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# Using soybean and corn processing co-products in different fermentation systems

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**Using soybean and corn processing co-products in different fermentation systems**

by

**Jun Yi Lio**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
**MASTER OF SCIENCE**

Major: Food Science and Technology

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2012

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**LIST OF ABBREVIATIONS**

ADF	Acid detergent fiber
ADL	Acid detergent lignin
AEP	Aqueous extraction processing
ARA	Arachidonic acid
CBH	Cellobiohydrolases
CCUR	Center for Crops Utilization Research
DDGS	Distiller's dried grains with solubles
DG	Distillers grains
DNS	3,5-dinitrosalicylic acid
EAEP	Enzyme-assisted aqueous extraction processing
EG	Endoglucanases
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FDA	U.S. Food and Drug Administration
FSO	Flaxseed oil
GRAS	Generally recognized as safe
NDF	Neutral detergent fiber
PUFA	Polyunsaturated fatty acid
RFA	Renewable Fuels Association
SBO	Soybean oil
SmF	Submerged fermentation
SSF	Solid-state fermentation
$\beta$ G	$\beta$ -glucosidase

## CHAPTER 1. GENERAL INTRODUCTION

### LITERATURE REVIEW

#### Enzyme-assisted aqueous extraction processing of soybeans

Soybean is known as the most widely produced oilseed around the world. In 2010, soybeans represented 58% of world oilseed production, with 35% of the soybeans was produced in United States (Soy Stats, 2011). In United States, 68% of the edible consumption of fats and oil is from soybeans (Soy Stats, 2011). The processing of soybeans commonly uses hexane as the organic solvent to extract oil from soybean seeds. However, the use of hexane has drawn negative attention as it poses environmental and safety hazard. Therefore, alternatives to solvent extraction have been sought.

Aqueous extraction processing (AEP) is an environmental friendly technology in which the oil extraction is based on the insolubility of oil in water rather than on the dissolution of oil in organic solvent (Johnson and Lucas, 1983). AEP extracts oil and protein simultaneously and appears to be a good alternative to solvent extraction. However, the low yield of oil, difficulties in demulsifying the emulsions, and the lack of resulting aqueous effluent (skim) usage challenged the use of AEP (Rosenthal et al., 1996).

Enzyme treatment was later shown as a great way to increase the oil yield in AEP of soybeans. Rosenthal et al. (1996) reported that carbohydrases (pectinase, cellulase, and hemicellulase) are specific in hydrolyzing cell wall and allow more oil to release into the aqueous medium. On the other hand, Lamsal et al. (2006) suggested flaking and extruding as forms of mechanical pre-treatment of oil-bearing materials could help to distort the cell wall

in order to achieve higher oil recovery from AEP and enzyme-assisted aqueous extraction processing (EAEP) of soybeans. Protease was further studied on the effect of demulsifying the cream fraction and was found to help in recovering more oil (de Moura et al., 2008).

EAEP of extruded soybean flakes resulted in three distinct fractions: insolubles fraction that rich in fiber, liquid fraction (skim) that rich in protein, and the fraction with cream and free oil (de Moura et al., 2008).

In order to reduce the amount of water used in EAEP at the same time improving oil, protein, and solids extraction yields, two-stage countercurrent EAEP was developed (de Moura and Johnson, 2009). In brief, the insoluble fraction from the first AEP extraction was subjected to the first EAEP extraction and the skim from the first EAEP extraction was recycled to the second EAEP extraction to enable the reuse of enzyme. The laboratory scale of two-stage countercurrent EAEP was later moved to the pilot-plant scale to examine the feasibility of large scale soybeans EAEP using the similar equipment that industry would use (de Moura et al., 2011). Fig.1 shows the process flow diagram for the pilot-plant scale of integrated two-stage countercurrent EAEP of soybeans.

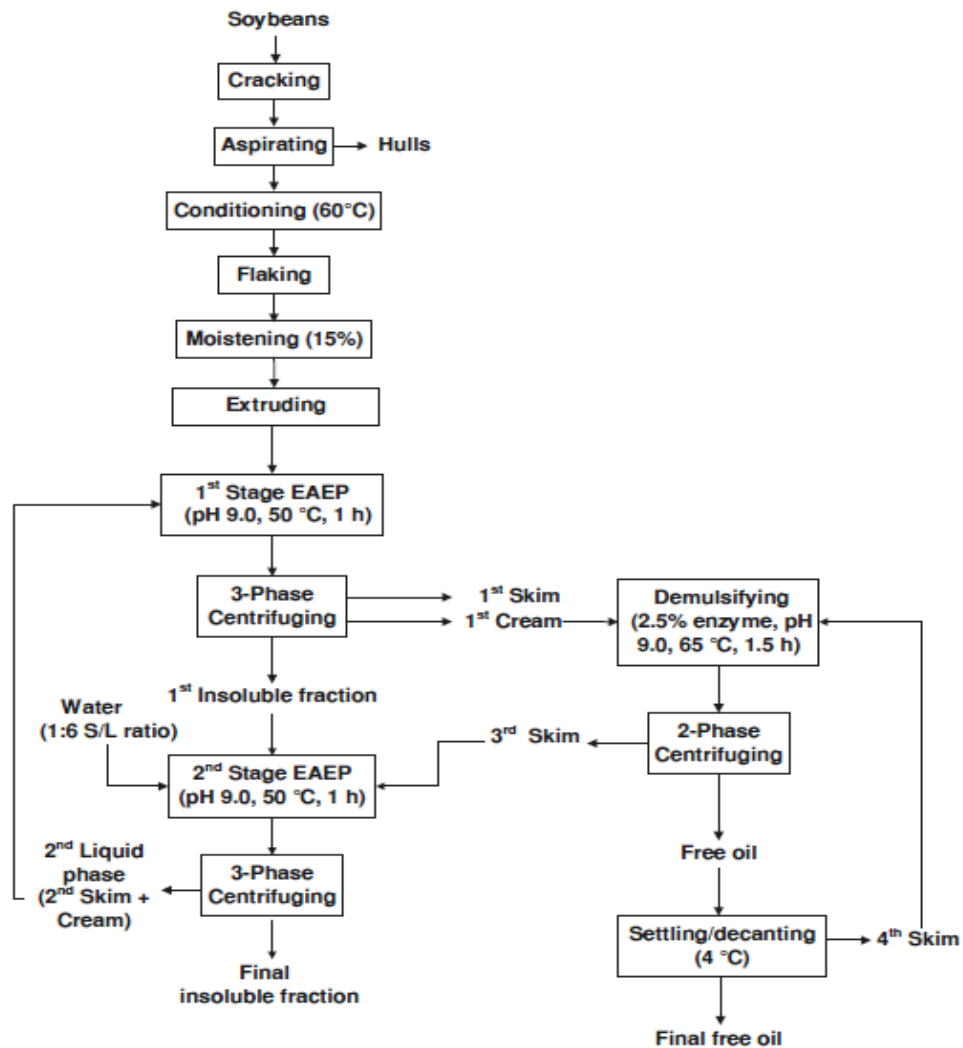


Fig.1. Process flow diagram for integrated two-stage countercurrent EAEP of soybeans.

(adapted from de Moura et al., 2011)

According to de Moura et al. (2011), for every liter of oil extracted from soybeans, 28.7 L of soy skim and 4.4 kg of insolubles were produced. Thus, the economic feasibility of the EAEP of soybeans depends on maximizing the values of all the three fractions. Since the cream fraction could be enzymatic demulsified to free oil, the other two fractions, protein-

rich skim and fiber-rich insolubles, need to be explored in order to facilitate the industrial adoption of soybean EAEP.

Pictures and proximate composition of the insoluble fiber and skim fractions (Yao et al., 2012) from EAEP are presented in Fig. 2 and Table 1. The insoluble fraction is rich in fiber and it was shown to be a good substrate for solid-state fermentation (SSF) by lignocellulose-degrading fungi to break down its fiber fraction (Yang et al., 2012). In addition, the protein-rich skim fraction was successfully used as an excellent nutrient source for corn ethanol fermentation by Yao et al. (2011). These two co-products can be used as suitable substrates by different fermentation systems and their values can be further maximized through more studies.



Fig. 2. Insoluble fiber (left) and skim (right) fractions from EAEP of soybeans.

Table 1. Proximate composition of two co-products from EAEP of soybeans.

	Solid content (%)	Composition (% dwb)			
		Oil	Protein	Ash	Carbohydrate
Insoluble fiber	15.0	3.3	6.4	4.3	86.0
Skim	11.0	9.0	56.0	13.0	22.0



### **Solid-state fermentation**

Solid-state fermentation (SSF) refers to the fermentation process in which microorganisms are grown on solid substrates without the presence of free liquid (Lonsane et al., 1985). It has been gaining attention in industry due to the low waste water production and operating expenses, simpler fermentation media requirement, superior productivity, and easier prevention of bacterial contamination (Deschamps et al., 1985; Chahal, 1991; Hema et al., 2006) compared to the submerged fermentation (SmF), in which the nutrients and microorganisms are present in a large amount of water. Another attractive advantage of SSF is the utilization of lignocellulosic residues as substrates to relieve the wastes disposal problem, as well as to enhance their values for other applications.

As SSF occurs when microorganisms grow on solid materials without the presence of free water, it can only be carried out by limited number of microorganisms. Among all, fungi are shown to be well adapted to SSF as their hyphae can grow on substrate surfaces and penetrate into the inter-particle spaces, thereby colonizing solid substrates (Santos et al., 2004). Lignocellulose-degrading fungi such as *Aspergillus* spp., *Trichoderma* spp., and white-rot fungi are commonly used to decompose the lignocelluloses in SSF (Pandey et al., 1999; Tengerdy and Szakacs, 2003) by producing enzymes such as cellulases, hemicellulases, and ligninases.

Optimization of SSF conditions is critical in supporting the growth of microorganisms and maximizing the production yield. Key parameters for optimization of SSF include the carbon and nitrogen sources, compatibility of strains and substrates, initial pH of the growth medium, incubation temperature and period, aeration, mixing, moisture

content, and water activity in the substrate (Pandey, 2003; Bhargav et al., 2008). Each of the parameters plays an important role in developing a complete system for SSF.

Although SSF has shown a great potential in producing valuable products, some difficulties including the lack of homogeneity in substrate, difficulty in scale-up, and requirement for controlling process variables are yet to be solved (Bhargav et al., 2008). SSF can have a crucial role in future biotechnologies if these drawbacks are given enough attention to overcome.

### **Mixed culture SSF**

Co-culture with two fungi has been reported to enhance enzyme production in SSF, especially when lignocellulosic residues are used as the substrates. The complexity of these substrates often requires the action of multiple enzymes and the interaction among different microbes to accomplish the biodegradation (Muhannad et al., 2001). *Trichoderma reesei*, for instance, is known as an efficient cellulose producer that secretes high amount of endoglucanases and cellobiohydrolases. Nevertheless, its low amount of secretion of  $\beta$ -glucosidase (Ryu and Mandels, 1980) may be supplemented by  $\beta$ -glucosidase from another microbial source such as *Aspergillus spp* (Grajek, 1987) to facilitate the bioconversion of lignocelluloses.

By using sugarcane baggase as substrate for SSF, higher cellulase activity was shown in mixed culture of *T. reesei* and *Aspergillus spp*, including *A. niger*, *A. terreus*, and *A. phoenicis* in different studies (Gutierrez-Correa et al., 1999; Muhannad et al., 2001; Gutierrez-Correa et al., 1997). Brijwani et al. (2010) also found that total cellulase,  $\beta$ -glucosidase, and endocellulase increased considerably when *T. reesei* and *A. oryzae* were

employed in a static tray bioreactor under SSF by using a soybean hull substrate supplemented with wheat bran. Enzyme productions of both cellulase and xylanase were enhanced considerably when *T. reesei* and *A. niger* were co-cultured in SSF of water hyacinth (Deshpande et al., 2008).

In addition, mixed culture of *T. reesei* and *P. chrysosporium* was also shown to facilitate the degradation of lignocellulosic residues through secretion of different enzymes. Kumar and Shweta (2011) reported the combination of *T. reesei* and *P. chrysosporium* to be the best in lignocellulosic decomposition of timber waste compared to other fungi mixtures. Such combination was also used in other studies in composting different solid wastes (Haddadin et al., 2009; Raut et al., 2008).

## **Substrates for SSF and SmF used in this research**

### **1. Soybean cotyledon fiber**

Soybean is a dicot plant, with seeds consisting primarily cotyledon cells and surrounded by a seed coat and pericarp layer (Campbell et al., 2011). Since most of the soybean protein and oil are stored in the cotyledon cells, the fiber-rich insoluble fraction after AEP or EAEP of soybeans is referred as soybean cotyledon fiber. Karki et al. (2011) demonstrated the potential use of soybean cotyledon fiber as lignocellulosic feedstock for ethanol production because of the high carbohydrate content. By enzyme hydrolysis, soybean cotyledon fiber could produce fermentable sugars that can be used in bio-ethanol production or as specialty food and feed ingredients. Pretreatments of soybean cotyledon fiber with ammonium hydroxide, sodium hydroxide, and sulfuric acid were also studied and shown to enhance the glucose production after enzyme hydrolysis (Karki et al., 2011). Although the

soybean cotyledon fiber recovered from EAEP was suggested to be used as animal feed (de Moura et al., 2009), the high fiber content limits its use as non-ruminant feed. Non-ruminants lack the ability to breakdown the fiber due to the absence of a special stomach as in ruminants. Yang et al. (2012) showed potential of using soybean cotyledon fiber as a substrate for SSF for improved digestibility. SSF of soybean cotyledon fiber by using different fungi promoted the enzyme production, resulted in decreased neutral detergent fiber (NDF) and acid detergent fiber (ADF). SSF thus showed a great potential for improving the digestibility of soybean cotyledon fiber as non-ruminant feed and may enhance the value of this fiber-rich fraction recovered from EAEP of soybeans.

## **2. Soy skim**

Soy skim is a liquid fraction from soybean EAEP and contains approximate 11% of dry matter, of which 56% (dwb) is partially hydrolyzed protein and 9% (dwb) is oil (Yao et al., 2012). Soy skim was shown to increase the ethanol production rate when it was used as water replacement in dry-grind corn fermentation (Yao et al., 2011). The final distiller's dried grains with soluble (DDGS) product from such fermentation also resulted in higher protein content, which may justify its use as animal feed. The promoting effect of soy skim on the fermentation rate was again confirmed by Yao et al. (2012) based on the evaluation of factors such as water-to-solids ratio, corn particle size, addition of urea, and type of skim on the corn-soy co-fermentation performance. Others have used thin stillage derived from corn ethanol fermentation as medium for fungal growth (Liang et al., 2012; Mitra et al., 2012) but there has been no report on suitability of using soy skim to produce fungal biomass. Fungal fermentation by using soy skim medium is yet to be explored.

## **3. Distiller's dried grains with soluble**

According to Renewable Fuels Association (RFA, Ethanol Industry Outlook, 2012), United States fuel ethanol production from grains increased from 1.63 billion gallons in 2000 to 13.2 billion gallons in 2010, with 2011 marking another production record for U.S. ethanol with an estimated 13.9 billion gallons. Being an alternate supply of petroleum fuel, this phenomenal grow in fuel ethanol production from grains is mainly driven by the increasing demand of transportation fuels.

Wet milling and dry-grind are the two major ways used in industry to produce ethanol, with over 80% is processed with the dry-grind method (RFA, 2012). The dry-grind processing starts with a whole corn kernel and the schematic diagram of a conventional dry-grind ethanol production is shown in Fig. 3 (Han and Liu, 2010). Approximately two-thirds of the corn is consisted of starch, which is converted to ethanol and carbon dioxide during the fermentation and thus, the concentration of unfermentable materials such as protein, oil, minerals, and vitamins are increased in the residue about three times. These concentrated residues become a co-product, which is known as distiller's dried grains with soluble (DDGS) and is a major type of distillers grains (DG). It is reported that the 13.9 billion gallons of ethanol produced in 2011 yielded a totally of 35.7 million metric tons of distillers grain, with 48% of it consumed by beef, 32% by other dairy, 11% by swine, and 8% for poultry (RFA, Ethanol Industry Outlook, 2012).

Although DDGS has been used as feed ingredient, its high compositional variation can reduce profitability in livestock operations and challenge the inclusion level of DDGS in animal feed. In a review by Liu (2011), the updated information on the chemical composition of DDGS, changes throughout the dry-grind processing, and causes for large variation such

as raw materials, effect of fermentation yeast, difference in process methods, and inconsistent analytical methods were provided.

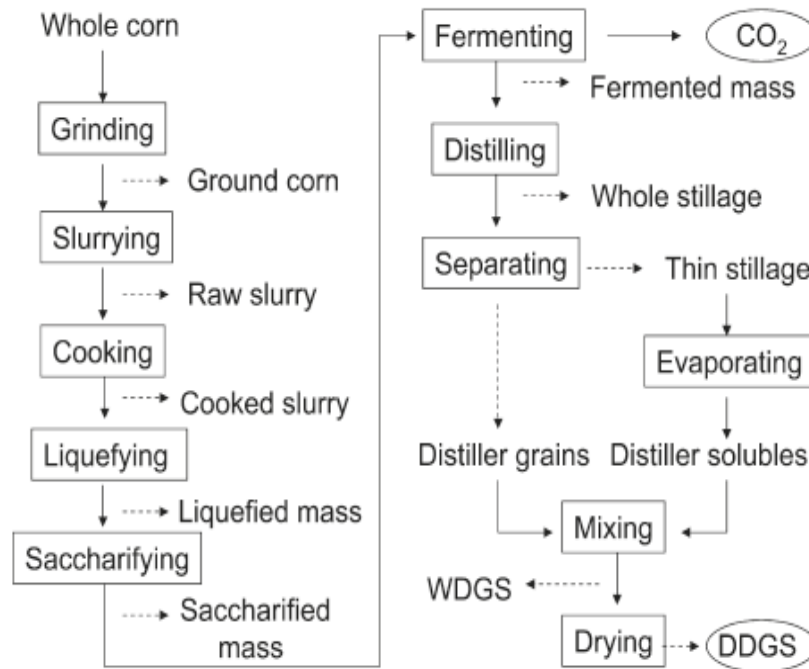


Fig. 3. Schematic diagram of a conventional dry-grind ethanol production. (adapted from Han and Liu, 2010)

Based on the concentrated amounts of fiber and protein, SSF of DDGS by two fungi, *Aspergillus oryzae* and *Rhizopus oligosporus*, was studied (Hoskins and Lyons, 2009). The fermented DDGS was used as an enzyme complex supplement in corn mash fermentation and ethanol yield was improved considerably. In addition, DDGS was proven to be a suitable substrate for SSF with only moderate changes found in its nutritional profile after SSF (Hoskins and Lyons, 2009). Yang et al. (2012) also demonstrated that with some

supplementation, DDGS could be used as a substrate for SSF to reduce its cellulose and hemicellulose content, showing a potential for its use as non-ruminant feed.

### Cellulose and its degrading enzyme

Cellulose is a linear polysaccharide which composed of D-glucose subunits linked by  $\beta$ -1,4 glycosidic bonds. As shown in Fig. 4, the dimer cellobiose is the basic repeating unit of cellulose. The cellulose chain ends are different with non-reducing end having closed ring structure and reducing end with aliphatic structure and a carbonyl group in equilibrium with cyclic hemiacetals (Kontturi et al., 2006).

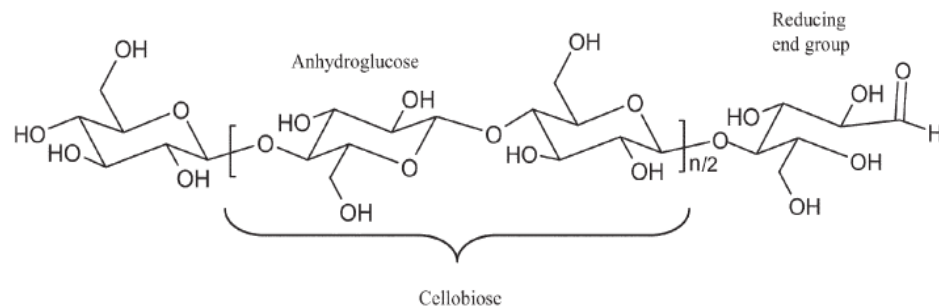


Fig. 4. Molecular structure of cellulose. (adapted from Kontturi et al., 2006)

Cellulolytic microorganisms are mainly found in eubacteria and fungi, although some protozoa are capable of degrading cellulose. These microorganisms are able to interact synergistically with non-cellulolytic species in mixed populations to degrade cellulose. The complete degradation of cellulose will convert the substrate into carbon dioxide and water under aerobic conditions (Béguin and Aubert, 1994).

Cellulases hydrolyze  $\beta$ -1,4-D-glucan linkages in cellulose and produce cello-oligosaccharides, cellobiose, and glucose as products. A cellulase complex contains three major components that work in concert, including endoglucanases (endo-1,4- $\beta$ -glucanase, EGs), cellobiohydrolases, (exo-1,4- $\beta$ -glucanase, CBHs) and  $\beta$ -glucosidase ( $\beta$ G). EGs are capable of hydrolyzing amorphous regions of cellulose fibers and releasing new terminal ends. CBHs cleave the existing or terminal ends generated by EGs, with CBH I acting on the reducing ends and CBH II acting on the non-reducing ends, liberating cello-oligosaccharides and cellobiose units. Both EGs and CBHs can degrade cellulose in amorphous regions, but with some exceptions, CBHs are the only enzymes that degrade crystalline cellulose. Cellobiose is broken down by  $\beta$ G, releasing two glucose molecules (Beguin and Aubert, 1994; Pérez et al., 2002).

As indicated in Fig. 5, all these enzymes act cooperatively to hydrolyze cellulose to glucose, which serve as carbon and energy sources for microorganisms in the system where cellulose is degraded.

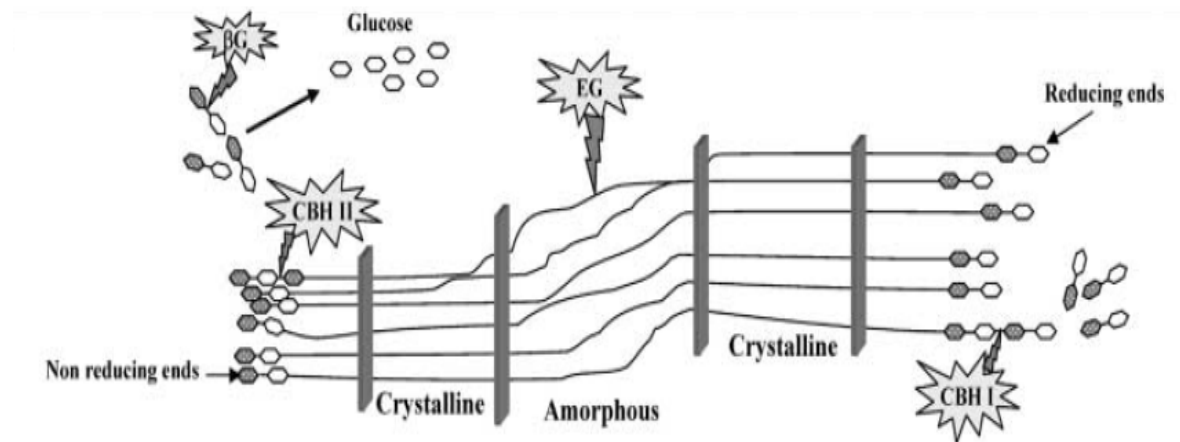


Fig. 5. Schematic diagram of enzyme hydrolysis of cellulose to glucose. EG: endoglucanases; CBH: cellobiohydrolases;  $\beta$ G:  $\beta$ -glucosidase. (adapted from Pérez et al., 2002)



## Hemicelluloses and its degrading enzyme

Hemicelluloses are heterogenous polymers consist of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (Zhang et al., 2011). Xylan, the most abundant component in hemicelluloses, is a complex polysaccharide consisting of a linear  $\beta$ -1,4 linked xylopyranose backbone with acetyl, glucuronosyl, and arabinosyl side chains (Fukuda et al., 2009).

Many species of bacteria and fungi, as well as several plants are capable of producing hemicellulases. Xylan, as the main component in hemicelluloses, requires a cooperative action of several enzymes in order to reach a complete degradation. It is hydrolyzed by endo-1,4- $\beta$ -xylanase to xylooligosaccharides, following by  $\beta$ -xylosidase to produce xylose from non-reducing ends. In addition, some accessory enzymes such as acetylxylan esterase,  $\alpha$ -glucuronidase, and  $\alpha$ -arabinose are required to degrade glucuronoxylans (Pérez et al., 2002; Fukuda et al., 2009). Fig. 6 shows a schematic diagram of enzymatic degradation of glucuronoxylans (Pérez et al., 2002).

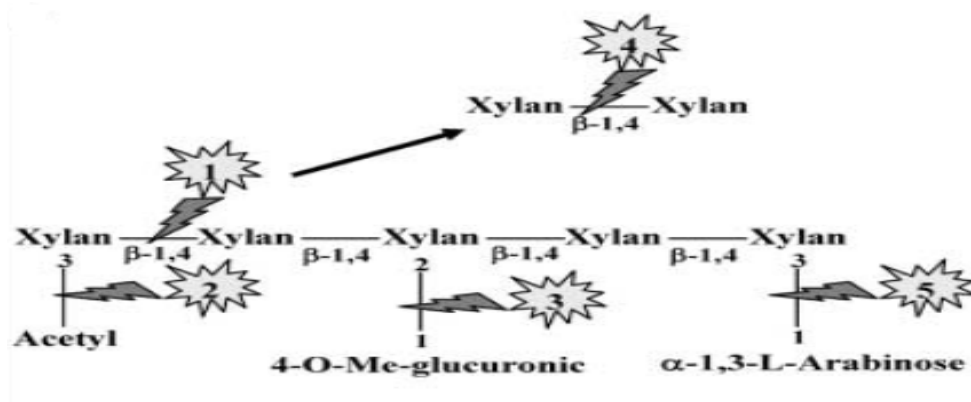


Fig. 6. Schematic diagram of enzyme hydrolysis of glucuronoxylans. 1: endo-1,4- $\beta$ -xylanase; 2: acetylxylan esterase ; 3:  $\alpha$ -glucuronidase; 4:  $\beta$ -xylosidase; 5:  $\alpha$ -arabinose. (adapted from Pérez et al., 2002)

## Enzyme producing fungi

### 1. *Aspergillus oryzae*

*Aspergillus oryzae* is a filamentous fungus that has been used in the traditional food fermentation in Japan for more than 1000 years for the production of soybean paste, soy sauce, and rice wine (Bennett, 2001). Its extensive uses in food fermentation industries have prompted it to be listed as the Generally Recognized as Safe (GRAS) strain by the U.S. Food and Drug Administration (FDA) (Tailor and Richardson, 1979). Based on its ability to secrete a wide range of different enzymes, *A. oryzae* has been used for commercial enzyme production.

Researches focusing on both cellulase and xylanase secreted by *A. oryzae* using agricultural by-products have been conducted intensively based on its good fermentation capability, particularly in producing wide range of enzymes for degradation of plant cell wall polysaccharides (de Vries and Visser, 2001). For instance, SmF of cheese whey (Youssef, 2011) and spent sulphite liquor (Chipeta et al., 2008) can induce the production of xylanase,  $\beta$ -xylosidase, endoglucanase, and exoglucanase.

In addition to SmF, SSF by *A. oryzae* has also been intensively studied. Szendefy et al. (2006) utilized eucalyptus and bagasse pulps as carbon feedstock for *A. oryzae* growth in SSF and reported that the xylanase produced could be used more efficiently in biobleaching of paper pulp compared to using commercial enzyme. It was also shown that *A. oryzae* produced at least three kinds of cellulases and two kinds of xylanases when using wheat bran as a substrate in SSF and SmF, with higher enzyme yield achieved in solid-state culture compared to submerged culture (Yamane et al., 2002).

## 2. *Trichoderma reesei*

*Trichoderma reesei* is a mesophilic fungus that is commonly used as a source of cellulases and hemicellulases in industry to hydrolyze plant cell wall polysaccharides (Martinez et al., 2008). It is recognized as GRAS strain by FDA and has been shown to produce considerable amount of endoglucanases, cellobiohydrolase,  $\beta$ -glucosidase, xylanase, and  $\beta$ -xylosidase (Dashtban et al., 2009). Based on its ability to secrete large amounts of cellulose-degrading enzymes, *T. reesei* has been subjected to extensive mutagenesis studies. For instance, *T. reesei* QM 9414 and RUT-30 are the common mutant strains that can produce more cellulase than the wild-type strain (QM 6a) (Dashtban et al., 2009).

The use of agricultural co-products as substrates has drawn interest in industry to reduce the cellulase production cost as commercial cellulose sources are expensive. SSF has been reported as an attractive process to produce enzymes due to its low waste water production and operating expenses (Deschamps et al., 1985). In addition, studies have been carried out to compare cellulase production between SSF and SmF. Tengerdy (1996) reported that production cost in crude fermentation by SmF was higher compared to SSF. Chahal (1985) also showed a higher yield of cellulases from *T. reesei* by SSF compared to SmF.

As a result, extensive researches have been conducted on using different agricultural residues such as rice bran (Latifan et al., 2007), wheat bran (Cen and Xia, 1999), sugarcane bagasse and rice straw (Sukumaran et al., 2009), wood chips (Xin and Geng, 2010), and kinnow pulp (Oberoi et al., 2010) for SSF by *T. reesei* to produce enzyme, in particular cellulases.

## 3. *Phanerochaete chrysosporium*

*Phanerochaete chrysosporium* is the most intensively studied white rot fungus that possesses the unique ability to degrade lignin, which is the most recalcitrant component of plant cell walls, to gain access to cellulose and hemicelluloses. It has been reported as a GRAS grade fungus (Ajila et al., 2011) and can simultaneously degrade cellulose, hemicelluloses, and lignin (Larrondo et al., 2005).

*P. chrysosporium* has been commonly employed in fermentation as it is also able to secrete multiple cellulases and hemicellulases such as endoglucanases, cellobiohydrolase,  $\beta$ -glucosidase, xylanase, and  $\beta$ -xylosidase (Wymelenberg et al., 2005; Pérez et al., 2002). Elshafei (1990) reported that corn stover can be utilized as substrate by this fungus for a range of cellulases and hemicellulases production by SmF. Szakacs-Dobozi et al. (1992) showed that corn stalk was a great medium in secreting xylanase by SmF using *P. chrysosporium*.

Various benefits from SSF have also drawn the interest in employing *P. chrysosporium* in SSF using agro-industrial residues for enzyme production. For instance, SSF of cotton seed coat fragments by *P. chrysosporium* was shown to secrete several enzymes that efficiently bleached the linen fabric (Csiszar et al., 2008). In addition, high endoglucanase and filter paper activities were shown from fermented sunflower and corn residues by *P. chrysosporium* SSF (Safari Sinigani et al., 2009).

Recently, some studies have been conducted using different agricultural by-products such as cotton stalks (Shi et al., 2009), corn fiber (Shrestha et al., 2008), and corn stover (Vincent et al., 2011) in *P. chrysosporium* SSF as a pretreatment for ethanol production. Enzymes produced from *P. chrysosporium* SSF assisted the releasing of reducing sugars and showed to promote the ethanol production.

### **Enzyme supplementation to animal feed**

Enzyme production by SSF has been well documented. These enzymes could be either extracted from the fermented substrates or be used together with the substrates after SSF for improving animal digestibility purposes. Regalado et al. (2011) reported that enzymes extracted from corn stover after SSF increased the *in vitro* cow's ruminal and true digestibility. Solid-state fermented sorgum stover (Akinfemi, et al., 2010), wheat straw (Shrivastava et al., 2011), and paddy straw (Sharma and Arora, 2011) by different enzyme producing fungi were also reported to enhance the *in vitro* digestibility of ruminants. In addition, various studies on the effect of enzyme supplementation to animal feed on animal performances have also been reported. For instance, improvements in average daily gain, feed conversion ratio, body weight, and apparent crude protein digestibility were found when enzyme extracts obtained from *A. niger* SSF was supplemented to the wheat-based diets for broilers (Wang et al., 2005). Meanwhile, *A. oryzae* fermented soybean meal as compared to the unfermented soybean meal was shown to be beneficial to the growth performance of broilers (Feng et al., 2007) and piglets (Feng et al., 2007) by increasing the digestibility of dietary components, average daily gain, and feed intake. On the other hand, no adverse effects were detected on broilers' growth performance, apparent nutrient digestibility, and serum biochemical constituents when maize-based diets were replaced by up to 40% of the *A. niger* fermented fruit meal (from the tropical *Terminalia catappa* tree) (Apatha, 2011). SSF of agro-industrial residues has shown promising results in enzyme secretion, as well as feed improvement for the animal production industry.

### **Polyunsaturated fatty acid production from oleaginous microorganisms**

The use of long chain omega-3 and omega-6 series of polyunsaturated fatty acids (PUFA) has been increased dramatically due to their tremendous potentials as food additives and pharmaceuticals for relieving heart and circulatory disorders and cancers, as well as inflammatory diseases (Jang and Yang, 2008). The addition of PUFA in feed also becomes popular in animal industry as improved feed conversion efficiency (Newman et al., 2002), increased body weight (Schreiner et al., 2005; López-Ferrer et al., 2001), and reduced inflammatory response (Korver and Klasing, 1997) were reported when n-3 PUFA was added to poultry diets. PUFA supplementation to diets in poultry could also contribute to PUFA enriched meat (Kehui et al., 2010; Geier et al., 2009) and egg yolks (Lewis et al., 2001; Schreiner, et al., 2004; Oliveira et al., 2010), adding the values for these products.

Arachidonic acid (ARA, C20:4, n-6) is an essential PUFA that acts as a precursor of important eicosanoids such as prostaglandins, thromboxanes, and leukotrienes (Gill and Valivety, 1997; Dong and Walker, 2008). Animal livers, egg yolks, and fish oil are the main sources of ARA but the relatively low concentration is prompting the industry to seek for other alternatives (Dong and Walker, 2008; Singh and Ward, 1997).

Eicosapentaenoic acid (EPA, C20:5, n-3) is an essential omega-3 fatty acid that has unique biological activities in the prevention and treatment of a number of human diseases and disorders (Cheng et al., 1999). Marine fish oil being a major source of EPA faces some challenges, such as the objectionable taste and odors, high cholesterol content, and heavy metal pollutants that are yet to be resolved. Thus, alternatives to fish oil are needed to supply the increasing demand for PUFA and avoid the current shortcomings.

A number of microorganisms possess the ability to accumulate lipids in their cells when cultivated under suitable conditions (Subramaniam et al., 2010). Microorganisms that could accumulate lipids at more than 20% of their biomass are classified as oleaginous species (Ratledge and Wynn, 2002). The oleaginous microbial species has gained attention in food and feed industry as great alternatives to plant and animal sources for long chain PUFA.

Several distinct pathways are involved in the biosynthesis of microbial PUFA, such as *de novo* synthesis of fatty acids from glucose, the incorporation of exogenous fatty acids into lipid structures, and PUFA formation through a series of desaturation and elongation (Certik and Shimizu, 1999). The biosynthetic pathways of PUFA in oleaginous species are illustrated in Fig. 7.

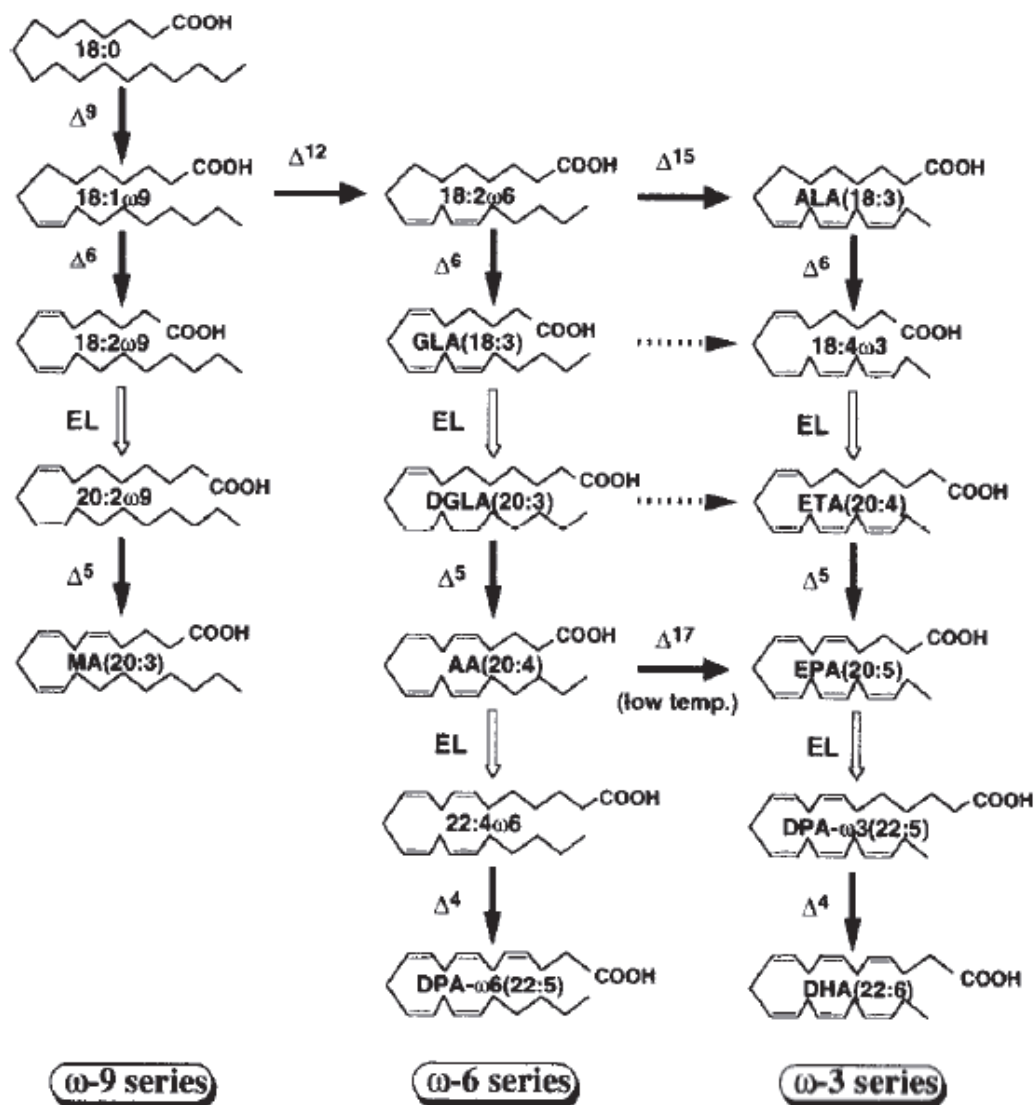


Fig. 7. Biosynthetic pathways of PUFA in oleaginous species. EL: elongase;  $\Delta^4$ ,  $\Delta^5$ ,  $\Delta^6$ ,  $\Delta^9$ ,  $\Delta^{12}$ ,  $\Delta^{15}$ ,  $\Delta^{17}$ : desaturase. (adapted from Certik and Shimizu, 1999)

### Oleaginous fungus - *Pythium irregulare*

*Pythium irregulare* is a filamentous fungus and was identified as a potential EPA producer through a specific screening of selected genera of fungi (Wessinger et al., 1990).

SmF has been commonly used as the means for *P. irregulare* to synthesize EPA. Stinson et



al. (1991) employed this fungus and studied the effect of cultural conditions on EPA production. It was shown that lowering the incubation temperature from 25° C to 12° C and addition of glucose during fermentation increased the EPA yield.

Several studies were carried out with different agricultural by-products as the media for *P. irregulare* to produce EPA. For example, sweet whey permeate from dairy industry was shown to be a great lactose source for the fungal growth and accumulation of EPA (O'Brien et al., 1993). Crude soybean oil, sucrose waste stream, and soymeal waste were used as oil and carbon sources for the production of EPA by *P. irregulare* (Cheng et al., 1999), and it was shown that soybean oil combined with glucose, with the presence of emulsifier, could be an excellent source for PUFA production.

Athalye et al. (2009) later reported that crude glycerol from biodiesel production could be used as carbon source in SmF by *P. irregulare* for EPA production with the removal of two impurities, soap and methanol, which inhibited the fungal growth. The study also showed that addition of flaxseed and soybean oil could increase the fungal biomass and total EPA yield. In addition, Liang et al. (2011 and 2012) demonstrated the feasibility of using rendered animal proteins and thin stillage, which were derived from meat processing and dry corn milling respectively, as feedstocks for *P. irregulare* in SmF. The small peptides and free amino acids produced from enzyme hydrolysis of rendered animal proteins were shown to be a great nitrogen source for the fungal growth. On the other hand, thin stillage could provide nutrients for *P. irregulare* in SmF. The fungal culturing resulted in a nutrient-depleted liquid after the fermentation.

Agricultural co-products have been utilized in different ways to maximize their values, as well as solving the waste disposable problems. SSF and SmF are two processing

technologies that can be employed to increase the values of these co-products. Soybean cotyledon fiber may contain usable or digestible proteins during EAEP processing that can support the growth of fungi. DDGS contains concentrated amounts of unfermentable materials from corn ethanol fermentation such as protein, fat, minerals, and vitamins that can be the great nutrient sources for the fungal growth. These two agro-industrial residues can be used as substrates in SSF. Their fiber contents could be potentially decreased by mixed culture SSF of various lignocellulose-degrading fungi to improve their values as animal feed. In addition, soy skim also contains a high amount of protein and peptides that may be utilized by oleaginous fungus in SmF to produce PUFA. Therefore, this study focuses on the utilization of these materials in both SSF and SmF by various fungi to enhance their values as feeds.

## **THESIS ORGANIZATION**

This thesis consists of a general introduction chapter, followed by two manuscripts of research papers and a general conclusions chapter. The papers are in the required formats of the corresponding journal.

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## CHAPTER 2. SOLID-STATE FERMENTATION OF SOYBEAN AND CORN PROCESSING CO-PRODUCTS FOR POTENTIAL FEED IMPROVEMENT

A modified manuscript submitted to Journal of Agricultural and Food Chemistry

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### ABSTRACT

Two agro-industrial co-products, soybean cotyledon fiber and distiller's dried grains with solubles (DDGS), were used as the substrates to evaluate the effect of co-culturing three different fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium*, on enzyme production by solid-state fermentation (SSF). When soybean fiber was used as the substrate, maximum xylanase activity of 757.4 IU/ g and cellulase activity of 3.2 IU/ g were achieved with the inoculation and incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae* for additional 108 h. This inoculation scheme also resulted in the highest xylanase activity of 399.2 IU/g compared to other fungi combinations in the SSF of DDGS. A large scale SSF by this fungi combination produced fermented products that had xylanase and cellulase activity of 35.9-57.0 and 0.4-1.2 IU/g, respectively. These products also had 3.5-15.1% lower fiber and 1.3-4.2% higher protein contents, suggesting a potential feed quality improvement.

**KEYWORDS:** Cellulase, distiller's dried grains with solubles, solid-state fermentation, soybean cotyledon fiber, xylanase

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## INTRODUCTION

Solid-state fermentation (SSF) is a fermentation process in which microorganisms are grown on solid substrate without the presence of free liquid<sup>1</sup>. SSF has been studied intensively for the production of enzymes, antibiotics, surfactants, and other value-added products.<sup>2</sup> Compared to the submerged fermentation in which nutrients are present in dissolved form in a large amount of water, SSF shows a great commercial potential due to its lower waste water production and operating expenses, simpler fermentation media requirement, superior productivity, and easier prevention of bacterial contamination.<sup>3,4</sup>

Various agro-industrial residues, such as sugarcane bagasse, cereal straws, brewer's spent grain, and corn stover, have been used as substrates for SSF to maximize their utilizations and to address the waste disposal issues. The enzyme-assisted aqueous extraction processing (EAEP) of soybeans developed by Iowa State University's Center for Crops Utilization Research (CCUR) is an environmental friendly method for soybean oil extraction. EAEP produces soybean cotyledon fiber and soy skim as co-products.<sup>5</sup> The low-valued soybean cotyledon fiber has high fiber content and this limits its use for non-ruminant feed. Thus, SSF may have a great potential in producing enzymes to breakdown its fiber to improve its digestibility. In addition, conventional dry-grind corn ethanol fermentation produces significant amount of distiller's dried grains with solubles (DDGS) as a co-product. DDGS contains high amount of protein and fiber and is commonly used in ruminant feed. The soy liquid skim from EAEP contains partially hydrolyzed protein and it has been shown to be a good nutrient and water source for corn ethanol fermentation.<sup>6</sup> The research showed an increased ethanol production rate as well as a final DDGS product with higher protein contents. The DDGS produced with such skim incorporation is referred as soy-enhanced

DDGS. The use of DDGS and soy-enhanced DDGS as non-ruminant feeds should be facilitated if the fiber content could be reduced by the enzymes produced by fungi through SSF.

As SSF occurs when microorganisms grow on solid materials without the presence of free water, it can only be carried out by a limited number of microorganisms. Fungi are well adapted to SSF as their hyphae can grow on particle surfaces and penetrate into the inter particle spaces and thereby, colonizing solid substrate.<sup>7</sup> Three different fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium* were chosen for the SSF of soybean cotyledon fiber, DDGS, and soy-enhanced DDGS based on their capability in producing enzyme, mostly xylanase and cellulase. *Aspergillus oryzae* has been studied for its ability to produce different enzymes by SSF of agro-industrial residues. *T. reesei* has also been used widely for its cellulase production and is commonly used in SSF studies. White rot fungi such as *P. chrysosporium* is known to secrete cellulase and xylanase enzymes<sup>8,9</sup> and it is also capable of producing lignin-degrading enzyme.<sup>10</sup>

Co-culturing of *T. reesei* and different *Aspergillus* species in SSF has been shown to enhance xylanase<sup>11,12</sup> and cellulase production.<sup>13-16</sup> The action of multiple enzymes and the interaction among different fungi are believed to be necessary to decompose complex substrates.

SSF of soybean cotyledon and DDGS with the three individual fungi *A. oryzae*, *T. reesei*, and *P. chrysosporium* were previously investigated by Yang et al.<sup>17</sup> but the effect of co-culturing using various combination of the fungi was unknown. Thus, the objectives of this study were: (1) to investigate if there is any synergistic effect among the three fungi based on the xylanase and cellulase production; (2) to determine the best combination of

fungi for large scale SSF; and (3) to examine the compositional change in soybean cotyledon fiber, DDGS, and soy-enhanced DDGS after large scale SSF.

## MATERIALS AND METHODS

**Microorganisms, Medium, and Culture Preparation.** *Aspergillus oryzae* (ATCC 1003), *Trichoderma reesei* (ATCC 13631), and *Phanerochaete chrysosporium* (ATCC 24725) were provided by Professor Hans van Leeuwen of Department of Civil, Construction and Environmental Engineering, Iowa State University. All three microorganisms are generally recognized as safe<sup>18-20</sup> and suitable for animal feed applications. The strains were cultured on potato dextrose agar plates, and the plates were incubated at 30° C for 7 days until complete sporulation. The spores from the plates were suspended in 15% sterile glycerol in water. The suspensions were used as cultures and were kept in vials at -20 °C until use.

**Substrate Preparation and Chemicals.** Soybean cotyledon fiber (6.4% protein dwb) was produced in the pilot plant of CCUR, Iowa State University via two stage counter-current enzyme-assisted aqueous extraction processing (EAEP).<sup>5</sup> To obtain DDGS or soy-enhanced DDGS, the first batch of corn ethanol fermentation was performed in the Iowa State University BioCentury Research Farm according to the conventional method<sup>19</sup> with 100% water. A second batch of corn fermentation was performed by the similar condition except that 50% of the water was replaced with soy skim fraction that was obtained from EAEP. Following the corn ethanol fermentation, downstream concentration and drying in the pilot plant, DDGS (34.3% protein dwb) and soy-enhanced DDGS (44.5% protein dwb) were

produced. The schematic diagram of this integrated soybean-corn biorefinery system is shown in Figure 1. The initial enzyme activities and composition of all three substrates were measured as describe below.

All chemicals and medium ingredients were purchased from Fisher Scientific (Pittsburgh, PA), Sigma Chemicals (St. Louis, MO) or BD (Franklin Lakes, NJ). Soybean hulls were provided by MicroSoy® Corporation (Jefferson, IA).

**Solid-State Fermentation.** Two culture loops of *A. oryzae*, *T. reesei*, and *P. chrysosporium* were transferred to 250 mL Erlenmeyer flask containing 50 mL of yeast extract peptone dextrose medium made of 2% yeast extract, 1% peptone, and 2% dextrose. The flasks were shaken at 150 rpm in a MAXQ Mini 4450 orbital shaker (Thermo Scientific, Asheville, NC) at 30°C for 24 h for *A. oryzae* and *T. reesei*, and 48 h for *P. chrysosporium*. Inoculums of each fungus at 5% (v/w) was used to inoculate a 40 g (as-is) of substrates in mixed fungi culture, whereas a 10% (v/w) inoculums was used in the single fungus culture. Soybean fiber with original moisture content of 85% was adjusted to moisture content of 75% by using soybean hulls and the pH was adjusted to 5.0 as reported.<sup>17</sup> DDGS was hydrated to 85% of moisture and was adjusted to moisture content of 75% with soybean hulls, using the same amount as for soybean fiber. Soybean hulls improve the porosity of substrates for better fungal growth as demonstrated in our previous study.<sup>21</sup> The pH of DDGS was adjusted to 5.0 as well. SSF was done by incubating the substrates at 30° C for 6 days. No nutrients or minerals were supplemented to the substrates in this study. All the substrates were sterilized by holding them at 121 °C and 103 kPa in autoclave for 20 min. The number of replicate that was used on each treatment is shown in each section below. A water



reservoir was placed in the incubator to maintain the relative humidity thus moisture level of the substrate.

**Effect of Dual and Trio Fungi Combination SSF on Enzyme Activities of Soybean Cotyledon Fiber.** Fungi was inoculated in dual and trio combinations to investigate if there is synergistic effect among the fungi. Soybean fiber was inoculated with single fungus as a comparison. Xylanase and cellulase activities of the fermented soybean fiber were measured after 6 days of SSF at 30° C. All treatments were repeated two times.

*Effect of Different Inoculation Time of A. oryzae and T. reesei.* Due to the absence of synergistic effect among different fungi when inoculated at the same time, inoculation time of *A. oryzae* and *T. reesei* was adjusted to determine the effect of inoculation sequence on enzyme activities. *A. oryzae* was inoculated at time 0, followed by inoculation of *T. reesei* to the 36 h *A. oryzae* fermented soybean fiber, which was expressed as A 36h+T. Selection of 36 h as incubation time was based on previous studies.<sup>13,14</sup> Treatment with *T. reesei* inoculation and incubation for 36 h, followed by inoculation of *A. oryzae*, expressed as T 36h+A, was investigated as well. Treatments of single fungus inoculation with *A. oryzae* and *T. reesei*, co-culture inoculation with *A. oryzae* and *T. reesei* (A+T) at the same time were used as comparison. Xylanase and cellulase activities were measured. All treatments were repeated three times.

*Effect of Different Inoculation Time of P. chrysosporium and T. reesei.* The inoculation sequence of *T. reesei* and *P. chrysosporium* was tested with the same manner as the SSF with *A. oryzae* and *T. reesei*. All treatments were repeated three times.

*Effect of Different Inoculation Time of A. oryzae and P. chrysosporium.* For *A. oryzae* and *P. chrysosporium* with different inoculation sequence, same approach was used as the combinations above. All treatments were repeated three times.

*Effect of Different Inoculation Time of A. oryzae, T. reesei, and P. chrysosporium.* To confirm and further compare the results obtained from different inoculation sequence with various dual fungi combinations, combinations with the expected high enzyme activities from previous trials were made and evaluated. Two new treatments were used, which were incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae*, expressed as (T&P) 36h+A, and incubation of *T. reesei* for 36 h, followed by *A. oryzae* and *P. chrysosporium*, expressed as T 36h+(A&P). For comparison, single culture inoculation of *T. reesei* and *T. reesei* inoculation for 36 h, followed by *A. oryzae* (T 36h+A) were also included. All treatments were repeated three times.

#### **Effect of Dual and Trio Fungi Combination SSF on Enzyme Activities of DDGS.**

SSF with the DDGS substrate was conducted by using different dual and trio fungi inoculation with simpler design based on the results from the previous batches of soybean fiber SSF. Single fungus inoculations were included in the same batch of SSF as comparisons. Based on soybean fiber SSF observation and other studies, treatment with inoculation of *T. reesei* and *A. oryzae* (T+A) was replaced by incubation of *T. reesei* for 36 h, followed by inoculation of *A. oryzae* (T 36h+A). The best fungi combination from soybean fiber SSF that had the highest enzyme activity, (T&P)36h+A, was used in the SSF of DDGS. Xylanase and cellulase activities of the fermented DDGS were measured after 6 days of SSF at 30° C. All treatments were repeated three times.

**Effect of Large Scale SSF on Enzyme Activities and Composition Change in the Three Fermented Materials.** In order to produce large quantities of solid-state fermented materials for preliminary feeding trials, large scale SSF, (300 g, as-is) of soybean fiber, DDGS, and soy-enhanced DDGS were conducted. Since high amount of soybean hulls was not desirable for feeding trials, soybean cotyledon fiber was freeze dried prior to SSF to reduce the moisture content to minimize the use of soybean hulls. The moisture content of soybean fiber and DDGS was adjusted to 50% with the addition of 5% (dwb) soybean hulls to improve their porosity. Since the moisture content of 50% in soy-enhanced DDGS was too high based on visual observation of the presence of free water, soy-enhanced DDGS was adjusted to moisture content of 40%. All substrates had water activity at above 0.90.

The pH of the substrates was adjusted to 5.0. A 5% (v/w) of fungal inoculums was used to inoculate a 300 g (as-is) of substrates, with the inoculation and incubation of *T. reesei* and *P. chrysosporium* for 36 h, follow by inoculation of *A. oryzae*, expressed as (T&P) 36h+A. This inoculation scheme was chosen from the previous batches of small scale SSF. SSF was done by incubating the samples at 30° C for 6 days, for the duplicate SSF of the three different substrates. Dry matter mass, xylanase and cellulase activities of the fermented substrates were measured after the SSF. All the fermented substrates were dried at 80 °C until completely dry and the replicates of the same substrates were mixed together for fiber, protein, oil, and ash content analyses as described below.

**Enzyme Activity Assays.** Enzymes were extracted by suspending 3 g (as-is) of fermented samples in 30 mL of 0.2 M sodium acetate-acetic buffer (pH 4.8) overnight at 4°C.

Following the centrifugation at 3,000 x g for 10 min with a IEC Centra CL3 centrifuge (Thermo Electron Corporation, Waltham, MA), the supernatant was collected and used as the enzyme extract for different enzyme assays.

Xylanase activity was assayed by measuring the reducing sugar from birch wood xylan (Sigma Chemicals, St. Louis, MO) which was dissolved by 0.2 M sodium acetate–acetic acid buffer (pH 4.8) according to Bailey et al.<sup>19</sup> with minor adjustment. The reaction mixture containing 1.8 mL of 1% (w/v) xylan solution and 0.2 mL enzyme extract (diluted by the same buffer if needed) was incubated at 50°C for 60 min. The reaction was stopped by adding 2 mL of 3,5-dinitrosalicylic acid (DNS) reagent and was incubated for 10 min in a boiling water bath for color development. After cooling, the mixture was diluted to a total of 15 mL with distilled water and was then measured against a reagent blank at 550 nm with a DU® 720 UV/ Vis spectrophotometer (Beckman Coulter, Brea, CA). The international unit (IU) per g of dried SSF sample was determined by the equation of

$$\begin{aligned} \text{Xylanase activity (IU per g of sample)} \\ = (R \times V_t \times 6.66) / (V_r \times T \times W) \end{aligned}$$

where R is the reducing sugar released in the reaction (mg), V<sub>t</sub> is the total volume of the enzyme extract (mL), V<sub>r</sub> is the volume of enzyme extract used in the reaction (mL), T is the time of reaction (min), W is the weight of the dried SSF sample used (g), and 6.66 represents the micromole (μmol) of xylose. One unit of xylanase activity is defined as the amount of enzyme needed to release one μmol of reducing sugar (equivalent to xylose) from birch wood xylan per minute under our assay conditions.

Cellulase activity was measured using the filter paper activity assay<sup>20</sup> with some modification. Briefly, 0.5 mL of enzyme extract and 1.5 mL of 0.05 M sodium citrate-citric

acid buffer (pH 4.5) were incubated for 10 min at 50 °C in a water bath. Whatman No. 1 filter paper of 50 mg was then added into the mixture and incubated at 50 °C for 60 min. The reaction was stopped by adding 1.5 mL of DNS reagent and the mixture was incubated for 5 min in a boiling water bath for color development. The mixture was then cooled and diluted to a total of 20 mL with distilled water. The mixture was measured against the reagent blank at 540 nm with a spectrophotometer. The international unit (IU) per g of dried SSF sample was determined by the equation of

$$\begin{aligned} & \textit{Cellulase activity (IU per g of sample)} \\ & = (R \times V_t \times 5.56) / (V_r \times T \times W) \end{aligned}$$

All parameters are as explained under xylanase activity, except that 5.56 represents the micromole ( $\mu\text{mol}$ ) of glucose. All assays were performed in duplicate.

**Fiber Content Quantification.** Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) analyses were performed according to the methods of Goering and Van Soest.<sup>21</sup> All samples were analyzed in duplicate.

For NDF determination, 0.5 g of oven-dried sample was heated in 100 mL of neutral detergent with addition of 0.5 g of sodium sulfite and 2 mL of decahydronaphthalene to boiling for 1 h in a Erlenmeyer flask. Sample was filtered through pre-weighed filter paper, followed by 3 times of washing with boiling water, then twice with acetone. The residue was then dried overnight at 105 °C and weighed after cooling in a desiccator. The NDF is calculated as percentage of residue relative to initial sample weight.

For ADF determination, 1 g of oven-dried sample was heated in 100 mL of acid detergent with addition of 2 mL of decahydronaphthalene to boiling for 1 h in a Erlenmeyer

flask. Sample was treated in the same way as in NDF determination. The ADF is calculated as the percentage of residue relative to initial sample weight.

For ADL determination, the ADF procedure was used as a preparatory step. The dried filter paper with ADF from the previous step was transferred into a 30 mL pre-weighed fritted crucible (coarse porosity). The content in the crucibles was covered with 72% sulfuric acid and stirred with a glass rod occasionally to break the lumps and refilling the content at hourly interval was done as the acid drained away. The acid was filtered after 3 h at ambient temperature, followed by 3 times of washing with boiling water. The crucible was dried overnight at 105 °C and weighed after cooling in a desiccator. The crucible was then heated in the Thermolyne furnace (Thermo Scientific, Waltham, MA) at 500 °C for 3 h for ash determination. The percentage of ADL is calculated as the difference between the acid insoluble residue and ash relative to initial sample weight.

ADL represents the lignin content. The difference of NDF and ADF is an estimate of hemicellulose, whereas the difference of ADF and ADL is an estimate of cellulose.

**Determination of Dry Matter Mass, Protein, Oil, and Ash Contents.** The dry matter mass was determined by weighing after oven drying at 105 °C overnight. Protein content was determined using the Dumas nitrogen combustion method using an ElementarVario MAXCN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) with a conversion factor of 6.25, and oil content by acid hydrolysis method (AOAC official method 922.06). Ash content was determined by heating the samples at 550 °C overnight (AOAC 923.03). The other carbohydrate content was obtained by subtracting the protein, oil, fiber, and ash contents from 100%.

**Statistical Analysis.** All treatments were repeated as described under each experiment. The data were analyzed by ANOVA (Analysis of Variance) using SAS (Version 9.1, SAS Institute Inc., Cary, NC) and the Least Significant Difference (LSD) mean comparison was used to compare the treatment mean differences at  $P = 5\%$ .

## RESULTS AND DISCUSSION

**Effect of Dual and Trio Fungi Combination SSF on Enzyme Activities of Soybean Cotyledon Fiber.** The enzyme activities of unfermented soybean fiber were assayed as control, and it had xylanase activity of 8.0 IU/g and cellulase activity of 0.3 IU/g as shown in Table 1. Figure 2 shows both xylanase and cellulase activities among different combinations of fungi after SSF at 30° C. Soybean fiber with mixed inoculation of *T. reesei* and *P. chrysosporium* had higher activity for both enzymes compared to other mixed fungi inoculated samples. However, its xylanase activity was lower than the *T. reesei* inoculation alone. Fermented soybean fiber with other combinations of fungi all showed lower activity for both enzymes compared to the single fungus inoculation. The low enzyme activities obtained from this mixed fungi culture SSF was unexpected, especially with the combination of *T. reesei* and *Aspergillus* species, which was shown to have synergistic effects in producing xylanase and cellulase.<sup>11-16</sup> Fungi may have responded differently to different substrate and growing conditions and our soybean fiber SSF conditions might not be the optimum for observing the synergistic effect among fungi. On the other hand, the inoculation sequence of different fungal strains might play a significant role in stimulating the enzyme production. Castilo et al. reported that mixed fungal inoculation sequence of *T. reesei* and *A.*

*niger* must be adjusted carefully due to the differences in individual fungal growth rates, enzyme production rates, and the possible dominance of one fungus over the other.<sup>11</sup>

*Effect of Different Inoculation Time of A. oryzae and T. reesei.* Investigation on different inoculation time of *A. oryzae* and *T. reesei* was conducted to determine its effect on enzyme production. Since Dueñas et al.<sup>23</sup> reported that mixed culturing was not beneficial when *Trichoderma reesei* and *Aspergillus phoenicis* were inoculated together, and knowing that *T. reesei* produces reducing sugars through cellulose hydrolysis that may accelerate the growth of *Aspergillus*, we conducted sequential inoculations. As shown under experiment 1 in Figure 3, xylanase activity of 1445.7 IU/g was obtained in the fermented soybean fiber with the inoculation of *T* 36h+A and this activity was significant higher than all the other treatments. To validate the synergistic effect observed, the SSF experiment with the same treatments was repeated and the results are presented as experiment 2 in Figure 3. Again, the highest xylanase activity of 855.8 IU/g was obtained in the fermented soybean fiber with the inoculation of *T* 36h+A. Xylanase are mainly produced by *Aspergillus* and *Trichoderma* spp.<sup>24</sup> Our findings showed that synergistic effect occurred when these two fungi were inoculated with an appropriate sequence that stimulated higher xylanase production. Besides xylan (hemicellulose), studies have shown that xylanase activity could be induced when *T. reesei* was grown in cellulose.<sup>16</sup> Our soybean fiber contained high level of both hemicellulose (26.5%) and cellulose (22.3%), so this may explain the high level of xylanase activity in soybean fiber inoculated with *T. reesei*, as well as mixed culture of *T. reesei* and *A. oryzae* with a proper inoculation sequence. Unlike the xylanase activity, no significant synergism was found in the cellulase activities with the mixed fungi of *A. oryzae* and *T. reesei*.



Although a basal medium of Mandal & Reese<sup>25</sup> containing all nutrients has been commonly added to solid substrates to give optimum fungal growth and increase the cellulase production, we did not add any external nutrients in this experiment. We intended to test if this substrate alone can simply be solid-state fermented to improve its compositional profile.

Both xylanase and cellulase activities of *A. oryzae* fermented soybean fiber from Figure 2 were lower than the enzyme activities shown in Figure 3. Also, inconsistency happened to enzyme activities of *P. chrysosporium* fermented soybean fiber shown in Figure 2 compared to the enzyme activities obtained from other batches of SSF as discussed below. Such inconsistencies were unexpected and they might be caused by the viability variance among samples in the individual inoculum vials, handling errors, and the complexity of biological system.

*Effect of Different Inoculation Time of P. chrysosporium and T. reesei.* To determine if different inoculation time would result in synergistic effect with fungi combination of *P. chrysosporium* and *T. reesei*, SSF of soybean fiber with this two fungi inoculation was done. As shown under experiment 1 in Figure 4, fermented soybean fiber with the inoculation of *T+P* and *T 36h+P* showed the highest xylanase activities among all fermented samples. However, these two combinations did not always contribute to the highest cellulase activities. Fermented soybean fiber with both the inoculation of *P* and *P 36h+T* had higher cellulase production. Due to the large standard deviation from this batch of SSF, another batch of SSF with the same treatments was conducted. The results shown under experiment 2 in Figure 4 indicate no significant differences among inoculation of *T*, *T+P*, and *T 36h+P* in xylanase activities. Large standard deviation was again found in enzyme activities. The lack of

homogeneity in fungal growth on the substrate might have contributed to the large differences. Nonetheless, cellulase activities measured from the repeated SSF showed similar trend compared to experiment 1. Based on the results obtained from both experiments, the treatment of *T+P* was identified to have a great potential in producing high level of xylanase and cellulase. As discussed earlier, the high hemicellulose and cellulose content in soybean fiber might help induce xylanase production in *T. reesei* inoculated substrate and also increase xylanase activity in treatment of *T+P*. The combination of *T+P* was also found to be the best in lignocellulosic decomposition of timber waste.<sup>26</sup> Such combination was also used in other studies in composting different solid waste.<sup>27,28</sup> As a result, this combination was used in our study for further investigation.

*Effect of Different Inoculation Time of A. oryzae and P. chrysosporium.* SSF of soybean fiber with inoculation of *A. oryzae* and *P. chrysosporium* at different inoculation time was investigated as well. Xylanase and cellulase activities of various fungi treatments and with different inoculation time are shown in Figure 5. Based on the two enzyme activities, no significant synergistic effect was observed, and xylanase activity among these combinations was lower compared to the other two fungal combinations.

*Effect of Different Inoculation Time of A. oryzae, T. reesei, and P. chrysosporium.* The treatments that gave high enzyme activities from each dual combination of SSF were further combined and such combination effects on xylanase and cellulase activities were evaluated. As shown in Figure 6, both enzyme activities from *T. reesei* inoculated soybean fiber were the lowest among all the treatments. No significant difference was found in

xylanase activities among the three combinations, *T* 36h+A, (*T&P*) 36h+A, and *T* 36h+(A&*P*). Considering *P. chrysosporium* as lignin-degrading enzyme producer<sup>29,30</sup>, the combination including this fungus should be chosen because soybean fiber contains lignin. In addition, lignin degradation enables the exposures of hemicellulose and cellulose for degradation. Summarizing data gathered from all previous batches of soybean fiber SSF, combination of (*T&P*) 36h+A was chosen as the final best fungi combination.

Our soybean cotyledon fiber SSF with the inoculation of *T. reesei* did not produce significant amount of cellulase, although *T. reesei* has been reported as a promising strain in producing cellulase. The cellulolytic activity of *T. reesei* wild-type strain (QM 6a) that was used in our study has been improved by different mutagenesis methods that have resulted in mutants such as QM 9414 and RUT-C30 that can produce 4-5 times more cellulase than the wild-type strain.<sup>31</sup> This might partially explain the low cellulase activity of the mixed fungal fermentation using *T. reesei* compared to other studies that employed *T. reesei* mutants.

Some batch to batch differences in enzyme activities were found in the fermented soybean fiber. These may be caused by the lack of homogeneity in the substrate, soybean cotyledon fiber, that was produced from pilot-plant scale EAEP.<sup>5</sup> Approximately 45 kg (as-is) of soybean fiber was produced in each trial of EAEP and the hand-mixing might not have been adequate to obtain a homogenous mixture for all treatments and replicates. Having a more effective means of mixing during production or fermentation in the future may help minimize the variability in SSF.

#### **Effect of Dual and Trio Fungi Combination SSF on Enzyme Activities of DDGS.**

The enzyme activities of unfermented DDGS were assayed as control and are shown in Table

1. Inoculation with different fungi combinations for DDGS SSF was done in a simpler way by using the results from soybean fiber SSF. The two enzyme activities of the fermented substrates are presented in Table 2. The fermented sample with the inoculation of (*T&P*) 36h+A, which was the best combination for soybean fiber SSF, showed the highest xylanase activity compared to the inoculation with single fungus and with other fungi combinations. Meanwhile, the fermented DDGS with inoculation of *P. chrysosporium* was identified to produce high xylanase activity of 364.8 IU/g. This is appreciably higher than all the batches of soybean fiber SSF inoculated with *P. chrysosporium*. In addition, this sample also showed the highest cellulase activities of 7.3 IU/g. This suggests that in contrast to soybean fiber, DDGS might have a better composition to support the growth of and enzyme production from *P. chrysosporium* in SSF. According to Leštan et al.<sup>32</sup>, the addition of linseed oil to the growth medium strongly stimulated mycelium biomass production of *P. chrysosporium*. DDGS has higher content of oil compared to soybean fiber as shown in Table 1, and this may explain the better growth of *P. chrysosporium* in DDGS, thus contributing to the higher enzyme production. Considering the high hemicellulose content of 24.9% compared to the low cellulose content of 11.6% in DDGS, xylanase activity is considered to be more important in breaking down the substrate. Therefore, inoculation of (*T&P*)+36h A was chosen as the best combination for further study. This same best fungi combination as for soybean fiber allows the future comparison of various substrates under the same fermentation condition.

**Effect of Large Scale SSF on Enzyme Activities and Composition Change in the Three Fermented Materials.** The scale 300-g scale SSFs of soybean fiber, DDGS, and soy-

enhanced DDGS with the best fungi combination, (*T&P*) 36h+A, were conducted, and xylanase and cellulase activities of the fermented materials are shown in Table 3. Compared to the SSF of soybean fiber and DDGS with the same fungi inoculation that were done on small scale (40 g), the enzyme productions from the large scale SSF were much lower. The lower amount of soybean hulls (5%, dwb) added in the substrates for the 300-g scale SSF may have contributed to the lower enzyme activities because all enzyme activities are presented as IU for one gram of dried substrate without the inclusion of the soybean hulls. The higher amount of soybean hulls (47%, dwb) used previously in the substrates for small scale SSF, the lower amount of dried soybean fiber or DDGS were included in the calculation, thus contributing to the higher enzyme activities. The other factor may be that the lower amount of soybean hulls used in the large sale of SSF caused a compact texture of the substrates, thus reduced the fungal growth and hyphae penetration. As shown in Table 3, both enzyme activities indicated a similar trend, with fermented soybean fiber having the highest activities, followed by DDGS and soy-enhanced DDGS. However, the xylanase activity of soybean fiber and DDGS was not significantly different due to the large standard deviation found in fermented soybean fiber. Consistent with the results from Yang et al.<sup>17</sup>, soybean fiber is shown to be a better substrate for SSF compared to DDGS. This may be due to the limited nutrient availability in DDGS for fungal growth. The high amount of the other carbohydrate content in soybean fiber (Table 1) compared to DDGS and soy-enhanced DDGS could be a good carbon source to support the growth of fungi and to produce enzyme. As shown in Table 3, soy-enhanced DDGS demonstrated low enzyme activities after SSF. The high ash content in soy-enhanced DDGS as shown in Table 1 indicates the high level of

salt, and this may be a reason of its poor fungal growth. Salt was shown to have adverse effects on microorganisms and resulted in reduced biological activity.<sup>33</sup>

Substrates weight reduction for 300-g scale fermentation was recorded. Approximately 20.4% reduction in dry matter mass was found in fermented soybean fiber, followed by reduction of 9.9% in DDGS, and 6.4% in soy-enhanced DDGS. The dry matter reduction reflected the vigor of fungi growth, with higher reduction indicating better fungal growth. The dry matter reduction of the three fermented samples correlated well with the enzyme production.

Table 4 shows the composition of unfermented and fermented soybean fiber, DDGS, and soy-enhanced DDGS. The theoretical values are those with the disappearance of dry matter taken into consideration. Fiber content decreased substantially in each fermented samples with hemicellulose content showed a greater reduction compared to cellulose and lignin content. This trend corresponded well to the xylanase and cellulase activities in the fermented samples. The reductions of hemicellulose content in the fermented soybean fiber, DDGS, and soy-enhanced DDGS were 13.4, 11.5, and 4.5%, respectively. The degree of reduction is proportional to the increase in xylanase activity. Cellulose contents in fermented soybean fiber and soy-enhanced DDGS increased after the SSF. The reduction in dry matter by SSF as a result of the metabolism of substrate by microorganisms may have contributed to the increase in cellulose content. By taking the dry matter reduction into consideration, the theoretical value of cellulose showed a 1.6 and 2.2% decrease in fermented soybean fiber and DDGS and a slight increase of 0.4% in fermented soy-enhanced DDGS. Lignin content in all the fermented materials decreased after SSF, with the highest reduction in DDGS, followed by soy-enhanced DDGS, and soybean fiber. This finding could be explained by the high oil

content in DDGS and soy-enhanced DDGS that may have stimulated the growth of *P. chrysosporium* as discussed earlier.

Protein content showed a slight increase after SSF, and this could be explained by the concentrating effect caused by microorganism metabolizing the substrates. The higher protein content in the fermented product is expected to improve its nutritional value as animals feed. Oil content of the three substrates did not change significantly after SSF, unlike other report that suggested that *Aspergillus* and *Rhizopus* metabolized a portion of fat in the DDGS.<sup>34</sup> Liu reported that DDGS composition varied with the differences in feedstock and composition, process methods and parameters, and the fermentation yeast.<sup>35</sup> The composition of DDGS used in this study may be different from the one used in the other study, and this may have caused the difference in fungal growth and substrate composition change. The slight increases of the total oil content in soybean fiber, DDGS, and soy-enhanced DDGS after SSF could be from the concentration effect.

In summary, synergistic effect was found among the three fungi used in the SSF of soybean cotyledon fiber and DDGS. The inoculation sequence of different fungi was identified as a important factor to allow the best interaction among the fungi to achieve better growth and higher enzyme production. Combination of fungi with the incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae* showed the best results in soybean cotyledon fiber SSF. The fermented soybean fiber has maximum xylanase activity of 757.4 IU/ g and cellulase activity of 3.2 IU/ g. This inoculation scheme also led to the highest xylanase activity of 399.2 IU/g in DDGS SSF. The fermented materials produced from the 300-g scale SSF showed 3.5-15.1% reduction fiber content and 1.3-4.2% increase in protein content, demonstrating the potential for non-ruminant feed improvement.

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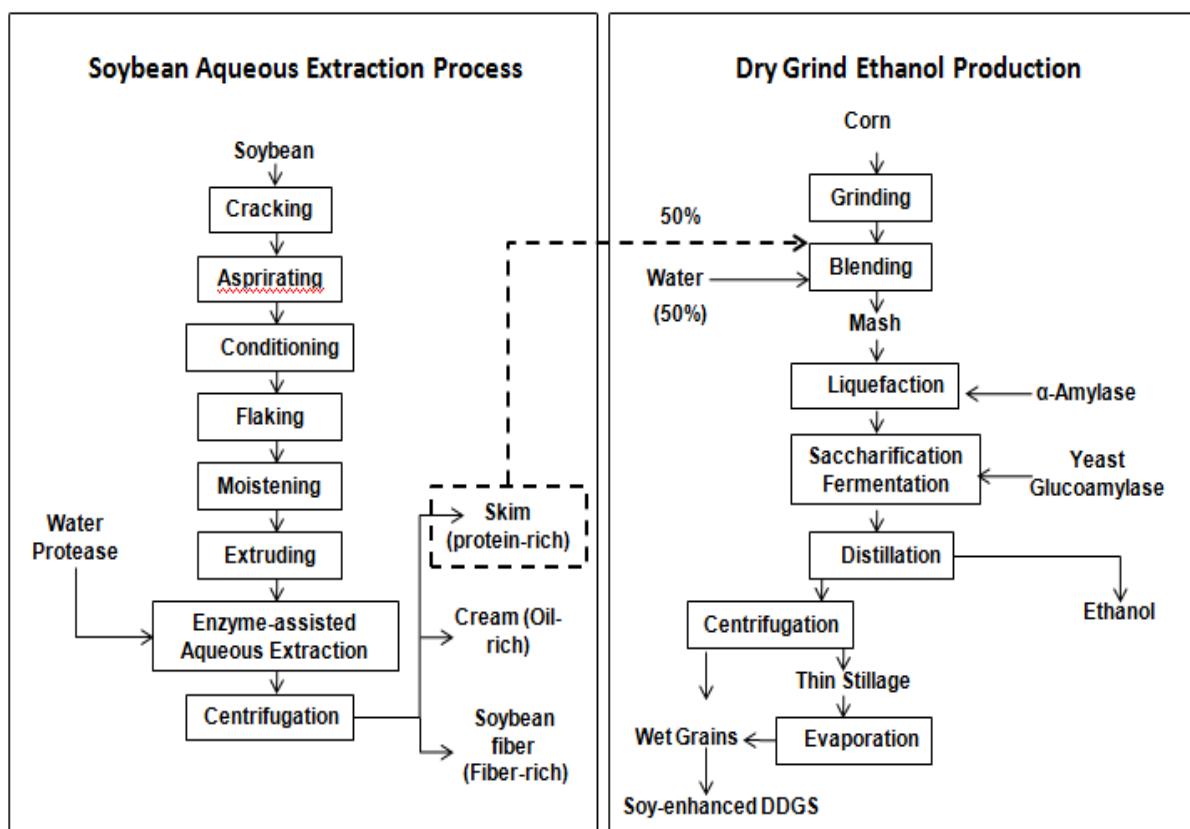


Figure 1. Schematic diagram of integrated soybean-corn biorefinery system.

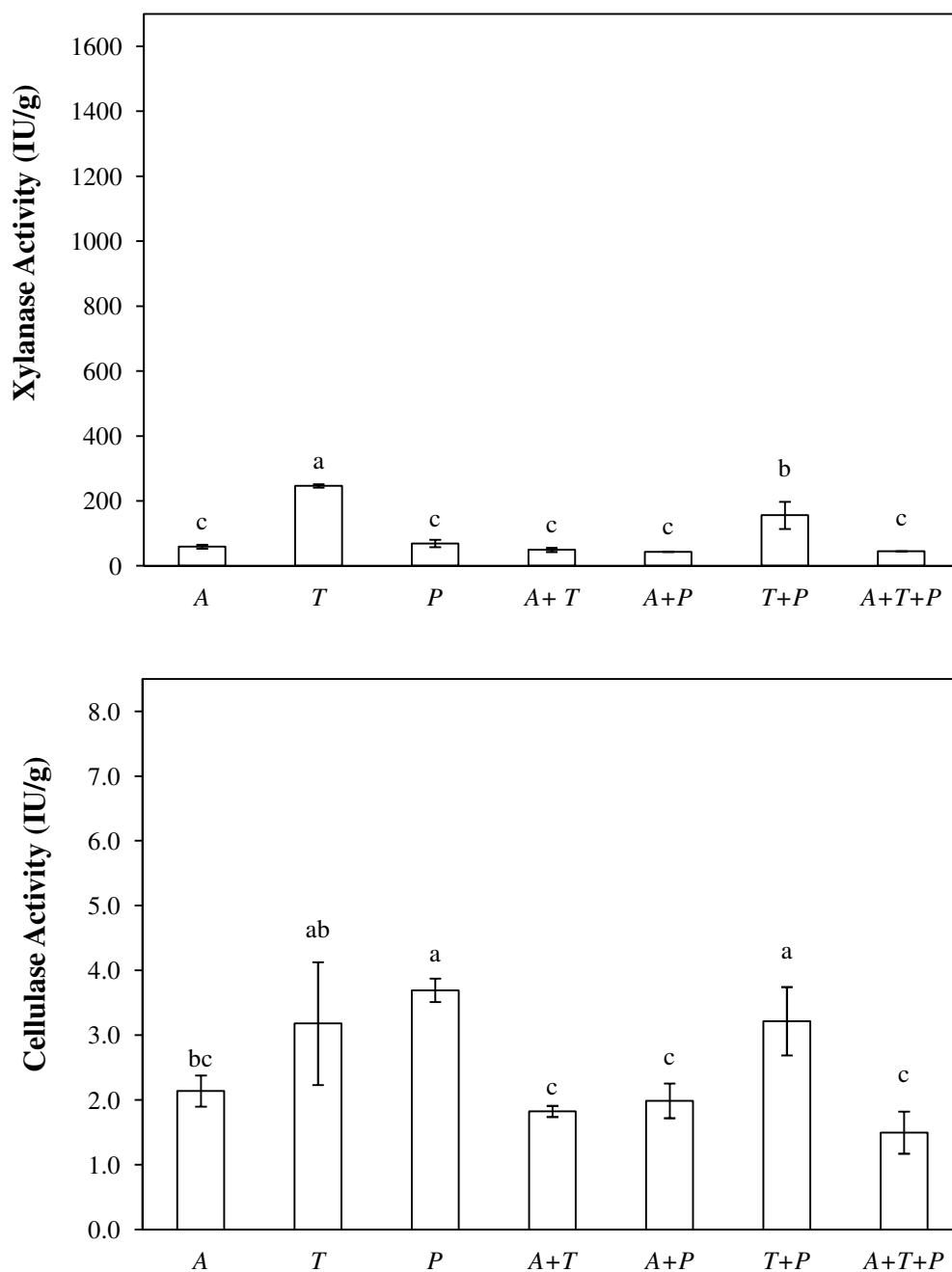


Figure 2. Effect of different fungi and combination on enzyme activities of soybean cotyledon fiber. N=2. The means followed by the same letter are not significantly different at P=5%.  $LSD_{0.05} = 39.6$  for xylanase activity and  $LSD_{0.05} = 1.1$  for cellulase activity. A: *A. oryzae*; T: *T. reesei*; P: *P. chrysosporium*.

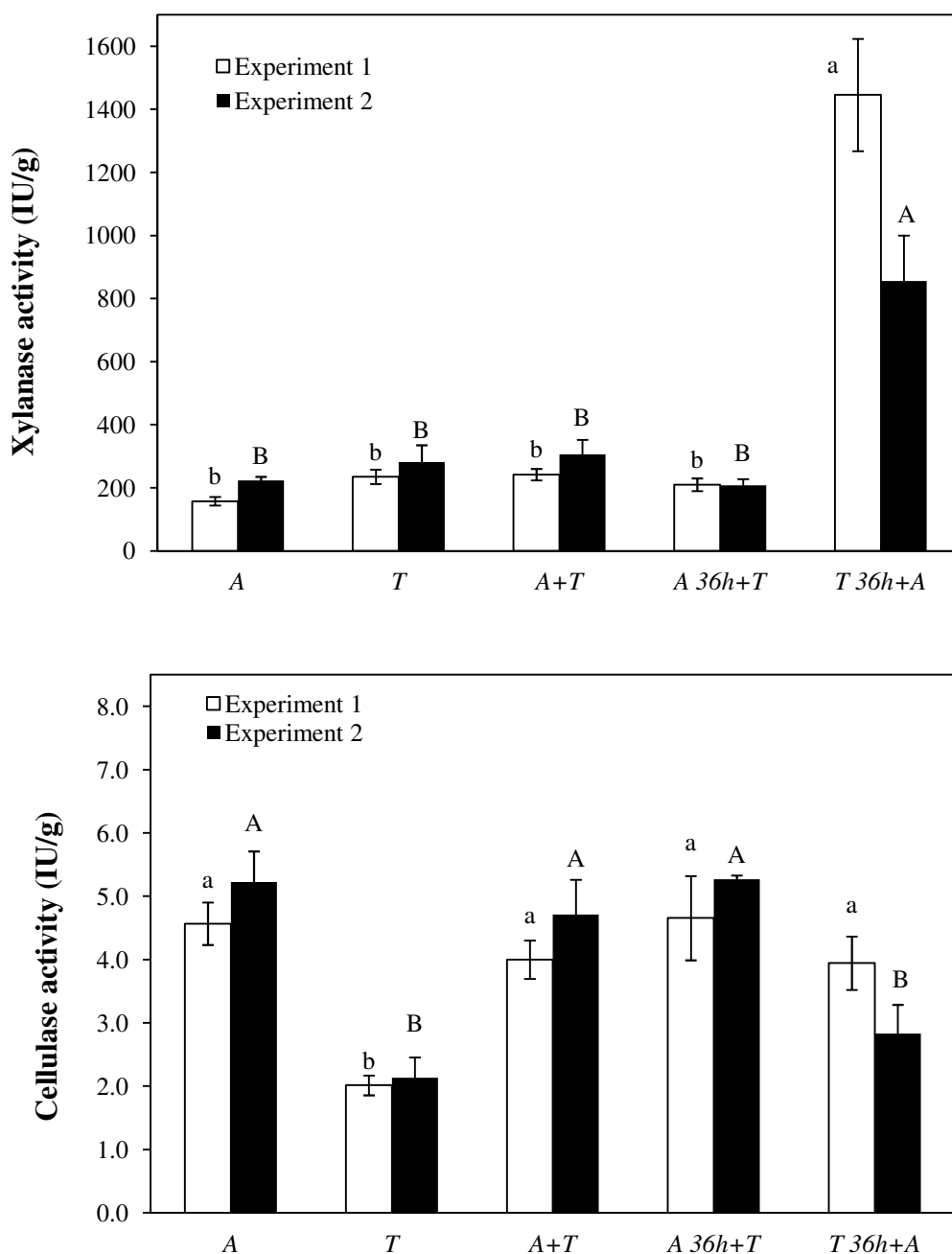


Figure 3. Effect of different inoculation time of *A. oryzae* and *T. reesei* on enzyme activities of soybean cotyledon fiber. N=3. The means followed by the same letter within each experiment are not significantly different at P=5%.  $LSD_{0.05} = 148.2$  for xylanase activity and  $LSD_{0.05} = 0.7$  for cellulase activity in experiment 1.  $LSD_{0.05} = 131.7$  for xylanase activity and  $LSD_{0.05} = 0.8$  for cellulase activity in experiment 2. A: *A. oryzae*; T: *T. reesei*; A 36h+T: *A. oryzae* for 36 h, followed by *T. reesei*; T 36h+A: *T. reesei* for 36 h, followed by *A. oryzae*.

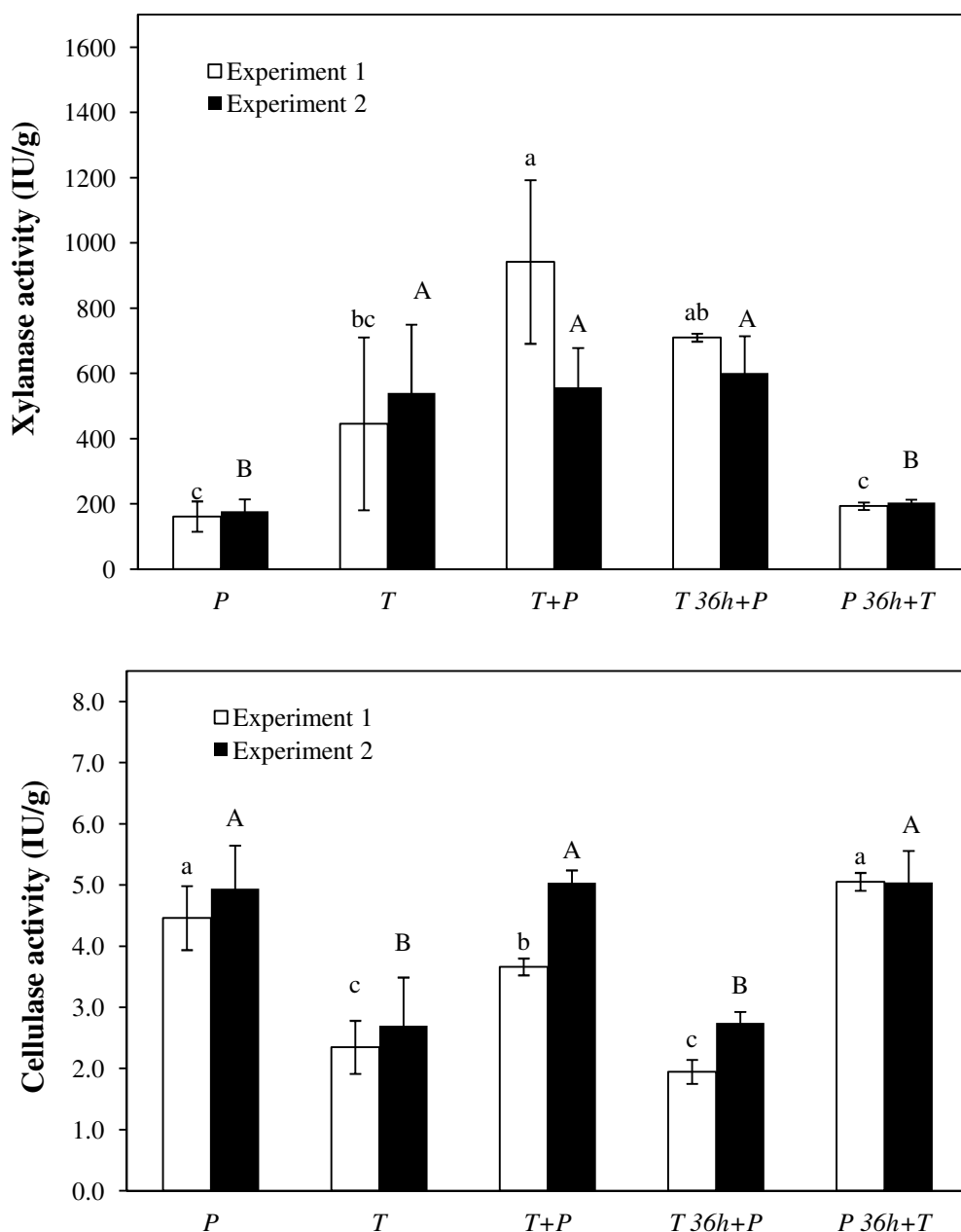


Figure 4. Effect of different inoculation time of *P. chrysosporium* and *T. reesei* on enzyme activities of soybean cotyledon fiber. N=3. The means followed by the same letter within each experiment are not significantly different at P=5%.  $LSD_{0.05} = 299.4$  for xylanase activity and  $LSD_{0.05} = 0.6$  for cellulase activity in experiment 1.  $LSD_{0.05} = 219.3$  for xylanase activity and  $LSD_{0.05} = 1.0$  for cellulase activity in experiment 2. T 36h+P: *T. reesei* for 36 h, followed by *P. chrysosporium*; P 36h+T: *P. chrysosporium* for 36 h, followed by *T. reesei*.

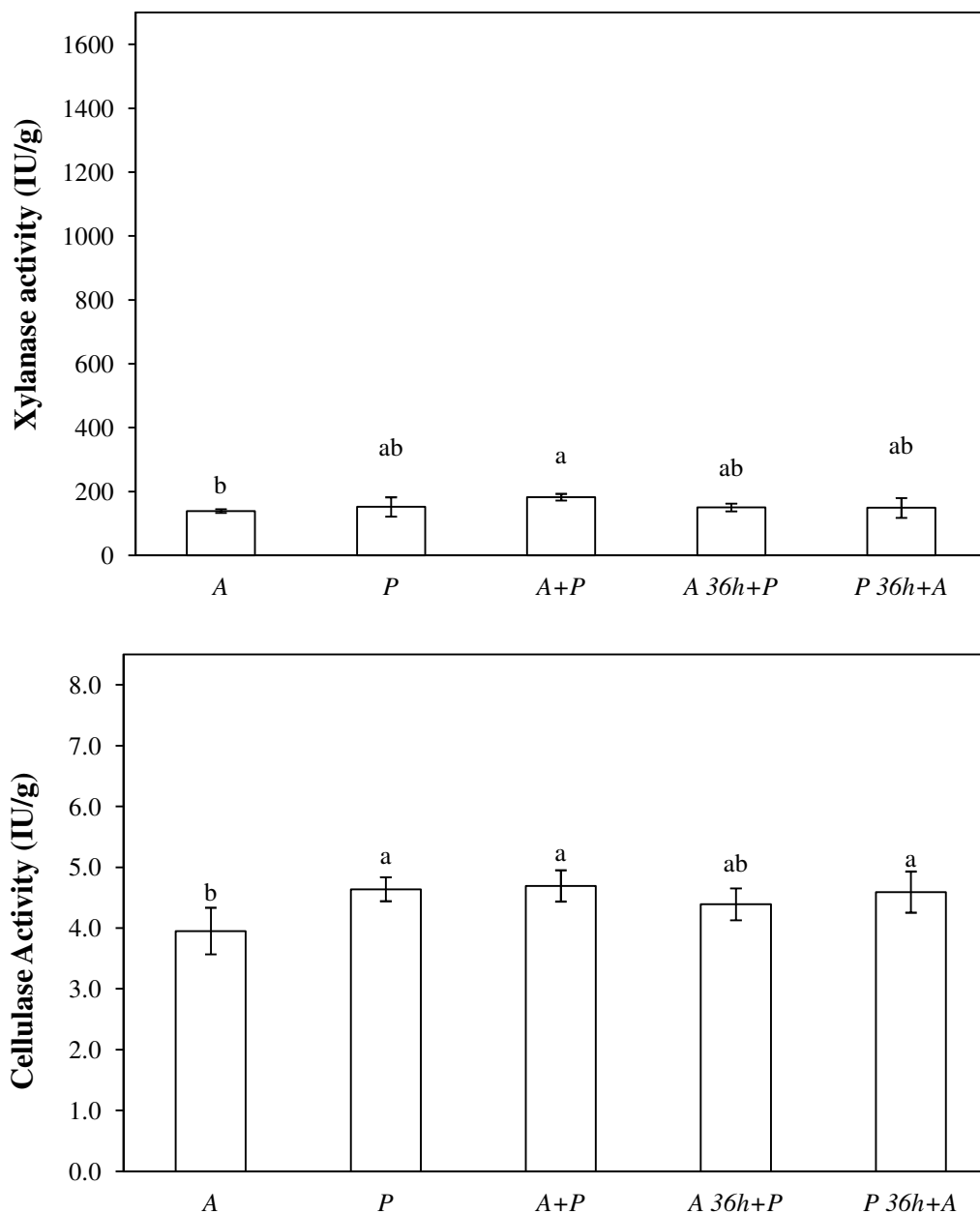


Figure 5. Effect of different inoculation time of *A. oryzae* and *P. chrysosporium* on enzyme activities of soybean cotyledon fiber. N=3. The means followed by the same letter are not significantly different at P=5%.  $LSD_{0.05} = 37.9$  for xylanase activity and  $LSD_{0.05} = 0.5$  for cellulase activity. A 36h+P: *A. oryzae* for 36 h, followed by *P. chrysosporium*; P 36h+A: *P. chrysosporium* for 36 h, followed by *A. oryzae*.



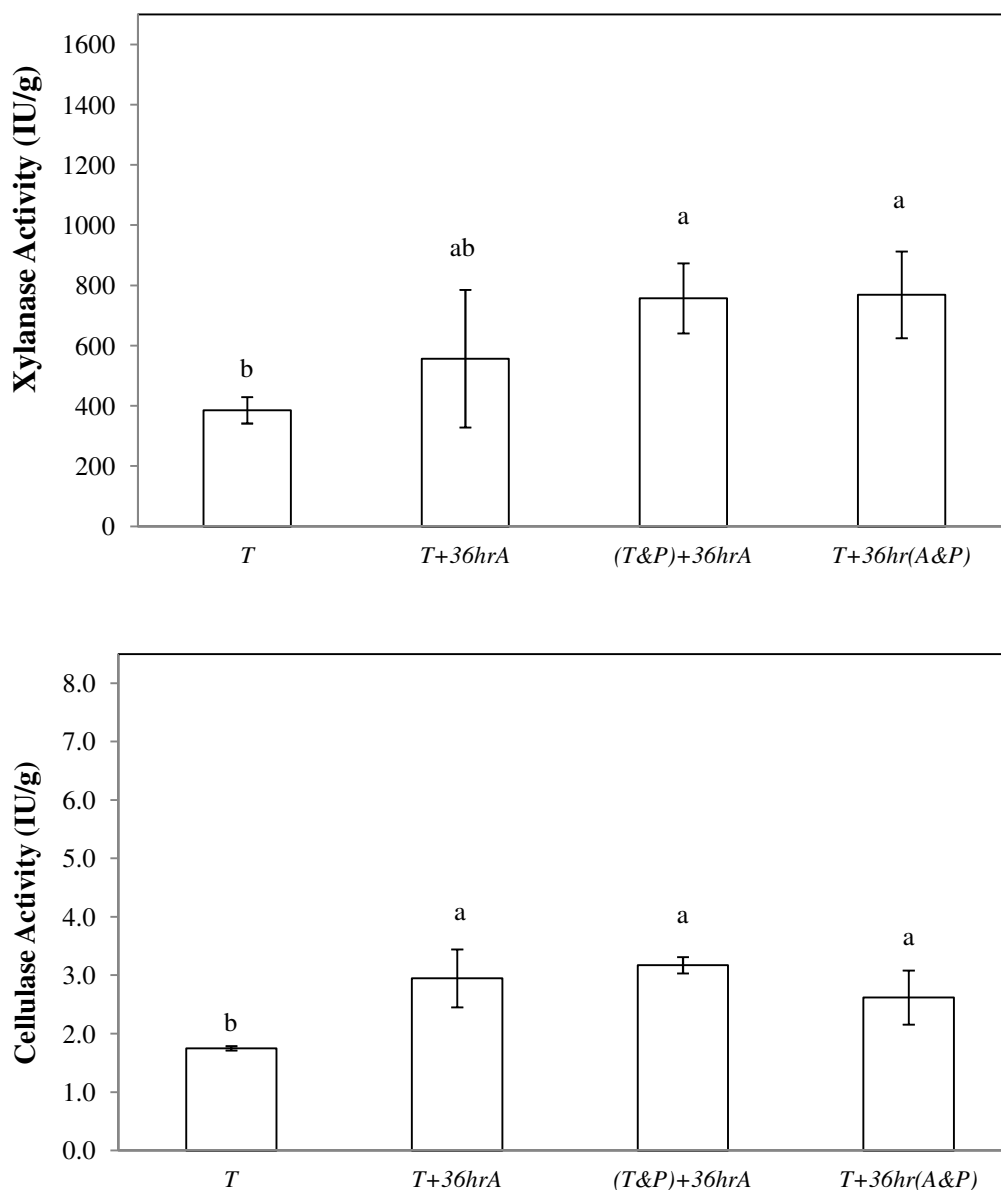


Figure 6. Effect of different inoculation time of *A. oryzae*, *T. reesei* and *P. chrysosporium* on enzyme activities of soybean cotyledon fiber. N=3. The means followed by the same letter are not significantly different at P=5%.  $LSD_{0.05} = 279.8$  for xylanase activity and  $LSD_{0.05} = 0.7$  for cellulase activity. T 36h+A: *T. reesei* for 36 h, followed by *A. oryzae*; (T&P) 36h+A: *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae*; T 36h+(A&P): *T. reesei* for 36 h, followed by *A. oryzae* and *P. chrysosporium*.

Table 1. Enzyme activities and compositions of soybean cotyledon fiber, DDGS, and soy-enhanced DDGS.

	Enzyme activities (IU/g)		Composition (% , dwb)						
	Xylanase	Cellulase	Hemicellulose	Cellulose	Lignin	Oil	Protein	Ash	Simple carbohydrate
Soybean fiber	8.0	0.3	26.5	22.3	1.9	3.3	6.4	4.3	35.3
DDGS	1.9	0.2	24.9	11.6	7.8	14.0	34.3	4.3	3.2
Soy-enhanced DDGS	1.7	0.3	22.9	5.5	1.6	11.8	44.5	7.2	6.5

Table 2. Effect of different fungi *combination* on enzyme activities of DDGS.

	Fungi combination								LSD <sub>0.05</sub>
	A	T	P	T 36h+A	T+P	A+P	(T&P) 36h+A	A+T+P	
Xylanase activity (IU/g)	88.3 ± 5.1 f	218.3 ± 24.6 d	364.8 ± 22.3 b	293.1 ± 7.8 c	157.0 ± 25.3 e	114.8 ± 5.7 f	399.2 ± 31.4 a	120.0 ± 11.5 f	33.4
Cellulase activity (IU/g)	2.1 ± 0.5 b	2.3 ± 0.7 b	7.3 ± 0.4 a	1.8 ± 0.2 b	2.0 ± 0.4 b	1.9 ± 0.4 b	2.0 ± 0.2 b	1.7 ± 0.1 b	0.7

N=3. Data are presented as Mean ± SD. The values followed by the same letter in the same row are not significantly different at P=5%. A: *A. oryzae*; T: *T. reesei*; P: *P. chrysosporium*; All the different sequences are described in Fig. 6.

Table 3. Effect of SSF using the best combination of fungi on enzyme activities of soybean cotyledon fiber, DDGS, and soy-enhanced DDGS .

	Soybean fiber	DDGS	Soy-enhanced DDGS	LSD <sub>0.05</sub>
Xylanase activity (IU/g)	57.0 ± 9.7 a	49.3 ± 3.0 ab	35.9 ± 2.2 c	19.1
Cellulase activity (IU/g)	1.2 ± 0.1 a	0.6 ± 0.0 b	0.4 ± 0.1 c	0.2

N=2. Data are presented as Mean ± SD. The values followed by the same letter in the same row are not significantly different at P=5%.

Table 4. Composition of unfermented and SSF soybean cotyledon fiber, DDGS, and soy-enhanced DDGS.

Substrates		Composition (%), dwb				
		Hemicellulose	Cellulose	Lignin	Protein	Oil
Soybean fiber	Unfermented*	22.0	25.5	1.2	8.3	3.5
	Fermented	10.8	30.1	0.9	12.5	5.0
	Theoretical value	8.6	24.0	0.8	9.9	4.0
	Increase (+) or decrease (-)	-11.2	4.5	-0.2	4.2	1.5
	Theoretical increase (+) or decrease (-)	-13.4	-1.6	-0.4	1.6	0.5
DDGS	Unfermented*	30.5	13.8	5.7	33.0	11.3
	Fermented	21.0	12.8	1.1	36.3	14.6
	Theoretical value	18.9	11.5	1.0	32.7	13.2
	Increase (+) or decrease (-)	-9.5	-1.0	-4.6	3.3	3.3
	Theoretical increase (+) or decrease (-)	-11.5	-2.2	-4.7	-0.3	1.9
Soy-enhanced DDGS	Unfermented*	21.2	8.9	3.7	43.0	10.5
	Fermented	17.9	10.0	2.3	44.4	11.1
	Theoretical value	16.8	9.4	2.2	41.5	10.4
	Increase (+) or decrease (-)	-3.3	1.1	-1.3	1.3	0.6
	Theoretical increase (+) or decrease (-)	-4.5	0.4	-1.5	-1.5	-0.1

\*All the unfermented materials contained 5% (dwb) soybean hulls.

### CHAPTER 3. ARACHIDONIC ACID (ARA) AND EICOSAPENTAENOIC ACID (EPA) PRODUCTION IN SOYBEAN PROCESSING CO-PRODUCTS BY *PYTHIUM IRREGULARE*

A manuscript to be submitted to Journal of Agricultural and Food Chemistry

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#### ABSTRACT

Arachidonic acid (ARA) and eicosapentaenoic acid (EPA) were produced by *Pythium irregulare* fungus using soybean cotyledon fiber and soy skim, two co-products from aqueous soybean processing, as substrates in different fermentation systems. Parameters such as moisture content, substrate glucose addition, incubation time, and vegetable oil supplementation were found to be crucial in solid-state fermentation (SSF) of soybean fiber, which is to be used as animal feed with enriched long chain polyunsaturated fatty acids (PUFA). Soybean fiber with 8% (dwb) glucose supplementation for 7-day SSF produced 1.3 mg of ARA and 1.6 mg of EPA in one gram of dried substrate. When soy skim was used as substrate for submerged fermentation, total ARA yield of 125.7 mg/L and EPA yield of 92.4 mg/L were achieved with the supplementation of 7% (w/v) soybean oil. This study demonstrated that the values of soybean fiber and soy skim could be enhanced through the long chain PUFA production by fermentations.

**KEYWORDS:** Arachidonic acid, eicosapentaenoic acid, polyunsaturated fatty acids, solid-state fermentation, soybean cotyledon fiber, soy skim, submerged fermentation

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## INTRODUCTION

Enzyme-assisted aqueous extraction processing (EAEP) of soybeans is a green technology that utilizes water and enzyme to recover oil from soybeans. It has been shown to be a great alternative to the traditional hexane oil extraction that may create pollution or safety problem. However, the feasibility of the soybean EAEP depends on the potentials of maximizing the values of two co-products, soybean cotyledon fiber and soy skim.<sup>1</sup> Soybean cotyledon fiber is a low-valued fraction that contains high amount of fiber. Our previous study showed that the fiber composition can be improved by enzyme-producing fungi through solid-state fermentation (SSF).<sup>2</sup> The fermented soybean fiber with reduced fiber and increased protein content can be used as non-ruminant feed. Soy skim is a liquid fraction from EAEP that contains partially hydrolyzed protein and has been shown to be an excellent nutrient source for corn ethanol fermentation.<sup>3</sup> The value of soybean fiber and soy skim could be increased considerably if they could be utilized to produce value-added products by fermentation.

Both omega-3 and omega-6 series of long chain polyunsaturated fatty acids (PUFA) have shown tremendous potential for use as food additives and in pharmaceuticals for relieving heart and circulatory disorders and cancers, as well as inflammatory diseases.<sup>4</sup> In addition to health-promoting effects in humans, PUFA has also shown to be beneficial to animals. Improved feed conversion efficiency<sup>5</sup>, increased body weight<sup>6,7</sup>, and reduced inflammatory response<sup>8</sup> were reported when omega-3 PUFA was added in poultry feed. PUFA supplementation in poultry feed could also contribute to PUFA enriched meat<sup>9,10</sup> and egg yolk<sup>11-13</sup>, adding values to these products. Arachidonic acid (ARA, C20:4, n-6) is an essential PUFA that acts as a precursor of important eicosanoids such as prostaglandins,

thromboxanes, and leukotrienes.<sup>14,15</sup> Animal livers, egg yolks, and fish oil are the sources of ARA but the relatively low concentration prompts the industry to seek for other alternatives.<sup>15,16</sup> Eicosapentaenoic acid (EPA, C20:5, n-3) is also an essential fatty acid that has unique biological activities in the prevention and treatment of a number of human diseases and disorders.<sup>17</sup> Marine fish oil, being a major source of EPA faces several challenges, such as the objectionable taste and odors, high cholesterol content, and heavy metal pollutants that are yet to be solved. Thus, ARA and EPA produced from fungi would be desirable.

*Pythium irregulare* is a filamentous fungus which is known to be a good EPA producer. It has been employed in submerged fermentation (SmF) using various industrial co-products such as sweet whey permeate<sup>18</sup>, sucrose waste stream and soy meal waste stream<sup>17</sup>, biodiesel-derived crude glycerol<sup>19</sup>, rendered animal protein<sup>20</sup>, and corn thin stillage<sup>21</sup>. Although *P. irregulare* has been shown to produce significant amount of EPA, it produces a certain amount of ARA as well.

We expected that soy skim from EAEP that contains partially hydrolyzed protein may provide the needed nitrogen nutrients to support the growth of *P. irregulare* in SmF and stimulate the production of ARA and EPA. Although several researchers reported the feasibility of using *P. irregulare* in SmF, SSF by this fungus has not been intensively studied. SSF, which is defined as the fermentation process that microorganisms are grown on solid substrate without the presence of free liquid<sup>22</sup>, has drawn interests in industry because of its low waste water production and operation expenses, superior productivity, and the use of agro-industrial solid residues as substrates. Thus, SSF of soybean fiber by *P. irregulare* for long chain PUFA formation may be a promising new use for this co-product to further



enhance its value as non-ruminant feed. Our research hypothesis was both soybean fiber and soy skim may be good substrates in different type of fermentation that will result in ARA and EPA enriched co-products. The objectives of this research were: (1) to study the effect of moisture content, incubation time, substrate glucose addition, and vegetable oil supplementation on long chain PUFA formation in soybean fiber by SSF, and (2) to investigate the effect of vegetable oil addition on the SmF performance of soy skim.

## MATERIALS AND METHODS

**Microorganisms and Culture Preparation.** *Pythium irregulare* (ATCC 10951) was provided by Dr. Zhiyou Wen of Department of Food Science and Human Nutrition, Iowa State University. The strain was cultured on agar plates containing 3% glucose, 1% yeast extract, and 1% agar. The plates were incubated at 25 °C for 6 days until complete sporulation. The spores from the plates were dislodged with sterile water containing glass beads. The suspensions were used as inoculums and were kept in 4 °C until use.

**Substrate Preparation and Chemicals.** Soybean cotyledon fiber and soy skim were produced in the pilot plant of Center for Crops Utilization Research (CCUR), Iowa State University via the two stage counter-current EAEP.<sup>23</sup> Soybean cotyledon fiber contained 82% moisture, 6.4% (dwb) protein, 3.3% (dwb) oil, and 4.3% (dwb) ash. The proximate analysis of soy skim was 11% solids, 56.0% (dwb) protein, 9.0% (dwb) oil, and 13.0% (dwb) ash.<sup>24</sup> Insoluble materials in soy skim were removed according to Yao et al.<sup>3</sup> to enable the recovery of only the fungal mycelia after SmF. Fully refined soybean oil and flaxseed oil were purchased from local grocery stores. All chemicals and medium ingredients were

purchased from Fisher Scientific (Pittsburgh, PA), Sigma Chemicals (St. Louis, MO) or BD (Franklin Lakes, NJ). Soybean hulls were provided by MicroSoy® Corporation (Jefferson, IA).

**Solid-State Fermentation.** Inoculum of *P. irregulare* at 10% (v/v) was transferred to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium made of 3% glucose and 1% yeast extract. The flask was shaken at 150 rpm in a MAXQ Mini 4450 orbital shaker (Thermo Scientific, Asheville, NC) at 25 °C for 2 days. The 2-day cultivated medium was homogenized with a bamix® M133 hand held homogenizer (Mettlen TG, Switzerland) and 10% (v/w) of homogenized medium was used to inoculate a 40 g (as-is) of substrate and the pH was adjusted to 6.0. SSF was conducted by incubating the substrates at 25 °C for different length of time as described below. A water reservoir was placed in the incubator to maintain the moisture level of the substrate. All the fermented materials were vacuum oven dried overnight and ground into powder in a coffee grinder for fatty acid analysis by GC. All treatments as discussed below were done in triplicate.

*Effect of Substrate Moisture Content on PUFA Formation.* The moisture content of freeze-dried soybean fiber substrate was adjusted to various levels (40, 50, 60, and 70%). Soybean hulls were added at 5% (dwb) to improve substrate porosity. The substrates were supplemented with 4% glucose (dwb). SSF was done at 25 °C for 7 days.

*Effect of Incubation Time with Limited Glucose (4%) and Soybean Oil (15%) Supplementation on PUFA Formation.* The freeze-dried soybean fiber was hydrated to moisture content of 70%. Soybean hulls were added at 5% (dwb). The substrates were

supplemented with 15% soybean oil (w/w, dwb) and 4% glucose (dwb) and incubated for 7, 12, and 20 days.

*Effect of Glucose Addition on PUFA Formation.* Soybean cotyledon fiber (as-is) with moisture content of 82% was adjusted to 75% using soybean hulls. Glucose was added to the substrates at concentrations of 4, 6, 8, and 10% (dwb) to examine PUFA production. The substrates were incubated at 25 °C for 12 days.

*Effect of Incubation Time with Sufficient Glucose (8%) Supplementation on PUFA Formation.* The moisture content of soybean cotyledon fiber was adjusted to 75% using soybean hulls. The substrates were supplemented with 8% glucose (dwb) and incubated for 7, 12, 17, 22, and 27 days at 25 °C to investigate PUFA formation.

*Effect of Vegetable Oil Supplementation on PUFA Formation.* The substrate moisture content was adjusted to 75% using soybean hulls. Flaxseed oil and soybean oil were added individually at the concentrations of 3, 6, and 10% (w/w, dwb) to examine PUFA formation. The substrates were incubated at 25 °C for 12 days.

**Submerged Fermentation.** Inoculum of *P. irregulare* at 10% (v/v) was transferred to 250 mL Erlenmeyer flask containing 100 mL of liquid medium. The pH of the medium was adjusted to 6.0 and the flask was shaken at 150 rpm in an orbital shaker at 25 °C for 7 days. The fungal mycelia were collected at the end of the fermentation by vacuum filtration through Whatman No. 1 filter paper and washed with distilled water. The washed mycelia were dried in vacuum oven overnight and ground into powder in a coffee grinder for fatty acid analysis.

*Effect of Glucose and Vegetable Oil Supplementation in Soy Skim on PUFA*

*Formation.* The control medium was made of 3% glucose and 1% yeast extract. Soy skim, 1:1 diluted with water was used in all treatments, with glucose added accordingly to the selected C/N ratio of 12. Soybean oil and flaxseed oil were added to the soy skim medium individually at levels of 3, 5, and 7% (w/v), along with 0.1% (w/v) of Tween 80<sup>TM</sup> as an emulsifier to allow the dispersion of oil in the medium. No additional nitrogen source was added in the soy skim medium. All treatments were done in duplicate.

**Fatty Acid Analysis.** The fatty acid composition of all fermented materials and fungal biomass was analyzed by GC. Briefly, 0.5 gram of ground solid-state fermented material was mixed with 6 mL of methanol containing 6% (by volume) sulfuric acid, whereas 3% sulfuric acid in methanol was used to react with 0.5 gram of ground fungal biomass from SmF. The mixture was heated at 60 °C for approximately 20 hours. The resulting fatty acid methyl ester (FAME) containing internal standard of 17:0 methyl ester was extracted with 2 mL of hexane and washed with 18 mL of distilled water. FAME sample of 1 µL was injected into an HP 5890 Series II GC (Hewlett-Packard, PA, USA) equipped with a SP-2340 fused silica column (60 m x 0.25 mm and 0.2 µm film thickness) (Supelco, Bellefonte, PA, USA). The injector and flame ionization detector were at 230 °C and the oven was programmed from 100 to 220 °C at a rate of 4 °C/min. The carrier gas (helium) flow rate was 2.9 mL/min and the split ratio was 50:1.

**Determination of Dry Fungal Biomass, Protein, Oil, and Ash Contents.** The dry fungal biomass was collected by filtration with Whatman No. 1 filter paper and determined

by weighing after vacuum oven drying overnight. Protein content was determined using the Dumas nitrogen combustion method with an ElementarVario MAXCN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) with a conversion factor of 6.25. Oil content was determined by acid hydrolysis method (AOAC official method 922.06). Ash content was determined by heating the samples at 550 °C overnight (AOAC 923.03). The total carbohydrate content was calculated by subtracting the protein, oil, and ash contents from 100%.

**Statistical Analysis.** All treatments were repeated as described under each experiment. The data were analyzed by ANOVA (Analysis of Variance) using SAS (Version 9.1, SAS Institute Inc., Cary, NC) to test significance of difference among all treatments at  $P = 5\%$ .

## RESULTS AND DISCUSSION

### Solid-State Fermentation

*Effect of Substrate Moisture Content on PUFA Formation.* SSF of soybean cotyledon fiber with various moisture contents was conducted and the fatty acid composition of the fermented materials is presented in Table 1. The total FAME in all the fermented materials was lower than that in the unfermented soybean fiber. According to Jacobs et al., oleaginous fungi utilized lipids as an energy source for growth under the substrate carbon limitation condition.<sup>25</sup> This suggests that the 4% (dwb) glucose added to the soybean fiber might not be sufficient to support the growth of *P. irregulare* and lipids contained in soybean fiber were consumed during SSF. The fungus in low moisture substrates was not able to grow and

synthesize PUFA. Moisture content was reported as one of the important parameters in SSF because low moisture content limits the growth and metabolism of microorganisms.<sup>26</sup> Both ARA and EPA were found only in the fermented soybean fiber with moisture content of 60% and 70%, with the later producing more PUFA. These results correlated to the visual observation of growth, where fungal mycelia covered only the high moisture substrates. Similar finding was reported that conversion of barley substrate into lipids increased with the increase of moisture level from 50% up to 75% in SSF by *Pythium ultimum*.<sup>27</sup> Large standard deviation was found in the treatment of 60% moisture content and this was mostly caused by the lack of homogeneity in the substrate. Compared to the previous batch of SSF (data not shown) without glucose addition, substrates supplemented with 4% glucose improved the SSF of soybean fiber with the production of PUFA. Optimization of glucose level in soybean fiber in SSF was then performed and the results are shown in the later discussion.

#### *Effect of Incubation Time with Limited Glucose (4%) and Soybean Oil (15%)*

*Supplementation on PUFA Formation.* Due to the low lipid (total FAME) content in the soybean fiber (3.3%, dwb), the addition of oil might serve as an energy source for fungal growth when the simple carbon source in substrate is depleted. Moreover, external vegetable oil was shown to act as precursor for the stimulation of PUFA in different SSF studies by *Mortierella* species.<sup>25,28,29</sup> High level of soybean oil, i.e. 15% (w/w, dwb), was used to supplement the substrate in this experiment. Investigation was done to examine PUFA production as affected by incubation time. As indicated in Table 2, the percentages of ARA and EPA increased with the increase of SSF incubation time. This trend demonstrates that this fungus was able to utilize the additional soybean oil as energy source to support its

growth and synthesize more PUFA when the sugar carbon source in substrate was becoming limiting during the long incubation period. The negative values of the theoretical new FAME of fermented soybean fiber further support this explanation. The theoretical new FAME was calculated by subtracting the oil (as FAME) added to the substrate from the total FAME in the fermented materials. The higher the negative value, the more lipids the fungus utilized to support its growth. Jacobs et al.<sup>29</sup> explained that lipid content after fermentation could be either decreased as lipids were utilized as energy source for cell growth or increased when lipids accumulated in the biomass, depending on the fungal strain and its growth phase. In this case, the external oil was consumed by *P. irregulare* to grow.

It is also shown in Figure 1 that the higher the ARA and EPA yield, the lower the total FAME in biomass were quantified after SSF. This fungus was able to utilize the additional lipids in substrate to synthesize PUFA. Based on the insignificant difference between the total yields of ARA and EPA in 12 and 20 days, 12 days of SSF was chosen for the next experiment.

*Effect of Glucose Addition on PUFA Formation.* Since substrates with high moisture content were more favorable (Table 1), the as-is soybean fiber with moisture content of 82% was used in the following experiments to avoid freeze drying step. Based on our previous study<sup>2</sup> of best fungus growth at moisture content of 75% or 70% as in Table 1, soybean fiber was adjusted to a moisture content of 75% using soybean hulls, which acted as inert materials to improve the porosity of the substrate and promoted better growth of fungi.<sup>2</sup>

Previous results showed that 4% glucose might not be sufficient for *P. irregulare* to grow, therefore, different level of glucose was added to the substrate to investigate PUFA

production. Table 3 shows the fatty acid composition of these treatments. The average values of ARA and EPA percentages increased with the increase of glucose level from 4% to 8%, but the 10% glucose treatment showed a lower PUFA production. However, no significant difference was found among the 6% to 10% glucose in the ARA and EPA percentages. The intermediate level of 8% glucose addition was chosen for the next experiment. The results from this experiment support our earlier finding that higher simple carbon source in substrate might be required to support the fungal growth during SSF.

*Effect of Incubation Time with Sufficient Glucose (8%) Supplementation on PUFA Formation.* The previous experiment showed that with 15% soybean oil and 4% glucose supplementation, increase of SSF incubation time favored PUFA production, and 12-day was an appropriate duration for incubation. In this study, the effect of incubation time on PUFA production was examined when high amount of glucose (8%, dwb) was supplemented to the substrate without the addition of oil.

The increasing trend of ARA and EPA percentages found in the previous SSF (Table 2) was not observed in this experiment, as shown in Table 4. *P. irregulare* was not able to further produce PUFA when the simple carbon source was quickly depleted without external oil supply. As shown in Figure 2, there were significant decreases in total FAME and ARA and EPA yields in the fermented soybean fiber incubated longer than 7 days. This trend demonstrates that significant amount of lipids in substrate was utilized to prolong the fungal growth. Cantrell and Walker also reported that the reduction in lipid and biomass during a long fermentation period could be caused by the potential death of cells after no available carbon source was provided.<sup>30</sup>



The percentages of ARA and EPA in the fermented materials that were incubated for 7 and 12 days in this experiment (Table 4) were much higher compared to the ARA and EPA values in Table 2. This indicates that when *P. irregulare* has to synthesize lipids *de novo* from the simple glucose carbon source, it naturally produces more ARA and EPA. When significant amount of external vegetable oil was used as in previous experiment, the unutilized oil in the substrate may dilute the ARA and EPA percentages in the final products. Therefore, the ARA and EPA yield (mg/g) in the final products may be a better indicator than their percentages when evaluating the effect of vegetable oil supplementation on PUFA formation. Even without oil supplementation, this fungus was able to produce ARA in a yield of 1.3 mg/g and EPA in a yield of 1.6 mg/g in 7-day fermented soybean fiber (Figure 2). These values were not substantially different from the ARA and EPA yield of 1.0 mg/g and 1.7 mg/g in the oil enriched substrate with the same SSF period as shown in Figure 1. Addition of oil might be beneficial only when the substrates are carbon limited or the samples are incubated for a long period.

*Effect of Vegetable Oil Supplementation on PUFA Formation.* In order to further investigate the effect of oil and glucose supplementation on PUFA formation, different levels of flaxseed oil and soybean oil were supplemented to the glucose sufficient (8%, dwb) substrate. The SSF was conducted for 12 days based on the previous result with the oil supplementation showing 12 days was a proper duration (Table 2). Flaxseed oil contains high level of  $\alpha$ -linolenic acid (C18:3) whereas soybean oil is rich in linoleic acid (C18:2). These fatty acids can act as precursors for PUFA production in SSF.

As shown in Table 5, the percentages of ARA and EPA in treatments with vegetable oil addition were lower compared to the control group with only glucose added. Flaxseed oil and soybean oil did not seem to stimulate the elongation and desaturation to produce more ARA and EPA. However, this could also be due to the dilution effect of the true vegetable oil that was not uptaken by the fungus. When the total FAME was examined, there were some increased formations with the increase of vegetable oil addition. This trend showed that the supplemented oil was not consumed by this fungus or simply being stored in the fungal mycelia without producing more PUFA. The earlier discussion (Table 2) showed that when the 4% glucose was supplemented to substrate, additional soybean oil was utilized and that led to the negative values of the theoretical new FAME. The positive values of the theoretical new FAME shown in this experiment indicate that the fungus may not have consumed significant amount of external oil when the simple carbon (sugar) was sufficient. However, the reducing trend in theoretical values indicates that the fungus may have still consumed more oils in the substrates that supplemented with higher level of oil. On the other hand, this reduction trend could also be explained by the high level of vegetable oil inhibited the fungal growth and decreased the ability to synthesize new oil.

ARA and EPA percentages in the fermented soybean fiber supplemented with 6% flaxseed oil were higher compared to the other two levels of supplementation but it showed no significant difference compared to control. On the other hand, no significant difference was found among the fermented substrates with 6% and 10% soybean oil addition. No ARA or EPA was found in the treatment with 3% soybean oil. The main drawback of SSF, the lack of homogeneity in substrate, may have affected the fungal growth and resulted in the large standard deviation in some treatments. Moreover, the added vegetable oil might not have

been well dispersed in the solid substrates and caused the inconsistency in the SSF. The higher ARA and EPA yield in the substrate treated with 6% flaxseed oil, 6% and 10% soybean oil compared to the control were due to the external oil addition and oil synthesis. Our results were different from the *Pythium ultimum* SSF study that showed increases in percentages as well as total yield of ARA and EPA in a nutrient enriched barley substrate with the addition of flaxseed oil.<sup>27</sup> Since only glucose was added to the soybean fiber in our study, other nutrients in soybean fiber might not be sufficient or in a form that was not readily available to support the fungal growth.

In general, we found that addition of vegetable oil did not stimulate the elongation and desaturation significantly for long chain PUFA formation by SSF when the substrate was supplemented with sufficient simple carbon source. Based on the overall results, soybean fiber with 8% (dwb) glucose supplementation for 7-day SSF produced 1.3 mg of ARA and 1.6 mg of EPA in each gram of dried substrate. Such fermented product was analyzed for proximate composition. As shown in Table 6, only moderate changes were found in the fermented substrate after SSF by *P. irregulare*. Figure 3 shows the vigorous fungal growth for this treatment.

Our previous study showed that fiber content in soybean fiber could be decreased significantly by a mixed culture SSF of lignocellulose-degrading fungi.<sup>31</sup> Results from this study further demonstrate that soybean fiber could be used as a substrate for *P. irregulare* SSF for long chain PUFA formation if a simple sugar is present in a sufficient quantity. Therefore, there is a great potential to combine these two types of SSF to produce value-added fermented product as animal feed.

## Submerged Fermentation

### *Effect of Glucose and Vegetable Oil Supplementation in Soy Skim on PUFA*

*Formation.* Although our results showed that soybean cotyledon fiber could be used as a substrate for *P. irregulare* SSF, the fungal biomass and substrate could not be separated from each other after SSF to allow the analysis of oil in fungus cells. To further investigate the oil quantity and composition of fungal biomass, SmF by using soy skim, the other co-product from soybean EAEP, was used as the nitrogen-rich liquid medium. According to Yao et al.<sup>3</sup>, replacing water with soy skim in corn ethanol fermentation resulted in an increased ethanol production rate. Soy skim with the insoluble materials removed contained 8.9% solids, of which 56.5% (dwb) was protein and a small amount of soluble sugars, mainly sucrose and stachyose.<sup>3</sup> Yao et al.<sup>3</sup> also indicated that 91% of peptides in soy skim were smaller than 17 kDa compared to 98% in peptone, with 70% of peptides in soy skim had molecular weight (MW) < 1.35 kDa. Since soy skim fractions were shown to contain adequate nitrogenous nutrients for yeast metabolism, no additional nitrogen was added to the soy skim medium prior to *P. irregulare* SmF.

In addition to nitrogen source, carbon source was reported as essential in the growth of *P. irregulare*.<sup>19</sup> Glucose was added to the soy skim medium to adjust the C/N ratio to 12 as in the control medium. Since SSF of soybean cotyledon fiber by *P. irregulare* showed that addition of vegetable oil was not beneficial in the presence of sufficient glucose supply, the soy skim media were supplemented with only one third of the simple carbon (sugar) as for the control for all the treatments with vegetable oil addition. This experiment allows a further investigation of the effect of vegetable oil on the long chain PUFA production in SmF

without the interference of the unused oil. The media information of all the treatments is included in Table 7.

As shown in Figure 4, no mycelia were present in the soy skim medium without oil addition. Even though the C/N ratio of the soy skim medium was identical to the control medium, the nutrients may not be utilized by *P. irregulare* or some inhibitory factors might be present in the soy skim fraction. The free amino acid and small peptides in soy skim that can be used by yeast may not be utilized by *P. irregulare*. Although the MW range of the small peptides in soy skim was similar to that of peptone<sup>3</sup>, the MW range of the peptides in peptone and yeast extract are different. Based on the BD Bionutrients™ Technical Manual (Franklin Lakes, NJ)<sup>32</sup>, approximately 62% of peptides present in yeast extract are in the MW range < 250 Da as compared to 15% in peptone. Most of the peptides (~67%) in peptone are in the MW range of 500-5000 Da. Bajpai and Bajpai reported that yeast extract was the best nitrogen source for ARA production by *Mortierella* fungi.<sup>33</sup> Liang et al. reported that rendered animal protein tend to be too large to be transported across microorganism cell membrane into cytoplasm and found that smaller peptides after protein hydrolysis favored the growth of *P. irregulare*.<sup>20</sup> Our findings also illustrated that *P. irregulare* might have only utilized the small peptides in yeast extract in the control medium and not the larger peptides in soy skim medium. The filtrates collected after harvesting the mycelia were characterized for nitrogen (N) content and the data is presented in Table 8. N content in control media decreased substantially after fermentation, indicating the fungus utilized it to grow. However, such decrease was not found in the soy skim media. Thus, N composition profile might be the limiting factor for the growth of *P. irregulare*. This may also be the reason for poor formation of ARA and EPA in SSF.

To determine if inhibitory factors or N composition profile was the main reason of the poor fungal growth in soy skim medium, an experiment was conducted by supplementing the soy skim medium with equal amount of yeast extract and glucose as for control medium. The fungal growth in the soy skim after 7 days of SmF was poor and the mycelia harvested from such medium was only 6.7% of the fungal biomass collected from control medium. This result confirmed the presence of inhibitory factors in soy skim and identification of the inhibitors are needed.

Even though some inhibitory factors may have prevented the growth of *P. irregulare* in soy skim fraction, the addition of vegetable oil was shown to overcome such factors. As shown in Figure 4, fungal biomass from the oil enriched media increased compared to that of the control. This fungus was able to utilize the external oil to grow and accumulate it in the mycelia by SmF. This observation agreed with our earlier findings in SSF that when carbon source was depleted, this fungus was able to utilize external oil as energy to grow. These results were also similar to the studies that showed increases in fungal biomass in the media with the addition of soybean oil and flaxseed oil due to the storage of excess oil in the fungal cells after *P. irregulare* SmF.<sup>17,19</sup> The fungal biomass from soybean oil enriched media increased with the increase of soybean oil concentration, with 7% (w/v) soybean oil resulted in biomass of 37 g/L after 7 days of SmF. Such trend was not observed in the media with the addition of flaxseed oil. Fungal biomass collected from the medium with 3% (w/v) flaxseed oil supplementation was significantly higher than the 5% (w/v) flaxseed oil but not significantly different from the medium with 7% (w/v) flaxseed oil addition.

Fatty acid composition of the fungal biomass harvested from different treatments after 7 days of SmF is presented in Table 7. Total FAME in all vegetable oil enriched media

were higher than in control, indicating that the fungus was able to accumulate part of the added oil into the mycelia. Nonetheless, the fatty acids were not extensively elongated or desaturated into long chain PUFA and caused the low percentages of ARA and EPA. The unmodified oil in the mycelia may dilute the ARA and EPA percentages. High levels of linoleic acid (C18:2) in soybean oil treatment and  $\alpha$ -linolenic acid (C18:3) in flaxseed oil treatment were the results of these oil accumulation in mycelia. Fungus might be able to grow better and synthesize more ARA and EPA if the inhibitors could be identified and removed from the medium. It also seems that the addition of vegetable oil partially relieved the inhibitory effect of the soy skim, since soy skim supplemented with yeast extract but with no oil had very poor fungal growth.

It should be noted in Table 7 that ARA contents were higher in the soybean oil enriched media and EPA contents were higher in the media with addition of flaxseed oil. This explains that *P. irregulare* utilized linoleic acid and  $\alpha$ -linolenic acid, respectively, as external precursors and synthesized ARA through the n-6 pathway and EPA through the n-3 pathway in PUFA biosynthesis.

Overall, soybean oil addition led to higher ARA and EPA yield compared to flaxseed oil as shown in Figure 4. Supplementation of 7% (w/v) soybean oil in the soy skim medium resulted in a total ARA yield of 125.7 mg/L and a EPA yield of 92.4 mg/L, which was no significantly different compared to the yield from the control medium. This treatment yielded a fungal biomass containing 0.35% ARA and 0.25% EPA. Fungal growth in such treatment is illustrated in Figure 3. These findings suggest soy skim from soybean EAEP could be utilized in fungal fermentation to synthesize ARA and EPA.

In general, *P. irregulare* was able to utilize the external oil to prolong the fermentation and synthesize long chain PUFA when simple carbon source in soybean fiber is limited in SSF. However, benefits from oil supplementation in stimulating PUFA formation were not apparent when the substrate was supplied with sufficient sugar carbon. On the other hand, this fungus was able to grow in the carbon-deficient soy skim with the oil addition by SmF. Oil seemed to make the fungus tolerable to the inhibitory factors that may be present in the soy skim. Further identification of inhibitors in soy skim is needed to develop a most suitable soy skim medium to generate ARA and EPA and at the same time make the full utilization of this nutrient-rich by-product from soybean processing. As a conclusion, both soybean cotyledon fiber and soy skim, co-products from aqueous soybean processing, have shown a promise in producing ARA and EPA by *P. irregulare* through different fermentation systems.

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Table 1. Effect of substrate moisture content on fatty acid composition of soybean fiber after 7 days of SSF.

Fatty acid	Unfermented material <sup>b</sup>	Moisture content (%)			
		40	50	60	70
14:0	0.1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	2.2 ± 1.4
16:0	15.8	15.9 ± 0.3	16.5 ± 0.9	17.5 ± 1.0	18.7 ± 3.5
16:1	0.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	1.0 ± 0.6
18:0	7.7	7.2 ± 0.3	6.8 ± 0.5	8.3 ± 0.1	6.8 ± 0.5
% of Total FAME					
18:1	28.4	27.5 ± 0.3	28.0 ± 0.4	29.7 ± 0.2	27.8 ± 0.2
18:2	41.7	42.2 ± 0.6	40.8 ± 1.6	36.9 ± 1.7	32.2 ± 4.9
18:3	4.2	4.8 ± 0.0	4.5 ± 0.2	4.0 ± 0.2	3.9 ± 0.4
20:4	ND <sup>c</sup>	ND	ND	0.1 ± 0.2	1.5 ± 0.5
20:5	ND	ND	ND	0.2 ± 0.3	2.0 ± 0.5
Others	1.9	2.3 ± 0.6	3.5 ± 0.8	2.8 ± 0.7	3.9 ± 1.7
Total FAME <sup>a</sup> (mg/g)	32.8	26.5 ± 3.8	23.7 ± 0.8	25.4 ± 0.6	27.9 ± 1.5
ARA yield (mg/g)	ND	ND	ND	0.0 ± 0.1	0.4 ± 0.1
EPA yield (mg/g)	ND	ND	ND	0.0 ± 0.1	0.5 ± 0.1

N=3. Data are presented as Mean ± SD.

<sup>a</sup> Total fatty acid methyl ester.

<sup>b</sup> Unfermented material composition (% dwb): Soybean fiber, 95; soybean hulls, 5.

<sup>c</sup> ND: Not detectable.

Table 2. Effect of SSF incubation time on fatty acid composition of soybean fiber with 4% glucose and 15% soybean oil supplementation.

Fatty acid	Incubation time (day)					
	7		12		20	
14:0	0.7	± 0.1	1.0	± 0.1	0.9	± 0.1
16:0	12.8	± 0.2	11.3	± 0.9	10.3	± 0.5
16:1	0.4	± 0.0	0.4	± 0.1	0.3	± 0.0
18:0	5.9	± 0.2	5.5	± 0.4	4.6	± 0.3
18:1	28.6	± 0.4	27.1	± 0.6	25.7	± 1.1
18:2	41.4	± 1.6	42.6	± 1.7	42.8	± 1.2
18:3 $\gamma$	0.3	± 0.1	0.3	± 0.0	0.2	± 0.0
18:3	4.6	± 0.4	4.2	± 0.1	4.0	± 0.1
20:4	0.9	± 0.3 a	1.4	± 0.0 a	2.6	± 0.3 b
20:5	1.5	± 0.5 a	2.8	± 0.2 b	4.6	± 0.6 c
Others	2.9	± 0.3	3.4	± 0.2	3.9	± 0.6
Total FAME <sup>a</sup> (mg/g)	113.7	± 2.5 a	107.3	± 16.4 a	66.4	± 4.9 b
Theoretical new FAME <sup>b</sup> (mg/g)	-1.1	± 2.5	-7.5	± 16.4	-48.4	± 4.9

N=3. Data are presented as Mean  $\pm$  SD. The values followed by the same letter in the same row are not significantly different at P=5%.

<sup>a</sup>Total fatty acid methyl ester.

<sup>b</sup>Total fatty acid methyl ester with the added oil (as FAME) subtracted.

Table 3. Effect of glucose addition on fatty acid composition of soybean fiber after 12 days of SSF.

Fatty acid	Unfermented material <sup>b</sup>	Glucose addition (% dwb)			
		4	6	8	10
14:0	0.1	0.8 ± 0.1	1.1 ± 0.4	1.4 ± 0.2	1.3 ± 0.2
16:0	17.0	13.2 ± 0.6	13.8 ± 1.4	14.0 ± 0.4	13.7 ± 1.1
16:1	0.2	0.5 ± 0.1	0.8 ± 0.3	1.0 ± 0.1	0.9 ± 0.2
18:0	9.3	5.5 ± 0.3	5.2 ± 0.2	4.9 ± 0.3	5.0 ± 0.1
% of 18:1	25.4	21.1 ± 0.6	21.5 ± 0.3	21.3 ± 0.7	21.3 ± 0.1
Total 18:2	39.4	42.8 ± 1.4	41.4 ± 4.5	38.6 ± 1.6	41.2 ± 3.3
FAME 18:3γ	ND	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:3	6.0	6.9 ± 0.5	6.4 ± 0.8	5.9 ± 0.4	6.5 ± 0.6
20:4	ND	2.6 ± 0.4 a	3.1 ± 1.1 a	3.9 ± 0.6 a	3.2 ± 0.9 a
20:5	ND	2.5 ± 0.4 b	2.8 ± 0.9 ab	3.8 ± 0.5 a	3.0 ± 0.7 ab
Others	2.5	4.0 ± 0.6	3.9 ± 0.9	4.9 ± 1.3	3.8 ± 0.5
Total FAME <sup>a</sup> (mg/g)	27.7	27.3 ± 3.1	28.9 ± 10.3	23.3 ± 3.2	32.5 ± 12.2
ARA yield (mg/g)	ND <sup>c</sup>	0.7 ± 0.0 d	0.8 ± 0.0 c	0.9 ± 0.0 b	1.0 ± 0.1 a
EPA yield (mg/g)	ND	0.7 ± 0.0 c	0.8 ± 0.1 bc	0.9 ± 0.1 ab	0.9 ± 0.1 a

N=3. Data are presented as Mean ± SD. The values followed by the same letter in the same row are not significantly different at P=5%.

<sup>a</sup> Total fatty acid methyl ester.

<sup>b</sup> Unfermented material composition (% dwb): Soybean fiber, 65.8; soybean hulls, 34.2.

<sup>c</sup> ND: Not detectable.

Table 4. Effect of SSF incubation time on fatty acid composition of soybean fiber with 8% glucose supplementation.

Fatty acid	Incubation time (day)					
	7	12	17	22	27	
14:0	3.0 ± 0.4	1.4 ± 0.2	1.4 ± 0.1	1.7 ± 0.2	1.4 ± 0.3	
16:0	16.9 ± 1.5	14.5 ± 1.0	14.5 ± 0.2	14.0 ± 0.3	14.3 ± 0.7	
16:1	2.2 ± 0.4	1.2 ± 0.1	0.8 ± 0.5	1.4 ± 0.2	1.1 ± 0.3	
18:0	4.9 ± 0.4	5.0 ± 0.2	5.2 ± 0.1	4.9 ± 0.1	5.1 ± 0.2	
% of						
Total	21.6 ± 1.0	20.8 ± 0.2	21.5 ± 0.1	21.5 ± 0.3	21.7 ± 0.2	
FAME	18:2	35.5 ± 2.6	38.3 ± 3.1	39.4 ± 0.9	38.8 ± 1.4	40.1 ± 2.2
	18:3γ	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
	18:3	5.9 ± 0.5	6.0 ± 0.8	6.3 ± 0.4	6.3 ± 0.6	6.6 ± 0.3
	20:4	2.7 ± 0.3 b	4.4 ± 0.9 a	3.6 ± 0.3 ab	3.5 ± 0.7 ab	2.9 ± 0.4 b
	20:5	3.3 ± 0.4 ab	3.8 ± 0.8 a	3.1 ± 0.2 ab	3.3 ± 0.8 ab	2.4 ± 0.4 b
	Others	3.7 ± 0.6	4.3 ± 0.8	4.2 ± 0.4	4.3 ± 0.5	4.4 ± 0.8
Total FAME <sup>a</sup> (mg/g)	47.2 ± 4.3 a	24.1 ± 5.2 b	23.1 ± 2.9 b	24.8 ± 3.1 b	23.5 ± 2.9 b	

N=3. Data are presented as Mean ± SD. The values followed by the same letter in the same row are not significantly different at P=5%.

<sup>a</sup>Total fatty acid methyl ester.

Table 5. Effect of vegetable oil supplementation on fatty acid composition of soybean fiber after 12 days of SSF.

Fatty acid	Treatment							
	Control <sup>c</sup>	3% FSO <sup>d</sup>	6% FSO	10% FSO	3% SBO <sup>e</sup>	6% SBO	10% SBO	
14:0	1.3 ± 0.1	0.5 ± 0.6	0.9 ± 0.2	0.2 ± 0.3	0.1 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	
16:0	12.6 ± 0.2	12.7 ± 0.2	10.5 ± 0.3	9.5 ± 0.3	13.3 ± 0.1	11.6 ± 0.2	11.4 ± 0.3	
16:1	1.1 ± 0.1	0.4 ± 0.5	0.7 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	
18:0	4.7 ± 0.1	5.8 ± 0.8	4.4 ± 0.1	5.0 ± 0.3	6.2 ± 0.0	5.1 ± 0.1	5.1 ± 0.1	
% of Total FAME	18:1	20.6 ± 0.2	24.5 ± 1.9	21.9 ± 0.3	23.6 ± 0.7	25.7 ± 0.1	24.0 ± 0.5	24.4 ± 0.5
	18:2	42.2 ± 0.8	34.7 ± 1.3	31.0 ± 1.7	27.8 ± 1.2	46.2 ± 0.2	46.1 ± 0.9	45.5 ± 0.7
	18:3 $\gamma$	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
	18:3	6.9 ± 0.1	16.7 ± 1.5	21.8 ± 0.7	30.5 ± 3.1	6.3 ± 0.1	6.1 ± 0.2	5.6 ± 0.2
	20:4	3.5 ± 0.4 a	1.0 ± 1.8 cd	2.7 ± 0.3 ab	0.6 ± 1.0 cd	0.0 ± 0.0 d	1.8 ± 0.3 bc	1.9 ± 0.3 bc
	20:5	3.2 ± 0.3 a	1.0 ± 1.8 cd	2.8 ± 0.4 ab	0.6 ± 1.0 cd	0.0 ± 0.0 d	1.6 ± 0.4 bc	1.9 ± 0.4 abc
	Others	3.8 ± 0.6	2.4 ± 1.0	3.0 ± 0.4	2.0 ± 0.7	2.1 ± 0.2	2.7 ± 0.3	3.1 ± 1.2
Total FAME <sup>a</sup> (mg/g)	33.1 ± 4.2	46.8 ± 5.1	61.3 ± 5.0	83.9 ± 8.2	55.4 ± 2.2	78.2 ± 9.4	92.3 ± 3.9	
Theoretical new FAME <sup>b</sup> (mg/g)	33.1 ± 4.2	23.2 ± 5.1	14.1 ± 5.0	5.2 ± 8.2	32.4 ± 2.2	32.3 ± 9.4	15.8 ± 3.9	
ARA yield (mg/g)	1.1 ± 0.0 ab	0.5 ± 0.9 bc	1.6 ± 0.1 a	0.4 ± 0.7 bc	0.0 ± 0.0 c	1.4 ± 0.2 a	1.7 ± 0.2 a	
EPA yield (mg/g)	1.1 ± 0.1 bc	0.5 ± 0.9 bc	1.7 ± 0.2 a	0.4 ± 0.8 bc	0.0 ± 0.0 c	1.2 ± 0.2 a	1.7 ± 0.3 a	

N=3. Data are presented as Mean ± SD. The values followed by the same letter in the same row are not significantly different at P=5%.

<sup>a</sup> Total fatty acid methyl ester.

<sup>b</sup> Total fatty acid methyl ester with the added oil (as FAME) subtracted.

<sup>c</sup> Fermented materials with 8% glucose added.

<sup>d</sup> FSO: Flaxseed oil.

<sup>e</sup> SBO: Soybean oil.



Table 6. Composition of soybean cotyledon fiber before and after *P. irregulare* SSF with 8% glucose addition for 7 days.

	Composition (% dwb)			
	Oil	Protein	Carbohydrate	Ash
Unfermented	4.9 ± 0.1	12.2 ± 0.3	78.7 ± 0.1	4.3 ± 0.3
Fermented	5.2 ± 0.2	10.4 ± 0.3	79.2 ± 0.7	5.2 ± 0.5

Table 7. Effect of glucose and vegetable oil supplementation in soy skim on fatty acid composition of fungal biomass after 7 days of SmF.

	Unfermented skim	Treatment								
		Control	Soy skim <sup>a</sup>	3% SBO <sup>b</sup>	5% SBO	7% SBO	3% FSO <sup>c</sup>	5% FSO	7% FSO	
Glucose (% w/v)		3	12.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Yeast extract (% w/v)		1	-	-	-	-	-	-	-	-
Soybean oil (% w/v)		-	-	3	5	7	-	-	-	-
Flaxseed oil (% w/v)		-	-	-	-	-	3	5	7	-
C/N ratio		12	12	9.1	12.9	16.7	9.1	12.9	16.7	-
Fatty acid composition										
	14:0	1.9	8.3 ± 0.3	ND	0.7	0.6 ± 0.0	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
	16:0	16.1	20.1 ± 0.5	ND	10.9	10.9 ± 0.1	11.0 ± 0.1	8.0 ± 0.0	7.8 ± 0.6	7.6 ± 0.6
	16:1	ND	7.1 ± 0.0	ND	0.4	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	18:0	6.7	3.3 ± 0.3	ND	4.2	4.3 ± 0.2	4.3 ± 0.0	4.8 ± 0.2	4.2 ± 0.1	4.1 ± 0.2
% of	18:1	18.3	17.2 ± 1.2	ND	23.1	23.2 ± 0.3	23.0 ± 0.0	25.6 ± 0.8	22.3 ± 0.0	21.3 ± 0.1
Total	18:2	48.8	16.4 ± 0.7	ND	50.1	50.6 ± 0.5	50.9 ± 0.7	17.9 ± 0.0	18.3 ± 0.7	17.6 ± 0.2
FAME	18:3 $\gamma$	ND	1.1 ± 0.0	ND	0.2	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
	18:3	6.4	2.7 ± 0.3	ND	6.7	6.7 ± 0.0	6.7 ± 0.0	40.2 ± 1.0	42.8 ± 0.2	44.9 ± 0.2
	20:4	ND	9.5 ± 0.1 a	ND	0.9 b	0.7 ± 0.0 bc	0.7 ± 0.2 bc	0.4 ± 0.1 d	0.6 ± 0.1 cd	0.6 ± 0.1 bcd
	20:5	ND	6.1 ± 0.3 a	ND	0.9 b	0.6 ± 0.0 b	0.5 ± 0.1 b	0.5 ± 0.1 b	0.9 ± 0.1 b	0.8 ± 0.3 b
	Others	1.9	8.0 ± 0.1	ND	2.0	1.8 ± 0.2	1.7 ± 0.1	1.9 ± 0.3	2.1 ± 0.3	1.9 ± 0.3
Total FAME <sup>d</sup> (mg/g)	2.2	175.3 ± 20.9	ND	433.1	479.3 ± 6.3	510.4 ± 5.0	341.6 ± 6.4	352.6 ± 31.9	374.4 ± 12.5	
ARA content (mg/g)	ND <sup>e</sup>	16.7 ± 2.2 a	ND	3.9b	3.4 ± 0.2 b	3.5 ± 0.9 b	1.3 ± 0.3 b	2.0 ± 0.0 b	2.3 ± 0.4 b	
EPA content (mg/g)	ND	10.7 ± 0.8 a	ND	3.9b	2.9 ± 0.1 bc	2.5 ± 0.5 bc	1.6 ± 0.3 c	3.0 ± 0.5 bc	3.1 ± 1.1 bc	

N=2. Data are presented as Mean  $\pm$  SD. The values followed by the same letter in the same row are not significantly different at P=5%. Only one replicate was presented in the treatment of 3% SBO.

<sup>a</sup> Soy skim was diluted with water at 1:1 ratio.

<sup>b</sup> SBO: Soybean oil.

<sup>c</sup> FSO: Flaxseed oil.

<sup>d</sup> Total fatty acid methyl ester.

<sup>e</sup> ND: Not detectable.

Table 8. Comparison of media N content before and after SmF.

	Treatment	N content <sup>a</sup> (g/L)
Unfermented media	Control <sup>b</sup>	1.1 ± 0.0
	Soy skim <sup>c</sup>	3.8 ± 0.0
Fermented media	Control	0.5 ± 0.4
	Soy skim	3.3 ± 0.2
	3% SBO <sup>d</sup> (w/v) + soy skim	3.3 ± 0.3
	5% SBO (w/v) + soy skim	2.8 ± 0.1
	7% SBO (w/v) + soy skim	2.7 ± 0.0
	3% FSO <sup>e</sup> (w/v) + soy skim	3.0 ± 0.0
	5% FSO (w/v) + soy skim	3.0 ± 0.1
7% FSO (w/v) + soy skim	2.8 ± 0.1	

<sup>a</sup> N content presented was determined by Dumas nitrogen combustion method with an ElementarVario MAXCN analyzer.

<sup>b</sup> Unfermented control medium contained 3% glucose with 1% yeast extract.

<sup>c</sup> Soy skim was diluted with water at 1:1 ratio.

<sup>d</sup> SBO: Soybean oil.

<sup>e</sup> FSO: Flaxseed oil.

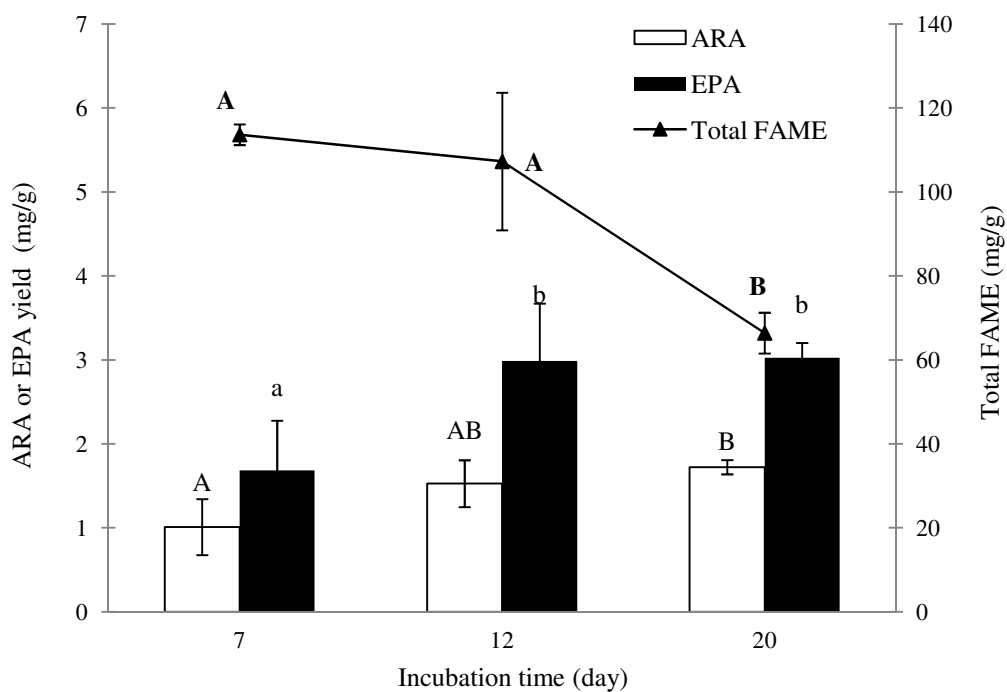


Figure 1. Effect of SSF incubation time on total FAME and yield of ARA and EPA of soybean fiber with 4% glucose and 15 % soybean oil supplementation. N=3. Data with the same letter within each series are not significantly different at P=5%.

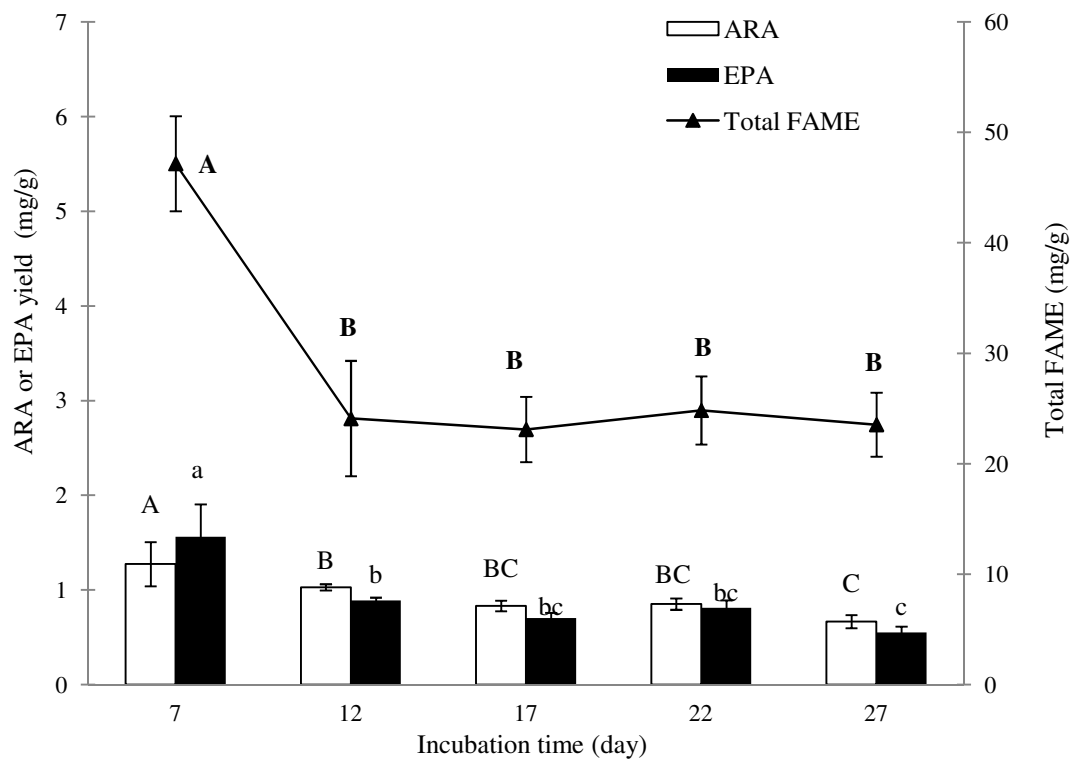


Figure 2. Effect of SSF incubation time on total FAME and yield of ARA and EPA of soybean fiber with 8% glucose supplementation. N=3. Data with the same letter within each series are not significantly different at P=5%.

(a)



(b)

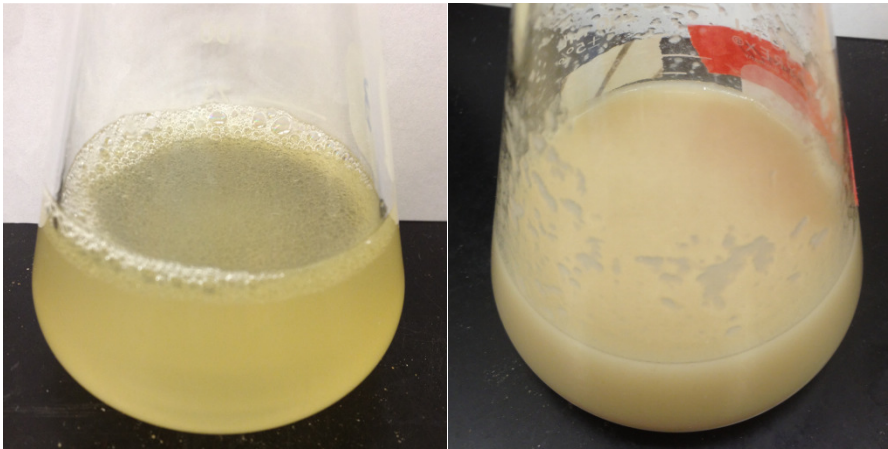


Figure 3. (a) Soybean fiber supplemented with 8% (dwb) glucose before (left) and after (right) 7 days of SSF. (b) Soy skim medium with 7% (w/v) soybean oil added before (left) and after (right) 7 days of SmF.

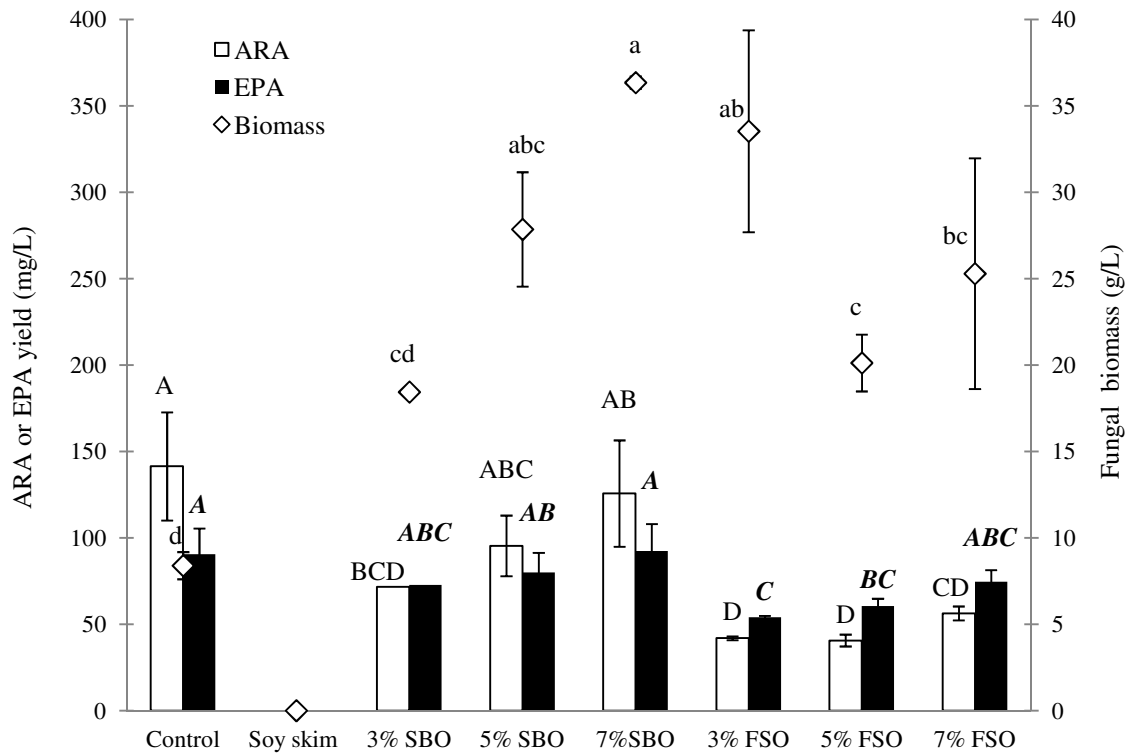


Figure 4. Effect of glucose and vegetable oil supplementation in soy skin on fungal biomass and yield of ARA and EPA after 7 days of SmF. N=2. Data with the same letter within each series are not significantly different at P=5%. Only one replicate was presented in the treatment of 3% SBO.



## CHAPTER 4. GENERAL CONCLUSIONS

Agro-industrial residues have been used in both solid-state fermentation (SSF) and submerged fermentation (SmF) to produce value-added products. Soybean cotyledon fiber is the fiber-rich fraction from enzyme-assisted aqueous extraction processing (EAEP) of soybeans and may contain all nutrients for SSF. Distiller's dried grains with soluble (DDGS) produced from corn ethanol fermentation contains concentrated amounts of unfermentable materials such as protein, oil, minerals, and vitamins, and it could be a good SSF substrate as well. The high fiber content in these co-products limits their use as non-ruminant feed. SSF by using mixed fungal culture may convert these materials to better feeds. We hypothesized that the nutrients in soybean cotyledon fiber and DDGS can support the growth of different fungi in SSF and the microbial enzymes secreted can potentially break down the fiber to improve their digestibility as non-ruminant feed.

In our first study, we found synergistic effect among the three fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium*, when they were inoculated in the soybean cotyledon fiber and DDGS in SSF. The inoculation sequence of different fungi was identified as an important factor to allow the best interaction among the fungi to achieve vigorous growth and high enzyme production. Combination of fungi with the inoculation of *T. reesei* and *P. chrysosporium* for 36 hours, followed by *A. oryzae* for additional 108 hours was shown to be the best for both soybean cotyledon fiber and DDGS SSF. Large scale SSF with the same inoculation sequence of fungi was conducted and the fermented materials had 3.5-15.1% lower fiber and 1.3-4.2% higher protein content after SSF, demonstrating the potential for non-ruminant feed improvement.

To further enhance the nutritional value of soybean cotyledon fiber and soy skim fractions, *Pythium irregulare* was employed in different fermentation systems for arachidonic acid (ARA) and eicosapentaenoic acid (EPA) production in the second study. When soybean fiber was used as the substrate in SSF by *P. irregulare*, parameters such as moisture content, substrate glucose addition, incubation time, and vegetable oil supplementation were found to affect the production of ARA and EPA. High moisture content and glucose supplementation in soybean cotyledon fiber favored the long chain polyunsaturated fatty acid (PUFA) production. The level of glucose in soybean fiber could affect the utilization of external oils by *P. irregulare* in producing PUFA in SSF. Soybean fiber with 8% (dwb) of glucose supplementation for 7-day SSF produced 1.3 mg of ARA and 1.6 mg of EPA in every gram of dried substrate. No substantial difference was found in other composition of fermented soybean fiber, demonstrating its potential use as enriched animal feed. When soy skim was used in the SmF, *P. irregulare* was able to grow in the carbon-deficient medium with the external oil addition. The data also suggests that oil overcame the inhibitory factors that may present in the soy skim. Total ARA yield of 125.7 mg/L and EPA yield of 92.4 mg/L were achieved with the supplementation of 7% (w/v) soybean oil in soy skim medium. This treatment also yielded a fungal biomass containing 0.35% ARA and 0.25% EPA. Overall, both fiber and skim co-products from aqueous soybean processing have shown promising results in producing ARA and EPA by *P. irregulare* through different fermentation systems. Further identification of inhibitors in the soy skim is needed to fully utilize this low-value co-product.

Although both soybean EAEP co-products were shown to be suitable substrate and medium in fermentations, the PUFA yield was unexpectedly low. The concentration of the

PUFA-rich oil used in different feeding studies was at least 1% of the total diet. The best treatments from this research led to the fermented soybean fiber containing 0.13% ARA and 0.16% EPA by SSF, and fungal biomass containing 0.35% ARA and 0.25% EPA by SmF. Therefore, optimization of strains and nutrient requirements of the fungus is needed to achieve meaningful PUFA enrichment in these soy co-products.

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