PRODUCTION OF CARBOHYDRASES FOR DEVELOPING SOY MEAL AS PROTEIN SOURCE FOR ANIMAL FEED

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PRODUCTION OF CARBOHYDRASES FOR DEVELOPING SOY MEAL AS
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ABSTRACT

Global demand for seafood is growing rapidly and more than 40% of the demand is met by aquaculture. Conventional aquaculture diet used fishmeal as the protein source. The limited production of fishmeal cannot meet the increase of aquaculture production. Therefore, it is desirable to partially or totally replace fishmeal with less-expensive protein sources, such as poultry by-product meal, feather meal blood meal, or meat and bone meal. However, these feeds are deficient in one or more of the essential amino acids, especially lysine, isoleucine and methionine. And, animal protein sources are increasingly less acceptable due to health concerns. One option is to utilize a sustainable, economic and safe plant protein sources, such as soybean. The soybean industry has been very prominent in many countries in the last 20 years. The worldwide soybean production has increased 106% since 1996 to 2010[1].

Soybean protein is becoming the best choice of sustainable, economic and safe protein sources. Defatted soybean flour contains about 53% proteins and 32% carbohydrates. In order to get rid of the un-digestible and anti-nutritional factors and enrich protein content, the soybean flour needs processing before consumption. The soy proteins can be concentrated by hydrolyzing the carbohydrates through an enzymatic separation process. The soy proteins produced by the enzymatic separation process have much higher protein contents and therefore will make better aquaculture diet
formulations. Furthermore, possible indigestion problems for young animals or fish can be avoided with the hydrolysis of carbohydrates. The leftover hydrolyzed soluble carbohydrates can be used as carbon sources for microorganisms to produce biofuel and other value-added products. The objective of this project is to produce effective enzymes for soybean hydrolysis developing fungal fermentation technology. The producer screening, culturing conditions, inducers, enzyme assay and stabilities were investigated and optimized to maximize the yields and productivities of the enzymes.

*Trichoderma reesei* is an efficient producer of carbohydrate-degrading enzymes, including cellulase, xylanase, and polygalacturonase. Therefore, this study investigated methods induce more effective enzyme production with *T. reesei*. The effects of inclusion of defatted soybean flour and a variety of soluble and solid carbon sources were evaluated for their enzyme inducing abilities. The fermentation pH effect was also investigated. The enzyme mixture from *T. reesei* achieved 68% total carbohydrate and 40% reducing sugar conversion.

Because of the low reducing sugar conversion achieved with *T. reesei*, a strain screening was conducted to select a better microorganism for enzyme production. The production of carbohydrase activities with soybean hull induction was tested for 16 different fungal strains. Of those tested, *Aspergillus niger* NRRL 322 and *Aspergillus foetidus* NRRL 341 were selected as the most promising for producing effective enzyme mixtures to upgrade the soybean flour. The selection was based on both enzyme productivities and soy carbohydrate hydrolysis efficiency.

To achieve sufficient enzyme production, culture media and growth conditions
were subsequently studied with these two strains. Different inducers, carbon and nitrogen sources, the carbon to nitrogen ratio, and other medium compositions were investigated for optimization. For this submerged process, the pH, temperature, and dissolved oxygen concentration were also investigated to find the optimum production conditions. With the enzyme produced by the selected *A. foetidus* strain, 78% total carbohydrate and 76% reducing sugar conversion were obtained in the enzymatic hydrolysis process.

More accurate, consistent and efficient assays for pectinase and polygalacturonase activities were also developed. The enzyme stability and storage time effect were also studied. Besides the soy meal hydrolysis, our enzyme mixtures were also applied to soy hull hydrolysis to produce syrup with high sugar concentrations. The enzymes contained low levels of protease. The effect of this presence of protease on protein degradation during soy meal hydrolysis was evaluated using soy protein analysis methods.

Overall, this research developed a highly efficient method for producing carbohydrases that can separate soy protein from soy carbohydrates in a soy meal. Soy protein concentrate (SPC) with at least 70% protein content and soy protein isolate (SPI) with higher than 90% protein content were obtained in the hydrolysis reactions with 1 ml enzyme broth per g soy meal, even under a high solid loading condition such as 250 g/L initial soy meal. These protein products had much higher protein contents than the original 53% protein in soy meal. Besides the high protein products, a hydrolysate of soluble carbohydrates was produced with 76% reducing sugar conversion. The hydrolysate can find value-added applications to further increase the economic value of soy meal.
I would like to express my heartfelt appreciation and gratitude to my advisor, Dr. Lu-Kwang Ju. With his great patience, profound knowledge, professional perspective, and careful scientific attitude he has taught me about bioprocess engineering and made this research possible. I also would like to thank my committee members: Dr. Gang Cheng, Dr. Lingyun Liu, Dr. Jie Zheng, Dr. Ge Zhang and Dr. Pei-Yang Liu for their valuable comments and guidance.

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CHAPTER I
INTRODUCTION

Global demand for seafood is growing rapidly, with more than 40% of the demand met by aquaculture. The National Marine Fisheries Service estimates the U.S. seafood demand will increase to 8.75 MMT by 2025 based on population growth, while the wild harvest will be at less than 5 MMT [2]. Conventional aquaculture diets use fishmeal as the protein source. Fishmeal is the most expensive major ingredient in aquaculture feed and it is widely used as the main source of dietary protein for most commercially farmed fish species [3]. The limited production of fishmeal cannot meet the increase of aquaculture production. Therefore, people are trying to partially or totally replace the fishmeal in fish feed with less-expensive protein sources, such as poultry by-product meal, feather meal blood meal, and meat and bone meal. However, these replacements are deficient in one or more of the essential amino acids, especially lysine, isoleucine and methionine [3]. And, animal protein sources are increasingly less acceptable due to health concerns from the spread of TSEs (transferable spongiform encephalopathies), salmonellae, mycotoxins, and veterinary drug residues from terrestrial animals to fish [4]. Based on these disadvantages, people transferred to sustainable, economic and safe protein sources, the plant proteins, especially soybean source [5]. Soy protein is plant protein. Unlike many sources of animal protein, soy is low in saturated fat
and naturally cholesterol-free [6]. Compared the menhaden fishmeal, except the sulfur amino acids (methionine and lysine) the amino acid composition of soy protein is equal to or even more [7]. Low-antigen and high protein level is an important concern for soy protein for aquatic feeds.

According to the protein content, there are three primary groups of soy protein products: 1) soy flour, protein content is about 50-65% (dry basis); 2) soy protein concentrate (SPC), with 65-90% protein content (dry basis); 3) soy protein isolate (SPI), with protein content of 90% (dry basis) [8, 9]. Currently, white flakes or soybeans after solvent extraction and crushing are generally the starting materials for SPC and SPI. Other soybean meal and soy flour can be used as starting materials for the different final products meeting specific protein standards and desired functional properties. Usually the goal is to get higher protein collection and recovery. There are different methods to prepare SPC and SPI products, and people generally use several standard methods to prepare them, such as acid-washed SPC, alcohol-washed SPC, and acid-washed SPI [10, 11]. These methods are based on the solubility of soy protein. Yet, the contents of enriched protein products from these methods are not high and the process is complicated. And, there are about 40% loses during protein enrichment, which increases the cost [12]. On the other hand, raw soybean cannot be directly used as animal or aquatic feed because of the anti-nutritional factors (ANFs). Soybeans contain some ANFs, which needs processing before consumption. The manufacturing methods typically include alcohol extraction, enzyme degradation and extrusion. The different processes leads to slightly different anti-nutritional factor levels [13]. The heat can destroy the trypsin inhibitors and lectins easily. Some heat stable factors, except oligosaccharides and
antigens, have low amounts in soybean and are not likely to cause problems in feed. The oligosaccharides and antigens can be removed by extraction or enzyme treatment [14]. Soy flour contains about 53% proteins and 32% carbohydrate [15]. The most anti-nutritional part is the complex carbohydrate. Therefore, it is necessary to develop a simple, economic and high efficient protein enrichment method. Enzyme hydrolysis is an easy method that does not require organic solvents or extraction. And, the enzymatically produced protein product will have less ANFs; lower oligosaccharide content will improve digestibility in fish than chemical extraction methods.

For the enzyme hydrolysis method, the foremost part is the enzyme production. Fungi are well-known producers of carbohydrases. The objective of this project was to select the optimum fungus to produce the carbohydrate-degrading enzymes and develop an economically favored and high-yield enzyme production system. Then, the enzymes were applied to separate the soy proteins from carbohydrates. The enriched proteins collected from the enzyme-facilitated separation can be used as high-quality feed. The soluble and hydrolyzed carbohydrates can be used as carbon sources for microorganisms to produce valuable bio-products. The soy proteins produced by the enzymatic separation process had much higher protein contents and, therefore, make better aquaculture diet formulations. Furthermore, the possible indigestion problem particularly for young animals/fish can be avoided through this enzymatic process with the hydrolysis of hemi/celluloses. The soy carbohydrates produced from the enzymatic separation process can be converted to biofuel (ethanol, butanol) or other value-added chemicals such as arabitol. Arabitol is one of the Department of Energy's (DOE) identified top 12 biomass-derivable building blocks [16].
In this study, the fungal strain screening and selection was based on both enzyme production and sugar released from soy flour. The strain with the higher enzyme production and releasing sugar concentration was further investigated. The strains screening was operated in simple shake flasks. According to other studies, some strains are known as good for producing carbohydrate-degrading enzymes and these strains were included during the strain screening [17-19]. *A. niger* strains from NRRL (322, 328, 334, 341, 348, 363, 566, 599, 2270, 13201, 13219, 62517), *A. aculeatus* from NRRL (2053) and *Trichoderma reesei* Rut C-30 from NRRL (11460) were studied through shaker cultivation, enzyme production analysis and soy carbohydrate hydrolysis efficiency analysis. Theses strains were all cultured in the same culture conditions (inoculation amount, medium, shaker speed, and temperature). Then, the enzyme activities were analyzed to find the best enzyme producer. Finally, the enzyme broths were applied to the soy flour hydrolysis systems to analyze the reducing and total sugar release during hydrolysis. The strain releasing highest amount of reducing sugars was considered. With combined enzyme production and carbohydrate hydrolysis results, the strains with best soy carbohydrate hydrolysis efficiency were selected.

After the strain screening, the best producer strains were tested in the fermentation process to produce enzyme broth. The culture conditions of these strains were optimized. First, a shake flask study was carried out to compare different medium such as different carbon sources, nitrogen sources and other nutrition parts. Then, an optimum medium recipe was applied to a fermentor scale to further evaluate and optimize the nutrition factors for enzyme production. Fungus is able to grow on a wide range of carbon and nitrogen sources. In order to induce and improve the soy
carbohydrate-degrading enzyme production, a soy based medium was investigated. Other carbon sources and nitrogen sources were also be studied to compare with soy-based sources. It has been reported that cellulase and xylanase production can be induced by sophorose, cellobiose, xylobiose, D-xylose, L-sorbose, and lactose [20, 21]. And, pectinases production can be induced by some natural sources with high pectin content, such as sugar beet pulp [22]. The effect of fermentation medium pH is also very critical in inducing special products. Some studies have shown lower pH is good for cellulase production, whereas higher pH is good for xylanase [23]. Therefore, it is necessary to find the optimum pH control to harvest all these carbohydrate degrading enzymes. The dissolved oxygen and agitation are also important factors in submerged fermentation. Mixing by agitation in stirred-tank bioreactors not only contributes to the oxygen transfer, but also the shear damage to fungi cells [24]. Therefore, selecting an optimum agitation and oxygen control is very important in submerged fermentation. Different strains have their own optimum culture temperatures. Some A. niger species have highest production of cellulase at pH 4.0 at 35°C under submerged conditions. The growth and enzyme production of fungi are affected by variations in temperature [25]. In summary, to develop the fungi fermentation for enzyme production, the study of nutrition sources, inducer, fermentation condition such as pH, dissolved oxygen, agitation and temperature was investigated.

In all these analyses, the most important part is the enzyme production analysis; therefore, standard enzyme assays are really helpful. Seven groups of enzymes were analyzed: cellulase, xylanase, pectinase, polygalacturonase, α-galactosidase, protease, and sucrase. Cellulase, xylanase, and pectinase are respectively responsible for cellulose,
hemicellulose, and pectin parts of the soy carbohydrate. Polygalacturonase can break down the polygalacturonic acid backbone of pectin to monomers or oligomers. Sucrase and \( \alpha \)-galactosidase are used for breaking down the oligosaccharides, such as sucrose, raffinose, stachyose. It is necessary to find or develop standard enzyme assays. *A. niger* was reportedly the producer of protease and the effect of protease on soy protein was also investigated [26, 27]. After enzyme hydrolysis, the soy carbohydrates were degraded into digestible and soluble monomers and the soy protein was enriched. The objective is to enrich and collect as more protein as possible, so the protease effects on the protein content and properties was also evaluated.

In summary, the objective of this project was to produce effective enzymes for soybean hydrolysis by fungal fermentation. In order to achieve the main objective, different studies were investigated. Firstly, the enzyme production of *T. reesei* is explained in Chapter IV, based on the hypothesis that *T. reesei* is an efficient producer of carbohydrate-degrading enzymes cellulase, xylanase, and polygalacturonase. The objective of this section is to induce more effective enzyme production with *T. reesei*. The effects of inclusion of soybean hull, defatted soybean flour and a variety of soluble and solid carbon and nitrogen sources were evaluated. The pH effect on the enzyme production was also investigated. Optimum inducers were selected such as soy hull from this study. When the produced enzyme from optimum *T. reesei* fermentation was used for soy meal hydrolysis, the conversions of total carbohydrate and reducing sugar were investigated. The sugar conversion especially the reducing sugar conversion was too low with *T. reesei* enzymes. Therefore, the strain screening was investigated in Chapter V, which tried to select the optimum strain for better enzyme production to hydrolyze the
soy carbohydrate. This study was the first to evaluate and compare among different *Aspergillus* species and strains for the production of various carbohydrase activities under soybean hull induction. Based on both measurements of their enzyme productivities and soy carbohydrate hydrolysis efficiencies, best producers were selected as the most promising strains for producing the enzyme mixtures more effective for enzymatic upgrading of soybean meal. After the selection of optimum producer, the culture conditions to induce more enzyme production were investigated. In Chapter VI, the enzyme production from *A. niger* fermentations were evaluated to find the optimum nutrition and bioreactor control conditions. This study was the first to induce productions of five group carbohydrases simultaneously. And, a novel dissolved oxygen and pH gradient control was investigated in this study. The optimum enzyme production strategy for optimum producer was developed. And, better total sugar and reducing sugar conversion were obtained with these enzymes, which were much higher than the *T. reesei*. Yet, protease was also found in the fermentation product. Therefore the effect of protease on soy protein hydrolysis was studied in Chapter VII. In order to develop a concentrated carbohydrate syrup with enzyme hydrolysis, explained in Chapter VIII, the soy hydrolysis with our enzyme mixtures was studied. Our *A. niger* enzymes degraded all the oligosaccharides and disaccharides, which would be beneficial for syrup application with organisms that cannot digest those saccharides. In order to establish the standard enzyme analysis and store the enzymes in proper conditions, Chapter IX explains the enzyme assays that were investigated and the stabilities of enzymes during storage. Finally, in order to further induce the pectinase and α-galactosidase production, in Chapter X the effect of pH and temperature on pectinase and α-galactosidase production
by *A. niger* 322 fermentation was investigated, which also provided a new direction for future study.
2.1 Soybean

Soybean has been widely used as both a food and industrial feedstock, due to its sustainability, safety and low cost. There are a lot of valuable soy-based products, including dyes, oils, fillers, and protein.

2.1.1 Soybean Introduction

The soybean, together with wheat, maize, rice, barley, sugarcane, sorghum, potato, oats, cassava, sweet potato, and sugar beet, are a primary food source for mankind since ancient ages [28]. In human diets, soybean has been used as a protein resource for a long time. The history of soybean is not clear because there are not many records. There is still some evidence suggesting soybean was domesticated during the Zhou dynasty in China about 1000 years ago. Since then, soybean has become more familiar and popular to people in the worldwide. There are plenty of advantages such as versatile applications, multiple nutritional values, good taste, sustainability and so on.

Many researchers have focused on the nutrition and health functions of soybean in the past 20 years. Soybean is good at preventing osteoporosis [29], heart disease [30] and
cancer [31, 32]. Governments in America and Europe have encouraged people to eat more plant sourced foods to prevent the risk of diseases. Soy-based food is the very good choice, without any doubts. For example, soy can provide a more healthy protein source to meet the needs of people, instead of meat, milk and egg. Soy isoflavones is one kind of phytoestrogens that have beneficial functions for human health [33]. The further processing has an effect on the level of isoflavones. Raw soybeans, roasted soybeans, soy flour have similar isoflavone content, but soy protein concentrate and soy protein isolate have lower amounts because of the loss during processing. For example, the ethanol washing method to collect soy protein will lead more isoflavone loss than the water washing method [34]. There are some other nutritional components of soybean, such as soy peptide, soybean lecithin, soy oligosaccharide, and soy saponin [28]. Besides the nutritional and food usage, many products derived from soybean are used for the chemical industry.

The soybean industry has been very popular in many countries the last 20 years. The world soybean production has increased by 106% has increased 106% since 1996 to 2010 [1]. In summary, the soy-based products comprised oil products like refined oil and lecithin; whole bean products like full-fat flour and roasted soybean; soy protein based product like soymeal, SPC and SPI; and soy hull based product like some fibers [35].

2.1.2 Soybean Application

Soybeans were first domesticated in northeastern China about 5000 years ago. The Chinese were the first to crush soybeans into oil and cake by using mechanical presses. Soybean contains about 20% fat, about 40% of the protein, and vitamins together
with other nutrients, which can be made for sauce, soy sauce, and a variety of soy foods besides direct eating. The stems, leaves, soybean meal and crude soybean meal can be used for fertilizer and excellent livestock feed [36]. The processed proteins like tissue protein, protein concentrates, and fibrin are the raw material for a variety of foods, such as artificial meat [37], casein, monosodium glutamate, paper making [38], plastic industry [39], man-made fibers [40], gunpowder and other raw materials. Soybean oil is mainly for food, and it is also used for lubricating oil [41], paint [42], soap, enamel, synthetic rubber [43], preservatives and other important raw materials. There are many important and functional ingredients after soybean oil extraction, such as phospholipids and the use of stigmasterol provides cheap sterol hormone materials for the pharmaceutical industry [44]. The industry application of soybean is far more than these.

2.1.3 Soybean protein

Soy protein is a plant protein. It has a similar amino acid composition as milk protein. Except the methionine content is slightly lower, the contents of other essential amino acids in soy protein are equivalent to those in animal protein. Soy protein is thus complete in nutritional value derivable from proteins. Unlike many sources of animal protein, soy is low in saturated fat and naturally cholesterol-free [6]. According to FAO/WHO (1985), the results of human trials showed that soy protein is more suitable for meeting the human needs for essential amino acids [45]. According to differences in age, sex, weight, and type of work, people’s need of protein varies. In order to guide people's meals, all countries in the world combined with the actual situation of their own, respectively, figure out the recommended daily dietary supply amount (RDA). The U.S.
Food and Drug Administration (FDA) reported consumption of 25 grams of soy protein everyday will help to reduce the probabilities of cardiovascular and cerebrovascular diseases [46].

Soy protein can be used for many industrially useful products. In order to reduce trans-fat in deep-fried foods, a soy protein film coating was developed and evaluated, which is made of soy protein isolate [47]. Because of the finite petrochemical resources, it is necessary to develop eco-friendly materials and polymer materials made from agricultural processing products such as soy protein. Soy protein based plastic [48], adhesives [49] and coating are used for industrial applications. Soy protein adhesive was first investigated in 1923 by researchers, when a soy-based glue patent was granted [50]. Later, soy-based adhesives have become increasingly popular in many industrial applications such as wood and paper. Soy proteins are used in binders in coating and paints and as emulsifiers in colloidal rubber products, textile fiber, foams for fire extinguishers and lubricants [51]. Some novel soy protein based surfactants have been developed by the reaction of hydrolyzed soy protein and alkyl succinic anhydride. Advantages of these novel surfactants include surface active properties in lowering surface tension, foaming, emulsifying, wetting power and buffer ability [52]. Aquaculture industry is growing very fast in the world’s animal agricultural industry. Global demand for seafood is growing rapidly and more than 40% of the demand is met by aquaculture. Conventional aquaculture diets used fishmeal as the protein source [53]. The limited production of fishmeal cannot meet increase of aquaculture production. Therefore, people try to partially or totally replace fishmeal with less-expensive protein sources in fish feeds, such as poultry by-product meal, feather meal, blood meal, and meat and bone
meal [54-56]. However, these feeds are deficient in one or some of the essential amino acids, especially lysine, isoleucine and methionine. And, animal protein sources are increasingly less or un-acceptable due to health concerns[57]. As people transfer to sustainable, economic and safe protein sources, the plant proteins, especially soybean proteins are growing in application [5, 58]. Soybean meal is much cheaper (by about 30%) than corn gluten meal, which is the main competitor for the aquaculture feed market as the non-fishmeal protein source.

Soy protein products have become increasingly popular. Based on the treatment methods, there are two kinds of soy protein products: soy protein powder and textured soy protein. The soy protein powder is obtained by the removal or partial removal of carbohydrate from defatted soybean. According to the different protein content, there are three primary groups of soy protein products: 1) soy flour, protein content is about 50-65% (dry basis); 2) soy protein concentrate (SPC), with 65-90% protein content (dry basis); 3) soy protein isolate (SPI), with protein content of 90% (dry basis) [8, 9]. Textured soy protein (TSP) is the product made from powdered soy protein products with an extrusion cooking process, which is a meat structure-like product. It is usually made to resemble the structure of all kinds of meat when hydrated. According to the protein content, it can be divided into two kinds: first one is textured soybean protein flour, which has 50-65% protein content (dry basis); and the other one is textured soy protein concentrate, with a protein content about 70% (dry basis) [59].

Currently, soybean white flakes are made with solvent extraction and are usually further processed for SPC and SPI. To obtain specific protein standards and required functional properties, other soybean meal and soy flour can be used as starting materials.
for the different final products. Another starting materials for SPC and SPI is the extruded and expelled (EE) processed soybean meal, which is processed by mechanically oil removal from soybeans. The first step of extrusion can reduce or remove trypsin inhibitors because of the heating treatment. An expeller can be used to totally or partially remove oil after extrusion. Then, the soy meal with different oil contents and heat-denatured proteins can be obtained after these processes with different control operation conditions [60]. There are some advantages from the EE process, such as no organic solvent, low cost and easy to operate for small scale processing.

SPC and SPI are the most popular soy protein products for industrial application. Usually, the goal of their preparation is to get higher protein collection and recovery. There are different methods to prepare SPC and SPI products, and people commonly chose several standard and easy methods. Wang et al. investigated different methods like acid-washed SPC, alcohol-washed SPC, and SPI preparation methods using hexane-defatted soy white flakes and two EE soy protein meals with different degrees of protein denaturation as starting materials [11]. After these treatments, the protein content of SPC was about 60-70% and the protein content of SPI was about 80-87%. And, the anti-nutritional factor oligosaccharide amount in SPC from these methods was higher than enzyme treated SPC [13]. These methods are not economically efficient and environmentally because of the low protein content and recovery, complicated and costly process, and bringing in solvent and impurities. Jung et al. reported the enzyme method to enrich soy protein proteins without causing protein degradation. The cellulase and pectinase were confirmed as effective in increasing the protein contents and reducing sugar amount from the defatted soy flakes [61]. With different treatments of de-hulled
and defatted soybeans, the water-soluble and non-protein ingredients will be removed to produce soy protein concentrates.

The soy protein products are used as food ingredients because of their specific properties that can influence the behavior of proteins in food systems during manufacturing, storage, cooking, preparation and eating. According to the mechanism of action, the specific functional properties of soy protein can be divided into three groups, which included the hydration property, protein structure and rheological characteristics, and protein surface activities. With different treatment or processing, these functional properties will be varied [62]. For different food systems and applications, some functional properties of soy proteins are more important, which deserved more study. In order to develop the functional properties of soy protein, much research is being done by using different treatments. SPI with the higher protein content has the best water-binding capacity. The protein solubility will increase after defatting process [63]. In the rheological properties of proteins, solubility, hydrodynamic properties, and microstructure of proteins have been reported to be essential factors [63]. During the product formation, the viscosity and flow properties of protein dispersions play very important roles [64]. Soy flour and SPC can form soft and fragile gels, however SPI can form firm and hard gels [62]. Surface activity of proteins can be affected by flexibility, conformational stability, and distribution abilities. There some other external factors including pH, ionic strength, temperature, and adsorption abilities which have effects [65]. As people become more and more interested in healthy foods, it is necessary to understand the functional properties of proteins and how they can be modified to achieve the special functions for different applications. The properties of soy protein can be
changed with different processing treatments, which include enzyme treatment, solvent purification, heating treatment, and pH adjustment [34]. More researches will be focused on modifying manufacturing and processing methods.

Soy protein products are not only for human food, but also for animal food and feed products. The SPC are also reported to be used in milk replacer feed for animals [13]. The high protein content, appropriate amino acid constituent, various nutrient content, no health concern, sustainability and economical efficiency are the reasons for the extensive application in animal feed. Because the aquaculture industry is growing very fast in the world’s animal agricultural industry, finding a more efficient and sustainable source to cultivate aquatic species and meet rapid growing market needs become more and more important. SPC has many nutritional properties that are well suited for aquaculture as a protein substitute. It is a renewable and sustainable plant protein product that can help reduce pressure on common fish feed such as fishmeal. Most of the amino acid ingredient of SPC is equal to or even more than menhden fishmeal [7]. And, for aquatic feeds, the low-antigen SPC and high protein level are the crucial features. Although soy protein is popular in animal feed, it cannot be directly fed as raw material without pretreatments because of the anti-nutritional factors (ANFs). Soybeans contain some ANFs, which needs processing before consumption. In order to obtain soy protein with low ANFs, the different manufacturing methods were studied. And different methods bring different products and slightly different anti-nutritional factor levels (Table 2.1) [13].
Table 2.1 Comparison of the anti-nutritional factor levels in various soy products [13]

<table>
<thead>
<tr>
<th>Best Products</th>
<th>Soybeans</th>
<th>Soybean meal</th>
<th>SPC enzyme treated</th>
<th>SPC alcohol extraction</th>
<th>SPC extrusion</th>
<th>Low-antigen SPC</th>
<th>SPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease Activity (pH rise)</td>
<td>SPC SPI</td>
<td>2.0</td>
<td>0.05-0.5</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>0.01-0.02</td>
<td>0.02-0.03</td>
</tr>
<tr>
<td>Trypsin Inhibitor (mg/g)</td>
<td>SPI</td>
<td>45-50</td>
<td>1-8</td>
<td>1</td>
<td>2</td>
<td>&lt;1.25</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Glycinin Antigen (ppm)</td>
<td>SPC</td>
<td>180</td>
<td>66</td>
<td>&lt;30</td>
<td>&lt;30</td>
<td>&lt;5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>β-conglycinin Antigen (ppm)</td>
<td>SPC</td>
<td>&gt;60</td>
<td>16</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lectins (ppm)</td>
<td>SPI</td>
<td>3.5</td>
<td>10-200</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Oligosaccharides (%)</td>
<td>SPI</td>
<td>14</td>
<td>15</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Saponins (%)</td>
<td>SPC SPI</td>
<td>0.5</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The ANFs will affect the nutritional quality for feeds, which may bring some digestion issues of animals (Table 2.2) [14]. So, people do some studies and try to remove or destroy these anti-nutritional factors by different processes and pretreatments. The heating step can easily destroy or remove the trypsin inhibitors and lectins. Except oligosaccharides and antigens, other some heating stable factors with trace amount in soybean are not likely to bring problems for animal feeding. And the enzyme methods and extractions can effectively remove the oligosaccharides and antigens, more details are listed in Table 2.2. Compared with different anti-nutritional factors and treatment
methods, the soy protein with enzyme treatment will be more digestible for fish feed than other methods, which is simple, fast, green and economic.

**Table 2.2 Anti-nutritional factors found in soybeans [14]**

<table>
<thead>
<tr>
<th>Anti-nutritional factor</th>
<th>Mode of action</th>
<th>Method of detoxification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lectins (Phytohaemagglutinins)</td>
<td>Agglutinates red blood cells</td>
<td>Heat treatments.</td>
</tr>
<tr>
<td>Anti-vitamin factors (rachitogenic factor and anti-vitamin B12 factor)</td>
<td>These factors render certain vitamins (e.g. vitamins A, B12, D, and E) physiologically inactive.</td>
<td>Cooking. Supplementation of vitamins.</td>
</tr>
<tr>
<td>Goitrogens</td>
<td>Enlargement of the thyroid</td>
<td>Heat treatment in some cases. Administration of iodide.</td>
</tr>
<tr>
<td>Metal-binding factors (phytate)</td>
<td>These factors decrease availability of certain minerals (e.g. P, Cu, Fe, Mn, Zn)</td>
<td>Heat treatment. Addition of chelating agents. Use of enzymes.</td>
</tr>
<tr>
<td>Saponins</td>
<td>Bitter taste, hemolyze red blood cells</td>
<td>Fermentation.</td>
</tr>
<tr>
<td>Estrogens</td>
<td>Cause an enlargement of the reproductive tract.</td>
<td></td>
</tr>
<tr>
<td>Cyanogens</td>
<td>Cause toxicity through the poisonous hydrogen cyanide.</td>
<td>Cooking.</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Impair digestion (e.g. intestinal cramps, diarrhoea, and flatulence)</td>
<td>Ethanol/water extraction.</td>
</tr>
<tr>
<td>Antigens (glycinin and β-conglycinin)</td>
<td>Cause the formation of antibodies in the serum of calves and piglets. Prevent proliferation of beneficial bacteria in the GIT.</td>
<td>Ethanol/water extraction.</td>
</tr>
</tbody>
</table>
2.1.4 Soybean carbohydrate

Soybean flour contains about 53% proteins and 32% carbohydrate [15]. The compositions of soybean flour and carbohydrate are shown in Figure 2.1. In order to enrich the protein content, the carbohydrate needs removed before consumption.

Figure 2.1 Soybean Flour and Soy Carbohydrate compositions
Soybean carbohydrates are composed of 58% polysaccharides, 20% oligosaccharides and 22% sucrose, and the polysaccharides include 6% cellulose, 23% hemicellulose, 28% pectin and 1% starch. It is composed of arabinogalactan, araban, and some acidic polysaccharides. The backbone is composed of rhamnogalacturonan and poly-galacturonic acid, while the arabinogalactan and arabinose formed the branches [66]. This fraction is generally used as a dietary fiber supplement and foam stabilizer in food industry application [67, 68]. In addition, the soluble soybean polysaccharide helps regulate blood sugar and blood lipids levels, which can accelerate intestinal absorption and excretion of harmful substances [69]. It also has other advantages such as anti-cancer, anti-oxidation, anti-bacteria, anti-viral, immune regulation and other aspects [70-72]. In addition, soy carbohydrates are also used for animal feed and microorganism fermentation feed [73, 74].

The soy carbohydrates are commonly divided into two groups, one group is nonstructural carbohydrates including low molecular weight sugars (mono- and disaccharides), oligosaccharides and storage polysaccharides, and the other group is structural polysaccharides and dietary fiber components [75]. In soybean, in the total carbohydrate constituents, the low molecular weight sugars accounts for about 45%, and this content increases to 50% when soybean are processed into soybean meal. The primary low molecular weight sugars are disaccharide including sucrose, and oligosaccharides including the stachyose, raffinose and verbascose which have nutritional contribution to soybean. The stachyose, raffinose and verbascose range from 3.1-5.7%, 0.50-0.74%, and 0.12-0.20%, respectively, and total to about 5% and 7-8% in processed
soybean meal. The storage polysaccharides are mainly comprised of nonstructural polysaccharides. The concentrations of theses sugars are presented in Table 2.3 [28].

Table 2.3 Composition of Total carbohydrates in Dehulled Soybean Meal[28]

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>% (dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total olio- and monosaccharides</td>
<td>14.8±3.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>6.3±3.2</td>
</tr>
<tr>
<td>Stachyose</td>
<td>4.7±1.7</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0.95±0.4</td>
</tr>
<tr>
<td>Verbascose</td>
<td>0.21±0.09</td>
</tr>
<tr>
<td>Total nonstarch polysaccharides</td>
<td>19.3±2.9</td>
</tr>
<tr>
<td>Noncellulosic</td>
<td>14.9±1.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>4.35±1.45</td>
</tr>
<tr>
<td>Starch</td>
<td>0.73±0.47</td>
</tr>
</tbody>
</table>

The structural polysaccharides are also called non-starch polysaccharides (NSP), which can be divided into two groups: cellulosic and non-cellulosic types. And, the non-cellulosic polysaccharides are comprised of glucose, mannose, xylose, arabinose, galactose, and uronic acids which were listed the content information below (Table 2.4) [28].

Table 2.4 Monosaccharide Composition of Nonstarch, Noncellulosic Polysaccharides in soybean meal [28]

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>% (dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>0.31±0.09</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>Arabinose</td>
<td>2.85±0.25</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.04±0.36</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.65±0.35</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.39±0.13</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>3.7±0.8</td>
</tr>
</tbody>
</table>
The structural carbohydrates are composed of cotyledon polysaccharides and soybean hull polysaccharides. The cotyledon polysaccharides consist of arabinogalactan and an acidic polysaccharide complex. Soybean hulls are a by-product from soybean meal processing and contain about 50-86% complex carbohydrates, comprised of 6-15% pectin, 10-20% hemicellulose, 1-18% lignin and 29-51% cellulose [28, 74, 76].

During the production and process of soy protein products, the sugar can be collected after extraction or enzyme hydrolysis. And different sugar profiles can be obtained in different soy protein products. The carbohydrate constituents of soybean meal were summarized below in Table 2.5 [75]. The most components of carbohydrates of soybean meal are the oligosaccharides and pectic polysaccharides. Soybean meal and soy flour has similar composition and the only difference is the size. The size of soy flour is smaller than that of soy meal.

Table 2.5 Carbohydrate composition of defatted soybean meal[75]

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight percent (moisture-free basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharides</td>
<td>15</td>
</tr>
<tr>
<td>Sucrose</td>
<td>6-8</td>
</tr>
<tr>
<td>Raffinose</td>
<td>1-2</td>
</tr>
<tr>
<td>Stachyose</td>
<td>4-5</td>
</tr>
<tr>
<td>Verbascose</td>
<td>trace</td>
</tr>
<tr>
<td>Pectic Polysaccharides</td>
<td>15-18</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1-2</td>
</tr>
<tr>
<td>Starch</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Soy molasses is also rich in sugars, which is normally considered as the waste after water extraction of soy flour. The nutrient profile of soy molasses is listed in Table
2.6, which was from ADM. Soybean hulls are also a by-product from soybean meal processing and contain about 86% complex carbohydrates, which can provide plenty of carbon sources for microorganism growth and biochemical production [74]. The compositions of soy hulls are listed in Table 2.7. For instance, soy hulls are an optimum medium component for T. reesei and A. niger growth and carbohydrate degraded enzymes production. Because of the existence of some polysaccharides in soy carbohydrates, it is desirable to degrade them into small digestible sugars for further use. As shown in this work, the enzymes produced by A. niger have ability to degrade those polysaccharides and oligomers into small digestible sugars.

Table 2.6 Nutrient profile of soy molasses

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein*</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Moisture</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Ash</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium (ppm)</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Potassium (ppm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Xylose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Fructose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Raffinose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Stachyose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Glucose</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Total Sugars</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>DM</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
</tbody>
</table>

* Crude protein= percentage N X 6.25.

W/W%= grams per 100 grams of sample, ppm=parts per million, or milligrams per kilogram. Results are expressed on an "as is" basis.
Table 2.7 Composition of soybean hulls and selected herbaceous biomass [74]

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
<th>Pectin</th>
<th>Protein</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean hulls</td>
<td>40±11</td>
<td>15±5</td>
<td>2.5±1.5</td>
<td>10.5±4.5</td>
<td>11.5±2.5</td>
<td>2.5±1.5</td>
</tr>
<tr>
<td>Corn stover</td>
<td>36±5</td>
<td>27±7</td>
<td>19.5±3.5</td>
<td>0</td>
<td>6.5±2.5</td>
<td>6±2</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>38±7</td>
<td>28.5±6.5</td>
<td>20±2</td>
<td>0</td>
<td>3±1</td>
<td>6.5±2.5</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>40.5±8.5</td>
<td>31±8</td>
<td>12±7</td>
<td>0</td>
<td>4±2</td>
<td>5.5±4.5</td>
</tr>
</tbody>
</table>

2.2 Carbohydrases

Industrial enzymes consist of two major categories: hydrolase and non-hydrolytic enzyme. Carbohydrases are enzymes that degrade the carbohydrate molecules into simple sugars. These small soluble sugars are easily and readily digested for animals and fishes. Carbohydrases are the fastest growing enzymes in the hydrolase market and will continue expanding in the future. Actually seven groups of enzymes were analyzed in this study: cellulase, xylanase, pectinase, polygalacturonase, α-galactosidase, protease, and sucrase. Cellulase is responsible for breaking down cellulose. One of the most common hemicellulosic polysaccharides is xylan, which consists of a backbone of β-1,4-linked xylose residues, and xylanase is responsible for breaking down xylan. Pectinase and polygalacturonase are responsible for breaking down pectin. Sucrase and α-galactosidase are respectively responsible for sucrose and oligosaccharides hydrolysis. The production and activity of protease was also analyzed as the guide for protein degradation in future studies.

2.2.1 Cellulase

The main component of the plant cell is cellulose, which is a linear homopolymer.
Cellulose is composed of β-1,4-glycosidic bonds linking D-glucopyranose units. The cellulose composition is 44.4% carbon, 6.17% hydrogen, and 49.39% oxygen, with \((C_6H_{10}O_5)_n\) as chemical formula (n represents the number of glucose groups, which is the degree of polymerization) [77].

The cellulase enzyme’s aim is to break down the cellulose of the soybean carbohydrate into digestible mono sugars -glucose. Cellulase enzymes fall into three major classes: endo-glucanases (1,4-β-D-glucan glucanohydrolase), exo-glucanases (1,4-β-D-glucan cellbiohydrolase) and β-glucosidases (cellobiase), and the enzymatic degradation of cellulose to reducing sugars is accomplished by the cooperative action of these enzymes. The endo-glucanase cut cellulose chains into glucose and cellular-oligo saccharides. The exo-glucanase is end-chain attack on the non-reducing end of cellulase with cellobiose as the primary product. The cellobiase is used to hydrolyze cellobiose into glucose [78].

Fungi are the main cellulase-producing microorganisms, though a few bacteria and actinomycetes can also produce cellulase. T. reesei and A. niger are the most common cellulase producers, which can be used for agriculture. T. reesei mainly produce endo-β-glucanase and exo-β-glucanase, but only low amounts of β-glucosidase. A. niger mainly produce endo-β-glucanase and β-glucosidase with less exo-β-glucanase production [78].

2.2.2 Xylanase

Besides cellulose, there is another main component of plant fiber: hemicellulose. The basic chain of hemicellulose contains D-glucose, D-xylose, D-mannose, and D-
galactose, which is linked to glycosyls as branched chains. The main components of hemicellulose include: xylan, xyloglucan, glucomannan, manna, galactomannan, and callose [77]. Xylan is the major part, which has 1,4-β-D-xylopyranose backbone and 4-oxymethylglucuronic acid branch chains. The main chain is linear homopolymer, which was composed of D-xylose[77].

The xylanase breaks down the xylan of the soybean carbohydrate into digestible mono sugar-xylose. The xylanase system is commonly comprised of endoxylanase (endo-1,4-β-xylanase), β-xylosidase (xylan-1,4-β-xylosidase), α-glucuronidase (α-glucosiduronase), α-arabinofuranosidase (α-L-arabinofuranosidase), and acetylxylan esterase[79]. These enzymes work cooperatively to break xylan into its constituent sugars. The endoxylananase is the most important one, which directly cleaves the glycosidic bonds and delivers short xylooligosaccharides [80].

The fungi, actinomycetes and bacteria have been found to produce complete xylanolytic enzyme systems, such as Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia, and Bacillus [80, 81]. Fungi are mainly producers of xylanases and other xylan-degrading enzymes, which can excrete the enzymes and have high-level enzyme production [80, 82].

2.2.3 Pectinase

Pectin, a complex polysaccharide composed primarily of esterified D-galacturonic acid units in α-(1-4) linear chain. The acid groups are mainly in esterification state with methoxy groups[83]. Acetyl groups can also be present on the free hydroxy groups. Rhamnose groups are not usually present in the galacturonic acid main chain. In side
chains, other neutral sugars have been found, such as xylose, galactose and arabinose [84].

The pectinase falls into three major groups: protopectinases, esterases and depolymerases (pectinesterase, polygalacturonase, galacturan 1,4-\(\alpha\)-galacturonidase, exopoly-\(\alpha\)-galacturonosidase, pectate lyase, pectate disaccharide-lyase, oligogalacturonide lyase, pectin lyase) [85]. Pectinase is a group of multiple enzymes that catalyze different reactions at various sites of pectin. The most studied and well-known enzyme is polygalacturonase, which can degrade the polygalacturonic acid backbone into galacturonic acids [86]. Other enzyme such as pectin lyase and pectinesterase respectively catalyzes the different sites of pectin backbones or make the pectin more accessible to the other enzymes [85, 87, 88]. The various pectinase enzymes act cooperatively to degrade the pectin into mono sugar [89].

Pectinase is produced by fungi of the genera *Aspergillus*, *Rhizopus*, *Penicillium*, and *Neurospora*, and by bacteria of *Bacillus* and *Streptomyces* [90-96]. Polygalacturonase is commonly produced by *Aspergillus*, *Saccharomyces*, *Thermoascus*, and *Fusarium* fungi and by *Bacillus* bacteria[97]. Pectin lyase is also produced by *Aspergillus*, *Thermoascus*, *Fusarium* and *Penicillium* fungi and *Bacillus* bacteria. Pectinesterase is usually produced by *Aspergillus* fungi and *Erwinia* bacteria [85, 98-101].

2.2.4 \(\alpha\)-galactosidase

There are certain amounts of raffinose and stachyose in the soybean carbohydrate, which are non-digestible short-chain oligosaccharides. Stachyose is a teraccharide, which
is composed of one α-D-glucose unit, and one β-D-fructose unit sequentially linked as gal(α1→6)gal(α1→6)glc(α1↔2β)fru. Raffinose is a trisaccharide, which is composed of galactose, glucose, and fructose. The α-galactosidase can break them down to monomers. The *Aspergillus* was the most reported α-galactosidase producer, such as *Aspergillus foetidus* [102], *Aspergillus oryzae* [103], and *Aspergillus niger* [104]. Some other fungi can also produce α-galactosidase, such as *Penicillium chrysogenum* [105]. Some bacteria can also produce α-galactosidase, such as *Lactobacillus plantarum* [106].

2.2.5 Sucrase

There is 22% sucrose in the soybean meal carbohydrate. Sucrose is a disaccharide consisting of glucose and fructose. Sucrase, also called invertase, breaks down sucrose into monomers. Fungi are reported the producers of sucrase, such as *Aspergillus niger*, *Aspergillus awamori* [107].

2.3 Enzyme Production with Fungus

Enzymes were produced not only by fungus, but also bacteria and yeast. However, fungus was the most efficient producer for carbohydrase production.

2.3.1 Strain screening

Khairnar et al. reported pectinase production in submerged fermentation with different strains of *Aspergillus niger* and various substrates [108]. They did strain screening through comparing the diameter of zone of clearance around fugal growth in the pectin agar plates. Although the operation was easier, the results did not reflect the
enzyme productivity in submerged fermentation. They used pectin and wheat bran as substrate. However, the pure pectin is much more expensive than raw biomass substrate. And the total carbohydrate of wheat bran is only 12%, which is much lower than soy hull with 50-70%.

Due to the complex structure of soybean carbohydrate with various kinds, complex systems of enzymes are required to breakdown those carbohydrates into digestible mono sugars. The enzymes used for hydrolysis can be produced by fungal fermentation, as people reported that fungi were good carbohydrate degrading enzyme producers [109, 110]. *Trichoderma reesei* is one of the best-studied producers of cellulase, xylanase and pectinase [109, 111, 112]. In a preliminary study, *Trichoderma reesei* RUT-C30 tested for its ability to produce extracellular enzymes capable of degrading soybean polysaccharides. But, because of low pectinase production and a defect in the ability to degrade oligosaccharides, *T. reesei* is not competent for the enzymatic process and other species of fungus needed to be investigated. *Aspergillus* is one of the best-studied fungi to produce polysaccharides degrading enzymes [113]. To select the optimum fungi to hydrolyze soy carbohydrates of soy flour, *Aspergillus niger*, *Aspergillus aculeatus* and *T. reesei* were studied because of their carbohydrase-producing abilities [114-116]. *T. reesei* preferred to form filamentous morphology in submerged fermentation which is an anamorph of *Hypocrea jecorina*. *T. reesei* was the most studied strain with ability to produce large amounts of cellulase [117]. *A. niger* is one of the most popular and important microorganisms in industry application. It has been used for productions of extracellular enzymes and citric acid for many years [118]. Different
strains were studied through shaker cultivation, enzyme production analysis and soy carbohydrate hydrolysis efficiency analysis.

2.3.2 Carbon sources, nitrogen sources and inducers

In order to increase enzyme production, it is necessary to design and modify the fermentation conditions for fungus. First, the culture medium is of most importance in fermentation conditions, which can provide nutrients for microorganism. Carbon is the structural backbone of the organic compounds that make up a living cell. During the synthesis of amino acids, DNA, RNA and ATP in the cells, nitrogen plays the crucial role. Different types of carbon and nitrogen sources for fungal fermentation have been studied. People used molasses and sucrose as carbon source and ammonium nitrate and ammonium sulfate as nitrogen source for citric acid production of *A. niger* [119]. In order to stimulate desired enzyme production, some people used biomass as carbon source, such as pectin for pectinase production [108]. The ratio of carbon to nitrogen also has strong effect on the growth and product production of fungus. The carbon limit will lead to fast cell dying, but too much carbon will lead to a lower pH condition. A nitrogen limit will lead to less cell growth and reduced product production, but too much nitrogen will lead to overly-quick cell growth and lower desired product production. Fungus is able to grow on a wide range of carbon source and nitrogen source. In order to induce the soy carbohydrate-degrading enzyme production, soy based medium was used. Other carbon sources and nitrogen sources were studied to compare with soy-based sources. It has been reported that cellulase and xylanase production can be induced by sophorose, cellobiose, xylobiose, D-xylose, L-sorbose, and lactose [20, 21]. And, pectinases production can be
induced by some natural sources with high pectin content, such as sugar beet pulp [22]. In addition to carbon source and nitrogen source, some other compositions provided the necessary nutrients to sustain the cell growth. For instance, the potassium phosphate can provide the phosphorus source, which is the necessary element for cell construction. And the surfactant in submerged fermentation can help to liberate the enzymes out of the cells.

2.3.3 Fermentation operation conditions

Besides the medium optimization, the fermentation operation conditions also play important role to induce or repress the desired product production such as pH, dissolved oxygen, mixing with different strategy, temperature and so on. The effect of fermentation medium pH is very critical in inducing specific products. Some studies have shown lower pH is good for cellulase and xylanase production in fungal cultures whereas higher pH is good for pectinase [23]. And the effect varied for different strains. Therefore, it is necessary to find the optimum pH control to induce and harvest all these carbohydrate-degrading enzymes.

The dissolved oxygen and agitation are also important factors in submerged fermentation. Mixing by agitation in stirred-tank bioreactors has not only contributed to the oxygen transfer, but also creates shear damage to fungal cells [24]. Besides the stirred-tank fermentation, airlift bioreactors are also widely used, which can bring less shear damage [120]. However, the stirred-tank bioreactors are suggested for high solid loading fermentations with better mixing, which used raw material such as soybean hull as carbon source in fermentations. Different agitation speed or propeller has effect on oxygen transfer. Therefore, the optimum agitation and critical oxygen concentration
control were studied in different submerged fermentations to provide proper oxygen supply.

Different strains have their own optimum culture temperatures. However, the optimum growth temperature may not be beneficial to enzyme productions because of over-quick growth and much consumption and conversion from food to cells rather than enzymes. Some *A. niger* species have higher production of cellulase in pH 4.0 at 35 °C under submerged conditions. The growth and enzyme production of fungi are affected with different temperature [25]. Therefore, different temperatures were investigated to optimize the enzyme productions rather than cell growth only.
CHAPTER III
MATERIALS AND METHODS

3.1 Cultivation

The fungi were stored and cultured appropriately to keep their activity and productivity. In order to achieve the best productivity, the optimized culture conditions were investigated.

3.1.1 Pre-culture

Fungal strains were stored on potato dextrose agar (PDA) at 4°C and subcultured regularly. A preliminary study was done to compare sucrose, soy molasses, lactose, and soy hull as carbon source. Results (not shown) clearly indicated soy hull as the best inducing substrate for production of hydrolytic enzyme for the complex soy carbohydrate.

All test conditions were inoculated with a pre-culture of *A. niger* in the base medium. The medium components and a magnetic stir bar were placed in a 250 mL Erlenmeyer flask covered with cheesecloth, autoclaved for 30 minutes, and cooled prior to adding the fungus. The fungal cells and spores were added using a sterile wire loop (LeStreak MP184-5, Decon Labs Inc., King of Prussia, PA) from an agar storage culture.
plate. Three loops of cells were added per 100 mL of culture medium. This culture was
allowed to grow for 3 days at room temperature while stirring at a setting of 8 on a
designated stirring plate (Corning PC-410, Corning, NY).

The pre-culture medium for the shake flask testing was 24 g/L potato dextrose
broth (Sigma-Aldrich). The pre-culture medium for the stirred tank fermentation was a
modified Mandels and Weber medium [94], which contained: 1.4 g/L (NH₄)₂SO₄, 2.0 g/L
KH₂PO₄, 0.3 g/L MgSO₄·7H₂O, 0.4 g/L CaCl₂·2H₂O, 0.3 g/L Urea, 1.0 g/L Proteose
peptone (Remel), 0.2 g/L Tween 80, 0.005 g/L FeSO₄·7H₂O, 0.0016 g/L MnSO₄·4H₂O,
0.0014 g/L ZnSO₄·7H₂O, 0.002 g/L CoCl₂·2H₂O, and 20 g/L Soy hull as carbon source.

3.1.2 Shaker culture and Fermentation

All of the fungal strains were stored on potato dextrose agar in petri dish at 4°C
and sub-cultured regularly. The flask medium was a modified Mandels and Weber
medium used in this laboratory [121] (Table 3.1). The spores were added to 50 ml seed
culture medium in 250 ml shake flask. And the cultures were incubated under 250 rpm
shaking (continuous orbital shaker with temperature control) for 2-5 days at room
temperature. Sucrose, soy molasses, lactose and soy hull were studied as carbon sources.
Note that soy hulls were used as the carbon source and, more importantly, the inducers
for enzymes hydrolyzing the complex soy carbohydrate. The basic fermentation medium
was same as flask medium. Different carbon source and nitrogen sources were applied in
different fermentation runs. And different concentrations of carbon and nitrogen sources
were applied also. The fermentation was made in a 3 L fermentor with 1.5 L working
volume. DO (dissolved oxygen concentration) was maintained at above 20% air
saturation throughout all the fermentation experiments. The broths harvested at the end of fermentation were centrifuged (12,227 g for 15 min) to collect the cell-free enzyme-containing solutions, which was stored at -20 °C for future use.

Table 3.1 Composition of medium used for cultivation in flasks

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.4</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.3</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>Urea</td>
<td>0.3</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>1</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.2</td>
</tr>
<tr>
<td>Trace Elements (1mL/L)</td>
<td></td>
</tr>
<tr>
<td>Soy hull</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3.1(b) Composition of Trace Elements Stock Solution

<table>
<thead>
<tr>
<th>Trace Elements</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>5</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>1.6</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1.4</td>
</tr>
<tr>
<td>CoCl₂·2H₂O</td>
<td>2</td>
</tr>
</tbody>
</table>

3.2 Enzyme Analysis

Methods are provided for analysis of seven groups of enzymes: cellulase, xylanase, pectinase, polygalacturonase, α-galactosidase, sucrase, and protease. As commonly defined, at one unit of activity the enzyme catalyzes the reaction to give the target product at the rate of 1 µmol/min.
3.2.1 Cellulase

The cellulase assay used is the modified method reported by Ghose [122]. It is best applied to samples with cellulase activities in the range of 0.05 FPU/mL to 3 FPU/mL. Samples with higher FPU activities should be properly diluted. The analysis is done with the following procedure: (1) Cut Whatman No. 1 filter paper into pieces of 6 x 1 cm, ~ 50 mg/piece. Roll and insert a piece (1 cm in height) into a 25 mL test tube, then add 100 µL enzyme-containing sample and 1.4 mL 0.05 M sodium citrate buffer, pH=4.8. The filter paper is completely immersed in the solution. (2) Prepare the blank in the same way but without the filter paper. (3) Incubate the samples and blanks in a water bath at 50°C for 1 h. (4) Add 3 mL regular DNS (3,5-dinitrosalicylic acid) solution to each sample and blank to stop the enzyme reaction. (5) Incubate the DNS-added tubes in boiling water (100°C) for 10 min. (6) Add deionized water to make the total volume of 25 mL, mix, and then measure the absorbance of reaction supernatant at 540 nm with a spectrophotometer. Determine the amount (mg) of reducing sugar released, using the pre-establish calibration with pure glucose solutions of different concentrations as standards. The cellulase activity is then calculated using the following equation:

\[
\text{Cellulase (FPU/mL)} = \frac{\text{glucose released (mg)}}{(60 \text{ min})(0.1 \text{ mL enzyme sample})} \times \frac{1 \text{ mmol}}{180 \text{ mg}} \times \frac{1000 \text{ µmol}}{1 \text{ mmol}}
\]

\[
= 0.925 \times \text{glucose released (mg)}.
\]
3.2.2 Xylanase

The xylanase assay used is the method reported by Bailey et al [123]. The method is best applied to properly diluted samples with xylanase activities in the range of 0.5-2 U/mL. The procedure is as follows: (1) Prepare 1 wt% substrate solution/suspension: mix 2 g beechwood xylan (Sigma Aldrich, St. Louis, MO) in 180 mL 0.05 M sodium citrate buffer (pH 5.3); heat the stirred mixture till the water vapor became apparent but not boiling; turn off heating and stir the mixture overnight; add 20 mL 0.05 M sodium citrate buffer (pH 5.3); and then store the substrate mixture at -20°C for future use. (2) Add 100 µL enzyme-containing sample and 900 µL xylan substrate mixture to a 25 mL test tube. (3) Prepare the (enzyme-free) blank with only 900 µL xylan substrate. (4) Incubate the samples and blanks in a water bath at 50°C for 5 min. (5) Add 3 mL regular DNS solution to each sample and blank to stop the enzyme reaction. (6) Add 100 µL enzyme-containing sample to the corresponding blank (to account for the potential turbidity introduced by the sample). (7) Incubate the (DNS-added) tubes in boiling water (100°C) for 10 min. (8) Add deionized water to make the total volume of 25 mL, mix, and then measure the absorbance of reaction supernatant at 540 nm. Determine the amount (mg) of reducing sugar released, using the pre-establish calibration with pure D-xylose solutions of different concentrations as standards. Calculate the xylanase activity by the following equation:

\[
\text{Xylanase (U/mL)} = \frac{\text{xylose released (mg)}}{(5 \text{ min})(0.1 \text{ mL enzyme sample})} \times \frac{1 \text{ mmol}}{150.13 \text{ mg}} \times \frac{1000 \text{ µmol}}{1 \text{ mmol}}
\]

\[= 13.32 \times \text{xylose released (mg)}.\]
3.2.3 Pectinase

The polygalacturonase method reported by Wang et al. is modified to conduct the assay at pH 4.8 and 50°C, the same condition used for our soy flour hydrolysis [124]. Different reaction times from 5 min to 60 min have been tested; 30 min is chosen for giving the most consistent activities for a practicable range of enzyme activities (0.3-0.7 U/mL). Different substrate concentrations from 1 g/L to 40 g/L have also been tested; 5 g/L is chosen as the best compromise between substrate limitation and inhibition. The procedure is the same as that for the xylanase assay with four modifications. First, the substrate solution/suspension is prepared by mixing 0.5 g polygalacturonic acid (Sigma Aldrich, St. Louis, MO) in 100 mL 0.1 M sodium citrate buffer and then adjusting the pH to 4.8. Second, the samples and blanks are incubated at 50°C for 30 min (instead of 5 min as in the xylanase assay). Third, the DNS solution used does not contain sodium-potassium tartrate to prevent precipitation of residual substrate. Fourth, the calibration for determining the released amount of reaction product(s) is made with standard solutions of D-galacturonic acid (monohydrate). The polygalacturonase activity is calculated according to the following equation:

$$\text{Polygalacturonase } \left( \frac{U}{mL} \right) = 1.57 \times \text{galacturonic acid released (mg)}$$

The pectinase assay used is modified from the method reported by Solis-Pereyra et al [125]. The assay is exactly the same as that for polygalacturonase activity except that the substrate solution is prepared with citrus pectin (Sigma Aldrich, St. Louis, MO) instead of polygalacturonic acid. Heating is necessary to prepare more homogeneous solution/suspension of the pectin substrate in citrate buffer.
3.2.4 α-galactosidase

The assay used is modified from the method reported by Mukesh Kumar et al [126]. Test samples should be adjusted to have α-galactosidase activities of 0.05-0.2 U/mL. The procedure is as follows: (1) Prepare the substrate solution by dissolving 0.03333 g p-nitrophenyl-α-D-galactopyranoside (Sigma Aldrich, St. Louis, MO) in 100 mL 0.1 M sodium citrate buffer and adjusting the pH to 4.8; (2) Mix 100 µL enzyme-containing sample with 900 µL substrate solution; (3) Prepare the (enzyme-free) blank with only 900 µL substrate solution; (4) Incubate the samples and blanks at 50°C for 10 min; (5) Add 2 mL 0.5 M sodium carbonate (pH 9.8) to each sample and blank to stop the reaction and show the color from released p-nitrophenol; (6) Add 100 µL enzyme-containing sample to the blanks; and (7) Measure the absorbance at 405 nm. p-Nitrophenol standards are used for quantitation of the enzyme-released p-nitrophenol. The α-galactosidase activity is calculated by the following equation:

\[
\alpha - \text{Galactosidase } \left( \frac{U}{mL} \right) = 7.19 \times p - \text{nitrophenol released (mg)}
\]

3.2.5 Sucrase

The assay used is modified from the method reported by Uma et al [127]. The method is best for samples with sucrase activities of 0.2-2 U/mL. The procedure is very similar to that for the pectinase assay, with the following differences: (1) sucrose is used for substrate solution; (2) the enzyme reaction at 50°C is allowed for 20 min; and (3) the regular (tartrate-containing) DNS solution is used. Glucose standards are used for calibration. The sucrase activity is calculated as:
Sucrase \( \left( \frac{U}{mL} \right) = 2.78 \times \text{glucose released (mg)} \).

3.2.6 Protease

The Pierce Fluorescent Protease Assay Kit (Thermo Scientific, Number 23266/23267) was used for protease assay. The kit included FTC-casein (\( \kappa \)-casein labeled with fluorescein isothiocyanate to yield the fluorescein thiocarbamoyl (FTC) derivative) and TPCK trypsin (trypsin treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)). FTC-casein was the protein substrate. TPCK trypsin was the reference protease for comparison/calibration. FTC-casein (2.5 mg) was dissolved in 500 \( \mu \)L ultrapure water to make a 5 g/L stock solution, 20 \( \mu \)L of which was then mixed with 10 mL 0.05 M sodium citrate buffer (pH 4.8) to make the substrate solution for the assay. The substrate solution (50 \( \mu \)L) was added with a 20 \( \mu \)L enzyme sample (or a TPCK trypsin standard) to a 96-well black plate (Greiner Bio One, Number 655076), mixed, and then allowed to react at room temperature for 20 min. The reaction mixture was next added with 200 \( \mu \)L 1 M Tris-HCl (pH 9.0), to provide the high pH required for emission of the fluorescein label. Fluorescence was measured using a multimode microplate reader (Infinite 200 PRO, TECAN) at excitation and emission wavelengths of 485 and 538 nm, respectively. A calibration curve of fluorescence changes versus TPCK trypsin concentrations used (0-50 mg/L) was generated by the same procedure. Accordingly, the fluorescence change generated by the enzyme sample was converted to the equivalent TPCK trypsin concentration, and subsequently to BAEE U/mL by multiplying the factor of 16,273.
3.3 Carbohydrate Analysis

The reducing sugar analysis was based on the DNS (3,5-dinitrosalicylic acid) test method [128]. The procedure has been described earlier in the enzyme analysis section. For the soy flour hydrolysis samples, the calibration was established with standard glucose solutions of 0-1 g/L. The total carbohydrate analysis was based on the phenol-sulfuric acid test method [129]. The hydrolysate sample was first diluted to a concentration of no more than 0.1 g/L. One mL diluted sample, 1 mL 5% phenol solution, and 5 mL concentrated H$_2$SO$_4$ solution were gently mixed in a test tube for 10 min reaction. Absorbance was then measured at 490 nm and converted to glucose-equivalent concentration using the calibration established with standard glucose solutions of 0-0.1 g/L.

3.4 Protein Analysis

Protein contents in the defatted soy flour and enriched protein products were determined by measuring the total nitrogen contents using the Kjeldahl method [130] and then multiplying them by the factor of 6.25. Briefly, samples were completely digested with a solution containing sulfuric acid, potassium sulfate and cupric sulfate and then distilled under high pH to collect the released ammonia by absorption in 0.1 N boric acid. The absorbed ammonia amount was measured by titration using 0.1 N sulfuric acid and converted to the total nitrogen and protein concentrations in the starting sample.

3.5 Fungal Cell Concentration Determination

The intracellular protein was measured to estimate the cell growth in systems
grown on sucrose. This was done by collecting three 1 mL samples in micro centrifuge tubes. Each sample was centrifuged for 10 minutes at 8,000 rpm (Eppendorf 8150, Hauppauge, NY) to separate the cells from the enzyme containing broth. The liquid fraction was collected using a glass pipet while preserving the cell pellet. The broth was frozen at -20°C while awaiting enzyme activity testing. Then, 1 mL of deionized water was added to each micro-centrifuge tube containing the cell pellet. The samples were then shaken to disperse the fungal cells and re-centrifuged at for 10 minutes and 8,000 rpm. The wash liquid was removed and discarded so that the cells were isolated. This process reduced extracellular protein that could interfere with further testing. Then, 1 mL of 0.2 M NaOH was added to each micro-centrifuge tube containing a cell pellet and were then heated at 100 °C for 30 minutes to release the intracellular protein. After being cooked, the samples were removed from the heating block and cooled to room temperature (~25 °C). Once cooled, the samples were centrifuged for 10 minutes at 8,000 rpm to remove the solids from the liquid protein suspension.

Intracellular protein concentration was assayed, in triplicate, with a commercially available protein kit (Bio-Rad Protein Assay Kit #500-002, Bio-Rad Laboratories, Hercules, CA). Briefly, 20 µL of cellular digest liquid was dispensed into a micro-plate well along with 200 µL of prepared dye The micro-plate was allowed to sit for exactly 10 minutes for color formation. It was then measured for optical density using a spectrometer (Tecan i-Control infinite 200 Männedorf, Switzerland) set at a wavelength of 595 nm, the average of four readings per well was taken.

*A. niger* doubling times were calculated from the measured intracellular protein, assuming that the fungal cells doubled for each division rather than tripled or quadrupled.
This relation is shown in Equation 1, with variable $x$ representing the cell concentration at a given time and $T_d$ corresponding to the time needed for each division.

$$T_d = \log(2) \left( \frac{t_1 - t_0}{\log \left( \frac{x_1}{x_0} \right)} \right)$$
CHAPTER IV

ENZYME PRODUCTIONS OF TRICHODERMA REESEI FERMENTATION

(Co-work with Anthony M. Coffman)

4.1 Introduction

*T. reesei* is an efficient producer of carbohydrate-degrading enzymes such as cellulase, xylanase, and polygalacturonase. The effects of defatted soybean flour, soybean hull and a variety of soluble and solid carbon sources and nitrogen sources were evaluated. The enzyme broth was used in hydrolysis of soymeal polysaccharides, which results in an enriched soy protein product. In order to improve the hydrolysis efficiency, the enzyme productions are the essential part. The effects of different carbon and nitrogen sources and different culture conditions on the enzyme production of *T. reesei* were investigated to improve the enzyme production.

4.2 Materials and Methods

Four different fermentations were carried out at constant pH 6.0 which were Fer20, Fer21, Fer22, and Fer23. Fer20 was the control with non-soy nitrogen source and lactose as carbon source, and the other 3 systems used soybean hulls as the only carbon source. In different pH control run, two 1 L fermentations and one 1.5 L fermentation
were started with 20 g/L soybean hulls as the carbon source, which were respectively Fer24, Fer25, and Fer26. Fer24 was operated at initial pH 4.5 and then let the pH itself drop to 4 as the cell growth and then kept 4 as constant. Fer25 was operated at constant pH 6.0. And Fer26 was operated at initial pH 6.0 and then let the pH itself drop to 4 during the growth and then kept 4 as constant. It wasn’t wellknown the optimum pH range to induce the enzyme productions, therefore different pH conditions were studied. Besides the soy hull, the other carbon sources were also investigated. Two 1.5 L fermentations were carried out with different carbon source, which were Fer27 using avicel as carbon source) and Fer28 with lactose as carbon source. Fer27 was operated at constant pH 6.0 and Fer28 was operated at constant pH 4.5.

4.3 Results and Discussion

The Enzyme production results of T. reesei fermentations were shown below (Table 4.1).
Table 4.1 Enzyme productions of *T. reesei* Fermentations

<table>
<thead>
<tr>
<th>Fermentation System</th>
<th>Nitrogen Source</th>
<th>Carbon source</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Polygalacturonase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fer20 (pH6.0) (10 days)</td>
<td>(NH₄)₂SO₄ - 1.4g/L Urea - 0.3g/L Protease peptone-1g/L</td>
<td>Lactose - 10g/L</td>
<td>2.4±0.1 (5 days) 2.2±0.1 (5 days)</td>
<td>69.4±2.4 (10 days)</td>
<td>7.8±0.2 (9 days) 7.8±0.2 (10 days)</td>
</tr>
<tr>
<td>Fer21 (pH6.0) (10 days)</td>
<td>Soy flour – 7.04g/L</td>
<td>Lactose - 8.6g/L</td>
<td>2.1±0.0 (9 days) 2.1±0.1 (10 days)</td>
<td>69.5±1.2 (9 days) 66.7±2.7 (10 days)</td>
<td>18.1±1.2 (8 days) 17.2±0.7 (10 days)</td>
</tr>
<tr>
<td>Fer22 (pH6.0) (10 days)</td>
<td>Soy flour – 7.04g/L</td>
<td>Avicel - 8.6g/L</td>
<td>2.9±0.1 (4 days) 2.8±0.2 (10 days)</td>
<td>142.4±12.4 (8 days) 131.6±1.6 (10 days)</td>
<td>3.8±0.4 (2 days) 3.8±0.5 (10 days)</td>
</tr>
<tr>
<td>Fer23 (pH6.0) (10 days)</td>
<td>Soy flour – 7.04g/L</td>
<td>Soy hulls -17.2g/L</td>
<td>2.7±0.1 (5 days) 2.6±0.1 (10 days)</td>
<td>269.9±5.7 (8 days) 235.2±6.9 (10 days)</td>
<td>5.9±0.2 (8 days) 5.4±0.2 (10 days)</td>
</tr>
<tr>
<td>Fer24 (pH4.5-4.0) (9 days)</td>
<td>NH₄)₂SO₄ - 1.4g/L Urea – 0.3g/L Protease peptone -1g/L</td>
<td>Soy hulls -20g/L</td>
<td>2.2±0.1 (6 days) 2.2±0.1 (9 days)</td>
<td>55.8±1.2 (8 days) 53.8±3.1 (9 days)</td>
<td>11.6±0.3 (8 days) 10.8±1.3 (9 days)</td>
</tr>
<tr>
<td>Fer25 (pH6.0) (9 days)</td>
<td>Same above</td>
<td>Soy hulls -20g/L</td>
<td>3.2±0.1 (7 days) 3.2±0.1 (9 days)</td>
<td>248.9±26.5 (8 days) 243.6±8.4 (9 days)</td>
<td>7.9±0.3 (9 days)</td>
</tr>
<tr>
<td>Fer26 (pH6.0-4.0) (11 days)</td>
<td>Same above</td>
<td>Soy hulls -20g/L</td>
<td>2.2±0.1 (6 days) 2.2±0.2 (11 days)</td>
<td>108.3±4.2 (7 days) 91.3±4.2 (11 days)</td>
<td>9.96±0.78 (11 days)</td>
</tr>
<tr>
<td>Fer27 (pH6.0) (9 days)</td>
<td>Same above</td>
<td>Avicel -10g/L</td>
<td>3.6±0.1 (6 days) 3.1±0.1 (9 days)</td>
<td>170.6±6.0 (9 days)</td>
<td>1.39±0.06 (8 days)</td>
</tr>
<tr>
<td>Fer28 (pH4.5) (9 days)</td>
<td>Same above</td>
<td>Lactose -10g/L</td>
<td>1.5±0.1 (7 days) 1.5±0.1 (9 days)</td>
<td>17.8±2.9 (4 days) 17.0±3.8 (9 days)</td>
<td>10.7±0.6 (5 days) 10.7±0.4 (9 days)</td>
</tr>
</tbody>
</table>
Cellulase production in Fer20 and Fer21 was almost the same even with different nitrogen source. In Fer23, soybean hulls, which contain approximately 40 wt% cellulose, gave similar results to Fer22 with avicel as carbon source. Xylanase production in Fer20 and Fer21 was similar even with different nitrogen source. The soybean hulls strongly induced the production of xylanase based on Fer23 results. It was likely polygalacturonase production was really lower in cultivations with large proportions of non-pectic polysaccharides based on Fer22 results. Much higher polygalacturonase production was obtained in Fer21 with soy flour and lactose separately as nitrogen source and carbon source.

![Figure 4.1 Cellulase production trend of *T. reesei* fermentation](image)

The cellulase production trends of *T. reesei* fermentations were shown in Figure 4.1. The nitrogen source did not affect the cellulase production really much. And the soy hull with approximately 40 wt% cellulose as carbon source strongly induced cellulase
production, which gave similar results to Fer22 using avicel as carbon source. And the cost of soy hull was much lower than avicel..

Figure 4.2 Xylanase production trend of T. reesei fermentation

The xylanase production trends of T. reesei fermentations were shown in Figure 4.2. The nitrogen source did not have obvious effects on the xylanase production. And the soy hull with approximately 20 wt% hemicellulose as carbon source strongly induced xylanase production, which might be the inducer function of xylan in hemicellulose.
Polygalacturonase production was much lower in cultivations with large proportions of non-pectic polysaccharides because of absence of inducer ingredient pectin. There was much higher polygalacturonase production in Fer21 with soy flour and lactose, which may be contributed from the high pectin amount in soy flour carbohydrate.

At pH 6.0, more cellulase and xylanase were produced with soybean hulls. There was a small difference in polygalacturonase production at different pH, which indicated lower pH was preferred for polygalacturonase. Lower cellulase and xylanase were obtained at pH 4.5. Higher pH induced the cellulase and xylanase production of *T. reesei* fermentation. Soy hull had the same effect as avicel to induce cellulase production, no matter which nitrogen source was applied. The soy hulls as carbon source induced the complex enzyme production than pure sugar carbon source. The soy flour did not have
any obvious effect on cellulase and xylanase production when cultured with the same carbon source. The soy flour induced more polygalacturonase when cultured with pure carbon sources like lactose and avicel, but not obviously in the systems with soy hull as carbon source. This may due to the inducer role of high amount of pectin in soy flour. With the presence of soy hull, some other inducers may induce other enzyme productions rather than polygalacturonase.

4.4 Conclusion

The soy flour did not have any obvious effect on cellulase and xylanase production when cultured with the same carbon source at the same pH. The soy flour induced more polygalacturonase when grown with pure carbon sources like lactose and avicel, but not obviously in the systems with soy hull as carbon source. The other study of soy hull as carbon source has proved the inducer function of soy hull, which can induce the complex enzyme production than pure sugar carbon source. The study of pH factor indicated lower pH was good for polygalacturonase but higher pH would be good for cellulase and xylanase production. Therefore, the suitable pH control strategy was to use higher initial pH and then gradually decrease pH.
CHAPTER V

STRAIN SCREENING FOR ENZYME PRODUCTION TO SEPARATE SOYBEAN
CARBOHYDRATE AND PROTEIN FOR INCREASED SOY MEAL VALUE

5.1 Introduction

Aquaculture industry has been growing rapidly, meeting more than 40% of the
global seafood demand [131]. Fishmeal is the preferred protein source for aquaculture
feed [53] but fishmeal production has long reached the sustainable limit [132].
Heightened demand has also caused fishmeal price to rise dramatically [133]. There have
been developments in using other animal proteins as alternative sources, such as poultry
by-product meal, feather meal, blood meal, and meat and bone meal [54-57]. However,
these animal protein sources are increasingly less acceptable due to health concerns [4].
Plant proteins, especially soy protein, are potentially more sustainable, economic and safe
alternatives [5, 58]. Soy protein products such as soy protein concentrate (SPC) are at
least as good as menhaden fishmeal in providing all amino acids except the sulfur amino
acids methionine and lysine [7].

Defatted soy flour/meal contains about 53% protein and 32% carbohydrate [15,
134]. It offers higher protein content than other plant sources such as wheat, lupin, peas
and barley. This protein content is however significantly lower than that of fishmeal, i.e.,
65-72% [135]. Soybeans also contain some anti-nutritional factors. Trypsin inhibitors and lectins can be easily destroyed by heat. Most others are present in low amounts, unlikely to cause problems in soy-based feed [14]. Only the indigestible oligo-/poly-saccharides pose potential anti-nutritional concerns. Processing is required to enrich protein content and reduce indigestible, anti-nutritional factors. Existing processes for producing soy protein products such as SPC and soy protein isolate (SPI) are largely based on physical methods [10, 11], which produce SPI with low yield and produce SPC with no polysaccharide removal and often incomplete oligosaccharide removal [12]. An alternative enzyme-based method was reported [136, 137], where enzyme hydrolyzes and solubilizes carbohydrate into aqueous hydrolysate while protein remains predominantly insoluble near isoelectric pH. Hydrolysate and protein-enriched solids could be separated; the former could potentially be used as fermentation substrate and the latter as protein source for aquaculture feed.

Carbohydrate in soy flour is however complex, including water soluble sucrose (6-8% soy flour), raffinose (1-2%), stachyose (4-5%) and verbascose (trace), and insoluble polysaccharides (15-18%) such as cellulose (2%), hemicellulose (5%), pectin (10%) and a small amount of starch (0.5%) [12, 75]. Effective and economic hydrolysis of the carbohydrate requires complex enzyme with properly proportioned activities, particularly if to maximize monomerization. The fungus Trichoderma reesei has been widely studied for production of enzyme that degrades cellulosic polysaccharide [121, 138]. Aspergillus niger and Aspergillus aculeatus are also known to produce carbohydrase [114, 116, 139, 140]. In this study, strains of these fungi were screened for production of protease and various carbohydrases. Totally seven groups of enzyme
activities were compared: cellulase, xylanase, pectinase, polygalacturonase, \( \alpha \)-galactosidase, sucrase and protease. Cellulase breaks down cellulose. Xylanase hydrolyzes xylan which is a major hemicellulose component with a \( \beta \)-1,4-linked xylose backbone. Pectinase activity indicates the overall ability of pectin hydrolysis while polygalacturonase is specifically for breaking down pectin’s polygalacturonic acid backbone. Sucrase and \( \alpha \)-galactosidase are responsible for hydrolyzing the large amounts of sucrose and galactose-containing side chains in, for example, raffinose, stachyose and verbascose. Protease was included in the study to indicate potential protein loss due to the enzymatic treatment. Direct comparison was further made for the effectiveness of fungal broths in releasing soluble carbohydrate from soy flour.

5.2 Materials and Methods

The materials and methods were divided into three parts, which were described below.

5.2.1 Materials and equipment

All strains used in this study were obtained from Agricultural Research Service Culture Collection of the United States Department of Agriculture. They included A. aculeatus NRRL 2053, T. reesei NRRL 11460 (Rut C30), Aspergillus foetidus NRRL 341, Aspergillus phoenicis NRRL 363, Aspergillus cinnamomeus type strain NRRL 348, and 11 other A. niger strains, i.e., NRRL 322, 325, 328, 334, 566, 599, 2270, 3122, 13201, 13219 and 62517. (The A. foetidus, A. phoenicis and A. cinnamomeus strains also belong to the A. niger aggregate [141].) Soy hull and flour samples were provided by
the Archer Daniels Midland Company (Decatur, IL, USA). Unless specified otherwise, chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). In-house equipment used in the study included UV-visible spectrophotometer (Shimadzu UV-1601), orbital shaker with temperature control (Thermo Scientific SHKA5000-7), centrifuge (Eppendorf Centrifuge 5415D), and water bath (Boekel Scientific ORS-200).

5.2.2 Cultivation and strain screening

Fungal strains were stored on potato dextrose agar (PDA) at 4°C and subcultured regularly. To start the screening study, the strains were grown on PDA plates for 72 h at room temperature and then the plates were washed with autoclaved deionized water containing 0.05% Tween 80 to collect spores. The same concentration of spores (3 × 10^5 spores/mL) were inoculated to 250 mL flasks containing 50 mL fresh medium, which was a modified Mandels and Weber medium used in this laboratory [121]: 20 g/L soybean hull, 1 g/L proteose peptone, 0.3 g/L urea, 1.4 g/L (NH₄)₂SO₄, 2 g/L KH₂PO₄, 0.4 g/L CaCl₂·2H₂O, 0.3 g/L MgSO₄·7H₂O, 0.2 g/L Tween 80, and 1 mL/L trace element solution (5 g/L FeSO₄·7H₂O, 2 g/L CoCl₂, 1.6 g/L MnSO₄·H₂O and 1.4 g/L ZnSO₄·7H₂O). A preliminary study was done to compare sucrose, soy molasses, lactose, and soy hull as carbon source. Results (not shown) clearly indicated soy hull as the best inducing substrate for production of hydrolytic enzyme for the complex soy carbohydrate.

Six batches of strain screening were carried out. The cultures were incubated at room temperature in a shaker operating at 250 rpm. The strains evaluated and properties measured (mostly daily) are summarized in Table 5.1. Originally only polygalacturonase
activity and pH profile were compared; more enzyme activities were included in latter batches. For the last 3 batches, the broths after 72 h were centrifuged (9300 g) to collect supernatants, which were used for the soy flour hydrolysis test in addition to being analyzed for various enzyme activities. The goal was to select the strains with highest carbohydrase activities, lowest protease activities, and release maximal amounts of reducing sugar and total carbohydrate from soy flour in the hydrolysis test.

Table 5.1 Six batches of strain screening conducted in this study

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Strains tested (NRRL #)</th>
<th>Properties measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>322 · 325 · 328, 334, 341, 348, 363, 566, 3122, 62517, 2053, 11460</td>
<td>Polygalacturonase and pH</td>
</tr>
<tr>
<td>2</td>
<td>334, 348, 2053, 3122, 11460</td>
<td>Polygalacturonase and pH</td>
</tr>
<tr>
<td>3</td>
<td>325, 599, 2270, 13201, 13219</td>
<td>Cellulase, xylanase, polygalacturonase, and pH</td>
</tr>
<tr>
<td>4</td>
<td>328, 334, 341, 348, 363, 566, 2053, 11460</td>
<td>Cellulase, xylanase, polygalacturonase, and soy flour hydrolysis test</td>
</tr>
<tr>
<td>5</td>
<td>322, 328, 334, 341, 348, 363, 566, 599, 2270, 13201, 13219, 62517, 2053, 11460</td>
<td>Cellulase, xylanase, polygalacturonase, pectinase, α-galactosidase, sucrase, protease, pH, and soy flour hydrolysis test</td>
</tr>
<tr>
<td>6</td>
<td>322, 341, 2053, 11460</td>
<td>Cellulase, xylanase, polygalacturonase, and soy flour hydrolysis test</td>
</tr>
</tbody>
</table>

5.2.3 Soy flour hydrolysis test

Ten mL cell-free enzyme broth (or water, for the enzyme-free control system) was diluted with 30 mL water and pH adjusted to 4.8. The solution was added to 10 g
defatted soy flour and the mixture was reacted for 49 h at 50 °C in a shaker operating at 250 rpm. Samples were taken at 1, 24, and 49 h. Samples were centrifuged to separate the hydrolysate from the enriched soy protein insoluble at this pH. Hydrolysate was analyzed for soluble carbohydrate released by the reaction, as both reducing sugar and total carbohydrate concentrations. Enriched soy protein was analyzed for the protein content.

5.3 Results and Discussion

The comparison results of these strains were described below, which included the comparison of cell growth, enzyme productivities, enzyme hydrolysis efficiency and protein product.

5.3.1 Growth observations in soy hull-based medium

In the soy hull-based medium, strains NRRL 328, 334, 363 (A. phoenicis), 566, 2053 (A. aculeatus), 2270 and 13201 grew in shake flasks as fine pellets of approximately 1 mm in diameter. (Strains given by only NRRL numbers are all A. niger strains.) Strains NRRL 348 (A. cinnamomeus), 599, and 13219 formed larger pellets, about 5-10 mm in diameter. The other 6 strains grew in the filamentous form in shake flasks. NRRL 3122 grew clearly slower than other strains, with very slight changes in medium turbidity and visual appearance. Strain NRRL 325 was later found to be isolated from an ear infection. These two A. niger strains were therefore not considered in later experiments.
The presence of soy hull solids made it impossible to follow cell growth quantitatively by dry weight measurement. Intracellular protein and DNA measurements [142, 143] were tried but also failed due to the protein and DNA introduced with hulls. pH was one factor that could be easily followed. pH profiles for the remaining 14 strains (excluding *A. niger* NRRL 325 and 3122) observed from all batches of screening experiments are summarized in Figure 5.1.

![Figure 5.1 pH change profiles of 14 fungal strains during growth in the soy hull-based medium](image)

Figure 5.1 pH change profiles of 14 fungal strains during growth in the soy hull-based medium

*A. niger* is known to produce organic acids such as citric, oxalic and acetic acids [144-147]. pH dropped rapidly to below pH 3 within 2 days with all *A. niger* strains screened in this study. The strain NRRL 334 showed the fastest pH decrease in the first day. *A. aculeatus* NRRL 2053 grew with active acid production, next only to the strain NRRL 334. It was noticed that all of the strains that showed faster first-day pH decreases,
i.e., in the descending order, NRRL 334, 2053, 328, 566 and 13201, all belonged to those growing with fine pellets (~ 1 mm). The *A. cinnamomeus* type strain NRRL 348 grew with slightly slower pH drop than the other *A. niger* strains. pH drop also occurred later, after 2 days, for *T. reesei* NRRL 11460. Among all the screened strains, *A. foetidus* NRRL 341 grew with the least pH drop; pH remained above 5 till 96 h.

5.3.2 Enzyme production

The enzyme activities produced, at 72 h, by the fungal strains evaluated are given in Table 5.2. Figures were plotted to identify potential correlation between any two groups of enzyme activities produced by these strains. Correlation could only be seen between \( \alpha \)-galactosidase and sucrase, and only tentatively, with \( R^2 = 0.52 \) (Figure 5.2). These two enzymes are important for hydrolysis of soy oligosaccharides such as raffinose and stachyose; \( \alpha \)-galactosidase removes the galactose residues and sucrase hydrolyzes the remaining sucrose to glucose and fructose. It is not too surprising to find a correlation between the production of these two enzymes under the induction of soy hulls as substrate.
Table 5.2 Enzyme activities produced at 72 h cultivation in the soy hull based medium

(all values reported in U/mL)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cellulase</th>
<th>Xylanase</th>
<th>Pectinase</th>
<th>Polygalacturonase</th>
<th>α-Galactosidase</th>
<th>Sucrase</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>322 (An)</td>
<td>0.31±0.0</td>
<td>101.7±1.</td>
<td>6.36±0.3</td>
<td>15.6±0.3</td>
<td>4.50±0.0</td>
<td>3.35±0.1</td>
<td>140.5±2.</td>
</tr>
<tr>
<td>328</td>
<td>0.24±0.0</td>
<td>56.2±5.0</td>
<td>4.95±0.0</td>
<td>7.5±0.4</td>
<td>3.33±0.0</td>
<td>1.71±0.1</td>
<td>225.2±4.</td>
</tr>
<tr>
<td>334</td>
<td>0.26±0.0</td>
<td>87.7±3.0</td>
<td>4.73±0.0</td>
<td>7.8±0.5</td>
<td>1.75±0.0</td>
<td>2.29±0.1</td>
<td>247.3±2.</td>
</tr>
<tr>
<td>566</td>
<td>0.26±0.0</td>
<td>53.5±12.</td>
<td>5.10±0.0</td>
<td>8.5±0.6</td>
<td>3.21±0.0</td>
<td>2.11±0.2</td>
<td>230.2±2.</td>
</tr>
<tr>
<td>599</td>
<td>0.19±0.0</td>
<td>38.3±1.1</td>
<td>4.13±0.0</td>
<td>6.4±1.0</td>
<td>3.47±0.0</td>
<td>2.27±0.3</td>
<td>290.1±4.</td>
</tr>
<tr>
<td>2270</td>
<td>0.26±0.0</td>
<td>79.6±1.4</td>
<td>3.80±0.0</td>
<td>14.7±0.1</td>
<td>2.20±0.0</td>
<td>1.4±0.1</td>
<td>125.3±2.</td>
</tr>
<tr>
<td>13201</td>
<td>0.37±0.0</td>
<td>86.1±3.1</td>
<td>4.34±0.0</td>
<td>12.5±1.0</td>
<td>0.43±0.0</td>
<td>1.46±0.0</td>
<td>111.9±1.</td>
</tr>
<tr>
<td>13219</td>
<td>0.22±0.0</td>
<td>58.5±12.</td>
<td>4.42±0.0</td>
<td>13.8±1.4</td>
<td>2.71±0.0</td>
<td>2.01±0.3</td>
<td>97.0±15.</td>
</tr>
<tr>
<td>62517</td>
<td>0.29±0.0</td>
<td>94.6±22.</td>
<td>3.49±0.0</td>
<td>5.1±0.1</td>
<td>4.25±0.0</td>
<td>3.34±0.0</td>
<td>217.3±1.</td>
</tr>
<tr>
<td>341 (Af)</td>
<td>0.37±0.0</td>
<td>77.6±2.7</td>
<td>4.54±0.5</td>
<td>2.4±0.2</td>
<td>4.78±1.6</td>
<td>2.22±0.7</td>
<td>101.3±2.</td>
</tr>
<tr>
<td>348 (Ac)</td>
<td>0.31±0.0</td>
<td>129.9±9.</td>
<td>3.50±0.0</td>
<td>10.7±0.4</td>
<td>0.45±0.0</td>
<td>1.44±0.3</td>
<td>96.1±19.</td>
</tr>
<tr>
<td>363 (Ap)</td>
<td>0.26±0.0</td>
<td>63.4±1.9</td>
<td>3.75±0.0</td>
<td>10.4±0.1</td>
<td>4.77±0.1</td>
<td>2.11±0.1</td>
<td>299.2±7.</td>
</tr>
<tr>
<td>2053 (Aa)</td>
<td>0.54±0.0</td>
<td>14.5±3.6</td>
<td>5.91±0.9</td>
<td>9.4±0.4</td>
<td>1.66±0.3</td>
<td>0.19±0.0</td>
<td>200.7±4.</td>
</tr>
<tr>
<td>11460 (Tr)</td>
<td>1.38±0.1</td>
<td>109.3±1</td>
<td>1.33±0.2</td>
<td>3.7±0.2</td>
<td>0.12±0.0</td>
<td>0.03±0.0</td>
<td>64.9±0.6</td>
</tr>
</tbody>
</table>

Note: The first 9 strains are *A. niger* (An); the last 5 strains are *A. foetidus* (Af), *A. cinnamomeus* (Ac), *A. phoenicis* (Ap), *A. aculeatus* (Aa) and *T. reesei* (Tr), respectively.

For easier strain selection, the top 5 or 6 producing strains for each carbohydrase activity are highlighted in bold font; for protease activity, the bottom 5 producing strains are highlighted (because higher carbohydrase and lower protease are desirable).
Figure 5.2 Tentative correlation between $\alpha$-galactosidase and sucrase productions in soy hull-based medium by the fungal strains evaluated

No correlations could be found between any other two groups of activities (figures not shown), not even between polygalacturonase and pectinase. Being responsible for breaking down the polygalacturonate backbone of pectin, polygalacturonase is an important component of pectinase activity. The two activities were originally thought to correlate somewhat. However, for examples, \textit{A. niger} NRRL 2270 had the second highest polygalacturonase activity ($14.7 \pm 0.1$ U/mL) in Table 2 but had only $3.80 \pm 0.01$ U/mL pectinase, among the lowest in all \textit{Aspergillus} species and strains tested; on the other hand, \textit{A. foetidus} NRRL 341 had the lowest polygalacturonase activity ($2.4 \pm 0.2$ U/mL) but a significantly higher pectinase activity ($4.54 \pm 0.57$ U/mL).
Presumably the lack of correlation between these two enzyme activities reflected the complex structure of citrus pectin used as substrate in the pectinase assay. The steric protection of side chains and presence of calcium crosslinks in pectin [148, 149] may render the polygalacturonate backbone inaccessible or resistant to enzyme attack so that high polygalacturonase activity does not necessarily translate into high pectinase activity; on the other hand, effective side-chain hydrolysis can give relatively good pectinase activity even when the enzyme is low in polygalacturonase activity. The complex structure also made pectin more difficult to hydrolyze to high extents than polygalacturonic acid, as suggested in Table 2 where the maximum polygalacturonase activity (15.6 ± 0.3 U/mL) attainable with all evaluated strains was significantly higher than the maximum pectinase activity (6.36 ± 0.32 U/mL).

Despite the complexities of substrate and enzymes, for the immediate purpose of this study the strain screening can be focused on the strains that have higher carbohydrase activities and lower protease activity, so as to maximize carbohydrate hydrolysis and minimize protein hydrolysis. Accordingly, the top 5 or 6 producing strains for each carbohydrase activity and the lowest 5 protease-producing strains are highlighted in Table 5.2. According to this approach, A. niger NRRL 322 was found to perform well on 6 out of the 7 tested activities: it was among the top strains for every carbohydrase activity but was not among the low protease producers. It produced 140.5 ± 2.1 BAEE U/mL protease, which was still below the average protease activity, 182 (± 78) BAEE U/mL, produced by all tested strains. Next, A. foetidus NRRL 341 was good in 5 activities and A. niger NRRL 13201 and A. cinnamomeus type strain NRRL 348 were both good in 4 activities. The latter 3 strains were all low in protease production. Strain 341 gave a
higher pectinase activity while strains 348 and 13201 produced significantly more polygalacturonase. As discussed earlier, polygalacturonase is only a component in the pectinase enzyme group; so, strain 341 might be more effective than the other two for overall pectin hydrolysis. Further, for potential use of hydrolysate as fermentation feedstock, it is desirable to maximize monomerization of soy carbohydrate. Therefore, it is beneficial to have higher activities of the oligosaccharide-degrading $\alpha$-galactosidase and sucrase. Both strains 348 and 13201 produced very low $\alpha$-galactosidase and sucrase activities. *A. foetidus* NRRL 341 is likely much more effective than the other two strains for the intended application.

*A. phoenicis* NRRL 363 and *A. aculeatus* NRRL 2053 performed well in only 2 activities. *T. reesei* Rut-C30 NRRL 11460 produced much more cellulase than all other strains and was among the highest xylanase producers; it also produced the lowest level of protease. The *T. reesei* strain, however, produced very low levels of enzymes for hydrolysis of pectin and soy oligosaccharides. More direct results about soy flour carbohydrate hydrolysis are given in the next section. Nonetheless, the above analysis of enzyme activities suggests that *A. niger* NRRL 322 and *A. foetidus* NRRL 341 are the two most promising strains for the intended application of separating soy flour/meal carbohydrate and protein.

5.3.3 Comparison of soybean hull induced enzyme profiles: *T. reesei* versus Aspergillus strains

*T. reesei* Rut-C30 (NRRL 11460) had very different carbohydrazide production profiles than the *Aspergillus* strains. Each enzyme activity produced by *T. reesei* Rut-C30
after 72 h cultivation was compared with the average activity from all *Aspergillus* strains (Figure 5.3). Despite the somewhat large variations among the activities from different *Aspergillus* strains, it is clear that *T. reesei* Rut-C30 produced much more cellulase than *Aspergillus* and was among the highest xylanase producers. *T. reesei* Rut-C30, however, produced very low levels of pectinase and polygalacturonase for pectin hydrolysis, and produced almost no $\alpha$-galactosidase and sucrase. It is clearly not an optimal enzyme producer for hydrolyzing biomass with high contents of galacto-oligosaccharides and other galactose- or sucrose-rich carbohydrates. *T. reesei* also produced the lowest level of protease.

Figure 5.3 Comparison of enzyme activities produced by *T. reesei* Rut-C30 after 72 h cultivation with the corresponding average activities from all of the *Aspergillus* species and strains (error bars for *T. reesei* are standard deviations of data from all repeated experiments made with this strain, those for *Aspergillus* are standard deviations of the average activities from all *Aspergillus* strains)

5.3.4 Comparison of soybean hull induced enzyme profiles among Aspergillus strains

The enzyme activities produced at 72 h cultivation of different *Aspergillus* strains
are compared (Figure 5.4). Different activities differ significantly in scale. For easy presentation and comparison, individual activities are averaged for all *Aspergillus* strains and then the activities of each strain are presented as % deviations (+/-) from the averages. For the x-axis, the strains are arranged in the descending order of carbohydrase productivity, in terms of their numbers and, secondarily, magnitudes of positive deviations from average carbohydrase activities. Protease activities for these strains are also shown (Figure 5.4). For the example of using the enzyme for soy flour protein-carbohydrate separation, low protease activities are more desirable to minimize soy protein degradation. The strains’ growth morphology, i.e., filamentous, fine-pellet and large-pellet forms, are also given to examine its possible correlation with the enzyme productivity (Figure 5.4).

![Graph showing enzyme activities](image)

Figure 5.4 Comparison of enzyme activities produced at 72 h cultivation of different *Aspergillus* species and strains. Activities for each strain are presented as % deviations (+/-) from the average activities calculated for all *Aspergillus* strains. Strains (x-axis) are
arranged according to decreasing carbohydralase productivity, mainly by their numbers of positive deviations from average carbohydralase activities and secondarily by the magnitudes of positive deviations. Strains are given in their NRRL numbers, plus a letter abbreviation for Aspergillus species: n for A. niger, f for A. foetidus, c for cinnamomeus, p for A. phoenicis, and a for A. aculeatus.

A. niger NRRL 322 was the top performer, producing every carbohydralase activity at an above-average level (by + 40-70%, except the +4% for cellulase). This strain also produced protease at about 23% below average. Next, A. foetidus NRRL 341 produced 4 carbohydralase activities at above-average levels (i.e., cellulase, xylanase, α-galactosidase and sucrase), 1 carbohydralase activity (pectinase) at the average level, and only 1 carbohydralase activity (polygalacturonase) at a below-average level. This strain was a very low protease producer, at about 45% below average. A. niger NRRL 13219 appeared to be the lowest carbohydralase producer, giving only 1 activity (polygalacturonase) at an above-average level.

The complete, approximate order of soybean hull-induced carbohydralase productivity is: A. niger NRRL 322 > A. foetidus NRRL 341 > A. niger NRRL 62517 > A. niger NRRL 13201 > A. cinnamomeus NRRL 348 > A. niger NRRL 566 > A. niger NRRL 334 > A. phoenicis NRRL 363 > A. niger NRRL 2270 > A. aculeatus NRRL 2053 > A. niger NRRL 328 > A. niger NRRL 599 > A. niger NRRL 13219. In this order, A. foetidus, A. cinnamomeus, A. phoenicis and A. aculeatus strains are dispersed among the A. niger strains. There is no clear evidence of species-dependent carbohydralase productivity among these Aspergillus cultures.
On the other hand, the growth morphology appeared to have a more consistent effect on the soybean hull induced carbohydrase productivity. The top 3 producers (\textit{A. niger} NRRL 322, \textit{A. foetidus} NRRL 341 and \textit{A. niger} NRRL 62517) all grew in the filamentous form; both of the 2 lowest producers (\textit{A. niger} NRRL 599 and 13219) formed large pellets; and the fine-pellet strains were the middle producers. The only clear exception was \textit{A. cinnamomeus} NRRL 348, which had large-pellet morphology but produced carbohydrase at higher productivity than most of the fine-pellet strains. This finding of correlation between carbohydrase productivity and growth morphology may be a very useful tool for preliminary strain screening in future studies.

5.3.5 Soy flour hydrolysis

The carbohydrate content of soy flour was measured in this study according to the NREL method [150], with both DNS (reducing sugar) and phenol-sulfuric acid (total carbohydrate) methods for analysis of the released carbohydrate, using glucose as the standard. One g soy flour was found to contain $0.25 \pm 0.01$ g glucose-equivalent reducing sugar or $0.26 \pm 0.01$ g glucose-equivalent total carbohydrate. The total carbohydrate content was given as 32 wt\% in the manufacturer’s product sheet. The seemingly lower measured values are believed to reflect the use of glucose as standard while different carbohydrates have different correlation coefficients in the DNS and phenol-sulfuric acid methods [129]. According to these measured values, complete hydrolysis of 250 g/L soy flour in the enzymatic hydrolysis tests could release up to 62.5-65.0 g/L soluble (glucose-equivalent) reducing sugar and total carbohydrate into the hydrolysate.
Summarized in Table 5.3 are the (glucose-equivalent) concentrations of soluble reducing sugar and total carbohydrate released from soy flour after 24 and 49 h hydrolysis by 4-fold diluted cell-free culture broths obtained in the above strain screening experiments. The strains are arranged in the descending order of average reducing sugar concentration obtained after 24 h hydrolysis. According to the analysis by Tukey’s pairwise comparison method with 95% confidence, the enzyme broths from *A. niger* NRRL 322 and *A. foetidus* NRRL 341 released the highest, statistically comparable concentrations of reducing sugar after 24 h. Four other *A. niger* strains, i.e., NRRL 13219, 62517, 2270 and 13201, joined this top performing group of reducing sugar release after 49 h. The decreasing difference with time was reasonable as the more readily accessible and hydrolyzable substrate diminished. The difference in release of total soluble carbohydrate was even less distinguishable. For both results at 24 and 49 h, only the enzyme broths from 3 strains, i.e., *A. niger* NRRL 2270, *A. aculeatus* NRRL 2053 and *T. reesei* NRRL 11460, caused statistically inferior release of total carbohydrate. The enzyme activities responsible for more complete monomerization (i.e., higher reducing sugar concentrations) are the most important differentiating feature among the enzyme broths from these fungal cultures. The soy flour hydrolysis tests confirmed that *A. niger* NRRL 322 and *A. foetidus* NRRL 341 are the two most promising strains for the intended application, which is consistent with the finding by the previous enzyme activity analysis.
Table 5.3: Concentrations of reducing sugar and total carbohydrate released after 24 and 49 h hydrolysis by 4-fold diluted cell-free culture broths

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reducing sugar (g/L)</th>
<th>Total carbohydrate (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>49 h</td>
</tr>
<tr>
<td>322</td>
<td>33.2±0.1</td>
<td>36.2±1.5</td>
</tr>
<tr>
<td>341 (Af)</td>
<td>32.1±0.2</td>
<td>36.7±1.7</td>
</tr>
<tr>
<td>13219</td>
<td>31.2±0.4</td>
<td>34.2±1.3</td>
</tr>
<tr>
<td>62517</td>
<td>30.3±0.1</td>
<td>34.1±0.2</td>
</tr>
<tr>
<td>334</td>
<td>30.1±0.3</td>
<td>33.2±0.5</td>
</tr>
<tr>
<td>2270</td>
<td>29.9±0.1</td>
<td>33.6±1.6</td>
</tr>
<tr>
<td>348 (Ac)</td>
<td>29.8±0.1</td>
<td>33.0±0.9</td>
</tr>
<tr>
<td>328</td>
<td>29.1±1.0</td>
<td>33.0±0.7</td>
</tr>
<tr>
<td>13201</td>
<td>29.0±0.4</td>
<td>33.5±0.3</td>
</tr>
<tr>
<td>599</td>
<td>27.4±0.4</td>
<td>33.1±1.6</td>
</tr>
<tr>
<td>566</td>
<td>27.0±0.5</td>
<td>33.1±1.9</td>
</tr>
<tr>
<td>363 (Ap)</td>
<td>26.9±0.4</td>
<td>31.2±0.6</td>
</tr>
<tr>
<td>2053 (Aa)</td>
<td>10.6±0.2</td>
<td>15.7±0.2</td>
</tr>
<tr>
<td>11460 (Tr)</td>
<td>7.5±0.2</td>
<td>8.9±0.2</td>
</tr>
<tr>
<td>Control</td>
<td>3.5±0.0</td>
<td>4.3±0.1</td>
</tr>
</tbody>
</table>

Note: Strains are arranged in the descending order of average reducing sugar concentration obtained after 24 h hydrolysis. *A. niger* strains are given only by NRRL numbers. Others are indicated with abbreviations: *A. foetidus* (Af), *A. cinnamomeus* (Ac), *A. phoenicis* (Ap), *A. aculeatus* (Aa) and *T. reesei* (Tr). In each column, the boldfaced values indicate the group of strains giving the highest reducing sugar or total carbohydrate concentrations, identified by the Tukey pairwise comparison method with 95% confidence. At least 1 strain in this group gave statistically higher hydrolysis results than at least 1 strain in the next group.
The soy flour carbohydrate hydrolysis tests confirmed that *A. niger* NRRL 322 and *A. foetidus* NRRL 341 are the two most promising strains for the intended application, which is consistent with the results of enzyme activity analysis. *T. reesei* NRRL 11460 produced the least effective enzyme, despite its highest cellulase and xylanase activities. The poor performance must be related to its very low pectinase and polygalacturonase and almost zero $\alpha$-galactosidase and sucrase. The second least effective strain, *A. aculeatus* NRRL 2053, also produced the lowest sucrase and very low $\alpha$-galactosidase among the *Aspergillus* strains although it had significantly above average (~ +30%) pectinase activity and near average polygalacturonase activity. The $\alpha$-Galactosidase and sucrase appeared to be very important to the hydrolysis of soy flour carbohydrate. Further, the soy flour carbohydrate hydrolysate may be used as fermentation feedstock. For this application, it is desirable to maximize monomerization of the carbohydrate. Therefore, it is beneficial to have higher activities of the oligosaccharide-degrading $\alpha$-galactosidase and sucrase.

5.3.6 Enriched protein product

To evaluate the effect of enzymatic treatment on soy protein enrichment, the enzymatic hydrolysis test systems using the cell-free broths of *A. foetidus* NRRL 341 and *T. reesei* NRRL 11460 were subjected to further analysis as examples. Hydrolysate and remaining protein-enriched solids were separated by centrifugation. The enriched protein products from these systems had different protein contents: 69% from the system with the *A. foetidus* NRRL 341 broth [151] but only 64% from the system with the *T. reesei* NRRL 11460 broth. The lower protein enrichment in the latter system was consistent with the
earlier finding of less complete soy carbohydrate hydrolysis by the *T. reesei* NRRL 11460 broth. Moreover, the carbohydrate released into the hydrolysate by the *T. reesei* broth was less completely monomerized and, thus, had a lower reducing sugar concentration. This was because the *T. reesei* broth contained extremely low α-galactosidase and sucrase activities. Although the less complete conversion of soy carbohydrate to reducing sugar is not detrimental to the purpose of protein enrichment (i.e., 64%, compared to 69% from the processing with more effective enzymes in the *A. foetidus* broth), it may more negatively affect the quality of hydrolysate produced when it is subsequently used as fermentation feedstock.

5.4 Conclusion

*T. reesei* and *Aspergillus* strains grew on soybean hulls with different morphologies: filaments – *T. reesei* Rut C30 (NRRL 11460), *A. foetidus* NRRL 341, and *A. niger* NRRL 322, 325, 3122 and 62517; fine pellets (~ 1-mm diameter) – *A. aculeatus* NRRL 2053, *A. phoenicis* NRRL 363, and *A. niger* NRRL 328, 334, 566, 2270 and 13201; and large pellets (~ 5-10 mm) – *A. cinnamomeus* NRRL 348 and *A. niger* NRRL 599 and 13219. Growth morphology was found to correlate with the pH profile exhibited and carbohydrase produced during growth on soybean hulls. Filamentous strains caused slowest pH decrease and, among *Aspergillus*, produced highest carbohydrase activities. The strains grew with fine pellets were the middle group of carbohydrase producers but
caused the fastest pH decrease. The strains forming large pellets tended to be the lowest carbohydrate-producing group.

*T. reesei* Rut-C30 (NRRL 11460) had very different carbohydrate production profiles than *Aspergillus*. *T. reesei* produced far more cellulase than *Aspergillus* and was among the highest xylanase producers. However, it produced very low pectinase and polygalacturonase activities for pectin hydrolysis, and produced almost no α-galactosidase and sucrase. Accordingly, *T. reesei* Rut-C30 produced the least effective enzyme for protein-carbohydrate separation of soy flour which had high contents of pectin and galactose- and sucrose-rich carbohydrates (sucrose, raffinose and stachyose).

*A. niger* NRRL 322 and *A. foetidus* NRRL 341 were the most potent strains, under soybean hull induction; they produced maximal carbohydrases and minimal protease. In soy flour hydrolysis tests, the enzymes from these two strains gave the fastest and most complete carbohydrate hydrolysis. Treatment with 4-fold diluted broth of *A. foetidus* NRRL 341 enriched the protein content from 53% (in original soy flour) to 69% in solids readily separated from the hydrolysate.
CHAPTER VI
EFFECTS OF CARBON SOURCES NITROGEN SOURCES OTHER NUTRIENTS AND FERMENTATION CONDITIONS FOR ENZYME PRODUCTION THROUGH *ASPERGILLUS NIGER* FERMENTATION

6.1 Introduction

Carbon is the structural backbone of the organic compounds that make up a living cell. Nitrogen plays vital role in the cell synthesis of amino acids, DNA, RNA, ATP and so on. Different types of carbon and nitrogen sources for fungal fermentation have been studied. People used molasses and sucrose as carbon source, ammonium nitrate and ammonium sulfate as nitrogen source for citric acid production of *A. niger* [119]. In order to stimulate desired enzyme production, some people used biomass as carbon source, such as pectin for pectinase production [108]. The carbon sources, nitrogen sources and other nutrients can strongly affect the enzyme production. The inducers play the most important role to induce the complex enzyme production. The enzymes were used to hydrolyze the soybean carbohydrate, therefore the soybean carbohydrate was good inducer to stimulate the right enzymes. Soy hull, soy flour, and soy molasses are good inducers, and they are rich in carbohydrates. Soy flour is not only rich in carbohydrate but also protein, which can also be part of the nitrogen source. Besides carbon sources
and nitrogen sources themselves, the ratio of carbon to nitrogen is really important. Carbon limiting or nitrogen limiting both cause problems to cell growth and enzyme production. Therefore, the optimum carbon to nitrogen ratio was studied. Some other nutrients also had effects on enzyme production. The potassium and tween 80 were studied in this project.

In order to increase enzyme production, it is necessary to design and modify the fermentation conditions for fungus. One important factor is nutrition conditions, and the other one is operation conditions like pH, dissolved oxygen, mixing, temperature, shear force and fungal morphology. During the nutrient utilization of fungus, some acidic or alkaline chemicals release to the fermentation broth, which can lead to pH fluctuation. In order to keep stimulating some desired product production, it is necessary to keep certain pH value. Therefore, it is essential to find the optimum pH environment for enzyme production of fungus. The fungus fermentation process was aerobic, which used oxygen to convert carbon source into carbon dioxide, water and energy. And most of energy is contributed to cell growth and product. Therefore, dissolved oxygen is crucial in the fermentation process. Usually oxygen limitation affected cell growth and product productivity [152]. Mixing not only help oxygen transferring, but also help form the uniform system. Moderate mixing was considered, which because too low mixing is poor for oxygen transfer but too high mixing leads to high shear force, which may break the cell. There are different optimum temperatures for different fungus. Not only the growth temperature, but also the optimum temperature for enzyme production is more important. Therefore, these fermentor operation conditions were investigated to induce the enzyme production.
6.2 Materials and Methods

The materials and methods were divided into two parts, which included shaker study and fermentor scale study, and described below.

6.2.1 *A. niger* 341 shaker study

First, the flask study of optimum strain was investigated in different combination of carbon source and nitrogen source at same initial pH. Soy hull, sucrose, soy molasses, and soy flour were investigated in flask study. Besides the carbon source and nitrogen source, some other nutrients were also investigated. In order to save cost, the culture medium was modified without effects on the enzyme production. Potassium phosphate was the expensive salt in the medium, which provided the necessary nutrition to keep growth. The surfactant tween 80 can help the enzyme releasing, which is costly. Therefore the study of the optimum amount of potassium phosphate and tween 80 was evaluated. Finally, one of the most important factors in fermentation was the ratio of carbon and nitrogen source. It’s important to make the carbon and nitrogen sources reach the balance during fermentation. Therefore, different carbon to nitrogen ratio was applied to the culture medium to find the optimum ratio.

The fungi were cultured in potato dextrose agar for 72 hours in the room temperature then stored in 4°C for one month. The flask study was carried out in the shaker. The selected optimum fungus was incubated for 5 days in the shaker with 250 rpm speed in room temperature. The Daily samples were taken to analyze the cell growth, pH and enzyme productivities. The experimental designs were listed in Table 6.1. Based
on the analysis of enzyme productivities with different factors, the one with higher enzyme productivity was the better treatment factor.

Table 6.1 The experimental designs of optimization of fermentation nutrition

<table>
<thead>
<tr>
<th>Experiment design</th>
<th>Treatment factor</th>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carbon source (soy hull, sucrose, soy molasses)</td>
<td>Enzyme productivities</td>
</tr>
<tr>
<td>2</td>
<td>Nitrogen source (soy flour versus protease peptone)</td>
<td>Enzyme productivities</td>
</tr>
<tr>
<td>3</td>
<td>Carbon/Nitrogen ratio (different ratio amount)</td>
<td>Enzyme productivities</td>
</tr>
<tr>
<td>4</td>
<td>Phosphorus (different amount of Potassium phosphate)</td>
<td>Enzyme productivities</td>
</tr>
<tr>
<td>5</td>
<td>Surfactant (with/without tween 80)</td>
<td>Enzyme productivities</td>
</tr>
</tbody>
</table>

6.2.2 *A. niger* 341 fermentation study

Through the shaker study of *A. niger* 341 fermentation, different carbon sources, nitrogen sources and other nutrients were studied. To further modify the medium composition and increase the enzyme productivities, different concentrations of carbon source and nitrogen source were studied in fermentation. Other nutrient such as potassium was also investigated in fermentation scale. Besides the medium optimization, the fermentation conditions were also studied. From the *T. reesei* fermentation pH study, more cellulase and xylanase were produced with soybean hulls as carbon source with
higher pH (pH=6.0). The polygalacturonase production preferred lower pH. Cell enzyme production for cellulase was lower at 4.5. Higher pH induced the cellulase and xylanase production of *T. reesei* fermentation. But these experiences cannot applied to different strain, because the behavior of *A. niger* was different from *T. reesei*. Different pH control was applied in *A. niger* fermentation such as constant pH, low constant pH, and high initial pH with low ending pH or versa. Changing pH control, and synthetic pH and dissolved oxygen strategy were investigated in *A. niger* 341 fermentation. Continuous air with 20% critical oxygen was supplied to previous *T. reesei* study, which is good for keeping enough dissolved oxygen, however with foaming issue. Therefore, continuous air and occasionally oxygen supply was investigated in optimum fungus fermentation. In previous study, all the temperature control is under room temperature. Actually, the optimum temperatures were different for different enzyme production. In order to induce complex enzyme production, the temperature study was investigated to find how it affects the enzyme production. The fermentations were operated in 3L fermentor with 1-1.5 L working volume under dissolved oxygen, pH, agitation, temperature, and foaming control. Daily samples were taken to analyze the enzyme productivities.

6.3 Results and Discussion

The enzyme production results of different conditions were described below, which included the shaker and fermentor study.

6.3.1 *A. niger* shaker study

The shake study of *A. niger* was investigated in different combination of carbon source and nitrogen source at initial pH 5.0, which were system 1 to system 4. The
enzyme production was best in system 4. The enzyme production results were listed in Table 6.2. And the pH trends, enzyme production trends were shown in Figure 6.1, 6.2, 6.3, and 6.4.

Table 6.2 Enzyme productions of *A. niger* shaker study

<table>
<thead>
<tr>
<th>Fermentation System</th>
<th>Nitrogen Source</th>
<th>Carbon source</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Polygalactosidase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System1</td>
<td>(NH₄)₂SO₄ - 1.4g/L Urea – 0.3g/L Proteose peptone - 1g/L</td>
<td>Sucrose - 10g/L</td>
<td>0.09±0.01</td>
<td>0.28±0.27</td>
<td>0.42±0.10</td>
</tr>
<tr>
<td>System2</td>
<td>soyflour –7.04g/L</td>
<td>Sucrose - 7.75g/L</td>
<td>0.13±0.01</td>
<td>6.8±1.1</td>
<td>0.37±0.08</td>
</tr>
<tr>
<td>System3</td>
<td>(NH₄)₂SO₄ - 1.4g/L Urea – 0.3g/L Proteose peptone - 1g/L</td>
<td>Soyhulls - 20g/L</td>
<td>0.44±0.03</td>
<td>44.7±1.6</td>
<td>5.04±0.20</td>
</tr>
<tr>
<td>System4</td>
<td>Soyflour –7.04g/L</td>
<td>Soyhulls - 15.5g/L</td>
<td>0.37±0.01</td>
<td>39.3±3.1</td>
<td>1.27±0.20</td>
</tr>
</tbody>
</table>
Figure 6.1 pH trends of *A. niger* shaker study

Figure 6.2 Cellulase production trends of *A. niger* shaker study
Different nitrogen sources were studied. System 1 was the original medium with protease peptone, urea and ammonia sulfate. System 2 applied ammonia sulfate as nitrogen source. System 3 was soy flour and urea mixture. System 4 was soy flour and
urea. System 5 contained only soy flour. In system 3 all the medium compositions were autoclaved together and most of the soy flour were splashed to the flask walls, which cannot be consumed by fungus. In system 4 and 5 the soy flour were autoclaved separately, and all the soy flour were in the medium liquid, which can be consumed by fungus. The extrocellular proteins were presented as the total enzyme concentration, which was showed in Figure 6.5.

![Figure 6.5 Extrocellular proteins at different nitrogen source systems](image)

Figure 6.5 Extrocellular proteins at different nitrogen source systems

The original medium with organic nitrogen source was better than inorganic nitrogen source medium. The soy flour system had the similar production as original medium, which can substitute peptone and urea. However, the soy flour with urea as nitrogen source were better than only soy flour. With organic nitrogen source, about 30%-123% higher extrocellular protein productions were obtained than only inorganic nitrogen source medium. Although inorganic nitrogen source cannot instead all the organic nitrogen sources, some less cost nitrogen sources can be the alternatives. Soy
flour with lower price provided 14.3% higher extracellular protein production than protease peptone as nitrogen source. Because urea was not expensive but contributed to enzyme production, it was still used as the ingredient of nitrogen source.

Some oligosaccharides like stachyose and raffinose, which cannot be digested by fish, it’s necessary to degrade them completely during the hydrolysis process. And $\alpha$-galactosidase was the known enzyme aims to break down these oligosaccharides. In order to induce more production of this enzyme by $A. niger$, the soy molasses with certain amount of stachyous (6.9%) and raffinose (1.4%) were used for stimulating the enzyme production.

Different ratio of soy molasses and soy hull as carbon source were studied to find how to reach the higher enzyme productivity and extracellular protein productivity by changing the carbon source. The results were present in Table 6.3, 6.4.

Table 6.3 Enzyme and extracellular protein productivity of $A. niger$ 322 and 341 flask study

<table>
<thead>
<tr>
<th>Strain 322</th>
<th>Soyhull/Soymolasses carbohydrate ratio(g/g)</th>
<th>Extrocellular protein (g/L)</th>
<th>$\alpha$-galactosidase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5/0</td>
<td>0.16±0.02</td>
<td>0.45±0.00</td>
</tr>
<tr>
<td></td>
<td>0.25/0.25</td>
<td>0.10±0.00</td>
<td>0.44±0.00</td>
</tr>
<tr>
<td></td>
<td>0/0.5</td>
<td>0.03±0.01</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td></td>
<td>0.5/0</td>
<td>0.24±0.00</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td></td>
<td>0.25/0.25</td>
<td>0.19±0.01</td>
<td>0.45±0.00</td>
</tr>
<tr>
<td></td>
<td>0/0.5</td>
<td>0.06±0.00</td>
<td>0.28±0.01</td>
</tr>
</tbody>
</table>
Table 6.4 Enzyme and extracellular protein productivity of *A. niger* 322 flask study

<table>
<thead>
<tr>
<th>Strain 322</th>
<th>Soyhull/Soymolasses carbohydrate ratio (g/g)</th>
<th>Extracellular protein (g/L)</th>
<th>α-galactosidase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5/0</td>
<td>0.12±0.00</td>
<td>5.18±0.05</td>
</tr>
<tr>
<td></td>
<td>0.25/0.5</td>
<td>0.10±0.01</td>
<td>1.25±0.06</td>
</tr>
<tr>
<td></td>
<td>0.25/0.25</td>
<td>0.11±0.01</td>
<td>3.46±0.10</td>
</tr>
<tr>
<td></td>
<td>0.4/0.1</td>
<td>0.12±0.02</td>
<td>4.39±0.10</td>
</tr>
<tr>
<td></td>
<td>0.45/0.05</td>
<td>0.13±0.01</td>
<td>4.49±0.17</td>
</tr>
<tr>
<td></td>
<td>0.45/0.1</td>
<td>0.15±0.01</td>
<td>4.91±0.09</td>
</tr>
</tbody>
</table>

Figure 6.6 Extracellular protein production of *A. niger* 322
Based on the results of extracellular protein and α-galactosidase production of strain 322 and 341, the more soy hull content in the carbon source provided better protein and enzyme production. Soy hull can continuously stimulate α-galactosidase production, but soy molasses cannot. The reason should be the different consumption rate of them. More readily free sugars in soy molasses played less inducer role, which can’t induce more enzyme production. However the soy hull with more oligosaccharides and polysaccharides can continuously induce enzyme productions.
Strain 322 and 341 were selected as the test producers for this study based on the strain screening results. These two strains were cultured in different systems. Different amount of potassium phosphate and nitrogen source were investigated. The potassium phosphate concentration varied from 0.2 g/L to 2 g/L (0.2, 0.4, 1, 2). The results were present in Figure 6.8. For strain 322, the extrocellular protein production increased as potassium phosphate increased. The protein production didn’t increase after potassium phosphate reached 1 g/L. For strain 341, the extrocellular protein production was similar from 0.2 to 1g/L potassium phosphate, but increased by 21% with 2 g/L potassium phosphate.
For *A. niger* 322, the extracellular protein production with tween80 was higher than the medium without tween80 by 1.4%. For *A. niger* 341, the extracellular protein production with tween 80 was higher than without tween 80 by 13%. Although tween 80 as the surfactant in the medium had some benefit than without tween 80 systems, the differences were not too large compared to the nitrogen source effects. Therefore, whether using tween 80 depends on how many enzyme productions can be sacrificed in the industry application.

No matter carbon limiting or nitrogen limiting would affect the cell growth and enzyme production. It’s important to control the balance of carbon and nitrogen sources in fermentation.

Therefore, different carbon to nitrogen ratio was applied to the *A. niger* 341 culture medium. The flask study was evaluated with sucrose as sole carbon source and ammonia sulfate as sole nitrogen source. The ratio of sugar concentration to nitrogen
concentration varied from 17 to 100. The daily cell dry weight and intracellular protein were analyzed.

Figure 6.10 Ammonia sulfate amount of different systems(Sugar/N ratio:1-17,2-20,3-25,4-29,5-33,6-100)

Figure 6.11 Cell dry weight of different systems(Sugar/N ratio:1-17,2-20,3-25,4-29,5-33,6-100)
Figure 6.12 Introcellular protein of different systems (Sugar/N ratio: 1-17, 2-20, 3-25, 4-29, 5-33, 6-100)

Figure 6.13 Ammonia sulfate amount of different systems (Sugar/N ratio: 1-29, 2-40, 3-67, 4-100)
Figure 6.14 Cell dry weight of different systems (Sugar/N ratio: 1-29, 2-40, 3-67, 4-100)

Figure 6.15 Introcellular protein of different systems (Sugar/N ratio: 1-29, 2-40, 3-67, 4-100)
Figure 6.16 (A)-(B) The maximum intracellular protein and dry weight results as different N concentration

From this study, nitrogen limiting happened when the sugar to nitrogen ratio was above 40 for strain 341 using sucrose as carbohydrate in shaker study. The conclusion was based on the results of cell growth, so the nitrogen limiting might happen earlier than ratio 40 in fermentor system for enzyme production. In the fermentation systems, sugar to nitrogen ratio should be controlled below 25.

During the fermentation, there was not obvious protease production. And the
protease was mostly accumulated from pre-culture. Therefore this study was to check if the medium composition and working volume (dissolved oxygen varied in different working volume) affect the protease production. It is necessary to decrease the protease production during the pre-culture. In systems 1 and 2, the soy hull was the carbon source. In system 3 and 4, the sucrose was the carbon source. The nitrogen sources of soy hull and sucrose systems were ammonium sulfate, protease peptone and urea. The other nutrition parts in system 1-4 were the same as usual. The system 5 used only potato dextrose broth as medium. The working volume of system 1 and 3 was 50mL in 250mL flask, and the volume of system 2 and 4 was 25mL in 250mL flask. The strain was incubated for 3 days in the shaker at 250rpm speed in room temperature.

![Graph](image)

Figure 6.17 The protease production of different systems

The protease production of systems 1-4 during pre-culture with different medium did not differ too much. Therefore, it can be concluded the working volume did not give
obvious effects on protease production. The culture time with 24 hours was not enough for cell growth from the observation because the cell concentration was too low. And 72 hours were too long, that the cells were too old reflecting yellow color. From these five systems, the best one with least protease was potato dextrose broth in 48 hours, which gave the lowest protease 50.7±7.1 U/mL. The reason of higher protease production in systems 1-4 than 5 might be the nitrogen source effect. In order to get clear conclusion, the different nitrogen sources study was suggested in future study.

6.3.2 A. niger 341 fermentation study

The fermentations with different soy hull amounts were investigated. In FerA1, A2 and A4, the soy hull was 20 g/L, and it’s 40 g/L in FerA3. FerA1 was operated at initial pH=4.5 without control later. FerA2 was operated at constant pH=4.5. FerA3 and FerA4 were operated at constant pH=5.0. And at the same time, strain 2053 was investigated in FerA5 compared to strain 341. The other strain 13201 was also tested in fermentations. Two 1.5L fermentations were tested with 20g/L soy hulls as the carbon source, which were FerA6, and FerA7. The pH of FerA6 was operated at pH=6.4 and decreased to 3.5 by strain itself then kept constant. FerA7 was operated at initial pH=6.4 without control later.
Table 6.5 Enzyme productions of *A. niger* fermentations

<table>
<thead>
<tr>
<th>Fermentation System</th>
<th>Carbon Source</th>
<th>Nitrogen Source</th>
<th>Sugar/N ratio</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Polygalacturonase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FerA1 (pH4.5-no control) (8 days)</td>
<td>Soy hulls -20g/L</td>
<td>(NH$_4$)$_2$SO$_4$ -1.4g/L Urea – 0.3g/L Proteose peptone -1g/L</td>
<td>23.8</td>
<td>0.32±0.0 (4 days) 0.30±0.01 (8 days)</td>
<td>32.5±1.5 (4 days) 27.2±0.8 (8 days)</td>
<td>2.6±0.1 (3 days) 0.7±0.1 (8 days)</td>
</tr>
<tr>
<td>FerA2 (pH4.5) (7 days)</td>
<td>Same as A1</td>
<td>Same as A1</td>
<td>23.8</td>
<td>0.35±0.00 (7 days)</td>
<td>50.95±1.46 (7 days)</td>
<td>2.5±0.1 (6 days) 2.1±0.2 (7 days)</td>
</tr>
<tr>
<td>FerA3 (pH5.0) (9 days)</td>
<td>Double</td>
<td>Same as A1</td>
<td>47.6</td>
<td>0.64±0.03 (9 days)</td>
<td>142.4±1.6 (6 days) 114.7±1.7 (9 days)</td>
<td>4.1±0.2 (8 days) 4.0±0.2 (9 days)</td>
</tr>
<tr>
<td>FerA4 (pH5.0) (9 days)</td>
<td>Same as A1</td>
<td>Double</td>
<td>11.9</td>
<td>0.62±0.02 (5 days) 0.61±0.03 (9 days)</td>
<td>65.1±1.7 (4 days) 60.1±4.7 (9 days)</td>
<td>2.6±0.1 (8 days) 2.5±0.1 (9 days)</td>
</tr>
<tr>
<td>FerA5 (2053) (pH6-4) (7 days)</td>
<td>Same as A1</td>
<td>Same as A1</td>
<td>23.8</td>
<td>0.56±0.03 (7 days)</td>
<td>20.1±1.0 (3 days) 11.4±2.1 (7 days)</td>
<td>27.4±0.6 (5 days) 27.4±0.3 (7 days)</td>
</tr>
<tr>
<td>FerA6 (13201) (pH6.4-3.5) (4 days)</td>
<td>Same as A1</td>
<td>Same as A1</td>
<td>23.8</td>
<td>0.47±0.09 (3 days) 0.47±0.09 (4 days)</td>
<td>83.8±8.9 (4 days)</td>
<td>11.7±0.1 (2 days) 8.6±0.4 (4 days)</td>
</tr>
<tr>
<td>FerA7 (pH 6.4-no control) (4 days)</td>
<td>Same as A1</td>
<td>Same as A1</td>
<td>23.8</td>
<td>0.28±0.03 (4 days)</td>
<td>57.6±3.2 (3 days) 44.7±4.1 (4 days)</td>
<td>10.1±0.2 (2 days) 6.2±0.6 (4 days)</td>
</tr>
</tbody>
</table>

From the results of A1 to A4, doubling carbon source increased production of all three enzymes almost two times, and doubling nitrogen source only doubled cellulase production. A5 is fermentation of *A. aculeatus* 2053, which is good producer for polygalacturonase. From the results of A6 and A7 of strain 13201, pH control
fermentation gave better enzyme productions than no pH control one. They differed in cellulase and xylanase but not in polygalacturonase.

Six 1L fermentations were investigated with 20g/L soy hulls as the carbon source. FerA8 was operated at constant pH=5. FerA10 was operated at constant pH=4. FerA11 was operated at initial pH=5 then increased to pH=6 (2 days) by itself and then kept constant. Fer12 was operated at constant pH=6.0. Fer14 was operated at initial pH=6.5 then forced pH gradually decrease to 5 in 4 days and kept constant. Fer15 was operated at initial pH=6 then forced pH gradually decrease to 5 in four days and kept constant.

Table 6.6 Enzyme productions of A. niger fermentations

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH</th>
<th>C/N source</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Polygalacturonase (U/mL)</th>
<th>Pectinase (U/mL)</th>
<th>α-galactosidase (U/mL)</th>
<th>Sucrase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FerA8</td>
<td>pH=4</td>
<td>same</td>
<td>0.58±0.02</td>
<td>98.9±1.05</td>
<td>1.00±0.02</td>
<td>2.51±0.26</td>
<td>1.97±0.03</td>
<td>3.18±0.03</td>
</tr>
<tr>
<td>(341)</td>
<td>(5 days)</td>
<td></td>
<td>0.57±0.03</td>
<td>90.5±3.2</td>
<td>(4 days)</td>
<td>2.19±0.22</td>
<td>(5 days)</td>
<td>(4 days)</td>
</tr>
<tr>
<td>FerA12</td>
<td>pH=6</td>
<td>same</td>
<td>0.34±0.04</td>
<td>41.1±7.1</td>
<td>1.64±0.1</td>
<td>3.43±0.47</td>
<td>3.85±0.01</td>
<td>3.68±0.03</td>
</tr>
<tr>
<td>(341)</td>
<td>(5 days)</td>
<td></td>
<td>0.34±0</td>
<td>0.95±0.06</td>
<td>(4 days)</td>
<td>2.70±0.29</td>
<td>(4 days)</td>
<td>(5 days)</td>
</tr>
</tbody>
</table>

Fer10 was operated at constant pH=4 (5 days). Fer8 was operated with Soy hulls (NH4)2SO4 Urea Peptone (23.8) (6 days). Fer12 was operated at initial pH=5 (3 days). Fer14 was operated at initial pH=6.5 (4 days). Fer15 was operated at initial pH=6 (5 days).
<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>FerA 11 (341) (5 days)</th>
<th>FerA 15 (341) (5 days)</th>
<th>FerA 14 (341) (5 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>same</td>
<td>0.46±0.03 (4 days)</td>
<td>0.52±0.03 (5 days)</td>
<td>0.54±0.03 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.44±0.04 (5 days)</td>
<td>109.2±1.6 (5 days)</td>
<td>159.6±6.5 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67.2±2.8 (5 days)</td>
<td>2.13±0.07 (5 days)</td>
<td>2.74±0.08 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.52±0.05 (5 days)</td>
<td>2.89±0.28 (4 days)</td>
<td>3.96±0.40 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.44±0.33 (3 days)</td>
<td>2.15±0.27 (5 days)</td>
<td>4.18±0.26 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.79±0.35 (5 days)</td>
<td>2.15±0.27 (5 days)</td>
<td>5.22±0.08 (5 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.59±0.09 (5 days)</td>
<td>2.15±0.27 (5 days)</td>
<td>3.46±0.07 (5 days)</td>
</tr>
</tbody>
</table>

The pH 6-6.5 was good for initial (~3 days) production of pectinase and polygalacturonase, and pH 5-6 was good for α-galactosidase production. The pH 4-5 favored xylanase and cellulase production and enzyme stability. Sucrase production was less pH dependent. The higher initial pH (6.5-7) induced faster early pectinase and α-galactosidase production, and lower later pH (5) was good for cellulase production.
Figure 6.18 Cellulase production trends of different constant pH systems

Figure 6.19 Xylanase production trends of different constant pH systems
Figure 6.20 Polygalacturonase production trends of different constant pH systems

Figure 6.21 Pectinase production trends of different constant pH systems

Figure 6.22 α–galactosidase production trends of different constant pH systems
Figure 6.23 Sucrase production trends of different constant pH systems

The profiles of cellulase, xylanase, polygalacturonase, pectinase, α–galactosidase and sucrase production for constant pH systems were shown in Figure 6.18-6.23. The cellulase production rate of FerA10 and FerA8 was similar before 24 hours, which was faster than FerA12. After 24 hours, the production rate of FerA10 was fastest. The production rate of FerA8 and FerA12 was similar. After 72 hours, the production rate went to almost flat trends. This means lower pH can accelerate the cellulose hydrolysis to be the food for fungus, which can induce more cellulase production. On the contrary, the hydrolysis rate of cellulose to be the food was too slow at higher pH, which also led to slower cellulase production rate.

The xylanase production rate of FerA10, FerA8 and FerA12 was similar before 24 hours. After 24 hours, the production rate of FerA10 was fastest. The production rate of FerA12 was slowest. After 48 hours, the production rate of FerA12 went to almost flat trends. FerA10 reached maximum productivity at 72 hours, but FerA8 reached maximum
productivity at 140 hours. This means lower pH can accelerate the hemicellulose hydrolysis to be the food for fungus, which can induce more xylanase production. On the contrary, the hydrolysis rate of hemicellulose to be the food was too slow at higher pH, which caused slower xylanase production rate.

The polygalacturonase production rate of FerA12 was fastest before 48 hours. After 48 hours, the rate went to almost flat trends. And after 96 hours, the polygalacturonase activity decreased. This might be due to the dying cells, which consumed the self-proteins as food. The production rate of FerA10 and FerA8 was too slow. Although the final activity of FerA8 was higher, too long time would increase the utility fee and labor operation fee then increase the cost.

The pectinase production was similar to polygalacturonase trends. FerA12 was best in pectinase production.

For the α–galactosidase and sucrase production, the middle pH system FerA8 performed best. Higher pH system FerA12 was second. The lower pH system FerA10 performed worst.
Figure 6.24 Cellulase production trends of different changing pH systems

Figure 6.25 Xylanase production trends of different changing pH systems
Figure 6.26 Polygalacturonase production trends of different changing pH systems

Figure 6.27 Pectinase production trends of different changing pH systems
The profiles of cellulase, xylanase, polygalacturonase, pectinase, α–galactosidase and sucrase production for changing pH systems were shown in Figure 6.24-6.29. The cellulase production rate of FerA11 was fast before 24 hours because of the low initial pH. After 24 hours, the production rate of FerA15 and A14 was much faster. From 48
hours- 96 hours, the production rate was similar of these 3 systems. After 96 hours, the production rate of Fer#5 and Fer#6 was much faster, which might due to the pH drop.

The xylanase production rate of FerA11 was fast before 24 hours because of the low initial pH. After 24 hours, the production rate of FerA15 and A14 was much faster.

The polygalacturonase production rate of FerA15 and A14 was fastest before 24 hours. After 24 hours, the rate of FerA15 went slower but FerA14 was still fastest. After 48 hours, the polygalacturonase production rate went to almost flat trends. This may due to the food limitation. After 96 hours, the production rate bounced back again.

The pectinase production rate of FerA15 and A14 was fastest before 24 hours. After 24 hours, the rate of FerA15 went slower but FerA14 was still fastest. After 72 hours, the pectinase production rate went to almost flat trends. This may due to the food limitation.

The α–galactosidase production rate of the low initial pH system FerA11 was fast before 24 hours. After 24 hours, FerA14 and A15 performed better. The sucrase production was comparable for these three systems.

FerA16 and 20 were operated at initial pH=7 then forced pH gradually decrease to 6 in 3 days and then forced pH gradually decrease to 5 at 5 days. FerA22 was operated at constant pH=6.5. FerA17 and 18 were operated at initial pH=7 then forced pH gradually decrease to 6 in 5 days. FerA19 was operated at initial pH=7 then forced pH gradually decrease to 6 in 3 days and then kept constant.
Table 6.7 Enzyme productions of *A. niger* fermentations

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>pH</th>
<th>C/N source</th>
<th>Sugar/N ratio</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Pectinase (U/mL)</th>
<th>α-galactosidase (U/mL)</th>
<th>Sucrase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FerA 14 (341) (5 days)</td>
<td>pH=6.5-5</td>
<td>Hull/(NH₄)₂SO₄/urea/Peptone</td>
<td>23.8</td>
<td>0.54±0.03 (5 days)</td>
<td>159.6±6.5 (5 days)</td>
<td>3.96±0.40 (5 days)</td>
<td>5.22±0.08 (5 days)</td>
<td>3.46±0.07 (5 days)</td>
</tr>
<tr>
<td>FerA 16 (341) (5 days)</td>
<td>pH=7-5</td>
<td>2hull/2(NH₄)₂SO₄/2urea/2Peptone</td>
<td>23.8</td>
<td>0.53±0.01 (5 days)</td>
<td>126.0±5.8 (5 days)</td>
<td>7.51±0.34 (4 days)</td>
<td>7.02±0.10 (5 days)</td>
<td>3.57±0.08 (5 days)</td>
</tr>
<tr>
<td>FerA 22 (341) (5 days)</td>
<td>pH=6.5</td>
<td>2hull/2(NH₄)₂SO₄/2urea/2soy flour</td>
<td>23.8</td>
<td>0.39±0.06 (5 days)</td>
<td>82.3±4.3</td>
<td>3.57±0.59</td>
<td>4.11±0.02</td>
<td>5.56±0.12</td>
</tr>
<tr>
<td>FerA 17 (341) (5 days)</td>
<td>pH=7-6</td>
<td>hull/flour/(NH₄)₂SO₄/urea</td>
<td>23.8</td>
<td>0.34±0.02 (5 days)</td>
<td>37.3±8.1 (5 days)</td>
<td>5.23±0.22 (5 days)</td>
<td>3.21±0.02 (5 days)</td>
<td>2.41±0.07 (5 days)</td>
</tr>
<tr>
<td>FerA 19 (341) (4 days)</td>
<td>pH=7-6</td>
<td>Hull/flour/(NH₄)₂SO₄/urea/lowK H₂PO₄/no tween 80</td>
<td>23.8</td>
<td>0.34±0.01 (4 days)</td>
<td>62.9±3.2 (4 days)</td>
<td>3.95±0.23 (4 days)</td>
<td>2.68±0.03 (4 days)</td>
<td>1.89±0.02 (2 days)</td>
</tr>
<tr>
<td>FerA 21 (341) (5 days)</td>
<td>pH=7-6</td>
<td>2hull/2flour/2(NH₄)₂SO₄/2urea</td>
<td>23.8</td>
<td>0.29±0.04 (4 days)</td>
<td>36.1±2.8 (4 days)</td>
<td>4.12±0.18 (3 days)</td>
<td>3.69±0.03 (4 days)</td>
<td>4.65±0.12 (5 days)</td>
</tr>
</tbody>
</table>
### Table 1: Enzyme Productions

<table>
<thead>
<tr>
<th>FerA 20 (341) (5 days)</th>
<th>pH=7-5</th>
<th>3Hull/2flour/(NH4)2SO4/2urea</th>
<th>35.7</th>
<th>FerA 18 (341) (5 days)</th>
<th>pH=7-6</th>
<th>2hull/2flour/(NH4)2SO4/urea</th>
<th>37.5</th>
</tr>
</thead>
<tbody>
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<tr>
<td>(5 days)</td>
<td>6</td>
<td>22</td>
<td></td>
<td>(5 days)</td>
<td>4.72±0.09 (4 days)</td>
<td>2.41±0.11 (5 days)</td>
<td></td>
</tr>
<tr>
<td>(5 days)</td>
<td>.25</td>
<td>6.71±0.15 (5 days)</td>
<td></td>
<td>(5 days)</td>
<td>.09</td>
<td>4.57±0.02 (5 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.72±0.09 (4 days)</td>
<td>2.41±0.11 (5 days)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>.09</td>
<td>4.57±0.02 (5 days)</td>
<td></td>
<td>(5 days)</td>
<td>.11</td>
<td>4.57±0.02 (5 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.72±0.09 (4 days)</td>
<td>2.41±0.11 (5 days)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>.09</td>
<td>4.57±0.02 (5 days)</td>
<td></td>
<td>(5 days)</td>
<td>.11</td>
<td>4.57±0.02 (5 days)</td>
<td></td>
</tr>
</tbody>
</table>

Changing pH strategy with initial pH = 7.0 and ending pH = 5.0 was the best pH strategy to induce all the enzyme productions. Low phosphate and without tween 80 system had lower pectinase and sucrase production. Protease peptone as nitrogen source favored enzyme production than soy flour as nitrogen source. Higher sugar/nitrogen ratio favored enzyme production except pectinase with soy flour as nitrogen source. Doubled soy hull and total nitrogen source can increase the pectinase and α-galactosidase production with protease peptone as nitrogen source. Doubled soy hull and total nitrogen source can increase the sucrase production with soy flour as nitrogen source. 3×soy hull can partly increase the pectinase production with soy flour as nitrogen source. The nitrogen limiting might happen when sugar/nitrogen ratio was larger than 40 for strain 341 using sucrose as carbohydrate in shaker study. The sugar/nitrogen ratio trends of A17 and A18 were shown in Figure 6.30 and 6.31. From the figures, there was not obvious
nitrogen limit in A18. So the nitrogen limit happened when sugar/nitrogen ratio was 37.5 in fermentation.

Figure 6.30 The NH₃-N trends of A17

Figure 6.31 The NH₃-N trends of A18
Two 1L fermentations were evaluated with soy hulls as the carbon source at different temperature. They were operated at initial pH=7 then forced pH gradually decrease to 6 in 3 days and then forced pH gradually decrease to 5 at 5 days. The temperature control of FerA24 was 30 ℃ and the others were in room temperature. The pre-culture of FerA24 was in 30 ℃, and the FerA25 was in 24 ℃. The high temperature 30 ℃ was good for cellulase, α-galactosidase and sucrase, but low temperature was good for xylanase and pectinase. The better strategy should be low seed culture temperature about 24 ℃ and high fermentation temperature about 28 ℃.

Table 6.8 Enzyme productions of *A. niger* fermentations

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH</th>
<th>C/N source</th>
<th>Sugar/N ratio</th>
<th>Cellulase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Pectinase (U/mL)</th>
<th>α-galactosidase (U/mL)</th>
<th>Sucrase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FerA24 (341) 30℃ (5 days)</td>
<td>pH=7-5</td>
<td>2hull/(NH₄)₂SO₄/2urea/2Peptone</td>
<td>23.8</td>
<td>0.75±0.03 (4 days)</td>
<td>152.8±8.4 (4 days)</td>
<td>4.63±0.15 (5 days)</td>
<td>8.88±0.18 (4 days)</td>
<td>6.30±0.03 (5 days)</td>
</tr>
<tr>
<td>FerA25 (341) 24℃ (5 days)</td>
<td>pH=7-5</td>
<td>2hull/(NH₄)₂SO₄/2urea/2Peptone</td>
<td>23.8</td>
<td>0.65±0.01 (5 days)</td>
<td>301.9±8.5 (5 days)</td>
<td>8.16±0.14 (4 days)</td>
<td>7.80±0.24 (5 days)</td>
<td>4.91±0.08 (5 days)</td>
</tr>
</tbody>
</table>
The enzyme production, pH and DO trends of optimum pH control of FerA 25 were shown in Figure 6.32. The first 72 hours with high pH induced pectinase production. The gradually decreasing pH induced the other enzyme productions. The dissolved oxygen jumped two times separately at 3\textsuperscript{rd} day and 4\textsuperscript{th} day, which might due to the food limitation or switching from consumption of pectin to cellulose and hemicellulose.

The dissolved oxygen and pH synergetic control program were designed to control the acid addition when dissolved oxygen increased to make proper soy hull hydrolysis for \textit{A. niger} carbon source support. Four fermentation runs A26, A27, A28 and A29 were carried out with soy hulls as the carbon source. They were operated at initial
The pH was set to 7 and then followed the dissolved oxygen and pH synergetic control program. The difference of these four runs was the acid addition rate according to increased dissolved oxygen. The pH step of A26 is 0.1/h, 0.05/h for A27, 0.1/h for A28 and 0.2/h for A29. The carbon sources of A26 and A27 were doubled concentrations of soy hull, and the nitrogen sources were also doubled. The carbon sources of A28 and A29 were 3 times concentration of soy hull, and 2.5 times nitrogen source.

Table 6.9 Enzyme productions of *A. niger* fermentations

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH</th>
<th>C/N source</th>
<th>Sugar/N ratio</th>
<th>Cellelase (U/mL)</th>
<th>Xylanase (U/mL)</th>
<th>Pectinase (U/mL)</th>
<th>αgalactosidas (U/mL)</th>
<th>Sucrase (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FerA2 6</td>
<td>pH step =0.1</td>
<td>2Hull/2(NH₄)₂SO₄/2urea/2peptone</td>
<td>23.8</td>
<td>0.45±0.03</td>
<td>181.0±5.6</td>
<td>8.49±0.23</td>
<td>7.58±0.09</td>
<td>4.57±0.09</td>
</tr>
<tr>
<td>(341) (5days)</td>
<td></td>
<td></td>
<td></td>
<td>(5 days)</td>
<td>(5 days)</td>
<td>(5 days)</td>
<td>(5 days)</td>
<td>(5 days)</td>
</tr>
<tr>
<td>FerA2 7</td>
<td>pH step =0.05</td>
<td>2Hull/2(NH₄)₂SO₄/2urea/2peptone</td>
<td>23.8</td>
<td>0.31±0.01</td>
<td>104.1±4.3</td>
<td>8.76±0.36</td>
<td>5.66±0.11</td>
<td>3.19±0.08</td>
</tr>
<tr>
<td>(341) (5days)</td>
<td></td>
<td></td>
<td></td>
<td>(4 days)</td>
<td>(5 days)</td>
<td>(3 days)</td>
<td>(5 days)</td>
<td>(5 days)</td>
</tr>
<tr>
<td>FerA2 8</td>
<td>pH step =0.1</td>
<td>3Hull/2.5(NH₄)₂SO₄/2.5urea/2.5peptone</td>
<td>28.6</td>
<td>0.29±0.01</td>
<td>55.6±2.4</td>
<td>7.36±0.48</td>
<td>9.02±0.15</td>
<td>4.17±0.07</td>
</tr>
<tr>
<td>(341) (5days)</td>
<td></td>
<td></td>
<td></td>
<td>(4 days)</td>
<td>(4 days)</td>
<td>(4 days)</td>
<td>(4 days)</td>
<td>(4 days)</td>
</tr>
<tr>
<td>FerA2 9</td>
<td>pH step =0.2</td>
<td>3Hull/2.5(NH₄)₂SO₄/2.5urea/2.5peptone</td>
<td>28.6</td>
<td>0.44±0.01</td>
<td>225.1±5.9</td>
<td>7.17±0.25</td>
<td>11.58±0.10</td>
<td>5.29±0.09</td>
</tr>
<tr>
<td>(341) (5days)</td>
<td></td>
<td></td>
<td></td>
<td>(5 days)</td>
<td>(5 days)</td>
<td>(5 days)</td>
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<td>(5 days)</td>
</tr>
</tbody>
</table>
The final pectinase production in A26 and A27 was similar. A27 reached higher pectinase activity earlier than A26, which was in 72 hours. The reason should be the slow pH decrease of A27, because high pH can induce pectinase production. The pectinase rate of both runs became almost flat after 72 hours which maybe the carbon limit. The lower pH can induce more a-galactosidase production. The other enzymes: cellulase, xylanase, and sucrase of A26 were better than A27, because fast pH drop in A26. Higher initial soy hull and nitrogen source amounts in A28 and A29 did not give better pectinase production, which might due to nitrogen limitation or more free sugar presence.

6.4 Conclusion

The soy hull was the best carbon source to induce the complex enzyme production from the shaker study. The best combination of nitrogen source was (NH₄)₂SO₄, protease peptone and urea. The soy flour can be the substitute of protease peptone. The optimum concentration of potassium phosphate was 2 g/L, however 1 g/L was acceptable in order to further reduce the cost. Although tween 80 as the surfactant in the medium had some benefit than without tween 80 systems, the differences were not too huge compare to the nitrogen source effects. Therefore, whether using tween 80 depends on how many enzyme productions can be sacrificed in the industry application. From the shaker study, nitrogen limit happened when the sugar to nitrogen ratio was above 40 for strain 341 using sucrose as carbohydrate in shaker study. The conclusion was based on the results of cell growth, so the nitrogen limit might happen earlier than ratio 40 in real system for enzyme production.
From the fermentation study, doubled concentration of carbon sources and nitrogen sources induced more enzyme production without mixing problem. And the nitrogen limiting happened when sugar/nitrogen ratio was 37.5 in fermentation. The soy flour did not provide better enzyme productions than protease peptone as nitrogen source. Low phosphate and no tween 80 system gave lower pectinase and sucrase production. The low pH induced more cellulase and xylanase production, but the high pH induced more pectinase and polygalacturonase production. The a-galactosidase and sucrase preferred medium pH. The use of a fixed condition cannot achieve optimization for more than two enzymes in a single run. Yet, a pH gradient can selectively move the fungal biomass though multiple optimum conditions. The best changing pH strategy which was operated at initial pH=7 then pH gradually decreased to 6 in 3 days and then gradually decreased to 5 at 5 days. When using the optimized gradient, the enzyme concentration can be scaled as a function of nutrient concentration. A control scheme that uses the change in dO₂ was presented as method to account for different medium compositions and direct the system to pass through the main optimal pH conditions.
CHAPTER VII
EFFECT OF PROTEASE ON SOY PROTEIN BY HYDROLYSIS WITH

ASPERGILLUS NIGER BROTH

7.1 Introduction

Barac et al. reported the impact of protease on soy protein concentrate properties and some nutritional properties [153]. After the treatment of trypsin and pepsin, better extractability was obtained. Hrckova et al. reported the effects of three proteases on soy protein functional properties. Better foaming, and gelation properties were achieved after protease treatments [154]. The presence of protease would bring changes on the soy protein properties, but it will also degrade part of protein. Therefore, it’s necessary to control the degradation degrees.

_A. niger_ was reportedly the producer of protease, therefore, it is necessary to find the protease effect on the soy protein [26, 27]. After enzyme hydrolysis, the soy carbohydrates were degraded into digestible and soluble monomers therefore the soy protein was enriched. But under protease treatment, the properties of soy protein might change such as solubility, molecular weight. So it’s necessary to investigate the properties of soy protein after protease treatment. And this knowledge can help to develop the soy protein application. In the soy protein collection process, there were two products: one
was soy protein concentrate (SPC), the other one was soy protein isolate (SPI). Through protein analysis, the distribution of soy protein in these two products got much more clear.

7.2 Materials and Methods

The hydrolysis procedure was that enzyme broth (cell-free) mixed with defatted soy flour, and water at pH=4.8, which incubated in shaker with 250 rpm speed at 50℃. After hydrolysis, almost all soy carbohydrates were expected to become soluble, and the majority of soy proteins would remain insoluble in the enzymatic solution at pH 4.8, because the pH was around their pl (isoelectric points) of 4.2-4.8. The soluble carbohydrates and the insoluble proteins can be easily separated by centrifugation. The insoluble proteins are the SPC. Then the soluble supernatant included soluble carbohydrates and left soy protein. After heating treatment, the left soy protein become insoluble in the liquid and can be separated as SPI from carbohydrates by centrifugation. The final liquid was hydrolysate, which was composed of soluble and digestible sugars. In order to find the distribution of soy protein in SPC and SPI, the study of soy protein analysis in both protein products was investigated. SDS-PAGE was the method for separating proteins by molecular weight. Protein samples were prepared using SDS, β-mercaptopethanol (thiol reducing agent). SDS-PAGE was applied to analyze the SPC, SPI and hydrolysate with enzyme treatment and without enzyme treatment. Then the effects of protease in the enzyme solution on protein were investigated. If there were more proteins in hydrolysate with enzyme treatment, protease production would be inhibited in fermentation because of protein lost. If there were more proteins in SPI with enzyme treatment, which means the protease can break soy protein into small fragments.
Therefore the proteins were more digestible and can be used for food application.

7.3 Results and Discussion

The major proteins were glycinin(11S) and β-conglycinin (7S), which were separately 52% and 35% in soy protein. The molecular weight of β-conglycinin (7S) was around 150-180 kDa, which was composed of three subunits - α', - α- and - β-. And the molecular weights of these subunits were respectively about 72kDa, 68 kDa and 52 kDa. The molecular weight of glycinin(11S) was about 360 kDa, and it’s compact quaternary structure constructed with disulfide, electrostatic and hydrophobic bonds. It was composed of six A-SS-B subunits, and except for the acid polypeptide -A4-, each subunit was composed of an acid and basic polypeptide, which was linked by single disulfide bond [155]. From the SDS-PAGE analysis, the major proteins distribution in SPC and SPC were clear.
During the sample preparation, SPC and SPI products were found not easy to be dispersed and soluble in water. That should be the reason of why there were not clear bands in Lane2 and Lane 3. But clear bands were found in Lane11-12 SPI, because there was not heating dry step in this SPI product. The proteins with size larger than 70kDa were not found in SPI any more. In hydrolysate, there were only two bands around 35kDa and 20kDa.

The molecular weights of soy protein products were investigated by SDS-PAGE. F14, 16, 18 were different hydrolysis systems, which respectively used FerA14, 16 and
18 fermentation enzyme broths. And in control one, the water without enzyme was applied to soy flour. In FerA14, 16 and 18, the protease was respectively 73, 139 and 123 BAEE/mL.

Figure 7.2 SDS-PAGE of different soy protein product (Lanes 1-4: SPC: Ct (4×), F14 (8×), F16 (8×), F18 (8×); Lanes 5-8: SPI, Ct (4×), F14 (40×), F16 (4×), F18 (4×); Lane 9: Marker; Lanes 10-12: Hydrolysate: Ct (2×), F14 (10×), F14 (20×))
In the SPC and SPI products, the subunits - α', - α- of 7S and acid peptides around 37k Da of 11S disappeared in F16 and 18. There were new bands around 25k Da in F16 and 18. In the hydrolysate, some peptides of 11S were detected in all these systems except 14 because of the too low protein concentration. It can be concluded that the protein degradation happened in system 16 and 18.

The molecular weights of soy protein products were analyzed by SDS-PAGE. No. 14, 16, 18 were hydrolysis systems with deactivated enzyme broth, which used FerA14, 16 and 18 fermentation deactivated enzyme broths. The objective was to study if the ion
strength in different fermentation systems caused protein degradation. And in control one, the water without enzyme was applied to soy flour.

Figure 7.4 SDS-PAGE of different soy protein product (Lanes 1-6: SPC: Ct, 14-1×, 16-1×, 18-1×, 14-4×, 16-4× (4× dilution); Lanes 7-12: SPI: Ct, 14-1×, 16-1×, 18-1×, 14-4×, 16-4× (4× dilution); Lane 13: marker)
From this study, with the deactivated enzyme broth hydrolysis in different ion strength, there wasn’t obvious protein degradation from SDS-PAGE, which supported the previous hypothesis. The most reason of disappearance of 7s subunits was the protease function. In order to prevent the soy protein degradation, it’s necessary to decrease the protease production in future study.

7.4 Conclusion

There are clear protein band disappearances after enzyme hydrolysis, which represents the protein degradation during the hydrolysis. Based on the verification results, this
protein degradation was not from the ion strength. The protease in the enzyme mixture broth caused the protein degradation.
CHAPTER VIII
SOY HULL HYDROLYSIS WITH ASPERGILLUS NIGER AND TRICHODERMA REESEI COMPLEX EZNYME

8.1 Introduction

The United States Department of Agriculture (USDA) reported that the Global Soybean Production on 2016 would be 336.09 million metric tons, which was around 22.89 million tons or 7.31% increase than last year. And there are 118.7 million metric tons, which were produced in the US. Soybean hull constitutes about 5% of soybean weight [156]. Compared to soy oil, soy meal and other high-value products, soy hull was considered a waste product. Soy hulls were typically used to feed to cows with compressed pellets or as is [156]. Soybean hull has been gradually used in plastic, metal biosorption, biochemical production [157-160].

Soy hull contains about 86% complex carbohydrates [161]. Soy hulls contain about 20-50% cellulose, 10-20% hemicellulose and 6-15% pectin [74]. Upon hydrolysis, the monomeric sugars can be converted by bioprocesses to other valuable products such as ethanol [74] and butanol [162]. Conversion of the carbohydrates of soy hull to valuable products will result in new markets. However, the traditional hydrolysis method
such as hot water and steam to addition of alkali, solvents, and dilute acid, which can simply hydrolyzes the hemicellulose to monomeric sugars and short chained sugar oligomers [74]. In order to get completely hydrolysis, the enzymatic hydrolysis is necessary. Accordingly, for hydrolysis of soy hull carbohydrate, cellulase is likely the most important enzyme, to hydrolyze the dominant component – cellulose. Xylanase and pectinase are also important to hydrolyze the hemicellulose and pectin components, respectively.

The enzymes produced from \textit{A. niger} and \textit{T. reesei} in our study were applied to hydrolyze the soy hull carbohydrate. The different pretreatment and enzyme loading were studied. This part was a preliminary study of soy hull hydrolysis comparison with different enzyme broth, which can be a guide for future experiment and design.

8.2 Materials and Methods

\textit{T. reesei}(Batch A4) and \textit{A. niger}(Batch 26) enzyme broth with different loadings were applied to soy hull hydrolysis research. The soy hull loading was 2.5 g which mixed in 50 mL water and operated at pH=4.8, and $T=50^\circ$. Eight systems were investigated. First, the comparison between \textit{A. niger} and \textit{T. reesei} was studied. System 2 and 5 were separately loaded with 10 mL \textit{A. niger} and \textit{T. reesei} broth. Then the \textit{A. niger} enzyme broth with different loading were studied. System 1, 2, 3 and 4 were separately loaded with 5 mL, 10 mL, 20 mL and 30 mL \textit{A. niger} enzyme broth. The un-pretreated and pretreated soy hull was studied in system 5 with un-pretreated soy hull and system 6 with 10 min autoclaved soy hull. In order to evaluate the size difference of soy hull, the grinded (100-200μm) and un-grinded soy hull (500-1000μm) were compared in different
systems. System 1 (grinded soy hull) and system 7 (un-grinded soy hull) both were loaded with 5 mL *A. niger* broth.

### 8.3 Results and Discussion

*A. niger* enzyme broth with different loadings were applied to soy hull hydrolysis research. The released reducing sugar during hydrolysis results were shown in Figure 8.1. The system 5 had most reducing sugar, which used 10 mL *T. reesei* broth. This might due to the high cellulase amount in system 5. System 6 with pretreated soy hull had 10 g/L released reducing sugar. The least one was system 7, which used least enzyme loading and unground soy hull. Based on these results, the pretreated soy hull was preferred.

![Figure 8.1 Reducing sugar profiles during hydrolysis](image)

The stachyose and raffinose profiles were shown on Figure 8.2 and 8.3. Only system 5 with *T. reesei* broth still had stachyose and raffinose left. The *A. niger* systems did not have any stachyose and raffinose left. Because the α-galactosidase was rich in *A. niger* broth, which used to break down stachyose and raffinose.
The sucrose profiles were shown on Figure 8.4. Only system 5 with *T. reesei* broth still had sucrose left. The *A. niger* systems did not have any sucrose left. Because the *A. niger* broth had sucrase to consume the sucrose.
Figure 8.4 The sucrose trends during hydrolysis

The glucose and xylose profiles were shown on Figure 8.5 and 8.6. System 5 released most glucose and xylose because of higher cellulase and xylanase in *T. reesei* broth. For the *A. niger* systems, the more enzyme loading system released more glucose and xylose. The system 6 with pretreated soy hull had comparable glucose release as 30 mL *A. niger* enzyme loading system, and comparable xylose release as 20 mL *A. niger* enzyme loading system. The system 7 with unground soy hull had least sugar release. This implied the pretreatment of soy hull was preferred.
The galactose profiles were shown on Figure 8.7. The trends of all the systems were similar.
The arabinose profiles were shown on Figure 8.8. The system 6 with pretreated soy hull gave best release. The other systems were similar. The mannose and fructose profiles were shown on Figure 8.9 and 8.10. All the systems were similar.
The released sugar results of all systems were summarized in Table 8.1. The system 6 with *T. reesei* broth gave most released sugar. The total released sugar of pretreated soy hull system ranked second. The total released sugar of the *A. niger* systems
increased as the enzyme loading increased. The unground soy hull system gave the worst total sugar release.

Table 8.1 HPLC sugar analysis

<table>
<thead>
<tr>
<th></th>
<th>Sta (g/L)</th>
<th>Raf (g/L)</th>
<th>Suc (g/L)</th>
<th>Glu (g/L)</th>
<th>Xyl (g/L)</th>
<th>Gal (g/L)</th>
<th>Ara (g/L)</th>
<th>Man &amp; Fru (g/L)</th>
<th>Total (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Contr ol</td>
<td>0.00495</td>
<td>0.06688</td>
<td>0.28366</td>
<td>0.70023</td>
<td>1.29432</td>
<td>0.828792</td>
<td>0.13846</td>
<td>2.089895</td>
<td>5.407211</td>
</tr>
<tr>
<td>1-5A</td>
<td>0.0147</td>
<td>0.34555</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
</tr>
<tr>
<td>2-10A</td>
<td>0.0147</td>
<td>0.34555</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
</tr>
<tr>
<td>3-20A</td>
<td>0.0147</td>
<td>0.34555</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
</tr>
<tr>
<td>4-30A</td>
<td>0.0147</td>
<td>0.34555</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
</tr>
<tr>
<td>5-10T</td>
<td>0.519696</td>
<td>0.2745</td>
<td>4.70719</td>
<td>4.632068</td>
<td>3.01092</td>
<td>0.781528</td>
<td>0.347608</td>
<td>1.867054</td>
<td>16.140564</td>
</tr>
<tr>
<td>6-10A-pre-10</td>
<td>0.394415</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-5A-unground</td>
<td>0.394415</td>
<td>0.666918</td>
<td>1.019584</td>
<td>0.76206</td>
<td>0.10076</td>
<td>2.292111</td>
<td>5.2358485</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

8.4 Conclusion

Based on the results of sugar analysis, it can be concluded the *T. reesei* enzyme broth released more reducing sugar releasing than the *A. niger*. But there was no stachyose, raffinose and sucrose left in the *A. niger* enzyme hydrolysis systems. Therefore, *T. reesei* had better efficiency than *A. niger* to break down cellulose and hemicellulose, but *A. niger* can break down the oligosaccharides and disaccharide better than *T. reesei*. 

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CHAPTER IX
ENZYME ASSAY DEVELOPMENT AND LONG TERM STORAGE AND DEFROSTING ON ENZYME STABILITIES
(CO-WORK WITH ANTHONY M. COFFMAN)

9.1 Introduction

Development of accurate, consistent, and efficient assays for enzyme activities is important. Unlike assays for cellulase and xylanase activities, there were no generally accepted procedures for the assays of pectinase and polygalacturonase activities. Among various pectin-degrading enzymes, this is especially true for polygalacturonase, since the other enzymes result in chemical changes that are easier to measure: pectinesterase activity is generally determined via titration or from the pH change resulting from deesterification of methyl ester portions of the galacturonan backbone, and pectin lyase is assayed by measuring the increase in absorbance at 235 nm that results from formation of a carbon-carbon double bond. Polygalacturonase depolymerizes the pectin backbone, and can therefore be assayed by measurement of increased sugar reducing ends or reduced viscosity [85]. Since quantitative correlation between viscosity reduction and the degree of hydrolysis was only empirical, it was decided to develop in this study an assay based on reducing end measurement, similar to the methods used for cellulase and xylanase assays.
The conditions reported in the literature for polygalacturonase and pectinase measurements vary in substrate, substrate concentration, temperature, pH, and incubation time. Substrates commonly include citrus pectin, sodium pectate, polygalacturonic acid (PGA), and galacturonic acid oligomers. The concentration varies widely between 0.09 and 1 wt%. Substrates are usually prepared in 0.05 to 0.1 M acetate or citrate buffers at different pH, from 4.0 to 5.5. Incubation time and temperature range from 5 to 60 min (or longer) and from 30 to 60 °C, respectively [22, 109, 163-166]. A summary of reported assay conditions is given in Table 1. Procedural descriptions in the literature often omit the quantity of substrate used and the enzyme activity ranges for which the assays were valid.

Considering the importance of these enzymes to biorefinery development and other industrial products, there was critical need of standard methods that give more reproducible activity measurements to allow meaningful comparison and compilation of knowledge about these enzymes. In light of the inconsistent assay techniques reported in the literature, a series of experiments were conducted in this study to develop polygalacturonase and pectinase assay methods that, if possible, yield reproducible results with low sensitivities to the assay parameters. This work was the first systematic study aiming at developing reproducible, and separate, assays for polygalacturonase and pectinase (including the use of a linear interpolation method for the pectinase assay to eliminate the sensitivity to unknown enzyme activity in the sample analyzed, as described later in detail). These assays can be the standard assays used in future research involving these enzymes. After enzyme harvest, the enzyme will be stored in -20°C, it is important to study how this storage time and method affect the enzyme activities.
9.2 Materials and Methods

The materials and methods were divided into three parts included pectinase and polygalacturonase assay study, enzyme storage study and soy enzyme assay, which were described below.

9.2.1 Pectinase and polygalacturonase assay study

The enzyme preparation, substrate preparation and concentration, enzyme concentration, and reaction time were investigated in pectinase and polygalacturonase assay study.

9.2.1.1 Enzyme preparation

Four different enzyme mixtures were used in this study. They were produced by either *Trichoderma reesei* (NRRL 11460) or *Aspergillus niger* (NRRL 341) fermentation with different carbon sources in the media. The fermentation was made in a 3 L fermentor with 1.5 L working volume. DO (dissolved oxygen concentration) was maintained at above 20% air saturation throughout all the fermentation experiments. Fermentations #1-#3 were made with *T. reesei*; Fermentation #4 with *A. niger*. Avicel cellulose (4 g/L) and soy molasses (11.8 g/L) were the carbon source for Fermentation #1, where pH was maintained at the range of 5.3-7.0. Soy molasses are the byproduct during manufacturing soy protein products, which are rich in carbohydrates[167]. The low commercial cost disaccharide lactose (10 g/L) was the carbon source for Fermentation #2 and pH was controlled at 6[168]. Fermentation #3 was made with 30 g/L soybean hull as carbon source and pH was 6 initially; it was allowed to drop naturally to 4 along with fungal growth and thereafter controlled at 4. Fermentation #4 was made
with 40 g/L soybean hull as carbon source and pH was controlled at 5 after being allowed to drop from the initial pH 7. The nitrogen sources in the media for Fermentations #1-#3 were 1.4 g/L (NH₄)₂SO₄, 0.3 g/L urea and 1 g/L protease peptone; the same nitrogen sources were used for Fermentation #4 but with doubled concentrations. The other medium ingredients were kept the same for all these fermentations: 2 g/L KH₂PO₄, 0.3 g/L MgSO₄·7H₂O, 0.4 g/L CaCl₂·2H₂O, 0.2 g/L Tween 80, and trace elements (0.005 g/L FeSO₄·7H₂O, 0.0016 g/L MnSO₄·H₂O, 0.0014 g/L ZnSO₄·7H₂O, and 0.002 g/L CoCl₂·6H₂O). The medium composition and fermentation operation were adopted and/or modified from those used previously in this laboratory for T. reesei fermentation[142, 169]. The broths harvested at the end of fermentation (#1 192 h, #2 166 h, #3 267 h, and #4 118 h) were centrifuged (12,227 g for 15 min) to collect the cell-free enzyme-containing solutions, which was stored at -20 °C for future use.

Soybean hulls and soy molasses were obtained from Archer Daniels Midland (Decatur, IL, USA). Avicel cellulose (PH-101, 50 µm particle size) was obtained from FMC BioPolymer (Philadelphia, PA, USA). All other chemical medium components were obtained from Sigma Aldrich (St. Louis, MO, USA).

9.2.1.2 Substrate

Polygalacturonic acid (PGA, 95% purity, average molecular weight 25,000-50,000 Da, Sigma Aldrich, Product Number 81325) was used as the substrate for polygalacturonase assay. Citrus pectin (galacturonic acid ≥ 74%, Sigma Aldrich, Product Number P9135) was used as the substrate for pectinase assay. Substrate solutions of 1 to 40 g/L were prepared in 0.1 M sodium citrate buffer at pH 4.8. The solid substrate was added gradually to the buffer while stirring to aid in formation of a uniformly opaque
suspension. In the event of clump formation, the mixture was stirred for an extended period of time, sometimes with heating, to break any clumps of substrate prior to the assay. Heating was typically necessary for preparing the citrus pectin suspension. The choice of citrate buffer was based on its use in the standard assays of cellulase and xylanase. Buffer pH of 4.8 was chosen based on the reported optimal range (4.2 to 5.1) [109, 166, 170] and the optimal condition (pH 4.8 and temperature 50 °C) established in this laboratory for the intended application of these enzyme mixtures to soybean meal treatment [121].

9.2.1.3 Enzyme analysis

Polygalacturonase/pectinase activity was determined from the increase in reducing sugar concentration after incubation of the enzyme-containing sample and substrate. Reducing sugars were measured following a modified version of the dinitrosalicylic acid (DNS) method, after observing that the addition of standard DNS reagent produced a cloudy precipitate of residual substrate. This effect was more pronounced in the enzyme blanks due to their higher concentrations of intact PGA/pectin. The modified DNS reagent omitted the 30 wt% sodium-potassium tartarate in the standard recipe [124].

To perform the enzyme assay, 100 µL enzyme broth and 900 µL PGA/pectin substrate solution were added to 25 mL glass graduated test tubes (Corning Life Science, Product Number 70075-25). Blanks containing only the substrate solution were also prepared. All tubes were sealed with Parafilm and incubated in a water bath at 50 °C for times ranging from 5 to 60 min. Reaction temperature was chosen based on the reported optimum range, 40 °C to 50 °C [22, 165], and the condition (50 °C) used for enzymatic
hydrolysis of carbohydrate in soybean meal. The enzyme reaction was terminated by
addition of 3 mL of a modified DNS solution that consisted of 10 g/L 3,5-dinitrosalicylic
acid and 16 g/L sodium hydroxide (NaOH). Enzyme broth was then added to the blanks,
to account for the turbidity introduced with the enzyme broth. All samples were boiled
for 10 min to develop color, diluted to a total volume of 25 mL, and inverted several
times to mix. The absorbance values were measured at 540 nm and calibrated against
standard solutions of D-galacturonic acid-H₂O at 0, 0.125, 0.25, 0.5, and 1 g/L. The final
activity calculation was based on the release rate of galacturonic acid-equivalent reducing
sugar from the PGA/pectin substrate. One unit (U) is equivalent to one µmol product
released per min. The enzyme activity (U/mL) is calculated according to the following
equation:

\[
\text{Activity (µmol/mL)} = \frac{\text{µg galacturonic acid released}}{(0.1 \times 194.1 \times t)}
\]

(1)

where “0.1” mL is the enzyme broth volume used in the assay, “194.1” is the
molecular weight of galacturonic acid, and \( t \) is the reaction time in min.

Based on these test procedure and conditions, a series of experiments was
designed to evaluate the effects of reaction time, substrate concentration, and enzyme
dilution on polygalacturonase assay. First, undiluted enzyme solution from Fermentation
#1 was incubated with 10 g/L PGA solution for 5 to 60 min to determine a suitable
reaction time. Next, the effect of substrate concentration ranging from 1 to 40 g/L was
studied in 3 sets of experiments using the enzyme solutions from Fermentations #1 and
#2, with different dilution factors, to determine the substrate concentration to use for the
assay. Then, multiple dilutions of the enzyme solution from Fermentation #3 were assayed with the determined PGA concentration to evaluate the range of enzyme activities that would give consistent results. The enzyme solution from Fermentation #3 was also used to validate the established reaction time and substrate concentration for polygalacturonase assay. Finally, the same assay conditions were evaluated and modified for applicability to pectinase assay using the enzyme solution from Fermentation #4. The effect of substrate preparation and storage was also examined.

Sample analyses were made in triplicates and the experiments were performed at least 3 times to ensure accuracy of the recommended assay procedures.

9.2.2 Enzyme storage study

Four fermentation broths were selected to study the pectinase and α-galactosidase activities after long term freezer storage and 3 times defrosting cycles. These four fermentation broths are from A17, A18, A24, and A25. The original activities of all these runs were analyzed with 1 time defrosting cycle after collecting fermentation broths, which stored in freezer less than a week. The activities of A17 and A18 were analyzed with 2 and 3 defrosting cycles, which stored in freezer for 13 months. And the activities of A24 and A25 were analyzed with 2 and 3 defrosting cycles, which stored in freezer for 11 months.

9.2.3 Soy enzyme assay

The goal of fermentation modification is to increase the enzyme productivity and hydrolyze the soy flour better. Although cellulase, xylanase, pectinase are the main
enzymes to break down soy flour, there are still other enzymes’ affects. If the method of using soy flour as substrate to directly analyze the whole enzyme efficiency to break down soy flour carbohydrate can be developed, it will be more accurate to give us the ideas. So different substrate concentration and enzyme loading were studied to find the optimum substrate concentration and enzyme loading. The reaction time is 1 hour for all the tests.

9.3 Results and Discussion

The results of development of pectinase and polygalacturonase assay were described below. The results of enzyme storage and development of soy enzyme assay were also discussed.

9.3.1 Pectinase and polygalacturonase assay study

Pectinase and polygalacturonase were separately investigated. The assays of them were similar but not exactly the same.

9.3.1.1 Polygalacturonase assay

Initial studies were conducted with the enzyme solution from Fermentation #1 ($T. reesei$ grown on Avicel and soy molasses). The effect of reaction time on the “observed” polygalacturonase activity was examined with the full strength (undiluted) enzyme solution using 10 g/L PGA as the substrate. The sugar release profile and the corresponding enzyme activities, calculated using Eq. (1), for different reaction times are shown in Figure 9.1. The observed enzyme activity rapidly decreases in the first 20 min, remains relatively constant till 30 min, and gradually decreases afterwards. One possible
explanation to the initial rapid decrease of activity, when plenty of substrate was available, is that polygalacturonase exhibits lower affinity toward galacturonic acid oligomers (with 2-8 residues) versus the longer-chain PGA, as reported in the literature [109]. So, as the long substrate chains are cleaved into shorter ones, the enzyme activity becomes lower. According to the results, a reaction time of 30 min was chosen, as a compromise between the high and low activities observed for short and long incubation times, respectively. In addition, by choosing 30 min, the obtained enzyme activity is no longer very sensitive to slight changes in reaction time (e.g., as compared to 20 min) and, thus, the assay gives potentially more reproducible results. Further, it was considered that most industrial pectinase/polygalacturonase applications would involve process time longer than 20 min, so this enzyme activity obtained with 30-min assay time would be more representative of hydrolysis performance in the applications.

Figure 9.1 Amounts of reducing sugar released and the observed polygalacturonase activities for the same enzyme solution from a T. reesei fermentation (#1) when assayed with different reaction times from 5 to 60 min
With the chosen 30 min reaction time, two sets of experiments were made with the enzyme solution from Fermentation #1: in Set 1 the enzyme solution was undiluted and PGA substrate concentrations were varied from 1 to 40 g/L; in Set 2 the enzyme solution used was 10-fold diluted and PGA concentrations were in the range of 1 to 10 g/L. In both sets of experiments the observed polygalacturonase activity increased with PGA concentration up to 10 g/L but, in the Set 1 experiments (high enzyme-high substrate) the activity dropped at higher PGA concentrations. (The polygalacturonase activities measured with different PGA concentrations are shown in Figure 9.2) The possibility of substrate inhibition to the enzyme was examined. Double reciprocal (Lineweaver-Burk) plots of the results are presented in Figure 9.3. The characteristic pattern of substrate inhibition is seen at PGA concentrations higher than about 10 g/L. Substrate inhibition of the polygalacturonase and polymethylgalacturonase enzymes of \( A. niger \) has been reported in the literature [171]. The polygalacturonase enzymes from \( T. reesei \) and \( A. niger \) act on PGA in a similar fashion [166], so it is not entirely surprising to observe the substrate inhibition behavior in this study.
Figure 9.2 Observed polygalacturonase activity at several substrate concentrations for undiluted and 10-fold diluted enzyme solutions from a *T. reesei* fermentation (#1)

Figure 9.3 Lineweaver-Burk plot for two sets of experiments examining the substrate concentration effect. Set 1 experiments were made with undiluted enzyme solution from
Fermentation #1; Set 2 with the 10-fold diluted enzyme solution. Substrate inhibition occurred at polygalacturonic acid concentration higher than about 10 g/L.

The above results dictate the use of lower than 10 g/L PGA as substrate. On the other hand, too low substrate concentrations could cause the enzymatically released reducing sugar concentrations to be low enough to negatively affect the accuracy of DNS analysis. A final set of experiments was therefore performed to more closely examine the effect of substrate concentrations between 5 and 10 g/L. The undiluted enzyme solution from another *T. reesei* fermentation (#2), with lactose as the inducing carbon source, was used in these experiments. (This change also served the purpose of probing the suitability of this PGA concentration range to different enzyme solutions.) The results (shown in Figure 9.4) showed that the observed polygalacturonase activity varied only slightly; increasing from 2.0 U/mL at 5 g/L PGA to 2.5 U/mL at 8 g/L PGA and then decreasing slightly to 2.2 U/mL at 10 g/L PGA. Based on the results from all these 3 sets of experiments, the PGA concentration of 5 g/L was chosen to be used for the assay, as a substrate level with sufficient separation from being affected by the substrate inhibition.
Figure 9.4 Observed polygalacturonase activities at different substrate concentrations in the range of 5 to 10 g/L for undiluted enzyme solution from Fermentation #2

Multiple dilutions of the enzyme solution from Fermentation #3 were analyzed for polygalacturonase activity. The assays were conducted for 30 min with 5 g/L PGA solution per the previous experimental results. Figure 9.5 shows the observed and adjusted enzyme activities at dilution factors from 1 (undiluted) to 80; adjusted activity = observed activity x dilution factor. It is seen that dilution to an observed activity range between 0.3 and 0.8 U/mL gives relatively consistent results once adjusted for the dilution factor, i.e., 8.5 ± 0.7 U/mL ($p = 0.28 > 0.05$). This activity range corresponds to the release of 0.17-0.45 g/L D-galacturonic acid and the substrate conversion of 3.7%-10%. At higher dilution factors the adjusted activity increases further, probably because the substrate-to-enzyme ratios were very high so the activity measured was primarily for hydrolysis of initial long-chain PGA without the effect of reduced enzyme affinity to shorter oligomeric substrate.
Optimal time (30 min), substrate concentration (5 g/L PGA), and enzyme activity range (0.3 to 0.8 U/mL) had been established by individual experiments as described previously. This additional set of experiments was to further validate these chosen assay parameters. The enzyme solution from Fermentation #3 was diluted by a factor of 20 to achieve an observed enzyme activity of 0.49 U/mL, within the range of values found to yield consistent results.

The reaction time and substrate concentration were then validated for the procedure. During 10 to 30 min the sugar release profile was found to be fairly linear and the observed polygalacturonase activities were consistent, at $0.50 \pm 0.04$ U/mL ($p = 0.90 > 0.05$). At 45 and 60 min, the observed enzyme activities and sugar release rates were slightly lower. The choice of 30 min incubation time was confirmed to be suitable.

(The released sugar amounts and corresponding enzyme activities obtained when the
analysis was allowed to proceed for different reaction times are shown in Figure 9.6) The effect of substrate concentration was validated at 5, 6, 7, 8, 9 and 10 g/L PGA. At all concentrations the observed activities were essentially identical at 0.45 ± 0.03 U/mL, indicating that the chosen assay procedure does not present substrate limitation or inhibition. (Results are shown in Figure 9.7)

![Graph](image)

Figure 9.6 Sugar release and polygalacturonase activity verification for incubations from 5 to 60 min with 20-fold diluted enzyme solution from Fermentation #3
Figure 9.7 Observed polygalacturonase activities of the 20-fold diluted enzyme solution from Fermentation #3 at different PGA substrate concentrations, for verifying the suitability of the chosen 5 g/L substrate concentration.

Overall, the validation experiments show that the polygalacturonase assay for 30 min with 5 g/L PGA at an enzyme activity of 0.49 U/mL (within the suggested range of 0.3-0.8 U/mL) yields consistent results. The developed procedure is rather insensitive to minor variations of the operating parameters and thus offers good reproducibility to allow for more reliable comparison across enzyme solutions from different fermentations.

9.3.1.2 Pectinase assay

Similar experiments were done to evaluate the applicability of the developed polygalacturonase assay procedure to measurement of pectinase activity. Citrus pectin was used as the substrate. The effects of reaction time, substrate concentration, and
dilution factors were examined. The enzyme solution used in these experiments was from an *A. niger* fermentation (#4) with 40 g/L soybean hulls as the inducing carbon source. Preliminary analysis using the previous assay procedure indicated that the 10-fold diluted enzyme solution would place the pectinase activity in the potentially suitable range of 0.3-0.8 U/mL. This diluted enzyme solution was first used to examine the effect of incubation time, with 5 g/L pectin as substrate. Encouragingly, the observed pectinase activities were confirmed to be consistent, at 0.60 ± 0.04 U/mL (p = 0.15), from 10 to 30 min. The trend was very similar to that shown earlier in Figure 9.8 for the polygalacturonase activities observed with the 20-fold diluted enzyme solution from Fermentation #3. Reaction time of 30 min was therefore considered to be suitable for the pectinase assay as well.

![Graph](image)

**Figure 9.8** Sugar release and polygalacturonase activity verification for incubations from 5 to 60 min with 20-fold diluted enzyme solution from Fermentation #3
The same 10-fold diluted enzyme solution was used with 30 min incubation time for evaluating the effect of pectin substrate concentration, varying from 1 to 40 g/L. The observed enzyme activities are shown in Figure 9.9. The observed activity exhibits the common saturation-type dependency on substrate concentration, without noticeable substrate inhibition (confirmed by the Lineweaver-Burke plot, not shown). While this observation indicates the possibility of using higher than 5 g/L pectin as substrate, it should be considered that pectin is complex material and the use of higher pectin concentrations means the pectinase activity is assayed at lower substrate conversions with increasing contributions from the reactions on structures that are easier to react. In that case, the activity obtained may be less representative to the combined effects of different pectinase components. Keeping the substrate concentration at 5 g/L is therefore considered to be reasonable.

![Graph showing observed pectinase activities of a 10-fold diluted enzyme solution from Fermentation #4 at different citrus pectin substrate concentrations](image-url)

Figure 9.9 Observed pectinase activities of a 10-fold diluted enzyme solution from Fermentation #4 at different citrus pectin substrate concentrations
Finally, the enzyme concentration range was analyzed for pectinase activity. The assays were conducted for 30 min with 5 g/L pectin and different dilutions of the enzyme solution from Fermentation #4. Figure 9.10 shows the observed and dilution factor-adjusted pectinase activities at dilution factors from 1 (undiluted) to 80. Unlike the results observed in Figure 9.5 for polygalacturonase activity, the pectinase test does not show a midrange of dilution that gives relatively consistent adjusted activities. The observed activity levels off only at the very high dilutions, where the substrate-to-enzyme ratios are very high and the activity observed may mostly come from the reaction of the easiest-to-react structures of the complex pectin substrate. An approach different from that established for the polygalacturonase assay is needed.

Figure 9.10 Observed and dilution factor-adjusted pectinase activities at multiple dilutions of the enzyme solution from Fermentation #4; reaction time: 30 min and substrate concentration: 5 g/L.
The approach used for standard filter paper unit (FPU) assay of cellulase is adopted [122]. In the FPU assay, the dilution factor of test enzyme sample that releases a fixed amount (2 mg) of glucose under the standardized assay condition is determined. Because 0.37 U/ml FPU is required to release that much glucose, the FPU of the enzyme sample is calculated and reported as (the dilution factor x 0.37 U/mL). Following the similar procedure, in Figure 9.11 the concentration of reducing sugar released (in D-galacturonic acid equivalent) is plotted against 1/(dilution factor), which corresponds linearly to the enzyme concentration. It is seen that the sugar release increases approximately linearly with (1/dilution) in the D-galacturonic acid equivalent concentration range of 0.2-0.6 g/L, obtained in the approximate range of 5- to 20-fold dilution. At lower dilution factors, the release rate decreases, presumably due to somewhat limited substrate availability; at higher dilution factors, the sugar release rate becomes higher, presumably due to higher availability of more easily reacted structures in pectin substrate. Release of 0.4 g/L D-galacturonic acid-equivalent reducing sugar, roughly midpoint of the linear range, is therefore chosen as the target concentration. This chosen target corresponds to 8.9% substrate conversion and 0.687 U/mL pectinase activity; the latter is found to be within the suggested range of 0.3-0.8 U/mL for polygalacturonase assay.
Figure 9.11 Released reducing sugar concentrations (in D-galacturonic acid equivalent) plotted against the reciprocal of different dilution factors used for the enzyme solution from Fermentation #4, for identification of the linearly proportional range and the near midpoint (0.4 g/L) as target for the suggested assay procedure. The corresponding observed pectinase activities are also shown.

Accordingly, the suggested procedure is to make multiple dilutions of the test enzyme sample and to have at least 2 of them in the linear sugar release range of 0.2-0.6 g/L to allow determination of the dilution factor that would give 0.4 g/L D-galacturonic acid equivalent. The pectinase activity of the enzyme sample is then determined as (the dilution factor $\times$ 0.687 U/mL).
9.3.1.3 Substrate preparation and storage

A heating step was found to be necessary for dissolving the pectin substrate in the buffer. The suggested procedure is to heat the suspension on a magnetic stirrer/hot plate for about 1-2 min, turn off the heating when thin water vapor just starts to appear, and continue mixing the suspension till the pectin is completely dissolved. The appearance of thus prepared substrate solution does not change for at least 24 h if it is kept frozen at -20 °C or refrigerated at 4 °C.

Storage of PGA solution has proved to be more troublesome. Large, seemingly random error in measured absorbance was initially observed for both the blanks and samples for the polygalacturonase assay. For example, when testing a single sample in triplicate at the same time under identical conditions, the observed error would occasionally be up to 30%. Early in the assay development, a large portion of PGA
substrate would be prepared in buffer. This substrate would be used to perform assays for several days and either frozen or refrigerated between experiments. Upon returning the PGA solution to room temperature, it was obviously cloudy in appearance with visible aggregates that were not observed in freshly prepared, uniformly translucent PGA solutions. Random variations in the assay results were believed to arise at least partially from inaccessibility of the aggregated substrate to the enzyme. For example, an experiment was made with the same enzyme sample (from Fermentation #2) incubated with either freshly prepared PGA solution versus the solution that had turned cloudy after 24 h refrigeration. For each substrate solution the assay was made in triplicate. The activity obtained with the fresh PGA solution was 1.98 ± 0.05 U/mL while that with the cloudy substrate was 1.75 ± 0.03 U/mL.

Therefore, several methods of PGA substrate preparation and storage were evaluated for their ability to overcome the formation of aggregates. It was hypothesized that the cold storage conditions and buffer pH near the pKa of PGA (approximately 3.5) supported the aggregation of uncharged PGA molecules. To see if the cold storage was responsible, one experiment were made with two aliquots of a freshly prepared PGA solution, one added with 0.5 g/L sodium azide (Na3N) to control potential microbial growth and the other without Na3N. Both were left at room temperature for 24 h. The Na3N-free aliquot was kept under magnetic stirring; the Na3N-added aliquot was not stirred. Both turned cloudy. The stirred, Na3N-free aliquot was less cloudy. So, storage either at cold or room temperature would lead to cloudy appearance and the cloudiness was not due to microbial growth because the Na3N-added aliquot was cloudier. Stirring may help but cannot completely prevent the aggregation. Then, to see if the low buffer
pH was responsible, the freshly prepared PGA solution was adjusted to neutral pH and then stored frozen or refrigerated. After thawed to room temperature, they were all cloudy. So, buffer pH is not the cause either. Nevertheless, reproducible results have been achieved with freshly prepared PGA solutions. The polygalacturonase assay is strongly recommended to be performed with freshly prepared PGA substrate solutions.

9.3.2 Enzyme storage study

The original enzyme activities and enzyme activity after long time storage and defrosting cycles were summarized in Table 9.1.

Table 9.1 The enzyme activities after long term storage

<table>
<thead>
<tr>
<th>System</th>
<th>Original Pectinase (1 cycle)</th>
<th>Pectinase (2 cycle)</th>
<th>Pectinase (3 cycle)</th>
<th>Original α-galactosidase</th>
<th>α-galactosidase (2 cycle)</th>
<th>α-galactosidase (3 cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A17</td>
<td>5.24±0.22</td>
<td>5.80±0.13 (13 months)</td>
<td>4.80±0.08 (13 months)</td>
<td>3.21±0.04</td>
<td>3.25±0.04 (13 months)</td>
<td>3.31±0.04 (13 months)</td>
</tr>
<tr>
<td>A18</td>
<td>5.50±0.11</td>
<td>5.46±0.17 (13 months)</td>
<td>4.59±0.21 (13 months)</td>
<td>8.02±0.29</td>
<td>7.62±0.15 (13 months)</td>
<td>7.57±0.07 (13 months)</td>
</tr>
<tr>
<td>A24</td>
<td>4.63±0.15</td>
<td>5.02±0.30 (11 months)</td>
<td>4.11±0.29 (11 months)</td>
<td>8.88±0.18</td>
<td>8.87±0.20 (11 months)</td>
<td>8.54±0.09 (11 months)</td>
</tr>
<tr>
<td>A25</td>
<td>8.16±0.04</td>
<td>7.91±0.30 (11 months)</td>
<td>7.14±0.19 (11 months)</td>
<td>7.80±0.25</td>
<td>7.44±0.08 (11 months)</td>
<td>7.50±0.07 (11 months)</td>
</tr>
</tbody>
</table>

For α-galactosidase activities, there are no significant differences from 1 - 3 defrosting cycles of all systems. The p-value of A17, 18, 24 and 25 are separately 0.06, 0.053, 0.074, and 0.057. Therefore, the α-galactosidase is stable after 3 defrosting cycles.
and 13 months freezer storage.

Compare the pectinase activities of these 4 systems, the pectinase activities are stable after 2 defrosting cycles and 13 months freezer storage. But after 3 defrosting cycles, the pectinase activities of these 4 systems have been degraded. The degradation percentages as original and 2 cycle’s pectinase activities of these systems are separately 8.4% to 17.2% of A17, 15.9% to 16.5% of A18, 11.2% to 18.1% of A24, and 9.7% to 12.5% of A25.

9.3.3 Soy enzyme assay

In order to simplify the enzyme measurement, the soy enzyme assay was developed to measure the complex enzyme activities instead of 6 different enzyme tests. Different substrate concentrations and enzyme concentrations were investigated.

Figure 9.13 Different substrate concentrations
Figure 9.14 Different dilution times of enzyme broth

Figure 9.15 (A) Different dilution times of enzyme broth
From this study, substrate limiting happened when the undiluted enzyme was used even though the substrate concentration is 10g/L. As the substrate concentration increases from 6g/L to 20g/L, the releasing sugar increased when the enzymes with lower than 10 times dilution were used, which means the 6g/L substrate was surplus when 10 times diluted or greater diluted enzyme was used. For the 1 hour reaction analysis, the conversion was 10% for 10 times diluted enzyme when substrate concentration is 6g/L or higher. In order to fix this method, it is important to fix the conversion. The different reaction times were analyzed to find how to keep the fixed reaction conversion.

In order to make sure all the enzymes play their role in the reaction, 50% conversion was defined as the standard. Different enzyme loading amount and different reaction time were studied to find out optimum enzyme loading and reaction time range to reach 50% conversion.
Figure 9.16 Conversion as different time and enzyme loading (Trial-1 Substrate 1.8g/L, Trial-2 Substrate 1.6g/L, Trial-3 Substrate 1.8g/L)

In trial 3, with 1.8g/L substrate and 700uL enzyme loading amount, 50% conversion happened in 20min. For future study, this enzyme loading and substrate concentration can be used as standard, and with different reaction time to find when will 50% conversion happen.

9.4 Conclusion

The procedures for polygalacturonase and pectinase enzyme assays were studied for test variables of substrate concentration, enzyme concentration, and reaction time. The optimized procedures are summarized as a flowchart in Figure 7. For the polygalacturonase assay with polygalacturonic acid as substrate, 30 min reaction time, 5 g/L PGA concentration, and proper sample dilution to 0.3 – 0.8 U/mL polygalacturonase activity were chosen and validated to give consistent assay results. For pectinase study with citrus pectin as substrate, the 30 min reaction time and 5 g/L substrate concentration were confirmed to be also suitable; however, unlike the polygalacturonase assay, no sample dilution to an activity range could be found to give relatively consistent assay
results. An approach similar to that used for FPU test of cellulase was adopted. Using multiple sample dilutions, the approach was to achieve (reaction-generated) reducing sugar concentrations of 0.2-0.6 g/L D-galacturonic acid equivalent with at least two of the dilutions, and then linearly interpolate the reducing sugar concentrations with the [1/dilution] values to get the dilution factor that would give the chosen target of 0.4 g/L D-galacturonic acid equivalent. The pectinase activity of the enzyme sample was then calculated as (0.687 U/mL × the dilution factor). Finally, by the substrate preparation and storage study, it was concluded that the pectin substrate solution can be stored without apparent changes for more than 24 h but the PGA substrate solution needs to be freshly prepared to avoid random error due to substrate flocculation/aggregation during storage.

There is no significant degradation of α-galactosidase after 13 months freezer storage and 3 times defrosting. The pectinase activities are stable after 2 defrosting cycles and 13 months freezer storage. But after 3 defrosting cycles, the pectinase activities have been degraded.
CHAPTER X

EFFECT OF PH AND TEMPERATURE ON ENZYME PRODUCTION (PECTINASE AND α-GALACTOSIDASE) BY ASPERGILLUS NIGER 322 SUBMERGED FERMENTATION

10.1 Introduction

Industrial enzyme production is used to manufacture a wide array of products that are used for food preparation and complex oligosaccharide hydrolysis. Among these are pectinase and alpha-galactosidase. Pectinase is commercially used to clarify food juices (i.e. apple, cranberry and grape), among other uses, by degrading the insoluble pectin components [172, 173]. This single enzyme class is generically used to describe a number of different enzyme components that work on the pectin substrate, including: endo-polygalacturonase, exo-polygalacturonase, pectin methyl esterase, and pectin lysate [174]. The production of pectinase is well studied, and commercially available from a number of different production methods. The most common is the fermentation of fungal strains [172].

Likewise, alpha-galactosidase is mostly found in commercial digestive aids, for human use, to degrade poorly digested sugars (i.e. raffinose and stachyose) and improve stomach comfort by reducing lower intestinal bacterial fermentation. The combination of
these enzymes has also been shown to improve chicken growth when used as a dietary supplement.

In this work, the use of *Aspergillus niger* 322 is explored as a means to produce these digestive enzymes in a submerged fermentation process using a low cost soy hull feedstock. The effects of temperature and pH are explored to determine if they influence enzyme production. Since solid particulates interfere with fungal cell growth determination, we are looking for a method to correlate measurable parameters (pH) with growth.

10.2 Materials and Methods

The materials and methods were divided into two parts included shaker study and fermentor scale study, which were described below.

10.2.1 Shake Flask Testing

To determine the temperature effects on the fungal growth rate and enzyme production on complex soy hull carbohydrates, a series of shake flask systems were tested. In one set, soy hull was used as the carbon source to provide for enzyme induction. Since the soy hull interfered with fungal cell concentration testing, a replicate set of flasks were grown on sucrose and used for growth rate testing. The flasks were prepared by transferring some of the pre-culture into new flasks with fresh, sterile, growth medium; the same as used for the pre-culture medium for the stirred tank fermentation except the different carbon sources for different systems. This achieved a dilution, where the pre-culture made up 10% of the new flasks starting volume.
The flasks were grown in a shaker at 20°C, 25°C or 30°C. All systems were grown in triplicate at 250 rpm in an orbital shaker (MaxQ 3000, Thermo Scientific, Dubuque, IA). Periodic 5 mL samples were taken at least every 24 hours to assess the pH, enzyme production or cell growth.

*A. niger* doubling times were calculated from the measured intracellular protein, assuming that the fungal cells doubled for each division rather than tripled or quadrupled. This relation is shown in Equation 1, with variable \( x \) representing the cell concentration at a given time and \( Td \) corresponding to the time needed for each division.

\[
T_d = \log(2) \left( \frac{t_1 - t_0}{\log\left(\frac{x_1}{x_0}\right)} \right)
\]

10.2.2 Stirred Tank Fermentation

To assess the effect of pH on enzyme production, four different pH conditions were tested. In fermentation Fer 1 the pH started at 7.0 ± 0.5, Fer 2 was initially at pH 6 ± 0.5, Fer 3 was initially at pH 4 ± 0.5 and Fer 4 was operated at a fixed pH 4.0 ± 0.5. In cases 1 through 3 the pH was allowed to drop naturally as the cells produced organic acids or raise with ammonia release. In case 4, the pH was controlled with automatic hydrochloric acid or sodium hydroxide addition and could only vary within the controller’s 0.05 deadband.

All pH controlled fermentations were done in a 3 L stir tank fermenter (Bioflo 110, NewBrunswick Scientific, Edison, NJ). The fermenters were operated with 1-1.5 L working volume with dissolved oxygen (DO), pH, agitation, temperature, and foaming...
control. The DO was maintained at above 20% air saturation throughout the fermentation experiments by automatic oxygen addition. The agitation rate was maintained at 400 rpm. A modified Mandels and Weber medium was used for growth [121]. This fermentation recipe was similar as shake flask medium except the doubled concentration of carbon and nitrogen sources. The composition was: 2.8 g/L (NH₄)₂SO₄, 2.0 g/L KH₂PO₄, 0.3 g/L MgSO₄·7H₂O, 0.4 g/L CaCl₂·2H₂O, 0.6 g/L Urea, 2.0 g/L Proteose peptone, 0.2 g/L Tween 80, 0.005 g/L FeSO₄·7H₂O, 0.0016 g/L FeSO₄·7H₂O, 0.0014 g/L ZnSO₄·7H₂O, 0.002 g/L CoCl₂·2H₂O, 40 g/L Soy Hull.

The pre-culture was made as previously described, except a 250 rpm shaker was used in place of a stir plate. The pre-cultures were added to the fermenter to provide a 10% inoculation. Daily samples were taken for enzyme analysis. Cell-free enzyme-containing solutions were stored at -20 °C prior to analysis or use.

10.3 Results and Discussion

The enzyme production results of different conditions were described below, which included the shaker and fermentor study.

10.3.1 Shake Flask Temperature Effects:

Figure 10.1(A) shows the measured pH for shake flasks which contained soy hull material as the carbon source. The initial rate of decrease in the pH was shown to increase with temperature. It can be seen that on average, the 25°C and 30°C systems reached a minimum pH before 24 h. The 20°C system reached a minimum by before 48 h. Assuming there is a slight amount of uncaptured lag phase in the 20°C system’s first data
point, the maximum rate of pH change is -0.078 ± 0.002 /h. At 25°C, the rate is -0.082 ± 0.007 /h, and at 30°C the rate is -0.099 ± 0.003 /h.

An increase in pH usually indicates depletion of carbon source as the cell switches to consuming proteins and releases excess ammonia. This behavior is seen at 25°C and 30°C systems and not at 20°C. It is possible that the rate of soy hull hydrolysis is reduced at lower temperatures, causing an extended period where the cells are limited by carbon source availability. This period would be indicated by the duration of depression in the pH; 8 h for 30°C, 28 h for 25°C, and more than 49 h for 20°C.
Figure 10.1 (A) The pH profiles for *A. niger* grown on Soy Hull (n=3) for different temperatures. (B) The pH profiles for *A. niger* growth on Sucrose (n = 3) at different temperatures. Showing a decreasing trend with respect to time.

When *A. niger* was grown on sucrose, the pH dropped as the cells grew. These trends are described in Figure 10.1(B). Assuming there is an uncaptured pH decrease from inoculation or lag phase, ignoring the first data point, the 20°C system has an average rate of pH decrease of -0.081 ± 0.001 /h, the 25°C system has -0.084 ± 0.004 /h, and the 30°C has -0.073 ± 0.005 /h. The observed minimum pH points were in the same time range as the soy hull system. When compared to the soy hull systems, 20°C and 25°C had the same rate of pH change (p-value=0.08 and 0.66), yet the 30°C sucrose system was slightly slower (p-value = 0.001). The similarity is important, as soy hull interferes with the cell concentration measurement and raises the starting pH to 6.0, effectively overwhelming the buffer and inoculum’s organic acid contributions. The goal is to use pH as a correlated stand-in for cell concentration. A similar rate of decrease
indicates that we might be able to neglect the initial starting pH differences for the purpose of calculating the pH-cell relations.

Intracellular protein was measured in the systems that used sucrose as a carbon source. The amount of protein at any given time was assumed proportional to the amount of fungal cells, roughly constant internal protein content. The growth profiles are depicted in Figure 10.2(A). The average calculated doubling times during the exponential growth periods are reported in Figure 10.2(B). All average doubling times were found to be significantly different (p<0.05) at their respective temperature.
Figure 10.2 (A) The intracellular protein is plotted in semi-log scale for the sucrose systems (n=3) at different temperatures. (B) The calculated doubling times and standard deviations taken for the average over the growth period.

Based on the results from Figures 10.1 and 10.2, the incubation temperature positively influenced the rate of change in pH and growth. As the fungus grows, it produces organic acids that decrease the system pH. When the carbon sources are depleted, internal proteins are consumed and excess ammonia is released, thereby increasing the pH.

The maximum intracellular protein is controlled by the medium composition, assuming either nitrogen-carbon balanced, or limited by carbon, the final cell concentration is fixed. Therefore, the intracellular protein measured values can be normalized by a constant scaling factor that represents the yield. Assuming that the pH
decrease is related to the cell growth and a constant organic acid yield, the pH can be normalized by the initial pH. These assumptions also rely on the temperature only changing the metabolic rate and not altering the processes over the narrow temperature range tested. A correlation can then be established that relates the change in pH to the cell growth. This relationship is shown in Figure 10.3. There is a significant linear relation (p-value = 2E-10), with the slope being -1.59 between the normalized pH and the predicted intracellular protein. This function can be used later to estimate the cell growth by using the pH decrease.

Figure 10.3 A relationship between the normalized-measured intracellular protein and the relative decrease in the pH can be established, to estimate fungal growth as a function of pH change, irrespective of the temperature.
The shake flask α-galactosidase activity profiles, shown in Figure 10.4, are based on the average activities (n=3) for a given temperature condition. These flasks were grown with soy hull as an inducing substrate. As shown, the *A. niger* grown at 25°C had the best performance, reaching $5.9 \pm 0.5$ U/mL. Systems grown at 30°C were the second best. Growth at 20°C had little enzyme production.

The enzyme activity positively correlates with temperature up 60 h. However, after this time the enzyme activity begins to plateau around a value of 3.4 U/mL in the 30°C system. From the pH profile, the pH had begun to increase at by 96 h, suggesting that the soybean hull was depleted and the fungus could no longer produce enzyme due to carbon limitation. An optimal below 30°C, with a decrease in activity at 30°C and a sharp decrease below the maximum, is consistent with other *A. niger* results [175]. The lower productivity at higher temperatures may be due to the enzyme instability at lower pH and higher temperatures [176]. It is also possible that a slightly higher enzyme activity at 30°C provided enough carbohydrate availably to reduce the induction effect, given that the cell growth would already be reduced by a lower pH by 24 h. Likewise, the poor enzyme production at 20°C may be caused by the lower activity, reducing the inducing effect. Looking at the pH decrease, it would appear the cells grew well on any remaining substrates available. This is reinforced by the extended period of pH, indicating the cells never ran out of carbon substrate at 100 h.
Figure 10.4 (A) α-Galactosidase Profiles for the Soy Hull Systems (n = 3) for each temperature shown with solid lines indicating the segments used for rate calculation. (B) The rate of enzyme production, with the 25°C system having the highest net positive production rate.
The pectinase activity profiles, shown in Figure 10.5, are based on the average concentration values (n=3) all grown at the same temperature. The maximum rate of production is also indicated and was taken during segments of growth were the enzyme was actively being produced. The 30°C system had a sharp decrease in activity after 71 h, suggesting either a sharp nutrient limitation as indicted from the pH profile or enzyme instability at elevated temperatures and low pH [140, 177]. Ignoring the last data points, increasing temperature yielded higher possible enzyme production.
Figure 10.5 (A) Pectinase profiles for *A. niger* grown on soy hull (n= 3) for each tested temperature. At 30°C a notable decrease in activity is observed after 71 h. The 20°C system had the poorest performance, yet the fastest instantaneous rate. (B) The net activity production rate is plotted, with 20°C and 30°C having the highest rates.

10.3.2 Fermentation Results for pH and DO controlled stir-tank fermenters:

While testing different growth conditions is convenient in shake flasks, the lack of DO control and pH monitoring can adversely affect the results. Here, fermentations fixed the starting or run pH to and corrected for any DO problems. A 25°C temperature was maintained, to correspond with the moderate growth rate and enzyme production. At this temperature pectinase degradation is not expected at later times and α-galactosidase should be close to the shake flaks optimal.
The pectinase and α-galactosidase production trends are shown in Figure 10.7 and Figure 10.8, respectively. As shown in Figure 10.7, the maximum obtained pectinase production correlates with the initial starting pH, with higher pH yielding better enzyme production. Regardless of the starting pH, at $28 \pm 1$ h all the pH-uncontrolled fermentations reached a minimum pH of $3.4 \pm 0.2$. In the case of Fer 4, the pH was not allowed to decrease and the enzyme production was poor compared to the cases where the pH was allowed to change. As plotted in Figure 6, the pectinase production follows an exponential trend during the first 48 h, with an average exponential term of $0.045 \pm 0.004$ /h. The pectinase activity doubles every 15 h, roughly three times longer than the growth rate. It is expected that the enzyme production is therefore a growth associated product, regardless of the starting pH. This finding is different from the shake flasks, where a clear linear enzyme release was observed. The difference is likely due to DO limitation that is slowing the growth after about 11 h, as shown in Figure 10.2(A). This limiter would stifle the enzyme release and would be hidden in the enzyme profiles shown in Figure 10.5(A) for very early times.

A starting pH of 5.5 to 6.2 has been previously reported for optimal enzyme production with *A. niger* [177, 178]. Yet, it was unclear to what extent the enzyme production was controlled by pH. In this data, it would suggest that a lower pH slows the growth (as in the pH 4 system) and reduces enzyme production (shown when all the systems eventually reach a lower pH). The conflicting part is that the all systems reach a lower pH at $28 \pm 1$ h, yet, the exponential enzyme production continues for another few hours until the lower pH catches up with the cell behavior and reduces activity. From the pH profiles (shown in Figure 10.6), there is an abrupt pH increase shortly after the
minimum followed by a gradual increase, suggesting a prolonged diminishing period of
difficult to hydrolyze soy hull substrate availability. In the case of Fer 3 and Fer 4, there
are two different segments where the pH temporarily increases, with the latter being at 45
h and 42 h respectively.

Figure 10.6 (A): pH profile for Fer 1, where the pH was initially at 7 and allowed to
fall with the production of organic acids and growth.

Figure 10.6 (B): pH profile for Fer 2, where the pH was initially set at 6 and allowed
to decrease with the growth and production of organic acids.
Figure 10.6 (C): pH profile for Fer 3, where the pH was initially set at 4 and allowed to fall with cell growth.

Figure 10.6 (D): pH profile for Fer 4, where the pH was fixed at 4.0. The only variation is within the pH controller's deadband.
While fungal cell concentration was not directly measured, the information would initially suggest that more cells had grown at higher pH conditions. The increased growth would speed the decrease in pH, allowing all the fermentations to reach the same pH at the same time, regardless of the starting condition. More cells would presumably release more enzyme. The maximum amount of enzyme obtained also correlates with the initial starting pH (correlation factor = 0.89).

![Figure 10.7 Pectinase activity profiles for the fermentation runs, showing that higher initial starting pH favors enzyme production. The maximum enzyme obtained correlates (correlation factor = 0.89) with the initial starting pH.](image)

The production of α-galactosidase is shown in Figure 10.7. As in the pectinase, the maximum α-galactosidase production was found when the starting pH was higher.
Starting at a pH of 7 produced the second highest activity vs starting at 6. The two lower pH 4 conditions had the worst behavior. All systems had the pH reach a minimum at about 28 ± 1 h (pH data in Figure 10.6).

It is shown that α-galactosidase production starts after a short lag phase, indicated by the slight deviation in profile slope from 0 to 20 h. In the case of Fer 3 there is an extended lag phase before enzyme is released. In all cases, the first 48 h of active release shows an exponential production of α-galactosidase. With an exponential term of 0.077 ± 0.007 /h. The activity doubles every 9 h, which is half rate of the cell growth. From this, the majority of the enzyme is co-produced during the exponential growth stage, and then has a slow constituent release in the later stages. Since the rates are same for all tested conditions, α-galactosidase production appears to be independent of the medium pH.

Figure 10.8 The α-galactosidase profiles for the fermentation runs, where a higher starting pH favors enzyme production. In all cases, during active growth, the enzyme
production follows an exponential release during the first 48 h of growth. The average exponential term is $0.077 \pm 0.007 / h$.

10.4 Conclusion

This work indicates that *A. niger* 322 performs well in submerged fermentation starting at elevated pH values without control. A relationship can be established between the decrease in the pH and the intracellular protein formed. At elevated temperatures or $30^\circ C$ and lower temperatures of $20^\circ C$, pectinase production is hindered. The $\alpha$-galactosidase production is not significantly affected by temperature. Regardless of the starting pH, both enzymes were found to follow an exponential release during the growth stage, with pectinase doubling every $15 \text{ h}$ and $\alpha$-galactosidase every $9 \text{ h}$ for a $25^\circ C$ condition with an estimated doubling time of $4.3 \pm 0.5 \text{ h}$. 
Due to the limited production of fishmeal with the increase of aquaculture production, the sustainable and economic substitutes of fishmeal become more significant and important. People studied some less-expensive protein sources in fish feeds, such as poultry by-product meal, feather meal blood meal, and meat and bone meal. However, because of the deficiency of the essential amino acids and many concerns on these animal protein sources, soybean protein was finally the best one. The United States Department of Agriculture (USDA) reported that the Global Soybean Production on 2016 would be 336.09 million metric tons, which was around 22.89 million tons or 7.31% increase than last year. The large amount of soybean will provide abundant protein source and other valuable soy-based products at the same time. Besides fish feed application, the soy protein source can also be used for animal feed. However, the soybean protein source should be pretreated other than directly feeding.

There are about 53% protein and 32% carbohydrate in defatted soy flour/meal [15, 134], which has higher protein content than other plant sources such as wheat, lupin, peas and barley. However, this protein content is significantly lower than that of fishmeal, i.e., 65-72% [135]. And there are some anti-nutritional factors in soybean flour, which need to be removed before feeding use. Some anti-nutritional factors can be easily
destroyed by heat such as trypsin inhibitors and lectins. The others with low amounts will not cause problems in soy-based feed [14]. The indigestible oligo-/poly-saccharides with large amounts pose potential anti-nutritional concerns. Processing is required to enrich protein content and reduce indigestible, anti-nutritional factors. Existing processes for producing soy protein products such as SPC and soy protein isolate (SPI) are largely based on physical methods [10, 11], which produce SPI with low yield and produce SPC with no polysaccharide removal and often incomplete oligosaccharide removal [12]. Therefore, an alternative enzyme-based method was applied in the dissertation, where enzyme hydrolyzes and solubilizes carbohydrate into aqueous hydrolysate while protein remains predominantly insoluble near isoelectric pH. The hydrolysate could potentially be used as fermentation substrate and the protein-enriched solids as protein source for animal feed. The core work of this dissertation is to select the optimum enzyme producer and establish the optimal conditions and procedures for maximal yields and productivities of soy carbohydrate-degrading enzymes.

In order to provide the high efficient enzymes, the selection of potential producer was investigated in the dissertation. Different fungi were investigated included T. reesei, A. niger, A. aculeatus, and A. foetidus. A. niger NRRL 322 and A. foetidus NRRL 341 were selected as the most promising producers for producing the effective enzyme mixtures for enzymatic soy protein concentration. The enzyme productivities and soy carbohydrate hydrolysis efficiencies were the selection standards. A. niger NRRL 322 and A. foetidus NRRL 341 were the most potent strains, under soybean hull induction; they produced maximal carbohydrases and minimal protease. In soy flour hydrolysis tests, the enzymes from these two strains gave the fastest and most complete
carbohydrate hydrolysis. Treatment with 4-fold diluted broth of *A. foetidus* NRRL 341 enriched the protein content from 53% (in original soy flour) to 69% in solids readily separated from the hydrolysate. Among the tested strains, *A. aculeatus* NRRL 2053 and *T. reesei* NRRL 11460 had the lowest potential for enzymatic upgrading of soybean meal/flour.

In order to stimulate desired enzyme production, different medium component was investigated and optimized. The carbon sources, nitrogen sources and other nutrients can strongly affect the enzyme production. In order to induce the complex enzyme production, the inducers play the most important role. Soy hull was the best carbon source to induce the complex enzyme production from the shaker study. The best combination of nitrogen source is (NH$_4$)$_2$SO$_4$, protease peptone and urea. The soy flour can be the substitute of protease peptone. From the fermentation study, doubled concentration of carbon sources and nitrogen sources can induce more enzyme production without mixing problem. And the nitrogen limiting happened when sugar/nitrogen ratio is 37.5 in fermentation. The soy flour did not provide better enzyme productions than protease peptone as nitrogen source. Fixed pH condition did not present advantage to multiple enzyme production. However, a pH gradient can selectively move the fungal biomass though multiple optimum conditions, which can induce different enzyme production at different phase. The best changing pH strategy of *A. niger* 341 which was operated at initial pH=7 then pH gradually decreased to 6 in 3 days and then gradually decreased to 5 at 5 days. When using the optimized gradient, the enzyme concentration can be scaled as a function of nutrient concentration. A control scheme that uses the change in dO$_2$ was presented as method to account for different medium
compositions and direct the system to pass through the main optimal pH conditions. The *A. niger* 322 performs well in submerged fermentation starting at elevated pH values without control. Regardless of the starting pH, both enzymes were found to follow an exponential release during the growth stage, with pectinase doubling every 15 h and α-galactosidase every 9 h for a 25°C condition with an estimated doubling time of 4.3 ± 0.5 h. The net pectinase production rate with 20°C and 30°C were the highest rates. However, the poor early growth at 20°C cause slower production at the beginning, the fast dying phase happened at 30°C due to the too fast growth. The α-galactosidase production is not significantly affected by temperature. *A. niger* 341 performed nice cell growth which did not cause high viscosity and poor mixing. However, the more control on pH and DO was necessary. *A. niger* 322 performed well without too much cost on condition control. However, the cell grows really fast which may cause the high viscosity and mixing problem. The balance of these two strains will depend on the economic cost. In order to further increase the enzyme productivities, fed-batch will be suggested in future study. The inducer soy hull as the carbon source will be gradually added into the fermentor to continuously stimulate enzyme production.

During the soy flour hydrolysis, the protein degradation happened due to the presence of protease in the enzyme broth. That is the reason of selection the strain with lower protease production. Because the degradation degrees were not clear, which may beneficial for animal feed digestion or not. The future study on soy protein degradation after hydrolysis is strongly recommended.
Different procedures of polygalacturonase and pectinase activities measurement have been used and reported in the literature. Considering the importance of these enzymes to biorefinery development and other industrial products, there is critical need of a standard method that gives more reproducible activity measurements to allow meaningful comparison and compilation of knowledge about these enzymes. In this dissertation, this is the first systematic study aiming at developing parameters and/or procedures to give reproducible assays for these enzymes. These assays can be the standard assays used in future research involving these enzymes. The variables of substrate concentration, enzyme concentration, and reaction time were studied. The polygalacturonase consistent assay were developed and validated with polygalacturonic acid as substrate, 30 min reaction time, 5 g/L PGA concentration, and proper sample dilution to 0.3 – 0.8 U/mL polygalacturonase activity. The pectinase assay with citrus pectin as substrate had same reaction time and substrate concentration as polygalacturonase. However, unlike the polygalacturonase assay, no sample dilution to an activity range could be found to give relatively consistent assay results. The linear interpolation method similar to that used for FPU test of cellulase was adopted. Finally, by the substrate preparation and storage study, the pectin substrate solution can be stored without apparent changes for more than 24 h but the PGA substrate solution needs to be freshly prepared to avoid random error due to substrate flocculation/aggregation during storage. The assays of cellulase, xylanase, α-galactosidase and sucrase were followed the literature standard methods. Multiple enzymes were analyzed in the dissertation, which is time-consuming. The enzyme broth will finally used to hydrolyze the soy flour. Therefore, the easier and timesaving method is to develop the soy enzyme assay, which
can give the total activity to hydrolyze the soy carbohydrates in soy flour. The study with soy flour as substrate was investigated at preliminary stage, which may be recommended for further study in future.

After enzyme production, the collected enzyme mixture will be stored in -20°C till using for hydrolysis. During longtime storage, it is really important to understand how the enzyme activities look like. In this dissertation, the more important two enzymes α-galactosidase and pectinase were investigated. There was no significant degradation of α-galactosidase after 13 months freezer storage and 3 times defrosting. The pectinase activities are stable after 2 defrosting cycles and 13 months freezer storage. But after 3 defrosting cycles, the pectinase activities have been degraded. The storage investigation of other enzymes will be recommended in future study.

In future work, fed batch fermentation is suggested to further increase the enzyme productivities. The inducer, soy hull, can be gradually added to continuously induce enzyme production. Better pH and dissolved oxygen control designs may also improve enzyme productivity. Soy protein degradation should be further investigated in future studies.
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