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# Improving the Nutritional Characteristics of Plant Feedstuff By-Products Using Fungal Metabolism

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# IMPROVING THE NUTRITIONAL CHARACTERISTICS OF PLANT FEEDSTUFF BY-PRODUCTS USING FUNGAL METABOLISM

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this thesis to my father and mother, grandmothers, and brother and sister. To my father Greg for teaching me the values of hard work, to my mother for teaching me the importance of learning, to my grandmothers for their constant support of my interests, and to my brother Nick, and sister Erin for setting positive examples contributing to my success.

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# ABBREVIATIONS

ADF- Acid-Detergent Fiber

AFEX- Ammonia Fibre Expansion

ANF- Anti-Nutritional Factors

CF- Crude Fiber

CP- Crude Protein

COD- Chemical Oxygen Demand

DM- Dry Matter

DS- Dissolved Solids

GG- Guar Gum

GKM- Guar Korma Meal

GRAS- Generally Regarded as Safe

GYE- Glucose Yeast Extract

GKMS- Guar Korma Meal Solubles

HPLC- High Performance Liquid Chromatography

LHW- Liquid Hot Water

MBP- Microbial Biomass Protein

NDF- Neutral-Detergent Fiber

PDA- Potato Dextrose Agar

RGKM- Raw Guar Korma Meal

SBM- Soybean Meal

SCP- Single-Cell Protein

SH- Sorghum Hominy

SLR- Solid-loading Rate

SmF- Submerged-State Fermentation

SSF- Solid-State Fermentation

TS- Total Solids

UDS- Undissolved Solids

WGKM- Washed Guar Korma Meal

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# ABSTRACT

IMPROVING THE NUTRITONAL CHARACTERISTICS OF PLANT FEEDSTUFF BY-PRODUCTS USING FUNGAL METABOLISM

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Plant feedstuff by-products such as soy processing wastewater, guar korma meal, and sorghum hominy are very different, however, all possess a rich nutrient profile. Lessening their value is the presence of lignocellulose, plant anti-nutritional factors, and unfavorable protein profiles. Fungal conversion processes are an attractive approach to improving the value of these by-products by degrading detrimental fractions of each while simultaneously creating nutrient-rich cell mass. The aim of this research was to improve the value of each by-product for potential application in monogastric diets such as fish. Fungal organisms, both yeast and filamentous fungi, were examined for their ability to enhance the nutritional value of each by-product.

Soybean processing supernatant contains a high amount of organic matter (carbohydrates and proteins) resulting in a high chemical oxygen demand (~55,000 mg/L), meaning that this by-product cannot be disposed of without treatment. Typically evaporation is used to concentrate the material from 7-10% solids to 40-50% solids which can be used in animal feeds. To improve the nutritional value of this material, eight fungal strains were tested for their ability to metabolize the soluble nutrients in the wastewater and produce protein-rich cell mass for potential application in animal feeds. Flask incubations trials of 100 ml total volume were conducted at 30°C and 150 rpm. *Trichoderma reesei, Paecilomyces variotii* and *Neurospora crassa* produced 51.7, 47.1

and 43.2 g/L of biomass while reducing solids present by 46.5, 48.9, and 49.1% respectively. The two best performing strains were further examined in fermenters using 3 L total volume. *Trichoderma reesei* and *Neurospora crassa* produced 55.5 and 62.0 g/L of protein-rich biomass while simultaneously reducing chemical oxygen demand levels by 10.53 and 23.04% respectively. Microbial processing produced a protein-rich animal feed ingredient while concurrently reducing organic matter present in the wastewater.

Sorghum hominy contains a high amount of starch (34.5%), fiber (7.6%), and low amount of protein (12.9%) which inhibits its inclusion into higher value feed industries such as aquafeeds. To improve the nutritional value of the sorghum hominy, eight fungal strains were assessed for their ability to degrade the carbohydrate fractions of the sorghum hominy while simultaneously improving the protein profile for potential use in aquadiets. Flask incubations were performed using 100 ml total volume at 30°C and 150 rpm. *Trichoderma reesei, Rhizopus oligosporus, Neurospora crassa* and *Aurebasidium pullulans* were the best performing fungi in flask trials, increasing the protein titer of sorghum hominy by 53.6, 48.0, 47.8, and 41.5% compared to the raw material. *T. reesei* and *A. pullulans* were further tested in 5 L benchtop reactors, where biomass yields of 65.1 and 73.6 g/L were obtained with similar increases in protein to the previous flask trials. Lastly, *T. reesei* was examined in 70 L and 150 L fermenters, increasing all amino acids in the range of 33.3-152.3%. Thus, *T. reesei* submerged fermentation effectively increased the nutritional value of sorghum hominy.

Guar korma meal contains a high amount of protein (55.6%), but also contains fiber (6.6%) and residual guar gum (0.77%) which inhibits its effectiveness as a feed ingredient in monogastric diets. To improve the nutritional value of guar korma meal, the

prospect of subjecting the guar korma meal to a centrifugal wash pre-fungal incubation was examined. For both the washed concentrate and washed solubles, five fungal strains were examined for their ability to create a protein-rich biomass for potential application as a feed ingredient, while degrading detrimental fractions of the washed concentrate. Flask incubations of 100 ml total volume were conducted at 30°C and 150 rpm. *T. reesei* was the most effective treatment on the washed guar korma meal as it reduced guar gum levels to 0.17% and increased the protein titer to 66.8% yielding 65.8 g/L of biomass. *A. pullulans* was the most effective treatment on the guar korma meal solubles, producing 12.8 g/L of protein-rich (40.6%) biomass. Together the two-step fungal incubation process yielded 78.6 g/L of biomass containing 61.5% protein.

# Chapter I- Literature Review

## 1.1 Overview

Research on pretreatment technologies of plant feedstuffs has been widely examined in academic and industrial settings. Typically, pretreatments are categorized as either physical, chemical, physicochemical, enzymatic, and microbial. Each possess their own advantages and disadvantages, as well as have been shown to be more effective on certain biomass. For this reason, it is important to understand each process to optimize the bioconversion of plant feedstuff by-products.

## 1.2 Physical pretreatments

Physical pretreatments increase the accessible surface area and decrease the crystallinity of lignocellulosic material. Processes applied for size reduction such as milling, chopping, and grinding on their own are effective in improving the accessibility of important nutritional fractions in feed; however, they are typically used as a precursor to increase the effectiveness of other pretreatment methods e.g. enzymatic, chemical, and microbial (Behera et al. 2014). These initial steps typically consume a large amount of energy, but are necessary to avoid mass- and thermal- transfer issues in downstream processing (Schell and Harwood 1994).

### 1.2.1 Dry Rolling

Dry rolling is a simple process commonly employed to increase the availability of starch in corn. This process can be conducted in a variety of ways, typically using a roller mill or set of roller mills to crack corn. The resulting fraction possesses smaller particles which has been shown to increase feed digestibility in cattle (Engel et al. 2015).

The dry rolling process is arguably one of the simplest and cheapest treatments of lignocellulosic biomass. Researchers estimated the cost of dry milling of corn to be $1.58 per metric ton in a 2006 study, compared to $4.71 and $9.57 for ensiling high-moisture and steam-flaked corn respectively (Macken et al. 2006).

### 1.2.2 Steam Flaking

Steam flaking is a process commonly applied in cereal grain processing to gelatinize starches, thus making them more digestible for the target animal. In areas where the availability of corn is limited, steam flaking is an attractive approach to increase the feeding value of the grain despite the higher processing costs than dry-rolling or high-moisture ensiling (Barajas and Zinn 1998).

The steam flaking process takes place in a steam chamber containing the grain and can be explained by three steps: conditioning, flaking, and drying. During conditioning, water is added to the steam chamber to soak the grain to approximately 21-22% moisture. Steam is then injected into the system to further increase moisture and to increase the temperature to 100°C. After the desired moisture and temperature have been achieved, the grain is kept for a period of time to allow proper heat distribution. Following conditioning comes the mechanical step of flaking using counter-rotating rolls. Starch granules are ruptured as well as size reduction of biomass, each improving the digestibility of the biomass. Lastly, the material is dried and cooled typically using forced air to evaporate water and transfer heat out of the system (Zinn et al. 2002).

Steam flaking has been shown to increase the feed value of corn in lactating cows by improvements in diet acceptability and digestibility of starch and nitrogen (Plascencia

and Zinn 1996). Also, steam flaking has been shown to increase starch digestibility compared to dry rolling (Barajas and Zinn 1998). However, this energy-intensive process has been shown to be more expensive compared to treatments such as dry rolling or ensilaging high-moisture corn (Macken et al. 2006).

### 1.2.3 Liquid Hot Water

Liquid hot water (LHW) uses a combination of hot water (160-220°C) and pressure to treat lignocellulosic biomass. Pressure is mainly used to keep biomass in contact with water and to reduce evaporation (Haghighi et al. 2013). This process has been commonly applied in ethanol bioconversion processes and is very similar to traditional sterilization cooks used in large-scale fermentations. Differences in the rates of hydrolysis between cellulose and hemicellulose have resulted in two-step LHW treatments to effectively degrade both fractions (Rabemanolontsoa and Saka 2016).

Many studies have examined the effect of LHW on hydrolysis of biomass containing large amounts of lignocellulosic material. LHW treatment of corn stover at 190°C for 15 min has been noted to solubilize up to 40% of total solids, mainly in the form of the hemicellulose xylan. Higher heat levels (>200°C) were shown to neither increase carbohydrate solubility nor increase sugar yields. Xylose levels were shown to increase when using high heat and increases in furfural were observed (Mosier et al. 2005). Higher temperatures as well as longer treatment times during LHW treatment result in the formation of degradation products (xylose -> furfural) inhibitory to fermentation (Klinke et al. 2004). As previously mentioned, two-step LHW treatments have been developed with the intention to first solubilize the hemicellulose fraction at lower temperatures while avoiding the formation of degradation products, then secondly

using higher temperatures on the non-soluble, cellulose-rich fraction to increase enzymatic reactivity. A two-step LHW treatment on *Eucalyptus grandis* recovered 96.6% of total sugars which proved to be more effective than single-step LHW and dilute acid pretreatments (Yu et al. 2010).

### 1.2.4 Extrusion

Extrusion processes have been used commercially for over 70 years to produce human food such as corn meal snacks. Since then, the use of extrusion processes has been applied in pet foods and more recently aquatic feeds (Rokey et al. 2010). Extrusion processes typically employ a combination of mechanical shear, moisture, pressure and temperature to alter characteristics of food and feed. Typically, extrusion processes are applied to blended feed rations to shape into pellets used for feed (Obaldo et al. 2000). Extruders consist of a hopper containing blended feed, a preconditioner, extruding cylinder, and die assembly. The extruding cylinder contains a flighted screw inside the compact cylinder (Rokey et al. 2010). An example can be seen in (Figure 1.1). Essentially there are two different mechanical types of extruders used in the feed industry; ie. single-screw and twin-screw extruders (Serrano 1997). As the feed is subjected to high pressures inside the extrusion cylinder, the feed product is subjected to high shear rates. As a result of shear, long molecules present in feed are cross-linked or restructured changing the overall texture (Serrano 1997).

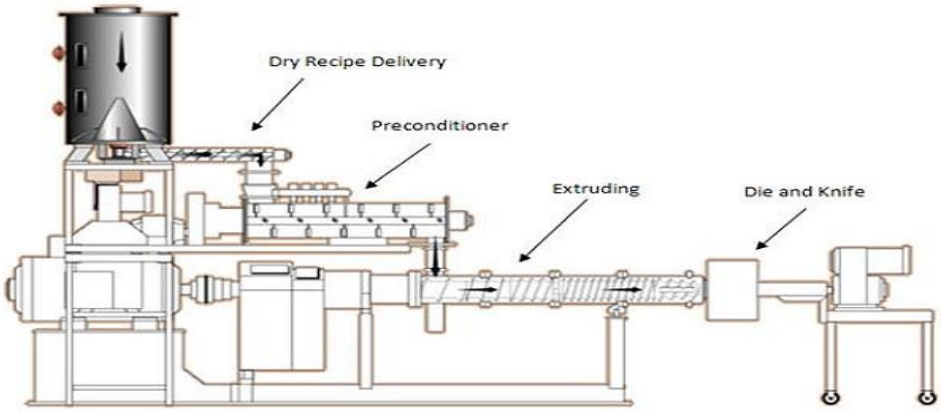


**Figure 1.1: Common feed extruder design** (Machine 2018)

The extrusion process consists of feeding, preconditioning, extrusion, and die-cutting steps. First the blended material is fed into the preconditioner from the hopper. The materials rate of entry into the preconditioner is controlled using an auger to ensure a consistent product. Next, the blended meal is mixed and preconditioned by the addition of steam to achieve moisture levels of 10-25% (Rokey et al. 2010). Preconditioning allows for the consistent alteration of physical and chemical properties of the biomass during the process (Obaldo et al. 2000). After preconditioning, the biomass is fed into the extruding cylinder which involves forcing the biomass through a barrel by the action of the screw. Pressure in the cylinder increases towards the end of the cylinder as output is restricted. Towards the end of the cylinder, the temperature is rapidly increased for several seconds prior to exiting the cylinder via the die. Temperature and pressures can reach over 125°C and 34 atm respectively at the end of the cylinder. Finally, the extruded material exits the cylinder through the die which controls the shape and expansion rates of the material (Rokey et al. 2010). Due to the process, both chemical and physical changes occur such as particle size reduction, starch gelatinization, and inactivation of enzymes (Cheng and Hardy 2003). Consequently, the nutrient digestibility, palatability,

pellet durability, and water stability of extruded feedstuffs are thought to improve (Barrows and Hardy 2000).

Overall, extrusion is a highly versatile and productive process with many advantages- especially in developing aquafeeds. For example, the nutritional characteristics of otherwise low-value proteins obtained from feed by-products can be improved via texturization through extrusion processes (Harper 1986). The effect of extrusion on availability of nutrients also has been explored. The extrusion of soybean meal increased digestibility of dry matter in soybean meal from 75-78% and digestibility of energy from 79% to 82% in rainbow trout measured *in vivo* (Cheng and Hardy 2003). Extruding wheat flour increased soluble fiber from 40% to 75% therefore increasing total digestible energy (Bjorck et al. 1984).

Although the extrusion process can be effective in breaking down negatively associated factors present in plant biomass, it also is known to degrade beneficial components. For example, extrusion temperatures, pressure and moisture are known to affect the stability of a variety of vitamins (Table 1.1). At milder extrusion temperatures (91-95°C) vitamin stability decreased only slightly except in the case for vitamin C. However, at higher temperatures (141-145°C) vitamin stability decreased another 7.1-36% for most vitamins and 91.2% in the case of vitamin C (Riaz et al. 2009). Also, the overall costs of operating a twin-screw extruder are typically 1.5 times the cost of a single-screw extruder (Hauck 1990).

**Table 1.1. Effect of extrusion temperature on different vitamins stability** (Asif et al. 2009)

| Fat-Soluble Vitamins | 91-95°C | 141-145°C | % Loss from 95 to 145°C |
|---|---|---|---|
| A, beadlet | 90 | 62 | 25.2 |
| D, beadlet | 96 | 86 | 9.6 |
| E, acetate | 95 | 81 | 13.3 |
| Water-Soluble Vitamins | | | |
| Thiamine HCL | 90 | 50 | 36 |
| Riboflavin | 98 | 91 | 7.1 |
| B6 | 93 | 73 | 18.6 |
| B12 | 97 | 86 | 10.67 |
| Pantothenic acid | 94 | 75 | 17.86 |
| Folic acid | 93 | 64 | 26.97 |
| Biotin | 93 | 63 | 27.9 |
| Niacin | 92 | 64 | 25.7 |
| Vitamin C | 57 | 5 | 91.22 |

## 1.3 Chemical Pretreatment

Chemical pretreatments methods tend to follow initial physical processes when treating lignocellulose. Application of acids, bases, and salts can remove lignin and/or hemicellulose therefore decreasing the crystallinity of cellulose (Arora et al. 2014). Instances of the application of sulfuric acid for the hydrolysis of cellulose dates back to the beginning of the 19$^{th}$ century (Braconnot 1819). Since the onset of this discovery, concentrated and dilute chemical treatments have been applied for the hydrolysis of lignocellulosic materials (Arora et al. 2014).

### 1.3.1 Acid Hydrolysis

A very popular method of lignocellulosic pretreatments involves using acids. This acid hydrolysis method first began using inorganic acids such as sulfuric, hydrofluoric, and phosphoric acids for biomass pretreatments. More recently, the use of organic acids

such as maleic, succinic, oxalic, and fumaric acids for lignocellulose degradation have been explored (Rabemanolontsoa and Saka 2016). On top of types of acids, acid hydrolysis also involves the use of concentrated or dilute acids to achieve desired effects; nevertheless, each possess their own advantages and disadvantages.

The use of concentrated acids allows for high yields of glucose from cellulose even at low temperatures. Subsequently, the use of concentrated acids results in indirect and negative effects on the lignocellulosic materials. For example, the hemicellulose fraction of lignocellulose is susceptible to degradation into furfural when using concentrated acids which may inhibit latter fermentation treatments as well as cause detrimental health effects in animals (Rabemanolontsoa and Saka 2016). Maximum levels of furfural in animal feeds for various species can be seen in (Table 1.2). Concentrated acids also contribute to the corrosion of equipment, and are toxic to the environment which requires expensive acid recovery processes (Jones and Semrau 1984). On the other hand, the use of dilute acids overcomes disadvantages such as corrosiveness and recovery processes needed for concentrated acids; however, heat must be applied to attain economically acceptable glucose yields from cellulose (Rabemanolontsoa and Saka 2016).

**Table 1.2: Max safe furfural concentrations in animal feeds for various species** (Commision 2016)

| Target Animal | Default values | | Maximum safe intake/feed concentrations | |
|---|---|---|---|---|
| | Body Weight (kg) | Feed Intake (g/day)[a] | Intake (mg/day) | Concentrations (mg/kg feed)[b] |
| Salmonids | 2 | 40 | 1.1 | 27 |
| Veal Calves | 100 | 2,000 | 54 | 27 |
| Cattle for fattening | 400 | 8,000 | 216 | 24 |

| | | | | |
|---|---|---|---|---|
| Pigs for fattening | 100 | 3,000 | 54 | 18 |
| Sows | 200 | 6,000 | 108 | 18 |
| Dairy Cows | 650 | 20,000 | 351 | 15 |
| Turkeys for fattening | 12 | 400 | 6.5 | 16 |
| Piglets | 20 | 1,000 | 11 | 11 |
| Chickens for fattening | 2 | 120 | 1.1 | 9 |
| Laying hens | 2 | 120 | 1.1 | 9 |
| Dogs | 15 | 250 | 8.1 | 29 |
| Cats | 3 | 60 | 1.6 | 24 |

[A] Complete feed with 88% DM, except milk replacer for veal calves (94.5% DM), and for cattle for fattening, dairy cows, dogs and cats for which are DM intake.
[B] Complete feed containing 88% DM, milk replacer 94.5% DM

Similar to other pretreatment methods, two-step processes have been developed to offset disadvantages seen in simpler single-step processes. In one such example, the first step consists of using a 70% sulfuric acid at 30-40°C to hydrolyze hemicellulose and decrystallize crystalline cellulose regions. Next, the acid is diluted to 30-40% with water and temperature is increased to 90-95°C to hydrolyze the decrystallized cellulose. Ion exchange columns are used to recover the acid from the hydrolyzed lignocellulose (Hogan 2009).

### 1.3.2 Alkaline Hydrolysis

Similar to using acids as catalysts, alkaline pretreatments have also been developed. Alkaline pretreatment processes commonly utilize non-corrosive chemicals such as ammonia, sodium hydroxide, sodium carbonate, and calcium hydroxide (Kim et al. 2016). Alkaline pretreatment using NaOH has been used heavily in the paper and pulp industries. It is believed the general function of alkaline processes is the disruption of lignin carbohydrate linkages, therefore increasing the total surface area and porosity of

the lignocellulosic biomass (Zheng et al. 2014). Alkaline hydrolysis processes and their subsequent effects can be seen in (Table 1.3).

**Table 1.3: Alkaline pretreatment processes and their effects on biomass (Kim et al. 2016)**

| Catalysts | Reaction types and conditions | Major effects | Remarks |
|---|---|---|---|
| Sodium Hydroxide | 0.5-10.0% NaOH 60-180°C, 5-60 min, SLR:10-30% | 50% hemicellulose dissolution, 60-80% delignification, difficulty in recovery of NaOH | High reaction rate |
| Sodium carbonate | 1-30% $Na_2CO_3$, 60-180°C, 5-60 min, SLR:10-30% | 20-40% hemicellulose dissolution, 40-60% delignification, Easier recovery than NaOH | Green liquor |
| Ammonium hydroxide | 5-30% ammonia, 3-210°C, 5-60 min, high pressure (2-17 atm), SLR: 10-50% | 10-50% hemicellulose solubilization, 0-80% delignification and profound swelling Lignin removal or modification | ARP, SAA, LLA |
| Anhydrous gaseous ammonia | Gaseous ammonia 25-80°C, ~72 h, SLR: 50% | No need for washing low liquid loading and mild reaction condition | LMAA |
| Liquid anhydrous ammonia | 70-90°C, ~5 min, high pressure (15-20 atm), anhydrous ammonia, SLR: 60-90% | No hemicellulose dissolution. No lignin removal. Rapid evaporation and liquefication of ammonia | AFEX |
| Lime | 25-130°C, 1 h-8 weeks 0.05-0.15 g $Ca(OH)_2$/g biomass, SLR: 5-20% | 20-40% hemicellulose dissolution. 60-80% delignification, de-acetylation. Low energy requirement | Simple and low-cost process |

Alkaline hydrolysis pretreatment processes hold their own advantages and disadvantages. Compared to acid hydrolysis processes, alkaline pretreatments generally require milder reaction conditions. Alkaline hydrolysis is known to be an effective method in both solubilizing lignin and altering the overall structure. Due to alkaline processes strong effects on lignin, the overall effectiveness of these processes are connected with the total lignin content of the biomass (Zheng et al. 2014).

## 1.4 Physicochemical Pretreatments

Pretreatments combining both physical and chemical processes can result in highly synergistic effects. These physicochemical processes can dissolve the hemicellulose fraction of lignocellulose and alter lignin structure, therefore increasing enzymatic access downstream during the overall pretreatment process (Behera et al. 2014).

### 1.4.1 Steam Explosion

Steam explosion has been one of the most commonly used physicochemical methods in the pretreatment of biomass. During the process, biomass is subjected to high-pressures (100-600 psi), heat (160-260°C), and steam for several seconds to minutes. After reaching the desired combination of treatment parameters, the pressure is rapidly reduced to normal atmospheric pressure. The rapid reduction in pressure results in an explosive decompression. Overall, the process effectively solubilizes hemicellulose and degrades lignin, therefore promoting access for secondary treatments such as enzymes to degrade cellulose (Kumar et al. 2009).

Grous et al. observed only 15% enzymatic hydrolysis of untreated poplar wood chips, but 90% hydrolysis was attained when chips were subjected to steam explosion prior to enzyme treatment. Although efficient hemicellulose solubility and lignin degradation can be achieved solely with heat, pressure, and steam; the addition of acid to the process has been explored. Studies have shown the addition of $H_2SO_4$ or $CO_2$ in steam explosion processes can decrease the overall time and temperature needed, and improve hydrolysis (Stenberg et al. 1999, Ballesteros et al. 2006).

Steam explosion has several advantages compared to conventional and alternative methods of biomass pretreatment. Holtzapple et al. (1989) determined that conventional methods required 70% more energy than steam explosion treatments to obtain the same particle size. However effective steam explosion has been shown regarding hemicellulose degradation and lignin transformation, increases in cellulose digestibility have only been weakly correlated with the pretreatment (Kumar, Barret et al. 2009).

### 1.4.2 Ammonia Fiber Expansion

The ammonia fiber expansion (AFEX) pretreatment, very similar to steam explosion, consists of exposing lignocellulosic material to heat (90°C), pressure, and liquid ammonia for a period of time. Like steam explosion, the pressure is suddenly reduced at the end of the pretreatment. Unlike steam explosion, hemicellulose is not solubilized, rather it is degraded to smaller oligomers and acetylated (Gollapalli, Dale et al. 2002).

AFEX pretreatments have been shown to have limited effectiveness on high-lignin substrates. For example, enzymatic hydrolysis yields of newspaper and aspen chips

were 40 and 50% respectively (McMillan 1994). Also, the cost of ammonia itself and ammonia recovery drives up costs as typically 1-2kg ammonia/kg of biomass are needed for an effective process (Kumar, Barret et al. 2009).

### 1.4.3 Ball Milling and Dilute Acid Hydrolysis

The combination of mechanical ball milling to decrease cellulose crystallinity followed by dilute acid hydrolysis for cellulose degradation has been applied. As the crystallinity of cellulose limits access for reactants or catalysts to β-1,4-glycosidic bonds connecting glucose in cellulose, increasing the non-crystalline fraction of cellulose increases glucose yields from dilute acid hydrolysis (Zhao, Kwak et al. 2006). Secondly, increasing access to β-1,4-glycosidic bonds potentially allows for milder reaction conditions such as high heat (~200°C) that contribute to glucose degradation (Mosier, Ladisch et al. 2002) and cellulose modification (Bouchard, Abatzoglou et al. 1989). Ball milling applied for a 6 d period decreased the crystallinity index of cellulose from 0.773 to 0.523 resulting in more than double the cellulose hydrolysis rate (Zhao, Kwak et al. 2006). However effective this process was found to be, industrial application is hindered by high energy input.

## 1.5 Enzyme Treatments

Some of the most effective treatments available are enzymes known for their high specificity. Typically proteins, these biocatalysts accelerate chemical reactions by lowering the activation energy. Industrial applications are of wide variety, as enzymes are applied in the food, feed, textile, and cleaning industries for various purposes (Li et al. 2012). Enzymes commonly used in the animal feed industry can be seen in (Table 1.4).

Animal feed enzymes can have several effects such as increasing digestibility of nutrients, degrading undesirable fractions and reducing fecal matter nutrients on land (Li et al. 2012).

**Table 1.4: Enzymes used in animal feed industries (Li, Yang et al. 2012)**

| Enzyme | Technical Benefits |
|---|---|
| Cellulases, Xylanases | Degrading fiber in viscous diets |
| Phytases | Degrading phytic acid to release phosphorus, and liberating calcium, magnesium ions |
| Proteases | Degrading protein into its constituent peptides and amino acids to overcome antinutritional factors |
| α-amylase | Digesting starch |

Several advances in technology have provided improvement in both availability and effectiveness in enzymes. First, the continuous improvement of fermentation and purification processes has made it possible to produce large amounts of desired enzyme at low cost. Secondly, the engineering of proteins and directed evolution of strains has contributed to the development of highly effective enzymes (Kirk et al. 2002). Today, the enzyme industry is highly competitive, technologically intensive, and has small profit margins. As of 2012, there were 400 minor suppliers of enzymes worldwide and 12 major suppliers. Over 75% of the entire amount of enzymes produced were by Novozymes (Denmark), DuPont (USA), and Roche (Switzerland) (Li et al. 2012).

### 1.5.1 Cellulase

A cellulase is any enzyme capable of cleaving cellulose into monosaccharides, oligosaccharides or polysaccharides. Complete cellulose hydrolysis is facilitated by three main types of cellulase enzymes which are endoglucanases, exoglucanases, and β-

glucosidases. Endoglucanase produces long chain oligomers by hydrolyzing glycosidic bonds at the amorphous regions of cellulose. Following the action of endoglucanase, exoglucanases cleave long-chain oligosaccharides into the short-chain oligosaccharide cellobiose. Lastly, β-glucosidase hydrolyzes cellobiose molecules into glucose (Juturu and Wu 2014).

Cellulases have predominantly been developed to degrade cellulose to produce fermentable sugars during ethanol processing (Thomas et al. 2013). Their use in feed has also been explored. Habib et al. found the addition of cellulase to broiler chicken diets significantly ($p<0.05$) increased weight gain and feed conversion ratio (Makhdum et al. 2013). Ranjan et al. (2013) conducted a compared four commercial carp diets, finding the diet containing cellulase to exhibit the highest dry matter and carbohydrate digestibility.

### 1.5.2 Ligninase

A ligninase is any enzyme capable of degrading lignin. The three lignin-modifying enzymes laccase, lignin peroxidase, and manganese peroxidase are known to play a role in lignin degradation (Hatakka 1994). Laccase is a copper-containing oxidase enzyme which oxidizes a variety of poorly-defined substrates such as lignin. Typically, laccase oxidizes phenolic lignin in the presence of mediators both synthetic and natural while subsequently reducing oxygen to water (Thurston 1994). Lignin peroxidase does not require mediators in contrast to laccase, but it is dependent on $H_20_2$ to degrade non-phenolic lignin (Martinez et al. 2005). Manganese peroxidase oxidizes $Mn^{2+}$ ions present in plant matter into highly reactive $Mn^{3+}$ ions, which attack phenolic or non-phenolic lignin compounds (Hofrichter 2002).

As lignin is the most plentiful aromatic polymer on earth, ligninase enzymes have many applications in chemical, fuel, food, agricultural, paper, textile, and cosmetic industries. Also, much research has been done on the degradation of dyes in wastewater streams by ligninase enzyme systems (Mendonça et al. 2010). Laccase has also been shown to degrade toxins such as aflatoxin $B_1$ (Alberts et al. 2009).

### 1.5.3 Amylase

Amylases are a family of enzymes that hydrolyze starch, eventually yielding glucose. Generally, three types of amylases are classified: α-amylase, β-amylase, and glucoamylase. The most researched and arguably most important amylases are α-amylases, which perform the initial hydrolysis of starch at random points yielding maltotriose, maltose, or glucose (Muralikrishna and Nirmala 2005). B-amylase cleaves starch from the non-reducing ends to produce maltose units. Glucoamylase hydrolyzes starch, maltotriose, or maltose from the non-reducing end to yield glucose (Evan et al. 2003, James and Lee 2007).

Amylase can be obtained from plants, animals, and microbes; however, microbial amylases are primarily used industrially today (Monterio de Souza and Oliveria de Magalhaes 2010). Commercial amylase usage first increased in the 1960s as amylase was produced on a large-scale from the bacterium *Bacillus subtilis* and the fungus *Aspergillus niger* (Muralikrishna and Nirmala 2005). Amylases have many applications in the food, fermentation, textile, paper, detergent, and pharmaceutical industries. Due to their wide availability, amylases have almost completely replaced chemical hydrolysis of starch and are estimated to make up as much as 25% of the global enzyme market (Monterio de Souza and Oliveria de Magalhaes 2010).

Monogastric diets supplemented with amylase have been studied. Owusu-Asiedu et al. (2002) found that supplementing a pea-based diet with amylase significantly increased ($p<0.05$) efficiency in weaning pigs. Kumar et al. (2006) found it was necessary to supplement α-amylase with non-gelatinized sources of starch.

### 1.5.4 Protease

Proteases are enzymes that cleave peptide bonds present in proteins. Proteases can be classified by their optimal pH being acidic, neutral or alkaline. Protease enzymes have major applications in food and feed, detergents, pharmaceutical, leather, diagnostics and silver recovery industries (Tremacoldi et al. 2004). Proteases made up over 60% of the global enzyme industry in 2002 and still are important in the market today (Gupta et al. 2002). Other than feed purposes, alkaline proteases are produced by bacteria such as *Bacillus sp.* and are widely used in the detergents industry (Tiwari 2015).

Some researchers have also brought attention to potential environmental benefits of the addition of protease in feed. The addition of protease to broiler chicken rations increased feed digestibility which in turn reduced the amount of nitrogen present in feces therefore reducing potential environmental impact (Oxenboll et al. 2011). Verlhac et al. (2012) found the addition of protease in rainbow trout diets to improve the utilization of all nutrients.

### 1.5.5 Phytase

Phytase enzymes are phosphatases that facilitate the breakdown of the antinutrient phytic acid commonly found in plant-based feed ingredients. Phytic acid otherwise known as phytate, is the primary storage form of phosphorus in plants (Figure 4).

Monogastric animals lack the ability to degrade phytic acid, leaving them unable to access phosphorus present in feed formulations. On top of the prospect of phosphorus deficiency, phytic acid is known to form a complex with proteins and chelate with minerals therefore reducing feed digestibility and feed efficiency (Francis et al. 2001). Subsequently, these problems are compounded as the undigested phosphorus is excreted and released into the environment contributing to pollution in the form of eutrophication (Bedford 2000). Plant proteins found in soybean meal, rapeseed meal, and sesame meal have been reported to contain phytic acid levels of 10-15, 50-75, and 24 g/kg respectively (Francis et al. 2001).

Phytases are produced via fermentation using fungal (Shivanna and Venkateswaran 2014) and bacterial (Singh et al. 2014) organisms. The first commercial phytase was produced by fungi in the early 1990s. Later it was found acid phosphatases produced by *E. coli* were more effective and are widely used today. The current global phytase market was estimated to be worth more than 350 million dollars annually, potentially accounting for more than 60% of the global enzyme market (Lei et al. 2013).

The effectiveness of phytase addition in animal feeds has been widely explored. Typically, phytases operate effectively at pH values of 2.5 and 5-5.5; therefore, phytase effectiveness greatly depends on the pH of the species digestive tract (Baruah et al. 2004). Forstera et al. (1999) found the addition of phytase to rainbow trout diets (canola protein concentrate) improved phosphorus utilization and the overall nutritive value. Other researchers have observed similar effects (Sajjadi and Carter 2003, Pham et al. 2008).

### 1.6 Microbial Treatments

Microbes such as algae, bacteria, and fungi have been intentionally and/or unintentionally fermented on plant biomass for thousands of years, producing products such as bread, alcohol, cheese and the biomass responsible for their creation (Tuse 1984). Over a century ago, research conducted in Berlin, Germany showcased the potential application of brewer's yeast as a feeding supplement for animals (Delbruck 1910). Several years later during World War 1, this information proved to be handy as Germany managed to replace as much as half of its imported protein sources as yeast (Hayduck 1913). Shortly after in 1919, Sak and Hayduck created the first "fed-batch" process, where substrate is fed to an aerated suspension of microbes rather than adding microbes to a substrate. The 1930's brought increased attention to the nutritive value of yeast and by the advent of World War 2, yeast was incorporated into army and civilian diets in Germany (Weitzel and Winchel 1932, Schulein 1937). Post war, the United States began experimenting with producing *C. utilis* for fodder on sulfite waste liquor (Harris 1949). During the 1960s, international organizations began to spearhead the task of solving world problems such as malnutrition. The Food and Agricultural Organisation of the United Nations (FAO) presented data showing 25% of the world was not receiving adequate protein in their diet. This information coupled with population growth estimates predicting the world's 2.5 billion people would increase to 5 billion by the year 2000, accelerated the notion to use microbial biomass as a source of feed protein (FAO 1960). As the technology of using microbes for potential food production became more popular, microbial protein was coined "single-cell protein" at the Massachusetts Institute of Technology (MIT) (Anupama and Ravindra 2000). As single-cell protein (SCP) was relatively low in price, low cost substrates such as cheese whey, molasses, starch, and

sulfite liquor were necessary for economic viability (Ugalde and Castrillo 2002). By the 1980s, large-scale production of SCP was underway in developed countries with plans to expand to other markets. As promising as the future of SCP looked, several problems arose. Technological advances such as improvement of crop yields provided cheaper sources of plant protein to directly compete with SCP for use in feed. On the geopolitical front, events such as the end of the Cold War led to the adoption of the General Agreement on Tariffs and Trade which de-regulated trade between nations, increasing the capability to trade agricultural goods (GATT 1986, FAO 2000).

Microbial treatments possess several advantages over non-biological methods, such as low energy requirements and high product yield. Timeliness of the process and obligatory care of growth conditions (monoculture) remain the main limiting factors of microbial treatments. As industry shifts towards more environmentally friendly methods of biomass treatment, interest in microbial treatments is on the rise (Behera et al. 2014).

### 1.6.1 Solid-state Fermentation

Solid-state fermentation (SSF) refers to the general absence of free water during the fermentation of solids (Pandey 2003). As the presence of water allows for diffusion of solutes and gas, inadequate levels of water limit the growth of most organisms. SSF uses water levels high enough to sustain growth of a target organism while simultaneously achieving a high substrate concentration (Gervais and Molin 2003, Holker et al. 2004). This technique more closely mimics the growth of organisms in a damp environment than the high amounts of water used in submerged state fermentation (SmF). Filamentous fungal organisms and yeast have been found to be best suited to lower moisture levels, although successful SSF have been conducted using bacterial cultures (Pandey 2003).

#### 1.6.1.1 Applications

Perhaps the simplest and most widely used SSF application is the ensiling of crops such as corn. High-moisture corn is harvested preferably between 30-50% dry matter (DM), milled, and stored in an oxygen-limiting structure to allow for anaerobic fermentation (Daynard and Hunter 1975). Upon the depletion of oxygen, anaerobic fermentation occurs, mainly using populations of lactic acid bacteria. Fermentation consumes available sugars in the silage producing mainly lactic acid, but also acetic acid, ethanol and carbon dioxide. The production of lactic acid is preferred as it lowers the pH of the silage preventing spoilage and preserving the silage until future use as feed (Rust et al. 1989). The overall nutritional value is increased as nutrients are concentrated due to the fermentation, and digestibility is increased (Johnson et al. 1999). The processing cost of ensiling corn was estimated to be $4.71 per metric ton which was cheaper than dry-rolling or steam-flaking corn (Macken et al. 2006).

Various researchers have reported successful production of high-value products other than feeds employing enzymes using SSF (Silva et al. 2005, Debing et al. 2006, Patil and Dayanand 2006, Murthy et al. 2009, Giraldo da Silva et al. 2012). Many different industrial enzymes are produced, mainly using filamentous fungal strains grown on feed processing by-products. Biopolymers, surface-active molecules, pigments and organic acids are also produced using SSF (Thomas et al. 2013).

#### 1.6.1.2 Advantages

Holker et al. (2004) summarized the general advantages and disadvantages regarding SSF (Table 1.5). In comparative studies, enzyme production levels are

commonly much higher in solid-state settings than in submerged incubations (Diaz-Godinez et al. 2001, Mrudula and Murugammal 2011). Much lower volumes of water allow for smaller reactor sizes and high concentrations of various secondary metabolites (Pandey et al. 1999, Jain, Morlok et al. 2013). Also, the absence of water vastly reduces the size of the fermentation vessel allowing for large substrate yields (Holker et al. 2004). Lastly, sterility is less of an issue with SSF as most yeast and bacteria are not as suited to grow in low moisture conditions as filamentous fungi which are primarily used in the process (Jain et al. 2013).

**Table 1.5: SSF advantages over SmF (Holker et al. 2004)**

| Advantages | Consequences | Problems to be solved |
|---|---|---|
| **Biological advantages** | | |
| Low water demand | Less waste water | Building of moisture gradients |
| High concentration of the endproduct | Lower down-stream costs | |
| Catabolite repression significantly lower or missing | Fermentation in the presence of glucose | |
| Utilisation of solid substrate | High concentration of the growth substrates | Building of substrate gradients |
| Lower sterility demands | Mixed cultures of fermenting microorganisms | |
| Solid support for microorganism | | |
| Stimulation of the natural environment | Better performance of cultivated microorganisms | |
| Fermentation of water-insoluble solid substrates | | |
| Mixed culture of microorganisms | Synergism of metabolic performance | |
| **Processing advantages** | | |
| High-volume productivity | Smaller fermenter volumes | |
| Low energy demand for heating | | Building of temperature gradients |
| Easy aeration | | Building of oxygen gradients on a large scale |

| | |
|---|---|
| Utilisation of otherwise unusable carbon sources | Cheap and abundant carbon sources |
| No anti-foam chemicals | No lost of microorganisms during fermentation |

1.6.1.3 Disadvantages

SSF technology has not enjoyed the same progress as SmF (Singhania et al. 2009). Issues with process scale-up hinder SSF's wide-spread industrial use. For this reason, past research has been devoted to developing continuous solid-state fermentation vessels for enzyme production with varying levels of success (Silman 1980, van de Lagemaat and Pyle 2001). Researchers attempted a continuous solid-state fermentation using an inclined, rotating, baffled tube and observed lower growth rates compared to a static control (van de Lagemaat and Pyle 2001). Silman (1980) has also suggested that even slow levels of rotation interfere with spore formation.

1.6.2 Submerged-state Fermentation

Submerged-state fermentations (SmF) utilize higher amounts of water during fermentation (<40% SLR) as compared to solid-state fermentation. The low substrate concentration ultimately results in greater substrate homogeneity due to better mixing, aeration, and overall mass transfer (Harvey and Mcneil 1994). SmF has been proven to be a viable, large-scale process due to enhanced control and ease of material handling. Bioreactors are vital to this process as they allow for consistent reaction conditions throughout the reactor (Shivanna and Venkateswaran 2014). SmF also requires a method to separate the desired fractions after the fermentation process. Typically, processes such as centrifugation concentrate insoluble components while filtration processes can capture

valuable soluble material. High-value products may employ chromatography methods for selective capture (Weuster-Botz et al. 2006).

#### 1.6.2.1 Applications

SmF using microbes such as bacteria, algae, yeast, and filamentous fungi has produced many successful products available today. Antibiotics, food additives, and enzymes are a few products still produced in vast quantities using SmF. Bioreactors allowing for consistent reaction conditions allow for the synthesis of desired products at constant rates (Harvey and Mcneil 1994).

#### 1.6.2.2 Advantages

Compared to SSF fermentation systems, SmF has enjoyed much more commercial success. Improvements in bioreactor technology have allowed for efficient mass transfer and a high rate of microorganismal growth (Jain et al. 2013). Even in the case of filamentous organisms which are subject to shear and require a high level of oxygenation, airlift reactors have been shown to be an effective tool as they do not exhibit stress on the organism and display high rates of mass transfer (Jin et al. 2001, Nitayavardhana et al. 2013). Due to the presence of high amounts of water, separation methods albeit expensive, are advantageous as they allow for the ability to capture material with high purity.

#### 1.6.2.3 Disadvantages

When using filamentous microorganisms, homogeneity of the bioreactor becomes an issue as certain conditions become more localized. This change in fermentation broth rheology is mainly attributed to the morphology of the filamentous microorganism which

can influence both the growth rate of the organism and subsequently product formation (Harvey and Mcneil 1994). Typically, filamentous organisms assume the form of freely suspended filaments or as pellets. Freely suspended filaments increase the viscosity of the culture medium which reduces oxygen and nutrient transfer, and cause blockages due to heavy growth around the impeller and process input lines (Grimm et al. 2005). Therefore, it is important to select filamentous organisms capable of assuming a pelleted morphology for industrial purposes. Lastly, sterility of the substrate prior to fermentation to sustain a monoculture is another issue when conducting SmF. High volumes of water used in the fermentation are expensive to heat.

### 1.6.3 Co-culture

Co-culture incubations consist of using more than one microorganism to achieve a desired effect. The use of more than one microorganism poses the advantage of low-cost industrial processes due to the potential lack of sterilization needs. However, it is also known that the simultaneous growth of multiple organisms can produce potentially toxic metabolites due to natural competition (Rateb et al. 2013) as well as reduce target yields and effectiveness of the overall process (Choi et al. 2016). To combat this issue, controlling fermentation parameters such as temperature or pH can favor the growth of one microorganism over another, resulting in the growth of a predominant species at desired times. For example, it is known that yeasts and fungi are generally more acid-tolerant than bacteria and commonly grow at pH values less than 4 (Temudo et al. 2007).

Researchers have found reported advantageous results using co-culture incubations. Brijwani et al. (2010) found that co-inoculating *T. reesei* and *A. oryzae* on soybean hulls resulted in an increase in total enzyme production. Interestingly, the strains

seemed to work in tandem as total enzyme titers lacking in monoculture for at least one of the organisms were increased during the co-culture method. Haq et al. (2005) found similar results using *T. viride* and *A. niger*. Zhao et al. (2014) found the co-inoculation of the microalgae *Chlorella sp*. and *Monoraphidium sp*. increased total biomass and the amount of oleic and linolenic acids.

### 1.6.4 Two-stage

Two-stage microbial treatments also utilize two microbes, but unlike co-culture inoculations the microbes are inoculated during different stages of the fermentation and typically there is sterilization between inoculations to allow for two separate monoculture incubations. Sterilizing biomass between incubations prevents the potential competition between microbes which can inhibit the breakdown of biomass as well as produce toxic metabolites (Rateb et al. 2013).

Several advantages of using separate monoculture incubations exist. Zhang et al. (2017) conducted a two-step incubation to utilize the advantages of both aerobic and anaerobic fermentations. It was found that fermenting a corn/soybean meal with *Bacillus subtilis* for 24 hours under aerobic conditions followed by fermentation with *Enterococcus faecium* under anaerobic conditions was found to decrease the size of peptides, and increase the crude protein (CP), ash and total phosphorus content of the feed. Similarly, researchers conducted a two-step fermentation of soybean meal using the robust enzyme-producing fungal strain *Aspergillus oryzae* followed by the lactic acid bacterial strain *Lactobacillus casei* (Chen, Shih et al. 2010). Overall, oligosaccharides such as raffinose and stachyose were significantly decreased ($p<0.05$), while protein utilization was improved as *in vitro* digestibility and soluble true protein content

increased. The ratio of small protein fractions (<16 kDa) increased in the *Aspergillus* and *Aspergillus* + *Lactobacillus* fermentations to 42.6% and 63.5% respectively compared to the untreated soybean meal with 7.2%. Inoculation with the *Lactobacillus* strain decreased the pH to a value of 4.5 and significantly ($p<0.05$) increased the lactic acid content. Lactic acid has been shown to be beneficial as a feed additive in some animal diets (Jorgensen et al. 2001). The wide-range of fungal strains available makes two-step fermentations an attractive approach to bioconversion processes; however, the need for additional sterilization steps- especially in batch fermentations- makes for a timely and expensive process (Choi et al. 2016).

## 1.7 Conclusion of pretreatment methods

The conversion of lignocellulosic material to effective feed ingredients is a complicated process. Reducing the physical size of biomass, increasing its susceptibility to degradation using various chemical pretreatment methods, saccharifying the indigestible carbohydrates using enzymes, and lastly exploiting microbial metabolism to create microbial protein and exogenous enzymes has shown to be effective in degrading invaluable fractions (Mosier et al. 2005). However, not all biomass is created the same. Therefore, wastewater streams and feedstuffs may require different individual treatments and combinations thereof for adequate conversions.

## 1.8 Feedstuffs

### 1.8.1 Sorghum Hominy

*Sorghum bicolor* is a resilient cereal grain able to resist drought-like conditions and even survive temporary water-logging while growing in semi-arid environments

across the world. Sorghum is native to Africa and Asia, however it is also grown in North, South, and Central America (Owuama 1997). Sorghum grain consists of the pericarp (6%), endosperm (84%), and germ (10%). Sorghum grain is a major ingredient in cattle, poultry, and swine feeds. It is generally accepted that sorghum contains ~95% of the digestible energy found in corn (Hancock et al. 1991).

#### 1.8.1.1 Sorghum Production

Sorghum is considered the fifth most important cereal grain worldwide, behind rice, wheat, corn and barley, and is the main cereal food for an estimated 750 million people living in Africa, Asia, and Latin America (Ramatoulaye et al. 2016). In traditional sorghum growing continents such as Asia and Africa, production is mostly small-scale and productivity yields vary yearly. In more developed settings, sorghum production can achieve consistently-high yields. Thus, the United States is now the world's largest producer of sorghum despite 80% of the total world land area devoted to sorghum being in developing countries (Table 1.6). Worldwide approximately 60 million metric tons were produced in 2010 (Srinivasa et al. 2014).

**Table 1.6: Sorghum production by country in 2010** (Srinivasa et al. 2014)

| Country | Area Harvested (m ha) | % of total area | Production (million ton) | % of total production |
|---|---|---|---|---|
| USA | 1.95 | 4.76 | 8.78 | 15.76 |
| India | 7.79 | 19.03 | 6.7 | 12.02 |
| Mexico | 1.8 | 4.40 | 6.94 | 12.46 |
| Nigeria | 4.74 | 11.58 | 4.74 | 8.51 |
| Argentina | 0.75 | 1.83 | 3.63 | 6.51 |
| Ethiopia | 1.62 | 3.96 | 2.97 | 5.33 |
| Burkina Faso | 1.98 | 4.84 | 1.99 | 3.57 |
| China | 0.54 | 1.32 | 1.73 | 3.10 |
| Australia | 0.51 | 1.25 | 1.6 | 2.87 |
| Brazil | 0.66 | 1.61 | 1.53 | 2.75 |
| Total | 22.25 | 54.58 | 39.74 | 72.88 |

#### 1.8.1.2 Sorghum Processing

The main goal of sorghum processing is to remove the outer tissues of the grain (germ and pericarp) therefore retaining the starchy endosperm portion of the grain. Traditionally this is achieved through two main processes. The first process removes the pericarp and germ using a conventional Beall-type degerminator, and then subsequently reducing the isolated endosperm into grits or flour. This method typically results in contamination of the endosperm with bran due to the nature of the grain. The second process commonly applied involves cracking the sorghum kernel and then grinding the endosperm from the bran using a roller mill. Both of these processes achieve a desired fraction purity through sieving or gravity separation (Kebakile 2008). A diagram of a sorghum dry-milling process and fractions can be seen in (Figure 1.2).

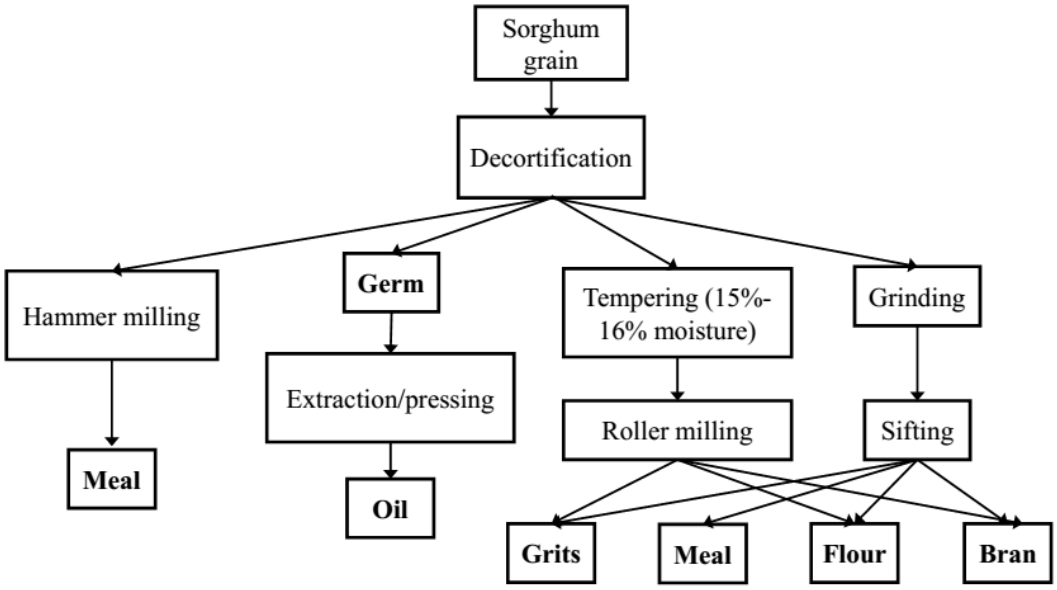


**Figure 1.2: Sorghum grain milling process and resulting fractions** (Rooney and Waniska 2000)

1.8.1.3 Current Uses

In Africa and Asia, 95% of sorghum is used for human food or animal feeds. In more developed regions of the world sorghum is primarily utilized as animal feed. As the biofuel industry continues to expand, research has also been performed on sorghum varieties for their potential to be used as a substrate for ethanol production (Sipos et al. 2009, Davila-Gomez et al. 2011). On top of use for food and feed, the demand for sorghum has been steadily increasing for use in brewing (Agu and Palmer 1998). Sorghum is also utilized as a substrate for the industrial production of starch, sugars and edible oils (Owuama 1997).

1.8.1.4 Limitations

Sorghum contains several antinutritional factors (ANFs) which inhibit its effectiveness in animal diets. Of the most prominent in sorghum is the phenolic

compound tannin. Tannins are known to bind to a wide variety of plant feedstuff components such as proteins and minerals which limits the overall digestibility of the feedstuff (Francis et al. 2001). On top of reduced digestibility, tannins have been reported to reduce palatability in aquafeed rations due to astringent taste (Francis et al. 2001).

Phytic acid (Figure 1.3) is another prominent constituent of sorghum grain that negatively affects feed performance. Phytic acid complexes with proteins and chelates minerals making them unavailable for digestion by monogastric organisms (Francis et al. 2001). Sajjadi and Carter (2004) found the addition of pure phytic acid significantly ($p<0.05$) reduced the protein digestibility of Atlantic salmon diets.

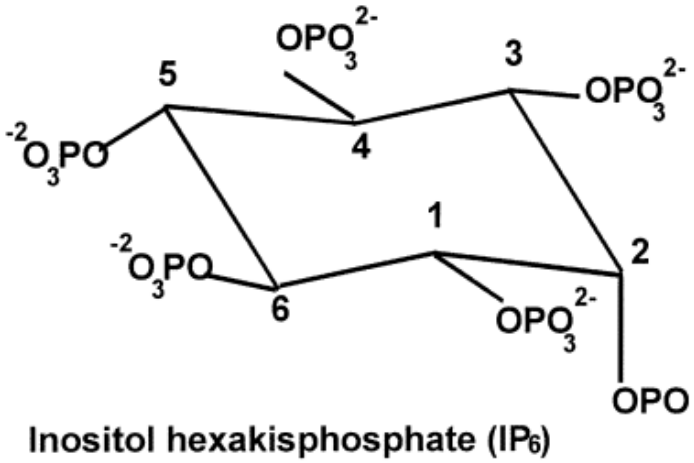


**Figure 1.3: Molecular structure of phytic acid** (Lei et al. 2013)

### 1.8.2 Gaur Korma Meal

Guar bean, otherwise known as clusterbean, is derived from *Cyamopsis tetragonoloba*, a legume native to India. Guar is tolerant to high temperatures, heavy rains and droughts, increasing its significance to farmers in arid areas (McLeary 1981). Although guar seed composition varies depending on growing region and season, guar

seed typically consists of hull (14-17%), endosperm (35-42%) and germ (43-47%) (Sharma 2010). The endosperm fraction contains high molecular weight galactomannans utilized in various industries due to their ability to easily dissolve in water creating a more viscous solution (Mudgil et al. 2014). Germ and hull fractions are combined at various ratios to produce two kinds of guar meal called “korma” and “churi”. The nutritive value of guar korma meal can be seen in (Table 1.7). All processed fractions of guar are considered safe for human consumption (Singh 2014).

**Table 1.7. Constituent of guar korma grain**

| **Constituent** | (Nidhina and Muthukumar 2015) | (Logaranjanai et al. 2015) | (Jannathulla et al. 2017) |
|---|---|---|---|
| Ash (%) | 5.1 | 5.87 | 5.4 |
| Fat (%) | 5.4 | 5.3 | 7.83 |
| Crude Fiber (%) | 4.9 | 7.1 | 7.94 |
| Protein (%) | 52.7 | 48.3 | 52.4 |

1.8.2.1 Guar Production

As of 2014, India is the top producing country of guar, providing 80% of the total guar in the world, while Pakistan provides 15% and other countries 5%. India produced 2.461 million metric tons of guar in 2012-2013 and increases in farmable area are expected to contribute to increased production (Singh 2014). Driving the increase in production is the use of guar gum in oil fracking procedures. Guar gum is added to increase the viscosity of fracking fluids to carry sand into fractures in rock, therefore increasing the efficiency of the overall process (Thombare et al. 2016). Although guar gum is the most industrially important fraction of guar seed, guar meal is still produced in great amounts as a by-product. Common guar seed production estimates are 1-1.6 million

tons per year, however, over the last 15 years, yields have ranged from 0.2-1.5 million tons (Sharma and Gummagolmath 2012).

#### 1.8.2.2 Guar Processing

Guar seed is separated using a combination of mechanical processing (Figure 1.4). Industrial processing of guar seed (Figure 1.4), produces guar gum (29.25%), guar meal (66.5%), and waste (4.25%). The endosperm is extracted by using a mechanical process of roasting, sieving, and polishing. First, the seeds are broken, separating the germ from the endosperm. Next, the two halves of the endosperm are collected and the hull is polished off to create refined guar splits. The germ and husk portions are combined at various ratios to produce guar meal. The refined splits containing the galactomannan guar gum are crushed using a mill and sieved to desired size (Mudgil et al. 2014). These commercial processes are not perfect as some of the endosperm is lost in the guar meal fraction, and likewise some of the germ and hull are left in the gum fraction. Higher levels of purity in the gum fraction allow for food grade status and do not have a significantly negative impact on the integrity of product (Thombare et al. 2016). In the case of guar meal, researchers have reported varying levels of 6-12% residual gum in the guar meal (Lee et al. 2004, Jannathulla et al. 2017).

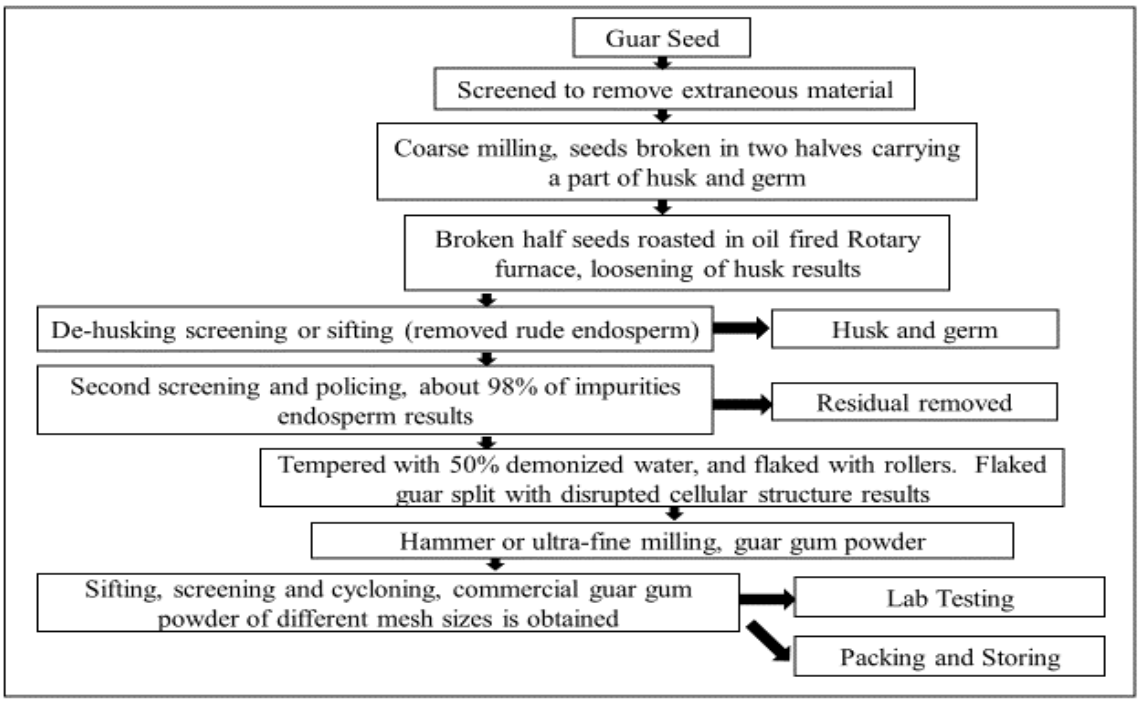


**Figure 1.4: Processing of guar bean** (Singh 2014)

1.8.2.3 Current Uses

Processed guar fractions have historically been used in broiler chicken feeds (Hussain et al. 2012, Dinani 2017), but inclusion rates have been limited due to the presence of anti-nutritional factors such as trypsin inhibitors, saponins, phytate and tannins (Nidhina and Muthukumar 2015). More recently, researchers have attempted inclusion of guar meal in rainbow trout diets as an alternative to soybean meal as guar contains similar levels of protein, amino acids, and fiber (Pach and Nagel 2017). Reported amino acid profiles of GKM can be seen in (Table 1.7).

MBP Group (Switzerland) and Cyamopsis Biotech (India) have commercialized guar meal for use as a high-protein ingredient (Biotech 2014, MBP Group 2014). Both

companies roast the guar meal to decrease levels of trypsin inhibitor and advertise their products as directly competitive with soybean meal.

**Table 1.7: Reported amino acid profiles of GKM**

| **Amino Acid** | (Jannathulla et al. 2017) | (Ahmed 1998) | (Lee et al. 2003) | (Nadeem et al. 2005) |
|---|---|---|---|---|
| Arginine | 6.19 | 5.34 | 4.03 | 4.76 |
| Alanine | 1.61 | 1.83 | 2.53 | 1.65 |
| Aspartic Acid | 4.31 | 4.1 | 4.4 | 3.91 |
| Cystine | 0.71 | 0.63 | NR | 0.52 |
| Glutamic Acid | 6.54 | 8.84 | 8.02 | 7.18 |
| Glycine | 2.77 | 2.28 | 4.24 | 2.11 |
| Histidine | 1.30 | 1.01 | 0.8 | 1.04 |
| Isoleucine | 2.15 | 1.21 | 1.26 | 1.19 |
| Leucine | 1.05 | 2.38 | 2.55 | 2.29 |
| Lysine | 2.06 | 1.72 | 1.7 | 1.66 |
| Methionine | 0.65 | 0.47 | 0.36 | 0.47 |
| Phenylalanine | 2.20 | 1.52 | 1.39 | 1.53 |
| Proline | 2.60 | 1.49 | 2.17 | 0.79 |
| Serine | 3.59 | 1.76 | 2.9 | 1.75 |
| Threonine | 2.47 | 1.26 | 1.51 | 1.16 |
| Tryptophan | 0.79 | NR | NR | 0.59 |
| Tyrosine | 1.91 | 1.48 | 1.01 | NR |
| Valine | 2.43 | 1.46 | 1.64 | 1.46 |

*NR- not reported by author

#### 1.8.2.4 Limitations

As India produces ~80% of the world's guar on an annual basis, growth of the guar industry is limited mainly due to inconsistency of environmental conditions. Most of the guar production (~60% of the country) occurs in the northwestern state of Rajasthan where growing seasons are heavily influenced by monsoons and rainy seasons. More recently, other states of India as well as other regions around the world have been increasing the amount of farmland dedicated to growing guar Ttable 1.8).

**Table 1.8: Area, production, and yield of guar in recent past in India** (Singh 2014)

| Year | Area (000 ha) | Production (000 tons) | Yield (kg/ha) |
|---|---|---|---|
| 2000-01 | 3497 | 659 | 188 |
| 2001-2002 | 2903 | 1090 | 375 |
| 2002-2003 | 975 | 199 | 204 |
| 2003-2004 | 2854 | 1513 | 530 |
| 2004-2005 | 2867 | 903 | 315 |
| 2005-2006 | 2956 | 1059 | 358 |
| 2006-2007 | 3344 | 1169 | 350 |
| 2007-2008 | 3863 | 1936 | 501 |
| 2009-2010 | 2996 | 595 | 199 |
| 2010-2011 | 3382 | 1965 | 581 |
| 2011-2012 | 3444 | 2218 | 644 |
| 2012-2013 | 5152 | 2461 | 478 |
| 2013-2014 | 5603 | 2715 | 485 |
| 2014-2015 | 4255 | 2415 | 567 |

Various antinutritional factors have been reported in guar korma meal such as trypsin inhibitors, saponins, phytate, tannins and residual gum (Hassan et al. 2010, Nidhina and Muthukumar 2015). Trypsin inhibitor has been reported to be the main inhibitory factor in guar meal by several researchers (Nidhina and Muthukumar 2015).

Residual guar gum levels have been shown to reduce nitrogen retention, energy utilization and fat adsorption resulting in decreased growth of broilers (Kamran et al. 2002). However, the galactomannan gum can prove beneficial in other species such as fish. Large droppings in aquaculture systems allows for cost-effective removal using mechanical methods such as sieves or sedimentation (Bergheim and Brinker 2003).

### 1.8.3 Soy Processing Wastewaters

With the growth of the soybean industry, also came the rise of soy processing wastewaters (SPW). Rich in nutrients, SPWs are created during the production of soy

protein concentrates and isolates (Yu 2015). The high titers of nutrients present are troublesome as they contribute to a high organic load in SPW. Any wastewater possessing a high organic load prior to being expelled into the environment can contribute to eutrophication. Therefore, wastewaters are commonly treated by both aerobic and anaerobic means (Nagadomi et al. 2000).

#### 1.8.3.1 Production

In 2015-2016, worldwide soybean production reached 313.7 million metric tons (USDA 2018). When soybeans are processed, 7-10 $m^3$ of wastewater is generated per 1000 kg soybeans. Although this number can vary between companies, countries such as China produced over 60,000 $m^3$ daily in 2012. Of this SPW, the chemical oxygen demand (COD) can reach values of 20,000 mg/L and above (Zhang et al. 2016). Due to the general need for more protein and changes in consumer perception in regard to plant proteins versus animal-based proteins, it is logical to assume this amount may increase.

#### 1.8.3.2 SPW Issues and Limitations

Biological treatment methods have typically utilized both aerobic and anaerobic methods to reduce COD. Anaerobic treatments utilize microbes to degrade organic material into $CO_2$, $H_2O$ and methane in the absence of oxygen. Aerobic treatments employ the use of microbes to utilize dissolved oxygen thus converting organic material to biomass (Barker et al. 1999). Legislation continues to decrease the acceptable level of organic matter in effluent streams, often as low as 25-150 mg/L (EPA 2012). With these stringent regulations, new technologies to decrease the levels of organic matter are needed.

## 1.9 Fungal Organisms

Selection of microbes for use in the conversion of plant feed by-products containing lignocellulose is critical to the success of the overall process. Microbial treatments must efficiently degrade indigestible lignocellulosic material while simultaneously producing protein-rich cell mass as well as be safe for consumption (Ortiz 1998).

Within the vast diversity of microbes across the planet, not all are created equal. Fungi are an attractive class of microorganisms as they have been eaten by both humans and animals for centuries. In terms of nutrition, fungi are competitive with animals and plants as adequate protein sources. Fungi contain about 20-30% crude protein on a DM basis (Moore and Chiu 2001) with some as high as 50% (Alriksson et al. 2014) while containing all the necessary amino acids for animal nutrition (Table 1.8). Also, important to be considered is the ability of a fungus to degrade unfavorable fractions of feedstuffs such as lignocellulose and ANFs. It has been widely documented that fungi produce robust enzymes capable of degrading these fractions to fuel their growth (Pandey et al. 1999).

**Table 1.7: Amino acid compositions of fungal strains**

| Amino Acid | *P. variotii* | | *F. venenatum* | | *R. oligosporus* | *T. reesei* |
|---|---|---|---|---|---|---|
| | (Udall et al. 1984) | (Wiebe 2002) | (Ahangi et al. 2008) | (Alriksson et al. 2014) | (Jin et al. 2002) | (Ghane m 1992) |
| Arginine | 6.1 | 2.9 | 7.3 | 2.5 | NR | 2.4 |
| Alanine | 5.8 | 2.8 | 6.3 | NR | 2.6 | 3.2 |
| Aspartic Acid | 8.1 | 2.9 | 10.3 | NR | 2.4 | 4.7 |
| Cystine | 0.8 | 0.4 | 0.8 | NR | 1.0 | 2.4 |
| Glutamic Acid | 10.8 | 5.2 | 12.5 | NR | 3.4 | 4.2 |
| Glycine | 4.4 | 2.1 | 4.3 | NR | 3.8 | 2.7 |
| Histidine | 1.9 | 0.9 | 3.5 | NR | NR | 1.9 |
| Isoleucine | 4.1 | 2.0 | 5.2 | 2.1 | 3.2 | 4.7 |
| Leucine | 6.5 | 3.1 | 8.6 | 3.1 | 3.9 | 3.5 |
| Lysine | 6.3 | 3.0 | 8.3 | 3.0 | 8.9 | 2.1 |
| Methionine | 1.5 | 0.7 | 2.1 | 0.8 | 1.5 | 0.4 |
| Phenylalanine | 5.8 | 2.8 | 4.9 | NR | 4.7 | 1.9 |
| Proline | 3.8 | 1.8 | 4.5 | NR | NR | 2.4 |
| Serine | 3.9 | 1.9 | 5.1 | NR | NR | 2.6 |
| Threonine | 4.1 | 2.0 | 5.5 | 2.0 | 2.8 | 2.5 |
| Tryptophan | 1.9 | 0.9 | 1.6 | 0.5 | 0.4 | NR |
| Tyrosine | 3.5 | 1.7 | 4.0 | NA | 1.1 | 2.3 |
| Valine | 4.9 | 2.4 | 6.2 | 2.5 | 3.1 | 3.4 |

NR- not reported

Fungi are known to produce secondary metabolites termed mycotoxins under certain conditions which can have potentially drastic effects on the health of humans and animals when digested. The first notable instance occurred in 1961 when a peanut meal included in livestock rations resulted in the death of thousands of animals. The mycotoxin-producing fungus was found to be *Aspergillus flavus* (Bosco and Mollea 2012). Due to this potentially serious issue, organizations such as the FDA are required to grant a generally regarded as safe (GRAS) affirmation petitions for legal use of microorganisms in food and feeds (Olempska-Beer et al. 2006). Today, many fungal organisms have received GRAS certifications for use in animal feeds (Wang et al. 2005).

### 1.9.1 *Neurospora crassa*

The ascomycete *Neurospora crassa* has represented filamentous fungi as a model organism for almost a century. The microbe was first described in 1843 after contaminating French bakeries and described as “red bread mold”. Later in 1901, *N. crassa* was used in the production of fermented soybean and peanut cake. First known for its incredibly fast growth, short life cycle and haploid genetics, it has now been identified as an adept producer of enzymes (Roche et al. 2014).

Aguado et al. (1999) examined the effectiveness of *N. crassa* to produce cellulase and cell mass using wheat straw as a substrate. It was found that enzyme production was highest at 30°C, but the mycelial cell mass exhibited the highest protein titer at 25°C. Kumar et al. (2012) found that an incubation temperature of 30 °C and supplementation of 0.8 g/l $CaCl_2$ optimized the cellulase production during the solid-state fermentation of wheat bran. Rodriguez-Jasso et al. (2012) found *N. crassa* to exhibit the highest hyphal growth rate (2-fold) when grown on coffee grounds compared to other carbohydrase-producing fungi. *N. crassa* also effectively degraded polyphenolic compounds and consumed reducing sugars. Yinbo et al. (1997) found *N. crassa* to be proficient in degrading cellulose and hemicellulose, however, it was relatively poor in degrading lignin.

Little research has been conducted on analyzing *N. crassa* cell mass. Mirnawati et al. (2015) found *N. crassa* fermented palm kernel cake could be included at levels of 13.05% in laying-hens diets without significant ($p<0.05$) decreases in feed performance. Perkins and Davis (2000) declared that *N. crassa* is a non-pathogenic fungal strain and is able to be used safely for feed applications.

### 1.9.2 *Trichoderma reesei*

The ascomycete *Trichoderma reesei* has been heavily researched due to its cellulolytic potential (Dashtban et al. 2009). The fungus was first identified as the culprit behind degrading cotton tents in 1944 during World War II in the Solomon Islands (Reese 1976). First named *Trichoderma viride*, the fungus was later named *Trichoderma reesei* after the name of the man credited to its discovery, E.T. Reese (Gusakov 2011). Since then, mutant strains producing up to several-fold increases in cellulolytic enzymes have been produced in academia (Ghosh et al. 1982, Gadgil et al. 1995). Although dated (1977), Mendels and Sternberg screened data from over 14,000 cellulase producing fungal strains and found no other competitors to *T. reesei*.

The morphology of *T. reesei* is known to impact the efficacy of certain applications. *T. reesei* can grow as pellets or suspended filaments during fermentations. Queiroz et al. (2000) found the spore concentration of the inoculum to be critical for pelleted growth in submerged culture. Spore concentrations of $10^5$ spores/ml developed pellets while concentrations of $10^7$ spores/ml developed flocs. The surfactant Tween 80 inhibited the formation of pellets. The nutritive profile of the growth substrate is also known to affect fermentation products. Fang and Xia (2015) found a lactose concentration of 15% and a carbon to nitrogen ratio of 7 produced the highest titers of cellulase. Cao et al. (2012) increased cellulase activity nearly 5-fold using a combination of wheat bran (24.63 g/L), avicel (30.87 g/L), and soybean cake (19.16 g/L) compared to a pure cellulose substrate. Less research has been performed on *T. reesei* fermented feed applications. Ghanem (2012) converted 35.7% of beet pulp to SCP containing a crude protein titer of 49.3% using *T. reesei*.

Commercial cellulase enzymes derived from *T. reesei* are produced by several companies worldwide (Gusakov 2011). T. *reesei* enzymes have had long-standing success in commercial enzyme with applications in food, animal feeds, pharmaceutical, textile, and pulp and paper industries. As the organism has not been shown to produce any toxins or antibiotics under enzyme production conditions, it is generally accepted that *T. reesei* is safe and non-toxic to man (Nevalainen et al. 1994).

### 1.9.3 *Aureobasidium pullulans*

*Aureobasidium pullulans* is an ascomycetous, black yeast-like fungus found ubiquitously around the world. *A. pullulans* has become well known and of biotechnological importance for its production of the valuable polysaccharide pullulan (Figure 1.5). The term "yeast-like" refers to the ability of this fungus to assume several different morphological forms; e.g. small elliptical yeast-like cells, large chlamydospores, and elongated branched septate filaments (Singh et al. 2008). The formation of these morphologies can be heavily influenced by environmental conditions including temperature, pH, and oxygen concentrations (Gaur et al. 2010). Catley (1973) noticed that enhanced pullulan production seemed to be consistent with a unicellular morphology. Since this discovery, many studies were conducted presenting mixed results. Thus, it is generally accepted that multiple factors interact in the formation of pullulan (Singh et al. 2008).

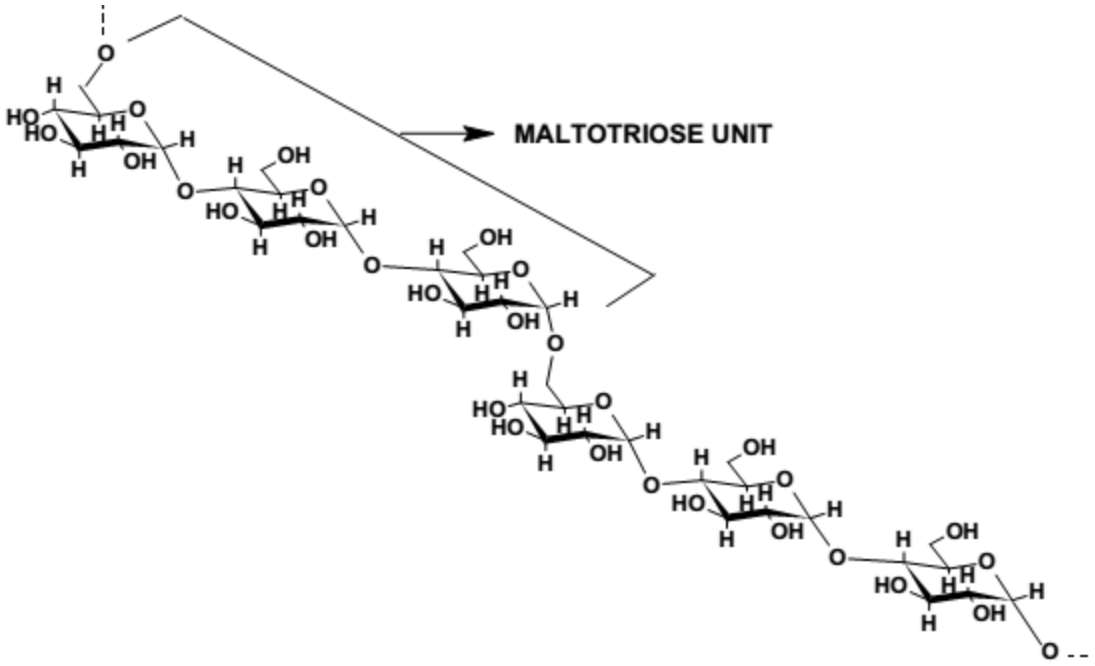


**Figure 1.5: Molecular structure of pullulan** (Singh et al. 2008)

*A. pullulans* is also an adept producer of enzymes including amylase, protease, cellulase, xylanase, and pectinase (Gaur et al. 2010). Silman et al. (1993) reported amylase production by using corn starch as a substrate. Pectinase was synthesized using a variety of pectin containing substrates, although D-galacturonic acid was found to be the best inducer (Biely et al. 1996). The optimal production parameters of an alkaline protease were found to be a pH of 6 and 24.5°C during a 30 h fermentation (Chi et al. 2007).

The production of pullulan by *A. pullulans* continues to fuel research. Since 1983, over 150 patents relating to pullulan applications have been filed. Patents have been mainly directed towards pharmaceutical, food, and personal care (Izutsu et al. 1987, Oguzhan and Yangilar 2013). In terms of food and feed applications, *A. pullulans* cell

mass is listed as a GRAS product for use in the food processing industry (Prajapati et al. 2013).

### 1.9.4 *Fusarium venenatum*

*Fusarium venenatum* was first isolated from soil samples in the United Kingdom in 1968. Originally named *Fusarium graminearum*, the strain was reclassified as *F. venenatum* after further studies on its morphological, molecular, and mycotoxin characteristics (Olempska-Beer et al. 2006). The strain gained commercial significance after the British company Rank Hovis McDougall (RHM) screened over 3,000 fungi over the course of a 3 year period, ultimately selecting *F. venenatum* for its texture, overall palatability, and ability to grow on cheap substrates such as starch and glucose (Wiebe 2002). Since 1985, *F. venenatum* has been mass produced as a source of microbial protein for humans under the brand name 'Quorn' by Marlow Foods, Inc (UK). *F. venenatum* mycoprotein was later given GRAS status by the FDA in 2001 and approved as safe for use in food as a meat substitute in the United States (Wiebe 2002).

*F. venenatum* is known to be non-pathogenic, though, reports of mycotoxin production have been published. To combat mycotoxin production, careful control of fermentation parameters is known to inhibit their production (O'Donnell et al. 1998). Other instances have been reported of allergenic reactions in certain individuals to 'Quorn' myco-protein (Katona and Kaminski 2002). Still, *F. venenatum* is a commercially available source of fungal protein for use in human foods.

Recently, attempts at including *F. venenatum* biomass in aqua diets has been explored. Alriksson et al. (2014) produced *F. venenatum* biomass containing 53%

protein, 7% fat, 26% carbohydrate, 5% ash and 9% moisture using a wood processing wastewater substrate. The produced biomass was used as a fishmeal replacement and inclusion rates of 38 and 66% performed similar to the fish meal control.

### 1.9.5 *Rhizopus oligosporus*

The filamentous zygomycete *Rhizopus oligosporus* of the mucoraceae family has been used to produce tempeh, a fermented soybean delicacy, since ancient times (Kovac and Raspor 1997). The fermented food was first referenced in 1821 and cultures have since been passed to new generations. Long used in human applications, *R. oligosporus* fermented soybean has also been found to increase feed efficiencies in animal feed. Other *Rhizopus ssp.* have been reported to produce mycotoxins such as rhizoxins and rhizonins, however, *R. oligosporus* has been declared for safe use in food and feeds (Erickson 2012).

The long history of using *R. oligosporus* in fermented foods has contributed to a large body of available research. Several researchers have evaluated the effectiveness of *R. oligosporus* to treat wastewater streams. Jin et al. (1999) found *R. oligosporus* to be proficient in treating a starch processing wastewater, reducing the COD by 95% while simultaneously creating protein-rich biomass (46% CP) and a glucoamylase enzyme. Nitayavardhana et al. (2013) achieved 80% reduction of COD in a wastewater stream primarily composed of vinasse while producing fungal biomass with a protein titer of 50%. Other studies have investigated the production of enzymes and subsequent degradation of unfavorable fractions present in various substrates. Li (2013) reported the production of an acid protease effective at high temperatures when *R. oligosporus* was grown on soy protein isolate. Production of lipases, carbohydrases, and phytase has also

been reported (Erickson 2012). Vig and Walia (2001) reported *R. oligosporus* fermentation to reduce the ANF glucosinolate and phytic acid by 43.1% and 42.4% respectively. A decrease in CF (25.5%) and increase in CP was also observed. Egounlety and Aworh (2003) found *R. oligosporus* to be an adept reducer of phytic acid during the fermentation of soybean, cowpea, and groundbean. Interestingly, the tetrasaccharide stachyose was consumed before simpler sugars such as glucose, galactose, and sucrose.

### 1.9.6 *Paecilomyces variotii*

The filamentous ascomycete *Paecilomyces variotii* is ubiquitously found around the world and has been isolated from various substrates such as food, soils, air, and wood (Hussain et al. 2012). *P. variotii* is known to be a robust producer of enzymes including cellulase, amylase, tannase, and phytase (Battestin et al. 2012, Hussain, Shrivastav et al. 2012). *P. variotii* cell mass was determined to be safe for human and animal consumption (Udall et al. 1984), but instances of allergic reactions have been reported (Houbraken et al. 2010).

Battestin and Macedo (2007) investigated the effects of nitrogen supplementation on potential increases in enzyme production. Ammonium nitrate supplemented at a concentration of 1.2% (w/v) was found to increase tannase production by 125%. Sodium nitrate did not have any significant effects, while yeast extract inhibited tannase production. On the contrary, yeast extract outperformed sodium nitrate, potassium nitrate, urea, ammonium sulphate, peptone, and beef extract as a nitrogen source for xylanase production (Amutha et al. 2015). Peptone supplemented at 1% (w/v) increased invertase production by 3.7-fold, while $NH_4H_2PO_4$ inhibited production by 80-82% (Giraldo et al. 2012). Carbon sources have also been examined for their effects on enzyme production.

Starch was found to have no effect on tannase production while using a coffee husk and wheat bran (50:50) substrate (Battestin and Macedo 2007). Wheat bran was found to be a superior carbon source to rice bran, saw dust, rice straw, and cassava bagasse for xylanase production (Amutha et al. 2015). *P. variotii* consumed glucose, mannose, xylose, galactose, and arabinose concurrently from wood processing wastewater (Alriksson et al. 2014).

The nutritive value of *P. variotii* biomass has also been explored. Alriksson et al. (2014) produced *P. variotii* biomass containing 48% protein, 5% fat, 37% carbohydrate, 5% ash, and 5% moisture and acceptable mycotoxin levels for use as a fish meal replacer in Tilapia diets. Inclusion rates of 38% fungal biomass increased fish weights by 12% compared to fishmeal control.

## Chapter II- Introduction

Current estimates predict the global population of 7.6 billion people to reach 8.6 billion by 2030, and 9.8 billon by 2050 (UN 2017). In addition to large increases in population, humans are consuming more food while their diets are incorporating higher amounts of high-protein foods (Alexandratos and Bruinsma 2012). Therefore, a need exists to increase the amount of animal protein produced today and consequently the fodder used to feed animals. Pressure from environmentalist's stance on biodiversity and the increasing strain on regional supplies of fresh water negates the solution of simply increasing farmable acreage across the world. Thus, improvements in the production and processing technologies of food and feed must transpire to feed an additional 83 million people per year (Hertel 2015).

The aquaculture industry is an attractive platform to produce vast amounts of animal protein due to efficient feed-to-weight conversions, and low amount of land use. The industry has experienced rapid growth over the last 5 decades, with aquaculture only providing 7% of the fish consumed around the world in 1974 to over 44% in 2014 (FAO 2016). Remarkably, almost half the fish consumed in the world today are now farm-raised instead of wild-caught. Accompanying the swift rise in farmed-fish production is the increase in demand of aquafeeds. Aquadiets have long relied on fish meal as an effective nutritional supplement. However, an increase in the demand of fishmeal has depleted the supply available in the world's fisheries (Troell et al. 2014). Consequently, the price of fish meal has risen above $1500 in recent years (FAO 2016). Due to this matter, alternative feed ingredients must be developed in order for aquadiets to remain economically viable. Plant feedstuffs primarily composed of lignocellulosic materials are

a particularly attractive substitute to the finite resource of fishmeal. Soybean meal (Salze et al. 2010, Troell et al. 2014) has already proven to be an effective substitute which presents the opportunity for other plant feeds to be utilized (Gatlin et al. 2007).

Cellulose, hemicellulose, lignin and sometimes pectin interact to form a complex structure known as lignocellulose (Sindhu et al. 2016). These plant structural carbohydrates form a complex structure naturally resistant to degradation. Cellulose, which is the most abundant polymer on earth, contains repeating units of the disaccharide cellobiose and is ultimately composed of anhydroglucose linked by β-glycosidic bonds (Zheng et al. 2014). Hemicellulose is a heteropolymer made of sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, and L-fucose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid). The exact composition of hemicellulose varies depending on the source of raw material and overall this polymer is easier to hydrolyze than cellulose (Brigham et al. 1996). Lignin is a complex aromatic polymer whose structure also varies between feedstocks (Vanholme et al. 2010). Some feedstuffs also contain the galacturonic acid-rich polysaccharide pectin. Hydroxyl groups on the anhydroglucose molecules allow for hydrogen bonding between adjacent cellulose chains forming microfibrils. Hemicellulose and pectin (if present) intertwine with the cellulose microfibrils to form the structural base of biomass, which typically is in the form of long fibers (Zheng et al. 2014). Lignin is wrapped around the fibers, providing protection against enzymatic hydrolysis (Vanholme et al. 2010). This complex structure is digestible by ruminant animals and hind-gut fermenters, but is not easily digested by monogastric organisms such as fish, due to their lower fermentative capacity and shorter

digestive tract (Choct and Kocher 2000). Thus, a large fraction of plant-based feedstuffs is rendered indigestible when included in monogastric diets.

On top of the indigestibility of lignocellulose present in plant feedstuffs, anti-nutritional factors can also adversely affect digestion and health of the animal (kahraman and Onder 2009). ANFs have been categorized into four groups based on their function: (1) ANFs affecting protein uptake; (2) ANFs affecting mineral utilization; (3) vitamin antagonists; and (4) miscellaneous substances such as allergens and toxins (Francis et al. 2001).

Together, these plant structural carbohydrates and ANFs commonly hinder plant feedstuff's effectiveness in aquadiets. However, many processes exist to degrade these various fractions in plant-based feedstocks to improve their effectiveness in non-rumen diets. Physical, chemical, combined physical/chemical, enzymes, microbes and combinations thereof have been used to degrade various plant components to improve the nutritional value of plant-based feedstuffs. Fungal fermentation is an attractive approach to increasing the nutritive value of plant by-products due to the large diversity of culturable microbes safe for consumption presently available. An assortment of fungal strains were tested for their ability to improve the nutritive value of a soybean meal processing supernatant, sorghum hominy, and guar korma meal under submerged fermentation conditions. In Chapter III, eight fungal strains were examined for the ability to reduce the chemical oxygen demand of a soybean-processing supernatant, while simultaneously producing protein-rich fungal biomass. In Chapter IV, eight fungal strains were examined for their ability to metabolize sorghum hominy carbohydrates therefore concentrating protein and improving the overall protein profile of the feedstuff. In

Chapter V, five fungal strains were screened for their ability to improve the nutritive value of guar korma meal as a whole and guar korma meal concentrate and supernatant fractions.

# Chapter III- Assessing various types of fungal strains to convert soybean processing industry wastewater into protein-rich animal feed


## Abstract

The aim of this study was to convert a soybean processing industry wastewater stream into a protein-rich animal feed via fungal bioprocessing while simultaneously reducing the potential environmental impact of the waste stream. Eight fungal strains were screened in flask trails for their ability to produce protein-rich biomass while simultaneously reducing solids found in the wastewater stream. *Trichoderma reesei, Paecilomyces variotii* and *Neurospora crassa* produced 51.7, 47.1 and 43.2 g/L of biomass in flask trials while reducing solids present in the supernatant fraction by 46.5, 48.9, and 49.1%, respectively. In bioflo fermenters, *Trichoderma reesei* and *Neurospora crassa* produced 55.5 and 62 g/L of protein-rich biomass while reducing chemical oxygen demand levels by 10.53 and 23.04% respectively. Microbial metabolic process led to the production of protein-rich animal feed ingredient and simultaneously reduced the level of organic matter in the wastewater stream.


## 3.1 Introduction

Biological wastewater streams continue to be a major economic and environmental factor in bioprocessing industry (Muga and Mihelcic 2008, Satyawali and Balakrishnan 2008). Conventional methods used over the past century such as aerobic and anaerobic wastewater treatments utilize bacteria to reduce organic matter present in these streams to acceptable levels (Chan, Chong et al. 2009). These methods possess little benefit for companies as focus is on removing necessary amounts of organic material to meet legislative demands on disposable wastewater (Barker, Manucchi et al. 1999,

Zhang, Jin et al. 2008). As a result of these processes, a large amount of low-value bacterial biomass otherwise known as “sludge” is produced which can account for up to 60% of wastewater plant operating costs when it needs to be discarded (Sankaran, Khanal et al. 2010). Alternative treatments of higher efficiency in removing environmental pollutants as well as recapture otherwise lost resources are of high interest.

It is well known fungi can produce valuable biochemical metabolites and single-cell protein (SCP) on a variety of substrates while simultaneously cleansing the waste (Jin, Hans van Leeuwen et al. 1999, Bellou, Makri et al. 2014). Single-cell protein of yeasts and filamentous fungi typically contains all of the necessary amino acids as well as they are easily digested (Ugalde and Castrillo 2002). The potential for SCP production to be used for a feed supplement in fish, broilers, and cattle is exceptionally attractive as the amount of food produced today is expected to need to increase by 100-110% by 2050 due to the increasing human population (Ugalde and Castrillo 2002, Tilman, Balzer et al. 2011).

Chemical oxygen demand (COD) is a concentration of organic material that can be chemically oxidized to inorganic products (Pisarevsky, Polozova et al. 2005). These organic carbon metabolites are produced by many industrial and domestic wastewater streams from humans and animals. When wastewater streams with a large amount of COD are released into natural waters, stark consequences can occur such as oxygen depletion and as a further result, the dying of fish due to lack of oxygen that was consumed by bacteria (Penn, Pauer et al. 2006). Hence, it is critical that this organic matter is reduced to a level deemed acceptable to be released into the natural environment. It has been observed that filamentous fungal organisms such as *R.*

*oligosporus* and *P. chrysosporium* were able to reduce COD in various wastewater streams by 95% and 74% respectively on effluents (Jin, Van Leeuwen et al. 1999, Kissi, Mountadar et al. 2001).

Soybean meal supernatant (SBMS) used in this study is a by-product of the local feed company, which uses microbial processes to upgrade soybean meal into a high-protein animal feed. This process produces a significantly large amount of SBMS and is currently discarded due to the lack of the economically viable methods for its utilization. With this soybean processing supernatant being rich in nutrients and minerals, we hypothesized that the metabolic activity of filamentous fungi may be utilized to achieve the simultaneous reduction in the nutrient loss as well as avoid environmental impacts of this waste stream (due to high chemical oxygen demand). The SBMS (Table 3.1) which is rich in protein (37.57%, dry basis) and residual carbohydrates would be converted into a more nutritional and highly digestible single-cell protein allowing for a potentially valuable feed ingredient. In this study, eight different fungal strains known to be generally regarded as safe (GRAS) by the FDA were screened to determine an optimal fungal treatment for converting soybean processing waste stream into high-protein animal feed.

## 3.2 Materials and Methods

### 3.2.1 Wastewater Sample

The waste stream of soybean meal (SBM) processing industry was obtained from a local feed company. The liquid stream of SBM processing industry is referred as SBMS throughout the manuscript. The nutrient composition of the SBMS is shown in (Table 3.1). The SBMS was transferred into closed containers immediately after harvesting and

stored in freezer (-20°C) until use. The samples were thawed at room temperature prior to using for experimental trials.

**Table 3.1: Proximate composition of SBMS**

| **Components** | **% (m/m)** |
|---|---|
| Moisture content | 92.6 |
| Dry matter (DM) | 7.38 |
| **All values below on DM basis* | |
| Crude fiber | 1.27 |
| NDF | 1.90 |
| ADF | 4.39 |
| Crude Fat | N/A |
| Crude Protein | 37.6 |

3.2.2 Cultures, Maintenance, and Inoculum Preparation

Different fungal strains used in this study are listed in (table 3.2). These strains were provided by the USDA National Center for Agricultural Utilization Research (Peoria, IL, USA). For short-term maintenance, strains were stored on Potato Dextrose Agar (PDA) plates and slants at 4°C. While lyophilization was used for long-term storage of organisms. Seed cultures were prepared in 250 ml Erlenmeyer flasks containing 100 ml of glucose yeast extract (GYE) media (5% glucose and 0.5% yeast extract). The pH of growth media was adjusted for each culture to their optimal pH (table 2) either by using 10M sulfuric acid or 10M sodium hydroxide prior to inoculation. The seed cultures were inoculated by transferring a previously cut square section of agar for filamentous fungi or isolated colonies for yeast –like organisms. Shake flasks were then incubated in a rotary shaker at 150 rpm and at 30°C for 72 h.

**Table 3.2: List of fungal strains and optimal pH of the growth media**

| Organism | Strain | pH |
|---|---|---|
| *Aureobasidium pullulans* | NRRL-Y-2311-1 | 3 |
| *Trichoderma reesei* | NRRL-3653 | 5 |
| *Fusarium venenatum* | NRRL-26139 | 5 |
| *Paecilomyces variotti* | NRRL-1115 | 5 |
| *Rhizopus microsporus var. oligosporus* | NRRL-2710 | 5 |
| *Neurospora crassa* | NRRL-2332 | 5 |
| *Mucor circinelloides** | N/A | 5 |
| *Pichia kudriavzeii** | N/A | 3 |

**These organisms were contaminants isolated from prior research and identified via 15s RNA analysis by Kerry O' Donnell's lab (USDA National Center for Agricultural Utilization Research, Peoria, IL, USA)*

### 3.2.3 Lab-scale Fermentation Using 250 ml Erlenmeyer Flask

Lab-scale fermentation of SBMS was carried out using 8 different fungal strains (table 2). Flask trials were performed in 250 ml Erlenmeyer flasks containing 100 ml SBMS. Prior to inoculating with seed culture, SBMS media for each culture was adjusted to their optimal pH (table 2) and sterilized using an autoclave for 20 minutes at 121°C. After cooling to room temperature, flasks were inoculated with 2 ml of inoculum of the desired microbe and incubated in rotary shaker at 150 rpm for 5 days at 30°C. After the completion of the incubation period, final pH of the fermented slurry was recorded and samples were harvested using centrifugation at 4,000 rpm for 10 min. Flask contents were then dried in an oven at 80°C for 24 h and total mass was recorded. Both the supernatant and solid fractions were used to determine protein titers. All the experiments were performed in triplicate.

### 3.2.4 Lab Scale Fermentation of SBMS using Bioflo Reactors (3 L)

The results from flask trials were used to down select the two best performing microbes for bioflo trials. Microbes that produced maximum cell mass and caused

significant reduction of solids in the supernatant were chosen to be tested under optimal conditions in a 5 L benchtop reactor (New Brunswick bioflo III, Edison, NJ, USA). Approximately 3 L of SBMS was added to the reactor and was pH adjusted to the optimal for the desired microbe (table 2). Reactors were sterilized using an autoclave at 121°C for 30 min. After cooling, reactor settings were fixed at an agitation rate of 250 rpm, 30°C, and an aeration rate of 0.8 v/v/min. Upon settings reaching equilibrium, reactors were inoculated with 30 ml of chosen 72 h culture and allowed to grow for 120 h. Daily samples of ~100 ml were collected and used to monitor pH, protein titers, mass balance calculations and reduction of solids in the supernatant. At the end of the incubation period, reactor contents were harvested and dried at 80°C until dry. All the treatments were performed in triplicate.

### 3.2.5 Analytical Methods

#### 3.2.5.1 Moisture Determination

The moisture content of liquid and solid samples were determined using the American Association for Clinical Chemistry (AACC) method (International). Where ~30 ml and ~0.5-1 g of sample respectively at 80°C for at least 24 h-48h or at 105ºC for at least 4 h.

#### 3.2.5.2 Total Protein

Total protein content of the samples was measured using a LECO model FP528 (St. Joseph, MI, USA). This method uses combustion method to measure the total nitrogen content in the sample. Roughly 0.25 g of sample fractions were placed in a tin foil cups for the combustion. Total protein titers were then calculated by multiplying the

total nitrogen content by a conversion factor of 6.25. Total moisture content of the sample was used to determine the protein content on dry basis. All the analysis was duplicated.

3.2.5.3 Chemical Oxygen Demand (COD) and Solids ($X_{TS}$, $X_{DS}$ and $X_{UDS}$)

COD was calculated using the closed reflux, titrimetric method APHA 5220c. A total solids fraction ($X_{TS}$) and a dissolved solids fraction ($X_{DS}$) were used to calculate an undissolved solids fraction ($X_{UDS}$). $X_{TS}$ was found by placing the harvested sample in a dryer at 105ºC for at least 4 h. $X_{DS}$ was found by centrifuging 10 ml of harvested sample at 4,000 rpm for 10 minutes and decanting. Then the decanted supernatant was centrifuged at 4,000 rpm for another 10 min and the resulting supernatant was placed in dryer at 105ºC for at least 4 h. The dry masses of both the $X_{TS}$ and $X_{DS}$ fractions were used to calculate $X_{UDS}$ using the formula below:

$$X_{UDS} = \frac{X_{TS} - X_{DS}}{1 - X_{DS}}$$

3.2.6 Statistical Analysis

All statistical analysis was performed using RStudio 1.0.1.143 (Team 2015). For Erlenmeyer flask trials the Bartlett's test was used to test for homogeneity between variances among treatments (Bartlett 1937). When homogeneity of variances was confirmed, Analysis of Variance (ANOVA) was applied to determine the significant differences between fungal treatments in the Erlenmeyer flask trials using the RStudio analysis package 'agricolae' (Mendiburu 2016). Significance differences between means were found *post hoc* using Duncan's new multiple range test (MRT) (Duncan 1955).

For 3 L benchtop reactor trials, an F-test for homogeneity was used to confirm the variances were not significantly different between treatments (Team 2015). A two-sample t-test was conducted if variances were not significantly different and a Welch

two-sample t-test was performed if variances were significantly different (Welch 1938, Cressie and Whitford 1986).

## 3.3 Results and Discussions

### 3.3.1 Proximate Composition of the SBMS

The proximate and mineral composition of the SBMS as determined are listed in the Table 3.1. The SBMS is mainly water (~92.62%), containing only about 7.28% of solid matter on dry basis. This by-product of the soybean processing industry is rich in protein (37.57%, db), fibers (4.39% ADF; 1.89% NDF), and essential minerals (iron, zinc, manganese, sulfur, and potassium etc.) and therefore, consisted of a high concentration of chemical oxygen demand (COD). Since the SBMS contains a high COD, it is critical to treat the effluent before discharging into the environment.

### 3.3.2 Fungal Fermentation using 250 ml Erlenmeyer Flasks

The first part of this study consisted of screening of eight fungal strains (Table 3.2) on the SBMS. After the 5 d incubation period, all fungi except for *P. kudriavzevii* produced significantly higher ($P<0.05$) amounts of biomass than the control. The respective biomass concentrations for the top performing strains ie. *T. reesei, N. crassa, F. venenatum and P. variotii* were 51.7, 47.1, 43.2, and 42.3 g/L. Whereas the rest of the fungal treatments yielded between 29.6-33.1 g/L biomass (Fig 3.1).

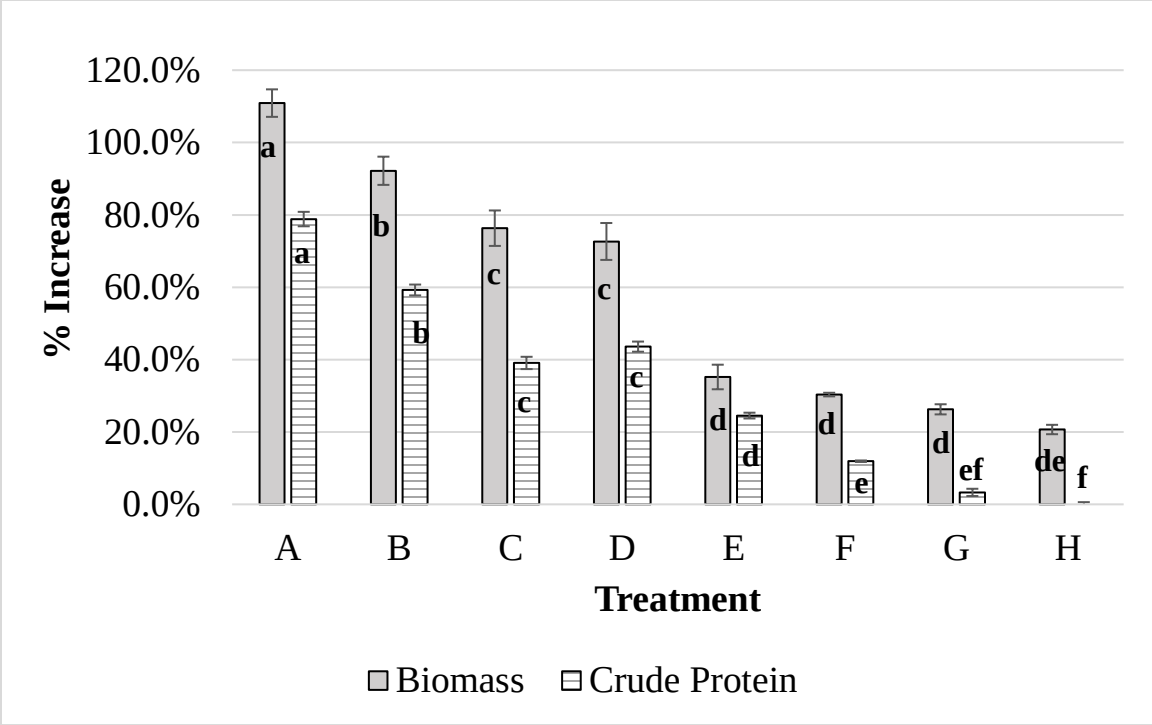


**Figure 3.1 Percent increases of biomass and crude protein yields after 5 d treatments. (A) *T. reesei* (B) *P. variotii* (C) *N. crassa* (D) *F. venenatum* (E) *R. oligosporus* (F) *M. circinelloides* (G) *P. kudriavzevii* (H) *A. pullulans***
*All values calculated from uninoculated control
*Mean value sharing the same letters are not significantly different from each other ($p$<0.05)

As explained in the above materials and methodology section, after the completion of the fermentation cycle, the solid and liquid fractions were separated using centrifugation and each fraction was then subjected to protein analysis. The protein titer of the solid fraction of all the treatments, including control are listed in the table 3.3. Protein titers analyzed on the treated product varied from 41.7-53.6% (table 3.3) whereas, the uninoculated control contained ~54.5% protein. Among the various strains evaluated, *P. kudriavzevii* yielded the highest protein titer in solid fraction, similar to that of un-inoculated control (~53.6% vs. 54.5%, Table 3.3). However, the protein titers in the rest of the fungal treated solid fractions were significantly lower than that of the control. This

could probably be explained by the fact that the extracellular enzymes produced by the fungi would lead to the degradation of the longer peptides into highly soluble form of protein, which would ultimately be lost in the supernatant during separation. Therefore, protein titers in the supernatant fractions were also determined and results showed that only with the exception of *T. reesei, P kudriavzevii,* and *A. pullulans*, all other fungal treatment resulted in the supernatant with high protein titers as compared to the control (data not shown).

**Table 3.3: Protein titers after 5 d incubation in Erlenmeyer flask trials**

| Treatment | Protein Titer (%) |
|---|---|
| Uninoculated Control | $54.5\pm1.0^{a}$ |
| *P. kudriavzevii* | $53.6\pm1.0^{a}$ |
| *M. circinelloides* | $46.8\pm0.6^{b}$ |
| *T. reesei* | $46.3\pm1.6^{b}$ |
| *F. venenatum* | $45.6\pm2.5^{bc}$ |
| *P. variotii* | $45.2\pm0.5^{bc}$ |
| *A. pullulans* | $44.9\pm0.6^{bc}$ |
| *N. crassa* | $43.1\pm2.1^{cd}$ |
| *R. oligosporus* | $41.7\pm2.6^{d}$ |

Mean value sharing the same superscript letters are not significantly different ($p<0.05$)

To fully understand the effectiveness of the overall process, mass balance calculations were conducted to determine protein and biomass yields (figure 3.1). As shown in figure 3.1, despite of being high in protein titers, the untreated control resulted in the lowest biomass and protein yield when compared to that of any fungal treatment. The protein yields for various fungal treatments ranged from 13.3-23.9 g/L, and the maximum yields were obtained by the *T. reesei* and *P. variotii* treatments at 23.9 and 21.3 g/L respectively (figure 3.1). Likewise, *N. crassa* and *F. venenatum* also resulted in

crude protein yields similar to that of *T. reesei* and *P. variotii* hence, improvement in the protein yields were achieved (figure 3.1). The total amount of supernatant solids reduced due to the fungal fermentation process was determined by subtracting the solids present in the supernatant fraction at the end of the fermentation from the supernatant solids present in the un-inoculated control. Percent solid reduction due to fungal treatment ranged from 6.6-49.1% (figure 3.2). Among all the fungal treatments, the maximum solid reduction of 49.1, 48.9, 48.4 and 46.5% were achieved respectively, with *N. crassa, P. variotii, F. venenatum and T. reesei* (figure 3.2). This study clearly indicated that among the eight strains that were evaluated, *T. reesei, N. crassa, F. venenatum and P. variotii* showed high potential for converting SBMS into protein rich feed. Previously, Simon et al. (2017) also reported the maximum protein yield on carinata meal using *N. crassa, P. variotii,* and *T. reesei.* From this study, T. *reesei* and *N. crassa* were chosen for the benchtop reactor trials for high biomass and corresponding protein yields as well as extremely high growth rate on the SBMS media in the case of *N. crassa*. The other two strains (*P. variotii* and *F. venenatum)* were excluded from the benchtop reactor trials as they visibly grew at a slower pace during the seed culture phase. Additionally, a literature review revealed instances of allergic reactions to *F. venenatum* protein (Katona and Kaminski 2002).

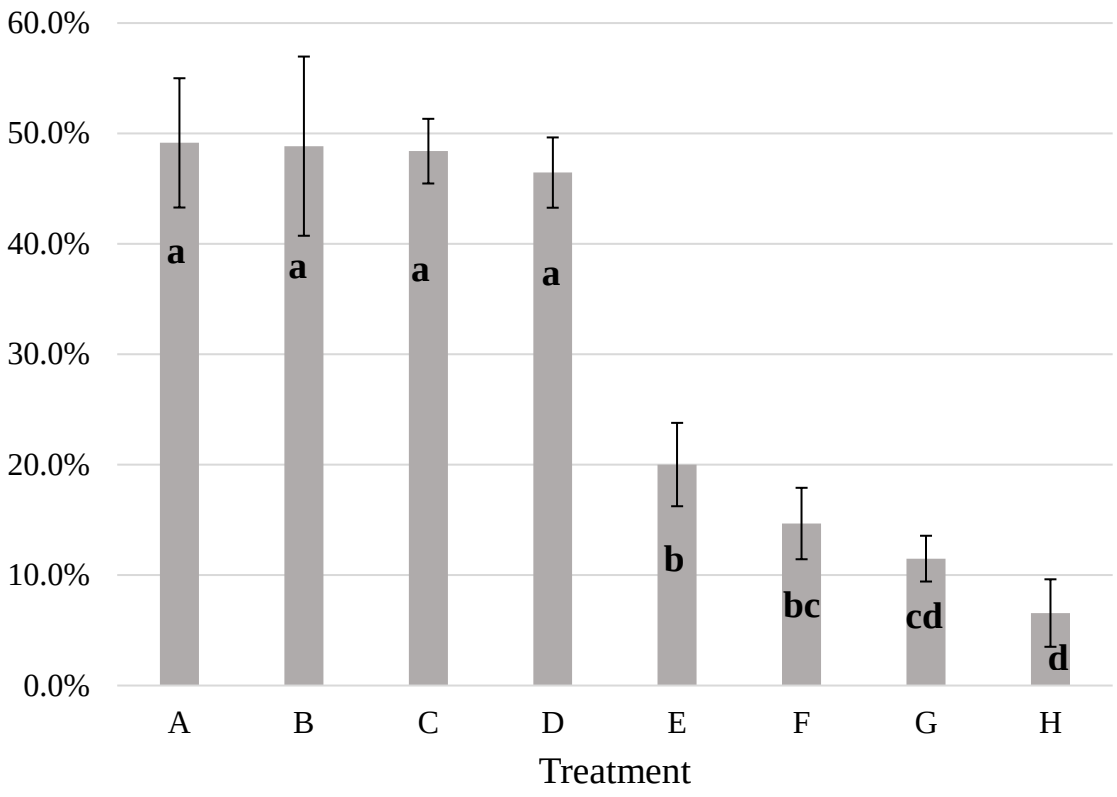


**Figure 3.2. Reduction in supernatant solids in flask trials compared to values obtained by comparing to uninoculated control. (A) *N. crassa* (B) *P. variotii* (C) *F. venenatum* (D) *T. reesei* (E) *R. oligosporus* (F) *A. pullulans* (G) *M. circinelloides* (H) *P. kudriavzevii***
*Mean value sharing the same letters are not significantly different from each other ($p$<0.05)

### 3.3.3 3 L Benchtop Reactor Trials Using *T. reesei* and *N. crassa*

Benchtop reactor trials conducted with the down selected treatments of *T. reesei* and *N. crassa* resulted in biomass yields of 55.5 and 62.0 g/L after a 5 d incubation under optimal conditions (table 3.4). The protein titers in the solids fraction of both trials (benchtop trials and flask trials) using *T. reesei* and *N. crassa* was consistent with *T. reesei* having ~3% higher titers in either scenario (table 3.3, table 3.4). However, an increase in biomass yield was observed for the benchtop trials when compared to the initial flask screening trials. This improvement in biomass yield could be attributed to the

aeration and agitation provided by the fermenter. *N. crassa* obtained higher and statistically significant ($p<0.05$) yields of 27.3-28.5 and 61.1-63.2 g/L of crude protein and biomass respectively despite a slightly lower protein titer compared to *T. reesei* (table 3.4). Yields and protein titers conducted in benchtop reactors were comparable to flask trials.

**Table 3.4: Biomass after 5 d incubation in benchtop reactors with 3 L total volume**

| Treatment | Biomass Yield (g/L) | Protein Titer % | Protein Yield (g/L) |
|---|---|---|---|
| *N. crassa* | 62.0±1.1 | 45.0±0.8% | 27.9±0.6 |
| *T .reesei* | 55.5±1.5 | 48.1±1.2% | 26.7±0.2 |

The mean $X_{TS}$ for either treatment were initially around 8.36-9.12% (figure 3.3) even though the material contained ~7.4% dry matter. Autoclaving the material resulted in a slight loss of (~300 ml) SBMS from the reactor which may have concentrated the amount of solids in the reactor. Throughout the incubation, *N. crassa* and *T. reesei* reduced mean $X_{TS}$ in the SBMS by 0.57% and 0.79% respectively. Differences in which solids were apparently utilized by each organism was seen between the two treatments. Mean $X_{UDS}$ remained unchanged throughout the study when treated with *N. crassa,* while mean $X_{DS}$ was reduced from 7.1 to 6.7%. On the contrary, *T. reesei* reduced mean $X_{UDS}$ from 1.5 to 0.8% and mean $X_{DS}$ from 7.7 to 7.6%. Thus, it appears *N. crassa* was more effective in reducing dissolved solids while *T. reesei* was more effective in reducing undissolved solids.

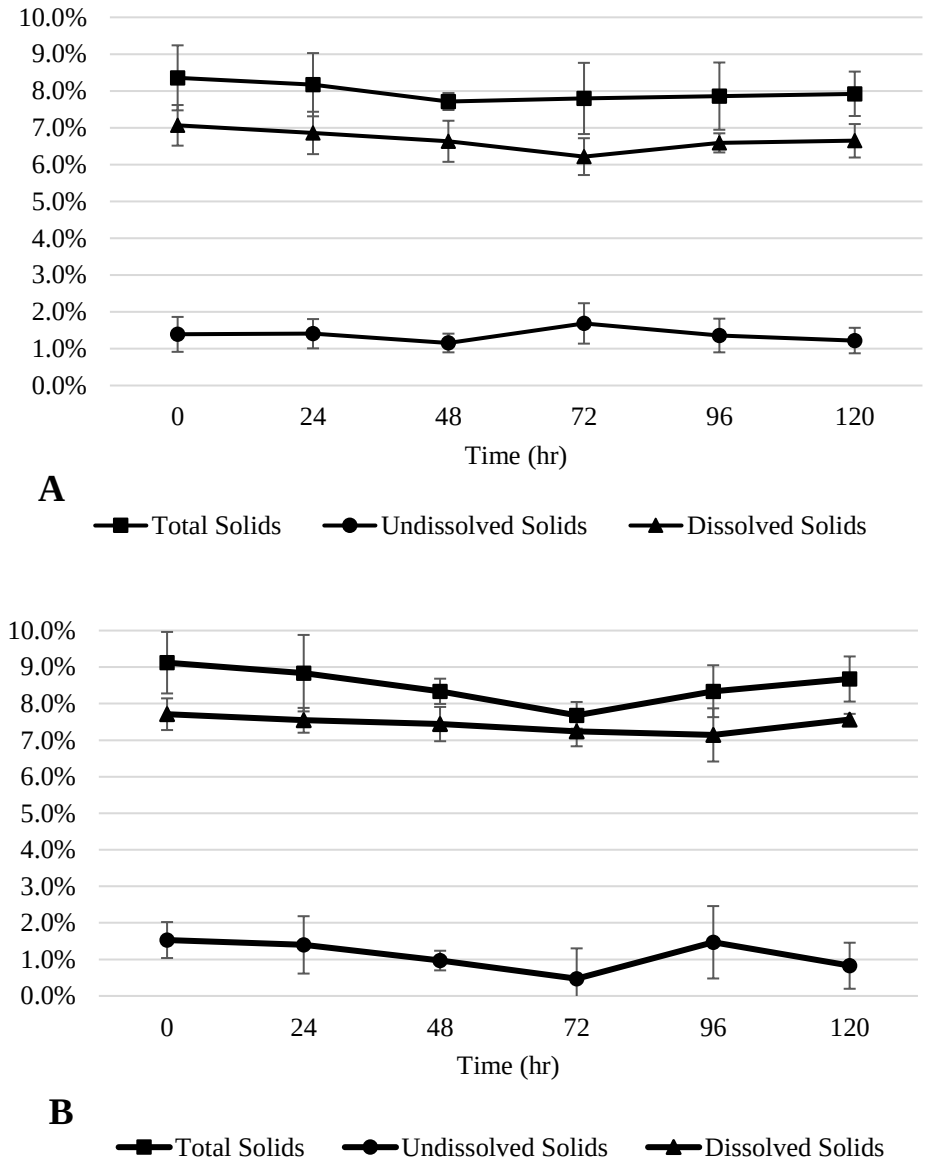


**Figure 3.3: Total Solids, Undissolved Solids and Dissolved Solids over the course of the 5 d incubation in benchtop reactors with 3 L total volume treated with (A) *N. crassa* (B) *T. reesei***

The pH value of the SBMS dropped slightly after autoclaving. Throughout the fermentation, the pH steadily rose. *N. crassa* obtained a pH value of 6.53-6.88 throughout the incubation while *T. reesei* obtained a pH value of 6.08-6.40 (figure 3.4). *N. crassa* and *T. reesei* are both known to produce extracellular enzymes capable of breaking down

the peptides. Hence, the production of the alkyl groups ($NH_3$, $NH_4$) during fermentation may have led to this slight increase in pH.

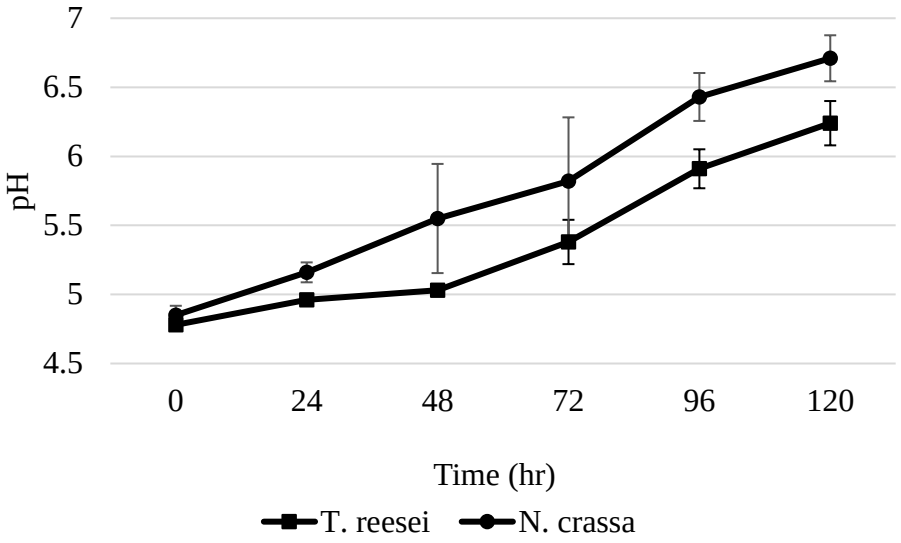


**Figure 3.4: Effect of fungal treatment on pH of media**

3.3.4 Change in COD Level of SBMS After Fungal Fermentation Using *T. reesei* and *N. crassa*

Using fungal fermentation to reduce the COD level of the wastewater from industrial processing has been studied previously (Lacina, Gourene et al. 2003, Sankaran, Khanal et al. 2010). SBMS used in this study was rich in protein resulting in COD measured at 57.64 g/L. We found that *N. crassa* and *T. reesei* reduced the COD level by 23.04% and 10.53% respectively after 5 d of incubation. This reduction in COD level would probably be improved further by lengthening the fermentation process. For example, a previous study reported that a longer incubation time (21 d after inoculation) proved to be beneficial in decreasing the amount of organic matter in the supernatant (Okoduwa, Igiri et al. 2017). At the same time, there are studies reporting more than 90%

of COD removal in less than a 24 h of inoculation. For instance, a starch processing wastewater treated with *R. oligosporus* NRRL-2710 reduced the COD by over 95% in just 16 h. Initial COD levels of the starch processing wastewater were 11.9 g/L to 18.9 g/L (Jin, Van Leeuwen et al. 1999). An additional study using olive mill wastewater found that COD values of 55-60 g/L inhibited the growth of *P. chrysosporium* and resulted in no decolorization activity due to the presence of a large amount of high-molecular weight aromatics (Sayadi, Allouche et al. 2000). Thus, the findings from the literatures and the results obtained in our study led us to conclude that there could be various parameters which would affect the COD reduction of the waste stream. Hence, further studies should be conducted focusing on optimizing process parameters to achieve adequate reduction of COD levels.

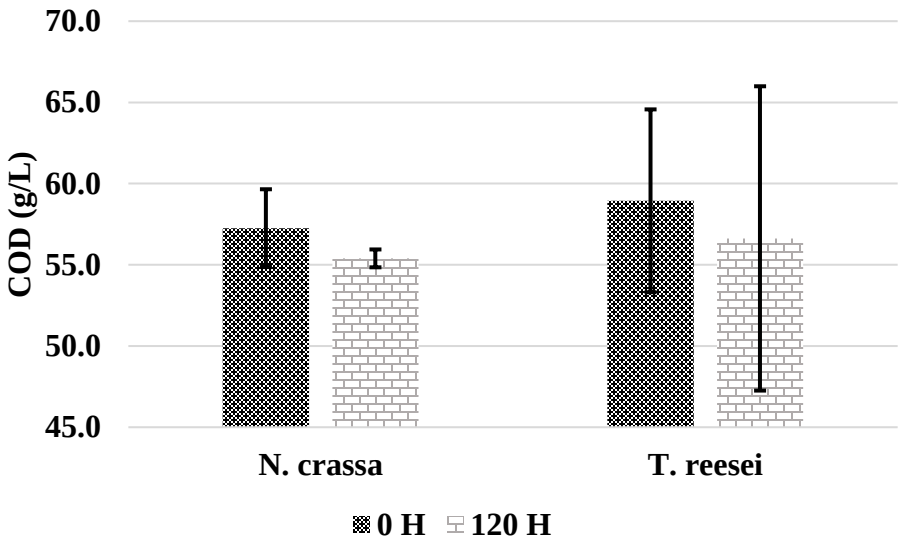


**Figure 3.5: COD measurements before and after fungal incubation**

## 3.4 Conclusions

Fungal incubation treatments produced protein-rich biomass for potential use in animal feed while simultaneously reducing various solids in the supernatant fraction of the soybean processing wastewater. *N. crassa* and *T. reesei* were down-selected for bioreactor trials as they outperformed other fungal strains tested in flask trials. *N. crassa* and *T. reesei* produced 62.0 and 55.5 g/L of biomass after 5 d incubation with 44.2-45.8% and 46.9-49.3% of that biomass existing as crude protein respectively. This study elucidated an interesting possibility of simultaneously producing protein-rich animal feed ingredient and reducing organic components in the SBMS by using fungal metabolism.

# Chapter IV - Microbial Processing to Increase Nutritional Value of Sorghum Mill Feed (Milo Hominy)


## Abstract

The objective of this study was to utilize fungal bioprocessing to increase the nutritional value of sorghum hominy. Eight fungal strains were screened for their ability to degrade plant feedstuff carbohydrates and concurrently produce microbial biomass protein (MBP). *Trichoderma reesei, Rhizopus oligosporus, Neurospora crassa* and *Aureobasidium pullulans* were the best performing fungi in flask trials and respectively increased the protein content of sorghum hominy by 53.6, 48.0, 47.8, and 41.47% compared to the raw feedstock. *A. pullulans* and *T. reesei* were selected for 5 L bioreactor trials, where biomass yield of 73.6 and 65.1 g/L were obtained for *A. pullulans* and *T. reesei* respectively. *T. reesei* was further selected for trials in 70 and 150 L fermenters and increased all amino acids in the range of 33.3-152.3%. The study showed a potential application of microbial processing to upgrade the sorghum hominy into a high protein feed ingredient for animal diets.


## 4.1 Introduction

Sorghum is a drought-resistant grain commonly grown in Africa and Asia for use in food and fodder (Ratnavathi and Patil 2013). The nutritional value of sorghum is comparable to corn, however, it typically contains more protein and less fat (Adebiyi, Adebiyi et al. 2005). It is generally accepted that sorghum contains about 5% less total digestible energy than corn (Hancock, Hines et al. 1991). Sorghum hominy (SH) is derived from a combination of grain sorghum bran, germ, and kernels (Rooney and Waniska 2000). Low levels of protein and amino acids in SH limits feed performance in

traditional applications and impedes entry into higher-level markets such as aquaculture feeds.

The growth of the aquaculture industry continues to outpace other sectors of world agriculture, with combined worldwide marine and inland harvests increasing at 6% per year from 2009-2014. Strong growth was even observed in the well-established Asian markets which represent 88% of the world's aquaculture production in 2014. Africa which represents a minor share (~2.3%) of the world's farmed-fish production has also strong growth in the last 20 years (FAO 2016). With the development of the aquaculture industry, demand has subsequently increased for aquaculture feeds. Aquaculture diets have long been reliant on fish meal as the main source of protein due to its high protein content and exceptional amino acid profile (Gatlin, Barrows et al. 2007). Fishmeal production has steadily decreased since peaking in 1994 due to strained supply and more strict measures implemented to protect fisheries. Despite it still existing as the most nutritious and digestible ingredients for farmed fish feeds, costs have risen up to ~1500 USD a ton in 2015 (FAO 2016).

Recent efforts have focused on utilizing alternative feedstuffs to fishmeal in aquafeed diets for increased economic viability of aquaculture feed rations (Gatlin, Barrows et al. 2007). The substitution of fish meal using lower cost animal protein sources such as blood and poultry meal (Nengas, Alexis et al. 1999, Zhou, Zhao et al. 2011, Aladetohun and Sogbesan 2013) along with plant-based protein sources such as soy protein concentrates (Deng, Mai et al. 2006, Salze, McLean et al. 2010, Li, Wang et al. 2015) has been widely examined. Plant-based protein sources are a particularly attractive substitute as they are in high supply and typically cheaper in price compared to

animal protein sources. However, insufficient levels of essential amino acids and the overall low digestibility of plant-based protein hinders inclusion in aqua diets (Francis, Makkar et al. 2001, Mamauag 2016).

Fungal fermentation is an attractive approach to improving the protein profile of plant feedstocks. During fermentation, fungi synthesize enzymes capable of degrading carbohydrates and produce highly digestible and protein-rich fungal biomass (Moore and Chiu 2001). Research on microbial fermentation of various oilseeds and cereal grains has been conducted (Han 2003, Chen, Shih et al. 2010, Azarm and Lee 2014, Plaipetch and Yakupitiyage 2014, Croat, Gibbons et al. 2016) and has been shown to concentrate protein and amino acids while simultaneously increasing digestibility. Numerous studies have also examined replacing fish meal and other proven ingredients with fermented soybean meal (Azarm and Lee 2014), canola meal (Plaipetch and Yakupitiyage 2014), and guar meal (Jannathulla, Dayal et al. 2017) for fish diets with varying levels of success.

The aim of this research was to evaluate if microbial processing could be used to enhance the protein titer of the milo hominy by using various fungal strains. Filamentous fungi and yeast are prime candidates for microbial biomass protein (MBP) production due to the fact that their respective biomass is easily digested, and high in protein content (Anupama and Ravindra 2000, Moore and Chiu 2001). Fungal incubations are commonly practiced in submerged (SmF) or solid state (SSF) settings (Pandey 2003). SSF has several advantages over SmF such as less water usage, higher enzyme titers and a lesser need for sterilizing pretreatment steps (Holker, Hofer et al. 2004). However, scale up of the process to industrial size can be problematic due to issues with mass and thermal

transfer, microbial resistance to agitation as well as damage to microbe due to shear, and issues with overall heterogeneity of the system (Durand 2003). On the other hand, SmF processes have the advantage of greater homogeneity of the substrate and greater mass transfer resulting in optimal growth of the microbe. As growth rates are of optimal concern in our process, a SmF process will be used for efficient mass transfer and thus quickly degrade available carbohydrates and produce MBP.

The specific objective of the study was to screen wide range of the yeast and fungal strains for their ability to metabolize carbohydrates including cell wall polysaccharides while simultaneously produce protein rich concentrate for aqua-diets. Higher protein titers obtained potentially allow for increased inclusion rates of microbial treated sorghum hominy biomass in fish diets.

## 4.2 Materials and Methods

### 4.2.1 Feedstock Preparation

Sorghum hominy was acquired from ADM milling (Overland Park, KS, USA). The hominy was milled using a Knife mill (FitzMill Model # S-DAS06, Elmhurst, Il, USA) and a 2 mm screen before use. Feedstock was stored at room temperature in an enclosed container throughout the study.

### 4.2.2 Cultures, Maintenance and Inoculum Preparation

Fungal strains provided by the USDA National Center for Agricultural Utilization Research (Peoria, IL, USA) are listed in (table 4.1). Maintenance cultures were stored on Potato Dextrose Agar (PDA) plates at 4°C and lyophilization was used for long-term storage of organisms. Seed cultures were prepared in 100 ml of glucose yeast extract

(GYE) consisting of 5% glucose and 0.5% yeast extract. Growth media was pH adjusted for each culture as shown in (table 4.1). Seed cultures were inoculated using a spore inoculum for filamentous organisms suspended in GYE and transferred with a pipette while isolated colonies were used for yeast -like organisms. Incubation took place in a rotary shaker at 30°C at 150 rpm for 72 h.

**Table 4.1*:* Fungal organisms tested**

| Organism | Strain | pH |
|---|---|---|
| *Aureobasidum pullulans* | NRRL-Y-2311-1 | 3 |
| *Trichoderma reesei* | NRRL-3653 | 5.6 |
| *Fusarium venenatum* | NRRL-26139 | 5.6 |
| *Rhizopus microsporus var. oligosporus* | NRRL-2710 | 5.6 |
| *Paecilomyces variotii* | NRRL-1115 | 5.6 |
| *Neurospora crassa* | NRRL-2332 | 5.6 |
| *Mucor circinelloides* | N/A | 5.6 |
| *Pichia kudriavzevii* | N/A | 3 |

### 4.2.3 Experimental Methods

#### 4.2.3.1 Preliminary Fungal Screening (5 d trials)

Trials were performed in 250 ml Erlenmeyer flasks with a working volume of 100 ml. A 10% (w/v) solid loading rate (SLR) was prepared with sorghum hominy and ~1 ml of antifoam. Media was pH adjusted to optimum for the various organisms as discussed previously. Flasks were topped with a foam stopper and aluminum foil prior to sterilization in an autoclave at 121°C for 20 min. Flasks were inoculated with a 1% (v/v) inoculum from previously prepared seed culture and placed in a rotary shaker at 30°C and 150 rpm. Daily observations were recorded for the 5 d incubation period. The flasks were harvested at the end of incubation for pH, mass balance, and protein analysis. All experiments were conducted in triplicate.

#### 4.2.3.2 Daily Flask Trials (7 d trials)

The four best performing microbes selected from the initial screening were chosen to be tested daily over the course of a 7 d incubation period. Trials were prepared in flasks identically to the initial screening in triplicate. A flask was harvested every 24 h (0-168 h) and contents were tested for pH, mass balance, and protein analysis.

#### 4.2.3.3 Benchtop Bioflo Reactor Trials (5 L)

The two best performing microbes selected from the secondary screening were chosen to be tested under optimal conditions in a 5 L benchtop reactor (New Brunswick Bioflo III, Edison, NJ, USA). Trials were prepared with a 10% SLR in reactors with a 5 L working volume. Reactors were sterilized using an autoclave at 121°C for 30 minutes. After cooling, reactors were set to a temperature of 30°C, agitation rate of 250 rpm, and aeration rate of 0.8 v/v/min. After reactor settings were attained, 50 ml of selected 72 h culture was inoculated into medium. Over the course of the 5 d incubation period 100 ml of sample was withdrawn daily for analysis of mass balance, pH, carbohydrates and protein titers. At the end of the fermentation period, reactor contents were dried at 80°C to moisture levels of less than ~5% moisture to avoid microbial spoilage during storage.

#### 4.2.3.4 70 L and 150 L fermentation trials

The best performing microbe was used to ferment material at larger scale. Two fermentations were conducted in a 70L vessel (New Brunswick IF-75, Edison, NJ) and one in a 150L vessel (New Brunswick VS-301, Edison, NJ) to produce enough fermented SH for feeding trials. Reactors were prepared identically apart from different working volumes which will be mentioned below. Reactors were causticized with UNIVAR

caustic soda beads (Silver Fern Chemical, Seattle, WA) for 30 min at 85°C. After caustic was removed by rinsing reactors, vessels were steamed at 121°C at 15 psi for 30 min. After cooling, 10% (v/v) solid-loading rates were obtained by the addition of water and SH in the respective vessels. The 70L and 150L vessels had final working volumes of 60L and 125L respectively. The pH was adjusted using 10M NaOH and allowed to equilibrate to a final pH value of 5.6. Following pH equilibration, the media was cooked at 121°C at 15 psi for 30 min. After sterilization, reactor contents were cooled to 30°C and inoculated with 0.8% (v/v) inoculum of *T. reesei* seed culture. The 70L and 150L fermenters were aerated at 0.8 (v/v/min), and operated at 30°C. Agitation rates of 25 Hz and 415 rpm used for the 70L and 150L respectively were predetermined to be the equivalent due to differences in reactor sizes and measured units. During fermentation, sample volumes of 100 ml were taken at the time of inoculation and every 24 h thereafter. Harvested samples were subjected to analysis for monoculture, pH, reducing sugars, solids fraction titers, and crude protein (CP). After a 72 h fermentation period, reactor contents were harvested and centrifuged at 4000xG for 5 min. Subsamples of both the pellet and supernatant fraction were kept for final analysis. The rest of the pelleted fraction was dried at 80ºC for 24 hr and milled through a 2mm screen.

### 4.2.4 Analytical Procedure

#### 4.2.4.1 Moisture and Proximate Analysis

Sample moisture content was determined using the American Association for Cereal Chemistry (AACC) method, where ~0.5-1 g of sample was dried in an oven at 105ºC for at least 4 h. The proximate composition of the sorghum hominy was determined by the SGS laboratory (Brookings, SD, USA) by using the standard protocol of Association of

Official Analytical Chemists (AOCS). The components of the proximate analysis are included in the Table 2. The amino acid profile of the material was determined by Midwest labs (Omaha, NE, 68144) using method AOAC method 994.12 (AOAC 1997).

4.2.4.2 Crude Protein

Crude protein (CP) of samples was found using a LECO model FP528 (St. Joseph, MI, USA). Combustion is used to measure total nitrogen content of sample. Approximately 0.25 g of sample was measured into tin foils for analysis. The nitrogen output was multiplied by a feed conversion factor of 6.25. Moisture calculations were then used to determine crude protein content of samples on a dry matter (DM) basis. All analysis was conducted in duplicate.

4.2.4.3 Total Solids, Dissolved Solids, and Undissolved Solids

After fermentation, three types of solids [total solids ($X_{TS}$), dissolved solids ($X_{DS}$) and undissolved solids ($X_{UDS}$)] were determined. $X_{TS}$ was found by placing ~1 ml of harvested sample in an oven at 105ºC for at least 3 hours. $X_{DS}$ was found by centrifuging the harvested sample at 4,000 rpm for 10 minutes and solids were separated from the liquid. Then the liquid fraction (~1 ml) was dried in an oven at 105ºC for at least 3 hours to estimate the $X_{DS}$. The undissolved solids fraction ($X_{UDS}$) was calculated using the formula below:

$$X_{UDS} = \frac{X_{TS} - X_{DS}}{1 - X_{DS}}$$

4.2.4.4 Residual Sugar Analysis

Glucose, sucrose, fructose, stachyose, and raffinose were quantified during the incubation. Residual sugars were measured using High Performance Liquid Chromatography (HPLC). Sugar standards of 99.9% purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gathered samples were first boiled for 10 min to inactivate potential enzymatic reactions and fungal metabolism and centrifuged at 10,000 $min^{-1}$. Following centrifugation, ~2 ml of supernatant was collected into a 2 ml microcentrifuge tube and frozen overnight. The supernatant was thawed and centrifuged again at 10,000 $min^{-1}$. The new supernatant was collected and filtered through a 0.2 µm filter and again frozen until analysis. The HPLC system (Agilent Technologies, Santa Clara, CA, USA) was equipped with a Waters size-exclusion chromatography column (SugarPak column I10 µm, 6.5 mm X 300 mm, Waters Corporation, Milford, MA, USA) and outfitted with a refractive index detector (Model G1362A) to measure sugar concentrations. De-ionized water was used for elution and mobile phase flow rate was 0.5 $ml/min^{-1}$ at 65°C for a 25 min run time.

### 4.2.5 Statistical Analysis

RStudio 1.0.1.143 was used to perform statistical analysis (Team 2015). The Bartlett's test was used to test for homogenous variances between the treatments for Erlenmeyer flask trials (Bartlett 1937). Analysis of variance (ANOVA) was used when variance homogeneity was confirmed to determine if a significant difference between treatments existed using the overall mean with the RStudio analysis package 'agricolae' (Mendiburu 2016). Duncan's new multiple range test tested for significant differences between means *post hoc* (Duncan 1955).

A F-test confirmed variances were not significantly different between treatments during 5 L bioreactor trials. Two sample t-tests were conducted for tested parameters if variances were not significantly different (Cressie and Whitford 1986).

## 4.3 Results and Discussion

### 4.3.1 Proximate Analysis

The sorghum hominy contained 90.3% dry matter (table 2). Hominy used in this study was found to have high amount of starch (34.5%) and fibers (crude fiber 7.6%, ADF 11.0%, NDF 22.0%). The protein level (12.9%) as obtained in this study was similar to the range (8-15%) reported by others (Shegro, Shargie et al. 2012). According to Shegro et al. genetic variation and environmental conditions may significantly influence and/or change the composition of the sorghum.

**Table 4.2: Nutritional value of sorghum hominy**

| Sorghum Hominy Proximate Composition | | |
|---|---|---|
| Parameter | Unit | Value |
| Moisture | %(m/m) | 9.7 |
| Crude Protein | %(m/m) | 12.9 |
| Crude Fiber | %(m/m) | 7.6 |
| ADF | %(m/m) | 11.0 |
| NDF | %(m/m) | 22.0 |
| Crude Ash | %(m/m) | 4.1 |
| Crude Fat | %(m/m) | 9.6 |
| Starch | %(m/m) | 34.5 |
| Available Starch | %(m/m) | 22.0 |
| Available Starch, % of Total Starch | % | 63.8 |
| Lignin | %(m/m) | 1.8 |
| Total Digestible Nutrients | %(m/m) | 85.2 |

* All values are on a dry matter (DM) basis

4.3.2 Preliminary Fungal Screening via SmF

Eight fungal strains were screened for their ability to increase the protein titers of the sorghum hominy (table 4.3). All fungal treatments resulted in significant ($p<0.05$) increase in protein titers compared to the control (no fungal inoculant). After the 120 hr incubation, the protein titers of the hominy increased in the range of 24.8 to 34.6% as compared to the un-inoculated control. *R. oligosporus*, *T. reesei*, *F. venenatum* and *N. crassa* outperformed the other strains and increased the protein titers by 60.2, 51.1, 45.9 and 37.6% respectively, compared to the un-inoculated control. The increase in protein content of the hominy after fermentation is attributed to the fungal metabolism. *T. reesei*, *N. crassa*, and *R. oligosporus* have been reported to produce high amounts of the cellulase and amylase enzymes (Pandey, Selvakumar et al. 1999, Duan, Qian et al. 2007,

Verma, kumar et al. 2012) possibly contributing to their high levels of conversion as observed in the present study (table 4.3). Fungal synthesis of hydrolytic enzymes such as amylases and cellulases degrade starch and fiber respectively into glucose providing the fungi with a utilizable carbon source. Fermentation of glucose releases $CO_2$ as a by-product therefore increasing the concentration of nitrogen in the MBP (Villas-Boas, Esposito et al. 2002). From this study, only four strains (*R. oligosporus, T. reesei, N. crassa* and *A. pullulans)* were selected for daily flask trials. Although effective in producing high protein titers sought after in this study, *F. venenatum* was excluded from the remainder of trials due to instances of allergic reactions to mycoprotein (Katona and Kaminski 2002). *A. pullulans* was selected over *P. variotii* and *M. circinelloides* because it is known to produce the valuable exopolysaccharide (EPS) pullulan with thickening properties (Gaur, Singh et al. 2010), which could also be observed in this study (data not shown).

**Table 4.3: 5 d protein titers of fermented sorghum hominy**

| Treatment | Protein Titer |
|---|---|
| Control | 13.3±0.2%[f] |
| *R. oligosporus* | 21.3±0.3%[a] |
| *T. reesei* | 20.1±1.0%[b] |
| *F. venenatum* | 19.4±0.4%[b] |
| *N. crassa* | 18.3±0.2%[c] |
| *P. variotii* | 17.9±0.1%[cd] |
| *M. circinelloides* | 17.3±0.4%[de] |
| *A. pullulans* | 16.7±0.5%[e] |
| *P. kudriavzevii* | 16.6±0.3%[e] |

*Letter indicates significance ($p<0.05$) between treatments

### 4.3.3 Daily Flask Trials (7 d)

To determine the full potential of the fermentation process, daily trials were conducted using the optimal fungal strains as identified in the preliminary study. Solid

and liquid fractions obtained from the daily flask trials were used for protein determination. Although protein titers of the solid fractions reached maximum within 48 to 72 h of fermentation, protein data of daily trails revealed that different fungal strains exhibited different trends during fermentation (figure 4.1.1). For example, there was a slow and consistent increase in protein content of the *R. oligosporus* and *N. crassa* fermented product whereas, *A. pullulans* and *T. reesei* protein titers displayed different tendencies. *A. pullulans* reached a maximum titer of 19.7% in 48 h, and thereafter, protein titers continued to decline for the rest of the incubation period. A rapid increase in protein concentration was also observed during the *T. reesei* treatment (19.84% in 48 h) and very minimal improvement was achieved afterwards (20.6% at 120 h). This observation clearly indicates the differences of fungal strains in terms of their metabolic processes and hence resulting into the production of MBP at different rates.

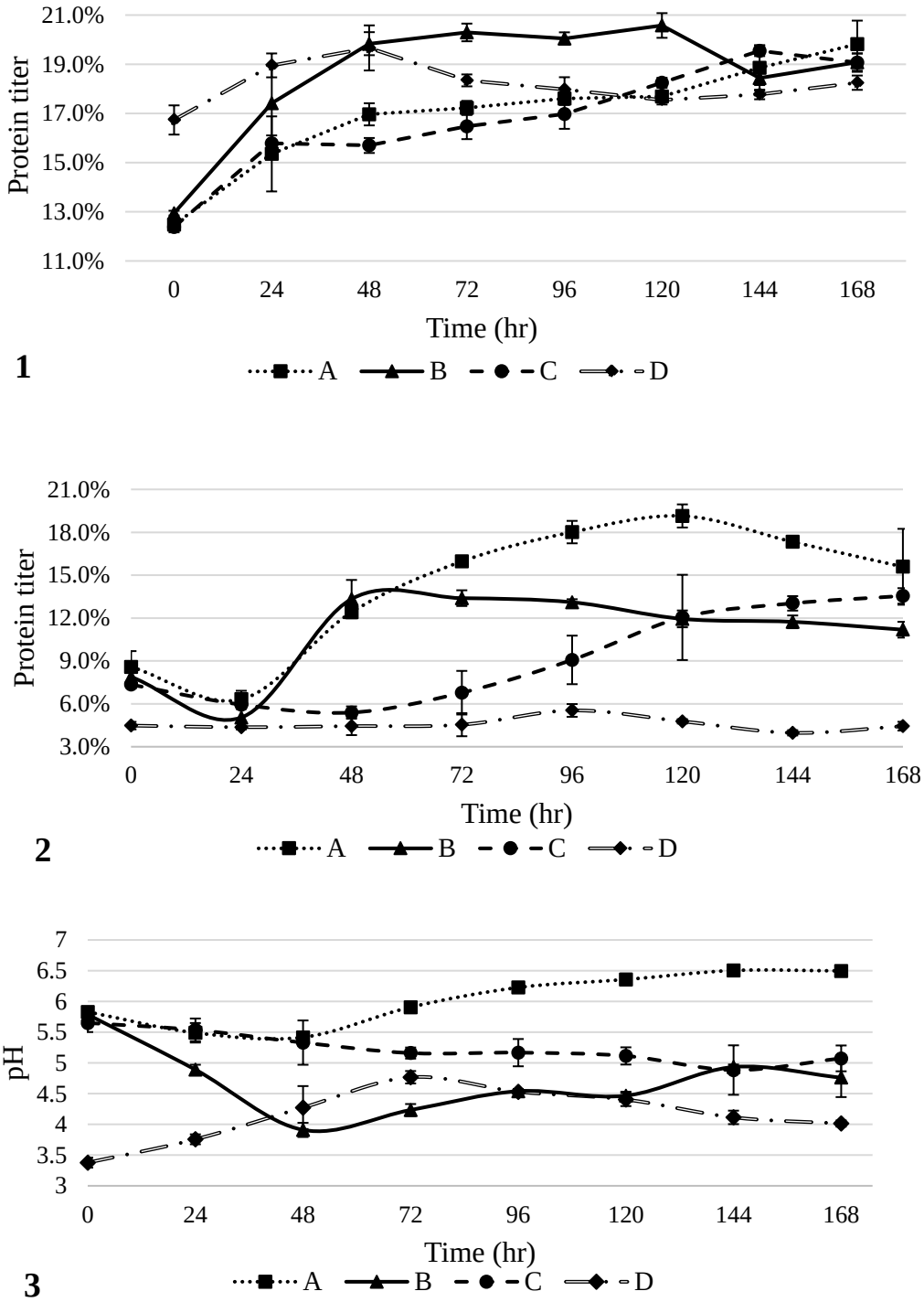


**Figure 4.1: (1) Solid Protein Titer (2) Liquid Protein Titer (3) pH during sorghum hominy fungal treatments. (A) *N. crassa* (B) *T. reesei* (C) *R. oligosporus* (D) *A. pullulans***

During large-scale fermentations, long incubation times result in higher production costs limiting the economic viability of the process. Thus, achieving maximum protein titers in minimal time is advantageous economically. Additionally, retaining the proteins in the solid fraction is highly significant for the process to be economically feasible. However, it is well documented in the literature that during fungal fermentation, hydrolytic activity of the enzymes including microbial proteases could lead to the partial breakdown of the longer chain polypeptides into short chain peptides and free amino nitrogen (FAN) (Ahmed, Zulkifli et al. 2014, Jannathulla, Dayal et al. 2017). Upon the completion of the fermentation process, when solids are recovered from the fermented slurry via centrifugation, short chain peptides and FAN concentration increases in the liquid fractions. Ultimately this leads to high protein titers in the liquid fraction. Therefore, in the present study, the protein titers in the liquid fractions of fermented slurry were determined. As shown in Figure 4.1.2, different trends were observed for the protein titers of different strains in the liquid fraction. During the first 24 h of incubation, protein titers in the liquid fraction dropped by 6-8% for all strains. However, increases in fermentation time resulted in increases in protein titers, with the exception of *A. pullulans* fermentation. The highest protein titer (18.6%) was found in *N. crassa* fermented liquid fraction followed by *R. oligosporous* (13.6%) and *T. reesei* (11.2%). As mentioned above, this observation is attributed to the hydrolytic activity of the fungal enzymes in breaking down the proteins into FAN and shorter chain peptides. When protein hydrolysis occurs the pH of the media also changes, which is also supported by the pH profile of different strain treatments (figure 4.2.3) during incubation period. During the incubation, *A. pullulans*- a yeast-like organism- showed the least

improvement in terms of protein titers of the solids as well as liquid fractions when compared to the filamentous fungal treatments. This may be due to the robust ability of the filamentous organisms to secrete extracellular enzymes (Guimaraes, Peixoto-Nogueira et al. 2006) compared to the yeast-like-organism.

Crude fiber, starch content, and the total biomass after fermentation were also measured (table 4.4). The crude fiber content of the control (un-inoculated) hominy increased from 7.6% to 10.6 and 8.5% respectively for pH 3 and pH 5 adjusted controls. Also, the crude fiber content of the fermented hominy increased from 7.6% to the range of 12.1-~15.5%. This increase in crude fiber was expected due to the concentration effect and a similar observation has been reported in a previous study (Croat, Gibbons et al. 2016). Among four strains that were evaluated, *N. crassa* resulted in the lowest level of crude fiber indicating the capability of releasing high amount of fiber degrading enzymes.

**Table 4.4: Carbohydrate and biomass titers after 7 d fungal treatment**

| Treatment | Fiber % (m/m) | Starch % (m/m) | Biomass g/100ml |
|---|---|---|---|
| Raw | 7.6 | 22.0 | NA |
| Control pH 3 | 10.6±0.19 | 15.2±0.59 | 6.21±0.17 |
| Control pH 5.6 | 8.50±0.14 | 25.4±2.2 | 8.11±0.20 |
| *N. crassa* | 12.1±0.35 | 5.52±0.59 | 5.51±0.14 |
| *A. pullulans* | 15.4±2.8 | 8.32±0.96 | 5.86±0.15 |
| *R. oligosporus* | 15.6±1.8 | 0.99±1.7 | 5.92±0.075 |
| *T. reesei* | 15.9±1.7 | 0.41±0.34 | 6.21±0.093 |

The starch content of the raw sorghum hominy was solubilized by mixing for seven days at pH 3, while mixing at pH 5.6 did not change the starch content (table 4.4). *T. reesei* and *R. oligosporus* utilized almost all of the available starch in sorghum hominy

(<1% remaining in the MBP), and thus resulted in the maximal protein titer of the solid fractions (table 3). It is interesting to observe that *N. crassa* utilized fiber when there was ~5% of starch available. *A. pullulans* was the least effective strain in utilizing the starch content with the residual starch content of 8.32%. Fiber concentrations were proportional to the 7 d biomass yields. Carbohydrates are a cheaper energy source than protein, however, fish species are known to have difficulty in digesting and metabolizing the carbohydrate fractions present in the diet. Carbohydrate digestibility varies depending on the fish species, source, inclusion level, and extent of processing applied (Krogdahl, Hemre et al. 2005). Although some fish species could use starch and monosaccharides to derive energy, non-starch polysaccharides particularly the fibers have adverse effect on the fish growth. Farmed carnivorous fishes such as salmon and trout are known to be least tolerant to carbohydrate rich diet and inclusion of carbohydrates beyond tolerable limit of the fish species reduces the starch digestibility and growth performance. Fermentation processing adds value to the nutritional profile of the feed ingredients by increasing the digestibility and improving the protein profile of the feedstuff.

*T. reesei* and *A. pullulans* were chosen again for further study in 5 L bioreactor trails. *T. reesei* was selected is due to its ability to quickly increase protein titers to ~20% and *A. pullulans* was selected to observe the performance of the yeast-like organism in the bioflo trials.

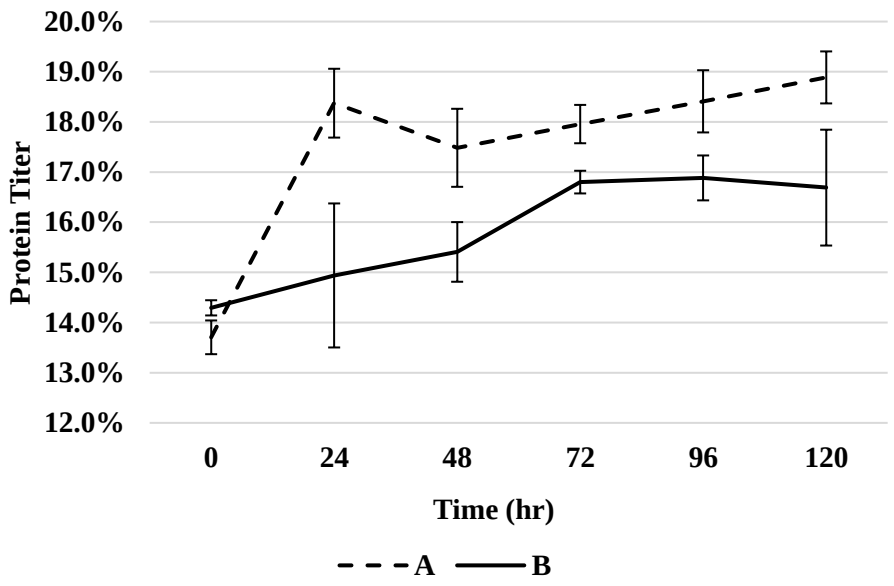


**Figure 4.2: Solid protein titers of (A) *T. reesei* and (B) *A. pullulans* in 5 L fermenters**

4.3.4 5 L Fermenter Trials

Fungal treatments conducted in 5 L bioreactors had the advantage of aeration (0.8 v/v/min) and greater mixing due to the unit's impeller compared to previous flask trials. The fungal treatments attained similar protein titers to flask trials as expected (figure 4.2). *A. pullulans* yielded significantly more ($p<0.05$) biomass than did *T. reesei* at 73.6 and 65.1 g/L respectively. This was inversely proportional to the protein titers obtained, as the *T. reesei* treatment resulted in a significantly higher ($p<0.05$) 5 d protein titer than the *A. pullulans* treatment at 18.9% compared to 16.7%. This can be explained by observing the distribution of solids fractions (figure 4.3) during fermentation period. Over the 5 d incubation, the $X_{TS}$ fraction of the *T. reesei* treated hominy decreased from 9.3-8.4% throughout the incubation while TS of the *A. pullulan* treated fraction increased from 9.6-10.5%. The greater increase in protein concentration for the *T. reesei* treated hominy may be due to the reduction in total solids. During the fermentation, *T. reesei* and other filamentous fungal strains are known to produce vast amounts of cell mass through

fermentation while expelling $CO_2$. Thus, the lost carbon may explain the loss of TS and also increased the concentration of protein in the treated biomass. This explanation does not explain the increase in $X_{TS}$ on the *A. pullulans* treated fractions. However, during the incubation water may be lost due to evaporation, which if greater than the amount of lost carbon, would cause an increase in $X_{TS}$. Both fungal treatments yielded 12.3 g/L of protein despite different biomass yields.

The observed pH value was similar to the daily flask trials. For either organism, extreme pH values were not reached allowing for the organism to grow at an optimal rate.

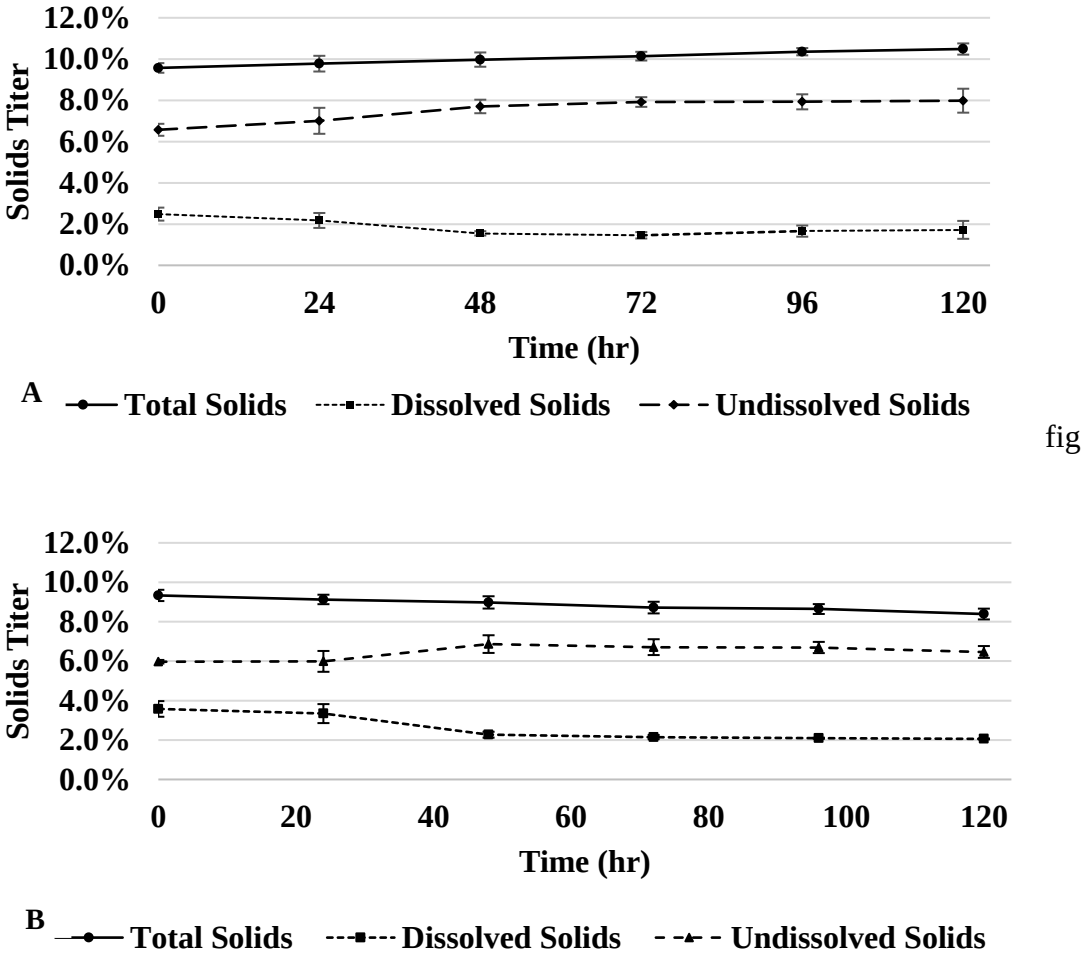


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**Figure 4.3: Various solids fractions of hominy during 5 L bioreactor trials. (A) *A. pullulans* (B) *T. reesei***

4.3.5 70 and 150 L Fermenter trials

The proximate nutritional profile of both the untreated and treated sorghum hominy can be seen in (table 4.5). Starch is the main constituent of SH and present in high amounts (34.5%). Crude fat (9.56%), crude fiber (7.60%), ash (4.07%), and CP (12.9%) also make up a large portion of SH. Of the primary fractions, starch experience the largest reduction and was reduced by 93.7% after the 72hr incubation to 2.13%. This lost biomass resulted in a concentration effect, therefore increasing the concentrations other fractions of the sorghum hominy. Other researchers have reported similar effects (Croat, Gibbons et al. 2016).

**Table 4.5: Nutritive value of untreated and treated SH**

| Constituent | Untreated | Treated | Unit |
|---|---|---|---|
| Crude Protein | 12.9% | 21.6% | % (m/m) |
| Crude Fat | 9.56% | 13.2% | % (m/m) |
| Crude Fiber | 7.60% | 16.1% | % (m/m) |
| Starch | 34.5% | 2.13% | % (m/m) |
| Ash | 4.07% | 5.07% | % (m/m) |
| Phosphorus | 0.62% | 1.01% | % (m/m) |
| Potassium | 0.80% | 0.49% | % (m/m) |
| Calcium | 0.05% | 0.11% | % (m/m) |
| Magnesium | 0.32% | 0.28% | % (m/m) |
| Sulfur | 0.12% | 0.24% | % (m/m) |
| Zinc | 37.6 | 53.9 | PPM |
| Manganese | 38.4 | 48.0 | PPM |
| Copper | 6.65 | 12.3 | PPM |
| Iron | 94.2 | 211 | PPM |

Compared to the untreated SH, the fungal treatment increased CP, crude fat, and crude fiber by 67.4, 38.1 and 112% respectively. Phosphorus and calcium concentrations were increased by 62.9 and 120% respectively. Potassium and magnesium concentrations decreased by 38.8% and 12.5%. Zinc, manganese, copper, and iron concentrations all increased in the range of 24.8-123%. All amino acid concentrations increased in the

range of 33.3-152.5% (table 4.6). Although *T. reesei* is known for its cellulolytic capabilities (Domingues, Queiroz et al. 2000, Yan, Cao et al. 2012), crude fiber was not significantly degraded during the incubation and concentrations increased to 16.1%. Crude fat and ash also increased to 13.2% and 5.07%.

**Table 4.6: Amino acid profile of both untreated and treated SH**

| Amino Acid | Untreated | Treated | Unit |
|---|---|---|---|
| Alanine | 0.87% | 1.36% | % (m/m) |
| Arginine* | 0.65% | 1.38% | % (m/m) |
| Aspartic Acid | 0.89% | 1.25% | % (m/m) |
| Cystine | 0.37% | 0.51% | % (m/m) |
| Glutamic Acid | 1.76% | 2.93% | % (m/m) |
| Glycine | 0.48% | 0.75% | % (m/m) |
| Histidine* | 0.27% | 0.49% | % (m/m) |
| Isoleucine* | 0.40% | 1.01% | % (m/m) |
| Leucine* | 1.06% | 2.32% | % (m/m) |
| Lysine* | 0.39% | 0.62% | % (m/m) |
| Methionine* | 0.19% | 0.30% | % (m/m) |
| Phenylalanine* | 0.48% | 0.87% | % (m/m) |
| Proline | 0.65% | 1.20% | % (m/m) |
| Serine | 0.49% | 0.74% | % (m/m) |
| Threonine* | 0.41% | 0.73% | % (m/m) |
| Tryptophan* | 0.15% | 0.20% | % (m/m) |
| Tyrosine | 0.41% | 0.72% | % (m/m) |
| Valine* | 0.57% | 0.91% | % (m/m) |

The pH of fermented SH and protein titers of both the solids and liquids fractions were observed after harvesting and centrifugation (figure 4.4). Sterilizing the SH and centrifuging the material increased protein titers to 16.1%. Protein titers continued to increase until the end of the 72hr incubation reaching 17.8%. Similar CP increases have been attained by other researchers using submerged fermentation processes (Croat, Gibbons et al. 2016) and solid-state fermentation processes (Han 2003, Imelda, Paulraj et al. 2008, Jannathulla, Dayal et al. 2017). The effect can be partially attributed to fungal

metabolism. During fermentation, fungi consume carbohydrates present in the SH and release $CO_2$ as a by-product, therefore increasing the overall concentration of nitrogen (Ugbogu and Ugbogu 2016). The material was then washed using water and centrifuged again to obtain a protein titer of 21.6%. The solids present in the liquid fraction contained a protein titer of 6.84% at inoculation. Throughout the incubation titers steadily decreased to 4.03% at 48hr and rose slightly to 4.59% at the end of the 72hr fermentation period. The pH of the media dropped from 5.55 to 3.40 throughout the incubation. Mixed results have been observed pertaining to protein solubility in submerged processes. (Chavan, Chavan et al. 1988) reported an increase in soluble proteins potentially due to microbial protease enzyme activity. A potential reason for the decrease in liquid protein in our process was the drop in pH value to ~3.3 observed throughout the incubation. (Adeyeye 2008) reported that sorghum proteins had an isoelectric point of 3.3 which would render the proteins insoluble and present in the solids fraction after centrifugation in the submerged process.

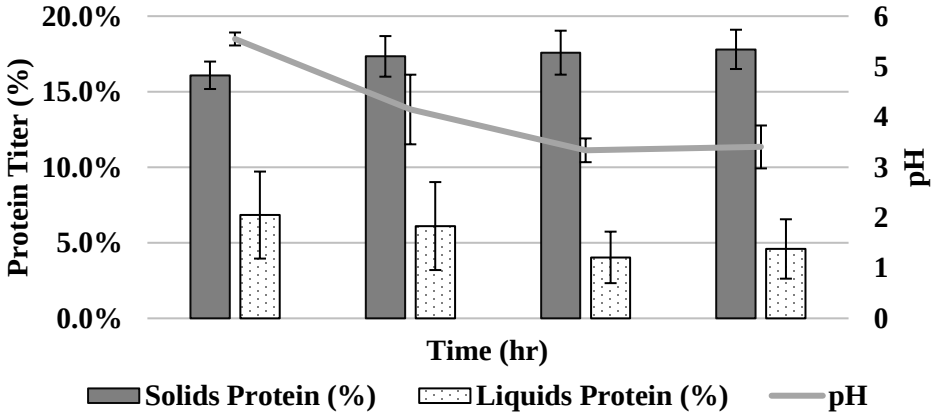


**Figure 4.4: Relationship between solids and liquid protein concentrations (DM basis) and pH**

Soluble sugar concentrations observed during the incubation can be seen in (table 4.7). Glucose was present at a concentration of 1.67 % at inoculation. After decreasing to 1.01 % at 24hr, concentrations rapidly increased to 8.41 % at 48hr and increased furthermore to 9.39 % at 72hr. The 0hr concentration of fructose was 0.86 %. Fructose titers decreased to 0.31 % at 24hr and then decreased to 0.23 % at the end of the incubation. Sucrose was initially present at a concentration of 1.48 % at inoculation. Sucrose concentrations on average slightly decreased, however, it is worth noting there was a large amount of deviation between readings. Stachyose titers of 0.43 % were observed at inoculation. Stachyose concentrations fluctuated slightly during the incubation in the range of 0.42-0.53 %. Raffinose concentrations of 0.48 % were observed at inoculation. Raffinose titers rose to 0.77 % at 24hr before plateauing to 1.1 % for the rest of the incubation.

**Table 4.7: Sugar profile of fermented SH supernatant**

| Time (hr) | Sugars (% m/m) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | | Fructose | | Sucrose | | Stachyose | | Raffinose | |
| 0 | 1.67 | ± 0.30 | 0.86 | ± 0.18 | 1.48 | ± 0.07 | 0.43 | ± 0.03 | 0.48 | ± 0.04 |
| 24 | 1.01 | ± 0.71 | 0.31 | ± 0.20 | 1.32 | ± 0.75 | 0.59 | ± NA* | 0.77 | ± 0.20 |
| 48 | 8.40 | ± 0.89 | 0.14 | ± 0.04 | 1.15 | ± 0.32 | 0.42 | ± NA* | 1.1 | ± 0.22 |
| 72 | 9.39 | ± 4.2 | 0.23 | ± 0.17 | 1.26 | ± 0.75 | 0.44 | ± 0.10 | 1.1 | ± 0.27 |

*Only 1 value was detected during sugar analysis

Soluble sugar concentrations fluctuated during the incubation as fungal growth ensued. The most notable increase in the measured sugar fractions was glucose as concentrations increased 585%. The increase can be partially explained by the large amount of starch present in sorghum hominy. As starch consists of repeating glucose monomers, it is likely that *T. reesei* produced a starch degrading enzyme such as an amylase to degrade starch resulting in increases in glucose molecules. *T. reesei* α-

amylase and glucoamylase production has been previously confirmed (Smits, Rinzema et al. 1996, Duan, Qian et al. 2007). Fructose concentrations decreased during the incubation by 55.8%. Although it is generally accepted that fungi typically consume glucose at higher rates over fructose, fructose is known to be consumed to some degree (Solaiman and Saito 1997). Sucrose concentrations decreased by 12.8% further explaining the increases in glucose and fructose concentration as sucrose is cleaved into fructose and glucose molecules by the invertase enzyme. Evidence of sucrose utilization and invertase production by *T. reesei* has been previously reported (Lieckfeldt, Kullnig et al. 2000). Overall, the sugars glucose, fructose, and sucrose present in SH were consumed by *T. reesei*. On the other hand, it was observed that the trisaccharide raffinose and tetrasaccharide stachyose were not consumed and raffinose levels increased 104% during the incubation. It has been previously reported that *T. reesei* is able to synthesize the enzyme α-galactosidase known to hydrolyze both these sugars (Zeilinger, Kristufek et al. 1993, Kachurin, Golubev et al. 1995). It is likely that the fungi consumed the widely available sugars of glucose, fructose and sucrose available in the SH and therefore did not need to degrade the more complex oligosaccharides raffinose and stachyose. However, other researchers have reported the utilization of stachyose due to fungal incubations. Egounlety & Aworh (Egounlety and Aworh 2003) observed a decrease in stachyose levels during the fermentation of soybean, chickpea, and groundbean using the fungi *R. oligosporus*.

Amino acids were increased in the range of 33.3-152.5% after the fungal treatment. Of the essential amino acids in fish nutrition, arginine, leucine and isoleucine concentrations experienced the greatest increases due to fermentation at 112.3, 118.9%,

and 152%. *T. reesei* cell mass has been shown to contain high levels of isoleucine and leucine in fermentation (Ghanem 1992). The sulfur-containing amino acids cysteine and methionine were increased by 37.8% and 57.9% respectively. Typically, cysteine and methionine are low in plant proteins and the latter is a common limiting factor in plant-based aquafeed diets (Craig and Helfrich 2009). The same is seen with lysine, which was increased from 0.39% to 0.62%, a 58.9% increase due to fermentation. Of the other essential amino acids, histidine, phenylalanine, threonine, tryptophan, and valine were increased by 81.5, 81.2, 78.1, 33.3, and 59.7% respectively.

The TS fraction was measured to be 8.34% after cooking. It is likely that during the cooking step, the SH became caked on the bottom of the reactor and was not resuspended upon agitation in the vessel. Therefore, it could not be measured. Throughout the incubation, TS concentrations decreased ultimately reaching 6.97% at 72hr. The decrease in measured TS during the incubation can be explained by utilization of solids due to fungal fermentation resulting in the subsequent loss of $CO_2$ into the atmosphere. Of the TS, 5.0% were DS at 0hr and 3.34% were UDS. The DS fraction decreased throughout the incubation attaining a 72hr concentration of 2.96%. The UDS fraction increased throughout the incubation reaching a 72hr concentration of 4.01%. The loss in DS can be attributed to fungal metabolism and likewise, the increase in UDS. Carbohydrate degrading fungi are known to effectively utilize DS in submerged-state fermentations (Nitayavardhana and Khanal 2010). The utilization of DS seen in the SH fermentation increased the concentration of UDS relative to TS, hence an increase was seen in UDS fractions.

## 4.4 Conclusions

The SmF process was found to be an effective tool in increasing the protein content of the sorghum hominy for its value-added application as a potential feed ingredient. Among the several strains evaluated, *T. reesei* (NRRL-3653) was determined to be the most effective fungal treatment. It increased the protein titer of the hominy to ~20% in 72 hr when grown in a 150 L fermenter. Low starch and high protein sorghum hominy after fungal fermentation could be promising alternative plant feedstuff in monogastric diets including aquadiets.

# Chapter V- Microbial bioprocessing of guar korma meal for development of fermented protein concentrate


## Abstract

The objective of the study was to compare the efficiency of a submerged fungal incubation process with a two-step submerged fungal incubation process for production of a fermented protein concentrate. Five fungal strains were assessed for their ability to improve the value of guar korma meal by measuring the degradation of crude fiber and guar gum fractions and the subsequent increases in crude protein concentration. It was found that creating two separate fractions of guar korma meal by washing with water via centrifugation results in a washed guar korma meal (WGKM) fraction high in crude protein (~65%) and another guar korma meal solubles (GKMS) rich in sugars. Fermentation of WGKM using *T. reesei* for 36 h produced 65.8 g/L of a protein-rich fraction (66.8%) in 5 L bioflo fermenters, while fermentation of GKMS using *A. pullulans* yielded 12.8 g/L of protein-rich (40.6%) biomass.


## 5.1 Introduction

Guar bean (*Cyamopsis tetragonoloba*) is a drought-tolerant legume commonly grown in the northwestern region of India and southeast region of Pakistan. Production of guar worldwide is estimated to be 1.0-1.6 million tons annually with Pakistan and India producing 15 and 80% of the world's total respectively (Hussain, Rehman et al. 2012). Due to this isolated area representing ~95% of the world's production, highly variable levels of precipitation have extreme effects on the annual production of guar, which has limited the growth of the industry (Sharma 2010). Recent increases in the demand of guar

gum have spurred interest in growing guar in countries such as China and Australia (Durgesh 2015).

Guar seed consists of a fibrous hull (14-17%), galactomannan-rich endosperm (35-42%), and protein-rich germ (43-47%) (Hussain, Rehman et al. 2012). Industrial processing of the seed typically yields guar gum (29.25%), korma (32%), churi (34.5%), and waste (4.25%) (Singh 2014). The guar gum, which is derived from the endosperm, remains the most valuable processed fraction of guar owed to its wide-range of applications such as in the oil, food, textile, agriculture, explosives and healthcare industries (Mudgil, Barak et al. 2014). Galactomannans contained in this fraction improve the viscosity of solvents such as water even at very low concentrations (Thombare, Jha et al. 2016). Guar churi meal and guar korma meal are different qualities of guar meal with crude protein content ranging from at 32-40% and 48-58% respectively (Pach and Nagel 2017). Although guar processing removes most of the galactomannan-rich endosperm, some residual gum remains in the feed fractions. Researchers have reported a wide-range of residual gum contents of guar meal ranging from 4-20% (Hussain, Rehman et al. 2012).

Guar korma meal (GKM) has primarily been used in livestock, and poultry feeds, however, inclusion rates have been limited by the presence of residual guar gum. It is generally believed that the gum residue increases the intestinal viscosity therefore decreasing the overall digestibility of the feed (Hussain, Rehman et al. 2012). Recently, interest has grown in utilizing GKM as a potential alternative to fish meal in aquadiets as it possesses a similar nutritional profile to soybean meal (SBM) which has been successfully been implemented in high-value aquadiets (Pach and Nagel 2017). However,

like other plant-based proteins, GKM inclusion rates are still restricted due to the presence of indigestible fibers, low protein titers, and plant antinutritional factors such as guar gum. Biological treatments such as microbial fermentation have been reported to increase the nutritional value of plant-derived feed ingredients by degrading indigestible fibers and antinutritional factors while simultaneously concentrating protein in feeds (Imelda, Paulraj et al. 2008).

Several researchers (Dinani 2017, Jannathulla, Dayal et al. 2017, Jannathulla, Dayal et al. 2017) have examined the efficacy of solid-state fermentation (SSF) to increase the nutritional value of various guar meal fractions with various levels of success. Less explored is the prospect of utilizing a submerged-state fermentation (SmF) process to improve the value of guar meal. SmF processes have the added advantage of utilizing a centrifugation-based wash step which concentrates insoluble feed components such as certain proteins similar to the acid or ethanol wash processes seen in soy protein concentrate (SPC) manufacturing (Ohren 1981). An unintended side-effect of SmF is the degradation of peptides via microbial enzymatic activity upon the depletion of available sugars. Although improvements in the digestibility of proteins has been found to increase (Kazanas and Fields 1981, Joshi and Sandhu 1996), they are also solubilized which renders them unable to be captured efficiently. Hence, microbial strains exhibiting carbohydrate degradation and to a lesser extent protein degradation are advantageous in submerged fermentations using centrifugation to concentrate protein.

We aim to determine the optimal submerged fungal process for improving the nutritional value of GKM by degrading indigestible fiber, reducing guar gum levels, and improving the overall protein profile. Said process will examine the effects of a standard

SmF process and the effect of washing the material prior to fermentation and conducting two-separate fermentations. Two-stage fermentations have been shown to be advantageous as different microbial strains can have beneficial effects on different substrates (Chen, Shih et al. 2010).

## 5.2 Materials & Methods

### 5.2.1 Feedstock Preparation

GKM was received from Sunita Hydrocolloids Pvt. Ltd. (Jodhpur, Rajasthan, India). GKM was milled to pass through a 2mm screen using a FitzMill model # S-DAS06 knife mill (Elmhurst, IL, USA). Milled GKM was stored in a sealed bucket throughout the duration of the study. GKM that was only milled is referred throughout the study as raw GKM (RGKM). A concentrated stream of GKM called washed GKM (WGKM) was prepared by creating a 10% (v/v) solid-loading rate (SLR) slurry of RGKM, adjusting the slurry to pH 4.7, and centrifuging the slurry at 4,000xG for 10 min. The supernatant was decanted, volumetrically measured, and kept at 4°C until fermentation preparation and is called GKM solubles (GKMS) throughout the study. The pelleted material is referred to as washed GKM (WGKM).

### 5.2.2 Cultures and Inoculation Preparation

*T. reesei* (NRRL-3653), *N. crassa* (NRRL-2332), *R. oligosporus* (NRRL-2710), *P. variotii* (NRRL-1115), and *A. pullulans* (NRRL-Y-2311-1) were obtained from the National Center for Agricultural Research (Peoria, IL, USA). Cultures were stored at 4°C on potato dextrose agar (PDA) plates. Filamentous fungal seed cultures were prepared by brushing fungal mycelium with a sterile loop to collect spores then inoculated into a 100

ml total volume of glucose yeast extract (GYE). Yeast organism seed cultures were prepared similarly using a sterile loop to transfer an isolated colony into 100 ml GYE. Flasks were incubated at 30°C and agitated at 150 rpm for 48 h in a New Brunswick Scientific Excella E24 rotary shaker (Hauppauge, NY, USA).

### 5.2.3 Experimental Procedures

#### 5.2.3.1 Initial Fungal Screening of Raw and Washed GKM

RGKM flasks were prepared at a 10% (v/v) SLR in 250 ml Erlenmeyer flasks using 100 ml total volume. WGKM flasks were prepared by adding the lost volume (~80 ml) from the wash in the form of dH2O and resuspending the material. All flasks were adjusted to pH 5 for filamentous fungal organisms and pH 4 for yeast organisms with 3.6 M sulfuric acid for optimal growth conditions. For each organism, 18 flasks were prepared for harvesting in triplicate every 24 h for a 0-120 h period. All flasks were sterilized via autoclave at 121°C and 15 psi for 20 minutes. After cooling, flasks were inoculated with 2% (v/v) desired seed culture. Controls flasks were set up identically to both filamentous and yeast organisms flasks, yet they were not inoculated with culture. Each day, three flasks were harvested entirely for analysis. Gram stains and streak plates were used to check for purity of sample. Flask contents were centrifuged at 4,000xG for 10 min and the supernatant was decanted. Both the pelleted and supernatant fraction were dried at 80°C for 24 h. Fractions were stored in sealed bags further analysis was conducted.

#### 5.2.3.2 Initial Fungal Screening of GKM Solubles

The collected GKM solubles were prepared in 250 ml Erlenmeyer flasks using 100 ml total volume. Flasks were adjusted to pH 5 for filamentous fungal organisms and pH 4 for yeast organisms with 3.6 M sulfuric acid for optimal growth. Eight flasks were prepared for each organism and autoclaved at 121°C and 15 psi for 20 minutes. Upon cooling, flasks were inoculated with 2% (v/v) fungal culture. Uninoculated control flasks were setup identically to fungal treated flasks. Gram stains and streak plates determine the purity of the sample. Flask contents were centrifuged at 4,000xG for 10 min and the supernatant was decanted. All fractions were dried at 80°C for 24 h, grinded and sealed in bags until further analysis.

5.2.3.3 Benchtop fermenter trials (5L) with Optimal Process

The two processes examined in the initial screening phases, RGKM or WGKM + GKMS were compared for their ability to retain protein in the pelleted fractions after fermentation. The best performing process (WGKM +GKMS) was further examined in 5 L benchtop reactors (New Brunswick bioflo III, Edison, NJ, USA). Two separate fermentations were conducted for each fraction; WGKM (5 L) and GKMS (3 L). The pH of the WGKM and GKMS fermenters were adjusted to pH 5 (*T. reesei*) and 4 (*A. pullulans*) respectively for optimal growth of down-selected microbes. All reactors were sterilized via autoclave for 40 min at 121°C and 15 psi. Reactor conditions were set to an agitation rate of 250 rpm, aeration of 0.8 v/v/min, and temperature of 30°C. After cooling, both the *T. reesei* and *A. pullulans* cultures were inoculated with 1% (v/v) seed culture. Fermentations were conducted for 48 hr, with 100 ml daily samples drawn every 12 hr, including a 0 hr sample. Harvested samples were checked for monoculture via gram stains and streak plates, pH recorded, and centrifuged at 4,000xG for 10 min.

Following centrifugation, the supernatant was decanted from the pellet, and 10 ml was drawn for sugars analysis via HPLC. Both the pellet and supernatant fraction had their masses recorded and were dried at 80°C for 24 hr. After drying, masses were again recorded and samples were grinded using a coffee grinder until further analysis.

### 5.2.4 Analytical Procedures

#### 5.2.4.1 pH

The pH of harvested samples was measured using an Oakton 110 series pH meter (Vernon Hills, IL, USA).

#### 5.2.4.2 Crude Protein and Moisture

Crude protein was found using a LECO FP528 (St. Joseph, MI, USA). Protein is quantified using combustion method which measures the total amount of nitrogen in the sample (AOAC Method 990.03). In duplicate, approximately 0.25 g of sample was measured into a foil for analysis. The nitrogen content was then multiplied using a conversion factor of 6.25 to determine crude protein. Moisture calculations were then used to determine crude protein on a dry matter (DM) basis.

#### 5.2.4.3 Fiber

Crude fiber was completed by SGS laboratories (Brookings, SD, USA). Crude fiber is a measure of cellulose and insoluble lignin after acid hydrolysis followed by alkaline hydrolysis. Analysis was completed using filter bag method (AOAC 962.09) using ANKOM Technology (Macedon, NY, USA).

#### 5.2.4.4 Guar gum

Guar gum content was using a galactomannan kit obtained from Megazyme (Leinster, Ireland, UK). By quantifying the hydrolysis of guar galactomannans to D-galactose and mannooligosaccharides using the enzymes ß-mannanase and α-galactosidase the amount of galactomannan gum can be calculated using the absorbance of processed sample (McCleary 1981). Approximately 0.1 g of sample was washed with 80% (v/v) EtOH, suspended in acetate buffer and then incubated in boiling water for 5 min. The sample is then cooled to 40°C and β-mannanase and centrifuged. The supernatant is then treated with α-galactosidase and β-mannanase to complete hydrolysis. The absorbance at 340 nm is taken to calculate galactomannan gum titers.

5.2.4.5 Sugars

Glucose, sucrose, fructose, stachyose, and raffinose were measured during the fermentation using High Performance Liquid Chromatography (HPLC). Sugar standards of 99.9% purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC system (Agilent Technologies, Santa Clara, CA, USA) was equipped with a Waters size-exclusion chromatography column (SugarPak column I10 µm, 6.5 mm X 300 mm, Waters Corporation, Milford, MA, USA) and outfitted with a refractive index detector (Model G1362A) to measure sugar concentrations. Supernatant (~10 ml) samples were boiled for 10 min to inactivate potential enzymatic activity and then centrifuged at 10,000xG for 10 min. Centrifuged sample supernatants were decanted into 2 ml microcentrifuge tubes and frozen overnight. After thawing, samples were filtered through 0.2 µm filter into an HPLC vial and again frozen until analysis.. De-ionized water was used for elution and mobile phase flow rate was 0.5 ml/min$^{-1}$ at 65°C for a 25 min run time.

## 5.3 Results and Discussion

### 5.3.1 Comparing RGKM and WGKM Nutritive Values

The material balance diagram of the wash can be seen in (Figure 5.1). RGKM contained 55.6% crude protein (CP), 6.61% crude fiber (CF), and 0.77% guar gum (GG) on a DM basis while the WGKM contained 63.2% CP, 8.27% CF, and 0.55% GG on a DM basis. Centrifugation increased the concentration of components of the GKM such as CP and CF by 13.7 and 25.1% respectively while it decreased GG concentrations by 28.1%. The GKMS contained approximately 2 grams of solids from the initial 10 g slurry of which 24.5% was CP.

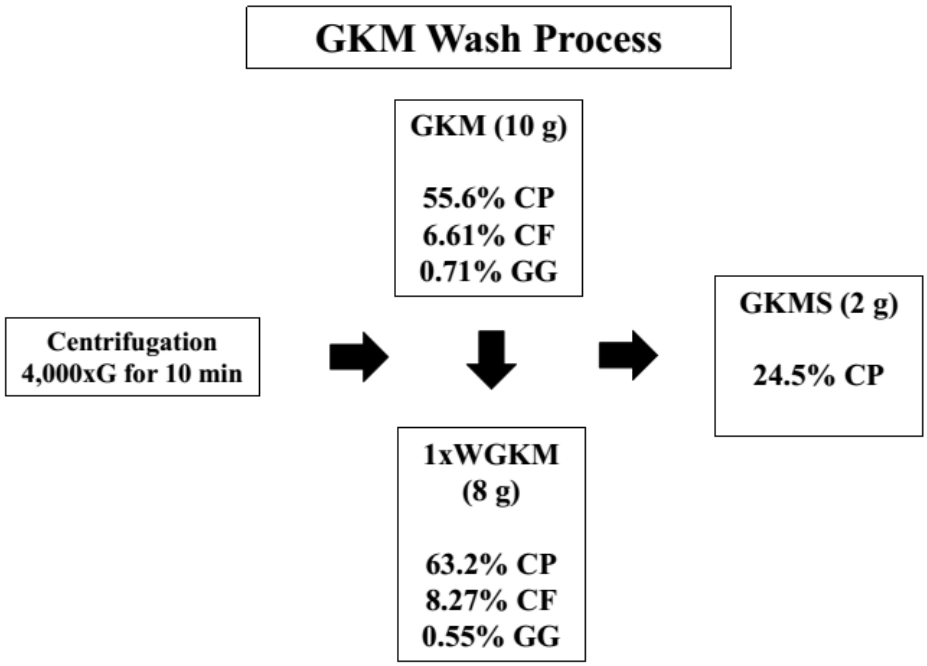


**Figure 5.1: GKM wash process and material balance**

### 5.3.2 Initial flask screening of RGKM

The process control (1xWash) for the RGKM increased fiber to 8.27% due to the wash. The five different fungal strains exhibited different metabolic tendencies on RGKM. *N. crassa* and *T. reesei* were the most effective in reducing fiber, obtaining CF values of 6.94 and 8.10% respectively. Both *N. crassa* and *T. reesei* are known to be robust producers of cellulase (Seiboth, Ivanova et al. 2011, Verma, kumar et al. 2012) which may explain their ability to effectively degrade the CF fraction of RGKM. *P. variotii, A. pullulans,* and *R. oligosporus* were not as effective in CF reduction and achieved values of 9.28, 10.5, and 11.12% respectively.

**Table 5.1: Fiber and guar gum fractions of GKM after flask trials**

| Treatment | | Crude Fiber %(m/m) | Guar Gum % (m/m) |
|---|---|---|---|
| Raw | | 6.61 | 0.77 |
| 1xWash Control | | 8.27 | 0.55 |
| 2xWash Control | | 8.61 | 0.52 |
| | Raw | 6.94 | 0.55 |
| *N. crassa* | Wash | 8.36 | 0.52 |
| | Raw | 8.10 | 0.20 |
| *T. reesei* | Wash | 10.7 | 0.17 |
| | Raw | 9.28 | 0.62 |
| *P. variotii* | Wash | 11.1 | 0.37 |
| | Raw | 10.5 | 0.03 |
| *A. pullulans* | Wash | 11.48 | 0.12 |
| | Raw | 11.12 | 0.50 |
| *R. oligosporus* | Wash | 14.23 | 0.32 |

The residual guar gum (GG) is known to decrease digestion and thus negatively impact feed efficiency in monogastric diets (Cheng and Prud'homme 2000), therefore degrading this polysaccharide could increase the value GKM. Concentrations of GG can be seen in (Table 5.1). The 1xWash control decreased the concentration of GG to 0.55%.

This could potentially be explained by the wash step, which may have solubilized a portion of the GG rendering it lost in the supernatant. Interestingly, *T. reesei* and *A. pullulans* decreased GG fractions to 0.20 and 0.03% respectively. Other studies applying fungal treatments have been unsuccessful in decreasing GG concentrations (Jannathulla, Dayal et al. 2017). The other fungal strains exhibited the inability to degrade GG.

Residual sugar concentrations after the 5 d incubation can be seen in (Figure 5.2). The 1x Wash control contained ~8.5% residual sugars which were reduced by all fungi in the range of 34.2-100%. *A. pullulans* consumed all available reducing sugars by 72 hr into the incubation (not shown). *P. variotii, N. crassa,* and *T. reesei* consumed 84.9, 84.0, and 60.4% respectively after 5 d.

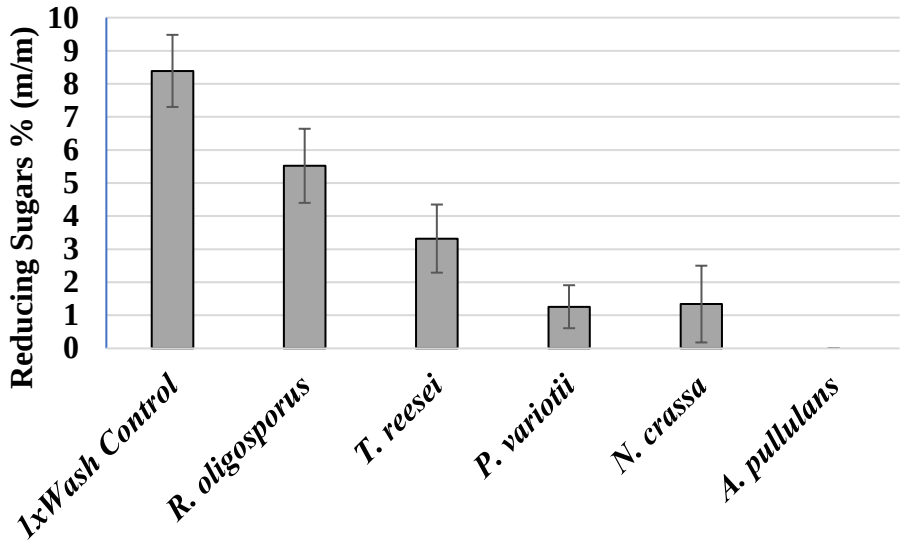


**Figure 5.2: Residual sugar concentrations following 5 d fungal incubation of RGKM**

The solids protein titer of RGKM was approxiametely 60% at 0 hr for all fungal treatments as anticipated. *T. reesei, P. variotii, R. oligosporus,* and *A. pullulans* managed slight increases in solid protein titers between 0-48 hr, however, all experienced a

decrease in solids protein between 48-72 hr during the fermentation (Figure 5.3). The *N. crassa* treatment caused a more pronounced protein titer increase in the liquid fraction, achieving a titer of ~65% in 24 hr. Increases in protein solubility are disadvantageous to the overall process as the protien lost in the superantant are more expensive to recover. Thus, strains that retain protein in the solids fraction are beneficial. Protein is commonly solubilized during fermentations due to microbial protease activity, which cleaves proteins into smaller more soluble peptides (Chavan, Chavan et al. 1988), or increases in the pH of the slurry. Guar proteins are known to have an isoelectric point of ~4.7, thus increases in pH during the fermentation could change the conformation of the protein rendering it more soluble (Tasneem and Subramian 1986).

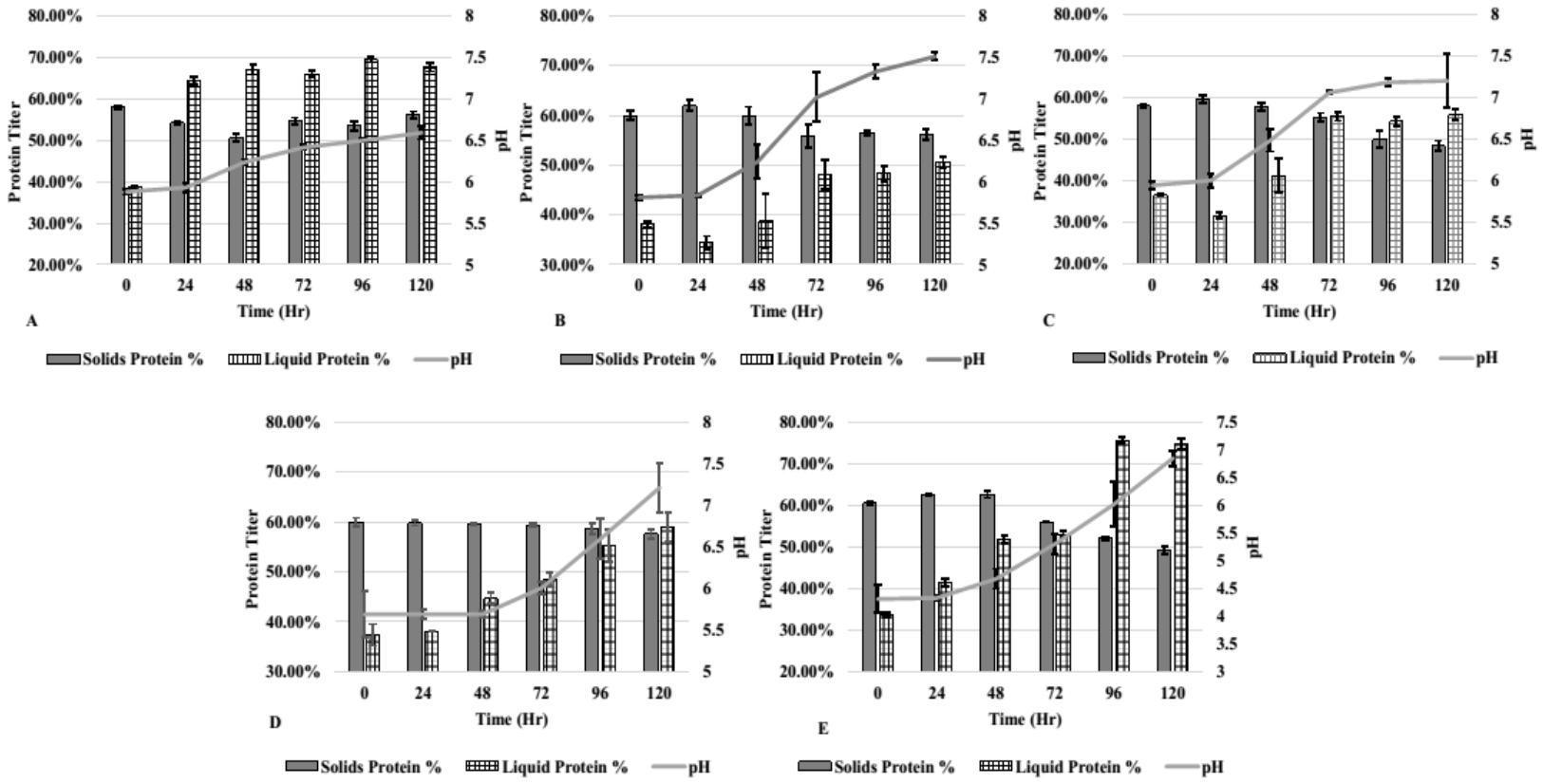


**Figure 5.3: Relationship between CP and pH in RGKM solids and liquids fractions when treated by (A) *N. crassa* (B) *T. reesei* (C) *R. oligosporus* (D) *P. variotii* (E) *A. pullulans***

### 5.3.3 Initial Flask Screening of WGKM

As the WGKM was originally subjected to a wash pre-incubation, CF titers were increased as soluble GKM constituents were expelled in the supernatant. The 2xWash control was compared to the WGKM fungal treatments as they both underwent a total of two centrifugal washes (Table 5.1). The 2xWash process control had a CF content of 8.67%. *N. crassa* was the only fungal treatment able to degrade fiber beyond that of the 2xWash control, attaining a concentration of 8.36%. All other fungal treatments resulted in CF titers of 10.7-14.3%.

The 2xWash control decreased the residual GG levels only slightly to 0.52%. This result is consistent with the first wash as some of the GG may be lost in the supernatant fraction. The residual GG concentrations of the fungal treated WGKM were similar to that of the fungal treated RGKM (Table 5.1). *N. crassa* and *T. reesei* had almost no change in GG levels, however, *P. variotii* and *R. oligosporus* achieved a greater reduction in concentrations (0.37% and 0.32%). The removal of soluble sugars due to the wash may have influenced *P. variotii* and *R. oligosporus* to consume the GG. On the other hand, *A. pullulans* was less effective in reducing GG levels, attaining a concentration of 0.12%.

Residual sugar concentrations after 24 h incubation of the WGKM can be seen in (Figure 5.4). The 2xWash control contained ~3.0% (m/m) residual sugars that were consumed entirely by all fungal treatments in 72 h. For this reason, Figure 5.4 illustrates sugar concentrations after 24 h into the incubation. *T. reesei* and *A. pullulans* consumed 79.6 and 58.5% of the sugars respectively. The other fungi decreased the residual sugars in the range of 24.1-31.4% respectively.

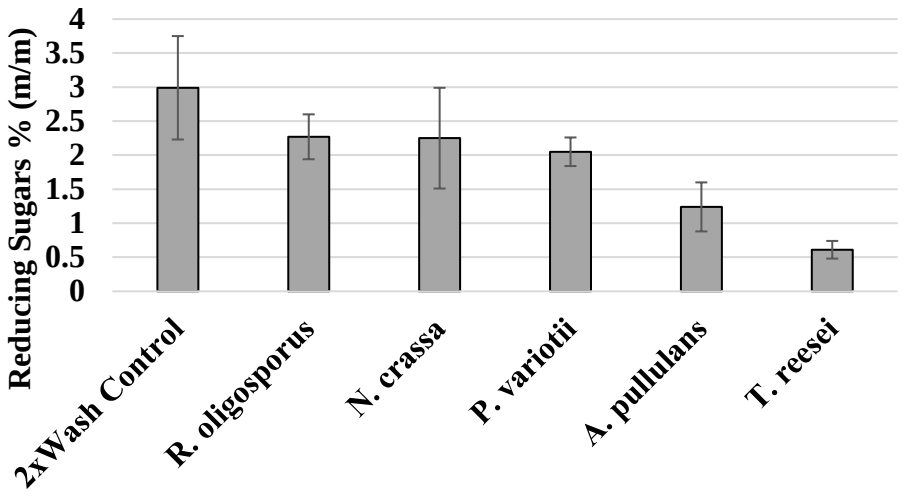


**Figure 5.4: Residual sugar concentrations after 24 h incubation of WGKM**

The WGKM had a 0 h protein titer of ~66% on a DM basis. Similar to the RGKM, protein titers of the solids fraction increased slightly early in the incubation then protein titers of the liquid fraction increased thereafter (Figure 5.5). However, changes in protein titers as well as pH were amplified which may potentially be explained by the absence of fermentable sugars lost in the wash. With no easily digestible carbohydrates,the fungi may haven taken to degrading available guar proteins to fuel their growth (Ugbogu and Ugbogu 2016). All fungi experienced a drastic increase in liquid protein after 24 h into the incubation. Interestingly, *A. pullulans* liquid protein titers reach ~90% at 72 h into the incubation. Despite liquid protein titers reaching over 50% 48 h into the incubation, *T. reesei* and *P. variotii* treated solids fractions did not experience a notable decline in solids protein titers. The same was not seen in *N. crassa* and *R. oligosporus* however, as their solids fraction protein titers decreased below 60% after 72 h incubation.

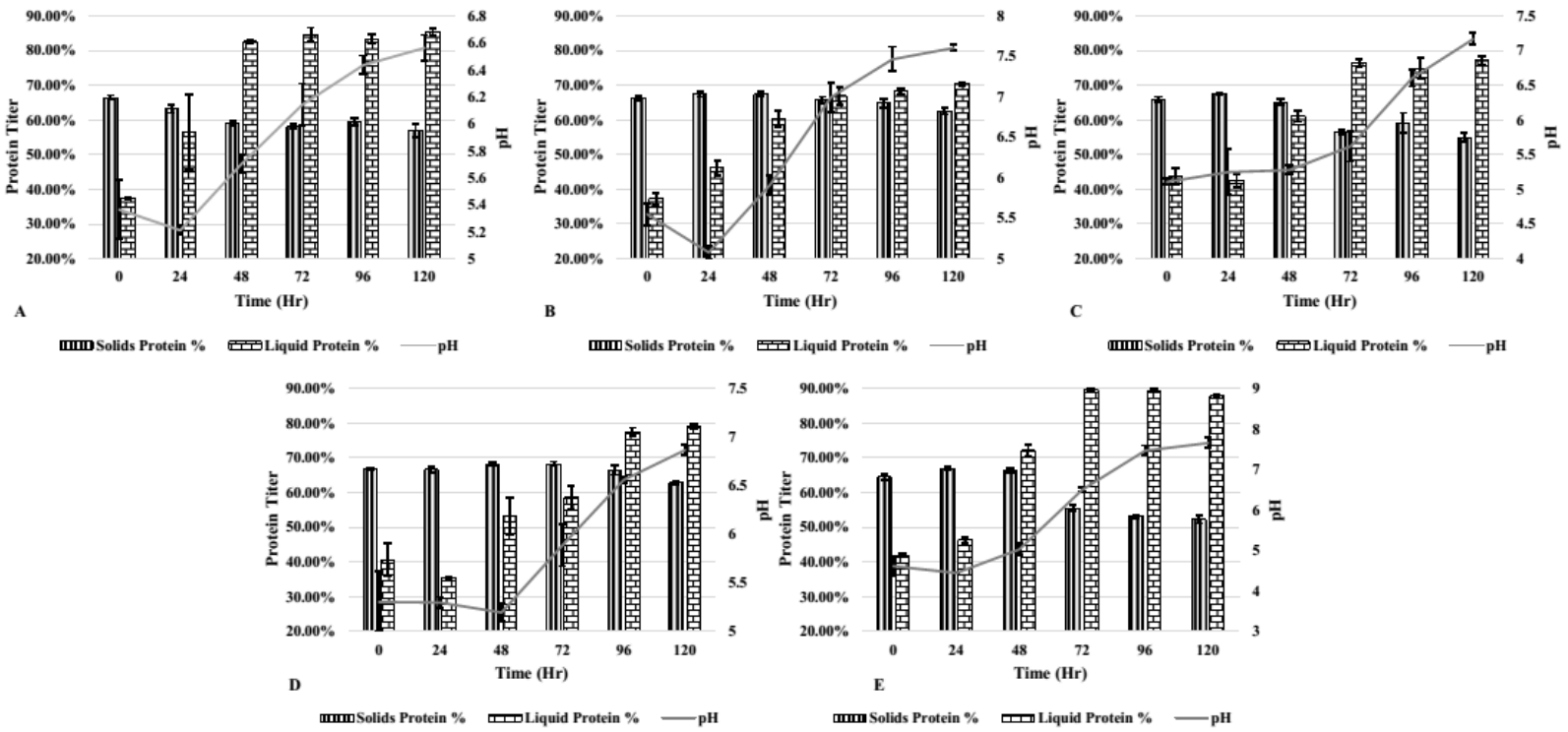


**Figure 5.5: WGKM solids and liquids fractions protein titers in relation to pH when treated by (A) *N. crassa* (B) *T. reesei* (C) *R. oligosporus* (D) *P. variotii* (E) *A. pullulans***

### 5.3.4 GKMS Initial Fungal Screening

The same five fungal strains examined for their ability to consume carbohydrates and concurrently produce protein-rich biomass on RGKM and WGKM were tested on the GKMS fraction. The solids fraction material balances can be seen in (Table 5.2). The uninoculated control was only able to recapture 6.2 g/L of biomass with a 33.6% protein titer. All fungal treatments increased the amount of biomass captured via centrifugation in the range of 38.7-70.9% compared to the uninoculated control. *T. reesei* produced the most biomass (10.6 g/L), however, the protein titer of said biomass was among the lowest at 33.7%. *A. pullulans* produced 9.4 g/L of biomass and also attained the highest protein titer of 43.2% resulting in a 90% increase in total protein yield compared to the uninoculated control.

**Table 5.2: Material balance of solids fraction of GKMS**

| Treatment | Biomass Yield (g/L) | Biomass Protein Titer (%) | Biomass protein (g/L) |
|---|---|---|---|
| Uninoculated Control | 6.2±0.1 | 33.6±0.28 | 2.1±0.1 |
| *N. crassa* | 8.6±0.4 | 34.5±0.57 | 3.7±0.2 |
| *T. reesei* | 10.6±0.1 | 33.7±0.60 | 3.1±0.3 |
| *A. pullulans* | 9.4±0.1 | 43.2±0.99 | 4.0±0.2 |
| *R. oligosporus* | 9.3±0.6 | 37.5±1.4 | 3.6±0.4 |
| *P. variotii* | 9.5±1.3 | 34.0±0.33 | 3.2±0.2 |

### 5.3.5 Comparing the RGKM and WGKM +GKMS Processes

The optimal treatment of RGKM and WGKM should degrade GG and CF fractions of the GKM as both inhibit the digestion of feed in monogastrics (Choct and

Kocher 2000, Saeed, Hassan et al. 2017) while minimizing the amount of proteinaceous loss in the supernatant. The fermentation of RGKM using *N. crassa* was able to reduce CF to a greater extent than the other fungal strains, however, GG was not reduced as well as protein was rapidly lost in the supernatant. On the other hand, *T. reesei* was able to achieve solids protein titers of ~62% while reducing CF and GG compared to the 1xWash control. Likewise, the fermentation of WGKM using *N. crassa* was also effective in reducing CF (8.36%), however, even greater extents of proteinaceous loss occurred in the supernatant resulting in liquid protein titers of ~80% in 72 h, thus, fungi with less impact on protein degradation such as *T. reesei* should be pursued for further trials despite being less efficient in degrading fiber. *T. reesei* reduced GG to levels of 0.17% while reaching a protein titer of almost ~70% after a 24 h incubation. The optimal strain for fermentation of GKMS would produce a protein-rich biomass with a high-titer as well as yield. *A. pullulans* was the best performing strain as it produced 9.4 g/L of biomass with a titer of 43.2%. Thus, final benchtop reactor trials were conducted using *T. reesei* to ferment WGKM for a shorter incubation time (36 hr) to lessen the proteinaceous loss, while *A. pullulans* was used to ferment the GKMS to produce a protein-rich biomass. The fermented GKMS biomass will be added to the fermented WGKM to evaluate the overall effectiveness of the process.

### 5.3.6 Benchtop Reactor Trials Using Optimal WGKM + GKMS Process

Benchtop reactor results obtained were similar to previous flask trials for both WGKM and GKMS (Figure 5.6). *T. reesei* fermented WGKM had a solids protein titer of ~66% at 0 h and peaked at ~67% at 48 h before beginning to decrease. Liquid protein

titers began at 42.3% and increased thereafter reaching over 60% by 48 h. Therefore, it was determined that 36 h was the optimal time for *T. reesei* fermentation of WGKM.

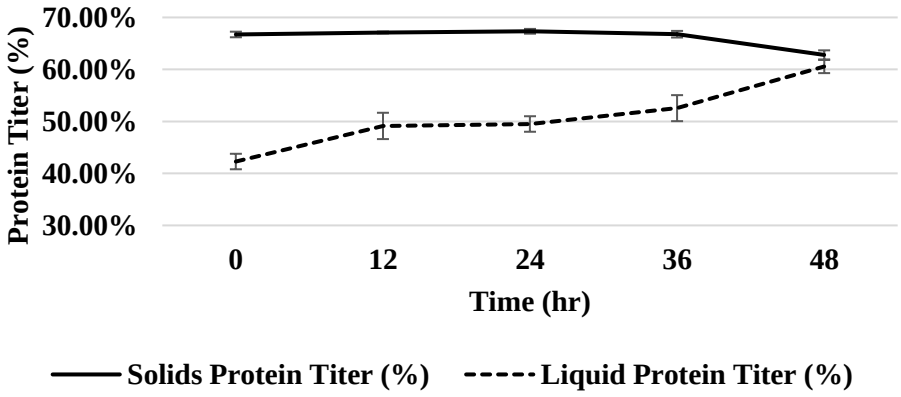


**Figure 5.6: Protein titer of solid and liquid fractions of WGKM in 5 L benchtop reactor**

Fermentation of GKMS using *A. pullulans* yielded 4.4 g/L at 0 h and quickly rose to 11.4 g/L at 12 h. The biomass yield peaked at 36 h reaching 12.8 g/L containing a protein titer of 40.6%. The pH value of the slurry steadily rose from 4.15 to 5.10 from 0 to 36 h, however, it quickly rose to 6.34 at 48 h.

The fungal treated 36 h WGKM and GKMS yields can be seen in (Table 5.3). The *T. reesei* treated WGKM yielded 65.8 g/L of fermented biomass containing a protein titer of 66.8%. The *A. pullulans* treated GKMS yielded 12.8 g/L of protein-rich cell mass containing a titer of 40.6%. To increase the efficiency of the overall process, both fermented fractions were combined to measure the maximum yield and titers. After combining, a biomass yield of 78.6 g/L was obtained resulting in a protein titer of 61.5%.

**Table 5.3: Biomass and protein yield of fermented WGKM +GKMS Process**

| Treatment | Fraction | Biomass Yield (g/L) | Protein Titer (%) | Protein Yield (g/L) |
|---|---|---|---|---|
| *T. reesei* | WGKM | 65.8±1.7 | 66.8±0.6% | 43.9±0.76 |
| *A. pullulans* | GKMS | 12.8±2.0 | 40.6±0.6% | 5.10±0.75 |
| WGKM+GKMS | | 78.6 | 61.5% | 48.3 |

## 5.4 Conclusions

A two-step submerged fermentation process proved to be more effective concentrating protein and increasing overall yield than a single submerged process. Using the filamentous fungi *T. reesei* to ferment a washed guar korma meal solids fraction, protein titers of over 66% were achieved while the antinutritional factor guar gum was reduced to 0.3% (m/m). A separate fermentation of the washed guar korma meal solubles produced 12.8 g/L of protein-rich biomass (40.6%).

## Chapter VI- Summary and Conclusions

With the world population expected to increase to 9 billion people by the year 2050 (Alexandratos and Bruinsma 2012), technologies to increase the nutritional value of plant feedstuff by-products containing lignocellulosic material are in need. Lignocellulose is a complex consisting of cellulose, hemicellulose, and lignin that is undigestible by monogastric organisms (Sindhu, Binod et al. 2016). Bioprocesses including microbial fermentation are an attractive lignocellulose bioconversion process as they possess the capability to produce robust amounts of enzyme thereby degrading undesirable fractions and rendering the overall feedstuff more digestible. At the same time, the microbes produce a protein-rich cell mass applicable in monogastric diets. Soy processing wastewater, sorghum hominy, and guar korma meal are all very different by-products; however, each can be improved through submerged microbial fermentations.

In chapter III, eight fungal strains were assessed for their ability to decrease the organic matter (55,000 mg/L) present in a soybean processing supernatant and concurrently produce protein-rich cell mass. *T. reesei* and *N. crassa* incubations conducted in benchtop reactors produced 55.5 and 62.0 g/L of biomass with potential applications as a feed additive while simultaneously reducing chemical oxygen demand levels by 10.53 and 23.04% respectively.

In chapter IV, eight fungal strains were examined for their ability to degrade sorghum hominy carbohydrates and produce microbial biomass protein. *T. reesei* and *A. pullulans* were the best performing fungi and yielded 65.1 and 73.6 g/L respectively in 5 L benchtop reactors. *T. reesei* fermentation in 70 and 150 L bioreactors increased all amino acids increased in the range of 33.3-152.3%.

In chapter V, guar korma meal was subjected to a centrifugal wash to produce a concentrated protein stream (WGKM) and a washed solubles stream (GKMS). Five fungal strains were incubated separately on each stream to increase the efficiency of the overall process. *T. reesei* was the most effective fungal treatment of WGKM as it degraded guar gum levels to 0.17% while yielding 65.8 g/L of protein-rich (66.8%) biomass. *A. pullulans* was found to be the most adept at consuming GKMS, producing 12.8 g/L of biomass. Washing the guar korma meal before fermentation was found to result in increased yields.

Future studies on the soybean processing supernatant should investigate the use of microfiltration to harvest soluble proteins not capturable by centrifugation or microbial metabolism. For sorghum hominy, as starch has some value in itself in monogastric diets whereas fiber does not, starch should be separated pre-fungal incubation to isolate protein and non-starch polysaccharides. Then enzymatic and microbial treatments should be applied to investigate effects on this new isolated fraction. The enzymatic saccharification of guar korma meal concentrate using cellulase enzymes should be investigated to attempt to decrease fibers pre-fungal incubation. In each of the three by-product fermentations, processes to decrease the amount of proteinaceous loss in the supernatant should be examined. Lastly, for all produced fungal biomass, extensive fish feeding trials should take place to investigate palatability, digestibility, and feed efficiency of the bioprocessed ingredients.

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