ENZYME BASED PROCESSING OF SOYBEAN MEAL: PRODUCTION OF ENRICHED PROTEIN PRODUCT AND UTILIZATION OF CARBOHYDRATE AS FERMENTATION FEEDSTOCK FOR ARABITOL PRODUCTION

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ABSTRACT

Soy protein is one of the major components of the diet of food producing animals and is increasingly important in the human diet as well. However, soy protein cannot be used as an ideal protein supplement in foods, because of the presence of high amount of indigestible carbohydrates in the soybean meal. Adverse nutritional and digestion effects have been reported in many animals and fish following the consumption of soybean meal and soybean meal derived products. To enhance the nutritional value of soybean meal in human food and animal feed, it is necessary to improve the protein content and remove the indigestible carbohydrates from the soybean meal during the processing of soy protein diets. This project aims to develop economically feasible technologies and processes for separating, enriching and upgrading soy proteins and carbohydrates from soybean meal. The objective is to separate proteins from carbohydrates (and other minor components) in soybean meal, facilitated by enzymatic hydrolysis of poly- and oligo-meric carbohydrates and other non-protein materials. The enriched proteins obtained are valuable for high-quality feed, food and industrial uses. The hydrolyzed carbohydrates could also be converted via fermentation into bio-fuel related products and other value added chemicals.
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CHAPTER I

INTRODUCTION

1.1 Background & Significance

As the human population continues to grow, finding food sources is one of the most important challenges. Improving economy and rising per capita income resulting in changes in dietary habits in developing countries has led to ever more demand for animal protein. IMPACT global food model developed at IFPRI (International Food Policy Research Institute) predicts that demand for meat could increase by 58 percent by the year 2020 where demand for poultry is expected to rise by 85 percent and that of pig meat by 55 percent of the current level.[1]. The agricultural sector will have to produce adequate amount of cereal crops to meet the world food energy requirements. However, cereals are low in protein content and lack many essential amino acids so it has to be supplemented with a protein source[2]. World aquaculture has grown dramatically in the last five decades. The contribution of aquaculture to global supply of fish has continued to grow, increasing from approximately 4.0 percent of total production by weight in 1970 to 36.0 percent in 2006[3]. Fish meal is the principal protein source of the fish diets with balanced amino acid composition and other nutritional factors. As aquaculture demand is increasing, clearly the future demand for fishmeal is on a rapidly increasing track. With depleting population of global fish stocks, the stage is set for a bottleneck that could severely limit industry growth. In order to head off such a bottleneck, the global aquaculture industry must eliminate its
dependence upon fishmeal (and fish oil) and develop a portfolio of sustainable dietary protein sources. Every industry that has been called upon to bridge the gap between demand and wild supply is presently dependent upon wild stocks of fish for the vast majority of its protein source.

Plant feed stuffs are one of the major sources of proteins that can be a potential alternative to human food and animal feed. Some plants that have proteins with a very good profile of essential amino acids can be important for consideration as potential animal feed. These protein sources also possess some other important characteristics such as high palatability and digestibility with wide availability. However, most plant proteins have high concentrations of insoluble fibers and lignins that cannot be digested by animals and fish preventing its use as an ideal feed.

Soybean with its reasonable amino acid profile can be one of the best sources of protein in animal feed and fish meal[4]. Soy protein products offer a lot more than the economic advantage that vegetable proteins have over animal protein sources. Advances in soy processing technology have resulted many desirable properties in the soy products such as emulsification, binding and texture. Inclusion of soy protein in the food products is increasing because of such functional properties, abundance and low cost.

Soy meal contains approximately 50% crude proteins and 30-35% carbohydrates. Soymeal carbohydrates are mainly composed of approximately 10% sugars and 20-25% non-starch polysaccharides (NSPs)[5]. NSPs are cellulose and some pectin polysaccharides. Soymeal products available commercially are typically of three categories: Soymeal, Soy Protein Concentrate (SPC) and Soy Protein Isolate (SPI). Current commercial processes for producing SPC and SPI use non-enzymatic methods. SPC is
currently produced by washing soy flour with water, possibly containing pH buffer and/or organic solvent. The water soluble carbohydrates (and other soluble and colloidal materials) are removed from the remaining protein-rich solids. SPC, thus produced, has a protein contents of 65-67% and retains the insoluble soy carbohydrates and other materials such as non-starch polysaccharides (hemi/cellulose and pectin) and lignin[4]. The protein yield (i.e., the portion of initial proteins retained in the product) for SPC production is high (90%-98% depending on the method), but it is less ideal for fish because of the presence of hard to digest polymers.

In young animals with still undeveloped digestive tract, feeding unprocessed soy protein results physiological and intestinal morphological changes. Although many herbivorous and omnivorous aquatic species seem to tolerate higher dietary level of soy products without any significant detrimental effect on the growth performance, Carnivorous fishes like atlantic salmons cannot digest high dietary content due to the lack of appropriate digestive enzymes. Accumulation of intestinal gas and flatulence in carnivorous animals results from the presence of alpha linked oligosaccharides mainly raffinose and stachyose which are non-reducing sugars composed of one or two galactose units linked to sucrose. Due to the lack of alpha 1,6-galactosidase enzymes in the intestinal mucosa of the monogastric animals, raffinose and stachyose, if present in the diet, remained unabsorbed in the small intestine and metabolized by the intestinal microflora in the large intestine resulting gas production which causes flatulence. During the processing of soy protein product removal of these indigestible oligosaccharides would contributes to a significant increase in the nutritional value of the product.
SPI, on the other hand, is prepared by first dissolving proteins in aqueous solution, together with water soluble carbohydrates and others. This causes disintegration of soy meal particles and allows removal of insoluble constituents by centrifugation. Proteins in the supernatant are then made insoluble by adjusting the pH of the protein containing solution and collected by centrifugation. The SPI product, thus prepared, has higher protein content (about 90%) but is costly to produce and the dry weight yield is only 30% (protein yield about 60%)[4]. Despite the protein content of SPC being comparable to that in the fish meal, the presence of considerable amount of indigestible fibers and cellulose during the SPC preparation by conventional method prevents it from being viable. So an economic process of producing SPC having higher digestibility to fish is necessary in order to effectively substitute fish meal by soy meal products. Digestibility of the SPC can be increased if cellulose and other indigestible carbohydrates can be hydrolyzed to soluble sugars.

The soy carbohydrates produced from the enzymatic separation process can be converted to biofuel (ethanol, butanol) and other valuable chemicals such as arabitol, succinic acid. Arabitol is one of the DOE identified top 12 biomass-derivable building blocks. It can be transformed to several families of high-value products, including unique polyesters and copolymer resins. We have developed a fermentation process to produce arabitol with yields higher than 50%. The proposed enzymatic separation process will enable coproduction of enriched soy proteins and new biobased chemicals such as arabitol.

The principle objective of this work is to enrich the protein content of soy meal and reduce the indigestible polysaccharides employing enzymatic treatment to make the soy meal an economically viable substitute for animal protein in the livestock and aquaculture
industry. To minimize waste and maximize profitability, the reducing sugar containing supernatant obtained as a result of enzymatic hydrolysis will be used to produce high value product arabitol, a five carbon low calorie sugar alcohol with unique antibacterial properties.

1.2 Objective

The primary goal of this work is to enrich the protein content of soybean meal and at the same time reduce the indigestible carbohydrates from enriched protein products. To achieve this goal, an enzymatic process was used for removal of the indigestible carbohydrates from the soybean meal and to increase the protein content of the resulting soy protein product. We hypothesize that the enzyme broth produced by fungal fermentation containing mixtures of cellulase, xylanase and pectinase will efficiently hydrolyzed the indigestible carbohydrates in the soy flour which will be separated into the hydrolysate from the proteins, precipitated at their isoelectric point. The protein content, thus will be increased and the enriched proteins collected from the enzyme-facilitated separation can be used for high-quality feed, food and industrial uses. Degraded soluble sugars in the hydrolyzate will be used as substrate for production of arabitol by *Debaryomyces hansenii* SBP-1 fermentation.

The whole study is divided into two parts, first is the study of enzymatic hydrolysis and the recovery of proteins after hydrolysis. Then, the second part is about the utilization of the carbohydrates rich hydrolysate as fermentation feedstock for arabitol production.
First part of the study is to hydrolyze the carbohydrates in soybean flour and enrich the protein content. Several important aspects will be investigated here. At first, the optimum hydrolysis conditions is investigated in shake flasks. It is well known that hydrolysis conditions e.g. pH, temperature, substrate and enzyme loading have significant impact on the rate of hydrolysis. Optimum working conditions for different enzymes alone are well documented in existing literature, however, the current study will employ a combination of enzymes so it is important to determine the optimum working condition for such a mixture. Once the optimum conditions have been identified, effect of the ratio of different types of enzymes in the mix on the rate of hydrolysis will be investigated under the optimized conditions. These experimental results will give more insights on the synergistic effects of different enzymes on the substrates, which will help in determining the optimum ratio of different enzymes to be used for hydrolysis. Along with the synergistic effect of enzymes, kinetic behavior of the hydrolysis will be investigated and an empirical model will be developed which would help in designing the hydrolysis for different concentrations of enzymes in the broth. Once an efficient hydrolysis is accomplished, insoluble protein product (SPC) and carbohydrate rich hydrolysate will be separated by centrifugation. Knowing the kinetics of the hydrolysis, further improvement can be investigated to develop a fed batch hydrolysis process to improve the sugar concentration and productivity. This process is expected to increase the sugar concentration in the hydrolysate. This hydrolysate will then be used as a fermentation feedstock for chemical production in the next part.

The objective in the second part is to use the hydrolysate effectively as a fermentation feedstock for arabitol production by Debaryomyces hansenii fermentation.
Fermentation conditions such as operating pH, dissolved oxygen concentration (DO) are highly critical for achieving high yield of the product. Operating conditions and the medium compositions will be investigated in the fermentation. Then a purification method of arabitol from the fermentation broth will be developed. And finally the application of arabitol as an anti-cariogenic agent for inhibiting growth and acid production by the oral bacteria will be investigated.

1.3 Experimental design & hypothesis

The studies in this work are mainly divided into seven main chapters. First four sections talk about the enzyme hydrolysis process and protein separation, more specifically optimization of operating conditions, kinetic of enzyme hydrolysis with different enzyme, fed batch enzyme hydrolysis process and refinement of enriched protein product and hydrolysate. On the other hand, last three sections talk about the production of arabitol from hydrolysate, its purification and application in reducing dental cavities.

First section is to understand whether enzyme produced by fungal fermentation can hydrolyze the carbohydrates in soybean flour and what are the operating conditions that affect the hydrolysis? Enzyme hydrolysis largely depends on the hydrolysis condition. Different operating parameters should be strictly maintained to achieve the highest degree of hydrolysis. This section sets out to answer some important questions, what are the important operating conditions to consider during the hydrolysis, what will be their ranges, which factors are more sensitive to affect the hydrolysis, are there any interaction effects between different factors?
Once the optimum operating conditions are known, further studies under optimum operating conditions are done to understand the effect of different enzyme activities. These are discussed in the second section. This chapter will teach about the kinetic behavior of hydrolysis in presence of different enzyme activities. Multiple experiments were designed to explain the fact that what enzyme activities are more important, what types of carbohydrates in soybean flour are more difficult to hydrolyze, what are the limiting enzymes? To put together all the understanding, a kinetic model is developed to explain the effect of different enzyme activities on hydrolysis.

With the guidance of kinetic model, fed batch and enzyme recycled processes are investigated in third section to increase the productivity of the hydrolysis. This section answered the fundamental questions, what type of fed batch process is more effective, whether enzymes can be recycled, if recycle process can improve the productivity, what are the factors that affect the recycle process? These knowledge would be used to develop a recycle process that can produce the hydrolysate with increased sugar concentration.

After hydrolysis of soybean flour carbohydrate, precipitated proteins (soy protein concentrate) are separated from the hydrolysate by centrifugation. However, protein content of the soy protein concentrate and the protein recovery will depend on the effectiveness of solid liquid separation. Protein recovery also depends on the fact whether proteins are hydrolyzed and solubilized in hydrolysate along with the carbohydrates. In this section, these aspects of the process are investigated. Finally the total material balance of the process is presented in this section.

Now the hydrolysate utilization as fermentation feedstock for arabitol production is investigated. Arabitol, a high value product, is produced by fermentation of the reducing
sugar in hydrolysate as a carbon source and residual protein as nitrogen sources by osmophilic microorganism *Debaryomyces hansenii* SBP-1. Srujana Koganti has already been successfully able to produce arabitol by this strain using glycerol from the biodiesel industry as substrate[6]. The soluble carbohydrate solution, with residual amounts of proteins and other nutrients, is ideal as fermentation feedstock. We have already demonstrated that the yeast strain can produce arabitol using glucose and other carbohydrates as substrate, with at least comparable effectiveness to the production using glycerol as substrate. Different fermentation parameters (pH, temperature, Dissolved oxygen) will be optimized to get maximum yield of the product. So this alternative sweetener production will give additional economic advantage of this integrated process.

Next section is the purification of the fermentation product arabitol from fermentation broth. Product purification is always very important for obtaining the desired product in purified form after the fermentation and accounts for a major portion of the total production cost. So effective process for purification with high product yield is very critical for the overall process economics. We have successfully developed a purification process of arabitol from fermentation broth containing glycerol. The similar process will be used to purify the arabitol from the fermentation broth after the production of arabitol using carbohydrates in the hydrolysate as substrates.

In the next section, application of arabitol will be discussed. Arabitol is a 2´-epimer of xylitol. Xylitol is widely known for its use as a non-cariogenic low calorie sugar substitute because it is not fermented by oral microorganisms including cariogenic bacteria such as streptococcus mutans and streptococcus sobrinus. It also inhibits the acid
production leaving the final pH neutral in dental plaque and leading to no enamel
demineralization. Chewing gums containing xylitol have been reported to reduce the dental
plaque and the number of mutans streptococci in saliva. Xylitol is currently produced by
chemical reduction of xylose derived from wood hydrolysate under alkaline condition. But
purification and separation of xylose required high pressure, temperature and expensive
catalyst which makes the process more expensive. Being an isomer of xylitol, Arabitol has
never been investigated for its potential to inhibit the oral bacteria as xylitol. We
hypothesize that arabitol also would have the potential to inhibit the oral microorganisms.
Moreover sweetness per caloric content for arabitol is much higher than that of xylitol
which could make it even better low calorie sugar substitute. The inhibitory effect of
arabitol on the growth of and acid production by the oral bacteria in presence of different
dietary sugars will be investigated.

Finally, these integrated processes were combined and evaluated by economic
analysis. The assessment was to determine if the overall integrated process is financially
viable. Does the use of enzyme to increase the value of protein and carbohydrate make
industrially sense?

The whole project can be simplified into a series of hypothesis or tested statements,
each pertaining to their respective section:

1. Can enzyme mixture produced by fungal fermentation is capable of hydrolyzing
   complex soybean carbohydrate and to what extent?

2. Whether hydrolysis and protein precipitation can be done at similar operating
   conditions and what are the applicable ranges of each operating conditions?
3. Whether fungal fermentation has right mixture of enzyme activities and if not what are be the limiting activities and how the limitation can be overcome?

4. Recycle of enzyme is a well-known technique to reduce the requirement of enzyme. In the current process, where multiple enzyme mixture is involved in the hydrolysis, is recycle also equally beneficial?

5. In conventional process, soy proteins are precipitated at isoelectric pH 4.8 after washing the soluble carbohydrate by acid or alcohol. Whether the protein recovery is also similar in enzymatic process?

6. If the protein content in the soy protein concentrate can be further increased by washing of carbohydrates and what would be the maximum protein content?

7. The hydrolysate is used for the production of arabitol. Whether the hydrolysate can be used as both carbon and nitrogen source?

8. Hydrolysate contains mixture of multiple hexose and pentose sugar. Does the yeast can consume all kinds of sugar and produce arabitol?

9. If hydrolysate can be used as both carbon and nitrogen source, dose the C/N ratio optimum to produce maximum arabitol production?

10. Produced arabitol is an enantiomer of anti-cariogenic sugar alcohol xylitol. Does arabitol can also be used as an anti-cariogenic agent as xylitol?

This work investigated a new process for protein enrichment and carbohydrate utilization from soybean flour. This new integrated process could be a major step forward to a soybean based bio-refinery. Using this process the protein content of the soybean flour in enriched and at the same time the indigestible carbohydrates are hydrolyzed and separated for value added use as fermentation feedstock. The similar enzymatic process
has the potential to be used for other soybean based by-products or wastes such as soybean hull, soybean molasses and soybean okara.
CHAPTER II

SOYBEAN COMPOSITION & INDUSTRIAL PROCESSING

2.1 Soybean

The soybean (*Glycine max*) is a legume crop native to East Asia, now grown worldwide because of its high content of protein and oil. Its world production level has been increasing continuously and reached 263.7 million metric tons in 2010/2011, which is more than double than that in 1992/1993. This continuing increase is due to the increasing demand to accommodate the food and fuel needs of the growing world population. Majority of soybean is produced in North and South America. The largest producer is the USA, with 34% of the global production, followed by Brazil (29%), Argentina (19%), China (6%), India (4%), and others (9%). Principal use of soybean is to extract the oil for human consumption. During the oil extraction process, remaining part of the cotyledon is processed as soybean meal. Soybean meal, with high protein content, is used for livestock feed. In Asian country soybean is also used for production of traditional foods, such as soymilk, soy sauce, tempeh, tofu and natto.

2.2. Composition

Composition of soybean is important for the processing for different products. Soybean seed is composed of two parts: soybean seed coat or hull and cotyledon. Cotyledon accounts for almost 90% of the seed weight. From the commercial point of
view, cotyledons are the most important parts of the seed because they are the storage of protein and oil. Soy protein and oil are packed into the subcellular structure called protein and oil bodies. They are located in the cytoplasm of cotyledon cells. Protein bodies are large spherical organelles with a diameter of 2-10 µm, whereas oil bodies are much smaller particles, ranging from 0.2 to 0.5 µm in diameter. Cell walls of the cotyledon cells are composed of polysaccharides and the main source of carbohydrates in the cotyledons. Protein content and carbohydrate contents in the soybean seed is around 38-40% and 25-28% respectively.

2.2.1 Soybean Proteins

Proteins in the soybean comprised of a broad range of components which are classified in terms of Svedburg sedimentation units, S. The smaller the Svedburg number, the smaller the protein molecular weight. Soy proteins are divided into four categories according to their size: 2S, 7S, 11S and 15S.

2.2.1.1 2S fraction

This fraction has been reported to contain 15-22% of soy protein with molecular weight ranging from 8 to 21.5 KDa. This fraction mainly consists of trypsin inhibitors and cytochromes enzymes[7]. The 2S fraction is a highly symmetrical protein composed of a number of tight rings. These rings are held together by 7 disulfide bonds[8]. This fraction exhibits good foaming capacity and water holding ability[9].
2.2.1.2 7S fraction (β-conglycinin)

β-conglycinin (7S) is one of the dominant storage proteins of soybean seeds, constituting about 30% of total soy protein. It is a trimetric protein with molecular weight of 140 – 210 KDa[7]. The 7S fraction contains approximately 85.06-90.17% protein, 1.38-1.96% fat, 4.8-5.91% ash, 1.82-8.81% carbohydrates and traces of fiber[10]. The protein is composed of three subunits, α (~67 KDa), α' (~71 KDa), and β (~ 50KDa). Several combinations of these subunits (α' β2, α β2, α α'β, α 2α', α3 and β) are known to exist and provide heterogeneity[11]. The α and α’ subunits are composed of extension regions and core regions and have acidic properties, whereas the β subunit consists of only the core region[11]. The thermal solubilities of individual subunits are different indicating that that they exhibit different physicochemical functions[12]. B-conglycinin has many properties, one of it is its ability to form disulfide – linked polymers, which contribute to insolubility of soy protein. These polymers cause turbidity and increased viscosity of soy protein 5 dispersions. Depolymerization can be achieved by adding of mercaptoethanol. A second property is sensitivity to ionic environment; it undergoes association- dissociation reactions with change in ionic strength.

2.2.1.3 11S fraction (glycinin)

Glycinin is one of the major constituents of soy protein representing ~ 35% of total protein[13, 14]. Native glycinin is a heterogeneous oligomeric protein, the molecular mass of which varies between 340-375 kDa. It is made up of six subunits, each consisting of a basic polypeptide (Bpolypeptide, MW 38 KDa) and acidic poly peptide (A-
polypeptide, MW 20KDa), which are connected by a single disulfide (SS) bond forming AB subunits[15]. The AB subunits associate into two hexagonal rings forming a hollow cylinder of 11 x 11 x 7.5 nm. At ambient temperature and at pH 7.6, glycinin forms hexameric complexes, whereas at pH 3.8 glycinin is mainly present as trimeric complexes[16]. Glycinin contains extensive disulfide bonds, which contributes to its insolubility. It undergoes association- dissociation reactions, with changes with ionic strength. The quaternary structure of the 11S molecule is disrupted by high and low pH, by high concentrations of urea, detergents, mercaptoethanol-urea mixtures and by temperatures above 80°C. The onset denaturation of glycinin is about 80°C at neutral pH. Thermal aggregation of glycinin at 80°C is mainly due to aggregation of the basic subunits after they have been thermally dissociated from the oligomeric structure. This aggregation is prevented in the presence of the other soy protein fractions. This is because of certain interactions between conglycinin and the basic subunits of glycinin, which may lead to formation of soluble complexes[17].

2.2.1.4 15S fraction

The 15 S is a minor component of soy protein (11%), with molecular weight of 506- 600 kDa. 15S either exists as a native protein in the seed or is an artefact formed during isolation of the proteins[18]. Conversion of 11S to 15S fraction occurs when 11S preparations are frozen and thawed or precipitated by dialysis against water and freeze-dried.
2.2.2 Soybean carbohydrate

2.2.2.1 Soluble sugars

The primary sugar found in the soybean meal is sucrose which can be as high as 25-35% of the total carbohydrates. In companion with sucrose other oligosaccharides are found in low concentrations, with stachyose as the main oligosaccharides followed by raffinose and verbacose[19]. Even though these oligosaccharides are low molecular weight sugars, they deserve special attention due to their structure and contribution to the nutritional attributes of the soybean meal. Stachyose, raffinose and verbacose are galacto-oligosaccharides which consists of a terminal sucrose linked with 1 (raffinose), 2 (stachyose) or 3 (Verbacose) galactose monomers by α-1,6 linkage and the bond between terminal sucrose and the galactose is α-1,3 (figure 2.1) [5].
2.2.2.2 Cellulosic and hemicellulosic polysaccharides

Cellulose is a linear insoluble unbranched polymer of β-1,4-linked D-glucose residues. One chain is associated with other cellulose chains by hydrogen bonding and Van der Waals forces. It is the main cell wall polymer and highly crystalline structure which is resistant to enzymatic attack. The insoluble hemicellulose are low molecular weight polysaccharides which contains xylan polymers in large quantities. They are linked by β-1-4 linkage and this type of glycan does not have large number of side chains and is, therefore, not soluble in water. The xylan structure are essentially linear due to the β-1-4 linkage between xylopyranose residues, and has the small proportion of glucuronic acid residues attached as monomeric side chains by 1-2 linkage.
2.2.2.3 Soybean pectic polysaccharides

Soybean pectic polysaccharides structures are very diverse and complex, which consists of both acidic and neutral polysaccharides. Acidic polysaccharides structure is more complex in nature. Neutral polysaccharides of soybeans are mainly the interior structure within the more complex acidic polysaccharides. Neutral polysaccharides are arabinogalactan chain which has fairly simple structure with linear 1-4 β-D-galactose chain that is branched at every fourth or fifth residue by two L-arabinose units. The ratio of arabinose to galactose in the arabinogalactan polysaccharides is approximately 1:2.8. The more complex acidic polysaccharides fraction were found to contain approximately 25-30% uronic acids. Compared to pectins from other sources such as citrus pectins, soybean acidic polysaccharides are highly branched with heterogeneous monosaccharide composition consisting mainly of galacturonic acid, galactose, arabinose, xylose, fucose and rhamnose. All these monosaccharides are arranged in mainly three types of structures: homogalacturanan, rhamnogalacturanan I and rhamnogalacturanan II.

Homogalacturonan chain consists of mainly α-(1, 4)-linked D-galacturonic acids, some of the carboxyl groups are partially methyl-esterified at C-6 and acetyl-esterified at positions O-2 and/or O-3. The linear units of HG in which more than 50% of the GalA are esterified with methyl (or methoxy) groups at the C-6 position are conventionally called high methyl-esterified HGs; otherwise they are referred as to low methyl-esterified HGs. The unmethylated HG is negatively charged and may ionically interact with Ca2+ to form a stable gel with other pectin molecules if 10> consecutive unmethyl-esterified GalA residues are coordinated.
Rhamnogalacturonan-I (RG-I) contains a backbone of the repeating disaccharide galacturonic acid and rhamnose:[α-(1,2)-D-GalA-α-(1,4)-L-Rha]n partially substituted O-4 and/or O-3 positions of α-L rhamnose residues with single neutral glucosyl residues and with polymeric side chains predominantly of α-(1,5)-L arabinans and β-(1,4)-D galactans, arabinogalactans-I(AG-I), arabinogalactans-II (AG-II) and possibly galacto-arabinans (figure 2.2) [22]. The backbone may be O-acetylated on C-2 and/or C-3 by α-L-rhamnose residues. The predominant side chains contain linear and branched α-L-arabinose and/or β-D-galactose residues [20] these two are linked to approximately half of the rhamnose residues of the RG-I backbone.

Figure 2.2: Structure of Rhamnogalacturonans-I, part of the complex structure of pectin, a soybean cell wall polysaccharide.
Rhamnogalacturonans-II are the most complex and branched polysaccharides of pectin. RGII is a minor pectic component of soybean cell walls. The RG-II has a characteristic structure of seven to nine residues of α-D-galacturonic acid backbone with four branches clearly differentiated (figure 2.3). RG-II has several kinds of substituents including 11 to 12 different glycosyl residues, some of them rare sugars in nature, like 2-\(O\)-methyl xylose, 2-\(O\)-methyl fucose, aceric acid, 2-keto-3-deoxy-D-lyxo heptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno octulosonic acid (Kdo) [27-30]. About 28-36 individual sugars, interconnected by more than 20 different glycosidic linkages that makes a highly complex polymer with an α-D-galacturonic acid backbone partially methyl esterified at C-6 with galactosyl residues and branched with oligosaccharide chains [31].

Figure 2.3: Structure of Rhamnogalacturonans-II, part of the complex structure of pectin, a soybean cell wall polysaccharide.
2.3 Processing of Soybean

Soybeans have been processed for thousands years for the recovery of fats and oils. Current processing of soybean are done mainly physical and chemical processing which involves hard screw pressing, prepress solvent extraction or direct solvent extraction. Prior to the oil recovery it is desirable to remove the hull that covers the cotyledon of meat. It contains much less oil and proteins than meat but high carbohydrates. Removing the hulls is mainly done to raise the protein level of the meal. After dehulling soybean flakes are processed by solvent extraction to extract the oil leaving soybean meal. The soybean meal contains high percent of proteins. These proteins can be used as the supplement in the animal feed and food. To further raise the protein content of the soybean meal, water extraction of the soluble sugars and acid precipitation at isoelectric point of the protein is done to produce high protein soy protein concentrate and soy protein isolate. Soybean okara is produced during the separation of soy protein isolate which contains mainly insoluble polysaccharides. On the other hand, during soy protein isolate production, soy molasses is separated which mainly consists of soluble sugars. A detail flow diagram is presented to better understand the processing of soybean in figure 2.4
Figure 2.4: Current processing of soybean for production of oil and protein products

The carbohydrate rich byproducts generated from the different part of the processing are yet to find valuable industrial applications. Although they can be valuable as a source of carbohydrates, they have minimal application to date. Principal carbohydrate rich byproducts from soybean processing are soybean hull, soybean meal, soybean okara and soy molasses.
2.3.1 Soybean hull

Even though soybean hull represents almost 8-10% of the whole soybean seed, it has not received noticeable attention as a low cost renewable feedstock for production of value added chemicals and its use is still limited to animal feed. Soy hulls are typically sold as is or as compressed pellets and fed to cattle and pigs. As a result its commercial value has been considered much less than oil and proteins of soybean. So it was no wonder that relatively fewer attempts have been made to study soy hull carbohydrates and their utilization other than animal feed. It is a lignocellulosic material containing mainly cell wall polysaccharides. A relatively small proportion of lignin in soybean hull compared to other agricultural residues could make its value much higher as a lignocellulosic material for production of high value chemicals. Since lignin is a major hindrance for the hydrolysis of lignocellulosic biomass to fermentable sugars. If carbohydrates of soybean hulls can be utilized for production of value added chemicals, it would add significant value to the soybean processing industry. Nutritional value and chemical composition of soybean hulls, however, is highly dependent on the nature of processing condition of soybeans. Overall amount of carbohydrates accounted in soybean hulls were reported from 64-70%. Cellulose, hemicellulose and k拉斯on lignin content varies from 40-51%, 14-20% and 1.4-6%, respectively. It has relatively lower pectin content than other soybean feed stocks[20].

2.3.2 Soybean Meal

Soybean meal, an abundant byproduct after oil extraction of soybeans has become increasingly important as a source of protein in animal diets, particularly for poultry,
swine, cattle and fish. Soybean meal is by far the dominant protein supplement used in livestock and poultry feeds in the United States and throughout the world. Soybean meal has excellent profile of essential amino acids that meets all the requirements as a supplement of animal proteins and the digestibility of amino acids is excellent among commonly available protein sources[21]. Soybean meal mainly consists of 50% proteins and 30-35% carbohydrates, 4-5% ashes and 3% fats[22].

2.3.3 Soybean molasses

Soybean molasses is a brown viscous syrup with a bittersweet flavor. It is a by-product of soybean processing that results from the aqueous alcohol extraction of soluble sugars from defatted soybean meal to produce soy protein concentrate. Soybean molasses is mainly composed of soluble carbohydrates, proteins, lipids, fibers and ash. The most abundant carbohydrates are sucrose (28%), stachyose (18%) and raffinose (9%)[23]. This carbohydrates rich syrup can be used as a useful feedstock for production of ethanol or other value added chemicals.

2.3.4 Soybean Okara

Okara is the fiber rich residue left from ground soybeans after extraction of the water soluble fraction used to produce soymilk, tofu, fried bean card and other protein rich food. About 1.1 kg of okara is usually produced from each kilogram of soybeans processed for soymilk production. It is typically considered an agricultural waste due to its difficulty in utilization, although many attempts have been made. This difficulty in utilization is mainly associated with the digestion and solubilization of high content of
complex polysaccharides. Large quantities of okara byproducts generated during the processing of protein rich food from soybeans involve an economic and environmental problem due to their high volumes and elimination cost. Moreover, due to the continuous increase in the worldwide production of soybean and consumption of soy products, accumulation of okara is also on the consequent increase. As a result, the search for the commercial exploitation of soybean okara is getting higher attention to the scientific community. Although the composition of okara depends on the processing condition and the product, the main component of okara by products are protein (28.5%), dietary fiber (55.5%) and fat (8.5%)[24]. Therefore okara has high potential to be incorporated as the valuable food ingredients or renewable feedstock. Okara is also reported to possess health benefits such as anticholesterol action[25] and prevention of liver fat accumulation[26, 27].
3.1 Soybean Meal Products

Soybean meal is considered one of the best available vegetable protein source for livestock industry. Soybean meal contains about 50% protein and 30-35% carbohydrate. Although it has almost 50% proteins, many of the animal feeds require sources with higher protein contents. The carbohydrates present in the soybean meal can be processed to improve the protein content. These carbohydrates contains insoluble and indigestible carbohydrates. The nutritional value of soybean meal is decreased by the presence of non-digestible oligosaccharides and non-starch cell wall polysaccharides. So to incorporate soybean meal in animal feed, removal of the major dietary fibers and indigestible components is often beneficial. So further processing of soybean meal to find products with higher protein content is necessary. Soybean products with higher protein content are generated to meet the requirement. There are two types of enriched protein products are available commercially: soy protein concentrate (SPC) and soy protein isolate (SPI).

3.1.1 Soy protein concentrate

SPCs with higher protein contents have been produced by washing the soluble carbohydrates from soybean meal and insolubilization of protein at isoelectric point (4.8)
at low temperature. The washing process is commonly done by one of the following methods [28-30]: (1) Hot water wash, (2) aqueous alcohol (20-80%) wash, and (3) dilute mineral acid precipitation.

3.1.2 Soy protein isolate

SPI is another soy protein product. Processing for making SPI involves dissolution of protein from soymeal at high pH, collection of supernatant by centrifugation, and then precipitation of protein at the isoelectric point [31, 32]. SPI has significantly higher protein content (85-90%) than SPC and contains almost no water-insoluble carbohydrates. However, the process gives low protein recovery and yield, making SPI expensive [33]. The compositions of soybean meal SPC and SPI are given in the table 3.1.

Table 3.1: Composition of different soy protein products: soybean meal, soy protein concentrate (SPC) and soy protein isolate (SPI)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Soybean meal</th>
<th>SPC</th>
<th>SPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>As is mfb</td>
<td>52-54</td>
<td>56-59</td>
<td>62-69</td>
</tr>
<tr>
<td>Protein(N×6.25)</td>
<td>0.5-1.0</td>
<td>0.5-1.1</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>Soluble fiber</td>
<td>2</td>
<td>2.1-2.2</td>
<td>2-5</td>
</tr>
<tr>
<td>Insoluble fiber</td>
<td>16</td>
<td>17-17.6</td>
<td>13-18</td>
</tr>
<tr>
<td>Ash</td>
<td>5-6</td>
<td>5.4-6.5</td>
<td>3.8-6.2</td>
</tr>
<tr>
<td>Moisture</td>
<td>6-8</td>
<td>0</td>
<td>4-6</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>30-32</td>
<td>32-34</td>
<td>19-21</td>
</tr>
</tbody>
</table>
3.2 Comparison of soy protein with other available protein source

Although animal proteins are preferred feed for the livestock industry, the supply of animal protein is limited. Fish meal is considered one of the important animal protein source. Due to limited supply vegetable protein sources are considered as alternative. Among all available vegetable protein sources soy protein is considered better due to its well-balanced amino acid profile and protein content. The content of different amino acid for soybean protein concentrate and fishmeal is presented in figure 3.1 and the composition of different vegetable protein source are presented in table 3.2.

![Figure 3.1: Comparison of amino acid composition between Soy Protein Concentrate and fish meal.](image-url)
Table 3.2: Composition of different vegetable protein meal after oil extraction.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Dry matter (%)</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
<th>Lysine (%)</th>
<th>Methionine (%)</th>
<th>Cystine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>92</td>
<td>72.0</td>
<td>8.4</td>
<td>10.4</td>
<td>5.57</td>
<td>2.08</td>
<td>0.74</td>
</tr>
<tr>
<td>Barley</td>
<td>88</td>
<td>14.9</td>
<td>2.1</td>
<td>2.9</td>
<td>0.44</td>
<td>0.16</td>
<td>0.24</td>
</tr>
<tr>
<td>Canola</td>
<td>93</td>
<td>38.0</td>
<td>3.8</td>
<td>6.8</td>
<td>2.27</td>
<td>0.70</td>
<td>0.47</td>
</tr>
<tr>
<td>Corn</td>
<td>88</td>
<td>8.5</td>
<td>3.6</td>
<td>1.3</td>
<td>0.25</td>
<td>0.17</td>
<td>0.22</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>91</td>
<td>60.4</td>
<td>1.8</td>
<td>2.1</td>
<td>1.11</td>
<td>1.63</td>
<td>1.20</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>92</td>
<td>41.7</td>
<td>1.8</td>
<td>6.4</td>
<td>1.89</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>Lupin</td>
<td>89</td>
<td>39.2</td>
<td>10.3</td>
<td>2.8</td>
<td>1.40</td>
<td>0.27</td>
<td>0.51</td>
</tr>
<tr>
<td>Field peas</td>
<td>89</td>
<td>25.6</td>
<td>1.3</td>
<td>3.4</td>
<td>1.50</td>
<td>0.21</td>
<td>0.31</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>90</td>
<td>48.5</td>
<td>0.9</td>
<td>5.8</td>
<td>3.08</td>
<td>0.68</td>
<td>0.75</td>
</tr>
<tr>
<td>Soy protein conc.:</td>
<td>90</td>
<td>64.0</td>
<td>3.0</td>
<td>1.5</td>
<td>4.20</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Wheat</td>
<td>88</td>
<td>12.9</td>
<td>1.7</td>
<td>1.6</td>
<td>0.36</td>
<td>0.21</td>
<td>0.27</td>
</tr>
</tbody>
</table>

3.3 Concerns associated with protein products by current processing

Soy protein processing has to meet other nutritional requirements. Both heat treatment and alcohol wash causes denaturation of the protein, resulting in low protein dispersibility[34]. SPCs produced by these two methods also have very low Protein Dispersibility Index (PDI) values, in the range of 10-15%, indicating lower functionality of the products[35]. Dilute acid precipitation method can produce SPCs with acceptable PDI but SPCs produced by all three method has significant amount of indigestible carbohydrates which cause digestibility problems in monogastric animals.
Soy flour contains approximately 30-35% of carbohydrates, predominantly non-starch polysaccharides (NSPs) and oligosaccharides[36, 37]. By the above methods significant amounts of carbohydrates remain insoluble and are retained in the SPCs produced [38]. These carbohydrates cause lower digestibility when SPCs are included in the animal feed[39, 40]. Appreciable portions of oligosaccharides mainly raffinose and stachyose are also not removed as they may be trapped by diffusion limitation inside the solid matrix. Their concentrations are important considerations for soy protein products because humans and other monogastric animals lack the α-galactosidase enzyme necessary to hydrolyze the α-galactosyl linkages in their structures[41, 42]. After entering the lower intestinal tract, they are metabolized by bacteria and the intestinal gas produced causes considerable discomfort[43, 44]. Removal of these indigestible oligosaccharides can significantly increase the nutritional value of soy protein products[45, 46]. Another important consideration is the presence of non-functional indigestible fibers in the SPCs produced by the above methods. These fibers can bind with proteins and limit protein digestibility [47].

3.4 Enzyme based processing of soybean meal carbohydrate

Enzyme based processing of soybean meal can bring significant advantage by degrading carbohydrates and improving protein content. Although enzyme based processing of soybean meal is not well investigated before, recently there are few attempt made to use enzyme in soybean processing. However, the complex structure of soybean carbohydrate made it difficult to hydrolyze with single enzyme. Recent progress in
enzyme based process and the enzymes required to degrade all types of carbohydrates is soybean is described below.

3.4.1 Enzymes required to degrade the carbohydrates

Due to the complexity of the soy cell wall a number of enzyme activities are needed for complete degradation. Enzymes required for degrading different group of carbohydrates are described below.

3.4.1.1 Oligosaccharides degrading enzymes

Stachyose, raffinose and verbacose are galacto-oligosaccharides which consists of a terminal sucrose linked with 1 (raffinose), 2 (stachyose) or 3 (Verbacose) galactose monomers by $\alpha$-1,6 linkage and the bond between terminal sucrose and the galactose is $\alpha$-1,3[5]. These can be hydrolyzed to D-galactose and sucrose by the $\alpha$-galactosidase ($\alpha$-GAL) enzymes (figure 3.2) [48]
3.4.1.2 Cellulose and hemicellulose degrading enzymes

Cellulose can be hydrolyzed by cellulase which is an enzyme complex composed of three major classes of enzymes: endo-gluconases, exo-gluconases and β-glucosidases. Enzymatic degradation of cellulose to reducing sugar is accomplished by the synergistic action of these three enzymes which consists the cellulase adsorption onto the surface of the cellulose, the biodegradation of cellulose to reducing sugars and desorption of the
cellulase. Hemi-cellulases are comprised of mainly xylanases which can break down the β-1,4-linked xylan polymers.

3.4.1.3 Pectic polysaccharides degrading enzymes

Pectic polysaccharides degrading enzyme is a group of enzymes broadly known as pectinases. Based on their mode of action and substrate specificity within the pectin structure, it can be divided into several categories. Based on the substrate portion of the pectins, they can be divided in two types: (1) homogalacturonan degrading enzymes and (2) rhamnogalacturonan degrading enzymes. Each of these two types can be divided into three categories based on their mode of action: (a) esterase, (b) hydrolases and (c) lyases.

Homogalacturonan degrading enzymes are responsible for degrading the main backbone of the pectins: polygalacturonic acid chains. This group of enzymes include deesterifying enzymes, hydrolase and lyase. Subclass deesterifying enzyme mainly include pectin methyl esterase (PME) and pectin ethyle esterase (PAE) which catalyzes deesterification of methyle group and acetye group forming pectic acid and formate and acetate (figure 3.3). Subsequent degradation of polygalacturonic acid requires the action of this enzyme group before as polygacaturonase and pectate lyase need non-esterified substrates. Polymethylgalacturonases (PMG) and polygalacturonases (PG) are hydrolytic enzymes which catalyzes the hydrolysis of α-1,4-glycosidic bonds in pectin backbone forming methyl galacturonate and galacturonate (figure 3.3). Pectate lyase (PGL) and Pectin lyase (PL) are under the lyase group of enzyme which break the α-1,4-glycosidic bonds via transelimination mechanism forming unsaturated galacturonate.
Figure 3.3: Catalytic action of pectinesterase (PE) and Polymethylgalacturonases (PMG) and polygalacturonases (PG)

Although homogalacturonan degrading enzymes group breakdown the main backbone of pectins, complete degradation of the substrate still requires enzymes that hydrolyse rhamnogalacturonan region and the side chains. The group of enzymes which are involved in the degradation of this rhamnogalacturonan region are rhamnogalacturonan hydrolase (RG hydrolase), rhamnogalacturonan lyase, rhamnogalacturonan rhamnohydrolase (RG rhamnohydrolase), rhamnogalacturonan galactohydrolase. RG hydrolase and RG lyase catalyzes the degradation of rhamnogalacturonan chain producing oligogalacturonates and rhamnose. RG rhamnohydrolases catalyzes hydrolytic cleavage of the rhamnogalacturonan chain at the nonreducing end forming rhamnose. RG galactohydrolases cleave rhamnogalacturonan chain at the nonreducing end producing monogalacturonate. However, there are other accessory enzymes involved in degradations of the side chains are required for complete degradation of
rhamnogalacturonan which include α-arabinofuranosidase, endoarabinase, β-galactosidase and endogalactanase. The structures and mechanism of action of these enzymes are hardly understood and detail investigation are required to understand them more clearly.

3.5 Recent progress in enzyme based soybean processing

There have been only a few studies on enzymatic degradation of the carbohydrate in soybean meal. The factors that affect the enzyme hydrolysis efficiency are discussed in the following subsections:

3.5.1 Pretreatment of soybean meal

Fisher et al. [49] investigated the effects of pretreatment by heating (autoclaving) on the subsequent enzymatic extraction of soybean meal carbohydrate, measured in terms of the percentage of total carbohydrate being extracted (removed) from the meal. They focused on the pretreatment at 125°C for 15 min, at 2 moisture levels. At the high moisture level, i.e., with 200 g soybean meal dispersed in 1800 ml water, the heat pretreatment improved the enzymatic extractability of carbohydrate by about 10%. On the other hand, with soybean meal containing only 15% moisture, the heat pretreatment did not give appreciable improvements over the control of no pretreatment. The heat pretreatment in presence of sufficient water was thought to loosen the intertwined structures between protein and carbohydrate, increasing the accessibility of carbohydrate to enzyme. Other pretreatment methods such as toasting and extrusion have also been
investigated[49]. Almost all of these pretreatments could improve the subsequent enzymatic hydrolysis, but to different extents.

3.5.2 Enzyme accessibility to different types of carbohydrates in soybean meal

Ouhida et al. [19] studied the enzymatic extractability of the water-unextractable residues of soybean meal before and after the residues were sequentially fractionated using chelating agents, dilute alkali (KOH), 1 M KOH, and 4 M KOH. After each fractionation, the solid and soluble fractions were separately collected and treated with pectinase and/or cellulase for 12 h at 1% dry mass loading and 40 °C. After the enzymatic reaction, the dissolved components were analyzed. Their results indicated that the sequential fractionation significantly increased the overall enzymatic degradation of carbohydrate but the reaction sequence with different enzymes was important, presumably because the enzyme accessibility to different polysaccharides varied. Pectin and arabinan chains were more accessible while xylan and cellulose were quite inaccessible. Even for pectin, some parts were also believed to be protected against enzymatic degradation by their interaction with the protein in meal. This protection was indicated by the following results: 20% carbohydrate was converted to monomeric sugars by pectinase when the water-unextractable meal residues were first contacted with a sodium dodecyl sulfate (SDS) solution to extract the protein; this conversion was significantly higher than the 11.4% conversion obtained without the prior protein extraction by SDS. Cellulose, the least accessible component, was found to become accessible to cellulase only after the protein-pectin interactions were destroyed and the pectin was largely degraded.
3.5.3 Effect of different carbohydrate enzymes

Besides the about 40% soluble oligosaccharides, the insoluble carbohydrate in soybean meal mainly contains pectin, hemicellulose, and a small amount of cellulose. For effective hydrolysis of all types of carbohydrate, the enzyme mixture should at least contain pectinase, xylanase, cellulase and α-galactosidase. It is also desirable to optimize the ratios among these enzymatic activities. Opazo et al. [50] studied the reduction of soybean meal carbohydrate using a mixture of cellulase and α-galactosidase produced by a solid-state fermentation using mixed cellulolytic bacteria isolated from different environments. The bacterial mixture included those in the genera of Streptomycetes, Cohnella and Cellulosimicrobium. The enzyme was found to reduce 83% stachyose and 69% raffinose but only 24% insoluble polysaccharides. Comprised mainly of cellulase and α-galactosidase, the enzyme could not break down pectin. A recent study in this laboratory investigated the effects of different ratios of these enzymatic activities on the hydrolysis of soybean meal carbohydrate using enzymes produced by Aspergillus niger and T. reesei. Even in the most effective enzyme produced by an A. niger strain selected through a screening study, pectinase was still found to be the limiting component in the enzyme mixture[51].

3.5.4 Effect of protease enzymes

As protein is the major constituent in soybean meal, the effects of protein and protein degrading enzymes were investigated for improving the extractability and hydrolysis of carbohydrate in meal. Fisher et al. [49] proposed that protein and carbohydrate are present as a complex matrix in the soybean meal which makes the
accessibility of carbohydrase to the substrate more difficult. They showed that protease could improve the effectiveness of carbohydrate extraction by carbohydrase. They studied the hydrolysis of soybean meal by two protease enzymes, i.e., Alcalase from *Bacillus licheniformis* and Flavourzyme from *Aspergillus oryzae*, and two carbohydrase enzymes, i.e., Energex from *Aspergillus aculeatus* and Biofeed Plus from *Humicola insolens*. (All these were commercial enzymes from Novozymes A/S, Bagsvaerd, Denmark.) Hydrolysis with the mixture of all these four enzymes removed 94% of the protein in meal and increased the carbohydrate extractability to 75%, from 64% by the two carbohydrases alone. The results indicated that the soybean meal protein hinders the accessibility of carbohydrase enzymes to their substrates. Although protease can improve the carbohydrate extractability and hydrolysis, the associated protein loss needs to be carefully considered from the viewpoint of overall economics.

In this work we investigated the enzymatic hydrolysis of soybean meal carbohydrate by mixture of enzyme and how to improve the processing of soybean meal to produce soy protein concentrate and soy protein isolate.
4.1 Enzyme hydrolysis

Hydrolysis experiments were made in 250 ml Erlenmeyer flasks; each flask contained a suspension of soy flour in 40 ml enzyme solution (not 40 ml in total volume). 0.5 g/L NaN3 was added to prevent microbial contamination. The enzyme solution was prepared by diluting the supernatant of fermentation broth to achieve the particular enzyme/soy flour ratio studied. The soy flour amount was also added according to the specific loading studied. Mixture pH was adjusted with 5M HCl to the studied pH. Hydrolysis was allowed to take place for 48 h in a shaker operating at 250 rpm. Different shaker chamber temperatures were set in different hydrolysis batches to study the temperature effect. Periodical samples were taken and centrifuged to separate the solids and the liquid supernatant; both fractions were frozen for later analyses.

4.2 Analysis of carbohydrate

4.2.1 Reducing sugar analysis

Reducing sugar concentration was measured with the dinitrosalicylic (DNS) acid method [52]. This method is based on the principle that 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid in the presence of reducing sugar. The DNS
reagent was prepared by dissolving 10 g 3,5-dinitrosalicylic acid, 16 g NaOH and 300 g sodium potassium tartrate (Rochelle salt) in 1 L deionized water. 3 ml DNS reagent and 1 ml supernatant sample were mixed in a test tube and then heated in a boiling water bath for 5 min. Deionized water was added to make the total volume in the tube 25 ml. After being cooled to the ambient temperature, the reacted mixture was measured for absorbance at 550 nm in the spectrophotometer. The reducing sugar concentration was then determined using the absorbance value according to a calibration curve established with standard glucose solutions.

4.2.2 Total carbohydrate analysis

Total carbohydrate concentrations were measured using the phenol sulfuric acid colorimetric method [53]. This method is based on the principle that carbohydrate reacts with sulfuric acid to produce furfural derivatives, which then react with phenol to develop a characteristic color. First, 1 ml sample was mixed with 1 ml aqueous phenol solution (5% v/v) in a test tube. 5 ml concentrated sulfuric acid was then added to the mixture. After 10 min reaction, the tube content was vortexed for 30 s and allowed to cool to room temperature. A reference solution was prepared in identical manner except that the 1 ml sample was replaced by deionized water. Then the absorbance at 490 nm was measured against the reference solution. The phenol used was redistilled and the 5% phenol solution was prepared fresh for each batch of analysis. Total carbohydrate concentration was determined from the absorbance reading according to a calibration curve obtained with standard glucose solutions, following the same procedure as described above.
4.2.3 Individual sugar monomer analysis

For individual sugar concentrations, samples were filtered (0.2 µm, Millipore, Danvers, MA) and analyzed using a high pressure liquid chromatography system (HPLC Shimadzu) with a refractive index detector. A carbohydrate column (Supelcogel Column Pb, 300 x 7.8 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA) was used at 80ºC. The mobile phase was HPLC grade water (Fisher Scientific, Pittsburgh, PA) at a flow rate of 0.5 ml/min.

4.3 Enzyme activity assay

4.3.1 Cellulase

The cellulase assay used was modified from that reported by Ghose[54]. It was found to be most suitable for 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 procedure was as follows: (1) Cut Whatman No. 1 filter paper into pieces of 6 × 1 cm, ~ 50 mg/piece. Roll and insert a piece (1 cm in height) into a 25 mL tube. Add 1.4 mL 0.05 M sodium citrate buffer (pH 4.8) and 100 µL sample under analysis to the tube. The filter paper should be 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 50ºC for 1 h. (4) Add 3 mL regular DNS solution that consisted of 10 g/L 3,5-dinitrosalicylic acid, 16 g/L sodium hydroxide (NaOH) and 300 g/L sodium-potassium tartarate to each sample and blank to stop the enzyme reaction. (5) Incubate the DNS-added tubes in boiling water (100ºC) for 10 min. (6) Add deionized water to
make the total volume 25 mL, mix, and then measure the absorbance of reaction supernatant at 540 nm with spectrophotometer. Determine the amount (mg) of reducing sugar released, using the pre-establish calibration with pure glucose solutions of different concentrations as standards. Steps (4)-(6) are the common procedure for reducing sugar analysis by the DNS method [52]. The cellulase activity was then calculated using the following equation:

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

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

4.3.2 Xylanase

The method reported by Bailey et al. [55] was adopted for the xylanase assay. For best results, samples should be diluted to have xylanase activities in the range of 0.5-2 U/mL. The procedure was 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 test 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. And, add 100 µL test sample to the corresponding blank (to account for the potential turbidity introduced by
the sample). DNS analysis was then done to determine the amount (mg) of reducing sugar released, using D-xylose solutions as standards. Calculate the xylanase activity by the following equation:

\[
\text{Xylanase } \left( \frac{U}{mL} \right) = \frac{\text{xylose released (mg)}}{(5 \text{ min})(0.1 \text{ mL enzyme sample})} \times \frac{1 \text{ mmol}}{150.13 \text{ mg}} \times \frac{1000 \text{ mmol}}{1 \text{ mmol}} = 13.32 \times \text{xylose released (mg)}.
\]

4.3.3 Polygalacturonase & Pectinase

A method was developed in this laboratory [56] using assay condition of pH 4.8 and 50°C, which was the same condition used in the soy flour hydrolysis test in this study. Samples should be diluted to the suitable enzyme activity range of 0.3-0.7 U/mL. The procedure was similar to that described above for the xylanase assay, with four differences. First, the substrate solution/suspension was 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 were incubated at 50°C for 30 min (instead of 5 min as in the xylanase assay). Third, the DNS solution used did 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 was calculated according to the following equation:

\[
\text{Polygalacturonase } \left( \frac{U}{mL} \right) = 1.57 \times \text{galacturonic acid released (mg)}
\]
The pectinase assay was the same as that for polygalacturonase activity except that the substrate solution was prepared with citrus pectin (Sigma Aldrich, St. Louis, MO). Also, heating was necessary to prepare more homogeneous solution/suspension of the pectin substrate in citrate buffer [56].

4.3.4. α-Galactosidase

A method modified from that reported by Mukesh Kumar et al. [57] was used. Test samples should be diluted to have α-galactosidase activities of 0.05-0.2 U/mL. The procedure was as follows: (1) prepare the substrate solution by dissolving 0.033 g p-nitrophenyl-α-D-galactopyranoside (Sigma Aldrich, St. Louis, MO) in 100 mL 0.1 M sodium citrate buffer (pH 4.8); (2) mix 100 µL test sample with 900 µL substrate solution; (3) prepare the (enzyme-free) blank with only 900 µL substrate solution; (4) incubate 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 develop the color from released p-nitrophenol; (6) add 100 µL test sample to the blank; and (7) measure the absorbance at 405 nm. Calibration established with pure p-nitrophenol standards was used for quantitation of the enzyme-released p-nitrophenol. α-Galactosidase activity was calculated by the following equation:

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

The Kjeldahl method [58] was used to measure the nitrogen contents of solid samples. The nitrogen content was multiplied by 6.25 [59] to estimate the protein content. A 50 ml sample containing 10 to 200 mg/l protein was added to a flask and digested with 10 ml reagent containing 134 ml/l concentrated sulfuric acid, 134 g/l potassium sulfate and 7.3 g/l cupric sulfate. The digestion was carried out to completion, until the reaction mixture became a clear solution. Then 30 ml water and 10 ml of a distillation reagent containing 500 g/l NaOH and 25 g/l Na₂S₂O₃·5H₂O were added to the digested sample. This mixture was then distilled using a distillation unit (RapidStill 1, Labconco, Kansas city, MO) to produce ammonia gas, which was absorbed in a 0.1 N boric acid solution. Then the boric acid solution was titrated using a 0.1 N H₂SO₄ to find the nitrogen concentration in the sample.

4.5 Determination of moisture content and ash in biomass

Ash and fatty acid content measurements were done by the University of Missouri Agricultural Experimental Station. Standard NREL method [60] was used for ash content analysis; Soxhlet hexane extraction method was used to measure crude fat content[61].

4.6 Strain for arabitol production and maintenance

The yeast culture of D. hansenii SBP-1 (NRRL Y-7483) was obtained from USDA ARS culture collection (Peoria, IL).
4.7 Cultivation of *Debaryomyces hansenii*

Culture was maintained on agar plates containing 10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 20 g/L agar. Same medium without the agar was used for preparing the inocula for the initial shake flask studies. A loop of cells was transferred from an agar plate to 50 ml medium in a 250 ml Erlenmeyer flask covered with cheese cloth sandwiched cotton. The culture was grown at 30°C temperature in a shaker rotating at 250 rpm for 24 h. The inoculum thus prepared was added at 5% of the final culture volume in the subsequent studies.

4.8 Determination cell concentration

The supernatants were collected and frozen for analysis of sugars and produced arabitol concentrations. The cell pellets were washed twice with deionized water and then used for measurement of cell dry weights. Dry cell weights were determined by drying the cells at 100°C for overnight in a hot air oven (Binder-world, Bohemia, NY).

4.9 Glycerol, xylitol and arabitol concentration measurement

For arabitol, xylitol and glycerol concentrations, the samples were filtered through 0.2-µm Millipore filters(Danvers, MA) and analyzed using the high pressure liquid chromatography (HPLC Shimadzu) with a refractive index detector.A carbohydrate column (SupelcoColumn H, 250 x 4.6 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA) was used at room temperature. The mobile phase was 0.1% H3PO4 in water at a flow rate of 0.14 ml/min.
4.10 Strains for studying effect of arabitol on growth and acid production

*S. mutans* (ATCC 10449), *S. salivarius* (NRRL B-3714), *S. sobrinus* QMZ 176 (NRRL B-4468), *L. acidophilus* (NRRL B-4495), and *L. fermentum* (NRRL B-1840) were used in this study. *S. mutans* was purchased from American Type Culture Collection (ATCC). The other four cultures were provided by USDA Agricultural Research Service (ARS) Culture Collection (Peoria, IL). The bacteria were maintained by weekly transfers on agar plates of trypticase yeast extract medium (Sigma Aldrich, St Louis, MO) containing 0.5% glucose and 1.5% agar.

4.11 Growth medium and culture conditions

All five strains of bacteria were cultured in the Brain Heart Infusion (BHI) Medium (Sigma Aldrich, St. Louis, MO) containing (in 1 liter solution) 17.5 g Calf Brain-Beef Heart Infusion, 10 g pancreatic digest of gelatin, 3 g NaCl, 2.5 g dipotassium phosphate (K$_2$HPO$_4$), 1 g monopotassium phosphate (KH$_2$PO$_4$) and 10 g glucose (Fisher Scientific, Hampton, NH). Xylitol and arabitol solutions were autoclaved separately and added to the sterile BHI medium to give 1% concentration. Control systems were prepared using same medium without any xylitol or arabitol. Cells were grown in 25 ml medium in a 125 ml Erlenmeyer flask inoculated with 5% (v/v) seed culture. (The seed culture was prepared by inoculating a single colony of cells from an agar plate into 25 ml BHI medium and incubating it overnight at 37 °C anaerobically.) Cultures were flushed with N$_2$ gas to make them anaerobic at the beginning and each time after collection of sample and closed by rubber stopper and incubated at 37 °C for 36 h in a shaker (Thermo
Scientific MaxQ 5000 Incubating/Refrigerating Floor Shaker, Asheville, NC) at 250 rpm.

Samples were taken periodically for analysis.

4.12 Growth profile and acid production monitoring

Growth of the bacteria was monitored by measuring absorbance at 660 nm\(^{23}\) (\(A_{660}\)) by a UV/VIS spectrophotometer (Model UV-1601, Shimadzu Corporation, Columbia, MD). Samples were diluted 10 times to ensure the absorbance value in the linear range and then shaken properly before placing in the spectrophotometer to get representative cell concentration reading. The pH readings of samples were taken to indicate acid production during the anaerobic growth of oral bacteria. All experiments were done in triplicate and the average values are reported.

4.13 Substrate consumption profile

For HPLC analysis the samples were filtered through 0.2-µm Millipore filters (Danvers, MA) and then analyzed using the HPLC system (LC 10AT, Shimadzu) with a refractive index detector. A carbohydrate column (Supelco Column H, 250 x 4.6 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA) was used at room temperature. The mobile phase was 0.1% \(\text{H}_3\text{PO}_4\) in HPLC grade water (VWR) at a flow rate of 0.14 ml/min.
CHAPTER V

OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF SOY FLOUR CARBOHYDRATE USING ENZYME PRODUCED BY ASPERGILLUS NIGER AND TRICHODERMA REESEI

5.1 Introduction

Soybean is considered as the best vegetable protein source among possible plant feed stuffs available, having well balanced and relatively constant amino acid profiles[2, 62]. It is used as a protein supplement for animal feed[63, 64] and fish meal alternative[65, 66]. Soy protein also plays an important role in reducing the risk of coronary heart disease [67-69] and this claim has been approved by the US Food and Drug Administration (FDA). These factors have attracted increasing interest in soy protein based foods and as supplement to animal protein. Soy flour, residue after oil extraction, is the main source of the soy protein. Besides protein (approximately 50 wt%), soy flour contains 30-35 wt% carbohydrate[70]. Despite the high content in soy flour, soy carbohydrate has not yet drawn much attention for value-added uses, such as the use as a major fermentation substrate. Moreover, the presence of carbohydrate can reduce the value of soy flour and soy protein product by two reasons: First, it lowers the protein content of soy flour, potentially to a level not high enough for use in certain animal feed [66, 71]. Second, the carbohydrate can raise concerns about the indigestibility of soy
flour-based animal feed [38, 72]. So it is desired to remove the carbohydrate for increasing the value of soy protein [73]. Currently, the commercial soy protein concentrate (SPC) is produced by removing the soluble carbohydrate from soy flour using an acid or alcohol wash process [30, 74]. This increases the protein content but all the insoluble carbohydrate (oligo- and poly-saccharides) still remains. Thus prepared SPC has been reported to cause indigestibility when fed to animal or fish [75, 76]. Among the soy oligosaccharides, stachyose and raffinose cannot be digested by the endogenous digestive enzymes of animal and fish [46, 77]. So, to increase the value of soy protein, it is important to reduce the indigestible carbohydrate components, more than to wash away the already soluble components [41, 78]. Further, if soy flour carbohydrate can be largely hydrolyzed to readily fermentable monosaccharides and separated from the protein, the hydrolysate can be collected and used as valuable fermentation feedstock for various bioproducts.

Soy flour carbohydrate is composed of approximately 10% sugars (mostly sucrose, raffinose and stachyose) and 20-25% non-starch polysaccharides (NSPs) [36, 79, 80]. NSPs are cellulose, hemicellulose and pectin polysaccharides. Microbial enzymes, particularly those from *Aspergillus niger* and *Trichoderma reesei*, have been used for hydrolyzing polysaccharides [81-84], but not for the complex carbohydrate present in the ill-defined composite with a large amount of various proteins in the soy flour. It was hypothesized in this study that if an effective enzyme mixture can be produced and used to hydrolyze the complex insoluble carbohydrate in soy flour while keeping the soy protein intact and insoluble, the hydrolyzed carbohydrate becomes soluble in the liquid hydrolysate and can be easily separated from the solid protein. The SPC produced by this
process can have a higher protein content than that by the existent method and have no indigestibility concern. Degradation of pectic substances also helps to reduce the viscosity of soy flour dispersion, making the SPC handing and processing easier.

Enzymatic hydrolysis efficiency largely depends on the reaction condition[85]. Temperature has a significant effect on enzymatic hydrolysis of polysaccharides; variation of 1 to 5 °C may change the reaction rate appreciably [86]. Generally the activity of enzyme increases with the rise of temperature till reaching the maximum level and then declines abruptly with further temperature increase, due to enzyme denaturation. pH is another important factor. Enzymes are amphoteric, containing both acidic and basic groups. By changing the charges of these groups [87], pH affects the intramolecular interaction forces in the enzymes, resulting in potential modification of the activity and shape of the active sites. There is an optimum pH range for each enzyme.

Substrate (soy flour) loading is yet another processing factor to consider. High loading is desirable from the viewpoint of energy and economic viability. Using a higher loading can raise the final concentration of soluble carbohydrate in the hydrolysate. Less water needs to be vaporized for concentrating or drying the hydrolysate for the subsequent uses, for example, as fermentation substrate. Using a higher loading also (1) helps to decrease the capital cost because a smaller reactor volume is needed for processing the same amount of soy flour, (2) lowers operating costs by consuming less heating/drying energy for the reactor and the product streams, (3) decreases the downstream separation/purification/processing costs with the higher product concentration, and (4) incurs lower wastewater treatment costs. However, there are limitations associated with high substrate loadings during hydrolysis. The high solid
concentrations can cause inadequate mixing or excessive mixing energy consumption in
the reactors because the slurry viscosity increases abruptly at higher solid concentrations
[88, 89]. At very low water contents, the transport of enzymes, reaction intermediates and
end products may all be affected.

In this study the hydrolysis of soy flour carbohydrate by the enzyme broths
produced by \textit{A. niger} and \textit{T. reesei} fermentation was investigated. The objective is to
identify the optimum hydrolysis condition for four factors: pH, temperature, enzyme-to-
soy flour ratio, and soy flour loading. Past research indicated that these factors often
interact with one another[90]. This study was designed to allow statistical analysis using
the response surface methodology (RSM). RSM is a collection of mathematical and
statistical techniques for the modeling and optimization of multiple variables [91-93].
This method can be used to determine the optimum process conditions for desirable
response [94]. A central composite design (CCD) is employed to fit an empirical, second-
order polynomial equation with a minimum number of experiments and, accordingly, to
determine the combination of variables for the optimal process response. In this study the
conversions (\%) of soy flour carbohydrate to soluble carbohydrate in hydrolysate,
measured as reducing sugar and total carbohydrate, respectively, are the responses to be
optimized.

5.2 Materials and Methods

5.2.1 Materials and equipment

Defatted soy flour (7B soyflour, product code: 063130) and soy hulls (product
code: 062611) were obtained from Archer Daniel Midland (Decatur, IL, USA). Water
used in the hydrolysis was Milli-Q water (18.2 MΩ cm at 25 °C; Milli-Q Direct 8, Millipore S.A.S., Molsheim, France). (NH₄)₂SO₄ (granular), KH₂PO₄ (99% purity), HCl (concentrated acid, 37.4%) and NaOH (98.8%) were purchased from Fisher Scientific (Waltham, MA, USA). Protease peptone (from meat, type I, for microbiology), MgSO₄·7H₂O (99%), MnSO₄·4H₂O (99%), ZnSO₄·7H₂O (ACS reagent grade), CoCl₂·6H₂O, FeSO₄·7H₂O (reagent grade), CaCl₂·2H₂O (reagent grade), Urea (98%), NaN₃ (>99%) and dinitrosalicylic acid (DNS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A. niger (NRRL 341) and T. reesei Rut-C30 (NRRL 3469) were obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. A 3 L Bioflo110 fermentor (New Brunswick Scientific Co., Edison, NJ) was used for enzyme production by fermentation. Absorbance was measured using a UV/Vis spectrophotometer (UV-1601, Shimadzu Corporation, Columbia, MD). The hydrolysis experiments were conducted in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating floor shaker, Ashville, NC). The centrifuge used was Sorvall Legend X1R from Thermo Scientific (Waltham, MA).

5.2.2 Enzymes

Fungal fermentation to produce enzyme broth was made with 1 L fresh medium containing 20 g/L soy hulls, 1 g/L proteose peptone, 1.4 g/L ammonium sulfate ((NH₄)₂SO₄), 0.3 g/L urea, 0.4 g/L calcium chloride (CaCl₂·2H₂O), 2 g/L monopotassium phosphate (KH₂PO₄), 0.3 g/L magnesium sulfate heptahydrate (MgSO₄·7H₂O), 5 mg/L FeSO₄·7H₂O, 1.6 mg/L MnSO₄·4H₂O, 1.4 mg/L ZnSO₄·7H₂O and 20 mg/L CoCl₂·6H₂O [95]. With an initial pH of 6.7, the medium was inoculated with a pre-grown A. niger or
T. reesei culture at an initial cell concentration of approximately 0.1 g/L. Temperature and agitation were maintained at 20 °C and 300-400 RPM. Dissolved oxygen concentration (DO) was maintained above 20% air saturation by automatic supplementation of pure oxygen as needed. pH was controlled at 6.0 ± 0.1 by automatic addition of 1 M NaOH or HCl. The fermentation was stopped after 5 days when the enzyme production rate decreased significantly. Supernatant was collected by centrifuging the fermentation broth at 8000 rpm (7440 g) for 10 min.

5.2.3 Enzyme hydrolysis

Hydrolysis experiments were made in 250 ml Erlenmeyer flasks; each contained a suspension of soy flour in 40 ml enzyme solution (with 0.5 g/L NaN₃ to prevent microbial contamination). The enzyme solution was prepared by diluting the supernatant of fermentation broth to achieve the particular enzyme/soy flour ratio studied. The soy flour amount was also added according to a specific loading studied. Mixture pH was adjusted with 5M HCl to the studied pH. Hydrolysis was allowed to take place for 48 h in a shaker operating at 250 rpm. Different shaker chamber temperatures were set in different hydrolysis batches to study the temperature effect. Periodical samples were taken and centrifuged to separate the solids and the liquid supernatant; both fractions were immediately frozen for further analyses. The factors investigated were in the following ranges: soy flour concentration, 5-25% w/v; enzyme/soy flour ratio, 0.1-2 ml/g; temperature, 40-60°C; and pH, 3.2-6.4.
5.2.4 Analytical Methods

The supernatants collected at the end of the hydrolysis were analyzed for concentrations of reducing sugar and total carbohydrate. Reducing sugar was measured with the dinitrosalicylic (DNS) acid method[52]. This method is based on the principle that 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid in the presence of reducing sugar. The DNS reagent was prepared by dissolving 10 g 3,5-dinitrosalicylic acid, 16 g NaOH and 300 g sodium potassium tartrate (Rochelle salt) in 1 L deionized water. Three (3) ml DNS solution and 1 ml supernatant sample were mixed in a test tube and then heated in a boiling water bath for 5 min. Deionized water was added to each tube to make the total volume 25 ml. After being cooled to the ambient temperature, the reacted mixture was measured for absorbance at 550 nm in the spectrophotometer. Reducing sugar concentration was determined according to a calibration curve obtained with standard glucose solutions. Total carbohydrate concentrations were measured using the phenol sulfuric acid colorimetric method[53]. This method is based on the principle that carbohydrate reacts with sulfuric acid to produce furfural derivatives, which then react with phenol to develop a characteristic color. First, 1 ml sample was mixed with 1 ml aqueous phenol solution (5% v/v) in a test tube. Five ml concentrated sulfuric acid was then added to the mixture. After 10 min reaction with mixing, the tube content was vortexed for 30 s and allowed to cool to room temperature. A reference solution was prepared in identical manner except that the 1 ml sample was replaced by deionized water. Then the absorbance at 490 nm was measured against the reference solution. The phenol used was redistilled and the 5% phenol solution was prepared fresh for each batch of analysis. Total carbohydrate concentration was determined according to a calibration
curve obtained with standard glucose solutions, following the same procedure as described above.

5.2.5 Experimental Design

Response surface methodology (RSM) was used for modeling and analyzing the enzymatic hydrolysis outcomes by the two enzyme broths used. Reducing sugar and total carbohydrate conversions (%) were the two separate responses of interest. pH, temperature, enzyme/soy flour ratio, and soy flour loading were the independent variables. Each variable was studied at five levels (-α, -1, 0, 1, +α) as listed in Table 5.1. Central composite design (CCD) of four independent factors was used as the experimental design approach. As summarized in Table 5.2, a total of 30 experiments were done, including 16 trials for factorial design, 8 trials for axial points, and 6 trials for replication of the central point. All experiments were done in triplicate and the average values of reducing sugar and total carbohydrate conversions were taken as the responses. Total carbohydrate and reducing sugar conversions were calculated as follows:

\[
\text{Reducing sugar conversion} \, (\%) = \frac{R_s}{T_c} \times 100
\]

\[
\text{Total carbohydrate conversion} \, (\%) = \frac{T_s}{T_c} \times 100
\]

Where \(R_s\) and \(T_s\) are concentrations of the reducing sugar and total carbohydrate, respectively, in the hydrolysate supernatant, and \(T_c\) is the initial total carbohydrate concentration introduced with the soy flour. The influence of variables on response was analyzed using the multiple regression method, which allows the development of a model
equation between one dependent response variable and multiple independent variables by fitting the experimental data to the equation. In this case, two quadratic polynomial equations in the form of equation (1) were proposed.

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon \ldots \ldots \ldots (1) \]

Where \( Y \) is the result of response from an experiment, either the reducing sugar or total carbohydrate conversion; \( \beta_0 \) is a general constant coefficient; \( \beta_i, \beta_{ii}, \beta_{ij} \) are coefficients for the linear, quadratic and interaction effects, respectively; \( x_i \) is a code level for an independent variable (i.e., pH, temperature, enzyme/soy flour ratio, or soy flour concentration); and \( \varepsilon \) is a random error. Analysis of variance was conducted to evaluate the effects of the variables and their potential interactions. Coefficients of the developed model were analyzed for significance and the insignificant terms were eliminated from the model. The reduced model was adjusted after all the insignificant terms were eliminated. The lack-of-fit test was used to determine whether the constructed models were adequate to describe the observed data. Response surface plots and the corresponding contour plots were then constructed according to the developed models to visualize the relationship between the responses and variables. Each response surface plot shows the relative effect of two variables on the conversion to soluble reducing sugar or total carbohydrate while other factors were kept constant at code level 0. The statistical software package Design-Expert 9 (Stat-Ease, Inc., Minneapolis, MN) was used for the aforementioned regression analysis of experimental data and for generating response surface plots.
Table 5.1: Code levels and actual values used for each of the four variables in the central composite experimental design for optimization of operating conditions of soy flour carbohydrates

<table>
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<th>Independent Variable</th>
<th>Unit</th>
<th>Symbol</th>
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</thead>
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<td>-α</td>
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<tr>
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<td>pH</td>
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<tr>
<td>Temperature</td>
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<tr>
<td>Enzyme/Soy flour</td>
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<tr>
<td>Soy flour loading</td>
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</table>
Table 5.2: Experimental design matrix for the four independent variables and two response variables, the reducing sugar and total carbohydrate conversions ($Y_{RS}$ and $Y_{TC}$) obtained after 48 h hydrolysis

<table>
<thead>
<tr>
<th>Run</th>
<th>Factors</th>
<th>A. niger</th>
<th>T. reesei</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH (°C)</td>
<td>T (°C)</td>
<td>E (ml/g)</td>
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5.3 Results & Discussion

5.3.1 Hydrolysis by *A. niger* Enzyme Broth

Results of soy flour carbohydrate conversions obtained from the 30 hydrolysis experiments made with the *A. niger* enzyme broth are shown in Table 5.2. ANOVA analysis of the initial quadratic models, prior to removal of the insignificant terms, for the reducing sugar and total carbohydrate conversions are used to determine the significant factors affecting the response. Summary of the ANOVA table for both reducing sugar and total carbohydrate conversion for both *A. niger* and *T. reesei* are shown in table 5.3. The large F values from the Fisher F-test (i.e., 34.39 and 40.01, respectively, for the models for reducing sugar and total carbohydrate conversions) and the very low \( p \) values, \(< 0.0001\), for both the reducing sugar and total carbohydrate models demonstrate the high significance of the models developed. The goodness of fit for the models is checked by the determination coefficient (\( R^2 \)) (table 3). In case of the reducing sugar model, the \( R^2 \) value is 0.987 and the adjusted \( R^2 \) value is 0.905, indicating that the model is statistically significant. Similarly, the \( R^2 \) value of 0.981 and adjusted \( R^2 \) value of 0.913 for the total carbohydrate model also confirm the statistical significance of the model. The variations of these conversions are therefore conclusively attributable to the variations of the four independent variables at more than 95% confidence level. The lack-of-fit analysis shows low F values (4.14 for reducing sugar and 3.13 for total carbohydrate) and high \( p \) values (0.13 for reducing sugar and 0.19 for total carbohydrate, both \( > 0.05 \)); thus, the lack-of-fit hypothesis is rejected. Figure 5.1 shows the correlation plots between the model predicted and experimental values; Figure 5.1(A) for the reducing sugar and total carbohydrate
conversion by *A. niger* enzyme broth and Figure 5.1(B) for the *T. reesei* enzyme broth.

Both plots exhibit satisfactory regression fitting.

Table 5.3: Goodness of fit and the lack of fit test analysis of the experimental design model from ANOVA analysis.

<table>
<thead>
<tr>
<th>Enzyme/conversion response</th>
<th>Model</th>
<th>Lack of fit test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted R²</td>
<td>F</td>
</tr>
<tr>
<td><em>A. niger</em> /reducing sugar</td>
<td>0.905</td>
<td>34.39</td>
</tr>
<tr>
<td><em>A. niger</em> /total carbohydrate</td>
<td>0.913</td>
<td>40.01</td>
</tr>
<tr>
<td><em>T. reesei</em> /reducing sugar</td>
<td>0.868</td>
<td>20.40</td>
</tr>
<tr>
<td><em>T. reesei</em> /total carbohydrate</td>
<td>0.857</td>
<td>11.82</td>
</tr>
</tbody>
</table>
Figure 5.1: Correlation of the model predicted and experimental conversions of soy flour carbohydrate to soluble reducing sugar and total carbohydrate using A. niger (a) and T. reesei (b) enzyme broth
The significance of model terms were also determined on the basis of their $p$ values. Significant effect of each term at more than 95% confidence level is indicated by a $p$ value of less than 0.05, together with a relatively high F value. Accordingly, for the model of reducing sugar conversion, the linear terms of all four independent variables, three quadratic terms ($pH^2$, $T^2$, and $E^2$) and two interaction terms ($T \times pH$ and $pH \times E$) were found to affect the response (conversion) more significantly; for the model of total carbohydrate conversion, the linear terms of all four independent variables, two quadratic terms ($pH^2$ and $T^2$) and two interaction terms ($pH \times E$ and $pH \times S$) were identified to be significant. The insignificant terms were removed from the model. The resultant reduced quadratic models, in terms of coded variables are given in equation (2) and (3) and after conversion of the coded levels to actual variable values, are given in Equations (4) and (5) for the conversions to soluble reducing sugar and total carbohydrate, respectively.

Reducing sugar conversion (%) 

$$= 61.89 + 5.35 \, pH - 0.64 \, T + 18.85 \, E - 4.29 \, S - 9.73 \, (pH \times T)$$
$$+ 8.06 \, (pH \times E) - 11.25 \, pH^2 - 6.78 \, T^2 - 4.02 \, E^2$$

Total carbohydrate conversion (%) 

$$= 68.47 + 1.86 \, pH - 0.076 \, T + 8.26 \, E - 4.63 \, S - 4.02 \, (pH \times T)$$
$$- 3.17 \, (pH \times E) - 4.54 \, (pH \times S) - 4.74 \, pH^2 - 4.65 \, T^2$$
$$- 1.34 \, E^2 \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots
**Total carbohydrate conversion (%)**

\[
= -903.7 + 142.24 \, pH + 23.4 \, T + 36.8 \, E + 0.22 \, S - 1.05 \, (pH \times T) \\
- 3.37 \, (pH \times E) - 0.056 \, (pH \times S) - 7.41 \, pH^2 - 0.186 \, T^2 \\
- 2.88 \, E^2 \ldots \ldots \ldots \ldots \ldots \ldots (5)
\]

5.3.2 Hydrolysis by *T. reesei* Enzyme Broth

ANOVA analysis showed that the quadratic models are also statistically meaningful for describing the hydrolysis achieved with the *T. reesei* enzyme broth, with high F values (11.82 and 20.40 for total carbohydrate and reducing sugar conversion model, respectively), low \(p\) values (< 0.0001 for both models), and close-to-1 adjusted R\(^2\) values (0.857 for total carbohydrate model and 0.868 for reducing sugar model). The high \(p\) values for the lack-of-fit test (0.85 for the total carbohydrate model and 0.40 for the reducing sugar model) confirmed the statistically significant fitting of experimental results to the models. The reduced models, after removal of insignificant terms, in terms of coded variables are presented in equations (6) and (7) and after translation of code levels to actual variable values, are given in equations (8) and (9):

**Reducing sugar conversion (%)**

\[
= 28.94 + 1.77 \, pH + 1.25 \, T + 4.43 \, C - 0.23 \, S - 3.38 \, (pH \times T) \\
+ 3.45 \, (pH \times E) + 1.92 \, (T \times E) + 0.96 \, (T \times S) - 4.35 \, pH^2 - 2.77 \, T^2 \\
- 3.92 \, E^2 \ldots \ldots \ldots \ldots \ldots \ldots (6)
\]

**Total carbohydrate conversion (%)**

\[
= 62.16 + 3.09 \, pH + 1.52 \, T + 4.31 \, E - 2.07 \, S - 2.15 \, (pH \times E) \\
- 3.15 \, pH^2 - 1.74 \, T^2 - 3.23 \, E^2 \ldots \ldots \ldots \ldots \ldots \ldots (7)
\]

65
Reducing sugar conversion (\%) 

\[
= -609 + 107 \, pH + 16.1 \, T - 41.65 \, C - 0.14 \, S - 0.9 \,(pH \times T) \\
+ 4.75 \,(pH \times E) + 0.53 \,(T \times E) + 0.0028 \,(T \times S) - 6.73 \,pH^2 \\
- 0.127 \,T^2 - 0.96 \,E^2 \text{ ............... (8)}
\]

Total carbohydrate conversion (\%) 

\[
= -286.3 + 54.1 \, pH + 7.24 \, T + 25.6 \, E + 0.02 \, S - 2.82 \,(pH \times E) \\
- 4.92 \,pH^2 - 0.07 \,T^2 - 1.5 \,E^2 \text{ ............... (9)}
\]

5.3.3 Model Predicted Maximum Conversions and Optimal Operating Conditions

The above model equations were used to determine the operating conditions, within the tested ranges of factor values, for achieving the maximum total carbohydrate and reducing sugar conversions. A numerical optimization method available with the software package Design-Expert 9 (Stat-Ease, Inc., Minneapolis, MN) was used. The optimal conditions and the maximum conversions achieved are summarized in Table 5.4. All maximum conversions were achieved at the highest enzyme/soy flour ratio (2 ml/g) and the lowest soy flour loading (150 g/L) tested. This finding is not surprising because the use of more enzyme is expected to enhance the reaction rate and completeness while a lower soy flour loading gives lower system viscosity and better mixing. There is also a report suggesting lower enzyme binding capacities at higher substrate concentrations [96]. The optimal pH and temperatures for maximum reducing sugar conversions were only slightly different from the conditions for maximum total carbohydrate conversions; and those optimal conditions also did not vary much when different enzyme broths were used. Within the tested ranges of variables, for the hydrolysis with \textit{A. niger} enzyme broth,
a maximum total carbohydrate conversion of 79.2% was predicted to be achieved at pH 4.79 and 50.5 °C, while the maximum reducing sugar conversion predicted was about 74.5% at pH 5.17 and 48.3 °C. For the hydrolysis with *T. reesei* enzyme broth, the maximum total carbohydrate conversion achievable was 68.9% under the condition of pH 4.77 and 50.1 °C while the maximum reducing sugar conversion achievable was only about 33.7% at pH 5.12 and 51.9 °C. These values confirm the observations made with Figure 5.1 that the *A. niger* enzyme broth was more effective than the *T. reesei* enzyme broth for hydrolysis of soy flour carbohydrate. The *T. reesei* broth gave substantially lower maximum conversions to both reducing sugar and total carbohydrate than the *A. niger* broth did. Furthermore, while the *A. niger* broth gave very similar maximum conversions to total carbohydrate and reducing sugar (79.2% versus 74.5%), the two maximum conversions differed very much (68.9% versus 33.7%) with the *T. reesei* broth. The large difference again indicates that the *T. reesei* broth was particularly deficient in the enzyme components required to hydrolyze the soluble oligomeric carbohydrate to monomers. In addition to the identification of optimal conditions in the tested variable ranges, it is important to know the sensitivity of these conversions to the variables near the optimal conditions. These dependencies and sensitivities are described in the following sections.
Table 5.4: Optimal operating conditions for maximizing reducing sugar (RS) and total carbohydrate (TC) conversions using both \textit{A. niger} and \textit{T. reesei} enzyme broth.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
<th>Optimum Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>\textit{A. niger}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RS</td>
</tr>
<tr>
<td>pH</td>
<td>3.2-6.4</td>
<td>5.17</td>
</tr>
<tr>
<td>Temperature (T, °C)</td>
<td>40-60</td>
<td>48.3</td>
</tr>
<tr>
<td>Enzyme/SF (E, ml/g)</td>
<td>0-2</td>
<td>2.0</td>
</tr>
<tr>
<td>SF loading (S, g/L)</td>
<td>150-350</td>
<td>150</td>
</tr>
<tr>
<td>Maximum conversion (%)</td>
<td></td>
<td>74.5</td>
</tr>
</tbody>
</table>

5.3.4 Model predicted single-factor effects

Effects of the single factors on reducing sugar and total carbohydrate conversions using, respectively, \textit{A. niger} and \textit{T. reesei} enzyme broths are presented in Figure 5.2: (a) pH effect, (b) temperature effect, (c) effect of enzyme-to-soy flour ratio, and (d) soy flour loading effect; the reference conditions used, when the factors are not varied, are pH at 4.8, temperature at 50 °C, enzyme-to-soy flour ratio at 1.5 ml/g, and soy flour loading at 200 g/L. For all 4 single-factor effects, the conversions by the \textit{A. niger} broth were consistently more responsive to the changes of these operating conditions, as compared to the conversions by the \textit{T. reesei} broth.

pH was found to have quadratic effects on the conversions. The enzyme broths from the fungal fermentations, as described earlier, contain the mixtures of pectic polysaccharide degrading enzymes, cellulolytic enzymes and oligosaccharide degrading enzymes. With these enzyme mixtures, the hydrolysis outcome depends on the
effectiveness of all the enzyme groups involved at the specific condition. Some previous reports indicated that the optimal pH are in the range of 5 – 6 for pectinase enzymes [97], 4.5 – 4.8 for cellulase and hemicellulase [98], and 4.8 – 5.0 for α-galactosidase [99]. As described in the previous section, in all cases examined in this current study the optimum pH values were in the range of 4.8 to 5.2. In Figure 5.2, the reducing sugar conversion by the A. niger broth is shown to be much more sensitive to pH change than the other three conversions. This suggests that the enzyme, such as α-galactosidase, responsible for degrading soluble oligosaccharides to smaller sugars is more sensitive to pH away from its optimum value. This is consistent with Awan et al.’s report that suggests α-galactosidase from A. niger has optimum activity at pH 4.8 to 5.0 and the activity is sensitive to pH changes beyond this range[100].

As shown in Figure 5.2 (b), temperature also had quadratic effects on the conversions. The optimum temperatures for both A. niger and T. reesei broths were in the similar range of 48 to 52 °C. For both broths, the reducing sugar conversions were slightly more temperature sensitive than their corresponding total carbohydrate conversions. According to Figure 5.2 (c), all of the 4 conversions increased clearly with increasing enzyme-to-soy flour ratio (E) in the range evaluated. The increasing trends were clearly higher with the A. niger broth than with the T. reesei broth. The trends indicate that significantly higher conversions can be achieved by using higher enzyme-to-soy flour ratios than the maximum of 2 ml/g included here, particularly with the A. niger broth. To confirm this, additional experiments were made at 3 ml/g and 4 ml/g ratios with A. niger broth keeping other conditions at reference conditions. The maximum conversions were found to reach 86% for the total carbohydrate and 83% for the reducing
sugar. The experimental results showed that the overly increased enzyme-to-soy flour ratio does not increase the conversion linearly.

Shown in Figure 5.2 (d) are the model-predicted effects of soy flour loading on the conversions. For hydrolysis using the *A. niger* enzyme broth, both the conversions to soluble total carbohydrate and reducing sugar are predicted to decrease linearly with increasing soy flour loading. Total carbohydrate and reducing sugar conversions at 350 g/L soy flour loading is about 14 and 9% lower respectively than the corresponding conversion obtained at 150 g/L soy flour loading. The essentially proportional decreases in the two conversions suggested that the negative effect is mostly on the release of soluble total carbohydrate, because whatever % of solid substrate becomes soluble, the enzyme components (such as α-galactosidase) acting on the soluble oligosaccharides can break them down to smaller sugar molecules (resulting in the proportional changes of the two conversions). Conceptually, the negative effect of increasing soy flour loading is more probably caused by the inhibited interactions between the solid substrate and the corresponding enzyme components. One of these inhibited interactions may be the reduced accessibility of enzyme to the solid substrate reactive sites that are increasingly blocked by the surrounding solids as the substrate loading increases [96]. For hydrolysis using the *T. reesei* enzyme broth, the conversion to total soluble carbohydrate also decreases linearly with increasing soy flour loading. On the other hand, the change of soy flour loading had an insignificant effect on the conversion to soluble reducing sugar by the *T. reesei* broth. This probably reflects again that the *T. reesei* broth is highly deficient in α-galactosidase and other enzyme components to hydrolyze the soluble oligosaccharides. In any case, even though high substrate loading negatively impacts the
hydrolysis yield, it can be beneficial for the process economics because of two reasons. First, high substrate loading reduces the working volume and potentially the operation cost. Second, the hydrolysate produced with a high substrate loading can have a higher carbohydrate concentration. This hydrolysate requires no or less costly concentrating step prior to its use as fermentation substrate. Future analysis on overall process economics is necessary to address these compromising effects of the soy flour loading level.
Figure 5.2: Effect of single factor (pH, temperature, enzyme/soy flour ratio, and soy flour loading) on the conversion of soy flour carbohydrate to soluble reducing sugar using *A. niger* enzyme broth; for each plot, the other three factors set at their optimal conditions: pH 4.8, Temperature 50°C, Enzyme to soy flour ratio 2 ml/g, soy flour loading 150 g/l.
5.3.5 Response surface plots

The three dimensional response surface plots and their corresponding two dimensional contour plots were drawn for the reducing sugar and total carbohydrate conversion to show the interaction effect of two factors while the other factors were kept constant (Figures 5.3-5.5). The graphical representation visually highlights the regression equation. A flat response surface indicates that the enzymatic hydrolysis of soy flour can tolerate variation in the processing conditions without responses being seriously affected. On the other hand a pointed surface indicates that responses would be highly sensitive to the processing conditions used.

5.3.5.1 Hydrolysis with *A. niger* enzyme broth

From comparison of the generated surfaces, both the conversions to reducing sugar and total carbohydrate were found to be more sensitive to the enzyme/substrate ratio. Figure 5.3a and 5.4a represents the total carbohydrate and reducing sugar conversion respectively as a function of hydrolysis temperature and pH at fixed Enz/sub ratio (2) and substrate concentration (150 g/L). A quadratic effect was observed for both variables, although the effect of pH had greater influence on the responses.

From Figure 5.3(b) represents the interaction effect of Enz/Sub (E) with pH keeping the Temperature (T) constant at 50°C and substrate concentration at 150 g/L. pH and Enz/Sub (E) has strong interaction effect. It is evident that effect of enzyme/substrate ratio is more sensitive at lower pH (4 – 4.8) than at higher pH (4.8-5.6). This probably
can be described by the effect of different enzyme groups involved in the enzyme mixture. The enzyme from the fungal fermentation broth contains the mixture of pectic polysaccharide degrading enzymes, cellulolytic enzymes and oligosaccharide degrading enzymes. Pectic polysaccharide degrading enzymes have been reported previously to have optimum pH between 5 and 6[97, 101]. On the other hand, optimum pH for cellulase and hemicellulase were reported to be between 4.5 and 4.8[102, 103]. Oligosaccharides degrading enzyme α-galactosidase was reported to be stable at pH between 5 and 6 and the optimal activity was found at pH between 4.8 and 5.0[104, 105] For the enzyme mixture, the hydrolysis outcome depends on the effectiveness of all the enzyme groups involved at the specific condition. So the condition of lower than pH 4.5 is bad for all enzyme groups, significantly depressing the hydrolysis of all types of insoluble soy flour carbohydrates, while at higher pH different carbohydrate types might be more effectively hydrolyzed and gives a less sensitive pH effect.

Figure 5.3c and 5.4c were generated to see the interaction effect of enzyme/substrate ratio and the substrate concentration keeping the pH (4.8) and temperature (50) constant. Interaction effect of Enz/Sub (E) and Substrate concentration (S) was not strong enough in case of total carbohydrates but reducing sugar has relatively strong interaction effect.

Figure 5.3d and 5.4d presents the interaction effects of substrate concentration and pH. At higher substrate concentration (350 g/L), conversion increased from from pH 4 to 4.8 and then it decreased with the further increase of pH but in case of lower substrate concentration, the conversion was not decreased with the increase of pH from
4.8 to 5.6. Although in case of reducing sugar, the decrease with the increase of pH from 4.8 to 5.6 was evident.

Figure 5.3(e-f) and 5.4(e-f) represents the interaction effects of temperature with Enzyme/substrate ratio and the substrate concentration for total carbohydrate and reducing sugar conversion respectively. Enzyme/substrate ratio has positive effect for the whole range of temperature for total carbohydrates, while the reducing sugar was saturated near the enzyme/substrate ratio 2. Substrate concentration had negative effect on both total carbohydrates and reducing sugar throughout the whole temperature range.

5.3.5.2 Hydrolysis with *T. reesei* enzyme broth

Figure 5.5 shows the interaction effect of pH, temperature and substrates on the reducing sugar and total carbohydrates conversion. As the substrate conversion effect is not significant so the graphical interaction effects are not shown. Like *A. niger* system, interaction effect of Enz/sub with pH is more significant than with the temperature in *T. reesei* system for both reducing sugar and total carbohydrate system. But the interaction effect of temperature and pH is more significant in case of reducing sugar than that in total carbohydrates. This is could be due to the sensitivity of the temperature and pH of the enzymes responsible for breaking down the oligosaccharides to monosaccharides. In case of *T. reesei*, unlike *A. niger* systems, more significant difference was found in the final conversion level of the total carbohydrates and reducing sugars.

In general, a higher enzyme-to-SF ratio results in better hydrolysis conversions as expected. However, an overly increase of enzyme loading does not linearly increase the hydrolysis. This phenomenon may be attributed to several factors. One is the decrease in
readily accessible reactive sites of soy flour to all the enzyme molecules at high enzyme-
to-SF ratios. Another factor is related to the nonhomogeneous reactive sites of SF: to
increase the conversion requires the enzyme to act on less reactive sites of the solid
substrate. Enzyme deactivation over time due to adsorption on nonreactive sites may also
occur to larger extents at higher enzyme-to-SF ratios. Similar range of pH (4.8 to 5.2) and
temperature (48-51) are optimal for both reducing sugar and total carbohydrate
conversions by both \textit{A. niger} and \textit{T. reesei} enzyme broths. Use of higher substrate
centralizations has some negative effect on soy flour carbohydrate conversion. But the
hydrolysates from these systems have higher soluble carbohydrate concentrations and,
therefore, require less or no further concentrating for subsequent use, e.g., as
fermentation feedstock. The \textit{T. reesei} enzyme broth is clearly inferior to the \textit{A. niger}
enzyme for the intended soy flour upgrading. This can be attributed to the different
compositions of these enzyme mixtures, because hydrolysis of different carbohydrate
structures requires different enzymes. Future study is warranted to investigate the effects
of individual enzyme activities on hydrolysis of carbohydrate in the complex soy
flour/meal and, accordingly, to identify the optimal enzyme composition. Results of this
current study enable more effective design of subsequent studies to achieve the overall
goal of optimizing soy flour/meal upgrading.
Figure 5.3: 3D response surface displaying relative effect of two variables on conversion of total soluble sugar (TS) from enzymatic hydrolysis of soyflour carbohydrates using *A. niger* enzyme system. The unit of response was percent (%) and the units of the variables of temperature, Enz/sub and substrate were °C, ml of enzyme broth per gram of soybean carbohydrates and g/L respectively.
Figure 5.4: 3D response surface displaying relative effect of two variables on conversion of reducing sugar (RS) from enzymatic hydrolysis of soy flour carbohydrates using \textit{A. niger} enzyme system. The unit of response was percent (%) and the units of the variables of temperature, Enz/sub and substrate were °C, ml of enzyme broth per gram of soybean carbohydrates and g/L respectively.
Figure 5.5: 3D response surface displaying relative effect of two variables on conversion of reducing sugar and total carbohydrate from enzymatic hydrolysis of soyflour carbohydrates using *T. reesei* enzyme system. The unit of response was percent (%) and the units of the variables of temperature, Enz/sub and substrate were °C, ml of enzyme broth per gram of soybean carbohydrates and g/L respectively.
5.4 Conclusion

A new enzyme-based process was evaluated for hydrolysis of soy carbohydrate in soy flour to enrich soy protein. Enzyme broths produced by *A. niger* and *T. reesei* were compared. For both enzyme broths, the effects of pH, temperature, enzyme-to-soy flour ratio, and soy flour concentration were determined. The *A. niger* enzyme broth was much more potent than the *T. reesei* enzyme broth. At optimal conditions the *A. niger* enzyme broth yielded very similar conversions to total carbohydrate and reducing sugar, while with the *T. reesei* enzyme broth the total carbohydrate conversion was much higher than the corresponding reducing sugar conversion.
6.1 Introduction

Soybean is the most widely used vegetable protein source for monogastric animals and the most prevalent oil seed crop in the world[7]. Soybean meal, the solid residue after oil is extracted, contains about 50% proteins and 30-35% carbohydrates. It is predominantly used as a source of animal protein replacement[22, 106]. In addition, carbohydrates are the predominant components in soybean hulls [36]. Although soybean contains nearly as much carbohydrates as proteins, the carbohydrates have so far added much less value due to their complex structures, presence of anti-nutritional factors, and low digestibility by the monogastric animals[70, 71, 107]. The carbohydrates in soybean meal are nonetheless a potential source of fermentable sugars for the production of biofuels and value-added chemicals.

Soy carbohydrates are usually divided into two categories, nonstructural and structural, based on their physiochemical properties[36, 108]. Each category makes up approximately half of the total carbohydrates. Nonstructural carbohydrates include three groups: monosaccharides, oligosaccharides, and storage polysaccharides [73, 109]. The structural polysaccharides, also known as non-starch polysaccharides (NSPs), include
dietary fiber components such as cellulose, hemicellulose and pectin [80, 110, 111]. Major parts of the mono- and oligo-saccharides are soluble. But the oligosaccharides, when used as animal feed, are reported to depress digestion efficiency. The problem is caused by the absence of α-galactosidase enzymes required for hydrolysis of these oligosaccharides. In addition, the microbial breakdown of oligosaccharides in lower intestine produces gas that causes flatus in rats, humans and swine, and may increase the possibility of diarrhea, abdominal discomfort and nausea [63, 77, 112, 113]. The usefulness of structural polysaccharides also depends on their fermentability [20]. Both ruminants and nonruminants are incapable of utilizing the structural polysaccharides without the assistance of microbial enzymes required to break down the polysaccharides[114, 115]. So, hydrolyzing soy oligosaccharides and non-starch polysaccharides is important. It increases the value of soy protein products by increasing their digestibility [78, 116, 117]. In addition, the hydrolyzed sugars may be separated from the proteins and fermented to produce biofuel and fine biochemical [118, 119].

Soy carbohydrate hydrolysis requires a complex enzyme system with at least pectinase, xylanase, cellulase and α-galactosidase activities [120]. Fungi have been reported to produce these hydrolytic enzymes. *Trichoderma reesei* and *Aspergillus niger* are among the most extensively studied species [102, 121, 122]. For example, Juhasz et al. [103] studied the enzyme production by *T. reesei* RUT C30 on various carbon sources. They reported that the fungus produced an enzyme mixture consisting of cellulases and hemicellulases, the latter include xylanase, mannanase, α-galactosidase, α-arabinosidase, β-xylosidase and acetyl xylan esterase. *A. niger* is a well-known producer of pectinolytic enzymes[123, 124]. While these enzymes can hydrolyze soy carbohydrates,
quantitatively describing the hydrolytic efficiency of complex enzyme mixtures on complex solid substrate is challenging. It has been recognized that for fungal enzyme mixtures, a model developed based on only one polysaccharide type does not reliably describe its performance on substrates with multiple polysaccharide types [125]. Many of the previous studies were done with lignocellulosic substrates, where cellulase was the focus enzyme group [126, 127]. Soybean meal carbohydrates are different from the common lignocellulosic biomass; the major portion of indigestible soy polysaccharides is pectin and only a small portion is cellulose [128]. Further, even though pectin is the major component, the significant amount of hemicellulose and the small amount of cellulose present are believed to exert significant restraints on the hydrolysis[80, 129]. It is probable that cellulose and hemicellulose restrict the access of pectinolytic enzymes to pectin. Different enzyme groups need to act synergistically to breakdown the complex polysaccharides.

It is obvious from the preceding discussion that studies are needed to better understand the hydrolysis of soy carbohydrates with different enzyme mixtures. As various changes take place concurrently during the course of hydrolysis, an approach to describe the phenomena is by using kinetic modeling. There has been no kinetic modeling study reported for soy carbohydrate hydrolysis. But there have been many mechanistic or empirical kinetic studies done on cellulosic biomass. Kinetic model studies of hydrolysis are helpful for substrate preparation, reactor design, and optimization of feeding profiles of substrate and/or enzyme in a fed-batch operation[130]. Most of the mechanistic models have been conducted using variations of Michaelis-Menten initial velocity kinetics[126]. But the Michaelis-Menten equation, which is
developed for enzymatic reactions in solution, may not describe the reactions on insoluble substrates like cellulose and pectin[131]. In this study, we developed an empirical kinetic model for the hydrolysis of soy flour carbohydrates using fungal enzyme mixtures containing various cellulase, xylanase, pectinase and α-galactosidase activities and soy flour concentrations. This model would be helpful for further reactor design, batch and continuous process design and optimization of overall economics.

6.2 Materials and Methods

6.2.1 Materials and equipment

Defatted soy flour (7B soy flour) and soy hulls were provided by Archer Daniel Midland (Decatur, IL). Water used in the hydrolysis was Milli-Q water (18.2 MΩ-cm at 25 °C; Milli-Q Direct 8, Millipore S.A.S., Molsheim, France). (NH₄)₂SO₄ (granular), KH₂PO₄ (99% purity), HCl (concentrated acid, 37.4%) and NaOH (98.8%) were purchased from Fisher Scientific (Waltham, MA). Proteose peptone (from meat, Type I, for microbiology), MgSO₄·7H₂O (99%), MnSO₄·4H₂O (99%), ZnSO₄·7H₂O (ACS reagent grade), CoCl₂·6H₂O, FeSO₄·7H₂O (reagent grade), CaCl₂·2H₂O (reagent grade), urea (98%), NaN₃ (> 99%) and dinitrosalicylic acid (DNS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). A. niger (NRRL 341), A. aculeatus (NRRL 2053), and T. reesei Rut-C30 (NRRL 3469) seed cultures were obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. Two 3 L Bioflo 110 fermentors (New Brunswick Scientific Co., Edison, NJ) were used for enzyme production by fermentation. Absorbance was measured using a UV/Vis spectrophotometer (UV-1601, Shimadzu Corporation, Columbia, MD). The
hydrolysis experiments were conducted in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating floor shaker, Ashville, NC). The centrifuge used was Sorvall Legend X1R from Thermo Scientific (Waltham, MA).

6.2.2 Enzymes

Commercial cellulase Spezyme CP (Dupont, Cedar Rapids, Iowa) and pectinase (Sigma-Aldrich, St. Louis, MO) were used in addition to the enzyme broths produced in the laboratory. Three different fungal species, *A. niger* (NRRL 341), *A. aculeatus* (NRRL 2053) and *T. reesei* Rut C-30 (NRRL 3469), were used to produce the enzyme broths. The fungal fermentation was performed in a 3 L fermentor containing 1 L of the following fresh medium: soy hulls, 20 g/L; proteose peptone, 1.4 g/L; (NH₄)₂SO₄, 4 g/L; K₂HPO₄, 0.32 g/L; KH₂PO₄, 0.21 g/L; and MgSO₄·7H₂O, 1 g/L. The initial pH was 6.7. Inoculation was done with a pre-grown culture at an initial cell concentration of about 0.1 g/L. Temperature and agitation were maintained at 23 °C and 350 rpm. The pH and DO (dissolved oxygen concentration) were allowed to vary naturally until they dropped to 6 and 20% (air saturation), respectively. Dissolved oxygen concentration (DO) was then maintained at 20% by automatic supplementation of pure oxygen as needed. pH was controlled at 6.0 ± 0.1 by automatic addition of 1 M NaOH or HCl. The fermentation was stopped after 5 days when the enzyme production rate decreased significantly. The enzyme broth used for hydrolysis study was the cell- and solid-free supernatant collected by centrifugation of the fermentation broth at 8000 rpm (9000 g) for 10 min (Sorvall RC 5C, DuPont, Wilmington, DE).
6.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in 250 ml flasks in a shaking incubator at 50°C and 250 rpm. Each flask contained 40 ml enzyme broth and an amount of soy flour depending on the designed substrate concentration. Two duplicate control systems, prepared with enzyme-free deionized water, were included in each batch of experiments. Dispersed soy flour in deionized water was warmed to 50°C. Enzyme broth and then deionized water was added. 1 M hydrochloric acid was used to initially adjust the pH to 4.8. During hydrolysis pH was checked every 4 h and adjusted to 4.8 with 1 M NaOH if required; pH had a slight tendency to decrease during the hydrolysis. Samples were taken at 0, 4, 8, 16, 22 and 48 h in triplicate and heated immediately for 10 min in boiling water to deactivate the enzymes. Samples were then centrifuged to separately collect the solids and supernatant. Supernatants were analyzed for concentrations of reducing sugars and total carbohydrates (methods described in the next section). Total carbohydrate and reducing sugar contents in the soy flour used were separately measured. The total carbohydrate conversion achieved was calculated by dividing the total soluble carbohydrates found in the solution by the total carbohydrate present in the soy flour initially added. The reducing sugar conversion achieved was determined similarly.

6.2.4 Analytical methods

Cellulase, xylanase and pectinase activities were measured according to the methods reported by Ghose et al. [132], Bailey et al. [55] and Li et al.[56] respectively. Assay reported by Kumar et al. [57] was used to measure the activity of α-galactosidase. Reducing sugar concentrations in the hydrolysates were measured by the dinitrosalicylic
(DNS) acid method [52]. DNS solution (3 ml) was placed in a test tube and mixed with 1 ml sample. Then the mixture was heated in a boiling water bath for 5 min. The tube was added with water to 25 ml total volume and cooled to ambient temperature. Absorbance was then measured at 550 nm with a spectrophotometer. The absorbance was converted to reducing sugar concentration according to the calibration curve obtained with glucose solutions as standards. Total carbohydrate concentrations were measured by the phenol-sulfuric acid colorimetric method[53]. A 1 ml sample was mixed with 1 ml aqueous phenol solution (5% w/w) in a test tube, followed by addition of 5 ml concentrated sulfuric acid. 5% phenol in water (w/w) was prepared immediately before the analysis. After 10 min reaction without mixing, the mixture was vortexed for 30 s, cooled to room temperature, and then measured for the absorbance at 490 nm. Blanks were prepared in the identical manner with 1 ml deionized water. Total carbohydrate and reducing sugar contents in the soy flour were determined by first processing the soy flour according to the NREL method[133] and then analyzing the acid hydrolysate by the phenol-sulfuric acid colorimetric method and dinitrosalicylic acid (DNS) method.

6.2.5 Experimental Design

Overall procedure followed in this study included: (1) running many hydrolysis experiments using different enzyme mixtures, soy flour (SF) concentrations, and enzyme-to-SF ratios; (2) fitting all final conversion results to an enzyme saturation-type model to determine the maximum conversions attainable from individual carbohydrate type (hypothetically grouped into pectin, xylan, cellulose, and oligosaccharides) by the corresponding enzyme activity used (pectinase, xylanase, cellulase, and α-galactosidase).
and to obtain the best-fit model parameters; and (3) fitting the conversions attained at different hydrolysis time to a kinetic (time-dependent) model that builds on the model parameters obtained from Step (2). The number of hydrolysis experiments made and the ranges of factors varied in the experiments are summarized in Table 6.1.

Table 6.1: The number of hydrolysis experiments made and the ranges of factors varied in the experiments, for fitting the conversions attained to the kinetic models developed in this study.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>No of systems</th>
<th>Cellulase (FPU/g SF)</th>
<th>Xylanase (U/g SF)</th>
<th>Pectinase (U/g SF)</th>
<th>α-Galactosidase (U/g SF)</th>
<th>Soy flour conc. (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger</td>
<td>32</td>
<td>0.04-2.0</td>
<td>9.5-456</td>
<td>0.4-19.2</td>
<td>0.5-22</td>
<td>50, 100, 125, 200, 250</td>
</tr>
<tr>
<td>T. reesei</td>
<td>24</td>
<td>0.2-2.0</td>
<td>4-191</td>
<td>0.03-1.37</td>
<td>0.01-0.32</td>
<td>100, 150, 250</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>8</td>
<td>0.35-1.2</td>
<td>14-119</td>
<td>2.6-13.3</td>
<td>0.5-3.6</td>
<td>150,250</td>
</tr>
<tr>
<td>A. niger &amp; T. reesei mixture</td>
<td>20</td>
<td>0.5-20.7</td>
<td>19.3-618.2</td>
<td>1.72-50</td>
<td>2.45-59.3</td>
<td>100, 200, 250</td>
</tr>
<tr>
<td>Commercial cellulase</td>
<td>4</td>
<td>2.0-30</td>
<td>17.5-122</td>
<td>0.12-1.6</td>
<td>0.05-0.75</td>
<td>250</td>
</tr>
<tr>
<td>Commercial pectinase</td>
<td>4</td>
<td>0.4-2.05</td>
<td>3.5-18.0</td>
<td>24-120</td>
<td>0.5-2.6</td>
<td>250</td>
</tr>
<tr>
<td>Cellulase + A. niger</td>
<td>6</td>
<td>2.5-29.5</td>
<td>70-190</td>
<td>1.4-6.5</td>
<td>0.44-8.2</td>
<td>250</td>
</tr>
<tr>
<td>Pectinase + T. reesei</td>
<td>6</td>
<td>0.5-2.5</td>
<td>10.5-112.2</td>
<td>14.5-60.2</td>
<td>0.4-1.9</td>
<td>250</td>
</tr>
</tbody>
</table>
6.2.6 Modeling

6.2.6.1 Modeling for maximum contributions from different hydrolytic enzyme groups

A saturation-type model was used to describe the final conversions achieved after 48 h hydrolysis at the optimized reaction conditions (pH 4.8, temperature 50°C). Conversions to two categories of soluble carbohydrates in the hydrolysate were determined: total carbohydrates and reducing sugars. Choice of the final condition was based on the hydrolysis profile when the total carbohydrate and reducing sugar concentrations no longer increase. Accordingly, 48 h was selected as the final conversion time. As mentioned earlier, the total carbohydrate conversion was calculated by dividing the total soluble carbohydrates found in the hydrolysate by the total carbohydrate present in the soy flour initially added. The soluble carbohydrates found in the hydrolysate include (1) the oligomeric and monomeric carbohydrates that are already water soluble without enzymatic hydrolysis and (2) those that become soluble by the enzymatic degradation of originally insoluble carbohydrates. Accordingly, total carbohydrate conversion was expressed by the summation of four portions: originally soluble carbohydrates and the 3 portions solubilized due to hydrolysis by individual enzymes (cellulase, xylanase, and pectinase), as shown in Equation (1):

\[
X_{TC} = \frac{TC_s}{TC_0} = \alpha_0 + \frac{\alpha_c E_c}{S K_c + \frac{E_c}{S}} + \frac{\alpha_x E_x}{S K_x + \frac{E_x}{S}} + \frac{\alpha_p E_p}{S K_p + \frac{E_p}{S}} \ldots \ldots \ldots (1)
\]

Here \(X_{TC}\) is total carbohydrate conversion, \(TC_s\) is the total soluble carbohydrate concentration measured in the hydrolysate after 48 h, and \(TC_0\) is the total carbohydrate concentration introduced with the initial soy flour. \(\alpha_0\) is the portion (fraction) of
originally soluble carbohydrates in TC₀. \( \alpha_c, \alpha_x, \) and \( \alpha_p, \) are the fractions generated by cellulase, xylanase and pectinase enzymes, respectively. E is the activity of each enzyme group. S is the total soy flour concentration used. K is the half-maximum constant for each enzyme activity. Subscripts c, x, and p denote cellulase, xylanase, and pectinase, respectively.

The originally soluble portion (\( \alpha_0 \)) consists of a small fraction of monosaccharides and a majority of oligosaccharides such as sucrose, stachyose and raffinose. Breaking down these oligosaccharides by \( \alpha \)-galactosidase (and sucrase, not included in the current modeling) gives higher reducing sugar concentrations (freeing more reducing ends in the carbohydrates). So, for modeling the reducing sugar conversion (\( X_{RS} \)), the soluble total carbohydrate portion (\( \alpha_0 \)) was divided into 2 portions: soluble monosaccharides (\( \alpha_{0RS} \)) and the \( \alpha \)-galactosidase generated reducing sugars (\( \alpha_g \)), as given in Equation (2):

\[
X_{RS} = \frac{RS_s}{TC₀} = \alpha_{0RS} + \frac{\alpha_c E_c}{K_c + E_c} + \frac{\alpha_x E_x}{K_x + E_x} + \frac{\alpha_p E_p}{K_p + E_p} + \frac{\alpha_g E_g}{K_g + E_g} \quad \ldots \ldots \quad (2)
\]

Here \( RS_s \) is reducing sugar concentration measured in the hydrolysate after 48 h and the subscript \( g \) is used to denote \( \alpha \)-galactosidase. Note that the model parameters for cellulase, xylanase and pectinase are kept the same in both equations. Using these equations, the maximum fraction of each carbohydrate type can be determined and the basic saturation-type dependency of conversion to the enzyme-to-substrate ratio for each enzyme group is also fitted.
6.2.6.2 Modeling for kinetic (time-dependent) hydrolysis performance

Different empirical models have been used to describe the kinetic performance of lignocellulose hydrolysis. During the initial stage, the rate is almost linear; however, at later stages it decreases continuously and ceases over time. In this study the empirical model described by Walseth[134] is chosen as the basis:

\[ X = A + B t^n \]

where \( X \) is the conversion (%), \( A \) is the percentage of readily soluble carbohydrates, \( t \) is time (h), and \( B \) and \( n \) are empirical constants. \( n = 0.5 \) is typically used[135]. \( B \) has been proposed as a function of the initial enzyme-to-substrate ratio (E/S):

\[ B = k \left( \frac{E}{S} \right)^m \]

Where \( k \) and \( m \) are empirical constants. The above model, however, fails at long reaction time; as the hydrolysis rate decreases, the parameter \( n \) deviates further from 0.5 with time. Accordingly, in one empirical model [136] the above equation is modified to the following:

\[ X = A + B t^{\left(0.5 - \frac{t}{\tau_d} \right)} \]

i.e., \( n = 0.5 - \frac{t}{\sqrt{\tau_d}} \)

Where \( \tau_d \) represents a characteristic time which describes how fast the hydrolysis deviates from the initial kinetics. This model was shown to describe the long-term hydrolysis well (before \( t \) is so large that \( n \) becomes negative).
In this study, different soy flour concentrations were used and the results indicated certain negative effect of increasing soy flour loading on the conversion achievable. Accordingly, a simple dependency of the enzyme-responsible conversion on the substrate concentration to a negative exponent \((r)\) is introduced to describe this negative effect of high soy flour concentration:

\[
X = A + Bt^{\left(0.5-\frac{T}{\sqrt{d}}\right)}S^{-r}
\]

With each enzyme-carbohydrate group being assumed to have independent kinetics, the hydrolysis of soy flour carbohydrates is modeled as follows:

\[
X_{RC}(\%) = \alpha_0 + k_c \left(\frac{E_c}{S_c}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_c}}\right)} + k_x \left(\frac{E_x}{S_x}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_x}}\right)} + k_p \left(\frac{E_p}{S_p}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_p}}\right)} \quad \cdots \quad (3)
\]

\[
X_{RS}(\%) = \alpha_{0rs} + k_c \left(\frac{E_c}{S_c}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_c}}\right)} + k_x \left(\frac{E_x}{S_x}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_x}}\right)} + k_p \left(\frac{E_p}{S_p}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_p}}\right)} + k_g \left(\frac{E_g}{S_g}\right)^m t^{\left(0.5-\frac{T}{\sqrt{d_g}}\right)} \quad \cdots \quad (4)
\]
Here $S_i$ is the concentration of carbohydrate group $i$, calculated as the fraction of total carbohydrate degradable by enzyme $i$. All other parameters have been previously defined. Note that the $k$, $m$ and $\tau_d$ values in the two equations may not be the same because, depending on the composition of each enzyme group, breaking down the soluble carbohydrates (measured as total carbohydrates) to monomers, resulting in higher reducing sugar conversions, may be the rate-limiting step. In this case, the kinetic parameters would be different for TC and RS conversions.

Model fitting of the above equations with experimental results, to determine the best-fit parameters, was carried out according to the non-linear regression optimization procedure, using the built-in Solver program in Microsoft Excel. An analysis of variance (ANOVA) was conducted using Excel. F and p values were used to indicate the significance of the developed models. $R^2$ values and adjusted $R^2$ values were determined to investigate how well the experimental results fit with the model described outcomes.

6.3 Results and Discussion

6.3.1 Hydrolysis profiles

Before the modeling results are presented, some profiles depicting the release over time of soluble carbohydrates from solid soy flour substrate into the liquid hydrolysate are shown in Figures 6.1 and 6.2 as examples. (Symbols in these figures are experimental data; the lines drawn are based on the kinetic model equations, (3) and (4), with best-fit parameters, as described in a later section.) The released soluble carbohydrates were measured respectively as total carbohydrates and reducing sugars. Figure 6.1 shows how different concentrations of an enzyme broth affected the
hydrolysis. The broth was produced by *A. niger* and was measured to have the following activities: 0.5 FPU/ml cellulase, 171 U/ml xylanase, 5.5 U/ml pectinase and 7.1 U/ml α-galactosidase. The broth was diluted to different strengths with dilution factors of 1 (undiluted), 2, 4 and 10. As expected, the conversions were lower and slower with increasing dilution factors. For examples, the undiluted broth gave maximally 80% conversions to both total carbohydrates and reducing sugars while the 10-fold diluted broth yielded only 45% reducing sugar conversion and 54% total carbohydrate conversion. Hydrolysis rates were also different. Total carbohydrate conversion increased significantly faster than the reducing sugar conversion. With the undiluted broth, total carbohydrate conversion reached the maximum level (80%) after about 10 h but the reducing sugar conversion increased slower and reached the same maximum level at the end of experiment (48 h). With diluter enzyme broths, both conversions increased slower. Total carbohydrate analysis does not differentiate between oligomeric and monomeric carbohydrates but the reducing sugar conversion increases with increasing degrees of monomerization. The same total carbohydrate and reducing sugar conversions achieved, after 48 h reaction, by the undiluted broth suggested all carbohydrates in that hydrolysate were monomers. The lower reducing sugar conversions and their slower increasing rates, compared to total carbohydrate conversions, suggested that hydrolysis of oligomeric carbohydrates in the hydrolysate was rate-limiting and that the enzyme broth used had a suboptimal composition of enzyme activities.

In Figure 6.2, the effects of substrate concentration (solid loading) on the total carbohydrate and reducing sugar conversions are presented. The increase in solid concentration has clearly negative effects on the conversions. Compared at 48 h the total
carbohydrate conversion dropped from 68% to 59% and the reducing sugar conversion dropped from 63% to 56% due to the increase of soy flour concentration from 50 to 200 g/L. Such an adverse effect of increasing solid substrate concentration on enzymatic hydrolysis is consistent with the findings reported in the literature for substrates such as lignocellulosic and pectic polysaccharides [89, 137, 138]. This confirmed the need to incorporate the substrate concentration effect in the kinetic modeling equations (3) and (4).
Figure 6.1: Increases of total carbohydrate conversion (upper) and reducing sugar conversion (lower) over time with a serially diluted enzyme broth (n=3); dilution factors – 1 (undiluted), 2, 4 and 10. 250 g/L soy flour was hydrolyzed at pH 4.8 and 50 °C. Undiluted *A. niger* enzyme broth was measured to have 0.5 FPU/ml cellulase, 171 U/ml xylanase, 5.5 U/ml pectinase and 7.1 U/ml α-galactosidase. Symbols are experimental data; lines are predicted from the kinetic model developed with best-fit parameters (Equations 3 and 4).
Figure 6.2: Increases of total carbohydrate conversion (upper) and reducing sugar conversion (lower) over time for hydrolysis of different concentrations of soy flour substrate: 50, 100, 125 and 200 g/L. Hydrolysis was done at pH 4.8 and 50 °C. The enzyme activities are same as stated in figure 6.1. Symbols are experimental data (n=3); lines are predicted from the kinetic model developed with best-fit parameters (Equations 3 and 4).
6.3.2 Modeling for maximum contributions from different hydrolytic enzyme groups

Table 6.2 shows the determined parameters for the model equations 1 and 2. These equations describe well the experimentally measured reducing sugar and total carbohydrate conversions (after 48 h reaction) as shown in Figure 6.3.

Table 6.2: Best-fit model parameters for Equations (1) and (2) describing the maximum fractions of contribution ($\alpha$) and half-maximum enzyme loading constants ($k$) for different substrate-enzyme groups

<table>
<thead>
<tr>
<th>Maximum fractions of contribution</th>
<th>Half-maximum constants (U/g SF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{0rs}$</td>
<td>0.118</td>
</tr>
<tr>
<td>$\alpha_{g}$</td>
<td>0.228</td>
</tr>
<tr>
<td>$\alpha_{c}$</td>
<td>0.044</td>
</tr>
<tr>
<td>$\alpha_{x}$</td>
<td>0.277</td>
</tr>
<tr>
<td>$\alpha_{p}$</td>
<td>0.333</td>
</tr>
<tr>
<td>$K_g$</td>
<td>1.31</td>
</tr>
<tr>
<td>$K_c$</td>
<td>31.4</td>
</tr>
<tr>
<td>$K_x$</td>
<td>3.49</td>
</tr>
<tr>
<td>$K_p$</td>
<td>13.4</td>
</tr>
</tbody>
</table>
Figure 6.3: Comparison of experimentally measured conversions of soy flour carbohydrates with the conversions predicted by model equations (1) and (2) with the best-fit parameters. Conversions were measured after 48 h reaction for the soluble carbohydrates (upper) and reducing sugars (lower) in the hydrolysate.
According to the modeling results shown in Table 6.2, about 12% of the soy carbohydrates are readily measurable as soluble reducing sugars in the hydrolysate ($\alpha_{ors}$). Another 23% ($\alpha_g$) are also measured as soluble carbohydrates (by the phenol-sulfuric acid based analysis) but would be measured as reducing sugars only after the enzymatic hydrolysis by $\alpha$-galactosidase (and sucrase also present in the enzyme broths, although not included in the modeling). These are mainly sucrose, raffinose and stachyose. Pectin (or pectinase-hydrolyzable carbohydrates, $\alpha_p$) and hemicellulose (or xylanase-hydrolyzable carbohydrates, $\alpha_x$) make up the major portions of the insoluble carbohydrates, 33% and 28%, respectively. They are the logical targets of enzymatic hydrolysis for separating the insoluble carbohydrates from the remaining soy proteins. For maximizing production of monomeric carbohydrates, either for easy animal digestion or for use as readily fermentable substrate, the high portion (23%) of $\alpha$-galactosidase-hydrolyzable oligosaccharides is also an important hydrolysis target. Among the enzymes for these 3 targets, i.e., pectinase, xylanase and $\alpha$-galactosidase, the best-fit value of half-maximum enzyme loading constant ($K$) is the highest for pectinase, requiring 13.4 U/g SF to achieve 50% conversion of the pectin present, as compared to 3.49 (U/g SF) xylanase and 1.31 (U/g SF) $\alpha$-galactosidase for 50% conversion of their responsible portions of soy carbohydrates. The low value of $K_g$ may be because $\alpha$-galactosidase acts on soluble substrates, which are more accessible and do not cause enzyme denaturation by irreversible binding as the other solid substrates do. High stability has been reported for the $\alpha$-galactosidase from $A. niger$ [104]. These $K$ values are valuable to know, as they offer a guideline for the optimal enzyme mixture composition to be produced in fungal fermentation and/or used for soy flour carbohydrate hydrolysis. The model-fitted $K$ value
for cellulase ($K_c$) is 31.4 FPU/g SF, even much higher than $K_p$. This is rather surprising since the cellulase-hydrolyzable portion ($\alpha_c$) is only 4.4% and, as a rule of thumb, about 10 FPU per g substrate is typically used for lignocellulosic hydrolysis[86]. There are several possible reasons for this high $K_c$ value: (1) pretreatment is normally required to lower the crystallinity of cellulose prior to enzymatic hydrolysis[139] while SF was not pretreated in this study; (2) the small amount of cellulose may be surrounded by proteins and other major carbohydrates, blocking its easy access by cellulase; and (3) cellulose hydrolysis contributes minimally to total conversions, rendering the model fitting inaccurate for $K_c$. Nonetheless, because of the small fraction of cellulose present, cellulase effect is the least important for the overall hydrolysis outcome.

6.3.3 Modeling for kinetic hydrolysis performance

The best-fit parameters of the kinetic models described by Equations (3) and (4) are given in Table 6.3. For both models the $r$ value is 0.114, indicating a relatively small but non-negligible effect of soy flour concentration on the carbohydrate hydrolysis. As described in the earlier Modeling section, the kinetic parameters can be different in the equations for TC and RS conversions. For each enzyme group, the TC conversion kinetics can be faster if the soluble oligomeric carbohydrates generated cannot be immediately hydrolyzed to monomers; in this case, the increase of reducing sugar conversion requires longer reaction time. This appears to be the case for xylanase- and pectinase-dependent hydrolysis in Table 6.3, where $k_x$, $k_p$, $m_x$, and $m_p$ values are larger for total carbohydrate conversion than for reducing sugar conversion. Among pectinase, xylanase and cellulase, pectinase have the largest $k$ and $m$ values, indicating that
pectinase has the strongest effect on total carbohydrate conversion. This is partly because pectin makes up the largest fraction of insoluble soy flour carbohydrates. In addition, Ouhida et al. [19] studied the fractionated extraction of soybean carbohydrates and reported that proteins and other carbohydrates are trapped by the pectic structural polysaccharides. Breaking down pectin is important to the accessibility of enzymes to more carbohydrates. The $m$ value for cellulase is particularly low, implying that cellulase is not very effective in hydrolyzing the cellulose in soy flour carbohydrates. This can be partly because the soy flour was not subjected to any pretreatment prior to the hydrolysis in this study, while pretreatment is generally required for enzymatic hydrolysis of cellulose (at least for lowering the crystallinity [139]. This is also consistent with the report by Ouhida et al. [19] that cellulose is the least accessible to enzyme hydrolysis; cellulose becomes accessible only after other carbohydrates are largely hydrolyzed. For the reducing sugar conversion model, $\alpha$-galactosidase has a much higher $k$ value: 8.22, as compared to the next highest value of 1.75 for pectinase. High $\alpha$-galactosidase activity is critical to monomerization of the relatively large fractions of stachyose and raffinose (and possibly other similar oligosaccharides released by other enzymes).
Table 6.3: Best-fit parameters for kinetic hydrolysis models given in Equations (3) and (4)

<table>
<thead>
<tr>
<th>Parameters in Eq. (3) for total carbohydrate conversion</th>
<th>Parameters in Eq. (4) for reducing sugar conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r ) 0.114</td>
<td>( r ) 0.114</td>
</tr>
<tr>
<td>( \alpha_0 (%) ) 34.6</td>
<td>( \alpha_{0rs} (%) ) 11.8</td>
</tr>
<tr>
<td>( k_c ) 0.074 ( m_c ) 0.091 ( \tau_{dc} (h) ) 1082</td>
<td>( k_c ) 0.074 ( m_c ) 0.091 ( \tau_{dc} (h) ) 1082</td>
</tr>
<tr>
<td>( k_x ) 2.32 ( m_x ) 0.272 ( \tau_{dx} (h) ) 4473</td>
<td>( k_x ) 1.38 ( m_x ) 0.113 ( \tau_{dx} (h) ) 4582</td>
</tr>
<tr>
<td>( k_p ) 2.37 ( m_p ) 0.468 ( \tau_{dp} (h) ) 235</td>
<td>( k_p ) 1.75 ( m_p ) 0.295 ( \tau_{dp} (h) ) 227</td>
</tr>
<tr>
<td>( k_g ) 8.22 ( m_g ) 0.263 ( \tau_{dg} (h) ) 2353</td>
<td>( k_g ) 8.22 ( m_g ) 0.263 ( \tau_{dg} (h) ) 2353</td>
</tr>
</tbody>
</table>

Characteristic time parameter \( \tau_d \) for each enzyme could give valuable insight on the enzyme stability or the substrate accessibility based on the structural changes or availability over time. \( \tau_d \) values for xylanase are the largest, > 4000 h. The values for \( \alpha \)-galactosidase (> 2000 h) and cellulase (> 1000 h) are also very large. The issues of enzyme stability and substrate change are therefore insignificant for the hydrolysis by these 3 enzymes, within the timeframe of interest to this study (no more than 48 h). The \( \tau_d \) values for pectinase are however much smaller: 235 h for total carbohydrate conversion and 227 h for reducing sugar conversion. This suggests that pectinase is less stable under the reaction condition (particularly the relatively high temperature, 50 °C) and/or the accessibility/hydrolyzability of pectin decreases more significantly with time. Pectinase has indeed been reported to be much less stable at higher temperatures (50°C)
than cellulase and xylanase[140]. The finding of relatively small $\tau_d$ values for pectinase is important if a fed-batch hydrolysis process is to be designed.

The model predicted conversions, with the best-fit parameters given in Table 6.3, are plotted against those experimentally measured in Figure 6.4 (with totally 155 sets of data points). The correlations are reasonably good, with $R^2$ values of 0.93 and 0.96 for total carbohydrate and reducing sugar conversions, respectively. The Fisher’s F-test gave very low p values ($<< 0.00001$) for both total carbohydrate and reducing sugar conversion models, also confirming the high significance of the models.
Figure 6.4: Comparison of experimentally measured conversions of soy flour carbohydrates with the conversions predicted by model equations (3) and (4) with the best-fit parameters. Conversions were measured for the soluble carbohydrates (upper) and reducing sugars (lower) in the hydrolysate.
6.3.4 Effect of enzymes from different fungal species

Figure 6.5: Increases of total carbohydrate conversions (upper) and reducing sugar conversions (lower) over time when 250 g/L soy flour was hydrolyzed at pH 4.8 and 50 °C by enzymes from different species (n=3): *A. niger*, *T. reesei*, and *A. aculeatus*, and from a mixed *A. niger* and *T. reesei* broth. The different enzymes used had the following measured activities: *A. niger* – 0.69 FPU/ml cellulase, 171 U/ml xylanase, 7.2 U/ml pectinase and 8.1 U/ml α-galactosidase.; *T. reesei* – 2.4 FPU/ml cellulase, 191 U/ml xylanase, 2.37 U/ml pectinase and 0.33 U/ml α-galactosidase; *A. aculeatus* – 0.71 FPU/ml cellulase, 14 U/ml xylanase, 6.9 U/ml pectinase and 1.8 U/ml α-galactosidase; and the mixed broth was a 50:50 mixture of *A. niger* & *T. reesei*. Experimental data are presented by the symbols and the model-predicted values are presented as lines.
As shown in Figure 6.4, the kinetic models with best-fit parameters describe the experimental results reasonably well for many systems. In Figure 6.5 the experimentally measured and model predicted hydrolysis conversion profiles are compared for enzyme broths produced by different fungal species: *A. niger*, *T. reesei*, and *A. aculeatus*, and a mixture of *A. niger* and *T. reesei* broths. The models are shown to describe the experimental results for enzymes from all three species with reasonable accuracy. However, the models consistently under-predicted the hydrolysis outcomes of the mixed enzymes of *A. niger* and *T. reesei*. This underestimation holds true for the mixed system with *A. niger* higher than 30% but lower than 80% (results not shown). Accordingly in figure 6.4, few systems were found to show higher underestimation than others in case of mixed enzymes of *A. niger* and *T. reesei*. This suggest that very low portion of either *A. niger* or *T. reesei* enzyme will undermine the synergy. Synergistic effect of *T. reesei* and *A. niger* enzyme mixtures on the hydrolysis of wheat straw and sugarcane bagasse was also reported earlier[141]. The synergy was driven by high cellobiohydrolase and β-glucosidase activities from *A. niger*. However, in the present study, the substrate contains higher pectinase and lower cellulase. This synergistic effect warrants further study for optimization of the soy carbohydrate hydrolysis.

In figure 6.5, both total carbohydrate and reducing sugar conversion by *T. reesei* enzyme system was found to be only 58% and 37% compared to that of 81% and 75% by *A. niger* system. Although *T. reesei* system have higher cellulase and xylanase, lower pectinase and α-galactosidase resulted lower conversion. As from the model equation (1) & (2), it was found that the half maximum enzyme loading constant for xylanase $K_x$ is only 3.49, xylanase activity of 171U/g of solid is already very high to degrade majority of
the hemicellulose and xylans. On the other hand, even though cellulase half maximum enzyme loading constant is very high (31.4), low cellulose content (3-4%) of soy flour made the effect of cellulase insignificant on the overall hydrolysis. Consequently pectinase and α-galactosidase are considered more important to get high conversion of total carbohydrate and reducing sugar respectively. Low α-galactosidase (0.33 U) in T. reesei resulted much lower reducing sugar of 37% compared to total carbohydrate of 58%. While in A. niger system reducing sugar conversion (75%) is very close to total carbohydrate conversion (81%). As the half maximum enzyme loading constant for α-galactosidase $K_g$ is 1.31, high α-galactosidase activity 8.1 U/g of solid lead to almost complete degradation of total soluble carbohydrate. However, Half maximum enzyme loading constant of pectinase (13.4) suggest that the highest available pectinase activity (8.1 U) in the A. niger system is not sufficient to breakdown half of the available pectic polysaccharides. Accordingly, the maximum conversion of 81%, achieved by A. niger enzyme system in spite of having satisfactory xylanase and α-galactosidase activity, was limited by the pectinase activity. To reach higher than 90% overall soluble carbohydrate and reducing sugar conversion, the pectinase activity should be increased to 90 U/g of solid. So according to the fitted half maximum enzyme loading constant from the model, an enzyme system with following activities per g of solid would be ideal to have higher than 90% conversion of the complex carbohydrates: 20 U/ml xylanase, 90 U/ml pectinase and 10 U/ml α-galactosidase. These comprehensive kinetic analysis could be very helpful for designing a process to fulfill a specific purpose.
6.4 Conclusion

Models were developed to describe the kinetic performance of batch enzymatic hydrolysis of soy flour carbohydrates. The performance was measured by both conversions to soluble reducing sugars and total carbohydrates in the liquid hydrolysate. The models considered the following factors: soy flour concentration and activities of cellulase, xylanase, pectinase and α-galactosidase. With same sets of best-fit parameters, the models described well the results from experiments made with enzymes from different fungal species: *A. niger*, *T. reesei*, and *A. aculeatus*, and over broad ranges of soy flour concentration (5-25%) and enzyme activities (per g soy flour): cellulase, 0.04-30 FPU; xylanase, 3.5-618 U; pectinase, 0.03-120 U; and α-galactosidase, 0.01-60 U. The models would be very useful for the improvement of enzyme mixture composition and the designs of reactor and process used for soy carbohydrate hydrolysis. The hydrolysis would produce soy protein products with higher protein contents and low/no indigestibility. It would also coproduce a liquid hydrolysate with fermentable, mostly monomerized carbohydrates, and add value to the large amount of soy carbohydrates present in soybean meal/flour.
CHAPTER VII

FED BATCH PROCESS AND RECYCLE OF ENZYME TO IMPROVE THE
CONCENTRATION AND PRODUCTIVITY OF REDUCING SUGARS IN SOY
FLOUR HYDROLYSIS

7.1 Introduction

Finding renewable biomass for effective conversion to fuels and chemicals have been attracted huge attention in recent past. Soybean is very well known for its high value oil and protein. However, it contains almost 25-28% carbohydrates[142] which has not been investigated well enough for high value application. One main reason is the difficulty to degrade the soybean carbohydrate to fermentable sugars[19]. There are few recent studies done to use soybean based carbohydrate from different soybean processing by-products such as soybean hull, soybean okara, molasses and soybean meal for the production of ethanol[143-145] and chemicals[146-149]. However, the main difficulty of the processes were to effectively hydrolyze the carbohydrate as multiple enzyme activities are required to completely hydrolyze all types of carbohydrates[19]. Recently we reported a study on the effects different enzyme activities in the enzyme mixture to maximize the hydrolysis of soybean meal[150]. Soybean meal carbohydrate content is about 30-35%[30]. The carbohydrate can be hydrolyzed to separate the protein in precipitated form. Then the hydrolysate can be used for fermentation to produce fuels and chemicals. But to achieve high productivity in the fermentation, high concentration of
sugar is important. With only 30-35% carbohydrate in the soybean meal, hydrolysis with high substrate loading is required to obtain high concentration of sugar in the hydrolysate. Moreover, hydrolysis of the high substrate concentration is beneficial because of its potential to give certain significant economic advantages over a conventional low substrate process which includes decreased capital cost of the process due to reduced working volume, lower operating costs due to less energy required for heating, lower downstream processing cost due to the high product concentration and, last but not the least, lower waste disposal and treatment costs[151, 152]. However, previous studies suggested that hydrolysis yield decreases with the increase in substrate loading [89, 138, 150]. As high solid concentration becomes problematic while mixing in the stirred tank reactors, Fed batch process could be helpful to minimize the mixing problem[153]. By optimizing solid loading and enzyme loading during the fed batch process, a feeding profile can be developed to maintain the concentration of insoluble solids at a manageable level throughout the hydrolysis reaction. By these approaches a relatively higher final sugar concentration can be obtained within the reactor without requiring high solid loading initially which is required if the process is operated in batch process. Recycle process is also advantageous to improve the effective utilization of the enzyme and at the same time increase the sugar concentration in the hydrolysate[154]. To further increase the sugar concentration obtained in the fed-batch process, recycle of the hydrolysate could be another viable approach.

In this current study, the fed batch processes were investigated to increase the hydrolysis of the soybean meal carbohydrate using enzyme mixture produced by *Aspergillus niger* fermentation. Different feeding profiles for the fed batch processes are
investigated to maximize the total soluble sugar and total reducing sugar concentration in the hydrolysate. After separating the supernatant, remaining solid containing precipitated proteins are also investigated for protein content. The effect of fed batch process on the protein content in the concentrate is compared. Effect of different solid loadings on the hydrolysis yield are then compared in both batch and fed batch process. To further increase the yield recycle of the hydrolysis is also investigated. Effect of enzyme loading, retention time and recycle rates are investigated on the total soluble sugar and total reducing sugar yield. And finally the volumetric productivity of the sugar and protein precipitates are compared for batch fed-batch and recycle process.

7.2 Materials and Methods

7.2.1 Materials and equipment

Defatted soybean meal was provided by Archer Daniel Midland (Decatur, IL). Water used in the hydrolysis was Milli-Q water (18.2 MΩ-cm at 25 °C; Milli-Q Direct 8, Millipore S.A.S., Molsheim, France). (NH₄)₂SO₄ (granular), KH₂PO₄ (99% purity), HCl (concentrated acid, 37.4%) and NaOH (98.8%) were purchased from Fisher Scientific (Waltham, MA). Proteose peptone (from meat, Type I, for microbiology), MgSO₄·7H₂O (99%), MnSO₄·4H₂O (99%), ZnSO₄·7H₂O (ACS reagent grade), CoCl₂·6H₂O, FeSO₄·7H₂O (reagent grade), CaCl₂·2H₂O (reagent grade), urea (98%), NaN₃ (> 99%) and dinitrosalicylic acid (DNS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). A. niger (NRRL 341) seed culture was obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. Two 3 L Bioflo 110 fermentors (New Brunswick Scientific Co., Edison, NJ) were used for
enzyme production by fermentation. Absorbance was measured using a UV/Vis spectrophotometer (UV-1601, Shimadzu Corporation, Columbia, MD). The hydrolysis experiments were conducted in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating floor shaker, Ashville, NC). The centrifuge used was Sorvall Legend X1R from Thermo Scientific (Waltham, MA).

7.2.2 Enzyme production

Enzyme mixture used was produced by the submerged fermentation of *A. niger* 341 strain. The fungal fermentation was performed in a 3 L fermentor containing 1 L of the following fresh medium: soy hulls, 20 g/L; proteose peptone, 1.4 g/L; (NH₄)₂SO₄, 4 g/L; K₂HPO₄, 0.32 g/L; KH₂PO₄, 0.21 g/L; and MgSO₄·7H₂O, 1 g/L. The initial pH was 6.7. Inoculation was done with a pre-grown culture at an initial cell concentration of about 0.1 g/L. Temperature and agitation were maintained at 23 °C and 350 rpm. The pH and DO (dissolved oxygen concentration) were allowed to vary naturally until they dropped to 6 and 20% (air saturation), respectively. Dissolved oxygen concentration (DO) was then maintained at 20% by automatic supplementation of pure oxygen as needed. pH was controlled at 6.0 ± 0.1 by automatic addition of 1 M NaOH or HCl. The fermentation was stopped after 5 days when the enzyme production rate decreased significantly. The enzyme broth used for hydrolysis study was the cell- and solid-free supernatant collected by centrifugation of the fermentation broth at 8000 rpm (9000 g) for 10 min (Sorvall RC 5C, DuPont, Wilmington, DE).
7.2.3 Batch hydrolysis

Enzymatic hydrolysis was conducted in 250 ml flasks in a shaking incubator at 50 °C and 250 rpm. Flask contained 40 ml enzyme broth and an amount of soybean meal depending on the designed substrate concentration. Dispersed soybean meal in deionized water was warmed to 50 °C. 1 M hydrochloric acid was used to initially adjust the pH to 4.8. 0.5% sodium azide was added to prevent from microbial contamination. Enzyme broth was then added to the dispersed soybean meal solution. During hydrolysis pH was checked every 4 h and adjusted to 4.8 with 1 M NaOH if required; pH had a slight tendency to decrease during the hydrolysis. Samples were taken at regular interval in triplicate and heated immediately for 10 min in boiling water to deactivate the enzymes. Samples were then centrifuged to separately collect the solids and supernatant for further analysis.

7.2.4 Fed batch hydrolysis

Two possible ways demonstrated in our work for improving the enzymatic processing effectiveness, particularly for processing reaction mixtures of high (total) soy meal concentrations, are to (1) add the enzyme in multiple batches along the processing and (2) add the enzyme and soy meal in multiple batches along the processing. Such operations involving multiple batch addition of materials into reactors are commonly termed as “fed-batch” operations. For the enzymatic processing, the fed-batch operation can be done in many different ways (figure 7.1), divided in three groups: (1) the total amount of soy meal to be processed can be added in multiple batches at different times along the enzymatic processing (figure 7.1b), (2) the total enzyme can be added in
multiple batches (figure 7.1a), and (3) both soy meal and enzyme can be added in multiple batches (figure 7.1c). All three groups were investigated in a series experiments. In these experiments the processing conditions such as temperature and pH were kept constant at 50 °C and 4.8, respectively. Total amounts of enzyme (10 ml) and soy meal (10 g) were also kept constant and the total liquid volume was fixed at 40 ml.

Figure 7.1: Experimental scheme for fed-batch hydrolysis of soybean flour carbohydrate: fed-batch addition of enzyme (a), fed-batch addition of soybean flour (b) and fed-batch addition of both enzyme and soybean flour.

7.2.5 Hydrolysis by recycling hydrolysate

Experimental scheme for hydrolysis of soybean flour carbohydrate by recycling of enzyme is shown in figure 7.2. A first batch of enzymatic hydrolysis was done with 10
g soy flour in 10 ml enzyme and 30 ml water, i.e., at a total liquid volume of 40 ml and a soy flour concentration of 250 g/L. 30 ml supernatant was collected, by separation from the remaining solids (termed soy protein concentrate, SPC, in the field). A fraction of this separated supernatant was collected as hydrolysate and remaining fraction was recycled back for the next batch of hydrolysis of soy flour. Recycle rate was termed as percent of the starting liquid recycled in the next batch. Recycled liquid was supplemented with 10 ml of enzyme and additional deionized water to make up the total liquid volume 40 ml. Similar recycle scheme is followed for the following batches. Following N number of batches, the hydrolysis operation reaches a pseudo-steady state operation where the liquid hydrolysate collected and soy protein concentrate have similar compositions. For each batch the enzymatic processing conditions were kept the same: 50 °C, pH 4.8, and desired batch processing time. Different batch processing time of 6, 8 or 12 hours were investigated.
7.2.6 Analytical method

7.2.6.1 Sugar analysis

The supernatants collected were analyzed for concentrations of reducing sugar and total carbohydrate. Reducing sugar concentration was measured with the dinitrosalicylic (DNS) acid method [52]. This method is based on the principle that 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid in the presence of reducing sugar. The DNS reagent was prepared by dissolving 10 g 3,5-dinitrosalicylic acid, 16 g NaOH and 300 g sodium potassium tartrate (Rochelle salt) in 1 L deionized water. 3 ml DNS reagent and 1 ml supernatant sample were mixed in a test tube and then
heated in a boiling water bath for 5 min. Deionized water was added to make the total volume in the tube 25 ml. After being cooled to the ambient temperature, the reacted mixture was measured for absorbance at 550 nm in the spectrophotometer. The reducing sugar concentration was then determined using the absorbance value according to a calibration curve established with standard glucose solutions. Total carbohydrate concentrations were measured using the phenol sulfuric acid colorimetric method [53]. This method is based on the principle that carbohydrate reacts with sulfuric acid to produce furfural derivatives, which then react with phenol to develop a characteristic color. First, 1 ml sample was mixed with 1 ml aqueous phenol solution (5% v/v) in a test tube. 5 ml concentrated sulfuric acid was then added to the mixture. After 10 min reaction, the tube content was vortexed for 30 s and allowed to cool to room temperature. A reference solution was prepared in identical manner except that the 1 ml sample was replaced by deionized water. Then the absorbance at 490 nm was measured against the reference solution. The phenol used was redistilled and the 5% phenol solution was prepared fresh for each batch of analysis. Total carbohydrate concentration was determined from the absorbance reading according to a calibration curve obtained with standard glucose solutions, following the same procedure as described above.

7.2.6.2 Protein content analysis

The Kjeldahl method [58] was used to measure the nitrogen contents of solid samples. The nitrogen content was multiplied by 6.25 [59] to estimate the protein content. A 50 ml sample containing 10 to 200 mg/L protein was added to a flask and digested with 10 ml reagent containing 134 ml/l concentrated sulfuric acid, 134 g/l potassium sulfate and 7.3 g/l cupric sulfate. The digestion was carried out to completion, until the
reaction mixture became a clear solution. Then 30 ml water and 10 ml of a distillation reagent containing 500 g/l NaOH and 25 g/l Na₂S₂O₅.5H₂O were added to the digested sample. This mixture was then distilled using a distillation unit (RapidStill 1, Labconco, Kansas city, MO) to produce ammonia gas, which was absorbed in a 0.1 N boric acid solution. Then the boric acid solution was titrated using a 0.1 N H₂SO₄. To find the nitrogen concentration in the sample.

7.2.6.3 Enzyme activity assay

**Xylanase**

The method reported by Bailey et al. [55] was adopted for the xylanase assay. For best results, samples should be diluted to have xylanase activities in the range of 0.5-2 U/mL. The procedure was 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 test 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. And, add 100 µL test sample to the corresponding blank (to account for the potential turbidity introduced by the sample). DNS analysis was then done to determine the amount (mg) of reducing
sugar released, using D-xylose solutions as standards. Calculate the xylanase activity by the following equation:

\[
\text{Xylanase (U/mL)} = \frac{\text{xylose released (mg)}}{(5 \text{ min})(0.1 \text{ mL enzyme sample})} \times \frac{1 \text{ mmol}}{150.13 \text{ mg}} \times \frac{1000 \text{ mmol}}{1 \text{ mmol}} = 13.32 \times \text{xylose released (mg)}.
\]

**Pectinase**

A method was developed in this laboratory [56] using assay condition of pH 4.8 and 50°C, which was the same condition used in the soy flour hydrolysis test in this study. Samples should be diluted to the suitable enzyme activity range of 0.3-0.7 U/mL. The procedure was similar to that described above for the xylanase assay, with four differences. First, the substrate solution/suspension was 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 were incubated at 50°C for 30 min (instead of 5 min as in the xylanase assay). Third, the DNS solution used did 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 was calculated according to the following equation:

\[
\text{Polygalacturonase (U/mL)} = 1.57 \times \text{galacturonic acid released (mg)}
\]

The pectinase assay was the same as that for polygalacturonase activity except that the substrate solution was prepared with citrus pectin (Sigma Aldrich, St. Louis, MO). Also,
heating was necessary to prepare more homogeneous solution/suspension of the pectin substrate in citrate buffer [56].

**α-Galactosidase**

A method modified from that reported by Mukesh Kumar et al. [57] was used. Test samples should be diluted to have α-galactosidase activities of 0.05-0.2 U/mL. The procedure was as follows: (1) prepare the substrate solution by dissolving 0.033 g p-nitrophenyl-α-D-galactopyranoside (Sigma Aldrich, St. Louis, MO) in 100 mL 0.1 M sodium citrate buffer (pH 4.8); (2) mix 100 µL test sample with 900 µL substrate solution; (3) prepare the (enzyme-free) blank with only 900 µL substrate solution; (4) incubate 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 develop the color from released p-nitrophenol; (6) add 100 µL test sample to the blank; and (7) measure the absorbance at 405 nm. Calibration established with pure p-nitrophenol standards was used for quantitation of the enzyme-released p-nitrophenol. α-Galactosidase activity was calculated by the following equation:

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

7.3.1 Improved enzymatic processing by multiple batch addition of enzyme and/or soy meal

**Group (a): Fed-batch addition of soy meal**

In this group, soy meal was added in multiple batches while the entire enzyme amount was added at the beginning of the enzymatic processing in a single batch. The multiple additions of soy meal are indicated in Figure 7.3, together with the comparison of soy carbohydrate conversions achieved by different addition schemes.
Figure 7.3: Effect of fed batch addition of soy flour on conversion of carbohydrate in soy flour to soluble carbohydrate in the hydrolysate. Total carbohydrate conversion with time for each system (n=3) (upper) and scheme of soy flour addition at different time for each system (lower).

Surprisingly, the results showed that the enzymatic processing performance was unfortunately poorer when the soy meal was added in multiple batches. Even though the
initial rates of hydrolysis were higher in the systems with fed batch addition of soy meal, the conversions were significantly lower after the second and third batches of soy meal were added. The results also suggested that the activity of the enzyme added at the beginning of enzymatic processing has become lower over time, prior to the second and third batches of soy meal addition. This enzyme activity decrease with time was presumably due to deactivation at the processing conditions (50 °C and pH 4.8) or irreversible binding of the enzyme to non-substrate solids. This could cause the lower hydrolysis rates observed after the second and third batches of soy meal addition.

Group (b): Fed-batch addition of enzyme

When enzymes were added in batches and soy meal was added initially in a single batch, the final hydrolysis yield was found to be improved over the control (simple batch), as shown in figure 7.4. Initially the hydrolysis rates in the fed-batch enzyme addition systems were lower than that in the control (because the control had a higher enzyme-to-soy meal ratio). After the addition of the second and third batches of enzyme, the conversion increased to levels higher than that in the control system.
Figure 7.4: Effect of fed batch addition of enzyme on conversion of carbohydrate in soy flour to soluble carbohydrate in the hydrolysate. Total carbohydrate conversion with time for each system (n=3) (upper) and scheme of enzyme addition at different time for each system (lower).
**Group (c): Fed-batch addition of both soil meal and enzyme**

When soy meal and enzyme were added simultaneously in multiple batches (Figure 7.5), the carbohydrate conversion was again higher than the control systems.

![Graph](https://via.placeholder.com/150)

**Figure 7.5:** Effect of fed batch addition of both soy flour and enzyme on conversion of carbohydrate in soy flour to soluble carbohydrate in the hydrolysate. Total carbohydrate conversion with time for each system (n=3) (upper) and scheme of soy flour and enzyme addition at different time for each system (lower).
In comparison, using fed-batch enzyme addition (to soy meal at a concentration of 250 g/L), the soy carbohydrate conversion was increased from 65% in control to 72%; using fed-batch addition of enzyme and soy meal simultaneously (to a final soy meal concentration of 250 g/L), the conversion was increased to 71%.

7.3.2 Comparison fed batch and batch at different solid loading

While these latter two fed-batch-addition approaches gave similar improvements in the final hydrolysis yield, simultaneous fed-batch addition of soy meal and enzyme is particularly beneficial for processing soy meal at cumulative (total) concentrations higher than 250 g/L. This is shown in an additional experiment where this fed-batch addition approach of both enzyme and soy meal was applied to different cumulative soy meal concentrations, from 100 g/L to 375 g/L. Compared in Figure 7.6 are the carbohydrate conversions achieved by the batch process and the fed-batch addition of both enzyme and soy meal at different final soy meal concentrations. The % improvement in carbohydrate conversion by the fed-batch operation is larger for the system with a higher soy meal concentration. More importantly, the negative effect of high solid loading can be substantially mitigated by the fed-batch operation.
Figure 7.6: Comparison of final total soluble carbohydrate conversion achieved by batch versus fed-batch (enzyme + soy meal) addition for different total soy flour concentrations (n=5): 100, 250 and 375 g/l.

7.3.3 Recycle of hydrolysate liquid containing enzyme

In the above batch and fed-batch processes, the maximum soluble carbohydrate concentration (measured by the common reducing sugar analysis) achievable was 65-70 g/L from processing a total of 375 g/L soy meal. To further increase the sugar concentration in the hydrolysate the recycle of the enzyme is investigated. For designing the recycle process enzyme degradation for pectinase and α-galactosidase over time in the batch process was investigated. The enzyme activity of pectinase and α-galactosidase in the batch process is shown in Figure 7.7. Pectinase activity was mostly removed from
the hydrolysate after 6 h (although a portion could be adsorbed on the solid substrate and continue act on the soy meal). The longer hydrolysis time helped to achieve degradation of soluble carbohydrate to reducing sugars. It was therefore thought that longer batch processing time was likely to have lower marginal benefit. So the recycle study is conducted to achieve additional benefit in the process. The effect of recycle rate, retention time, enzyme loading effects during the recycle process is further investigated.

![Enzyme activity](image)

Figure 7.7: Activity profiles of the enzymes pectinase and α-galactosidase in hydrolysate with time during the first batch of enzymatic hydrolysis (n=3).

7.3.3.1 Progression of recycled hydrolysis

With the progression of recycle, sugar concentration in the liquid build up. The development in the sugar concentration depends on the sugar conversion after each hydrolysis, the efficiency of the solid liquid separation and the rate of liquid hydrolysate recycle. For simplified calculation, the sugar concentration build up can be represented
by the geometrical series. The equation can be used for prediction of experimental results at different recycle rate.

\[ S_n = S_0 + S_0 r + S_0 r^2 + S_0 r^3 + \cdots + S_0 r^n \]

\[ S_n = S_0 \left( \frac{1 - r^{n+1}}{1 - r} \right) \]

\[ S_{max} = S_0 \left( \frac{1}{1-r} \right) \]

Where \( S_n \) is the sugar concentration of the hydrolysate after nth recycle, \( r \) is the recycle rate and \( S_0 \) is the sugar concentration of the hydrolysate after without any recycle. \( S_{max} \) represents the maximum sugar concentration achievable using a specific recycle rate, which will no longer increase with more recycle step.

Total soluble carbohydrate and reducing sugar concentration and activity of \( \alpha \)-galactosidase progression along with each recycle of hydrolysate is presented in the figure 7.8. The enzyme activity used in this process is cellulase 0.7 FPU, xylanase 180 U, pectinase 7.25 U and \( \alpha \)-galactosidase of 8.1 U per g of soybean flour. The recycle rate of 62.5% of the starting liquid and retention time of 8 hours for every batch were used in the recycle process. After 7 recycle system, sugar concentration of 104.28 g/l was achieved which is comparable to the predicted concentration of 107 g/l achievable by the equation. This suggests that there was no product inhibition at this high concentration of hydrolyzed sugar. From the figure it was found that in the first batch before recycle the reducing sugar concentration was only 27 g/l compared to the total carbohydrate concentration of 40 g/l. Reducing sugar concentration was lower because the retention time was only 8 hours and the \( \alpha \)-galactosidase present in the enzyme broth was not
capable to degrade all the soluble carbohydrate, mainly stachyose and raffinose, into monomer. However, the galactosidase activity is increased when it would be recycled in the following batch. The build-up of α-galactosidase activity helped to degrade the soluble carbohydrate to reducing sugar over progression of recycle process. After 5 recycle steps, the total carbohydrate and reducing sugar concentration became almost same. The increase of α-galactosidase activity to approximately 15.9 U/g of soy flour helped to neutralize the difference between total carbohydrate and reducing sugar. This indicate the benefit of using recycle process to degrade the total carbohydrate in the reducing sugar by building up the activity over recycle process within very short retention time.
Figure 7.8: Total soluble carbohydrate and reducing sugar concentration along with the progression of the recycle (n=3). The enzyme activities of the enzymes used are cellulase 0.7 FPU, xylanase 180 U, pectinase 7.25 U and α-galactosidase of 8.1 U per g of soybean flour. Soy flour concentration in each recycle was 250 g/l. The recycle rate of 62.5% of the starting liquid and retention time of 8 hours for every batch were used in the recycle process.

7.3.3.2 Effect of enzyme loading

Different enzyme loading in the recycle process is studied to understand the effect on the effective conversion of the total soluble carbohydrate to reducing sugars as hydrolysate value is higher with soluble sugars in the monomeric form. Total soluble carbohydrate and reducing sugar concentration with the progression of recycle process
using three different strength of enzyme is presented in the figure 7.9. Enzyme strength was designated as low medium and high strength. The activities are provided in figure 7.9. Total soluble carbohydrates are presented in the lines and the reducing sugars are presented in the bars to differentiate clearly. The total soluble carbohydrate concentration was comparable with all three strength of enzyme. With high strength enzyme the maximum concentration, 109 g/l, was slightly higher than that with the low and medium strength enzyme. However, the reducing sugar concentration differed significantly with the strength of enzymes. With high strength enzyme the reducing sugar concentration and total carbohydrate concentrations are almost similar from the very first batch of the recycle system. On the other hand, with low strength enzyme, the gap between total carbohydrate and the reducing sugars widens during the first 3 recycle steps, then the gap started to reduce in the subsequent recycles. This indicates that with low enzyme strength, difference initially was due to low α-galactosidase activity which was insufficient to breakdown the soluble carbohydrates, mainly oligosaccharides e.g. stachyose and raffinose, to reducing sugars. However, with the progression of the recycle, the α-galactosidase activity build up and after 4 recycle steps the increased α-galactosidase activity were able to close the difference between the soluble carbohydrates and reducing sugars. Using weaker enzyme strength, the total soluble carbohydrate and reducing sugar was not the same at steady state condition but with medium strength enzyme it was able to degrade all the soluble carbohydrate in reducing sugar in steady state. This suggests that if low enzyme strength is used then to reach degrade the total soluble carbohydrate to reducing sugar, longer retention time may be required.
Figure 7.9: Total soluble carbohydrate and reducing sugar concentration with the progression of recycle process using different strength of enzyme (n=3). The enzyme broth used has activities of cellulase 0.7 FPU, xylanase 180 U, pectinase 7.25 U and α-galactosidase of 8.1 U per ml. This enzyme broth was used by three different dilution (DF): DF 1, DF 2 and DF2. The recycle rate of 62.5% of the starting liquid and retention time of 8 hours for every batch were used in the recycle process.

7.3.3.3 Effect of recycle rate

The effect of the proportion of recycled hydrolysate was studied by comparing the recycle rate of 25%, 37.5%, 50% and 62.5% in hydrolysis. The maximum sugar concentration (total carbohydrate) achieved and the corresponding protein content in the soy protein precipitate is presented in the figure 7.10 for different recycle rate. The
Retention time was considered for 8 hours and the enzyme used with the activity of cellulase 0.7 FPU, xylanase 180 U, pectinase 7.25 U and α-galactosidase of 8.1 U per g of soybean flour. Highest sugar concentration achieved was 104 g/l using highest recycle rate of 62.5% as expected. The maximum sugar concentration for other recycle rate was decreased along with the decreasing recycle rate. At lowest recycle rate of 25%, the sugar concentration achieved was only 54 g/l. Higher sugar concentration is expected for higher value of hydrolysate, but it came in expense of the protein content decrease in the soy protein concentrate. The protein content can be decreased to as low as 58% in the highest recycle rate of 62.5%. In the lower recycle rate of 25%, the protein content is 66% compared to 69% in the batch process. The protein content decreased due to two factors. One is due to the trapped hydrolysate with high sugar concentration inside the wet protein. As the soluble carbohydrate concentration in hydrolysate increased with hydrolysate recycle, the amount of carbohydrate trapped in the wet solid mass also increased. Another reason is the lower retention time of the solids compared to batch process, which is responsible for not hydrolyzing the insoluble carbohydrates in the solid effectively. However, remaining hydrolyzed reducing sugar in the protein concentrate may be beneficial for its use as animal and aquaculture feed because the monosaccharides can serve as readily available energy source to the animals and fish without indigestion problem. But, it reduces the protein content in the concentrate. However, the protein content can be further increased by using a wash step to remove the soluble carbohydrate to increase the protein content. Whether the wash step to increase the protein content is required or not, it will depend on the protein requirement of the specific use of the soy protein concentrate.
Figure 7.10: The maximum sugar concentration (total soluble carbohydrate) achieved and the corresponding protein content in the soy protein precipitate for different recycle rate of enzyme: 25, 37.5, 50 and 62.5% (n=3). The retention time was considered for 8 hours and the enzyme used with the activity of cellulase 0.7 FPU, xylanase 180 U, pectinase 7.25 U and α-galactosidase of 8.1 U per g of soybean flour.
7.3.4. Volumetric productivity

Volumetric productivity is a critical factor in the process design as it defines the size of the reactor required for a designed output. The volumetric productivity of the different hydrolysis strategies discussed in the current study are shown in figure 7.11. In figure 7.11a, the volumetric productivity of sugars and protein concentrates are compared among batch, fed-batch and recycle process. In recycle process, volumetric productivity of both the sugar in the hydrolysate, 3.75 gl⁻¹h⁻¹, and protein, 10.25 g kg⁻¹h⁻¹, are significantly higher compared to the batch and fed batch hydrolysis. In a batch reaction, the total amount of liquid is inefficiently incubated with fixed amount of substrate throughout the complete reaction time, while in recycle process same amount of liquid is more effectively utilized for saccharification of multiple addition of solid substrate. The amount of water usage is also decreased significantly in recycle process. Sometimes viscosity of the liquid due to high solid loading can be inhibitory for the hydrolysis. To circumvent the high viscosity stage, gradual feeding of the substrate has been studied in the fed batch process. But in fed batch process the total solid to liquid ratio was kept constant which didn’t increase the volumetric productivity. But in recycle process, the fresh substrate was added after separating solids from the previous loading and therefore a lower viscosity can be maintained even after new addition of substrate.
Figure 7.11: Volumetric productivity of sugar in the hydrolysate and protein in the soy protein concentrate with different hydrolysis strategies (n=3): batch, fed-batch and enzyme recycle (a). Volumetric productivities at different retention time (6h, 8h and 12h) during the enzyme recycle process (n=3).
Retention time of the solids in the recycle process is also important factor in terms of volumetric productivity. Volumetric productivity of sugars and protein concentrate with different retention time of solids in the recycle process is presented in the figure 7.11b. Higher productivity is obtained for both sugars and protein concentrate with lower retention time. However, the lower retention time is responsible for lower protein content in the soy protein concentrate as discussed earlier. So the decision on the right retention time will depend on the protein content requirement in the soy protein concentrate product for the specific use.

7.4 Conclusion:

Soybean flour is mainly used for its high value protein. But the carbohydrate presents in the soy flour can also bring added value in the soybean flour if they can be effectively hydrolyzed and can be used as a fermentation feedstock. In this study, the comparison of enzymatic hydrolysis of the soy flour carbohydrate in batch fed-batch and recycle process is discussed. Fed batch process can be used to hydrolyze soy flour carbohydrate at relatively higher solid loading compared to batch process. Fed-batch process was beneficial to neutralize the negative effect of high solid loading hydrolysis in batch operation. However, to increase the sugar concentration further in the hydrolysate for its application as fermentation feedstock, recycle process was found useful. Recycle process can increase the sugar concentration up to 104 g/l in the hydrolysate. The volumetric productivity of sugars and protein concentrate were also increased in the recycle process. Ability of degradation of oligosaccharides to reducing sugars using low strength enzyme was another advantage of recycle process. The recycle process is
recommended for hydrolysis of soy flour carbohydrate with higher productivity of sugar and protein concentrate with low water usage.
CHAPTER VIII

REFINEMENT OF SOY PROTEIN CONCENTRATE, SOY PROTEIN ISOLATE AND FERMENTABLE SUGAR SYRUP

8.1 Introduction

To meet the requirement of growing population, low-cost vegetable sources of protein become increasingly more important to supplement animal protein. Soybean is the predominant source of vegetable protein and oil, supporting over 65% of the global demand of vegetable protein [2]. Soy protein contains suitable levels of all essential amino acids (except for methionine and leucine), which is an important advantage over many other vegetable protein sources [35, 155, 156]. Soy protein products can also offer important health benefits. They reduce the risk of heart disease if included in low-fat diets [67, 157, 158]. They are helpful for weight reduction and control programs [159, 160]. Soy protein products can play an important role in preventing osteoporosis [68, 161]. They may also help to reduce certain cancers [162, 163].

Based on protein contents, soy protein products are classified into three main categories: soy flour, soy protein concentrate (SPC), and soy protein isolate (SPI). Soy flour contains about 50% protein and 30-35% carbohydrate. Majority of the animal feeds require sources with higher protein contents. SPCs with higher protein contents (60-68%) have therefore been produced by partial removal of the carbohydrate in soy flour using,
for example, one of the following methods [28-30]: (1) hot water wash, (2) aqueous alcohol (20-80%) wash, and (3) dilute mineral acid precipitation. The heat treatment and alcohol wash methods may denature protein, resulting in SPCs with low protein dispersibility indices (PDI), 10-15% [34], and lower functionality [35]. In addition, SPCs still contain significant amounts of indigestible carbohydrate. Soy flour carbohydrate is predominantly non-starch polysaccharides and oligosaccharides [36, 37]. By the above methods, essentially all insoluble carbohydrate remains in the SPCs produced [38], which may cause lower digestibility when the SPCs are included in animal feed [39, 40]. Oligosaccharides, mainly raffinose and stachyose, are largely soluble in the wash solvents used. But, depending on process conditions, certain portions may remain trapped in the SPCs due to incomplete wash and the diffusional limitation of larger oligosaccharides from the solid matrix [43, 111]. Their concentrations are important to consider because humans and other monogastric animals lack the α-galactosidase enzyme necessary to hydrolyze the α-galactosyl linkages in their structures [41, 42]. After entering the lower intestinal tract, they are metabolized by bacteria and the intestinal gas produced can cause considerable discomfort [43, 44]. Ensuring more complete removal of these oligosaccharides may increase the nutritional value of soy protein products [45, 46]. Another consideration is the presence of non-functional indigestible fibers in the SPCs produced by the above methods. These fibers can bind with protein and lower the protein digestibility [47].

SPIs are another group of soy protein products. The process for making SPIs involves dissolving protein from soymeal at high pH, collecting supernatant by centrifugation, and then re-precipitating protein at the isoelectric point [31, 32]. SPIs
have significantly higher protein contents (85-90\%) than SPCs and contain almost no water-insoluble carbohydrate. However, the protein yield from the process can be as low as 45\%, making SPIs expensive to produce [33].

An alternative process that can produce SPC and SPI with similar or higher protein contents and no indigestible or poorly digestible carbohydrate is desirable. In earlier studies, we demonstrated the effectiveness of an enzymatic process to hydrolyze soy flour carbohydrate while keeping the majority of protein insoluble for easy separation[150]. The carbohydrate hydrolysis aspect of that process was well characterized but the protein enrichment and collection aspects were not. The objectives of this study were to develop and optimize the methods for separating and collecting protein-enriched products (SPC and SPI) after the enzymatic hydrolysis, and to address several remaining issues for completing this enzyme-based soybean biorefinery platform. The issues included (1) the potential change of amino acid profile by the enzymatic process, important for nutritional value of the protein products, (2) the resulting sugar composition of the hydrolysate, important for use as, e.g., fermentation feedstock, (3) material balances of the overall platform, critical for the future process economics analysis, and (4) the effects of using different enzyme broths in the process. A more complete summary of the experimental design is given in the following Materials and Methods section.
8.2 Materials and Methods

8.2.1 Study design

In an earlier study, pH 4.8 and 50 °C were found to be optimal for the enzymatic hydrolysis of soy flour carbohydrate (unpublished results). After the hydrolysis, a majority of the protein remained insoluble and could be easily collected by centrifugation. A small fraction of protein (and amino acids and small peptides) dissolved into the liquid, together with the hydrolyzed carbohydrate. If not recovered, this protein dissolution decreased the overall protein yield of the process. Also, for the following purposes: minimizing reactor size, obtaining hydrolysate of a high sugar concentration, and reducing water usage and wastewater treatment, it was desirable to use high soy flour loading in the process. But, with increasing solid loading, the large amount of wet mass of insoluble protein collected by centrifugation also contained more liquid with the hydrolyzed carbohydrate. This lowered the collectable volume of hydrolysate and the yield of fermentable sugar. Upon drying of the wet mass, the carbohydrate in the entrapped liquid became part of the product and reduced the protein content of the product.

According to the above observations, the following studies were conducted. (1) To gauge the extent of possible minimization of hydrolysate entrapment in the wet mass collected by centrifugation, the effect of different centrifugation speeds was investigated. (2) The centrifugation-collected wet mass was washed with different solvents to evaluate the effects on soluble carbohydrate removal and protein content increase. (3) To recover the proteins dissolved into the hydrolysate, different precipitation methods were studied.
And, (4) the above experiments were all done with the systems hydrolyzed with the enzyme broth from an *A. niger* fermentation. To investigate the effects of using different enzyme broths, one set of experiments was made to compare the outcomes with 5 enzyme broths using the optimized process established. Experiments made for these studies are described in more detail in the following sections.

8.2.2 Materials and equipment

Defatted soy flour (7B soy flour) and soy hulls were provided by Archer Daniel Midland (Decatur, IL). Water used in the hydrolysis was Milli-Q water (18.2 MΩ-cm at 25 °C; Milli-Q Direct 8, Millipore S.A.S., Molsheim, France). (NH₄)₂SO₄ (granular), KH₂PO₄ (99% purity), HCl (concentrated, 37.4%) and NaOH (98.8%) were purchased from Fisher Scientific (Waltham, MA). Proteose peptone (from meat, Type I, for microbiology), MgSO₄·7H₂O (99%), MnSO₄·4H₂O (99%), ZnSO₄·7H₂O (ACS reagent grade), CoCl₂·6H₂O, FeSO₄·7H₂O (reagent grade), CaCl₂·2H₂O (reagent grade), urea (98%), NaN₃ (> 99%) and dinitrosalicylic acid (DNS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). *Aspergillus niger* (NRRL 341), *Aspergillus aculeatus* (NRRL 2053), and *Trichoderma reesei* (Rut-C30, NRRL 3469) seed cultures were obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. Two 3 L Bioflo 110 fermentors (New Brunswick Scientific Co., Edison, NJ) were used for enzyme production by fermentation. Absorbance was measured using a UV/Vis spectrophotometer (UV-1601, Shimadzu Corporation, Columbia, MD). The hydrolysis experiments were conducted in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating floor shaker, Ashville, NC). The
centrifuge used was Sorvall Legend X1R from Thermo Scientific (Waltham, MA). The vacuum oven used was manufactured by Lab-line Instruments, Inc. (Melrose Park, IL).

8.2.3 Enzyme production

Fungal fermentation was performed in the 3 L fermentor containing 1 L medium of the following composition: soy hulls, 40 g/L; proteose peptone, 1.4 g/L; (NH₄)₂SO₄, 4 g/L; K₂HPO₄, 0.32 g/L; KH₂PO₄, 0.21 g/L; and MgSO₄·7H₂O, 1 g/L. The initial pH was 6.7. Inoculation was done with a pre-grown culture at an initial cell concentration of about 0.1 g/L. Temperature and agitation were maintained at 23 °C and 350 rpm. Dissolved oxygen concentration (DO) was allowed to drop naturally to 20% (air saturation); it was then maintained at 20% by automatic supplementation of pure oxygen. For the *A. niger* fermentation the pH was dropped linearly to 6.0 in the first 3 days by controlled addition of 1 M NaOH or HCl, then maintained at 6.0 ± 0.1 in the 4th day, and dropped linearly again to 5 in the 5th day. The fermentation was stopped after 5 days. The enzyme broth used for hydrolysis study was the cell- and solid-free supernatant collected by centrifugation of the fermentation broth at 8000 rpm (9000 g) for 10 min (Sorvall RC 5C, DuPont, Wilmington, DE).

8.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in 250 ml flasks in a shaking incubator at 50 °C and 250 rpm. Each flask contained 10 ml enzyme broth, 30 ml deionized water, and 10 g/l soy flour. The soy flour was first dispersed in deionized water and warmed to 50 °C. 1 M HCl was used to adjust the pH to 4.8. Then, the enzyme broth was added.
Duplicate control systems, prepared with enzyme-free deionized water, were included in each batch of experiments. Samples were taken at 0, 5, 10, 24 and 48 h in triplicate and heated immediately for 10 min in a boiling water bath to deactivate the enzymes.

8.2.5 Separation of SPC and liquid hydrolysate: Effect of centrifugation speed

Samples (20 ml) taken from hydrolysis reactors were solid-liquid two-phase mixtures. They were centrifuged in 50 ml tubes for 10 min to separately collect the wet solid mass and the supernatant. Supernatant was stored for further analysis. The wet solid mass collected was dried overnight in a vacuum oven at 60°C and then ground to powders. In this experiment, different centrifugal forces from 4000×g to 12000×g were compared for the resultant liquid amount remaining in the wet solid mass collected, determined from the weight difference between the wet and dried solid mass. The protein content was measured from the dried powders (method described later).

8.2.6 Collection of SPI from hydrolysate: Effects of different precipitation methods

The liquid hydrolysate collected after the enzymatic hydrolysis contained some dissolved protein. It was desirable to recover the protein as an SPI product with very high protein content. The following approaches were investigated in this study to precipitate out soluble protein from the hydrolysate: heating, ethanol addition, pH adjustment, and water addition. (Protein precipitation by water addition to the hydrolysate was accidentally observed during the project. It was thus included here for a more systematic study.) Other potentially more expensive membrane-based approaches such as ultrafiltration and electrodialysis [164] were not examined.
Heat-induced precipitation was studied with 10 ml hydrolysate in 50-ml vial in a water bath (Boekel Grant ORS 200, Feasterville, PA). Four temperatures, 60, 70, 80 and 95 °C, were studied for varying heating times from 10 to 60 min. Precipitation by ethanol addition was done at room temperature. Ethanol was added to 10 ml hydrolysate to give varying ethanol-to-hydrolysate ratios (v/v) of 0.2 to 2. Precipitation by water addition was similarly investigated but at dilution ratios of 2 to 10. For the pH-induced precipitation, hydrolysate was adjusted to a chosen pH in the range of 3.5 to 9 using 1 N HCl or NaOH. Precipitates from all these experiments were collected by 10-min centrifugation at 7500×g, dried at 100°C for 24 h, and then measured for dry weight to determine the recovery yield. Supernatants were stored for analysis of reducing sugar.

8.2.7 Effects of enzyme sources on the process

The effects of different enzyme sources on SPC and SPI recovery, protein contents of SPC and SPI, and the sugar compositions in the final hydrolysate were investigated. Five enzyme systems with different cellulase, xylanase, pectinase and α-galactosidase activities were chosen (Table 8.1). Two of the systems were mixtures of enzyme broths from two fungal fermentations. These systems were diluted 4-fold for use in the enzymatic hydrolysis studies. After enzymatic hydrolysis, the protein-rich precipitates were separated from the liquid hydrolysate by centrifugation at 7500×g for 10 min. The collected solids were then vacuum dried (48 h at 60°C) and ground to get SPC powders. Protein, carbohydrate, ash and fat contents of the SPC were determined. Separately, liquid hydrolysate was heat-treated at 95°C for 30 min to precipitate protein, which was collected by centrifugation, vacuum dried, ground to get SPI powders, and
analyzed for various contents, all by the same methods described above for SPC. After the SPI removal, the remaining hydrolysate (a sugar-rich syrup) was collected and stored for reducing-sugar composition analysis.

Table 8.1: Sources and key enzyme activities for the five enzyme systems evaluated for protein enrichment and carbohydrate hydrolysis

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Cellulase (FPU/ml)</th>
<th>Xylanase (U/ml)</th>
<th>Pectinase (U/ml)</th>
<th>α-galactosidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em></td>
<td>0.48±0.02</td>
<td>126.1±5.6</td>
<td>10.6±0.05</td>
<td>11.2±0.03</td>
</tr>
<tr>
<td><em>T. reesei</em></td>
<td>3.24±0.12</td>
<td>243.5±14.5</td>
<td>2.75±0.02</td>
<td>0.6±0.01</td>
</tr>
<tr>
<td><em>A. aculeatus</em></td>
<td>0.71±0.04</td>
<td>14.41±3.6</td>
<td>5.26±0.01</td>
<td>1.9±0.03</td>
</tr>
<tr>
<td><em>A. niger-A. aculeatus</em> mixture</td>
<td>0.59±0.03</td>
<td>70.25±4.7</td>
<td>8.75±0.04</td>
<td>6.5±0.06</td>
</tr>
<tr>
<td><em>A. niger-T. reesei</em> mixture</td>
<td>1.85±0.05</td>
<td>184.8±8.5</td>
<td>6.67±0.05</td>
<td>5.9±0.09</td>
</tr>
</tbody>
</table>

8.2.8 Refinement of SPC: Effect of SPC washing with different solvents

In the aforementioned SPC collection process, the wet solids collected by centrifugation included a significant amount of liquid hydrolysate which contained the soluble/hydrolyzed carbohydrate and others. It was desirable to further increase the protein content in the SPC by removing the soluble carbohydrate embedded. This would produce a more refined product for the applications where higher protein contents are necessary. This would also increase the yield of soluble carbohydrate from the process.
For this purpose, experiments were done to evaluate the effects of washing by 3 types of solvents: water, methanol-water mixtures with 20-70% (v/v) methanol, and ethanol-water mixtures with 20-70% ethanol. The washing was done with 5 g wet solid mass and varying solvent volumes in 125 ml flasks.

Water wash was done with the water-to-dry-SPC ratios of 5 to 20 (v/w). Water addition caused pH to increase. To reduce the associated protein loss, pH was adjusted back to 4.8 using 3 N HCl. The flask contents were then mixed for 1 h in a shaker operating at 25°C and 250 rpm. The mixture was then centrifuged at 7500×g for 10 min to separate the supernatant and wet solids. Protein and carbohydrate concentrations in the supernatant (wash) were measured to determine the percent carbohydrate removal and percent protein loss caused by the water wash.

For ethanol wash, two sets of experiments were done. The first set was done to determine the optimal ethanol concentration in the ethanol-water mixture for minimizing the protein loss by the wash. The second batch of experiments was conducted with the optimized ethanol-water mixture, to determine the optimal liquid-to-dry-solid-mass ratio (v/w) for maximizing the soluble carbohydrate recovery and minimizing protein loss. For the first set of experiments, 5 g wet solids with known water content were added to a 50 ml glass vial. Pre-calculated amounts of water and ethanol were added to make a total liquid volume of 15 ml (including the original water in the wet solids) and a chosen ethanol concentration varying in the range of 20% to 70% for different systems. pH was adjusted to 4.8 by addition of 1 N HCl. The subsequent operations, including mixing of the vial contents in the shaker, centrifugation, and measurements of protein and carbohydrate concentrations, were the same as those described above for water wash. For
the second set of experiments, 5 g wet solids in each 50 ml vial were again used. Calculated amounts of ethanol and water were then added to have the same optimized ethanol concentration in the final liquid phase but different liquid-to-dry-solids ratios (v/w) ranging from 6 to 15. Similar operations as described earlier were followed thereafter. The same design with two sets of experiments was also used for the study of methanol wash.

8.1.9 Analytical methods

Supernatants were analyzed for concentrations of dissolved reducing sugar and total carbohydrate. The former was analyzed by the dinitrosalicylic (DNS) acid method [165]; the latter by the phenol sulfuric acid method [165]. For individual sugar concentrations, samples were filtered (0.2 µm, Millipore, Danvers, MA) and analyzed using a high pressure liquid chromatography system (HPLC Shimadzu) with a refractive index detector. A carbohydrate column (Supelcogel Column Pb, 300 x 7.8 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA) was used at 80ºC. The mobile phase was HPLC grade water (Fisher Scientific, Pittsburgh, PA) at a flow rate of 0.5 ml/min.

The Kjeldahl method [58] was used to measure the nitrogen contents of solid samples. The nitrogen content was multiplied by 6.25 [59] to estimate the protein content. A 50 ml sample containing 10 to 200 mg/l protein was added to a flask and digested with 10 ml reagent containing 134 ml/l concentrated H₂SO₄, 134 g/l K₂SO₄ and 7.3 g/l CuSO₄. The digestion was carried out to completion, until the reaction mixture became a clear solution. Then 30 ml water and 10 ml of a distillation reagent containing 500 g/l NaOH and 25 g/l Na₂S₂O₃.5H₂O were added to the digested sample. This mixture was then
distilled using a distillation unit (RapidStill 1, Labconco, Kansas city, MO) to produce ammonia gas, which was absorbed in 0.1 N H₃BO₃. Then the H₃BO₃ solution was titrated using 0.1 N H₂SO₄ to find the nitrogen concentration in the sample. Ash and fatty acid content measurements were done by the University of Missouri Agricultural Experimental Station. Standard NREL method [60] was used for ash content analysis; Soxhlet hexane extraction method was used to measure the crude fat content [61].

8.3 Results and Discussion

8.3.1 Enzymatic hydrolysis

Enzymatic hydrolysis was done using a 4-fold diluted A. niger fermentation broth after clarification by centrifugation. The (undiluted) broth contained a complex mixture of enzymes with the following measured activities: 0.48 FPU/ml of cellulase, 126.1 U/ml of xylanase, 10.6 U/ml of pectinase and 11.2 U/ml of α-galactosidase. As shown in Figure 8.1, over 74% of the total carbohydrate in soy flour was released into the hydrolysate, measured both as dissolved total carbohydrate and reducing sugar, after 48 h reaction. Further, the equal concentrations of total soluble carbohydrate and reducing sugar obtained indicated that essentially all soluble carbohydrates were monosaccharides; otherwise, the glycosidic bonds in non-monosaccharides would consume the reducing end of an appreciable fraction of the bonded monomeric units. (Faster and higher carbohydrate release could be achieved by using a less diluted broth.) The hydrolysate and solids thus obtained were used as the starting materials for most of the experiments done in this work.
Figure 8.1: Time profiles of enzymatic conversion of soy flour carbohydrate to soluble carbohydrate, measured as reducing sugar and total carbohydrate in the hydrolysate, respectively (n=3). Enzyme broth contains the activity of 0.48 FPU/ml of cellulase, 126.1 U/ml of xylanase, 10.6 U/ml of pectinase and 11.2 U/ml of α-galactosidase.

8.3.2 Separation of post-hydrolysis liquid and solid streams at different centrifugation forces

After hydrolysis, the wet SPC solids were separated from the liquid hydrolysate by centrifugation. Preliminary filtration tests were also done but the presence of very small particles, < 20 μm, tended to clog the filter media. Centrifugal separation was easier. Different centrifugal forces from 4000×g to 12000×g, for 10 min centrifugation, were tested to compare their effects and estimate the maximum extent of separation obtainable. The volumes of liquid hydrolysate collected, the liquid volumes entrapped in
the wet SPC solid mass, and the water contents of the wet solids obtained at different centrifugal forces are presented in Figure 8.2. At 12000×g, the highest centrifugal force studied, 30 ml hydrolysate (out of the total liquid volume of 40 ml) was separated from the wet solids and the water content of the wet solids remained at about 68%. The separation improvement diminished with increasing centrifugal force, particularly at higher than 7500×g, which was therefore used in subsequent experiments. As an example, the compositions of starting soy flour and the SPC (prior to drying) and hydrolysate obtained by 10-min centrifugation at 7500×g are summarized in Table 8.2. The dissolved carbohydrate concentration in hydrolysate was 60 g/L which corresponded to the release of about 75% of the total carbohydrate present in the original soy flour. The protein concentration in hydrolysate was 49 g/L, part of which, as described later, would be recovered as SPI.
Figure 8.2: Effects of different centrifugal forces on separation of wet SPC solids from enzymatic hydrolysate, in volumes of hydrolysate collected versus entrapped and the weight % of water in wet SPC solids (n=3).

Table 8.2: Compositions of starting soy flour and the wet soy protein concentrate solids and hydrolysate obtained by 10-min centrifugation at 7500×g

<table>
<thead>
<tr>
<th></th>
<th>Soy flour, g (% DM)</th>
<th>Soy protein concentrate (SPC), g (% DM)</th>
<th>Hydrolysate, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>10 (100)</td>
<td>18.5±0.4 (100)</td>
<td>29.5±0.5</td>
</tr>
<tr>
<td>Moisture</td>
<td>0.8±0.2</td>
<td>13.0±0.7</td>
<td>26±1.0</td>
</tr>
<tr>
<td>Protein</td>
<td>5.2±1.1 (56)</td>
<td>3.8±0.2 (69)</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>3.1±0.9 (34.5)</td>
<td>1.3±0.1 (23)</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>0.5±0.1 (5.5)</td>
<td>0.3±0.1 (4.5)</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>Fat/oil</td>
<td>0.3±0.0 (3)</td>
<td>0.2±0.0 (3.6)</td>
<td>Not measured</td>
</tr>
</tbody>
</table>

Note: Total liquid volume – 40 ml, mixed with 10 g soy flour for hydrolysis; DM – dry matter

8.3.3 Collection of soy protein isolate

The hydrolysate collected after the enzyme hydrolysis contains soluble proteins. This soluble protein can be precipitated to get soy protein isolate.

8.3.3.1 SPI collection by heat precipitation

Heat precipitation was investigated for effects of temperature and time. Figure 8.3a shows the amount (g) of SPI collected after treating 10 ml hydrolysate. 95 °C, the highest temperature tested, was clearly more effective. 0.46 g SPI was collected by 15 min treatment at this temperature, while only 0.4 g SPI was obtained at 80 °C even after
60 min. 15 min at 95°C was considered a good SPI collection condition, if protein
denaturation would not be a major factor in the intended application.

8.3.3.2 SPI collection by ethanol precipitation

In Figure 8.3b, the SPI amounts precipitated from 10 ml hydrolysate are shown
for different ethanol-to-hydrolysate ratios (v/v), i.e., 0.2 to 2. SPI recovery was
significantly lower at ratios lower than 1; but, increasing the ratio beyond 1 did not
further improve SPI recovery. At the optimal ratio of 1, the maximum recovery obtained,
about 0.48 g per 10 ml hydrolysate, was the same as that found by heat treatment.

8.3.3.3 SPI collection by water addition

Figure 8.3c shows the precipitated amounts when 10 ml hydrolysate was diluted
by water at different dilution factors. While the salting-in effect on protein solubility was
known[166], it was rather surprising to find that almost all (0.46 g per 10 ml hydrolysate)
precipitable protein, compared to the 0.48 g obtained with ethanol or heat precipitation,
could be collected at the high dilution factor of 10. The negative effect of this procedure
is that the hydrolysate is diluted to such extents that the soy carbohydrate released cannot
be used as fermentation feedstock. Too much wastewater may be generated.

8.3.3.4 pH adjustment

Hydrolysate contains a mixture of proteins. Different proteins have different
isoelectric points. Although majority of the soy proteins had pI around 4.8, some had
different pI values and this might be the reason for their presence in the hydrolysate.
Figure 8.3d shows the effect of pH adjustment from 3.5 to 9 on SPI recovery from the
hydrolysate. The recovery was low, up to 0.1 g at pH 9, compared to the other precipitation methods studied. pH adjustment was not effective.

![Graphs showing recovery from different methods](image)

Figure 8.3: Soy protein isolate recovery from enzymatic hydrolysate evaluated by different methods – heat treatment (top left), ethanol precipitation (top right), water dilution (bottom left) and pH adjustment (bottom right) (n=3).

8.3.4 Material balances

According to the results described above, the process developed in this work included the following steps: enzymatic hydrolysis (48 h, 50°C, pH 4.8, solid loading 250
g/l, cellulase 0.48 FPU/g, xylanase 126 U/g, pectinase 10.6 U/g, α-galactosidase 11.2 U/g); separation of solid and liquid streams by centrifugation (10 min, 7500×g); SPI precipitation from hydrolysate (heat treatment 15 min, 95°C); and centrifugation to separate sugar syrup and SPI (10 min, 7500×g). Figure 8.4 shows the overall material balances of the process. From 100 g defatted soy flour, the process produces 53.5 g SPC with 70% protein, 9.1 g SPI with 89% protein, and 280 ml sugar syrup with 60 g/l reducing sugar. The process is a platform for soy-based biorefinery which used soy flour to produce two protein-rich products SPC and SPI with high protein contents and concentrated sugar syrup as potential fermentation feedstock. Note that the SPC generated using this method is a substantially improved product compared to the SPCs produced by the conventional methods. Potentially indigestible oligosaccharides are substantially eliminated and polysaccharides are greatly reduced.
Figure 8.4: Material balance of the process (without wash to remove entrapped hydrolysate from wet solids collected by centrifugation) shown with 100 g starting defatted soy flour

8.3.5 Amino acid composition

As shown in Figure 8.5, the amino acid compositions were comparable between the soybean flour and the soy protein concentrate obtained; given on the basis of total dry weight, all percentages were higher for SPC because of its higher overall protein content. Overall, the similar compositions suggested that the enzyme hydrolysis did not have any negative effects. When comparison was made on the basis of summed weight of only amino acids, the enzymatic hydrolysis was found to increase the proportion of hydrophobic amino acids (i.e., alanine, valine, leucine, isoleucine, glycine, methionine, phenylalanine, tyrosine and tryptophan) from 32% in soy flour to 36% in the SPC while
correspondingly decrease the proportion of hydrophilic amino acids (i.e., lysine, arginine, histidine, cysteine, glutamic acid, and aspartic acid) from 47% in soy flour to 44% in the SPC. The proportion of neutral amino acids remained relatively unchanged.

Figure 8.5: Amino acid compositions of defatted soy flour and the soy protein concentrate produced from enzymatic hydrolysis

8.3.6 Effect of different fungal enzymes on the process

Table 8.3 summarizes the protein contents and protein yields of soy protein concentrate and isolate products as well as the total protein and solid yields of the process. These process outcomes were evaluated using enzyme mixtures produced by different fungal fermentations (activities given earlier in Table 8.1) in the enzymatic hydrolysis step. The SPC yield was found to correlate with the hydrolysis efficiency as expected: more complete hydrolysis removed more carbohydrate, leading to lower yield (weight) of SPC (the remaining solids). So, the *A. niger* enzyme gave a lower SPC yield
than *T. reesei* enzyme. The opposite was true for the protein content of the resultant SPC: more complete carbohydrate removal by hydrolysis left behind SPC solids with a higher proportion of protein. So, the *A. niger* enzyme produced the SPC with the highest protein content (70%).

Table 8.3: Comparison of outcomes, i.e., total soluble carbohydrate concentration in liquid hydrolysate, yields and protein contents of soy protein concentrate (SPC) and isolate (SPI), and overall solid and protein yields of the enzymatic process using different enzymes

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Liquid</th>
<th>Solid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPC</td>
<td>SPI</td>
</tr>
<tr>
<td></td>
<td>Total carbohydrate (g/l)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>A. niger</td>
<td>59.6±0.9</td>
<td>53.5±0.5</td>
</tr>
<tr>
<td>T. reesei</td>
<td>49.7±0.8</td>
<td>59.5±0.8</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>50.9±0.5</td>
<td>59.9±1.0</td>
</tr>
<tr>
<td>A. niger &amp; A. aculeatus</td>
<td>55.6±0.6</td>
<td>57.3±0.7</td>
</tr>
<tr>
<td>A. Niger &amp; T. reesei</td>
<td>54.7±0.9</td>
<td>56.7±0.5</td>
</tr>
</tbody>
</table>

The yield of soy protein isolate was affected by the amount of protein solubilized into hydrolysate during the enzymatic reaction and the solubility of (partially) hydrolyzed protein in the hydrolysate. Higher SPI yields were obtained from the hydrolysate generated with *A. niger* (9.1%) enzyme and the enzyme mixture of *A. niger* and *T. reesei*.
(9.0%); the lowest SPI yield was from the hydrolysate of *A. aculeatus* enzyme (6.5%). The differences can be largely explained by the different protease activities of these enzymes: 200 U/ml in *A. aculeatus* enzyme, 122 U/ml in *A. niger* enzyme, and 68.9 U/ml in *T. reesei* enzyme. With the highest protease activity, *A. aculeatus* enzyme degraded more protein to small peptides that were too soluble to precipitate by the SPI collection method used, leading to the lowest SPI yield (and the lowest total protein yield 87.0%). Compared to the *T. reesei* enzyme, *A. niger* enzyme also had a higher protease activity but it did not appear to degrade protein to very small peptide so the hydrolyzed peptide could still be captured by precipitation during the SPI recovery step and the SPI and total protein yields in systems with *A. niger* enzyme remained high (8.8-9.1% SPI yield and 88.6-91.7% total protein yield). Protein contents, 86.1-89.3%, were similar in the SPIs generated in all systems. The lower than 90% protein contents were due to the soluble carbohydrate in the hydrolysate embedded in the wet SPI precipitates collected by centrifugation. A wash step, similar to that described in the next section for SPC wash, can effectively raise the protein content for applications where removal of soluble carbohydrate is desirable. Total solid yield ranged from 65% to 68%, depending predominantly on the degree of carbohydrate hydrolysis and slightly on protein hydrolysis. The *T. reesei* enzyme gave the highest solid recovery (68.4%) due to its lowest degradation of carbohydrate and protein.

8.3.7 Effects of solvent washing for SPC refinement

According to Table 8.2, the centrifugation-collected and then dried SPC had a carbohydrate content of 23%. A significant portion of the 23% was actually soluble
carbohydrate in the hydrolysate trapped inside the wet SPC solids during the centrifugation. Despite the incomplete removal of soluble carbohydrate, the protein content in thus prepared SPC was already 70%, which was in the higher end of the protein content (64-68%) of currently available commercial SPCs [111]. However, for applications that require even higher protein contents, such as for inclusion in some food or protein diets, further removal of the soluble carbohydrate may be desirable. Here we investigated the possibility of increasing the protein content by washing out the soluble carbohydrate in the SPC before drying.

8.3.7.1 Water wash

The effects of different water-to-solids ratios (v/w), from 5 to 20, on the water wash performance is presented in Figure 8.6a. As expected, higher ratios were more effective in removing soluble carbohydrate from the SPC but at the same time caused more significant protein dissolution. Increase of the ratio from 5 to 20 increased the removal of soluble carbohydrate from 61% to 91%. As a result, protein content of the SPC was increased from 67% before water wash to 77% after the wash at a ratio of 5 and to 81% at the wash ratio of 20. But the protein dissolution also increased from 11% to 19% along with the increasing water-to-solids ratio from 5 to 20. Another disadvantage of using high wash ratios was the generation of larger volumes of wash water with more dilute sugar concentrations; the sugar concentration was as low as 7 g/l at the highest ratio of 20. This would make protein and sugar recovery from this wash stream more costly.
8.3.7.2 Methanol and ethanol wash

It is desirable to minimize the protein dissolution (loss) caused by the solvent wash. The extent of protein dissolution was found to depend on the methanol or ethanol concentration in the alcohol-water mixture. For example, these alcohol concentration effects are shown in Figure 8.6b for the wash done with a solvent-to-solids ratio of 10. Including methanol or ethanol in the wash solvent was clearly beneficial. With only water, about 17% protein became dissolved. Methanol addition lowered the protein dissolution to the range of 8.6% to 12%, with the minimal dissolution found at 50% methanol. Ethanol addition was even more effective at all alcohol concentrations, reducing the protein dissolution to 6% - 9%, with the minimum obtained at 60% ethanol.

With 60% ethanol as the wash solvent, effects of the ratio of solvent volume to SPC dry weight was investigated for getting the highest recovery of SPC and protein (Figure 8.6c). As the solvent-to-SPC ratio was increased from 6 to 15, the SPC recovery decreased from 89% to 82% and protein recovery decreased from 96% to 90%; on the other hand, the protein content of washed SPC increased from 73% to 84%. Different protein contents are required for different applications. Results of this study provide a guide for producing different grades of SPC with protein contents tailored for the specific uses.
Figure 8.6: Soy protein concentrate wash to remove soluble carbohydrate in embedded hydrolysate and to maximize SPC protein content: (top) water wash – effects of water-to-dry solids ratio on wash outcomes; (middle) alcohol wash – comparison between methanol and ethanol on protein loss (dissolution) into wash solvent at different alcohol concentrations (vol%); (bottom) wash with 60% ethanol – effects of solvent-to-dry SPC ratio on wash outcomes, i.e., SPC recovery, protein recovery, and SPC protein content.

Material balance for the process including this wet SPC washing step is presented in Figure 8.7, again using the *A. niger* enzyme system as the example. The washing step could increase the protein content of washed SPC to as high as 80%. However, the ethanol wash process requires consideration of the capital and running costs for ethanol recovery, e.g., by distillation. Future economic analysis of the two process options is warranted to help decide the compromise between the increased protein content and higher production cost. The market value of a soy protein product of about 80% protein content is also yet to be assessed.
8.4 Conclusion

Soybean flour is an important plant protein source. It contains a high level of oligosaccharides and polysaccharides that have anti-nutritional concerns for certain applications. In the current study, an integrated process was developed to facilitate the incorporation of enzymatic carbohydrate hydrolysis for effective management of this property of soy flour. The integrated process enabled production of soy protein concentrate and soy protein isolate with the entrapped carbohydrate being largely hydrolyzed and essentially all soluble carbohydrate being monomerized. This could offer
improved digestibility to these soy protein products. The process included an optional step of washing the wet SPC solids to remove the entrapped hydrolysate. Without this step, the SPC protein content from this process was 70%, at the high end of commercial SPCs’ protein contents (64-68%). With the wash, the SPC could be 80% protein. In addition to the production of high-quality protein products, the process allowed collection of a majority of soy flour carbohydrate as monosaccharides in hydrolysate, which can be used as ready fermentation feedstock for manufacturing of other bioproducts.
CHAPTER IX

PRODUCTION OF ARABITOL FROM SOYBEAN FLOUR HYDROLYSATE BY

DEBARYOMYCES HANSENII FERMENTATION

9.1 Introduction

Arabitol is a five carbon sugar alcohol with one hydroxyl group on each carbon. It is an enantiomer of xylitol, a well-known low calorie sugar substitute. Researchers have long been interested on xylitol because its anti-cariogenic characteristics [167-170]. Arabitol, being an enantiomer of xylitol is also expected to show anti-bacterial properties. Recently we reported inhibitory effects of arabitol on the growth and acid production by cariogenic bacteria [171], which suggests arabitol could be potential contender of xylitol and other anti-cariogenic polyols such as sorbitol and erythritol[172-174]. Use of xylitol as anti-cariogenic agents in different commercial products such as chewing gums[175, 176], toothpastes[177], lozenges[169] and low calorie sweetener[178] is well established. However, arabitol having the anti-cariogenic effects could be also more advantageous because sweetness per caloric content for arabitol is much higher than that of xylitol [179-181]. Sweetness vary among sucrose and different sugar alcohols. The degree of sweetness may vary from about half as sweet as the same amount of sucrose to equally as sweet as sucrose. Compared to sucrose, sugar alcohols are slowly and incompletely absorbed and metabolized in the small intestine which makes their caloric contents lower than sucrose[182]. Among the sugar alcohols, arabitol and erythritol were reported to
have very low glycemic response and thus have very low caloric content compared to other sugars[182]. In addition to its low sweetness per caloric content, arabitol, like other sugar alcohols, can produce a noticeable cooling sensation in the mouth when consumed, due to the dissolution of arabitol being an endothermic reaction[179].

Arabitol can be produced by fermentation of a suitable medium by different osmophilic yeasts[183-186]. Production of D-arabitol by fermentation was first suggested by Binkley and Wolfrom [187] who detected the polyol in fermentation residue of cane blackstrap molasses by baker's yeast. During 1960’s, *Endomycopsis chodati* is found to be able to produce D-arabitol under suitable conditions of substrate, nutrient concentrations, temperature and aeration[188]. Peterson et.al[189], investigated the polyhydric alcohol-producing ability of 11 species of *Zygosaccharomyces* using glucose as carbon source. Later Arabitol was successfully produced from different sources of glucose or sucrose by various osmophilic yeast e.g *E. chodati* [188], *Saccharomyces rouxii* [190] or *S. mellis* [191], *Zygosccharomyneces* [192], *Hansenula* [193], *Debaryomyces* [194], and *Pichia* [195]. All species can produce arabitol but most of them also produce glycerol or other polyols along with arabitol. Koganti et al. [6] successfully produce arabitol as a sole polyol from biodiesel glycerol by osmophilic yeast *debaryomyces hansenii* SBP-1. Thorough strain screening was done to find out this *D. hansenii* species as a sole producer of arabitol. The optimum fermentation conditions were reported to produce arabitol from biodiesel glycerol as substrate. Optimum temperature Dissolved oxygen and pH were found to be 30°C, 5% and 3.5 respectively for effective production of arabitol from glycerol[196].
Lignocellulosic biomass is one of the most potential fermentation feedstock that has been used for producing different value added chemicals and biofuels by means of biological conversion[197-199]. Although much effort was taken to produce biofuels using lignocellulosic biomass, it can be a potential substrate to produce arabitol as well. However, lignocellulosic biomass differs in composition in both carbon source and nitrogen source based on the source. Majority of the lignocellulosic biomass are rich in cellulose and hemicellulose content[200]. Some of them, such as from soybean flour and hull, also contains pectic polysaccharides and galacto-oligosaccharides[5, 201]. Hydrolyzed biomass, thus, contains different ratio of hexose and pentose sugars. Also protein content of the hydrolysate obtained from hydrolyzed biomass could be an important factor to design the fermentation. These factors were not understood or reported for the production of arabitol from lignocellulosic biomass. Different fermentation conditions were previously investigated. Aeration, inorganic phosphate concentration, yeast extract concentration in the medium as nitrogen source are found to be an important factor for arabitol production[202, 203]. Initial glucose concentration had also significant effect on product yield. Nozaki et al. [204] investigated temperature and pH effect during arabitol production by Metschnikowia reukaufii AJ14787. Culture temperature seemed to be having significant effect on arabitol production. The optimal temperature and pH for D-arabitol production was found to be at 33°C and 5 respectively.

To use lignocellulosic substrate as fermentation feedstock, there are different factors that need attention. The ratio of carbon to nitrogen source would be a major factor as different biomass hydrolysate could have different combination of carbon source and nitrogen source. Nitrogen source is also an important factor whether it’s coming from
inorganic or organic nitrogen source. Operating conditions such as pH and dissolved oxygen is very well known to be an important factor for fermentation.

Soybean by-products are one of the rich sources of carbohydrates. These waste/byproducts include the hulls separated from beans before oil extraction [205], molasses generated from the processes for soy protein concentrate (SPC) production [206], and the soybean okara created during production of soy protein isolate (SPI) and soybean milk, respectively [207]. These soybean based carbohydrate rich by-products is highly valuable potential fermentation feedstock. We have recently reported effective hydrolysis of soybean flour carbohydrates using effective enzyme mixture containing activities of cellulase, xylanase, polygalacturonase, pectinase and α-galactosidase[150]. The hydrolysate contains a mixture of glucose, galactose, fructose, xylose and arabinose as carbon source. And it also contains soluble proteins as potential nitrogen source. In the current study, we consider soybean flour hydrolysate as model substrate for understanding the behavior of arabitol production by *D. hansenii*.

We have investigated the effect of carbon to nitrogen ratio (C/N), inorganic to organic nitrogen ratio I/O (N), different sugar substrates as carbon source in the medium for arabitol production. We also evaluated the effect of operating conditions, pH and dissolved oxygen, in the fermentation. This study would be a guide to produce arabitol from any lignocellulosic biomass hydrolysate.
9.2 Materials and Methods

9.2.1 Chemicals

Peptone, yeast extract, malt extract, K$_2$SO$_4$, CuSO$_4$, Na$_2$S$_2$O$_3$.5H$_2$O and MgSO$_4$.7H$_2$O were purchased from Sigma–Aldrich; agar, glucose, fructose, H$_3$BO$_3$, HPLC grade water and ammonium sulfate from Fisher Scientific; K$_2$HPO$_4$ from Merck; Arabinose from Acros organics and KH$_2$PO$_4$ from EMD Chemicals.

9.2.2 Soybean flour and soybean hull hydrolysate preparation

Soybean meal hydrolysate was prepared according to previously reported conditions. In short, soybean flour was hydrolyzed using the enzyme produced by the *Aspergillus niger* 341. The produced enzyme contained different enzyme activities including, but not limited to, cellulase, xylanase, pectinase, α-galactosidase and sucrose. At first, soybean flour was dry sterilized at 160°C for 2 hours. The enzyme containing liquid to dry sterilized soy flour ratio of 4 (v/w) was used for enzyme hydrolysis at 50°C and pH 4.8 for 24 hours. After enzyme hydrolysis is done, hydrolysate is separated from remaining solid by centrifugation at 7000 rpm for 10 mins. Similar procedure was also followed for soybean hull hydrolysis except soybean hull was autoclaved at 121°C for 15 min before addition of enzyme instead of dry heat sterilization in case of soybean flour.

9.2.3 Microorganism and maintenance

The yeast culture of D. hansenii SBP-1 (NRRL Y-7483) was obtained from USDA ARS culture collection (Peoria, IL). Culture was maintained on agar plates
containing 10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 20 g/L agar.

9.2.4 Inoculum preparation

Same medium without the agar was used for preparing the inocula for the initial shake flask studies. A loop of cells was transferred from an agar plate to 50 ml medium in a 250 ml Erlenmeyer flask covered with cheese cloth sandwiched cotton. The culture was grown at 30°C temperature in a shaker rotating at 250 rpm for 24 h. The inoculum thus prepared was added at 5% of the final culture volume in the subsequent studies.

9.2.5 Studies in the shake flask

9.2.5.1 Effect of the culture volume

Dissolved oxygen (DO) concentration is a very important factor for growth and product formation. It is difficult to monitor dissolved oxygen concentration in shake flask studies. However, different culture volume to flask volume ratio would be helpful to get an indication of the culture sensitivity to low DO and high DO. To evaluate the DO effect on the cell performance, a study was done with \textit{D. hansenii} SBP-1 in 500 ml flask containing following three different medium volume: 40, 75 and 100 ml. Under the same shaker rotation speed of 250 rpm, the flask with smaller volume was expected to have better oxygen transfer efficiency via surface aeration, resulting in higher DO condition in the culture of similar cell concentrations.
9.2.5.2 Effect of C/N ratio

Effect of carbon to nitrogen ratio (C/N) of the fermentation medium on cell concentration and product formation is very important factor to achieve high productivity. Shake flask studies to understand the effect of C/N ratio is conducted in a 250 ml flask with 50 ml working volume. Composition of media used to evaluate the C/N ratio is are given in the table 1. Media is formulated using the soybean flour hydrolysate (SFH) and additional sugar to the SFH. SFH combined reducing sugar concentration was 46 g/L and soluble protein concentration was 11.24 g/L. SFH was diluted to achieve the protein concentration reported in table 9.1 and sugar concentration was adjusted to reported concentration by addition of pure sugar. Pure sugar was added so that the ratio of glucose, fructose and galactose were the same in all the systems to minimize the effect of different sugar compositions. C/N ratio was calculated by dividing the total weight of carbon used in the medium over total weight of nitrogen (calculated by dividing protein concentration by 6.25) in the medium.

Table 9.1: Composition of the medium in the shake flask studies for different C/N ratio effect on the cell growth and arabitol production

<table>
<thead>
<tr>
<th>System</th>
<th>Sugar concentration</th>
<th>Protein concentration</th>
<th>Total Carbon</th>
<th>Total nitrogen</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.8</td>
<td>2.81</td>
<td>10.72</td>
<td>0.45</td>
<td>23.8</td>
</tr>
<tr>
<td>2</td>
<td>49.9</td>
<td>3.75</td>
<td>19.96</td>
<td>0.60</td>
<td>33.3</td>
</tr>
<tr>
<td>3</td>
<td>76.7</td>
<td>3.75</td>
<td>30.86</td>
<td>0.60</td>
<td>51.4</td>
</tr>
<tr>
<td>4</td>
<td>94.5</td>
<td>3.75</td>
<td>37.80</td>
<td>0.60</td>
<td>63.0</td>
</tr>
<tr>
<td>5</td>
<td>76.8</td>
<td>5.63</td>
<td>30.72</td>
<td>0.90</td>
<td>34.1</td>
</tr>
</tbody>
</table>
9.2.5.3 Effect of organic to inorganic nitrogen ratio

Nitrogen source is a very important factor for the cell growth in fermentation. Nitrogen from different organic or inorganic source have also impact in the cell growth behavior. We investigated the effect of nitrogen source coming from hydrolysate and additional nitrogen source provided by ammonium sulfate in the fermentation medium on the cell growth and product formation. Medium used to do this study is presented in table 9.2. Medium was designed by keeping the total nitrogen and carbon concentration same to maintain constant C/N ratio but changing the source of nitrogen. Organic nitrogen is coming from soybean flour proteins and inorganic nitrogen source is ammonium sulfate.

Table 9.2: Composition of medium in shake flask studies for different inorganic to organic nitrogen concentration ratio I/O (N) effect on the cell growth and arabitol production.

<table>
<thead>
<tr>
<th>System</th>
<th>Sugar</th>
<th>Protein</th>
<th>Ammonium sulfate</th>
<th>Organic nitrogen</th>
<th>Inorganic nitrogen</th>
<th>Total nitrogen</th>
<th>Inorganic/organic N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76</td>
<td>2.81</td>
<td>2.14</td>
<td>0.45</td>
<td>0.45</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>3.13</td>
<td>1.90</td>
<td>0.50</td>
<td>0.40</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>3.43</td>
<td>1.67</td>
<td>0.55</td>
<td>0.35</td>
<td>0.90</td>
<td>0.64</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>4.06</td>
<td>1.19</td>
<td>0.65</td>
<td>0.25</td>
<td>0.90</td>
<td>0.39</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>5.63</td>
<td>0</td>
<td>0.90</td>
<td>0.0</td>
<td>0.90</td>
<td>0</td>
</tr>
</tbody>
</table>

9.2.5.4 Effect of sugar composition

Different sugar has significant effect on the cell growth and product conversion by microorganisms. Here a study was designed to understand the effect of different
composition of available sugars in lignocellulosic hydrolysate. Lignocellulosic hydrolysate contains mainly hexose and pentose sugars. Among the hexose sugars, glucose, fructose and galactose are most common and among the pentose sugars xylose and arabinose are more frequently found. Here we investigated the effect of different ratio of sugars mainly in terms of glucose percentage and pentose sugar (combined xylose and arabinose) percentage. The nitrogen source was used only organic nitrogen from the soybean protein hydrolysate and total nitrogen concentration was kept constant at 0.9 g/L. Total carbohydrate concentration was maintained constant at 76 g/L but the composition of different sugars were changed by addition of different ratio of sugars. Responses were analyzed in terms of different percentage of glucose present in the medium.

9.2.6 Analytical methods

9.2.6.1 Cell concentration (cell dry weight) measurement:

Periodic samples were taken at regular intervals from the culture systems studied. The samples were then centrifuged at 10000×g for 10 mins using centrifuge 5415D (Eppendorf, Hauppauge, NY). The supernatants were collected and frozen for future analysis of sugars and produced arabitol concentrations. The cell pellets were washed twice with deionized water and then used for measurement of cell dry weights. Dry cell weights were determined by drying the cells at 100°C for overnight in a hot air oven (Binder-world, Bohemia, NY).
9.2.6.2 Substrate and product concentration

Sugars in the medium and produced arabitol concentrations were measured using a high performance liquid chromatography (HPLC) system (Shimadzu LC 10A) with a refractive index detector (RID-10A). A carbohydrate column (Supelgel column Pb, 30 cm × 7.8 mm) with a guard column (No. 59345, 50 mm × 4.6 mm) was used at 80°C temperature. The mobile phase was HPLC grade water at a flowrate of 0.5 mL/min. The retention times for glucose, xylose, galactose, arabinose, fructose and arabitol were 17.3, 18.3, 19.5, 20.7, 22.1 and 32.1 min, respectively. Calibration curves for converting the peak areas to concentrations were established with standard solutions of pure sugars and arabitol.

9.2.6.3 Protein concentration

The Kjeldahl method [58] was used to measure the nitrogen contents of solid samples. The nitrogen content was multiplied by 6.25 [59] to estimate the protein content. A liquid hydrolysate sample diluted to 50 ml, containing 10 to 200 mg/l protein, was added to a flask and digested with 10 ml reagent containing 134 ml/l concentrated H\textsubscript{2}SO\textsubscript{4}, 134 g/l K\textsubscript{2}SO\textsubscript{4} and 7.3 g/l CuSO\textsubscript{4}. The digestion was carried out to completion, until the reaction mixture became a clear solution. Then 30 ml water and 10 ml of a distillation reagent containing 500 g/l NaOH and 25 g/l Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}.5H\textsubscript{2}O were added to the digested sample. This mixture was then distilled using a distillation unit (RapidStill 1, Labconco, Kansas city, MO) to produce ammonia gas, which was absorbed in 0.1 N H\textsubscript{3}BO\textsubscript{3}. Then the H\textsubscript{3}BO\textsubscript{3} solution was titrated using 0.1 N H\textsubscript{2}SO\textsubscript{4} to find the nitrogen concentration in the sample.
9.3 Result and discussion

9.3.1 Culture volume effect

Different medium volumes (40, 75 and 100 ml) used in the systems studied were expected to cause different profiles of dissolved oxygen concentrations in the fermentation medium due to different surface to volume ratio. However, DO profiles were difficult to monitor in the shake flasks cultures. The cell concentrations, sugar consumption and arabitol produced were determined. Figure 9.1 represents the profile of cell concentration, arabitol production at different culture volume. To analyze the kinetic parameters, including specific growth rate (\( \mu \)), specific production rate (\( q_p \)) and specific consumption rate (\( q_s \)) were calculated by an interpolation method based on the data in fig. 9.1A-B, and presented in figure 9.1 C-E. Higher Dissolved oxygen concentration (DO) is clearly found better for high cell concentration. Maximum cell concentration was found as high as 25 g/L in case of high DO condition. It decreases with the decrease of lower dissolve oxygen and can be as low as 9 g/L at the low DO condition. Maximum specific growth rate, production rate and consumption rate all were highest at high DO conditions. Due to the highest growth rate, and consumption rate at high DO, the sugars were consumed completely within only 28 hours. And the arabitol production was also maximized at 20 hours with arabitol concentration of 12.8 g/l, which corresponds to the yield of 37%. In medium and low DO condition maximum arabitol concentration researched were 12.5 and 11.2 g/L respectively but it took 46 and 72 hours respectively. Maximum specific production rate and sugar consumption rate were also found higher at
high DO. High DO resulted highest volumetric productivity of 0.6 g l⁻¹ h⁻¹. So high DO is recommended for achieving higher volumetric productivity.
Figure 9.1: Profile for cell concentration (A), arabitol production (B), specific growth rate (C), specific production rate (D) and specific sugar consumption rate (E) during fermentation at different DO condition at different culture volume (n=3).

9.3.2 Sugar consumption profile

Figure 9.2A represents different sugar consumption profile in the fermentation system with low DO. From this figure, it was found that the sugars were not consumed simultaneously in the system. It switched from consumption of one sugar to another once
the preferred sugar are completely consumed. The sequence of consumption of hexose sugars were clearly found as Glucose>fructose>galactose. Pentose sugars were not consumed in this systems. Pentose sugars were only consumed in high DO conditions, after the complete consumption of hexose sugars and when cell were actively growing. Pentose sugars were not consumed during stationary phase. Figure 9.2B-C represent the consumption of xylose and arabinose at different DO conditions. Between xylose and arabinose xylose was consumed first and then arabinose.
The graphs illustrate the concentration of various sugars (Glucose, Fructose, Galactose, Xylose, Arabinose) and Xylose over time under different Dissolved Oxygen (DO) levels (Low, Medium, High). The concentration is measured in percentage and grams per liter (g/l) respectively. The graphs show a decrease in sugar concentration over time, with the low DO level showing the most significant decrease in Xylose concentration.
Figure 9.2: Sugar consumption profile (A), Xylose consumption profile at different DO condition (B), arabinose consumption profile at different DO condition (C) during fermentation (n=3).

9.3.3 Effect of carbon and nitrogen source concentration

C/N ratio of fermentation medium plays a vital role in production of arabitol. The C/N ratio of the fermentation medium was varied from 23 to 63 using nitrogen source from only from SFH and carbon source from SFH and additional sugars. The medium compositions are given in table 9.1. Cell concentration, arabitol production profile are presented in figure 9.3 A-B for different C/N ratio. Initial sugar concentration, total sugar consumed and yield of arabitol from the consumed sugar is presented in the bar chart in figure 9.3C. Cell concentration was, as expected, directly related to the nitrogen concentration. Highest cell concentration of 11.3 g/L was achieved with highest N concentration of 0.5 g/L in C/N 34.1 (high N) system and lowest cell concentration of 6 g/L was achieved in case of lowest concentration of 0.45 g/L present in C/N ratio of 23.3
(low N). For other three systems where nitrogen concentration were 0.60 g/L, cell concentrations were similar at around 7.5 to 8 g/L. Highest arabinol production of 36 g/L was achieved in case of C/N ratio of 63, which corresponds to highest yield of 47% among all the systems. In system C/N 23.8 and C/N 33.3, arabinol production stopped after 48 hours when the cell growth stopped. And then in system C/N 23.3, arabinol concentration started to decrease. This suggests that the arabinol was produced only in the stationary phase and after all the sugar consumption, cell started to consume arabinol until the nitrogen source run out. On the other hand, system C/N 51.4 and C/N 63 arabinol production continues after the cell growth stopped by limiting nitrogen source. Arabinol continued to be produced at the stationary phase utilizing available carbon source. The arabinol yield of these two systems were 43 and 47% respectively. Due to the production during stationary phase, arabinol production yield was found higher in this system compared to the two previous system. So C/N ratio of higher than 33.3 is required to produce arabinol in the stationary phase which is very important to increase the yield. In the other system where nitrogen concentration is higher but C/N ratio is 34.1, the yield is 40% which is lower. This lower yield was again because the arabinol was produced only during growth phase. In system C/N 63, only 77 g/L of sugar was consume of the available 94 g/L. So the yield would expected be increased even higher if the fermentation is continued until the complete consumption of the available sugars.
9.3.4 Effect of inorganic to organic nitrogen ratio

Nitrogen source is a very important factor in fermentation for cell growth. We investigated the ratio of inorganic to organic nitrogen source in case of arabinol fermentation. The medium composition studied is presented in table 9.2. The effect of Inorganic to Organic nitrogen, I/O (N), on the arabinol production and cell growth are shown in figure 9.4 A-D. Cell concentration profile were almost similar for all the system as the total nitrogen were same in all cases. However the maximum cell concentration was slightly higher when I/O (N) ratio is low. For I/O (N) from 0 to 0.64 the cell concentration were 10.6 where it was 9.3 for I/O (N) ratio of 0.8 to 1.0. The arabinol concentration were similar for all the systems during the growth phase. However the arabinol concentration differed during the stationary phase. Highest arabinol concentration

Figure 9.3: Cell concentration (A), arabinol production (B) and initial sugar, total sugar consumed after 120 hours and arabinol yield from the consumed sugar (C) during fermentation with medium containing different C/N ratio (n=3).
of 37.5 g/L was found at highest I/O (N) ratio of 1.0 and lowest arabitol concentration of 31 g/L was found when there was no inorganic nitrogen presents. This suggests that inorganic nitrogen source is helpful during the production of arabitol during the stationary phase.
Figure 9.4: Cell concentration (A), arabitol production (B), pH profile (C) and arabitol yield in the fermentation with medium containing different inorganic to organic nitrogen ratio I/O (N).
9.3.5 Effect of pH

pH is another important factor for cell growth and product formation due to the dependency of enzymatic reaction for product formation on pH. It is difficult to maintain the constant pH in shake flask studies. Nevertheless, the pH profile was monitored during the run with different I/O (N) ratio studies. The pH profile for different I/O (N) ratio is presented in figure 9.4 C and the arabitol yield achieved and the lowest pH recorded for each system is presented in figure 9.4D. It was found that the lowest pH in the system was dependent on the ratio of I/O (N). When the inorganic nitrogen was higher in the system the recorded lowest pH was also lower. Lowest pH found was 3.56 in the system I/O (N) 1.0 and in the system I/O (N) 0, where no inorganic nitrogen was present the lowest pH was found to be 6.3. Inorganic nitrogen consumption decreases the pH along with their consumption by the cell. However, when organic nitrogen source is higher, consumption of organic protein based nitrogen source release ammonia which increases the pH. From the figure 1C, it was found that the pH during the stationary phase was also different due to the different I/O (N) raito. From figure 1D, the arabitol yield is found to correlate with the lowest pH or the stationary phase pH in different systems. So at lower stationary phase pH, the arabitol yield was found higher. The highest arabitol yield of 48% was achieved when the stationary phase pH in the system was 4.3. This also correlates with previous study conducted in this lab to produce arabitol from glycerol by the same yeast. The optimum pH for higher arabitol production was found 3.5 to 4 during that study. So at the stationary phase lower pH helped to increase arabitol yield.
9.3.6 Effect of different sugar composition

Lignocellulosic biomass usually have different hexose and pentose sugars. So composition of different sugar may have effect on the arabitol production. In this study the effect of glucose composition on the cell growth and arabitol production is investigate. Arabitol yield and maximum specific growth rate for different glucose ratio in total sugar is presented in figure 9.5A. In figure 9.5B residual total sugar concentration is presented for system with different glucose percentage from 18.5% to 67%. Sugar consumption rate is dependent on the glucose composition. But final residual sugar concentration is dependent on the pentose (combined xylose and arabinose) sugar composition. Because the pentose sugars both xylose and arabinose were left unconsumed in the current systems. The Specific growth rate and arabitol yield both were dependent on the percent glucose in the total sugar consumption. Highest arabitol yield of 45% was achieved when the glucose composition was 67% in the total sugar.
Figure 9.5: Arabitol yield and maximum specific growth rate (A), total residual sugar percentage (B) in fermentation with medium containing different initial glucose composition.
9.4 Conclusion

In this study, effect of different medium compositions and operating conditions were investigated for arabitol production from lignocellulosic biomass by *D. hansenii* fermentation. From the above results and discussion, it is found that the several factors are important for the production of arabitols by *D. hansenii* using lignocellulosic biomass substrate: Dissolved oxygen concentration, C/N ratio, pH and glucose percentage in the fermentation medium. Hexose were consumed preferably over pentoses and ponose sugars were only consumed if the system is actively growing at high DO condition without nitrogen limitation. Highest yield was achieved at high DO condition, C/N ratio higher than 33, stationary phase pH of 4-4.5 achieved by I/O (N) ratio of 0.8 to 1 and glucose composition of 55% in the medium. The maximum yield obtained at this condition of 48% of the sugar consumed. In the shake flask study volumetric productivity of 0.38 gL⁻¹h⁻¹ and specific productivity of 0.04 gg⁻¹h⁻¹ was achieved.
CHAPTER X

PURIFICATION OF ARABITOL FROM THE FERMENTATION BROTH OF

DEBARYOMYCES HANSENII

10.1 Introduction

Arabitol, a five carbon polyhydric sugar alcohol, is an enantiomer of xylitol. Xylitol is a natural sweetener having anti-cariogenic characteristics [208] and is used as a sugar substitute in different food products that may cause dental carries [209, 210]. Arabitol as an isomer of xylitol is also expected to have the anti-cariogenic properties. Currently xylitol can be produced from xylose by chemical reduction under alkaline condition [211-213]. But chemical reduction process requires high pressure and temperature and expensive Raney-nickel catalyst [212, 214] causing the process less economically efficient. Xylitol can also be produced by yeast fermentation from xylose derived by enzymatic hydrolysis of lignocellulosic materials [215-218]. But both processes require hydrolysis and subsequently purification of xylose from the resultant sugar mixtures.

Onishi and Suzuki [219] reported a process of converting glucose to D-arabitol by *Debaryomyces hansenii* followed by Production of D-xylulose from D-arabitol by *Acetobacter suboxydans* and finally D-xylulose to xylitol by *Candia guilliermondii*. Recently Suzuki et al [220] reported a method for conversion of D-arabitol to xylitol with
Gluconobacter oxydans. So if arabitol can be produced from cheap substrates, xylitol can be produced easily by microbial conversion of arabitol.

Arabitol can be produced from glucose or sucrose by various osmophilic yeasts, e.g., Endomycopsis chodati [221], Saccharomyces rouxii [222], S. mells [223], Zygosaccharomyces [224], Hansenula [193], Debaryomyces [225], and Pichia [195].

Peterson et al [226] investigated the polyol-producing ability of several Zygosaccharomyces species. All could produce arabitol but most of them produced glycerol along with arabitol. Similarly arabitol and glycerol mixtures were produced from glucose by S. rouxii [227]. Falanghe and Caruso produced arabitol from sugarcane juice media as a cheap substrate by E. burtonii [228].

Recently an osmophilic yeast D. hansenii SBP-1 (NRRL Y-7483) has been found to produce arabitol as a sole polyol using glycerol, a byproduct from biodiesel production, as substrate [229]. But a significant amount of glycerol was left unconsumed when the cells stopped producing arabitol and switched to consume arabitol when the glycerol concentration became lower than the arabitol concentration. As both arabitol and glycerol are polyols most of their chemical properties are similar. It is more complicated to recover arabitol from the fermentation broth containing a significant amount of glycerol and other complex impurities present.

Podmore [230] reported arabitol recovery from fermentation broth where glucose or molasses was used as the carbon substrate. Hot n-butanol was used to extract arabitol at more than 103°C (melting point of arabitol) followed by cooling of butanol phase leading to crystallization of arabitol. But if large amount of glycerol was in the broth, butanol would extract glycerol as well. Arabitol could not be crystallized as it is soluble...
in glycerol. Therefore this process cannot help to separate arabitol from glycerol containing fermentation broth.

Recovery of xylitol from fermentation of hemicelluloses hydrolysates was proposed using membrane separation technology by Affleck [231]. Separation was based on the molecular size of the particles. The HG19 10000 molecular weight cut off polysulfone membrane was found to be most effective to allow over 87% of xylitol to permeate retaining about 50% of protein and impurities from the fermentation broth. However other sugars mainly arabinose and xylose also permeates along with xylitol due to its low molecular size. So to recover xylitol from those sugars urea and NaHSO₃ was added so that they can react with sugars to increase their molecular size and prevent permeation through the membrane. Permeates were then collected and crystallized to get 90% pure xylitol crystals. However due to the presence of impurities crystallization rate was significantly slow and took 14 days to accomplish that. But in present system because of the presence of glycerol this process would not be helpful. As the structure of glycerol is similar as arabitol (both are polyols), Urea and NaHSO₃ would not react either with arabitol or glycerol leaving both of them permeable through the membrane. So arabitol can not be crystallized if glycerol is present there.

Sampaio et al [232] reported crystallization of xylitol from culture media fermented by yeasts where xylitol was recovered from xylose containing fermentation broth. Fermented broth was treated with activated carbon to remove the impurities followed by the crystallization of xylitol in presence of residual xylose at -15°C. Presence of xylose in fact showed positive effect for crystallization process. But in current process in presence of glycerol xylitol cannot be crystallized as stated above.
Rivas et al [233] also reported a similar process of recovery of xylitol from the broth obtained from the fermentation of concorb hydrolysates. After adsorption of impurities a ethanol precipitation step was added before crystallization to precipitate the residual protein and impurities as the presence of impurities affect the final purity of xylitol crystals. Again glycerol was not present there. If it does like in this process glycerol cannot be removed from the broth by ethanol precipitation.

Gurgel et al [234] used anionic and cationic ion exchange resins to remove protein and impurities from the fermentation of the sugarcane baggase hydrolysate to facilitate crystallization but compromised to lose 46-57% of xylitol. However ion exchange resins cannot remove glycerol as it is neutral as arabitol.

In the present study a process for the recovery of arabitol from the fermentation broths containing arabitol, glycerol and other impurities was developed. Activated carbon adsorption was used first to remove the proteins and colored impurities (amino acids, peptides, nucleic acids, proteins or inorganic salts) either produced by cells or coming from nutrient media. This activated carbon treatment was suggested by the experiment done before without discoloring treatment that provided lower arabitol recovery than desired which was because of higher solubility of arabitol in acetone in presence of impurities during glycerol extraction and washing step. Gurgel et el [234] also reported that presence of different colored organics may interfere the separation of desired product from the fermentation broth negatively. Moreover crystallization of a desired product directly from fermentation broths is a difficult task [235] because of the presence of these complex organic substances [236-239]. So the removal of such unwanted colored organics becomes very important to have better arabitol recovery. Recently removal of
different such colored substance and dye by activated carbon has been investigated [240-245] in different studies. Then experiment was designed based on the hypothesis that after concentrating the fermentation broth by vacuum evaporation, hot n-butanol at 103°C would be added to extract arabitol from the remaining arabitol glycerol mixture. Then separated butanol phase would be cooled down at room temperature (21°C) to crystallize arabitol. Since arabitol and glycerol both are soluble in n-butanol, glycerol also was extracted to butanol phase along with arabitol which prevent the crystallization of arabitol. To facilitate the crystallization, glycerol must have been removed from the fermentation broth. A suitable solvent which can selectively extract glycerol from the glycerol arabitol mixture had to be found. But the solubility data of arabitol is not cited in the literature. Xylitol, being a stereo isomer of arabitol, was expected to have approximately similar properties as arabitol. Among many other solvents acetone was found to be the one in which glycerol is highly soluble but xylitol is very less. So commercially available xylitol was used to understand the behavior of 3-component (xylitol glycerol acetone) solution. Then based on the solubility and liquid liquid equilibrium data of 3 component mixture later experiment with fermentation broth was designed. After removing all glycerol from the fermentation broth arabitol was extracted ion hot butanol phase and butanol phase was cooled down to room temperature to get white arabitol crystals. The process is expected to be useful for recovery of xylitol from glycerol containing fermentation.
10.2 Materials and Methods

10.2.1 Materials & Chemicals

Glycerol (99%), n-butanol (99.4%) and 12-40 mesh activated carbons were purchased from Acros Organics (Morris plains, New Jersey). Xylitol (99+%) was purchased from Sigma Aldrich (St. Louis, Missouri), D-arabitol was purchased from MP Biomedicals LLC (Solon, OH, USA) and analytical grade acetone was obtained from EMD Chemicals (Gibbstown, New Jersey).

10.2.2 Collection of Supernatant

Fermentation was performed in a BIOFLO 110 (New Brunswick Scientific Co., Edison, NJ, USA) fermentor of 2L total capacity, maintaining % DO by varying agitation speed and keeping air flow constant, using 1L of the fermentation medium (Glycerol 120 g/l, Yeast extract 6g/l, Ammonium sulfate 4 g/l, Dipotassium phosphate (K₂HPO₄) 0.316 g/l, Monopotassium phosphate (KH₂PO₄) 0.211 g/l and Magnesium sulfate heptahydrate (MgSO₄·7H₂O) 1 g/l) inoculated with 0.1 g/L initial cell concentration of Debaryomyces Hansenii SBP1 yeast at optimized fermentation conditions (temperature 30°C, pH 3.5 and DO 5%). Fermentations were stopped after 140 hrs when approximately 25% (25-30 g/l) of the glycerol left (since the arabitol production rate decreased) and supernatant was collected by centrifugation of the fermentation broth at 8000 rpm for 10 min (Sorvall RC 5C, DuPont, Wilmington, DE, USA) to remove cells and other solid particles. More detail about the fermentation can be found in the literature[229].
10.2.3 Treatment with Activated Carbon

The supernatant obtained by centrifugation was treated with activated carbon (Calgon Carbon Corporation, Pittsburgh, PA, USA) to minimize the colored organics. Effect of different parameters including initial pH values (6 and 9), temperature (30, 45, 60) and concentration of activated carbon (3-20 g/l) were evaluated to have effective treatment. The supernatant was adjusted to different pH by adding 5N NaOH and fifty (50) ml of this were transferred to each 125 ml Erlenmeyer flask containing 3-20 g/l of activated carbon. The flasks were shaken at 200 rpm for 2 hrs in a shaker (Thermo scientific MaxQ 5000 Incubating/Refrigerating Floor shaker, Asheville, NC, USA) maintained at desired temperature. The activated carbon was removed by centrifugation at 8000 rpm (Sorvall RC 5C, DuPont, Wilmington, DE, USA) and treated liquor was collected for the subsequent glycerol removal and arabitol extraction and crystallization.

10.2.4 Solubility Determination

The solubility study was done in sealed testtubes shaken in a water-bath shaker (Boekel/Grant Orbital and Reciprocating Water Bath ORS 200, Boekel Scientific, Feasterville, Pennsylvania, USA) set at different temperatures ranging from 25 to 45°C for different batches of experiments. Acetone (5ml) was first added into each test tube. Glycerol was then added gradually and mixed till a small separate liquid glycerol phase appeared. At this condition the solvent phase had been saturated with glycerol. As stated earlier xylitol (instead of arabitol since pure arabitol is not readily available) was used in
the solubility study to understand the behavior of three component (xylitol glycerol acetone) mixture, xylitol (as solid powders) was added and mixed till not all the added xylitol could be dissolved and a small amount of xylitol solids remained. The test tubes were mixed in the shaker for two more days to ensure equilibrium. A small volume (approximately 1 ml) of the acetone phase and glycerol phase were separately collected and analyzed for the saturated (equilibrated) glycerol and xylitol concentrations in both the glycerol and acetone phases.

10.2.5 Water Removal and glycerol extraction

As both glycerol and arabitol are readily soluble in water, glycerol cannot be removed from supernatant without removing arabitol in presence of water. Supernatant, after treatment with activated carbon, was transferred into a Rotavapor flask. The Rotavapor (Buchi Rotavapor R200 including bath B490, BUCHI Corporation, New Castle, DE, USA) was operated at 36°C and 730mm Hg vacuum created by vacuum pump (Gem® Direct-Drive Vacuum Pump, model 8890, Gardner Denver Thomas, Inc., Niles, IL, USA) to remove all the water from supernatant. Acetone required to extract all the glycerol from supernatant was then added to the flask and the system was shaken (Thermo scientific MaxQ 5000 Incubating/Refrigerating Floor shaker, Asheville, NC, USA) at 250 rpm and desired temperature ranging from 25 to 45°C (to optimize temperature) for two days to ensure equilibrium. Amount of acetone required was initially estimated based on the solubility study with xylitol and later it was optimized for the mixture (arabitol and glycerol) present in the supernatant. The acetone phase was collected and fresh acetone (one fourth of the previous volume) was added for wash
(ensuring complete glycerol removal). After the wash acetone phase was collected, the
arabitol-rich remainder was dried (by air drying). The extract and wash acetone phase
were analyzed for the concentrations of arabitol and glycerol.

10.2.6 Arabitol extraction and crystallization

The dried (acetone-vaporized) sample was used for collection of pure arabitol.
Required amount of n-butanol to extract all the arabitol was added to the dried sample
and heated on a hot magnetic-stir plate. Amount of n-butanol required at particular
temperature was estimated based on the solubility of xylitol (expected to be similar being
a stereo isomer of arabitol) reported by Wang et al [246]. At higher temperature the
solubility of arabitol is higher so the temperature as high as possible was preferred until
thermal degradation of arabitol occurred. The system was kept in the optimized
temperature until all the arabitol was extracted. Butanol phase was separated while hot.
The crystallization of arabitol was performed by cooling the hot butanol phase to room
temperature for significantly reducing the solubility of arabitol [247] and improving the
purity of product arabitol crystals by leaving impurities in the mother liquor [248, 249].
The crystallization was allowed to take place for 48 hrs in a mildly shaken (50 rpm) water
bath (Boekel/Grant Orbital and Reciprocating Water Bath ORS 200, Boekel Scientific,
Feasterville, Pennsylvania, USA) kept at room temperature. Ground commercial arabitol
(0.5 g/l) was added as seeds to accelerate the crystallization. The white crystals formed
were collected, dried and weighted.
10.2.7 Analytical method

Samples of liquors were micro filtered through 0.2 µm Millipore filter paper (Danvers MA, USA) and analyzed for concentration of arabitol and glycerol using High Pressure Liquid Chromatography (HPLC Shimadzu) with a refractive index detector, using a different carbohydrate column (Supelco column H, 250 x 4.6 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA, USA) maintained at ambient temperature. The mobile phase used was 0.1% H₃PO₄ at a flow rate of 0.14 ml/min. Clarification of the liquor after treatment with activated carbon was quantified by measuring the absorbance at 580 nm, using a UV/VIS spectrophotometer (Model UV-1601, Shimadzu Corporation, Columbia, MD, USA).

10.3 Result and Discussion

10.3.1 Activated Carbon Treatment

A supernatant sample of fermentation broth with 40.2 ± 0.2 g/L of arabitol was used for tests. The sample was brown in color and had a high A₅₈₀ value (≈ 0.095 ± .002) due to the presence of colored substances and other organics such as proteins, peptides, and nucleic acids. The effects of activated carbon treatment at different carbon concentrations (up to 20 g/L), temperature (30, 45 and 60°C) and pH (6 and 9) are shown in Figure 10.1. The desired effect of removing colored impurities is clearly shown by the larger % reduction of A₅₈₀ with increasing activated carbon concentrations for each temperature and pH.
pH 6 was significantly more effective than pH 9 for reduction of A₅₈₀ (Figure 10.1), particularly at higher activated carbon concentrations; even the least favorable system (60°C) at pH 6 showed higher treatment effect than the most favorable system (30°C) at pH 9. The temperature effect was also very clear: within each pH group, increasing temperature from 30°C to 60°C caused lower % reduction of A₅₈₀ (and visibly higher residual color). This trend of temperature effect contradicted the results reported by Gurguel et al.[234], who observed lower residual color with increasing temperature (in the range of 35-80°C) while using activated carbon to remove colored organics from fermentation broth of sugarcane bagasse hydrolysate. They hypothesized that their observed trend was because the colored impurities became chemically bound to the adsorbent. On the other hand, the trend observed in the current study was in good agreement with that by How and More [250] who reported that activated carbon removed the colored substances from soy protein extracts by physical adsorption. An increase in temperature would cause intensified molecular vibration which leads to breakage of weaker van der Waals and dipole-dipole interactions and results in reduced adsorption. Sampaio et al.[232] also proposed that some nucleic acids and aromatic amino acids could be adsorbed on the activated carbon by physical adsorption. Accordingly, the most probable hypothesis for the trend observed in our study is that the colored organics in the arabitol fermentation broth adsorbed on activated carbon primarily by physical adsorption and were thus less effectively removed with increasing temperature. Regardless, our results suggested the use of pH 6 and 30°C for the activated carbon treatment.

As for the effect of activated carbon concentration, although addition of more activated carbon could remove more colored substances from the broth, it would also
remove more arabitol, the desired product, as shown in Figure 10.1. After removing most of the colored organics, each additional g of activated carbon could adsorb up to 0.7 g arabitol at 30°C (value could be calculated from the data shown in Figure 10.1).

Fortunately, the activated carbon had higher affinity to the colored organics than to arabitol, as suggested by the faster increase in % reduction of A580 than in % reduction of arabitol concentration, responding to the initial additions of activated carbon up to 8 g/L. At higher activated carbon concentrations, the increase in % reduction of A580 was slower and the increase in % reduction of arabitol concentration was faster. Accordingly, for this supernatant sample, the use of more than 8 g/L activated carbon was not advisable as it provided only marginal benefits of removing organic impurities at the expense of more significant loss of product (arabitol). Accordingly, the activated carbon treatment of the arabitol broth samples used in this study was carried out with 8 g/L activated carbon at 30°C and pH 6.

It should be noted that the activated carbon treatment was beneficial not only for color removal but also for reduction of the arabitol loss during the acetone extraction (data not shown). It appeared that arabitol had higher solubility in acetone when the impurities were not first removed.
Figure 10.1: Effects of treatment with different concentrations of activated carbon at different temperatures and pH, on the percent reduction of optical density and arabitol concentration, respectively. (Initial $A_{580}$ was 0.095 and arabitol concentration was 40.2 g/L. Standard deviations were in the range of 0.5-6% for $A_{580}$ and 0.3-2% for arabitol concentration.)

10.3.2 Acetone Extraction to Remove Glycerol

10.3.2.1 Solubility Study with Simulative Acetone-Glycerol-Xylitol Systems

Solubility of xylitol in acetone (with small amounts of glycerol) and glycerol (with small amounts of acetone) is given in Tables 10.1 and 10.2, respectively. It can be seen that the solubility of xylitol in acetone increased in the presence of glycerol (Table
10.1) and the solubility of xylitol in glycerol decreased in the presence of acetone (Table 10.2). The former trend suggested potentially higher arabitol loss during the acetone extraction for glycerol removal from fermentation broth.

Table 10.1: Effect of the presence of glycerol on the solubility of xylitol in acetone (values given as mass fractions)

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<td>0.0916</td>
<td>0.0148</td>
<td>0.8936</td>
</tr>
<tr>
<td>0.0673</td>
<td>0.0053</td>
<td>0.9274</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Glycerol solubility in acetone for xylitol-free systems was 9.5 % (by wt) at 25°C, 10.6% at 35°C, and 12.7% at 45°C, respectively.
Table 10.2: Effect of the presence of acetone on the solubility of xylitol in glycerol
(values given as mass fractions)

<table>
<thead>
<tr>
<th></th>
<th>25°C</th>
<th>35°C</th>
<th>45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycerol</strong></td>
<td><strong>Xylitol</strong></td>
<td><strong>Acetone</strong></td>
<td><strong>Glycerol</strong></td>
</tr>
<tr>
<td>0.9040</td>
<td>0.0960</td>
<td>0.0000</td>
<td>0.8680</td>
</tr>
<tr>
<td>0.8998</td>
<td>0.0249</td>
<td>0.0753</td>
<td>0.8460</td>
</tr>
<tr>
<td>0.8863</td>
<td>0.0138</td>
<td>0.0999</td>
<td>0.8387</td>
</tr>
<tr>
<td>0.8664</td>
<td>0.0126</td>
<td>0.1210</td>
<td>0.8354</td>
</tr>
<tr>
<td>0.8563</td>
<td>0.0090</td>
<td>0.1347</td>
<td>0.8271</td>
</tr>
<tr>
<td>0.8455</td>
<td>0.0053</td>
<td>0.1492</td>
<td>0.8227</td>
</tr>
<tr>
<td>0.8375</td>
<td>0.0000</td>
<td>0.1643</td>
<td>0.8194</td>
</tr>
</tbody>
</table>

10.3.2.2 Liquid-Liquid Equilibrium (LLE) Study with Simulative Acetone-Glycerol-Xylitol Systems

Experimental data for two xylitol-containing liquid (acetone-rich and glycerol-rich) phases in equilibrium are given in Table 10.3 for 25, 35 and 45°C, respectively. The corresponding ternary phase diagrams, including the results of both the solubility and LLE studies, are shown in Figure 10.2 for these acetone-glycerol-xylitol systems. Ternary phase diagrams are particularly helpful for design of the extraction process. For selective extraction of glycerol, from a mixture of glycerol and xylitol (or arabitol), the acetone should be added in such a way that a single liquid phase would form to dissolve all the glycerol and a minimal amount of xylitol, leaving the predominant majority of xylitol in a solid phase. As shown by the enlarged circles in Figure 10.2, the single acetone-rich
phase is a very small region, particularly at lower temperatures. Very minute amounts of xylitol would dissolve in this single phase, which is ideal for the intended purpose. The two-liquid-phase regime lies between the two single phases (acetone-rich and glycerol-rich) and below the topmost tie line. For extracting glycerol it is not recommended to operate in the two-phase regime because much more xylitol (or arabinol) would be lost into these liquid phases. Xylitol solids would be present in the regime above the single- and two-phase regimes.

Table 10.3: LLE data for acetone-glycerol-xylitol systems (mass fractions for glycerol and xylitol given, theremainders not shown for acetone)

<table>
<thead>
<tr>
<th></th>
<th>Acetone phase</th>
<th>Glycerol phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>xylitol</td>
<td></td>
</tr>
<tr>
<td>0.795</td>
<td>0.068</td>
<td>0.068</td>
</tr>
<tr>
<td>0.767</td>
<td>0.036</td>
<td>0.063</td>
</tr>
<tr>
<td>0.629</td>
<td>0.011</td>
<td>0.073</td>
</tr>
<tr>
<td>0.593</td>
<td>0.01</td>
<td>0.078</td>
</tr>
<tr>
<td>0.551</td>
<td>0.007</td>
<td>0.08</td>
</tr>
</tbody>
</table>
(B) 35°C

<table>
<thead>
<tr>
<th>Initial</th>
<th>Equilibrated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetone phase</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Xylitol</td>
</tr>
<tr>
<td>0.735</td>
<td>0.041</td>
</tr>
<tr>
<td>0.652</td>
<td>0.020</td>
</tr>
<tr>
<td>0.563</td>
<td>0.011</td>
</tr>
<tr>
<td>0.518</td>
<td>0.009</td>
</tr>
<tr>
<td>0.473</td>
<td>0.005</td>
</tr>
<tr>
<td>0.459</td>
<td>0.004</td>
</tr>
</tbody>
</table>

(C) 45°C

<table>
<thead>
<tr>
<th>Initial</th>
<th>Equilibrated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetone phase</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Xylitol</td>
</tr>
<tr>
<td>0.716</td>
<td>0.038</td>
</tr>
<tr>
<td>0.645</td>
<td>0.022</td>
</tr>
<tr>
<td>0.569</td>
<td>0.011</td>
</tr>
<tr>
<td>0.52</td>
<td>0.0087</td>
</tr>
<tr>
<td>0.468</td>
<td>0.0041</td>
</tr>
<tr>
<td>0.426</td>
<td>0.0040</td>
</tr>
</tbody>
</table>
Figure 10.2: Ternary phase diagram for Glycerol (1) + Xylitol (2) + Acetone (3) system at three different temperature namely 25°C, 35°C and 45°C

Consistency and reliability of the experimentally measured LLE data were ascertained by fitting the data with the Hand and Othmer-Tobias correlations [251]:

Hand correlation [252]
\[
\ln \left( \frac{x_2^\beta}{x_3^\beta} \right) = A + B \ln \left( \frac{x_2^\alpha}{x_1^\alpha} \right)
\]

(1)

Othmer–Tobias correlation [253]
\[
\ln \left( \frac{(1 - x_3^\beta)}{x_3^\beta} \right) = A_1 + B_1 \ln \left( \frac{(1 - x_1^\alpha)}{x_1^\alpha} \right)
\]

(2)
Where $x_i^\varphi$ stands for the mole fraction for component i (1 for glycerol, 2 for xylitol and 3 for acetone) in phase $\varphi$. $\alpha$ indicates the glycerol-rich phase, and $\beta$ the acetone-rich phase. The best-fit coefficients (A, B, A_1, and B_1) and correlation factors ($R^2$) are reported in Table 10.4. The correlations are shown in Figures 10.3 and 10.4 for Hand and Othmer-Tobias equations, respectively. The linearity of the best-fit lines, with $R^2$ values close to unity, indicates good consistency of the experimental data.

![Figure 10.3: Hand correlation of LLE data for glycerol (1) + xylitol (2) + acetone (3) systems at 25°C, 35°C and 45°C.](image-url)
Figure 10.4: Othmer-Tobias correlation of LLE data for glycerol (1) + xylitol (2) + acetone (3) systems at 25°C, 35°C and 45°C.

Table 10.4: Best-fit coefficients and correlation factors for Hand and Othmer–Tobias equations using the LLE data obtained with glycerol (1) + xylitol (2) + acetone (3) systems at 25°C, 35°C and 45°C.

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>Hand equation 1</th>
<th></th>
<th>othmer-tobias equation 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>R²</td>
<td>A</td>
</tr>
<tr>
<td>25</td>
<td>-4.264</td>
<td>0.518</td>
<td>0.979</td>
<td>-2.563</td>
</tr>
<tr>
<td>35</td>
<td>-3.737</td>
<td>0.604</td>
<td>0.967</td>
<td>-2.379</td>
</tr>
<tr>
<td>45</td>
<td>-2.736</td>
<td>0.771</td>
<td>0.9814</td>
<td>-1.878</td>
</tr>
</tbody>
</table>

10.3.2.3 Choice of Temperature for Glycerol Removal by Acetone Extraction

From the experiments for solubility determination, the results obtained with acetone as the extracting solvent are shown in Figure 10.5 (as the curves with symbols).
Acetone can clearly dissolve much more glycerol than xylitol and, thus, can be used to very preferentially extract glycerol from mixtures of glycerol and xylitol. For comparison, the solubility of glycerol in acetone, without the presence of xylitol, reported by McEwen [254] and the solubility of xylitol in acetone (without the presence of glycerol) reported by Wang et al. [246] are also included in Figure 10.5 (as the dashed lines). The presence of glycerol increases the solubility of xylitol in acetone while the presence of xylitol lowers the solubility of glycerol in acetone. It should be emphasized that there were no reported solubility data in the literature for the 3-component acetone-xylitol (or arabitol)-glycerol systems and there was no previous teaching for the use of acetone (or other solvent) for selective separation of polyols.
Figure 10.5: Solubility of xylitol and glycerol in acetone at different temperatures in two-component (xylitol-acetone and glycerol-acetone) and three-component (xylitol-glycerol-acetone) systems

The results shown in Figure 10.5 suggested that at temperatures higher than 30°C, the increase of xylitol solubility was faster than the increase of glycerol solubility, causing the undesirable loss of xylitol. Also, at temperatures lower than 30°C, the glycerol solubility decreases faster, requiring use of larger amounts of acetone to remove all glycerol (and higher associated capital and operating costs, particularly for recovering the solvent for reuse). 30°C appeared to be the optimal temperature for operating the extraction of glycerol by acetone. To be sure, calculations were done and shown for different temperatures (Column 1) in Table 10.5 for a hypothetical sample with 50 g/L xylitol and 20 g/L glycerol (Columns 2 and 3), similar to the concentrations typically
obtained in fermentation broths. The measured solubilities of xylitol and glycerol in acetone (as shown in Figure 10.4) are given in Columns 4 and 5. According to the glycerol solubility, the volume of acetone needed to extract the 20 g/L glycerol from the polyol mixture can be calculated (Column 6). To make sure complete removal of glycerol, an acetone wash with a quarter of the volume required for extraction is included in the purification procedure. The acetone volumes required for this wash are given in Column 7. The total acetone volumes needed for both extraction and wash are given in Column 8. According to the solubility of xylitol in acetone and the volume of acetone used at each temperature, the amount of xylitol removed in the acetone extraction and wash steps can be calculated. The calculated xylitol loss data are shown in Columns 9-11. It is seen that at 30°C the xylitol loss is indeed the lowest (2.1 g/L, corresponding to 4.2% of the 50 g/L xylitol present in the starting sample) and the acetone volume needed is significantly smaller than that at 25°C (for the second lowest xylitol loss).
Table 10.5: Comparison of acetone volume needed and xylitol loss at different temperatures

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>Concentration in Sample (g/L)</th>
<th>Solubility in Acetone (g/L)</th>
<th>Acetone Needed (ml)</th>
<th>Xylitol Loss (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylitol</td>
<td>Glycerol</td>
<td>Glycerol</td>
<td>Xylitol</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>20</td>
<td>51.8</td>
<td>3.7</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>20</td>
<td>64.2</td>
<td>4.3</td>
</tr>
<tr>
<td>35</td>
<td>50</td>
<td>20</td>
<td>66.9</td>
<td>6.5</td>
</tr>
<tr>
<td>40</td>
<td>50</td>
<td>20</td>
<td>73.9</td>
<td>8.9</td>
</tr>
<tr>
<td>45</td>
<td>50</td>
<td>20</td>
<td>76.3</td>
<td>12.4</td>
</tr>
</tbody>
</table>

10.3.2.4 Glycerol Removal from Real Arabitol Fermentation Broth

The experiment was done at 30°C according to the above simulative studies. The sample was measured to contain 37 g/L arabitol and 40 g/L glycerol (shown in Columns 2 and 3 of Table 10.6). An experiment was first done to evaluate how the presence of other organics and salts in the real sample, not completely removed by the activated carbon treatment, would affect the solubilities of glycerol and arabitol in acetone. For this evaluation, acetone was added at the volume required to extract the glycerol according to the glycerol solubility found from the previous study with the mixture of pure glycerol and xylitol (shown in Table 10.5), i.e., assuming there were no effects from the presence of other substances in the real broth sample. The equilibrated acetone phase was collected and analyzed by HPLC for arabitol and glycerol concentrations. It was found that compared to the solubilities obtained in the pure glycerol-xylitol mixtures, the glycerol solubility in acetone (33.8 g/L) was significantly lower and the arabitol solubility in
acetone (5.8 g/L) was higher when the real broth supernatant was extracted. The changes were found to be even larger if the broth supernatant was not treated with activated carbon; 27.8 g/L glycerol and 6.8 g/L arabitol were soluble in acetone. Later, the amount of acetone needed to extract all glycerol was calculated according to the solubility of glycerol in acetone measured for the real sample (Column 4) and the results of arabitol loss determined and reported in Table 10.6.

Table 10.6: Selective removal of glycerol by acetone extraction at 30°C and the associated arabitol loss for a real fermentation broth sample

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>Concentration in Sample (g/L)</th>
<th>Solubility in Acetone (g/L)</th>
<th>Acetone Needed (ml)</th>
<th>Arabitol Loss (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arabitol</td>
<td>Glycerol</td>
<td>Arabitol</td>
<td>Glycerol</td>
</tr>
<tr>
<td>30</td>
<td>37.3</td>
<td>40.2</td>
<td>33.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

10.3.3 Butanol Extraction and Crystallization of Arabitol

Butanol was selected for extracting arabitol according to the reported solubility of xylitol in different solvents. Solubility of xylitol in butanol is higher than in other solvents (acetone, pentanol, propanol, and toluene)[246]. The fermentation broth extracted had been treated with activated carbon, extracted with acetone to remove glycerol, and air-dried to vaporize residual acetone. Arabitol extraction by butanol was conducted at 90°C. Preliminary experiments done at 103°C and 95°C were found to leave blackish product. Higher temperatures appeared to cause thermal degradation of the product. The amount of butanol needed to extract arabitol completely was initially estimated from the reported solubility data of xylitol [246] in butanol by extrapolating the data to 90°C. The actual
solubility of arabitol in butanol, when the sample from real fermentation broth was used, was measured to be significantly lower, about 32 g/L at 90°C, instead of the 51 g/L estimated. It is yet unknown to what extent the solubility difference came from the presence of other materials versus from the difference of arabitol and xylitol. Nonetheless, the measured solubility was used to determine the needed butanol volume for extracting arabitol from the fermentation broth sample (about 900 ml butanol per liter of the starting fermentation broth).

Crystallization was then carried out at room temperature. White arabitol crystals were formed. The purity of recovered arabitol crystals was determined to be approximately 95%. The overall yield of arabitol crystals (24.6 g per L of broth supernatant) was 66%. Crystallization was also evaluated at lower temperatures. The purity of crystals obtained at 15°C and 10°C was found to be lower at 92% and 88%, respectively, but the product yield was higher at 70% and 73%, respectively. Lower crystallization temperatures appeared to negatively affect the product purity by precipitating other impurities along arabitol.

Finally it should be noted that in the real practice, the acetone phase from the extraction and wash steps would be collected. The acetone would be vaporized and collected for reuse. A small amount of water can be added to redissolve the glycerol and arabitol. This aqueous solution would be added back to the fermenter for the cells to consume the glycerol as substrate. Neither glycerol nor arabitol would really be lost or wasted.
10.4 Conclusion

A process for purifying arabitol from fermentation broth of *D. hansenii* was developed. Activated carbon was used to first remove some colored materials. The unconsumed glycerol in the broth was next removed by acetone extraction at 30°C. Arabitol was then extracted by hot butanol (at 90°C) and then crystallized in the butanol extract at room temperature. The activated carbon treatment is was found very important not only for color removal but also because these materials, if not removed, would reduce the glycerol solubility in acetone and increase the arabitol solubility in acetone, causing larger amounts of acetone required and more arabitol lost during the acetone extraction to remove glycerol. Solubility and liquid-liquid equilibrium data were also obtained for the 3-component xylitol-glycerol-acetone systems at different temperatures and used to optimize the selective glycerol removal by acetone extraction. The overall yield of arabitol crystals was found to be 66% and the arabitol purity in crystals was approximately 95%. Recovery and reuse of the solvents acetone and butanol and recycle of extracted glycerol and unrecovered arabitol to the broth would make this process even more cost effective.
CHAPTER XI

INHIBITORY EFFECT OF ARABITOL ON THE GROWTH OF AND ACID PRODUCTION BY ORAL BACTERIA

11.1 Introduction

Dental caries is one of the most prevalent chronic infectious disease in the world[255]. Although the widespread presence of dental caries has been reduced significantly over last few decades in some countries, the disease is still a major problem for both adults and children. Among the main factors causing dental caries are the metabolic activities of cariogenic microorganisms. Mouth contains a wide variety of bacteria; some of them are believed to cause dental caries: *Streptococcus mutans*, *Streptococcus salivarius*, and some lactobacilli are among them. There are many reports on which microorganisms are more dangerous for causing dental caries. Particularly for root caries and cavity formation in children’s teeth, *S. mutans* and *Lactobacillus acidophilus* are frequently identified as the most closely related bacteria[256]. These bacteria are found around the teeth and gums in a sticky, creamy-colored mass called plaque, which serves as an adhering biofilm. These bacteria are necessary but not sufficient for developing dental caries, which is exacerbated by different dietary factors[257, 258]. Excessive consumption of sugars, especially sucrose and glucose, is proved to be most responsible for the prevalence of dental caries regardless of the geography or age[259-261]. When fermentable carbohydrates such as glucose, sucrose,
fructose and galactose are taken into mouth, they are metabolized by these bacteria with production of organic acids such as lactic acid, acetic acid and formic acid. These acids diffuse through the plaque and into the porous enamel, dissolving the minerals, freeing calcium and phosphate into the solution (i.e., demineralization). The weakened enamel then collapses to form a cavity and the tooth is progressively destroyed[262-264].

This damaging acid production from fermentable carbohydrates has prompted the search for an easily available and acceptable sugar substitute. Xylitol is well-known for the anti-cariogenic property. It is not fermented by the cariogenic bacteria such as *S. mutans* and *Streptococcus sobrinus*[168, 265]. It also inhibits the acid production that would cause pH decrease in dental plaque and enamel demineralization. Chewing gums containing xylitol have been reported to reduce the dental plaque and the number of *S. mutans* in saliva[266]. Xylitol is currently produced by chemical reduction of xylose derived from wood hydrolysate under alkaline condition. But purification and separation of xylose as well as the required high pressure and temperature and expensive catalyst make the process less economical[267, 268]. Arabitol, a five carbon polyhydroxy sugar alcohol, is a 2´-epimer of xylitol. Arabitol however has never been investigated for its potential to inhibit the oral bacteria as xylitol. Moreover, sweetness per caloric content for arabitol is much higher than that of xylitol[179-181]. Sweetness vary among sucrose and different sugar alcohols. The degree of sweetness may vary from about half as sweet as the same amount of sucrose to equally as sweet as sucrose (Table 11.1).

Compared to sucrose, sugar alcohols are slowly and incompletely absorbed and metabolized in the small intestine which makes their caloric contents lower than sucrose. Among the sugar alcohols, arabitol has the lowest caloric content. In addition to its low
sweetness per caloric content, arabitol, like other sugar alcohols, can produce a noticeable cooling sensation in the mouth when consumed, due to the dissolution of arabitol being an endothermic reaction.\textsuperscript{36} Arabitol can be produced by osmophilic yeasts using sugars (e.g. glucose, arabinose) as substrate\textsuperscript{[192, 193]}. Recently, production of arabitol from glycerol, a biodiesel byproduct, by \textit{Debaryomyces hansenii}\textsuperscript{[6]} and subsequent purification process have been reported\textsuperscript{[269]}. These processes may make both arabitol and biodiesel production more economical.

Table 11.1: Comparison of sweetness and caloric content for different sugar substitutes, with sucrose as reference for sweetness\textsuperscript{[270]}

<table>
<thead>
<tr>
<th>Name</th>
<th>Relative sweetness (sucrose = 1.0)</th>
<th>Caloric content (kcal/g)</th>
<th>Sweetness per caloric content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabitol</td>
<td>0.7</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Erythritol</td>
<td>0.8</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Maltitol</td>
<td>0.9</td>
<td>2.1</td>
<td>0.43</td>
</tr>
<tr>
<td>Xylitol</td>
<td>1.0</td>
<td>2.4</td>
<td>0.42</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.5</td>
<td>1.6</td>
<td>0.31</td>
</tr>
<tr>
<td>HSH*</td>
<td>0.4–0.9</td>
<td>3.0</td>
<td>0.13–0.3</td>
</tr>
<tr>
<td>Isomalt</td>
<td>0.5</td>
<td>2.0</td>
<td>0.25</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.6</td>
<td>2.6</td>
<td>0.23</td>
</tr>
<tr>
<td>Lactitol</td>
<td>0.4</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.6</td>
<td>4.3</td>
<td>0.14</td>
</tr>
<tr>
<td>Sucrose</td>
<td>\textit{1.0}</td>
<td>\textit{4.0}</td>
<td>\textit{0.25}</td>
</tr>
</tbody>
</table>

*HSH = Hydrogenated Starch Hydrolysate

The objective of this study was to compare the purified arabitol with commercial xylitol in terms of the inhibitory effects on growth and acid production of oral bacteria.
First we investigated the effect of xylitol and arabitol, at a fixed 1% concentration, on 5 oral bacteria *S. mutans*, *S. salivarius*, *S. sobrinus*, *L. acidophilus* and *Lactobacillus fermentum*. Then the species found to be more inhibited by xylitol and arabitol, were further investigated for the inhibition effects in presence of different sugar substrates. Effects of different arabitol/xylitol concentrations were also investigated.

11.2 Materials & methods

11.2.1 Microorganisms

*S. mutans* (ATCC 10449), *S. salivarius* (NRRL B-3714), *S. sobrinus* QMZ 176 (NRRL B-4468), *L. acidophilus* (NRRL B-4495), and *L. fermentum* (NRRL B-1840) were used in this study. *S. mutans* was purchased from American Type Culture Collection (ATCC). The other four cultures were provided by USDA Agricultural Research Service (ARS) Culture Collection (Peoria, IL). The bacteria were maintained by weekly transfers on agar plates of trypticase yeast extract medium (Sigma Aldrich, St Louis, MO) containing 0.5% glucose and 1.5% agar.

11.2.2 Chemicals

Arabitol used in the experiment was produced in the laboratory by *D. hansenii* fermentation as described elsewhere. Arabitol obtained from fermentation broth was purified through the process of activated carbon treatment followed by vacuum concentration, acetone extraction of glycerol, butanol extraction, and crystallization, which yielded at least 95% pure arabitol. Xylitol (99+) was purchased from Sigma Aldrich (St. Louis, MO).
11.2.3 Inhibitory effect experiments on 5 bacterial species

All five strains of bacteria were cultured in the Brain Heart Infusion (BHI) Medium (Sigma Aldrich, St. Louis, MO) containing (in 1 liter solution) 17.5 g Calf Brain-Beef Heart Infusion, 10 g pancreatic digest of gelatin, 3 g NaCl, 2.5 g dipotassium phosphate (K₂HPO₄), 1 g monopotassium phosphate (KH₂PO₄) and 10 g glucose (Fisher Scientific, Hampton, NH). Xylitol and arabitol solutions were autoclaved separately and added to the sterile BHI medium to give 1% concentration. Control systems were prepared using same medium without any xylitol or arabitol. Cells were grown in 25 ml medium in a 125 ml Erlenmeyer flask inoculated with 5% (v/v) seed culture. (The seed culture was prepared by inoculating a single colony of cells from an agar plate into 25 ml BHI medium and incubating it overnight at 37 °C anaerobically.) Cultures were flushed with N₂ gas to make them anaerobic at the beginning and each time after collection of sample and closed by rubber stopper and incubated at 37 °C for 36 h in a shaker (Thermo Scientific MaxQ 5000 Incubating/Refrigerating Floor Shaker, Asheville, NC) at 250 rpm. Samples were taken periodically for analysis.

11.2.4 Effect of different sugar substrates

From the results of growth and acid production by all 5 bacteria, two of them, \textit{S. mutans} and \textit{S. salivarius}, were selected for investigating the effects of 4 different sugar substrates (glucose, galactose, fructose, sucrose) with or without 1% arabitol or 1% xylitol on the growth and acid production of these bacteria. In these experiments, for each bacterium studied, there were three systems for each substrate (e.g. 1% fructose): one control (without arabitol or xylitol), one with 1% arabitol, and another with 1% xylitol.
11.2.5 Effect of arabitol or xylitol concentration

*S. mutans* and *S. salivarius* were further investigated for the comparative effect of different concentrations of xylitol and arabitol, respectively. For each bacterium 5 different arabitol concentrations (0.01%, 0.1%, 0.5%, 1% and 2%) and, for selective comparison, 3 different xylitol concentrations (0.1%, 0.5% and 1%) were investigated.

11.2.6 Analytical Methods

Growth of the bacteria was monitored by measuring absorbance at 660 nm (A\textsubscript{660}) by a UV/VIS spectrophotometer (Model UV-1601, Shimadzu Corporation, Columbia, MD). Samples were diluted 10 times to ensure the absorbance value in the linear range and then shaken properly before placing in the spectrophotometer to get representative cell concentration reading. The pH readings of samples were taken to indicate acid production during the anaerobic growth of oral bacteria. All experiments were done in triplicate and the average values are reported. To establish the substrate consumption profile, 1 ml sample was collected at 8, 16, 24 and 30 h, centrifuged at 8000 rpm (7440 g, Sorvall RC 5C) to remove the cells, and then the supernatant was saved for analysis by high pressure liquid chromatography (HPLC). For HPLC analysis the samples were filtered through 0.2-µm Millipore filters (Danvers, MA) and then analyzed using the HPLC system (LC 10AT, Shimadzu) with a refractive index detector. A carbohydrate column (Supelco Column H, 250 x 4.6 mm, with a guard column, 50 x 4.6 mm, Bellefonte, PA) was used at room temperature. The mobile phase was 0.1% H\textsubscript{3}PO\textsubscript{4} in HPLC grade water (VWR) at a flow rate of 0.14 ml/min.
11.3 Results

11.3.1 Effects of 1% arabitol or xylitol on growth and acid production of 5 oral bacteria with glucose as the sugar substrate

Results obtained from the growth experiments of 5 oral bacteria on glucose in presence of 1% xylitol or 1% arabitol showed that growth of *Streptococcus* species is clearly inhibited while the inhibition effect on the *Lactobacillus* species is less significant. The profiles of cell growth \( (A_{660}) \) and pH decrease observed for *S. mutans*, which was found to be most inhibited by the two polyols, are shown in Figure 11.1 for examples. It is clear that arabitol and xylitol at 1% concentration had comparable effects on the bacterium by decreasing the growth rate, limiting the maximum cell concentration achieved, and significantly reducing the pH decrease.
Figure 11.1: Profiles of (a) cell growth (shown in $A_{660}$), (b) pH decrease, and (c) glucose consumption (filled symbols) and arabinol/xylitol nonconsumption (unfilled symbols) in the control and 1% arabinol- or xylitol-added systems of *S. mutans*, showing
clear and comparable inhibition effects of the two isomers on cell growth, acid
production and glucose consumption of this oral bacterium.

To allow easy comparison of effects of arabitol and xylitol on all of the bacteria
studied, the growth rate was compared by calculating the cell doubling time (Td, in h)
during the fastest growth period (assumed to be the exponential-growth phase): Td =
\[ \ln(2)/\mu_{\text{max}} \], where \( \mu_{\text{max}} \) (in h\(^{-1}\)) is the maximum specific growth rate determined as the
slope of the steepest increasing line in the semilogarithmic plot of \( A_{660} \) versus time.
Accordingly, results obtained for all 5 bacteria are summarized in Table 11.2, by
comparison of the doubling time (Td) and the maximum cell concentration (\( A_{660} \)) and the
lowest pH reached at the end of experiments (48 h for the experiments with \( S. \) salivarius
and \( S. \) sobrinus, 30 h for all others). The inhibitory effects of arabitol and xylitol were
comparable for all species and for all parameters compared (Td, maximum \( A_{660} \), and
minimum pH). The 5 bacteria can be divided into 3 groups, in order of their sensitivity to
these two polyols: \( S. \) mutans > \( S. \) salivarius, \( S. \) sobrinus > \( L. \) acidophilus, \( L. \) fermentum.
Table 11.2: Effects of 1% arabitol and xylitol, respectively, on growth and acid production of 5 oral bacteria compared in terms of cell growth doubling time (Td) during the fastest growth period and the maximum cell concentration (in A660) and lowest pH reached at the end of experiments (48 h for *S. salivarius* and *S. sobrinus*, 30 h for the others)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Control</th>
<th>1% Xylitol</th>
<th>1% Arabitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Td (h)</td>
<td>Max A660</td>
<td>Min pH</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>1.9 (±0.1)</td>
<td>0.28 (±0.02)</td>
<td>4.2 (±0.1)</td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td>1.7 (±0.1)</td>
<td>0.31 (±0.01)</td>
<td>5.1 (±0.1)</td>
</tr>
<tr>
<td><em>S. sobrinus</em></td>
<td>1.6 (±0.2)</td>
<td>0.35 (±0.03)</td>
<td>4.8 (±0.1)</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>1.9 (±0.1)</td>
<td>0.34 (±0.01)</td>
<td>4.5 (±0.1)</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>1.9 (±0.1)</td>
<td>0.36 (±0.01)</td>
<td>4.4 (±0.1)</td>
</tr>
</tbody>
</table>

Note: Average (± standard deviation) reported from no. of independent experiments (n):
n = 6 for *S. mutans*, n = 4 for *S. salivarius*, n = 3 for all others.

*S. mutans*

In case of *S. mutans*, the cell-growth doubling time (Td) was about 2 times longer in presence of 1% arabitol or xylitol (1.74 and 1.79 times for arabitol and xylitol, respectively). Maximum final growth (A660) was also significantly reduced, by 54% and 61% for arabitol and xylitol, respectively. Final pH was significantly closer to neutral, at 6.4 and 6.3 in presence of 1% arabitol and xylitol, respectively, as compared to the acidic
pH of 4.2 in the control. Significantly decreased acid production by the presence of arabitol and xylitol is clearly indicated.

*S. salivarius* and *S. sobrinus*

The inhibition effect of arabitol and xylitol on *S. salivarius* and *S. sobrinus* differed from the effect on *S. mutans* not only in the extents of inhibition but also in the patterns (i.e., potentially in the mechanisms). Cell growth profiles of *S. salivarius* are shown in Figure 11.2. Similar profiles were seen for *S. sobrinus* (data not shown). When compared with the growth profiles of *S. mutans* shown in Figure 11.1, two major differences (in patterns) are clear. The first difference is that for *S. salivarius* and *S. sobrinus* 1% arabitol or xylitol caused an approximately 8-h lag phase before the cells could assume growth, which was not seen for *S. mutans* or for any of the controls. Once they overcame the lag phase, *S. salivarius* and *S. sobrinus* grew with slower rates than in the uninhibited controls but, interestingly, the doubling times were not prolonged as much as in the case of *S. mutans*. The doubling time increases were about 1.5 times for *S. salivarius* and about 1.7 times for *S. sobrinus*, smaller than the about 2 times increase seen for *S. mutans* (Table 11.2). The second noticeable growth-profile difference between Figures 11.1 and 11.2 is that, under the inhibition of arabitol/xylitol, the growth of *S. mutans* tapered off after about 20 h while the growth of *S. salivarius* and *S. sobrinus* continued even during the period of 24 to 48 h. At the end of experiments, the maximum A660 values reached were only 19% and 16% lower for *S. salivarius* and 27% and 23% lower for *S. sobrinus* in presence of 1% arabitol and xylitol, respectively. These decreases were far lower than the 54% and 61% decreases seen for *S. mutans* (Table 11.2). The lower extents of long-term inhibition on *S. salivarius* and *S. sobrinus* than on *S. mutans*
were similarly reflected in the effect on final pH. In presence of 1% arabitol or xylitol, the final pH values for *S. salivarius* and *S. sobrinus* systems were in the range of 5.5 to 5.8, appreciably lower than the final pH of 6.3 or 6.4 for *S. mutans*. So the long-term inhibition on acid production by arabitol and xylitol was also clearly more severe for *S. mutans* than for *S. salivarius* and *S. sobrinus*.

Figure 11.2: Growth profiles of *S. salivarius* in control and the systems added with 1% arabitol or xylitol showing clear and comparable inhibition effects of the two isomers, including the clear lag phase to overcome prior to growth. Similar behaviors were observed for *S. sobrinus* (data not shown).

*L. acidophilus* and *L. fermentum*

Growth of these two lactobacilli was minimally inhibited by 1% arabitol or xylitol. Their doubling times were only increased by 10-20% and the maximum cell
Concentrations reached were reduced by only 5-10%. The overall growth profiles (not shown) were also similar to that of control. Arabitol and xylitol were still beneficial in reducing the acid production. The lactobacilli lowered the final pH to 4.4 to 4.5 in controls but only to the range of 5.0 to 5.3 in presence of 1% arabitol or xylitol. It should be mentioned that even though the lactobacilli produce lactic acid, they are considered less important for initial caries development. It has been reported that *L. acidophilus* has effects mostly in the progression of caries that have been already initiated by other acid producing bacteria such as *S. mutans*.

Glucose, arabitol, and xylitol consumption

Concentrations of glucose, arabitol and xylitol were measured by HPLC for the samples taken along the inhibition experiments on all 5 bacteria. It was found that neither arabitol nor xylitol was metabolized by these bacteria; for example, the profiles of arabitol and xylitol concentrations measured for the *S. mutans* systems are shown in Figure 11.1(c). Glucose was consumed, along cell growth and acid production (see Figure 11.1(c) for the profiles in the *S. mutans* systems). The inhibition effects of arabitol/xylitol on the amounts of glucose consumed, determined at the end of experiments, for these bacteria followed the same order as the inhibition on overall cell growth or acid production (pH decrease): *S. mutans* > *S. salivarius*, *S. sobrinus* > *L. acidophilus*, *L. fermentum*. Stronger inhibition resulted in lower glucose consumption. After 30 hours of incubation, *S. mutans* consumed 7 g/l glucose in the control but consumed only 2.5 and 2.3 g/l glucose in presence of 1% arabitol or xylitol, respectively. *S. salivarius* and *S. sobrinus* consumed more glucose, in the range of 5.1 to 5.9 g/l, in presence of arabitol or xylitol. For the two lactobacilli, glucose consumption was not
significantly different with or without arabitol or xylitol. The overall amounts of glucose consumed correlate with the extents of cell growth.

11.3.2 Effects of different sugar substrates on the inhibition by 1% arabitol/xylitol on S. mutans and S. salivarius

This study on sugar substrate effect was done with only S. mutans and S. salivarius, as arabitol and xylitol inhibited streptococci more clearly. The sugar substrates compared were 1% glucose, galactose, fructose, or sucrose. Experiments were carried out for 30 h. The results of doubling time (T_d), maximum cell concentration, and final pH are summarized in Table 11.3 for all systems with and without addition of the sugar alcohols. A few general observations can be made from the tabulated results: (1) In the controls without arabitol/xylitol, the 4 sugars supported similar growth for each of the two streptococci (but different between the two species). (2) Arabitol and xylitol at 1% concentration clearly inhibited cell growth and acid production from any of the 4 sugar substrates. (3) Between arabitol and xylitol, the inhibition effects caused by these two isomers were similar. (4) The inhibition effects varied for systems with different sugar substrates. To more easily see these different inhibition effects, Figure 3 is prepared by averaging the results from arabitol and xylitol and plotting them as % T_d increase, % maximum A_660 decrease, and final pH, caused by the 2 isomers on each of the 4 sugar substrates.
Table 11.3: Effects of 1% arabitol or xylitol on the growth and acid production of *S. mutans* and *S. salivarius* in media with different sugar substrates (experiment duration: 30 h)

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>S. mutans</em></th>
<th></th>
<th></th>
<th><em>S. salivarius</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T_d</em></td>
<td>Max <em>A</em></td>
<td>Final pH</td>
<td><em>T_d</em></td>
<td>Max <em>A</em></td>
<td>Final pH</td>
</tr>
<tr>
<td>Glu</td>
<td>1.9±0.1</td>
<td>0.28±0.02</td>
<td>4.2±0.1</td>
<td>1.7±0.1</td>
<td>0.31±0.01</td>
<td>5.1±0.1</td>
</tr>
<tr>
<td>Glu+Ara</td>
<td>3.3±0.1</td>
<td>0.13±0.01</td>
<td>6.4±0.1</td>
<td>2.6±0.1</td>
<td>0.21±0.02</td>
<td>5.9±0.1</td>
</tr>
<tr>
<td>Glu+Xyl</td>
<td>3.4±0.1</td>
<td>0.11±0.01</td>
<td>6.3±0.1</td>
<td>2.7±0.1</td>
<td>0.19±0.01</td>
<td>5.8±0.1</td>
</tr>
<tr>
<td>Gal</td>
<td>2.1±0.1</td>
<td>0.32±0.03</td>
<td>4.5±0.1</td>
<td>1.9±0.1</td>
<td>0.38±0.01</td>
<td>4.9±0.0</td>
</tr>
<tr>
<td>Gal+Ara</td>
<td>3.1±0.2</td>
<td>0.10±0.01</td>
<td>5.9±0.2</td>
<td>2.5±0.1</td>
<td>0.33±0.03</td>
<td>5.3±0.1</td>
</tr>
<tr>
<td>Gal+Xyl</td>
<td>3.2±0.1</td>
<td>0.11±0.01</td>
<td>5.7±0.1</td>
<td>2.5±0.1</td>
<td>0.31±0.02</td>
<td>5.3±0.1</td>
</tr>
<tr>
<td>Fru</td>
<td>2.3±0.1</td>
<td>0.27±0.02</td>
<td>4.3±0.1</td>
<td>1.7±0.1</td>
<td>0.36±0.03</td>
<td>5.0±0.1</td>
</tr>
<tr>
<td>Fru+Ara</td>
<td>3.1±0.1</td>
<td>0.13±0.01</td>
<td>4.9±0.1</td>
<td>2.2±0.1</td>
<td>0.29±0.02</td>
<td>5.4±0.2</td>
</tr>
<tr>
<td>Fru+Xyl</td>
<td>3.0±0.1</td>
<td>0.14±0.01</td>
<td>5.1±0.1</td>
<td>2.3±0.1</td>
<td>0.28±0.02</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>Suc</td>
<td>2.0±0.1</td>
<td>0.34±0.03</td>
<td>4.3±0.2</td>
<td>1.8±0.1</td>
<td>0.37±0.02</td>
<td>4.7±0.1</td>
</tr>
<tr>
<td>Suc+Ara</td>
<td>2.7±0.1</td>
<td>0.20±0.01</td>
<td>5.6±0.1</td>
<td>2.1±0.1</td>
<td>0.29±0.02</td>
<td>5.3±0.1</td>
</tr>
<tr>
<td>Suc+Xyl</td>
<td>2.8±0.1</td>
<td>0.20±0.02</td>
<td>5.7±0.1</td>
<td>2.1±0.1</td>
<td>0.31±0.03</td>
<td>5.2±0.2</td>
</tr>
</tbody>
</table>

Average ± standard deviations reported from 3 independent experiments
Figure 11.3. Effects of different sugar substrates (glucose, galactose, fructose, and sucrose, at 1% concentration) on the growth and acid production inhibition by 1% arabitol/xylitol on *S. mutans* and *S. salivarius*; growth inhibition shown by both the increase in doubling time \((T_d)\) and the decrease in maximum cell concentration \((A_{600})\), achieved at the end of 30-h experiments; acid production shown by the final (minimum) pH

Figure 11.3 clearly shows the stronger inhibition of arabitol/xylitol on *S. mutans* than on *S. salivarius*, as described earlier. For example, the inhibition on the maximum cell concentrations in the *S. mutans* systems was in the range of 40-65%, much higher than the range of 18-25% for the *S. salivarius* systems. More importantly, for the purpose
of this set of experiments, Figure 3 shows an approximate order of inhibition on metabolism of these sugars: glucose > galactose > fructose > sucrose (with some inconsistencies such as the higher inhibition on maximum \textit{S. mutans} concentration grown on galactose than glucose).

11.3.3 Effects of different arabitol/xylitol concentrations on growth and acid production of \textit{S. mutans} and \textit{S. salivarius}

Experiments were made for 30 h with 1% glucose as sugar substrate and with arabitol concentrations of 0.01, 0.1, 0.5, 1 and 2 g/L, and compared with xylitol at selected concentrations of 0.1, 0.5 and 1 g/L. Results of $T_d$, maximum cell concentration ($A_{660}$) and final pH obtained at different arabitol/xylitol concentrations are shown in Figure 11.4. Again, arabitol and xylitol at same concentrations gave comparable inhibition effects. The extent of inhibition depended on the arabitol/xylitol concentration, and the dependency was particularly strong for \textit{S. mutans}. Future work on the mechanistic model for the inhibition kinetics can use the quantitative data obtained in this study for estimating the model parameters and potentially providing more insights to the different behaviors of these streptococci at different arabitol/xylitol concentrations.
(a) *S. mutans*

- Arabitol/Xylitol Concentration (%)
- Maximum A$_{660}$
- Td, Final pH

(b) *S. salivarius*

- Arabitol/Xylitol Concentration (%)
- Maximum A$_{660}$
- Td, Final pH
11.4 Discussion

This study showed that, under anaerobic conditions, the growth and acid production of streptococci were clearly inhibited by the presence of arabitol and xylitol, and the inhibition effects were comparable between the two sugar alcohols. Inhibitory effect of xylitol on S. mutans has been studied by several investigators. The mechanisms involved in the growth inhibition and reduced acid production for glucose as the sugar substrate have also been proposed[272-274]. For S. mutans, xylitol is transported and phosphorylated across the cell membrane via the phosphoenolpyruvate–phosphotransferase system (PEP-PTS), forming intracellular xylitol-5-phosphate (X5P). Accumulated X5P is dephosphorylated to xylitol and expelled outside the cell body. This futile phosphorylation-dephosphorylation cycle wastes the PEP potential. Since PEP is a phosphoryl donor for ATP synthesis and is also required for glucose transport via the PEP-glucose-PTS system, this futile cycle can inhibit cell growth and acid production from glucose metabolism. In addition, the intracellular X5P inhibits phosphoglucose isomerase (PGI) and phosphofructokinase (PFK), the principal glycolytic enzymes responsible for converting glucose-6-phosphate (G6P) to fructose-1, 6-bisphosphate (FBP). This inhibited glycolysis can restrict cell growth. The above inhibition also causes lower FBP levels. FBP is a known activator for lactate dehydrogenase (LDH), the enzyme principally responsible for lactic acid production from pyruvate. The lower FBP
levels due to xylitol inhibition may cause reduced lactic acid production. In this study arabitol and xylitol are shown to have very similar inhibition effects on cell growth and acid production. It is highly possible that the two isomers share the same inhibition mechanisms.

Regarding the inhibition in media with different substrates, Kakuta et al. did a similar study[275]. In addition to the 4 sugars examined in this current work, they included also lactose and maltose in their study. But they studied only xylitol and only with *S. mutans*. They observed that the levels of xylitol inhibition in the systems with sucrose and fructose were clearly lower than those in the others, consistent with the order of inhibition observed in this current work. Kakuta et al. hypothesized that, compared to glucose, fructose has higher affinity for the PEP-PTS transport system and therefore can better out-compete with xylitol transport and minimize X5P (xylitol 5-phosphate) formation. This way not much PEP-PTS enzyme activity is wasted on the futile cycle of xylitol transport and the X5P-effected inhibition on glycolytic enzymes does not occur as much as in the case of growth on glucose.

As described in the Results section, arabitol/xylitol inhibition caused two major differences in the growth profiles observed between *S. mutans* and the other two streptococci examined, i.e., *S. salivarius* and *S. sobrinus*. For the initial, short-term (~ 8 h) inhibition, arabitol/xylitol at 1% concentration completely stopped the growth of *S. salivarius* and *S. sobrinus* but only slowed down the growth rate of *S. mutans*. In the experiments studying the effects of different arabitol/xylitol concentrations, the cell-growth lag phase observed for *S. salivarius* and *S. sobrinus* was not seen for *S. mutans* even at the higher 2% arabitol concentration (data not shown). Arabitol/xylitol clearly
had stronger short-term inhibition on *S. salivarius* and *S. sobrinus* than on *S. mutans*. But, for the longer-term inhibition, *S. salivarius* and *S. sobrinus* were seen to adapt and develop ways to more successfully, although still partially, overcome the inhibition mechanism(s) on them while *S. mutans* did not show apparent adaptation to the arabitol/xylitol inhibition. Even at the higher 2% arabitol concentration (Figure 11.4), the long-term (30 h) inhibition on *S. salivarius* did not become significantly more pronounced. *S. salivarius* (and probably *S. sobrinus*) could partially overcome the inhibition even at this higher concentration. So the long-term inhibition effect of arabitol/xylitol was clearly lower on *S. salivarius* and *S. sobrinus* than on *S. mutans*. No information is available for the inhibition mechanisms on *S. salivarius* and *S. sobrinus*. Nonetheless, results of this study are important when considering the intended applications of these sugar alcohols, if important to differentiate short- and long-term inhibition effects.

More study is needed to understand the 3 different levels and profiles of inhibition exerted by arabitol/xylitol on the 5 oral bacteria examined. A future study is also warranted to build a kinetic model that includes all the inhibitory mechanisms previously proposed and fit the model with all the quantitative data of glucose consumption, cell growth and acid production obtained in this work. The model can provide more insights on the inhibition mechanisms of arabitol/xylitol on oral bacteria.

Finally, two other relevant findings in the literature reports should be noted. First, regarding the higher pH observed in systems under arabitol/xylitol inhibition, it was reported that the concentrations of basic amino acids and ammonia increased when dental plaque was exposed to xylitol[276, 277]. So xylitol (and probably arabitol) may have
other beneficial mechanisms, in addition to the reduced acid production, to effect a less acidic environment in vivo. Second, Makien reported[278] that the invertase activity of human saliva in vivo was lower in presence of xylitol. As a result, xylitol could negatively affect the hydrolysis of sucrose to glucose and fructose in the saliva and thereby inhibit the growth of oral bacteria. Therefore, although arabitol and xylitol were found to have the least significant inhibition effects on sucrose metabolism by oral streptococci in this and Kakuta et al.’s[275] studies, the inhibition on sucrose utilization in vivo might still be strong due to the reduced invertase activity in the saliva.

11.5 Conclusion

Realizing the importance of arabitol in prevention of dental caries, present investigation focused on the comparative effects of arabitol and xylitol in inhibiting growth and acid production of oral bacteria. Arabitol and xylitol were found to have very similar effects on all parameters and profiles compared for all bacteria tested. When examined for bacterial growth in media with 1% glucose as sugar substrate, both had strong inhibition effects on *S. mutans* and relatively weak inhibition effects on *L. acidophilus* and *L. fermentum*. Their short-term inhibition effects, observed at 8 h, were even stronger (completely stopping cell growth at 1% arabitol/xylitol concentration) on *S. salivarius* and *S. sobrinus* than on *S. mutans*. But *S. salivarius* and *S. sobrinus* could adapt and partially overcome the inhibition (after 8 h) while no apparent adaptation was evident for *S. mutans*; consequently, the long-term inhibition was stronger on *S. mutans* than on the other streptococci. When compared for growth of *S. mutans* and *S. salivarius* on different sugar substrates, the two isomers showed the following approximate order of
inhibition strength: glucose > galactose > fructose > sucrose. The concentration
dependency of inhibition effects by these two sugar alcohols was also determined.
Arabitol is a new polyol that is found to be effective for inhibiting growth and acid
production of oral bacteria in this study. Given less expensive arabitol production by
yeast fermentation, the finding of this study could open up a new opportunity for
prevention of dental caries.
CHAPTER XII
CONCLUSION & RECOMMENDATION

12.1 Summary & conclusion

The summary and conclusions obtained from this research projects are described in this section.

1. An enzymatic process is developed to hydrolyze the soybean flour carbohydrate, which were separated in the solution and the remaining soy protein concentrated were enriched in protein content. This process improved the quality of soy protein concentrate by decreasing the indigestible carbohydrates.

2. The operating conditions of the enzymatic hydrolysis was optimized. Temperature and pH were the critical operating conditions which were found to be optimum at 50°C and 4.8 respectively for enzyme hydrolysis with enzyme broth produced by both A. niger and T. reesei.

3. Multiple enzyme activity effect on the enzyme hydrolysis were investigated including cellulase, xylanase, pectinase and α-galactosidase. After thorough investigation, a kinetic model is developed which was highly efficient to predict the hydrolysis yield both in terms of total soluble carbohydrate and reducing sugars. Solid loading was found to have negative effect on the hydrolysis which is also incorporated in the model.
4. Using the knowledge from kinetic model, efforts were made to develop improved process for enzyme hydrolysis including multiple fed-batch scheme and recycle of enzyme broth. Fed-batch process was found to improve the hydrolysis yield by mitigating the negative effect of high solid loading. When 375 g/l solid loading was used, hydrolysis yield can be increased from 65% in the batch process to 72-73% in the fed-batch process. However, fed batch process has the limitation to increase the sugar concentration beyond certain concentration. So recycle of enzyme process was developed to further increase the sugar concentration of the hydrolysate up to 109 g/l.

5. After developing effective hydrolysis process, separating the protein rich concentrate from liquid hydrolysate is investigate. Effective process was developed to maximize the protein recovery in the soy protein concentrate by further precipitating soluble proteins in the hydrolysate. The refinement of soy protein concentrate was investigated by washing off the entrapped soluble sugar from the concentrate and the final protein content of the soy protein concentrate was reached to 80%. Protein recovery was found to be more than 90%.

6. Sugar rich hydrolysate was used as the fermentation feedstock for the production of arabinol by *Debaryomyces hansenii* fermentation. For obtaining high yield of arabinol in the fermentation, different operating conditions and medium compositions were investigated. The soy flour hydrolysate can be used as both carbon and nitrogen source for the growth of yeast and production of arabinol. At high DO conditions, arabinol yield of alose to 50% of the initial sugar content was obtained from the fermentation. Arabinol concentration of 40 g/l was obtained from 80 g/l of initial sugar in the hydrolysate after 2 days of fermentation.
7. Arabitol purification process from the fermentation broth of *D. hansenii* was developed. The purification process consists evaporating the water and extraction of arabitol in the butanol phase and precipitating of arabitol at room temperater. The purification process yield was higher than 70% with more than 95% purity.

8. Finally arabitol was investigated for its applicability as preventing dental carries by inhibiting growth of and acid production by oral bacteria. 5 oral bacterial strains were investigated and arabitol was found to greatly inhibit the growth and acid production in bacteria. This suggests that arabitol, with its potential as low calorie sweetener, can be an effective agent to prevent dental caries formation.

Overall the process developed in the current process in described in figure 12.1.

![Figure 12.1: Integrated enzyme based processing of soybean flour for production of protein rich product and utilization of carbohydrate as fermentation feedstock for production of arabitol.](image)
12.2 Economic viability

The economic viability of this process is analyzed by one of my group mate Dr. Nicholas Callow. On a basis of 50,000 ton/year soy protein concentrate (SPC) product, the product price would be $0.99/kg. The price was obtained using net present value (NPV) of 0 after 20 years. Which is much lower than the current market price of $1.5 to $2 per kg of the soy protein concentrate. If the product price is set at current market price of $1.5 per kg than the NPV after 20 years would be 108 million dollars. The cash flow diagram for both cases are presented in figure 12.2 and 12.3. Moreover, this product would have competitive advantage as the protein content (72%) is higher than the protein content of currently available SPC and the indigestible carbohydrates are greatly reduced.

![Cash Flow Diagram](image)

**Figure 12.2:** Cash flow diagram of the project setting net present value (NPV) of 0
Figure 12.3: Cash flow diagram setting product price equal to current market price of $1.5 per kg.

12.3 Future recommendation

The total carbohydrate degradation of soy flour in the current study was around 70–75%. The complete hydrolysis could improve the protein content even higher and could be as high 90%. To get the nearly complete hydrolysis, enzyme composition need to be improved. As outlined in the modeling part, the enzyme composition is highly important to get effective hydrolysis of the soy flour carbohydrate. The enzyme composition, particularly higher pectinase and cellulase activity could be helpful for complete hydrolysis. Hydrolysis can be improved by other means such as investigating the structure of the protein and carbohydrates in the soy proteins. Whether protein is
preventing the enzyme to work on the substrates? The literature suggested that the calcium crosslinking with pectic polysaccharides made the hydrolysis of pectins difficult. If that’s the case, the use of chelating agent might be helpful for increasing the hydrolysis yield. These studies would be helpful to further increase the carbohydrate hydrolysis and protein content in the SPC.

Another aspects of the interesting future study would be the process to separate remaining proteins from the hydrolysate. This would help to improve the fermentation process for arabinol production. Currently the protein concentration in the hydrolysate is more than required as nitrogen source. This made it difficult to reach stationary phase during the growth of yeast. The stationary phase arabinol production yield is higher. If the protein content can be decreased so that the culture can reach to stationary phase, the arabinol production yield can be increased.
REFERENCES


72. Glencross, B.D., et al., *A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon* (*Salmo*...


Enzyme activity is one of the main concerns to optimize the hydrolysis process. Since the previous disclosure we have made significant progress in the fermentation process development to improve enzyme activity. From the optimization of enzyme composition it was found that pectinase activity is one of the main constraints for high productivity of pectinase. Productivity depends on medium composition and process conditions. Soyhull contains higher pectin and it has the potential to induce the pectinase production if it’s included in the medium. pH profile also affect the pectinase productivity to a great extent. If soyhull is used as the principle carbon source then pH would have even higher effect on the cell growth and pectinase activity. Because carbon source in the soyhulls presents mainly in the form of cellulose, hemicellulose and pectin. These complex carbohydrates needs to be degraded to smaller monosaccharides to be utilized by the fungi as carbon source. pH is crucial factor for the effective hydrolysis of soybean hull. Hydrolysis is more effective at pH from 4.8 to 5.6. On the other hand pectinase production is better at higher pH. So it is possible to control the pH at higher value so that the cell can grow utilizing the available substrates. When the consumption rate by the cells increased due to higher cell concentration, lowering pH would increase the hydrolysis rate of the substrate providing the required carbon source. This phenomena also would simultaneously induce
the cells to produce more enzymes. Control of this pH could help to increase the pectinase activity to higher extent.

For example, we have controlled pH through a new scheme that will help to produce pectinase and α-galactosidase at higher activity. For the fermentation, medium composition was presented in the table below:

Table A.1: Nutrient composition and trace element concentration in the fungal fermentation medium.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH4)2SO4</td>
<td>2.8 g/L</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>2 g/L</td>
</tr>
<tr>
<td>MgSO4-7H2O</td>
<td>0.3 g/L</td>
</tr>
<tr>
<td>CaCl2 - 2H2O</td>
<td>0.4 g/L</td>
</tr>
<tr>
<td>NH2CONH3 - Urea</td>
<td>0.6 g/L</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>2 g/L</td>
</tr>
<tr>
<td>Tween - 80 (1.08 g/mL)</td>
<td>1.85 ml</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>1 ml</td>
</tr>
<tr>
<td>Trans 280</td>
<td>1 ml</td>
</tr>
<tr>
<td>Soy Hulls</td>
<td>40</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>g/L</td>
</tr>
<tr>
<td>FeSO4 - 7H2O</td>
<td>0.005</td>
</tr>
<tr>
<td>MnSO4 - H2O</td>
<td>0.0016</td>
</tr>
<tr>
<td>ZnSO4 - 7H2O</td>
<td>0.0014</td>
</tr>
<tr>
<td>CoCl2</td>
<td>0.002</td>
</tr>
</tbody>
</table>
pH during the 5 day fermentation period was controlled from 7 to 5. For the first 3 days it was decreased from 7 to 5 at constant rate and then kept constant at pH 6 for 24 hours and then decreased to 5 from 6 in next 24 hours. Dissolved oxygen level was maintained over 20% for the whole period of time. After 5 days the cells were separated from the broth and the enzymes were analyzed for the activity of different enzymes. Activity of pectinase & α-galactosidase, pH and DO profile is presented in the figure below.

Maximum activity level of pectinase & α-galactosidase were found to be 8.15 U/ml and 7.8 U/ml respectively. This activity level is higher than previously obtained activity of 3-4 U/ml & 2-3 U/ml for pectinase & α-galactosidase respectively. This pH control was found to be effective for increasing the activity level for both enzymes.

α-galactosidase activity profile suggests that it was increased almost linearly from day 1 through day 5. On the other hand, pectinase activity stopped increasing after 3 days and started decreasing after 4 days. Although pH was decreased from 6 to 5 in the final day, DO level was found to be increasing after 3 days suggesting the cell activity was decreased. This DO level increase could be responsible for the reduction in the pectinase activity.
From suggested optimum enzyme mixture, it was found that the current α-galactosidase activity (7.8 U/ml) is sufficient to degrade majority of the oligosaccharides to monosaccharides. But the pectinase activity is still lower than suggested level by enzyme optimization. It was also found that pectinase activity was stopped increasing after 3 days and started decreasing after 4 days. This decrease was suggested due to the increase of DO level after 3 days. pH control could be instrumental for availability of reduced sugar. If the pH was decreased very fast then the higher hydrolysis rate could make the reducing sugar easily available and pectinase production may not be induced. On the other hand if the pH level is not decreased enough the availability of substrate may not be possible which could cause the death of the cells. So if the pH can be controlled based on the DO
level from the beginning that could solve this problem. pH decrease can be directed by the increase in the DO level. This way cell will have both available substrate and induction for pectinase production.

For example, we have done fermentation run with DO directed pH control within the level of pH 7 to 5. Medium composition and the other process conditions were kept same as the previous experiments. Pectinase profile is presented in the figure below. It was found that the pectinase activity continued to increase until 5 days. And final activity level of the pectinase (11.6 U/ml) is significantly higher than the earlier fermentation run (8.1 U/ml).

Figure A.2: Enzyme activity and process conditions (pH & DO) profile of the fermentation run with DO directed pH control.
APPENDIX B

STERILIZATION TECHNIQUES FOR SOYBEAN MEAL FOR SOY ENZYME HYDROLYSIS

1. Introduction:

Soy protein from soy beans have important applications in food industry, due to their high nutritional value, and in adhesive, cosmetics, paper coatings, textiles, water-based paints and textiles, due to their emulsifying properties. For applications in the food industry, soy proteins have several advantages over animal proteins: low cost vegetable source, high nutritional value as food recognized by FDA and USDA, and versatile functional properties such as binding, emulsifying, and flavor- and texture-improving qualities.

The enzymatic process used for enrichment of soy proteins is to be done at about 50 °C. The high temperature curbs the growth of common mesophilic microorganisms. Occasional growth of certain thermophilic bacteria has been observed, particularly if the process time is longer than 1 day. The process involves rich proteins and carbohydrates in aqueous media. Strict control of microbial growth during the process is essential. So different sterilization procedure were investigated.
2. Materials and Methods:

Defatted soy flour obtained from Archer Daniels Midland Company (Decatur, IL) was used for the enzymatic hydrolysis in this study. Defatted soy flour was mixed with an enzyme medium in a 250 ml Erlenmeyer flask such that the solid (soy flour) to liquid ratio was 1:4 w/v (i.e., 10 g soy flour in 40 ml medium) unless mentioned otherwise. Initial pH was adjusted to 4.8. After application of one of the sterilization methods described in the following sections, soy flour was enzymatically processed at 50 °C in a temperature controlled shaker at 250 rpm with the Erlenmeyer flask closed with a rubber stopper. Enzyme hydrolysis was performed for about 2 days with sampling for sugar release and pH measurement.

2.1 Sterilization Methods:

Different methods were tested. The procedures and conditions used in these methods are described in detail in the following:

(a) Liquid autoclaving:

Soy flour (10 g) and water (40 ml) were mixed in an Erlenmeyer flask and autoclaved at 121 °C for 15 min. 30 ml of enzyme solution, harvested carefully from fermentation broth by sterile procedures, was added to the autoclaved mixture (making a solid to liquid ratio of 1:7) to start the enzymatic hydrolysis. The larger liquid volume used in this set of experiments was because, after autoclaving, the soy slurry agglomerated into curd/cheese-like material. The additional liquid volume was thought to improve the re-suspension for better enzymatic reactions. The enzyme activities of the enzyme solution used in this study are listed in Table B.1 (Solution 1). Total enzyme activity loading used was 50 units/g of soy flour.
(b) Vacuum autoclaving:

10 g soy flour was vacuum-autoclaved for 15 min in a 250 ml Erlenmeyer flask. Vacuum-autoclaved soy flour was then mixed with the same sterile enzyme solution as in (a) and pH was adjusted to 4.8.

(c) Preservatives:

Sodium benzoate and sodium nitrite were individually evaluated for their use as preservatives, for controlling the growth of thermophilic bacteria during the enzymatic hydrolysis process. Concentration of these preservatives tested was 2 g/L. Soy flour was mixed with sterilized enzyme solution (enzyme broth was filtered with 2 µl nylon filter and water was autoclaved at 121 °C) in a ratio as described above (1:4) with 2 g/L sodium benzoate/sodium nitrite in a sterilized Erlenmeyer flask. The activity of the enzymes used in this study was as listed in Table 1. Enzyme loading used was 50 units/g of soy.

(d) Dry heat:

In dry heat sterilization method, soy flour was heat sterilized in drying oven (Model ED 53-UL from VWR international) at 3 different temperatures (150, 160 and 170 °C) for one or two hours before using in enzyme hydrolysis. After dry heat sterilization of soy flour, it was mixed with sterilized enzyme solution (enzyme broth was filtered with 2 µl nylon filter and water was autoclaved at 121 °C) in a sterilized Erlenmeyer flask. The activity of the enzymes used in this study was as listed in Table B.2. Enzyme loading used was 50 units/g of soy.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity, (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td>2.3</td>
</tr>
<tr>
<td>Xylanase</td>
<td>70</td>
</tr>
<tr>
<td>Pectinase</td>
<td>7.8</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
</tr>
</tbody>
</table>

Table B.1. Enzyme activities in the broth considered for enzyme hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity, (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td>0.35</td>
</tr>
<tr>
<td>Xylanase</td>
<td>60.6</td>
</tr>
<tr>
<td>Pectinase</td>
<td>2.1</td>
</tr>
<tr>
<td>Total</td>
<td>63.1</td>
</tr>
</tbody>
</table>

Table B.2. Activities of cellulase, xylanase and pectinase considered for the enzyme hydrolysis with soybean meal for heat sterilization studies.

2.3 Analytical Methods:
Reducing sugar concentration was measured by the dinitrosalicylic acid (DNS) method, based on the color formation of DNS reagent when heated with reducing sugars [52]. Total carbohydrate concentration was measured by the phenol sulfuric acid method [279].

3. Results and Discussion:
3.1 Sterilization Methods:
The main objective of this study was to select the best method to prevent microbial growth during the enzymatic hydrolysis process. In the preliminary
experiments without any attempt for microbial control, the microbial growth had been recognized to cause pH decrease and consumption of released sugars after certain period of hydrolysis (as the profiles shown in Figure B.1 for the non-sterilized system). Presumably, the pH decrease was due to organic acid production from microbial carbohydrate metabolism and the increase-then-decrease profile of released sugars was due to the initially faster hydrolysis rate than the microbial sugar consumption rate when microbial concentration was low and the reversed rate order caused by rapid microbial growth. Accordingly, the selection of best microbial control method in this study was based on the comparison of pH and reducing sugar release profiles observed in the different enzymatic hydrolysis experiments. As mentioned earlier in materials and methods section, two different enzyme solutions from different fermentation broths obtained under different medium and operating conditions were used for different sterilization methods. First, all the sterilization methods except for dry heat sterilization which had same enzyme broth were discussed.
The different methods used for sterilization of soy flour, were compared with control and non-sterilized systems. Control was the system with same solid to liquid ratio but with no enzyme. Non-sterilized system was also with same solid to liquid ratio but
with enzyme solution similar to the rest of the sterilized method systems but the enzyme solution used was not sterile. The pH profile for these systems was shown in Figure B.1(a). Initial pH of all the systems was adjusted to 4.8 before the hydrolysis began, which is the optimal pH for best activities of most of the enzymes such as cellulase, pectinase and xylanase. From the pH profile it was evident that except in case of preservatives, all the systems showed decrease in the pH to about 4 in two days. The decrease in the pH could be related to the release of organic acids released from the bacterial growth. Figure B.1(b). shows the reducing sugar release profiles for these systems. Ideally, in the absence of any bacterial growth during the enzyme hydrolysis, the sugar released should not be decreased in concentration throughout the hydrolysis process. From Figure B.1(b). except in case of use of preservatives, the reduced sugar concentration profile showed a decreasing trend in all the systems after 14 h. This decrease in the sugar concentration showed the bacterial growth from the consumption of released sugars during the enzyme hydrolysis. This comparison study clearly indicated that among the sterilization methods that were studied for the elimination of bacterial growth during the enzyme hydrolysis of soy flour, use of preservatives was the best method.

Another type of sterilization method that was studied was dry heat sterilization as described in materials and methods section. Three different temperatures (150, 160 and 170 °C) and two different time periods (1 and 2 h) were considered resulting in 6 systems. These systems could not be compared directly to other sterilization methods in terms of total reducing sugar amount released, as the enzyme broth used was different in these methods. However, the decrease in the reducing sugar concentration in the reducing
sugar profile was used as an indicator in identifying the system with bacterial growth during the hydrolysis. Figure B.2. shows the reducing sugar profile for the 6 systems with dry heat sterilization method. From this figure, all the soy hydrolysis systems except systems heat treated at 160 °C for 2 h and 170 °C for 2 h showed decrease in the reducing sugar concentration after 12 h. Dry heat sterilization at 160 °C for 2 h and 170 °C for 2 h performed almost similar in terms of total reducing sugar concentration released.

![Reducing Sugar Profile](image)

Figure B.2: Comparison of reduced sugar concentration profiles among heat sterilized systems at 150, 160 and 170 C for 1 and 2 h during soy flour hydrolysis.

It was found that heating soy flour inactivated trypsin inhibitor which is known as a growth depressor by reducing the digestibility of the proteins when used in food [280, 281]. Heat sterilization seems to be beneficial in reducing the activity of trypsin inhibitor but, also denatures proteins at these high temperatures. Though dry heat sterilization was effective in preventing any bacterial growth during soy enzyme hydrolysis, it was limited.
to amount of soy flour that could be heat sterilized in one batch. It was observed that after heat treatment of soy flour at 160 °C for 2 h or 170 °C for 2 h, soy flour turned from initial creamy white color to brown color after heating. This color change was presumably a result of Milliard reactions between proteins and carbohydrates in the soy flour at high temperatures. When large amount of soy flour was used for heat sterilization in an oven, soy flour was placed as bulk amount. After the heat treatment, it was observed that not all the soy flour turned to brown indicating that soy flour has to be placed as thin sheets for the heat to reach homogeneously. This was the only drawback in this process unless the soy flour is placed in a way that heat treatment is uniform throughout. Use of preservatives overcomes this disadvantage as the preservatives could be homogeneously dispersed in soy hydrolysis.

Soy flour when used in a fermentation process with *Streptomyces* Sp. No 6907, it was sterilized at 121 °C for 20 min suspended in water [282]. In that process, 4% soy flour was used. In our study, 20% soy flour was used and at this high solid concentration, soy flour suspension in water after sterilization at 121 °C for 15 min became agglomerated in to curd/cheese like material. Soy flour in curd form would be difficult to be used in enzyme hydrolysis process as it would be difficult for the enzyme to attack the substrate sites. This type of autoclave method at 121 °C would not be feasible for enzyme hydrolysis process. Another method for soy flour sterilization using steam injection of soy slurry with its pH around its isoelectric point for 3-30 sec at a temperature range of 225-400 °F [283]. Also, in this case, when temperature used is 350 °F, soy flour suspension turned in to heavy paste with viscosity more than 10,000 cps. This type of steam injection was able to avoid bacterial growth (bacterial count < 10,000), the change
in the physical form of the soy may not be beneficial for the enzyme hydrolysis. As discussed in our study, either dry heat sterilization (160 °C for 2 h or at 170 °C for 2 h) or use of preservatives would be ideal for eliminating bacterial growth during the enzyme hydrolysis of soy.