimum values, phytase activity decreased significantly.

Analysis of response-surface curves and contour plots indicated the necessary to optimize variable levels to achieve better results. The results predicted by CCD design showed that a combination of moisture 51% and temperature of 36 ^{o}C would favor maximum phytase production, yielding phytase activity 13.5 U. Although pH may be also an influencing factor, it was excluded, since the final product would be directly used for feed and we didn't want to introduce extra salt to the feedstuff.

Effect of temperature on phytase activity and stability

The optimal temperature for *A. ficuum* phytase activity was 50 ^{o}C (Fig. 2a). At 45 ^{o}C and 55 ^{o}C, phytase activities were also high, just right after that of temperature 50 ^{o}C. *A. ficuum* phytase stability was shown in Fig. 2b. At 35 ^{o}C to 45 ^{o}C, phytase stability was 100%. At 50 ^{o}C, phytase was still relatively stable, with 93% activity remaining. Ullah and Gibson (1987) studied *A. ficuum* extracellular phytase produced from synthetic medium. The phytase optimal temperature was 58 ^{o}C, with catalytically active for at least 3 hr, and phytase lost 60% of its activity when subjected to 68 ^{o}C for 10 min. Ullah used purified extracellular phytase, but our research used crude enzyme. That might result in the difference.

Phytic acid degradation of SM during temperature two-stage Aspergillus ficuum solid state fermentation

Phytase activity change during the two-stage temperature fermentation protocol was shown in Fig. 3a. Phytase activity was sluggish before 18 hr. But from 18 hr to 24 hr, phytase activity was highly enhanced, and kept rising when temperature was kept at 36 ^{o}C, but stayed constant at temperature of 50 ^{o}C, which indicated the good phytase stability. From our research, the optimal temperature for phytase was 50 ^{o}C and below 45 ^{o}C, phytase could keep 100% of its catalytical activity for more than 3 hr. Our former research showed that phytase from *A. oryzae* attained its peak activity by 25 hr and then lost its activity with fermentation time going on. Compared with *A. oryzae, A. ficuum* was a preferred fungus for degrading phytic acid due to its better phytase stability.

In consistent with phytase activity, phytic acid content before 18 hr was no change (Fig. 3b). Phytic acid in SM is located in the protein bodies and forms protein – phytate complex (Liu 1997). During fermentation, protease was secreted (Wang et al 2005). Hyrolysis of protein might ease phytic acid extraction. That was the reason that the highest amount of phytic acid was measured at 18 hr. Because of the good phytase stability, from 18 hr, phytic acid degradation was carried out and lasted till the end of fermentation at both temperatures. Fermentation at 50 ^{o}C achieved larger phytic acid degradation than that of 36 ^{o}C fermentation. At 50 ^{o}C, 89% the SM phytic acid was degraded, comparing with 45% at 36 ^{o}C, by 36 hr fermentation. Using temperature two – step protocol, 98% increase in phytic acid degradation level was achieved. Nair and Duvnjak (1990) once applied the extracted phytase to hydrolyze phytic acid in semi –

solid canola meal at 45 ^{o}C, and 58% of phytic acid was hydrolyzed in 20 hr. Considering the combination of production and hydrolysis, our temperature two-stage protocol had the higher efficiency.

Degradation of phytate in animal feed ingredients would alleviate the negative effect caused by phytate. The benefit of low phytate diet achieved by adding phytase has been reviewed (Chen et al 2010a). The enzymatic modified diet, which had around 50% phytate degradation (Selle and Ravindran 2007), could increase protein/amino acid availability, enhance phosphorous, zinc, calcium retention, and improve animals' growth performance. Hirabayashi et al. (Hirabayashi et al 1998a; Hirabayashi et al 1998b) also investigated the effect of *A. usamii* FSM on chicks. The *A. usamii* FSM could improve phosphorus, zinc and iron bioavailability in SM and enhance body weight gain of chicks.

Nutrient profile change of SM during temperature two- stage Aspergillus ficuum solid state fermentation

Fig. 4 showed the sucrose and oligosaccahrides changes during *A. ficuum* fermentation. After 18 hr, when sucrose was depleted, oligosaccharides degradation speed was highly enhanced. At 28 hr, SM oligosaccharides were totally removed. Total sugar and total non - reducing sugar content (Fig. 5) also kept decreasing, while total reducing sugar was increased. 87% of total structural polysaccharides was decreased by the end of fermentation. Sugar content changed largely during 12 hr to 24 hr fermentation, when fungus growth and enzyme secretion took place. Same trend was observed for protein change (Fig. 6). Protein content increased 6.7% totally, of which, from 18 hr to 26 hr, protein increased by 5.8%. From 26 hr to 36 hr, although phytase activity kept increasing, protein content had no significant change.

Removal of anti-nutritional factor could enhance nutritional value of SM. Oligosaccharides in soybean were mainly stachyose and raffinose. They represent approximately 4 – 6% of soybean dry matter (Liu 1997). Galacto-oligosaccharides in SM could cause gastrointestinal disturbance and influence the nutrient digestibility and energy availability of SM (Zhang et al 2003). Around 20% of non-starch-polysaccharides (NSP) are present in SM (Hollung et al 2006). These NSP reduce digesta passage rate, which in turn may lower feed intake and decrease growth performance (Dunshea et al 2001). The two-stage temperature protocol solid state fermentation by *A. ficuum* could not only achieve a higher phytate

degradation level, but also remove oligosaccharides, decrease non – reducing carbohydrates and increase protein content.

Conclusion

Two-stage temperature protocol for *A. ficuum* solid state fermentation had the high efficiency to degrade phytate in SM. Comparing with the constant temperature fermentation, this two-stage temperature protocol achieved a 98% increase in phytate degradation level. Besides phytate degradation, oligosaccharides were removed, and non-reducing polysaccharides content decreased by 87 %. Meanwhile, protein content increased by 6.7 %. The nutritional enhanced FSM would be better feed ingredients compared with original SM.

Table 4.1 Parameter levels for CCD design

Variables	Symbol	Coded level				
		-1.682 (-α)	-1	0	1	1.682 (α)
Moisture (%)	A	35.86	40	50	60	64.14
Temperature (^{o}C)	B	31.76	33	36	39	40.24

Table 4.2 Phytase activity under different fermentation conditions

Run Order	A	B	Phytase (U)
1	0	0	13.45
2	1	1	4.44
3	1	-1	4.92
4	0	0	14.70
5	0	1.682	6.00
6	1.682	0	4.81
7	-1.682	0	3.56
8	-1	-1	3.57
9	0	0	12.18
10	0	-1.682	1.97
11	-1	1	5.51

Table 4.3 ANOVA for the fitted quadratic model for phytase production as per CCD design

Souce	Sum of Squares	df	Mean Square	F value	p-value
Model	188.88	5	37.78	30.25	0.0010
A-Temperature	0.53	1	0.53	0.43	0.5429
B-Moisture	6.42	1	6.42	5.14	0.0727
AB	1.46	1	1.46	1.17	0.3293
A^2	114.20	1	114.20	91.44	0.0002
B^2	119.33	1	119.33	95.55	0.0002
Residual	6.24	5	1.25		
Lack of Fit	3.07	3	1.02	0.65	0.6548
Pure Error	3.17	2	1.59		
Cor Total	195.12	10			

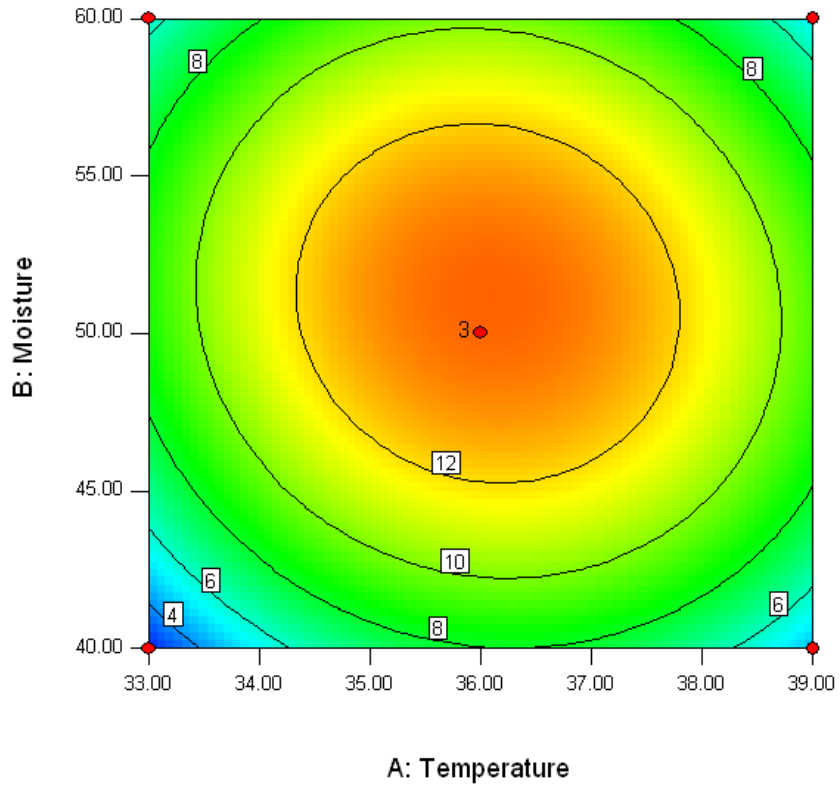


Figure 4.1 Contour plots illustrating the interaction of moisture and temperature on phytase production

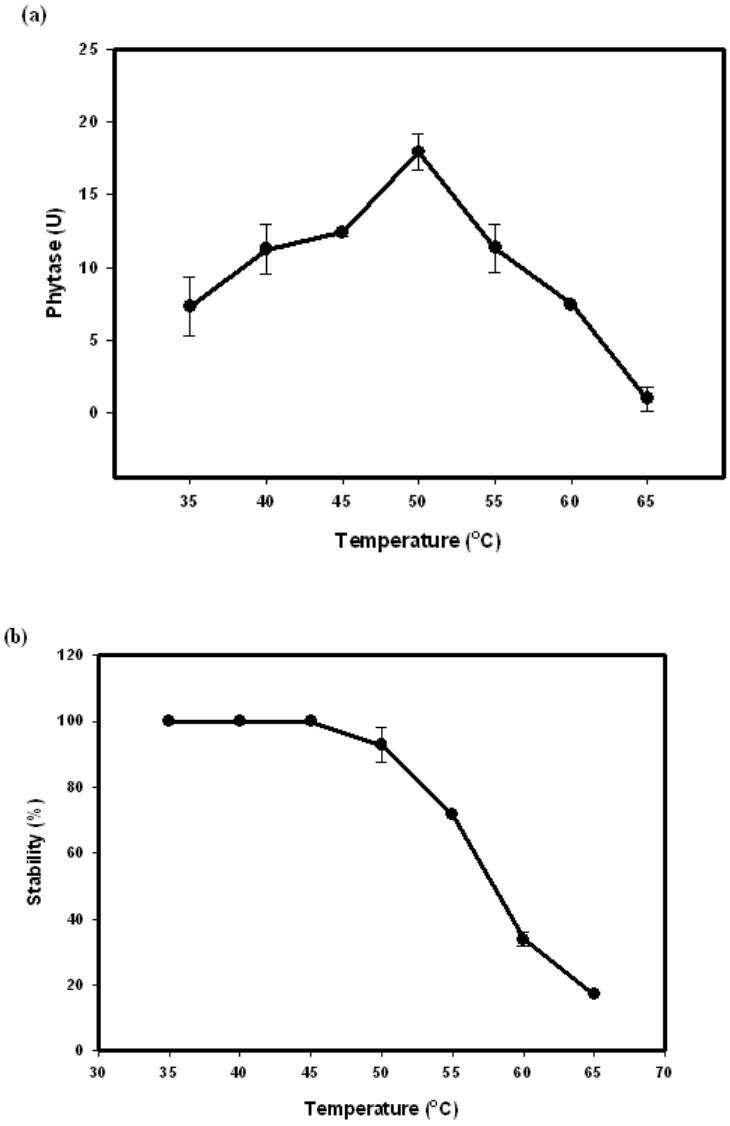


Figure 4.2 Effect of temperature on (a) phytase activity; (b) phytase stability

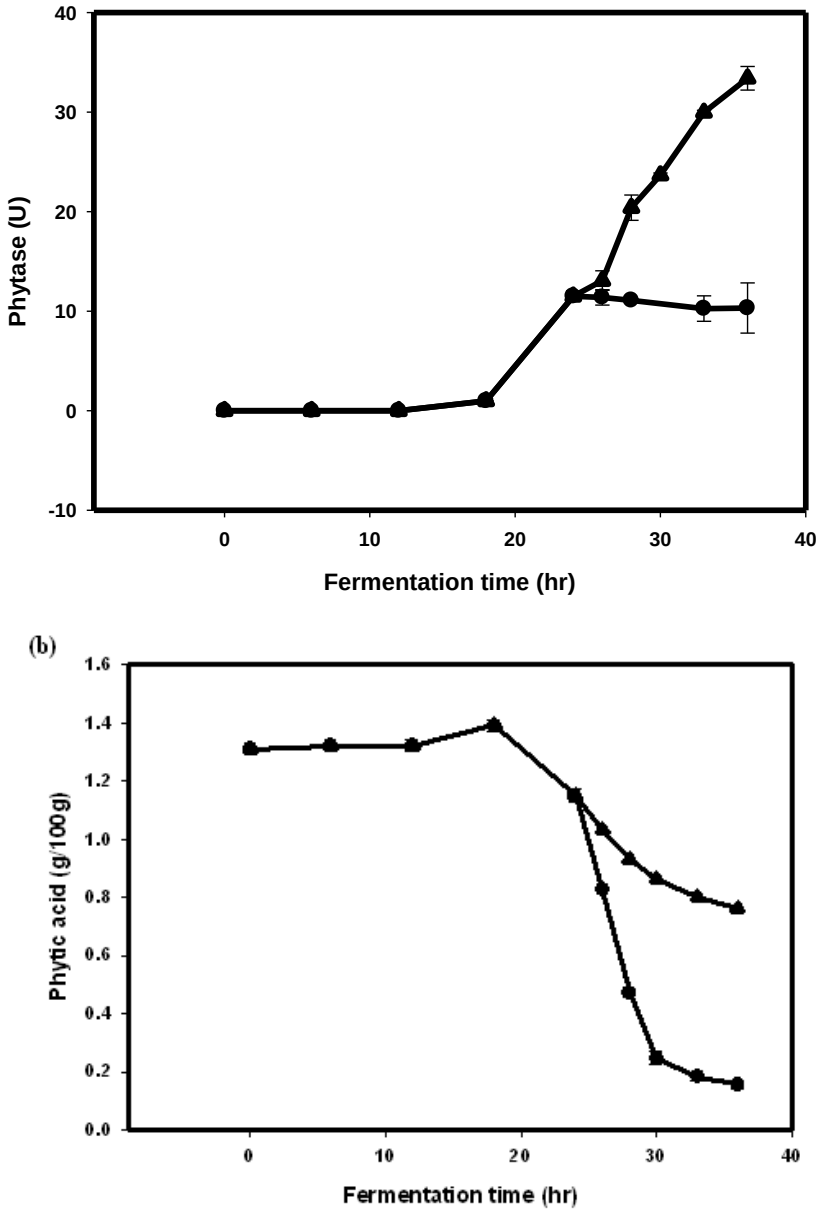


Figure 4.3 (a) Phytase activity and (b) phytic acid content with different fermentation temperature at different fermentation time (triangle: 36 ^{o}C; circle: 50 ^{o}C)

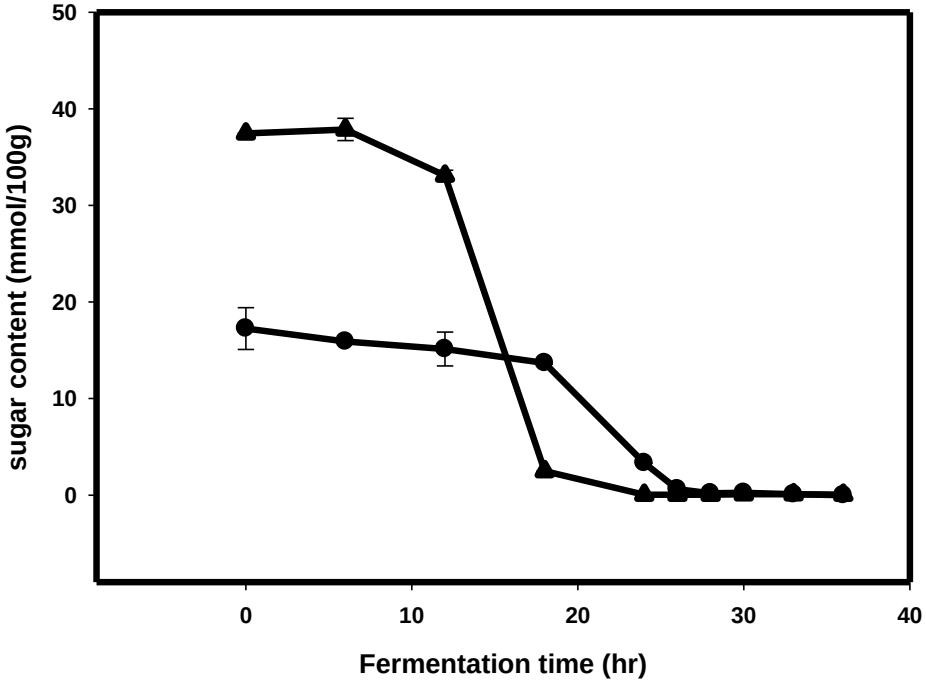


Figure 4.4 Oligosaccharides and sucrose content change of SM during *Aspergillus ficuum* solid state fermentation (triangle: sucrose; circle: oligosaccharides)

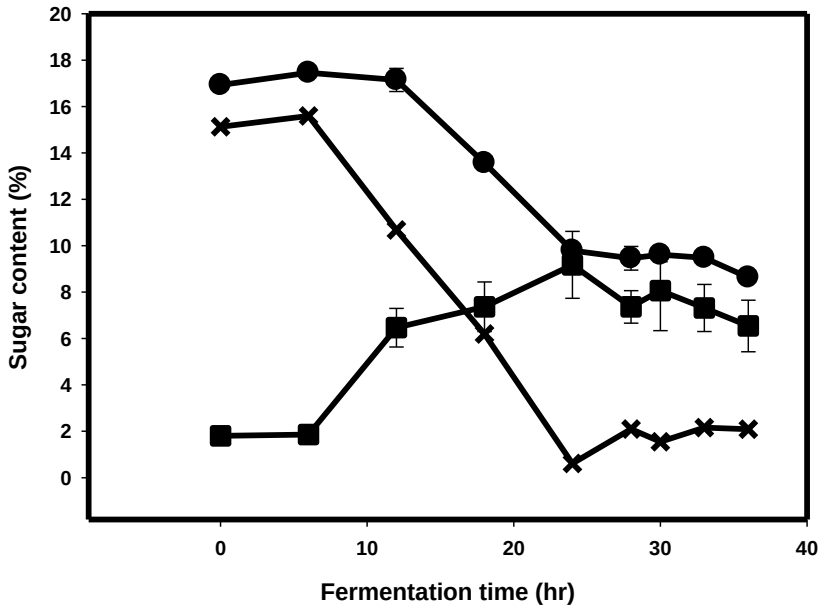


Figure 4.5 Total, reducing sugar and total structural polysaccharides content changes of SM during *Aspergillus ficuum* solid state fermentation (triangle: total non – reducing polysaccharides; circle: total carbohydrate; square: reducing sugar)

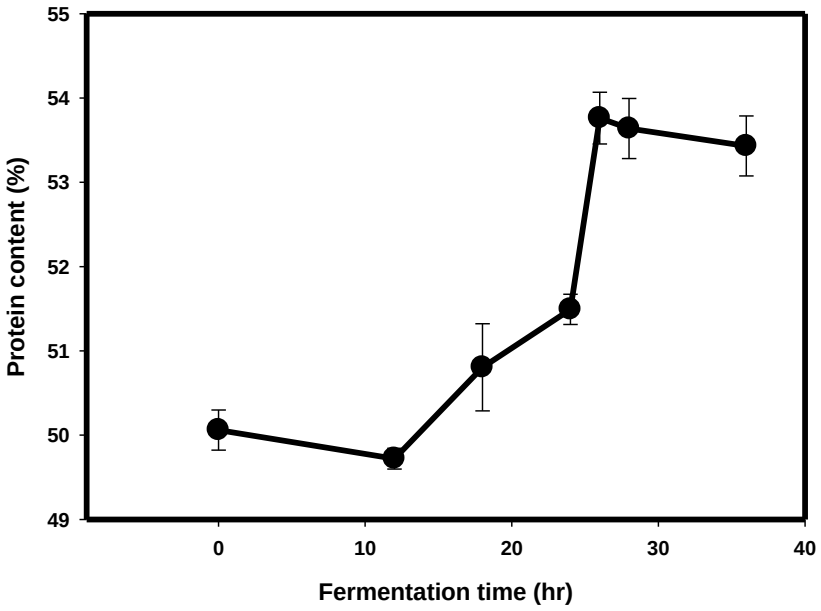


Figure 4.6 Protein content change of SM during *Aspergillus ficuum* solid state fermentation

Chapter 5 - Simultaneous high-efficiency degradation of phytic acid and soy protein *via* co-fermentation of *Aspergillus oryzae* and *Aspergillus ficuum*

Abstract

Fermentation was applied to simultaneously degrade phytic acid and large molecular size of soy protein in soy meal (SM). *Aspergillus oryzae* (ATCC 9362) with protease and *Aspergillus ficuum* (ATCC66876) with good quality phytase were co-fermented. Two-stage temperature protocol, with 0 hr to 28 hr fermented at 36.5 ^{o}C and 28 hr to 40 hr fermented at 50 ^{o}C, was used. Co-fermentation of *A. ficuum* and *A. oryzae* realized 17% increase in phytic acid degradation compared with *A. oryzae* fermentation and 72% increase in DH comparing with *A. ficuum* fermentation. Two-stage temperature fermentation obtained 27% increase in phytic acid degradation and 90% increase in DH compared with single stage fermentation. Two-stage temperature co-fermentation of *A. oryzae* and *A. ficuum* simultaneously degraded phytic acid and soy protein with high efficiency. The fermented soy meal with low anti-nutritional factors would be high quality feedstuff.

Introduction

Soy meal (SM) is mainly used as protein source for monogastric animals (Chen et al 2013). However, the anti-nutritional factors in SM, like trypsin inhibitor, oligosaccharides, phytic acid etc., lower down its nutritional value. Our former research showed that trypsin inhibitor and oligosaccharides could be easily removed *via* fermentation. But degradation of phytic acid and soy protein molecular size could not be obtained to a high level simultaneously.

Phosphorous in phytic acid form could not be absorbed by monogastric animals because they lack phytase. Phytic acid in SM combines with other nutrients, like protein and important minerals, to form phytic acid-protein or phytic acid-protein-phytic acid complicates etc. (Selle and Ravindran 2007). The existence of phytic acid influences the digestion of SM nutrients. Further, the unabsorbed phytate could result in excess phosphorous excretion in monogastric aniamls' manure and lead to europhication. For the protein part, only two to three peptides and amino acids could be directly absorbed by monogastric animal intestine cells. Young animals with limited stomach acid and enzymatic secretions in the small intestine can have difficulty in

digesting proteins with complex structures and large molecular weights (Kim 2010). But investigation has been committing to apply soy protein for chick broilers and newly weaned piglets, in order to decrease the cost of feedstuff (Feng et al 2007b; Feng et al 2007c).

Aspergillus ficuum (ATCC66876) and *Aspergillus oryzae* (ATCC9362) have been used to ferment soy meal to decrease anti-nutritional factors. Phytic acid degradation by these two cultures was up to 89% and 57%, respectively, in our former research. Phytase from *A. ficuum* have better stability, but *A. ficuum* doesn't secrect protease. *A. oryzae* has high protease activity, but its phytase stability is not as good as *A. ficuum*. In order to achieve the high level degradation of both phytic acid and soy protein, *A. ficuum* and *A. oryzae* were co-fermented in this research. Two-stage temperature solid state fermentation, which we initiated in former research, was applied. The final product is expected to have both good phytic acid degradation and soy protein degree of hydrolysis.

Materials and methods

Microbial cultures

A. oryzae (ATCC9362) and *A. ficuum* (ATCC66876) were preserved 15% glycerol and stored at -80 ^{o}C in 1 ml aliquots in Bioprocessing and Renewable Energy Lab in KSU. Before experiment, the frozen cultures were revived in potato dextrose broth twice. After revival, culture was inoculated on potato dextrose agar (PDA) slant, incubated at 30 ^{o}C for 7 days. Spores were collected from the slants by gently washing with 0.1% Tween 80 to obtain spore suspension of around 10^{7} spores/ml.

Substrate preparation and fermentation

Soy meal (SM) was procured from ADM Alliance Nutrition (Abilene, KS). Solid state fermentation (SSF) was carried out in 125-ml Erlenmeyer flasks. Moisture content of SM was adjusted with distilled water to 50% before autoclaving. Moisture contributed by inoculation was counted. Erlenmeyer flasks containing 10 g substrates (dmb) were autoclaved at 121 ^{o}C for 15 min. Eight sterilized samples were separated into four groups, inoculated with (1) AO: *A.orzyae* 3.2 ml; (2) AOF and AOFC: *A. oryzae* 1.6 ml and *A. ficuum* 1.6 ml; (3) AF: *A. ficuum* 3.2 ml.

The two-stage fermentation was committed by fermenting SM at 36.5 ^{o}C from 0 hr to 28 hr and then changed fermentation temperature to 50 ^{o}C from 28 hr to 40 hr. The current research

applied two-stage temperature solid state fermentation for samples AO, AOF and AF. AOFC was continuously fermented at 36.5 ^{o}C and worked as the single step fermentation control of AOF.

Protease activity

To extract protease, milled samples were diluted with distilled water at a ratio of 1:20 (w/v). The mixture was then placed in a 40 ^{o}C water bath and shaken at a speed of 200 rpm for 1 hr. After protease extraction, the mixture was centrifuged at 5000 rcf for 15 min at 4 ^{o}C. The supernatant was used as crude enzyme and diluted two times with pH 7.2 sodium phosphate buffer for protease analysis. Casein (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 ^{o}C for 10 min. The reaction was terminated by adding 0.5 ml of 0.4 M trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000 rcf for 15 min at 4 ^{o}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 reaction stood for 20 min at 40 ^{o}C; absorbance was read at 660 nm. Sample blank 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 to 1 ug of tyrosine per minute at 40 ^{o}C per gram fermented SM (FSM) (dmb).

Effect of temperature on activity and stability of A. oryzae protease

A. oryzae protease activity was analyzed at 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C, 60 ^{o}C and 70 ^{o}C to investigate temperature effect. To study protease stability at different temperatures, samples were stored at 36.5 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C, 60 ^{o}C and 65 ^{o}C. After 1 hrs, residue protease activities were analyzed. The percentage of residue protease activity to protease activity before storage was used to describe protease stability.

Phytase and phytic acid assay

Phytase was extracted by mixing FSM with 1:10 w/v of 2% $CaCl_2$ (w/v), shaking for 1 hr. The mixture was then centrifuged at 5,000 rcf for 20 min at 4 ^{o}C and the supernatant was used as crude enzyme preparation for further investigation. Phytase analysis was following the method of Gunashree and Venkateswaran (Gunashree and Venkateswaran 2008, for details

please see Chapter 3). Phytase activity was defined as the amount of enzyme that liberates 1 μmol of inorganic phosphate in 45 min. Phytic acid was analyzed with Phytic acid assay kit (Megazyme International Ireland, Bray, Country Wicklow, Ireland).

Degree of Hydrolysis

Protein degree of hydrolysis (DH) was analyzed using the trinitrobenzenesulfonic acid (TNBS) method, following the procedure of Adler-Nissen (1979) with modifications. Protein solution extracted for protease analysis was used for DH analysis. One milliliter sample solution was mixed with 2 ml of 1% (v/v) sodium dodecyl sulfate (SDS) solution (Sigma Aldrich, St. Louis, MO). The protease was first inactivated by putting the mixture in 75 ^{o}C water bath for 15min. After that, 0.25 ml aliquot of the mixture was transferred to test tubes containing 2.0 ml of sodium phosphate buffer (0.2M, pH8.2). 0.1% TNBS reagent (Sigma Aldrich, St. Louis, MO) was then added, followed by mixing and incubation in the dark at 50 ^{o}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 using a spectrophotometer. 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 taken as the L-Leucine equivalent in the extracted solution.

Statistics analysis

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

Results and discussion

To better degrade phytic acid and protein molecular size of SM simultaneously, two-stage temperature co-fermentation of *A. oryzae* and *A. ficuum* was applied. The first stage is to achieve high activities of both phytase and protease, and the second stage is to achieve best enzymatic hydrolysis *via* adjusting fermentation temperature to proximal temperature of enzyme activity and stability.

To realize the two-step temperature fermentation, the right time to change temperature need to be acquired first. Our preliminary experiment showed that after 28 hr, visible spores

would come out for both *A. oryzae* and *A. ficuum*. Too many spores might cause digestive sensation of monogastric animal digestive tract. So the specific time between 0 hr to 28 hr would be proper. Figure 1 shows the phytase acitivity of *A. ficuum* and protease activity of *A. oryzae* at different fermentation time. From 22 hr to 28 hr, phytase acitivity of *A. ficuum* was not significantly changed, but protease activity increased continuously with fermentation time going on from 0 hr to 28 hr. Considering all these factors, 28 hr was chosen as the turning point of fermentation temperature.

To choose the right temperature for two-step temperature fermentation, *A.orzyae* protease properties were studied and shown in Figure 2. The proximal temperature for *A. oryzae* is 55 ^{o}C (1094 U), while protease activity at 50 ^{o}C (922 U) and 60 ^{o}C (884 U) were a little lower than that of 55 ^{o}C. But with higher temperature, the stability was lower. Staying at 50 ^{o}C for 1 hr, there was 67% activity left, but at 55 ^{o}C for 1 hr, there was only 32% left. So 50 ^{o}C was the better choice to obtain both good *A. oryzae* protease activity and stability. Our former research about phytase showed that the optimal temperature for *A. ficuum* phytase was 50 ^{o}C, at which, *A. ficuum* phytase had 93% activity remaining after 3 hr. Comprehensively considering the proximal temperature for both *A. oryzae* protease and *A. ficuum* phytase activity and stability, 50 ^{o}C was chosen as the fermentation temperature for the second stage.

Phytase activities of FSM with different fermentation conditions are shown in Figure 3. Both *A. ficuum* and *A. oryzae* secreted phytase. *A. oryzae* phytase activity decreased when temperature was changed to 50 ^{o}C, while *A. ficuum* phytase activity kept increasing. Phytase from *A. ficuum* was possible spore related. When fermentation temperature was changed to 50 ^{o}C, mycelia stopped growth and spores were generated. The activity of *A. ficuum* spore related phytase increased. Phytase activity for co-fermentation samples was higher than that of *A. oryzae* and lower than that of *A. ficuum*.

Figure 4 shows the phytic acid changes of FSM with different fermentation conditions. Phytic acid degradation degree was significantly different for four different samples. AOFC had the lowest degradation degree (48%), while AF had the highest (72%). Phytic acid degradation for AOF was 61%, higher than AO (52%).

Although AOFC has higher phytase activity from 28 hr to 40 hr, phytate degradation degree was lower than both AO and AOF. Because phytase had much higher enzymatic activity at 50 ^{o}C than 36.5 ^{o}C, the two-stage fermentation obtained better result than single stage

fermentation. With better phytase stability, AOF had higher degradation degree than AO. Comparing AOF and AOFC, two-stage fermentation obtained 27% increase in phytic acid degradation. Co-fermentation of *A. ficuum* and *A. oryzae* realized 17% increase, derived by comparing AOF with AO.

Protease activities of FSM with different fermentation conditions are shown in Figure 5. No protease was secreted by *A. ficuum*. Co-fermentation of *A. oryzae* and *A. ficuum* had higher protease activity than *A. ficuum* but lower than *A. oryzae*. *A. oryzae* protease activity decreased at 50 ^{o}C with fermentation time going on.

Figure 6 shows the degree of hydrolysis of SM fermented at different conditions. AO had the highest DH. AOF had higher DH than AOFC and AF. Same thing as for phytase, although AOF had lower protease activity than AOFC from 28 hr to 40 hr, its DH was still higher than AOFC. This confirmed the effectiveness of two-stage temperature fermentation again. Comparing DH of AOF and AOFC, two-stage temperature fermentation obtained 90% increase in DH. According to Figure 5, *A. ficuum* didn't secret protease, but the DH for AF was still higher than AOFC. Phytic acid is located within the protein bodies, in the form of protein-cation-phytate complex (Prattley and Stanley 1982). As shown in Figure 3, *A. ficuum* had high amount and good quality of phytase. The degradation of phytic acid help release combined protein and peptides, therefore lead to DH increase. Comparing DH of AF and AOF, co-fermentation obtained 72% increase in DH.

Degradation of phytic acid and soy protein molecular size helps alleviate their negative effect on animal growth and environment (Chen et al 2013). The enzymolytic modified diet with phytase, which had around 50% phytate degradation (Selle and Ravindran 2007), could increase protein/amino acid availability, enhance phosphorous, zinc, calcium retention, and improve animals' growth performance. Hirabayashi (Hirabayashi et al 1998a; Hirabayashi et al 1998b) also investigated the effect of *Aspergillus usamii* FSM on chicks. The *A. usamii* FSM could improve phosphorus, zinc and iron bioavailability in SM and enhance body weight gain of chicks. Enzymolytic soy protein produced by fermentation and protease hydrolysis contains more soluble protein and small-size peptides than untreated soy meal. Protein hydrolysis could also lower soy protein immunoreactivity by hydrolyzing soy protein into peptides and amino acids and altering the structure of antigen epitopes, therefore reducing the IgE binding potential (Song et al 2008). Partial replacement of soy meal with protease hydrolyzed soy protein for

broilers from 1 to 21 days old increased feed intake, body weight gain, and nutrient digestibility of crude protein, Ca and P (Wang et al 2011). The FSM obtained in this research had both low phytic acid contend and high protein hydrolysis degree, its nutritional value was much higher than SM fermented with other methods. Animal experiments were needed to further test this.

Conclusion

Both *A. oryzae* and *A. ficuum* grew well while cultivated together on SM. Co-fermentation of *A. oryzae* and *A. ficuum* had higher phytase activity and better phytase stability than *A. oryzae* fermentation, and it also had higher protease activity than *A. oryzae* fermentation. Two-stage temperature fermentation obtained 27% increase in phytic acid degradation and 90% increase in DH compared with single stage fermentation. Co-fermentation of *A. ficuum* and *A. oryzae* realized 17% increase in phytic acid degradation compared with *A. oryzae* fermentation and 72% increase in DH when compared with *A. ficuum* fermentation.

Abbreviations used

ATCC, American Type Culture Collection; SM, soy meal; FSM, fermented soy meal; AO: two-stage fermentation of *A. oryzae*; AOF: two-stage co-fermentation of *A. oryzae* and *A. ficuum*; AOFC: single-stage co-fermentation of *A. oryzae* and *A. ficuum*; AF: two-stage fermentation of *A. ficuum*.

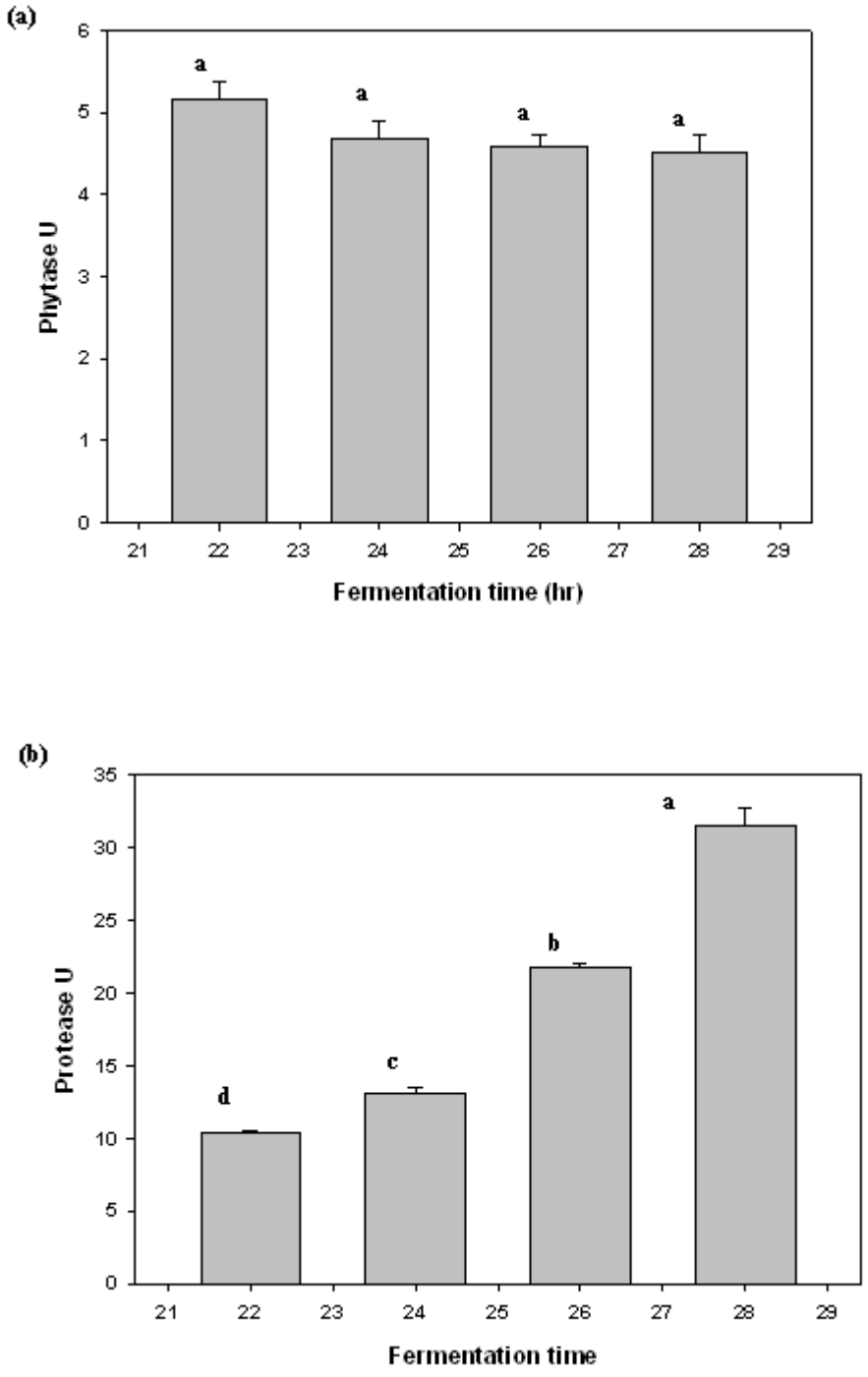


Figure 5.1 Phytase activity of *Aspergillus ficuum* (a) and Protease activity of *Aspergillus oryzae* (b) at different fermentation time

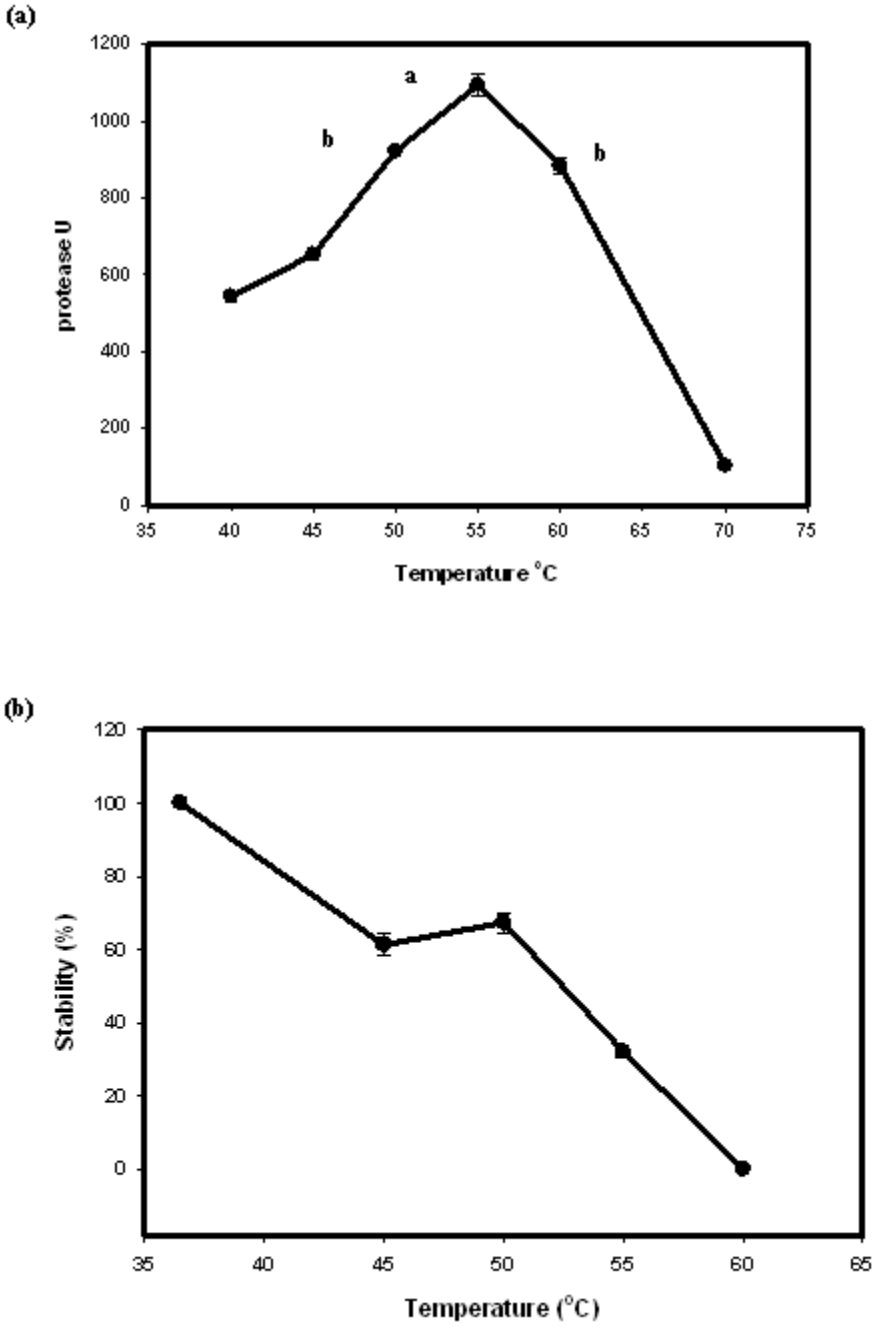


Figure 5.2 *Aspergillus oryzae* protease properties: (a) temperature influence (b) temperature stability

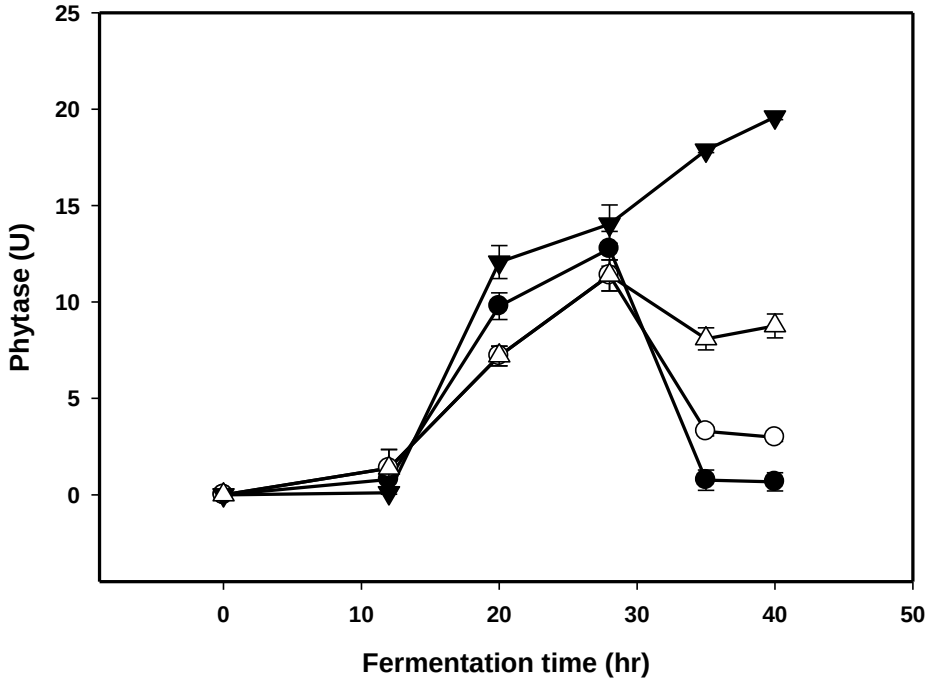


Figure 5.3 Phytase activities of FSM with different fermentation conditions (black dot: AO; white dot: AOF; inverted black triangle: AF; white triangle: AOFC)

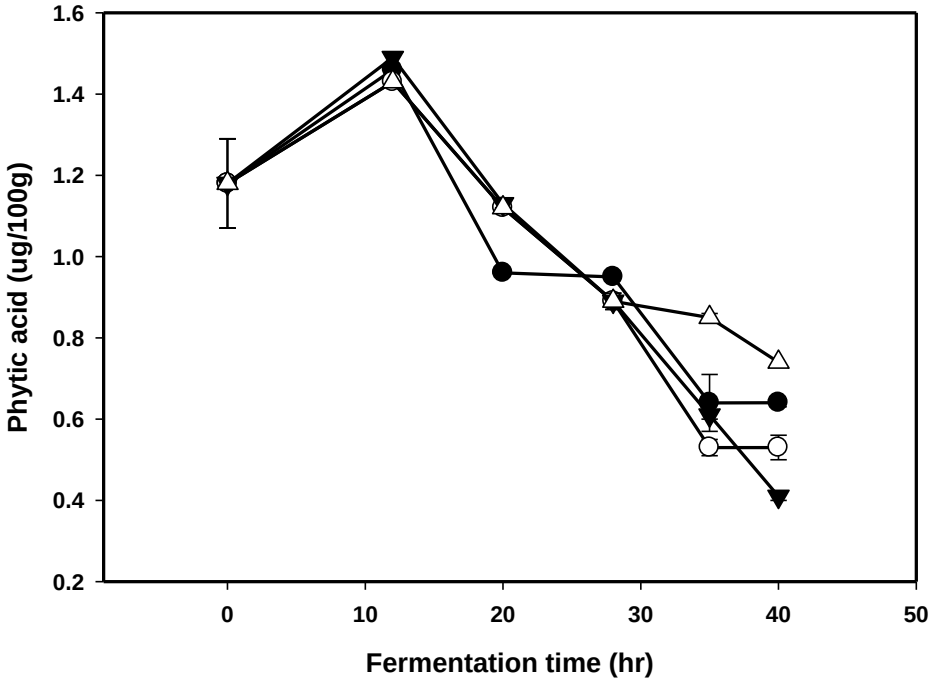


Figure 5.4 Phytic acid changes of FSM with different fermentation conditions (black dot: AO; white dot: AOF; inverted black triangle: AF; white triangle: AOFC)

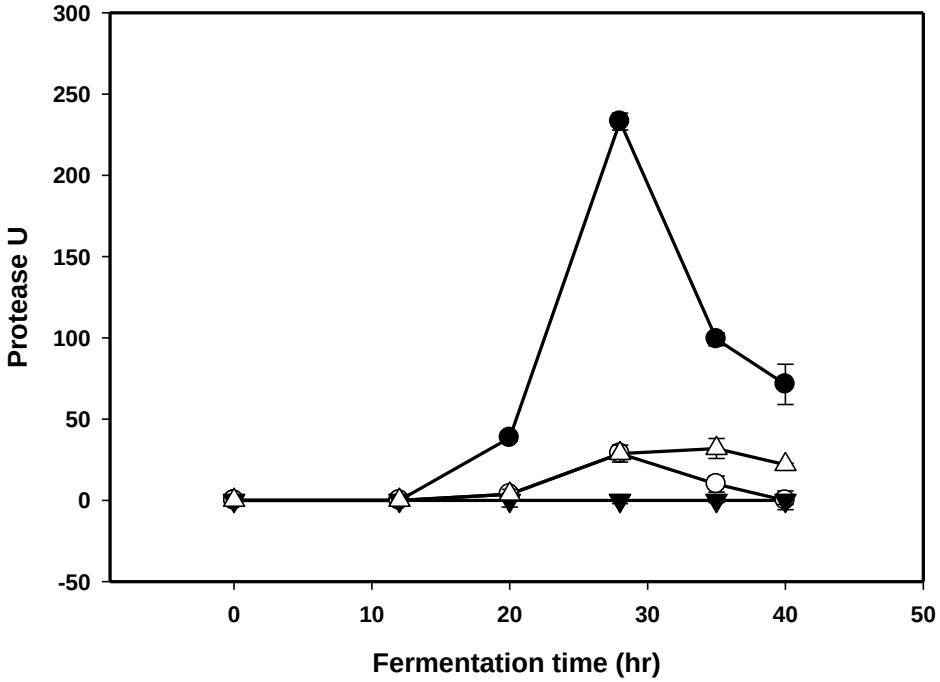


Figure 5.5 Protease activities of SM fermented at different conditions (black dot: AO; white dot: AOF; inverted black triangle: AF; white triangle: AOFC)

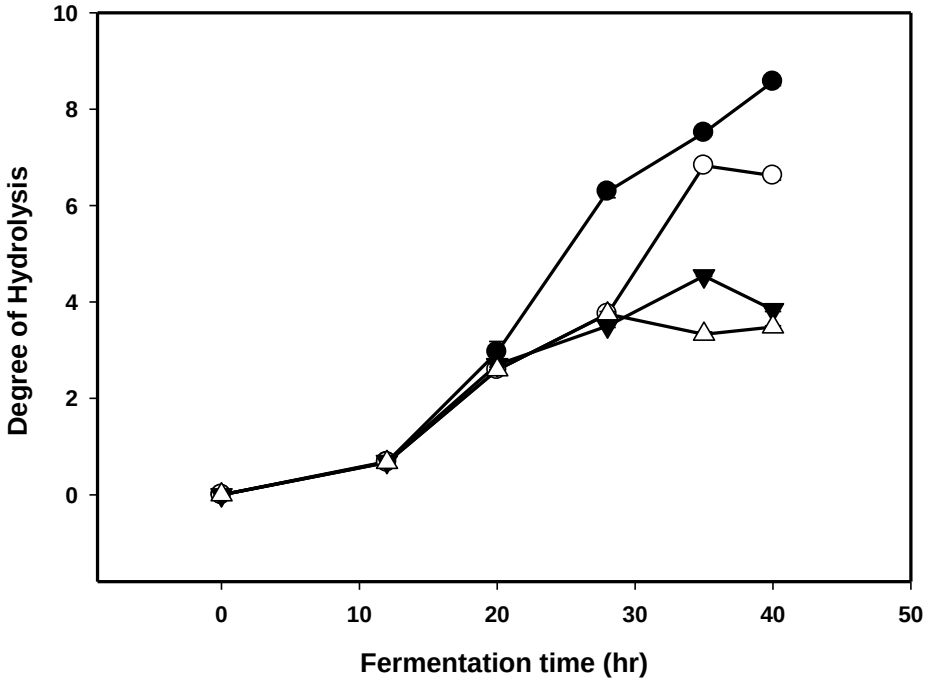


Figure 5.6 Degree of hydrolysis changes of FSM with different fermentation conditions (black dot: AO; white dot: AOF; inverted black triangle: AF; white triangle: AOFC)

Chapter 6 - Virginiamycin addition to investigate fermentation effect on immunoreactivity reduction and molecular weight degradation of soy protein

Abstract

Soy protein allergens and large molecular size restrict its usage as protein source for young mono-gastric animals. Fungi fermentation might be a way to degrade protein molecules and reduce its immunoreactivity. But substrate sterilization during fermentation process denatured protein and might contribute to the degradation of allergens. Virginamycin, as the bactericin, would inhibit the contaminating bacteria, and exclude the necessity of sterilization process. Our research first confirmed that using sterilized substrate would not elucidate the fermentation effect on protein. Virginiamycin was added to *Aspergillus oryzae* solid state fermentation of unsterilized soy meal (SM) to investigate how *A. oryzae* fermentation contributed to nutritional enhancement of SM. With the addition of virginiamycin, the benefits of *A. oryzae* fermentation could be clearly illustrated. *A. oryzae* solid state fermentation had the priority to degrade α and α' subunits of β-conglycinin and decreased the immunoreactivity of soy protein. Also, fermentation increased protein content and essential amino acids content in soy meal and produced the value added soy meal products.

Introduction

Soy meal (SM) is the main protein source for monogastric animals in the United States (Chen et al 2010a). But its inclusion in newly weaned pigs is limited because of some anti-nutritional factors and antigenic soy proteins causing hypersensitivity (Song et al 2010). To date, 34 soybean proteins have been identified as allergens (Wilson et al 2008). All three parts of the β-conglycinin, both acidic and basic subunits of glycinin and P34 have been identified as main allergens for young pigs (Frias et al 2008; Wilson et al 2008; Zhao et al 2008). Some studies indicate that incorporation of antigenic soy proteins such as pure glycinin or β-conglycinin to the diet lead to a reduced weight gain and feed efficiency as well as an increased incidence of diarrhea in pigs (Zhao et al 2008). Further, pepsin and trypsin cannot perform their entire

function until 3 weeks of age in piglets (Zhao et al 2008). Large molecular size protein is hard for them to digest.

Fermentation has been applied to remove soy protein immunity and degrade protein molecular size. Song (2008) found that natural fermentation, *Saccharomyces cereviseae*, *Bacillus lactis* fermentation of SM reduced in 80%, 77% and 77% immune response when using 97.5 kUA/l human plasma, respectively. Amnuaycheewa (2010) analyzed the profilin in fermented soy products. The reduction in profiling in natto fermented by *Bacillus natto* was 12.8% to 35.4% and for soy paste 12.8% to 46.3%, in comparison to soy flour. But as to the fungi fermentation effect on SM immunity, research is limited. Frias (2008) found that *Aspergillus oryzae*, *Rhizopus oryzae* solid state fermentation produced a reduction of immunoreactivity of 68%, 66% of soy meal, respectively. But both samples had been using 121^{o}C 15 min sterilized before and after fermentation. Since high heat could denature protein (Mills et al 2004) and reduce its immunity (Wilson et al 2008), it could not elucidate that the deduction of immunity was caused by fermentation. Fungi solid state fermentation of SM has been applied to enhance the nutritional value of SM as mono-gastric animals' feed (Chen et al 2010b). Animal test has already found that feeding FSM could decrease the immune response to soy protein in piglets (Liu et al 2007). But enzymes with different origins have different hydrolytic effect (Yamanishi et al 1996). There is a need to find the proper way to investigate whether and how a specific fungus solid state fermentation affects soy protein immunoreactivity.

Virginamycin is the bactericin to feed industry. Also it is used in ethanol production to prevent contamination of materials used for fermentation. In our research, we applied the virginiamycin to inhibit bacteria growth during *A.orzyae* solid state fermentation in unsterilized SM. Our preliminary research found that 0.05% (dmb) of virginamycin in SM could inhibit bacteria growth while *A. oryzae* could still grow well. Our research was to give the proof that using sterilized SM as substrate was not persuasive to study the fermentation effect on molecular degradation and protein immunireactivity reduction. With adding virginiamycin, fermentation of non-sterilized SM could provide the proper information.

Materials and Methods

Microbial culture

Lyophilized cultures of *A. oryzae* (ATCC 9362) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived in potato dextrose broth twice. After revival, culture was inoculated on potato dextrose (PDA) slant, incubated at 30 ^{o}C for 7 days, and later stored at 4 ^{o}C for short term preservation. For routine experiments, spore solution was used. Spores were collected from the slants by gently washing with 0.1% Tween 80 to obtain spore suspension of around 10^7 spores/ml. Additionally, spores were suspended in 15% glycerol and stored at -80 ^{o}C in 1 ml aliquots for long term preservation.

Substrate preparation and fermentation

SM was procured from ADM Alliance Nutrition (Abilene, KS). Solid state fermentation was carried out with three different kinds of substrates, which were original SM, SM autoclaved at 100 ^{o}C for 30 min (SM100C) and SM autoclaved at 121 ^{o}C for 15 min (SM121C). For SMV, virginiamycin was added at a ratio of 0.05% dry matter base of SM. Four milliliters of *A. oryzae* spore solution containing 10^7 spores/ml were inoculated into 20 g substrate. Moisture was adjusted before autoclave. Moisture contributed by inoculation was considered. And final moisture was 50%. The flasks were incubated at 35 ^{o}C for 36 hr. The fermented samples were then lyophilized and used for analysis.

Soy protein sample preparation

Lyophilized FSM was milled with mortar and pestle to flour. SM flour was dispersed in distilled water at a ratio of 1:10. The mixture pH was adjusted to pH8.2 by using 2N NaOH. After 2 hr of shaking at room temperature, the mixture was centrifuged at 5,000 rcf at 4 ^{o}C to remove insoluble residues. Soy protein in supernatant was precipitated by adjusting the supernatant pH to pH4.8.

Differential Scanning Calorimeter (DSC)

The denaturation of soy proteins was assessed with a differential scanning calorimeter (DSC) (DSC7, Perkin-Elmer, Norwalk, CT) calibrated with indium and zinc. Wet soy protein samples were hermetically sealed in a large-volume stainless pan. About 10 mg soy protein (dmb) with moisture around 60% was loaded. Samples were scanned from 10 to 150 ^{o}C at a heating rate of 10 ^{o}C/min. Denaturation temperatures (T_d) were determined from the peak temperatures.

SDS-PAGE

The precipitated wet protein was diluted with distilled water by adjusting pH to pH8.2 using 2 N NaOH. Protein concentration was determined with Bradford method. SDS-PAGE of soy protein samples was performed on a 4% stacking gel and 12% separating gel. Fifty milligrams of soy protein were inoculated into the gel for each sample. A prestained standard with molecular weight from 8 kDa to 250 kDa was used. Electrophoresis was performed at 120 V for 2 hr. The gel was stained in 0.1% Coomassie brilliant blue R-250 and destained in a solution containing 10% acetic acid and 40% methanol. Densitometry of the gel was analyzed by the Kodak 1D Image Analysis software, version 4.6 (Kodak, Rochester, NY).

Western Blot Procedures with Human Plasma

Western blot was performed according to the method of Frias (2008) with modifications. Human plasma used had soybean-specific IgE 10 kUA/L provided by PlasmaLab International (Everett, WA). After transferring, the membrane was stained with Poceau S for 5 min to check the transferring effect. Poceau S was then washed off with distilled water before proceeding to the next step. The saturation solution, primary and secondary antibodies were prepared in tris-buffered saline (TBS) instead in 0.01% Tween in TBS (TTBS) buffer, to avoid the dark background. The membrane was exposed to Kodak X-ray film.

Chemical analyses

The proximate composition was analyzed by the Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO) using the following methods:

crude protein (AOAC Official Method 990.03, 2006), crude fat (ether extraction, AOAC Official Method 920.39 (A)), crude fiber (AOAC Official Method 978.10, 2006), acid detergent fiber (ADF) (AOAC Official Method 973.18 (A-D), 2006), neutral detergent fiber (NDF), cellulose (AOAC Official Method 973.18 (A-D), 2006), pepsin digestibility (AOAC Official Method 971.09, 2006), amino acid profile (AOAC Official Method 982.30 E(a,b,c), chp.45.3.05, 2006), available lysine (AOAC Official Method 975.22, chp.45.4.03, 2006) (Official methods of analysis of AOAC international. 2006).

Statistics analysis

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

Results

Figure 1 shows the DSC results of soy protein samples pretreated at different temperatures and their corresponding fermentation products. SM and SMV both had two peaks, with the degradation temperatures (T_d) at around 79 ^oC and 96 ^oC. SM100C and SM100 each had one peak at temperature around 97^oC. SM121C and SM121 had one indiscernible peak at around 97^oC.

Figure 2 shows the SDS-PAGE results of soy meal samples with different pretreatment. SM contained 79% bands with molecular weight larger than 60,000 kDa, but SM100C only had 4% bands and SM121C only had 6% bands in that range. For SMV, SM100 and SM121, the three fermented samples, the molecular weight of all peptides were smaller than 60,000 kDa. All the fermented samples had 2% small peptides with molecular weight smaller than 10,000 kDa, which the controlling unfermented samples were not endowed. The α and α’ subunits of SM were not shown in SMV, and β-conglycinin was not shown in SM100, SM121 and their controls.

Figure 3 shows the Pauceau S staining of the membrane after transferring. Comparing with Figure 2, all bands in SDS-PAGE gel had been transferred to the membrane. Figure 4 shows the immunodominant proteins interacting with human plasma 10 kUA/L. SM presented the highest complexity protein profile and plasma immunoreactivity towards α- and α’- (75 kDa) and

β- (50 kDa) conglycinin subunits, P34 fraction, and glycinin basic (30 kDa) and acidic (22 kDa) subunits. Its corresponding fermented sample SMV didn't have immunoreactive proteins toward α- and α'- (75 kDa) conglycinin subunits. Compared with SM, the immunoreactions signals for β-conglycinin, P34 and acidic (22 kDa) glycinin subunits were weak for SM100C. There was no visible immunoreactivity for β-congycinin in SM100. SM121 and SM121C both only had immunoreactive protein towards basic (30 kDa) glycinin.

The composition changes of SM samples were illustrated in Table 1. Heat treatment did not significantly influence the content of crude protein, crude fat, crude fiber, total ash and pepsin digestibility, but decreased the content of crude fiber, acid detergent fiber, cellulose content, and increased the neutral detergent fiber. Also, heat treatment decreased the content of available lysine content. The higher the temperature, the lower the available lysine content of heat pretreated soy meal. The content of all components increased after fermentation, except that pepsin digestibility decreased. Fermented samples with higher heat treatment had higher available lysine.

Amino acid contents of fermented samples were shown in Table 2. Heat treatment didn't significantly affect essential amino acids content, except lysine. Fermentation increased the total amino acids content of SM, SM100C and SM121C by 7%, 8%, and 9%, respectively. Fermentation also increased all amino acids contents. The higher the temperature, the higher the amino acid content of fermented samples. Methionine, cysteine, and threonine in SM121 increased by 11.4%, 22.39% and 16.22% after fermentation, respectively. Lysine and tryptophan in SM121 increased by 13% and 30% comparing with SM121C.

Discussion

Soy protein denaturation is an endothermic process (Wool and Sun 2005), by rupture of inter- and intra-molecular bonds. Its denaturation degree could be shown by DSC. Undenatured soy protein has two peaks in the DSC curve, which represent the two main soy storage proteins – conglycinin and glycinin (Wool and Sun 2005). Glycinin is more heat stable than conglycinin (Wool and Sun 2005). The T_d for glycinin was in the range of 96.3 - 97.7 ^oC, while the T_d for conglycinin was in the range of 77.1 - 79.3 ^oC (Figure 1). Protein denaturation is a non-reversible process (Wool and Sun 2005). The denatured protein would not show peaks on the DSC

diagram. According to Figure 1, protein in SM and SMV were non-denatured; 7S subunits in SM100C and SM100 were denatured; in SM121C and SM121, 7S subunits were totally denatured while 11S were almost totally denatured.

SDS-PAGE is a common way to evaluate protein molecular size. But for heat treated samples, it didn't work well, according to our result. The denatured 7S subunit of soy protein was not shown on SM100C and SM121C lanes. This didn't mean that SM100C and SM121C had less large molecular weight protein. Protein denaturation was the change of the secondary, tertiary and quaternary structures. Thermal energy input disrupted the wean bonds stabilizing the native conformation, causing protein to unfold (Wang et al 2007). The denatured protein may form large aggregate (Utsumi et al 1984), which may become insoluble and would not be shown on SDS-PAGE. Wang (Wang et al 2007) also showed that 100 ^{o}C heating for 20 min resulted in the loss of protein bands on SDS gel. The addition of virginiamycin avoided the heat pretreatment of soy meal. *A. oryzae* solid state fermentation could degrade large protein molecules into smaller peptides, as shown by comparing the molecular weight of SM and SMV from Figure 2.

About 34 subunits of soy protein have been recognized as allergens (Wilson et al 2008). Our result also showed the strong signal of immunoreactive protein in SM. Heat treatment has been shown to affect allergen conformational epitopes and decrease its immunoreactivity (Maldonado et al 1998). Western blot has been used to illustrate protein immunoreactivity. Like SDS-PAGE, there was still the problem caused by heat induced protein denature. Subunits of soy protein, like β-conglycinin in SM100C and SM121C and acidic glycinin in SM121C, were not visible on western blot. This didn't mean that they didn't have immunoreactivity, but they were not present on SDS gel. The virginiamycin addition helped the investigation on fermentation degradaded soy protein immunoreactivity. The weaker immunoreactive signals for SMV, comparing with SM, illustrated that *A. oryzae* solid state fermentation could decrease immunoreactive soy protein.

Natural fermentation has been shown to degrade soy protein molecular size with non-heated samples (Frias et al 2008). In the natural fermentation, various kinds of microorganisms have been involved. Different proteases target different protein subunits (Tsumura 2009). In

order to achive better degradation, it is necessary to investigate the fermentation effect of specified microorganism. Virginamycin could inhibit bacterial growth while the inoculum of fungus could inhibit the growth of other contaminating fungi, according to our preliminary results. Virginiamycin addition excluded the necessity of heat treatment, which was beneficial to the protein size and immunoreactivity investigation.

A. oryzae secrets acidic and neutral proteases, which could degrade epitopes (Maldonado et al 1998). According to our research, *A. oryzae* fermentation provides means to degrade soy protein molecular size while decrease its immunoreactive protein. Soy protein with low molecular weight and weak or no immnoreactive protein have been added to newly weaned piglets' diet, to lower the feeding cost (Song et al 2010). Fermentation parameters, like temperature, moisture, fermentation time etc., need to be optimized to maximize the protein degradation and to produce hypoallergenic fermented soy products for young pigs.

Acid detergent fiber mainly includes cellulose and lignin. Neutral detergent fiber mainly includes hemicelluloses, cellulose and lignin. Heat treatment may degrade lignin or catalyze complex structure formation between hemicelluloses and lignin or hemicelluloses with other components, like lysine. Lysine is susceptible to react with other compounds, such as reducing sugars to form Maillard compounds, resulting in the loss of available lysine and reduction of nutritional value. *A. oryzae* secre various kinds of enzymes, such as α-amylase, carboxymethyl cellulase, pectinlyase, protease, endo-β-xylanase (EC 3.2.1.8) etc. (Chen et al 2013)to help utilize soy meal components to meet its growth needs. The enhancement of all components by fermentation was mainly because of the dry matter loss resulting from *A. oryzae* consumption, which concentrated the nutritional compounds. Proper heat could unfold protein structure and make protein easier to digest by proteases. The decrease of the pepsin digestibility may be partially contributed by the increased protein content.

Soy protein is rich in lysine and tryptophan, but lacking in methionine and threonine, compared with cereals proteins. SM and maize are main ingredients for monogastric animals' diet and provide complementary amino acid profiles. Amino acids deficiency may decrease feed efficiency, feed intake, cause weight loss and influence animals' growth performance (Moehn et al 2012). *A. oryzae* solid state fermentation enhanced the amino acid contents of all samples,

especially for the SM121. Heat treatment could expose inside peptide bonds and ease enzymatic hydrolysis. Proper heat treatment might benefit the subsequent fermentation process.

A. oryzae solid state fermentation could degrade large soy protein molecular size and decrease soy protein immunoreactivity. *A. oryzae* protease had priority for α and α' components of β-conglycinin. Virginiamycin facilitated the investigation by controlling competitive bacterial growth. Fermentation enhanced the nutritional value of soy meal, with higher protein content. Essential amino acids contents were also enhanced by fermentation. Proper heat treatment facilitated the fermentation process. The value added FSM should have a wider market than SM, such as use for newly weaned piglets.

Abbreviations used

ATCC, American Type Culture Collection; DSC, differential scanning calorimetry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ADF, acid detergent fiber; NDF, neutral detergent fiber; AOAC, Association of Official Analytical Chemists; SM, soy meal; FSM, fermented soy meal; SMV, fermented soy meal with virginiamycin added; SM100C, soy meal autoclaved at 100 ^{o}C for 30 min; SM100, fermented soy meal which was autoclaved at 100 ^{o}C for 30 min; SM121C, soy meal autoclaved at 121 ^{o}C for 15 min; SM121, fermented soy meal which was autoclaved at 121 ^{o}C for 15 min.

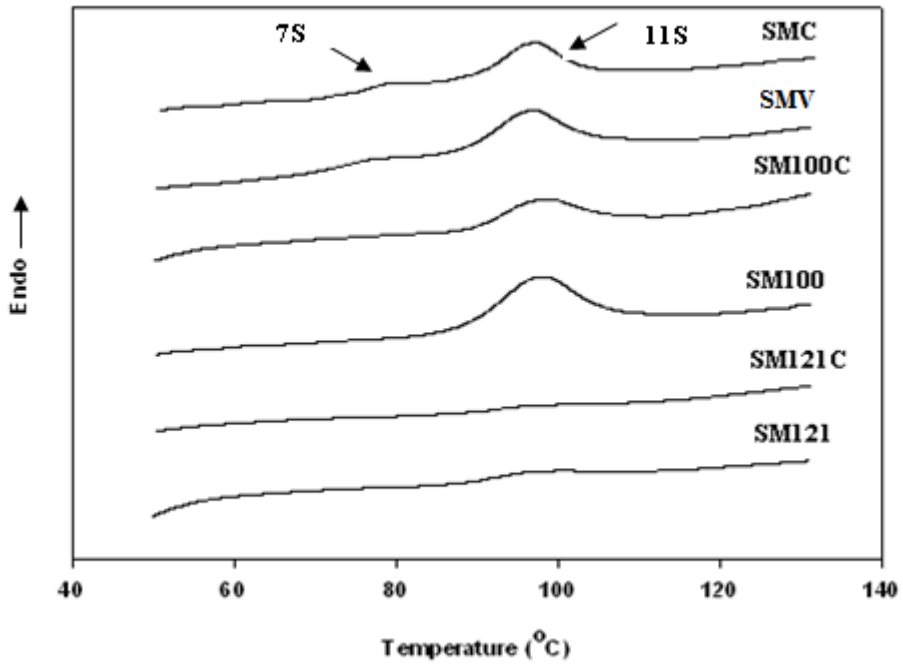


Figure 6.1 DSC thermogram of soy protein and fermented soy protein pretreated at different conditions

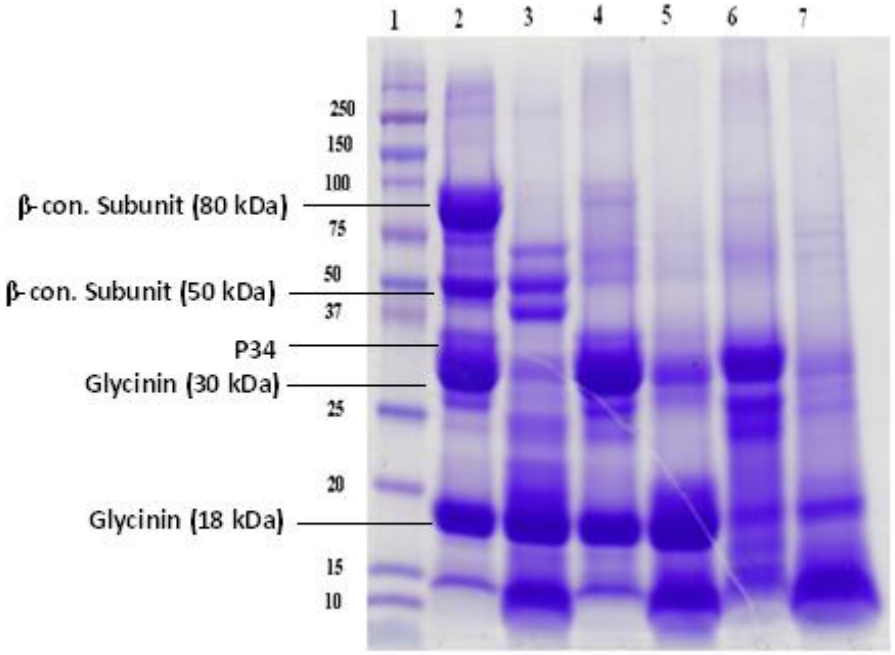


Figure 6.2 SDS-PAGE for soy protein and fermented soy protein pretreated at different conditions. Lane 1: prestained standard; lane 2: SM; lane 3: SMV; lane 4: SM100C; lane 5: SM100; lane 6: SM121C; lane 7: SM121

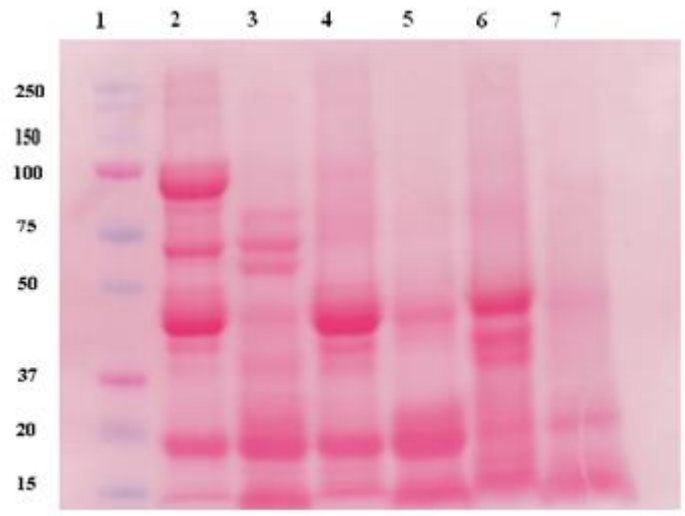


Figure 6.3 Pauceau S staining of the membrane after transferring. Lane 1: prestained standard; lane 2: SM; lane 3: SMV; lane 4: SM100C; lane 5: SM100; lane 6: SM121C; lane 7: SM121

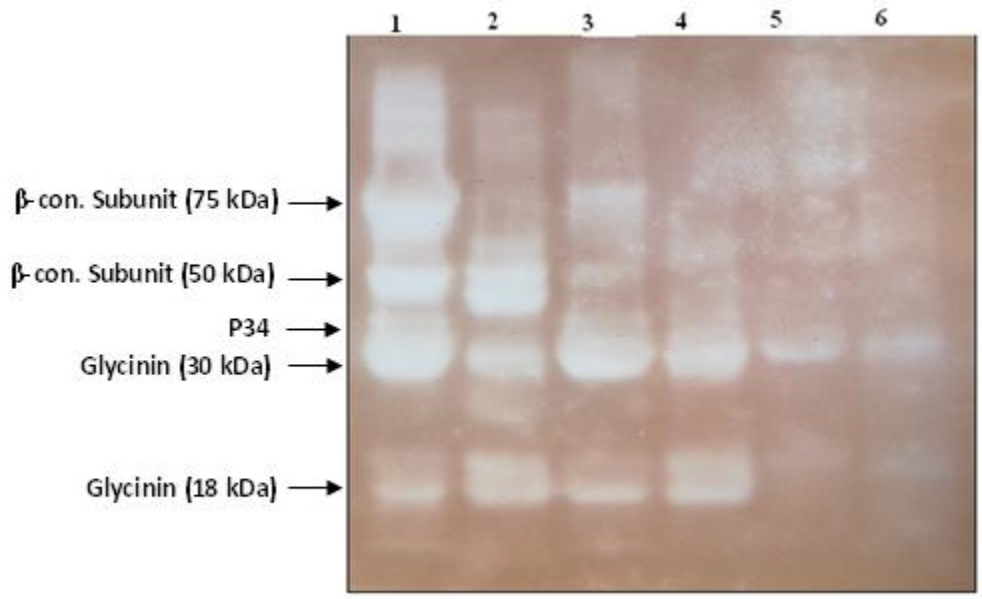


Figure 6.4 Western blot results of soy protein samples. Lane 1: SM; lane 2: SMV; lane 3: SM100C; lane 4: SM100; lane 5: SM121C; lane 6: SM121

Table 6.1 Proximates of soy meal samples

Samples	SM	SMV	SM100C	SM100	SM121C	SM121
Crude Protein	50.46[b]	58.78[a]	50.74[b]	58.82[a]	50.62[b]	58.47[a]
Crude Fat	2.93[a]	3.15[a]	2.06[b]	3.07[a]	2.24[b]	2.94[a]
Crude Fiber	3.32[b]	4.14[c]	3.11[c]	4.37[b]	3.02[c]	5[a]
Ash	6.68[d]	8.05[b]	6.93[c]	8.24[a]	6.66[d]	8.31[a]
ADF	5.48[c]	7.7[a]	4.74[c]	7.52[a]	4.67[c]	7.11[b]
NDF	8.18[b]	10.13[b]	12.38[b]	11.41[b]	18.15[a]	12.3[b]
Cellulose	5.41[c]	7.51[a]	4.75[d]	7.42[a]	4.62[d]	6.99[b]
Pepsin Digestibility	95.33[a]	93.07[c]	95.18[a]	94.1[b]	94.84[a]	93.33[c]
Available lysine	3.06[b]	3.07[b]	2.95[c]	3.08[b]	2.84[d]	3.26[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009)

Table 6.2 Amino acids changes of soy protein with different treatment

Sample	SM	SMV	SM100C	SM100	SM121C	SM121
Hydroxyproline	0^{b}	0.11^{a}	0.1^{a}	0.11^{a}	0^{b}	0.12^{a}
Aspartic Acid	5.77^{b}	6.27^{a}	5.62^{c}	6.24^{a}	5.66^{bc}	6.3^{a}
Threonine	1.93^{b}	2.09^{a}	1.92^{b}	2.17^{a}	1.85^{b}	2.15^{a}
Serine	2.14^{a}	2.58^{a}	2.34^{a}	2.57^{a}	2.04^{a}	2.29^{a}
Glutamic Acid	8.85^{ab}	8.91^{ab}	8.42^{b}	9.07^{a}	8.66^{ab}	9.02^{a}
Proline	2.53^{bc}	3.04^{a}	2.45^{c}	2.62^{bc}	2.51^{bc}	2.67^{b}
Glycine	2.14^{b}	2.28^{a}	2.05^{b}	2.38^{a}	2.07^{b}	2.39^{a}
Alanine	2.19^{b}	2.41^{a}	2.13^{b}	2.39^{a}	2.13^{b}	2.45^{a}
Cysteine	0.7^{b}	0.8^{a}	0.66^{b}	0.82^{a}	0.67^{b}	0.82^{a}
Valine	2.52^{d}	2.96^{ab}	2.63^{c}	2.89^{b}	2.51^{d}	3.01^{a}
Methionine	0.72^{d}	0.75^{c}	0.68^{f}	0.76^{b}	0.7^{e}	0.78^{a}
Isoleucine	2.39^{dc}	2.52^{b}	2.32^{d}	2.47^{bc}	2.33^{d}	2.63^{a}
Leucine	3.92^{c}	4.43^{a}	3.95^{c}	4.24^{b}	3.84^{d}	4.24^{b}
Tyrosine	1.84^{b}	1.94^{a}	1.78^{b}	1.91^{a}	1.77^{b}	1.93^{a}
Phenylalanine	2.53^{bc}	2.75^{a}	2.48^{c}	2.58^{b}	2.48^{c}	2.59^{b}
Hydroxylysine	0.03^{a}	0.02^{ab}	0.01^{c}	0.02^{ab}	0.02^{ab}	0.02^{ab}
Ornithine	0.03^{b}	0.04^{b}	0.03^{b}	0.05^{b}	0.04^{b}	0.07^{a}
Lysine	3.24^{b}	3.22^{b}	3.12^{c}	3.29^{b}	3.1^{c}	3.51^{a}
Histidine	1.32^{b}	1.38^{a}	1.27^{c}	1.37^{a}	1.27^{c}	1.4^{a}
Arginine	3.64^{a}	3.48^{c}	3.56^{b}	3.48^{c}	3.52^{bc}	3.57^{b}
Tryptophan	0.74^{c}	0.81^{b}	0.79^{b}	0.89^{a}	0.71^{c}	0.92^{a}

Total	49.13[b]	52.73[a]	48.28[b]	52.27[a]	47.84[b]	52.83[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009)

Chapter 7 - Conclusion

Conclusions of current research

Aspergillus solid state fermentation of soy meal was effective in removing anti-nutritional factors and enhancing nutritional value of soy meal (SM) as feed ingredients for monogastric animals. *Aspergillus oryzae* (ATCC 12892), *Aspergillus oryzae* (ATCC 9362) and *Aspergillus ficuum* (ATCC 66876) were used to detoxify SM and promising results were obtained.

Aspergillus grew well in SM as the sole substrate. Anti-nutritional factors in SM include trypsin inhibitors, GOS, structure polysaccharides and large molecular protein. All these factors decreased significantly *via A. oryzae* fermentation. After fermentation of *A. oryzae* (ATCC 12892), GOS and trypsin inhibitors were totally degraded within 36 hr fermentation. Non-reducing polysaccharidies decreased by 59% (w/w) and the degree of hydrolysis of SM protein increased from 0.9% to 7% (w/w) throughout the 7 d fermentation. Nutritional factors, including protein, amino acid and fatty acid, were also modified. Protein content increased from 50.47% (w/w) to 58.93% (w/w) after 36 hr fermentation. Amino acid content was significantly enhanced.

Regarding the degradation of phytic acid in SM, two-stage temperature induced solid state fermentation by *A. oryzae* (ATCC 9362) worked well. In the first stage, a combination of moisture of 41%, temperature of 37 ^{o}C and inoculum size of 1.7 ml in 5 g substrate (dmb) obtained via Central Composite Design (CCD) experiment favored maximum phytase activity 58.7 U. The second stage fermentation used temperature 45 ^{o}C and 50 ^{o}C, which were the optimal temperature for phytase activity, and 46% increase of phytate degradation was obtained at 50 ^{o}C compared with the fermentation at 37 ^{o}C. Besides phytate degradation, oligosaccharides were removed, and non-reducing polysaccharides content decreased by 67 %, with concomitant, protein content increased by 9.2 %.

Two-stage temperature protocol for *A. ficuum* (ATCC 66876) solid state fermentation had higher efficiency in degrading phytate in SM. Comparing with the constant single temperature fermentation, the two-stage temperature protocol achieved a 98% increase in phytate degradation level. Besides phytate degradation, similar to the effect of *A. oryzae* fermentation, oligosaccharides were removed, and non-reducing polysaccharides content decreased by 87 %. In addition, the protein content increased by 6.7 %.

Considering the fact that *A. ficuum* achieved higher phytic acid degradation than *A. oryzae*; however, *A. ficuum* could not secret protease. Co-fermentation of *A. oryzae* (ATCC 9362) and *A. ficuum* (66876) were applied to simultaneously degrade phytic acid and large molecular protein. Co-fermentation of *A. oryzae* and *A. ficuum* had higher phytase activity and better phytase stability than *A. oryzae* fermentation, and it also had higher protease activity than *A. oryzae* fermentation. The co-fermentation realized 17% increase in phytic acid degradation compared with *A. oryzae* fermentation and 72% increase in DH comparing with *A. ficuum* fermentation.

Sterilization distorted the results of fermentation effect on soy allergens and soy protein degradation. Virginiamycin, a kind of bacterisin, was added to *A. oryzae* solid state fermentation, to exclude the necessity of SM sterilization. Nonsterile, solid state fermentation using *A. oryzae* and virginiamycin showed the complete degradation of α and α' subunits of β-conglycinin and decreased immunoreactivity of soy protein.

The innovative features of the research

(1) Two-stage temperature protocol was developed to fungal solid state fermentation to achieve efficient enzymatic hydrolysis.

(2) Co-fermentation of *A. oryzae* and *A. ficuum* to achieve high efficiency of simultaneous degradation of phytic acid and protein in SM was developed.

(3) Virginiamycin addition to unsterilized substrate to inhibit the contaminating bacterial contamination during fungi solid state fermentation was found very effective, thereby enabling the investigation of heat-sensitive substrate component change during fermentation.

Future research

(1) Fermentation modeling is required to better understand the large scale process. In order to do this analysis, Aspergillus growth must be analyzed.

(2) Large scale production of fermented soy meal is required for feed trials. Heat dissipation and moisture control for large scale fermentation need to be investigated.

(3) Economic analysis of the cost for the large scale process is required.

(4) Animal feed trial, for newly weaned piglets and broilers and also for normal monogastric animals, is required to check the benefits of the enhanced nutritional value and to enhance the utilization of soy meal.

For animal test, the presence of mycotoxin needs to be investigated. *A. oryzae* is generally regarded as safe (GRAS) (Vishwanatha et al 2010). And none of the strains of *A. oryzae* for koji making produced aflatoxins (Hesseltine et al. 1966). But Blumenthal (2004) reviewed several mycotoxins from *A. oryzae* and summarized that mycotoxins production was related to specific strain, fermentation time and physical and chemical environment. By designing and controlling the fermentation conditions to favor the selective production of the product of interest, the presence of mycotoxins could be eliminated. There is no research about mycotoxins of *A. ficuum*. So for animal test, whether mycotoxin is a concern, needs to be evaluated.

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