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Improvement of Dried Distillers' Grains with Solubles Utilizing Pretreatments and Fungal Fermentation

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IMPROVEMENT OF DRIED DISTILLERS' GRAINS WITH SOLUBLES UTILIZING PRETREATMENTS AND FUNGAL FERMENTATION

BY

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

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I would like to dedicate this thesis to my family for their support and encouragement. Mom- you never let me anything quit when you knew it was for my best interest. To my husband Kevin – for supporting me in all the late nights and long weekends spent in the lab. And finally, to my Grandmothers Dorothy and Marguerite - for being role models and pushing me to follow my dreams.

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ABBREVIATIONS

ADF = Acid Detergent Fiber

ADL = Acid Detergent Lignin

AFEX = Ammonia-fiber expansion

ANF = Anti-nutritional factor

CHO = Carbohydrate

db = Dry basis

DDGS = Dried distillers' grains with solubles

DHA = Docoshexaenoic acid

EPA = Eicosapentoaenoic acid

GRAS = Generally recognized as safe

ISR = Intra species recycling

NDF = Neutral Detergent Fiber

NSP = Non-starch polysaccharides

NSPase = Non-starch polysaccharide enzymes

OTA = Ochratoxin A

PUFA = Polyunsaturated fatty acid

SCP = Single cell protein

WDDGS-PC = Wet dried distillers' grains with solubles -protein concentrate

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ABSTRACT

IMPROVEMENT OF DRIED DISTILLERS' GRAINS WITH SOLUBLES UTILIZING PRETREATMENTS AND FUNGAL FERMENTATION

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Limited inclusion of distillers' grains in animal feed is primarily due to low protein and high fiber content. Other elements, such as phytate phosphorus levels in DDGS are still an issue for monogastric animals such as swine, poultry, and fish. Furthermore, unabsorbed phytic acid in manure poses a high risk for environmental pollution, because bacteria can hydrolyze phytic acid into free phosphorus that can result in algal blooms and eutrophication of surface or ground water. The objective of this thesis was to improve the nutritional value of corn dried distillers' grains with solubles (DDGS). This was done utilizing submerged fungal fermentation, enzymatic hydrolysis, and a combination of extrusion pretreatment and fungal fermentation/enzymatic hydrolysis. All treatments served as a means of degrading fiber and phytic acid while improving protein content for animal feed applications.

Initially, fungal fermentations at 5, 10, and 20% solid loading rates (SLR, dry weight basis) were used to assess four fungal strains (*T. reesei*, *N. crassa*, *R. oligosporus*, and *A. pullulans*). Flask trials were incubated for 120 h at 30°C and 150 rpm. Un-inoculated control flasks were also included. Flasks were sampled at 24 h intervals, with solids recovered by centrifugation. A solubilization effect was observed at the 0 h sampling for all SLRs, as DDGS solubles fractionated into the centrate stream, while insoluble fractions such as protein and fiber were concentrated in the pellet. An increase

in protein content ~5% and crude fiber ~1.5% is seen at the 5% SLR in the uninoculated control; at higher SLR the increase in protein drops to ~3% and crude fiber increases about 0.5-1%. After fermentation *N. crassa* (NRRL-2332) at a SLR of 20% resulted in the greatest reduction in fiber (-0.5% equal to original grain), while increasing protein (+5%) and lowering phytic acid levels (~0.3g/100g). An increase in fiber was seen in all other fungal and SLR combinations. Higher SLR resulted in better protein content for each of the fungi.

Enzymatic hydrolysis under submerged conditions (10% SLR) was also tested to determine if fibrous components of the DDGS could be degraded into simple sugars. Trials were conducted in 250 ml flasks incubated for 24 h at 55°C and 150 rpm. Four commercially available enzymes (cellulase, xylanase, phytase, pectinase) at four dosages were tested at the recommended pH levels for individual testing. Trials were also conducted to assess synergistic effects of various enzyme combinations. The greatest reduction in crude fiber for the individual enzyme was seen in the cellulase 1 and 2 mg/g dosages while the greatest reduction in NDF and ADF seen in all four dosages of pectinase. When all four enzymes were combined the greatest reduction in all fibers (crude, NDF, and ADF) was achieved. The combination of 1mg/g of xylanase, phytase, and pectinase had the most effective releases of glucose and total sugars of all individual enzymes and combinations. Overall the most effective treatments were 1m/g of cellulase, xylanase, phytase, and pectinase and 1m/g of xylanase, phytase, and pectinase.

To increase the fiber hydrolysis, the use of extrusion pretreatment prior to fungal fermentation was examined. Extrusion was conducted using DDGS at a 12% moisture content, with a barrel compression ratio of 3:1 and length to diameter of 20:1 at 90-100°C

Extruded DDGS was then blended with water to a 15% SLR, autoclaved, and inoculated with *T. reesei*, *N. crassa*, *R. oligosporus*, or *A. pullulans*. Extrusion reduced crude fiber content and increased protein concentration. However, after the addition of water to create the submerged fermentation the fibers were increased. After 120h of fermentation crude fiber was significantly increased in *N. crassa* and *A. pullulans* trials. Protein was concentrated during fermentation by removal of the soluble fraction, but *N. crassa* was able to increase the protein ~10% from the original grain and ~5% from the un-inoculated control at 48 h. *A. pullulans* was also able to increase the protein significantly while *T. ressei* and *R. oligosporus* were not significantly different from the control.

Lastly, the use of fungal fermentation was conducted on DDGS that was pretreated via extrusion (12% moisture, barrel temperature 90-100°C, compression ratio 3:1, barrel length to diameter 20:1) and hydrolyzed via the combination of cellulase, xylanase, pectinase, and phytase, each at dosage of 1 mg protein/dry gram of DDGS and fermented using *N. crassa* using sequential (4h of hydrolysis then *N. crassa*) or simultaneous addition. Irrespective of extrusion the use of simultaneous addition of enzyme and fungi had the greatest reduction in fiber.

Chapter 1 - Literature Review

1.1 Fishmeal: gold standard for high protein, highly digestible feedstuffs for a broad range of animals and fish

1.1.1 Fishmeal

Of all the fish caught in the world, approximately 30% are turned into fishmeal and fish oil to be used in the animal feed industry (Barroso et al., 2014). Originally, fishmeal was the by-product of the extraction of fish oil which was extracted for production of margarine (A. Jackson & Shepherd, 2010; Yasufuku, Okafuji, Hasegawa, & Haga, 1988). A variety of portions of the fish are included in the fishmeal such as: viscera, heads, frames, skins, trimmings, blood, and belly flaps (Stevens, Newton, Tlusty, & Little, 2018). Initially fishmeal was primarily used as feed for poultry and swine due to its rich amino acid content, and high level of trace minerals (Stickney & McVey, 2002). Over the past 20 years, an increasing amount of fishmeal has been used in aquaculture diets due to the global growth of aquaculture.

1.1.2 Types and sources

Fishmeal can be derived from a multitude of sources such as whole pelagic fish, by-products of canning or surumi production (Hardy & Tacon, 2002). In 2011, almost 23 Mt of fish were destined to undirect human consumption (anchovy, herring, mackerel, and sardines) (Bene et al., 2015). Of the 23 Mt, 17 Mt were utilized for fishmeal and fish oil that were incorporated in poultry, aquaculture, and other livestock feeds (Bene et al., 2015). Currently, the world's largest producers of fishmeal are Peru and Chile supplying *Peruvian anchoveta* (Cashion, Manach, Zeller, & Pauly, 2017). The world's top

consumer of fishmeal is China, which also ranks number one in aquaculture production (FAO, 2018). The United States ranks 15th in total production of farmed fish and this is comprised of mainly finfish (channel catfish and rainbow trout) and shellfish (Moffitt & Cajas-Cano, 2014).

1.1.3 Composition

The fishmeal is an attractive source of animal nutrition due to its oil content, high protein content, properly balanced essential amino acid profile and lack of antinutritional factors (ANF). Table 1.1 lists the proximate composition of most commonly used commercial fishmeal (Cruz, 1997). Based upon the various parts of fish that are processed into fishmeal, protein concentration and quality can vary. Connective tissues and bone are commonly lower in protein. Because of this, fishmeal derived from by-products are about 10% lower in protein than fishmeal derived from whole fish such as anchovy (Cruz, 1997). Along with the high protein content, fishmeal is highly digestible by most animals with no negative side effects (Daniel, 2018; A. Jackson & Shepherd, 2010). Fishmeal therefore has become the major protein source used in aquaculture diets (Daniel, 2018). Fishmeal accounts for approximately 62% of the fish feed, with 20% wheat flour, 20% fish oil, 3.4% milk whey 2.1% vitamins and minerals, and 0.5% choline chloride (Delgado & Reyes-Jaquez, 2018). However, depending upon the species protein requirements can vary. Omnivores and herbivores protein content requirements are lower, whereas carnivorous species require higher protein content (Craig, Helfrich, Kuhn, & Schwarz, 2017). Example diets feed to rainbow trout are shown in Table 1.2.

Table 1.1: Proximate composition of some of the commercial fishmeal products taken from Cruz (1997)

Fishmeal Types	Dry matter (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	NFE (%)	Ca (%)	P (%)
Alaskan Pollack	91.53	71.94	9.58	0.10	5.32	13.10	-	-
Local mixed species	91.12	52.89	5.78	3.11	21.90	16.32	-	-
Peruvian	92.83	67.39	6.97	0.89	18.84	5.65	-	-
Tuna	93.24	52.04	10.37	1.56	24.15	7.81	3.30	2.44
White	93.63	68.68	6.78	1.46	20.16	3.73	4.40	3.3

Table 1.2:Proximate composition of reference diets feed to rainbow trout

	Kasiga and Brown (2019)	Voorhees, Barnes, Chipps, and Brown (2019)
Crude Protein (%)	44.3	43.18
Lipid (%)	16.9	15.91
Ash (%)	9.7	2.42
NFE (%)	25.9	20.48
Gross Energy (kJ/g)	22.1	16.5
Protein: Energy (g/MJ)	19.1	26.2

1.1.4 Feed applications

During the early years in the use of fishmeal as a feed additive, the poultry and pork industries consumed almost all this by-product (Asche, Oglend, & Tveteras, 2013). For instance, in 1988, 80% of the fishmeal produced globally, was fed to poultry and pork, while aquaculture only consumed 10% (Olsen & Hasan, 2012). From 1985 to 2006 the poultry and pork industry saw an annual growth of 4.9% and 2.8% respectively. However, during the same period, aquaculture industry grew rapidly with annual growth rate of 10.3% (Tveterås & Tveterås, 2010). Because of this substantial increase in growth, the aquaculture industry has become the leading consumer of fishmeal (Asche et

al., 2013). As of 2010, aquaculture consumed 73% of the fishmeal, followed by pork and poultry with 20% and 5%, respectively, and 2% utilized by others (Bene et al., 2015).

1.1.5 Supply and demand

Prior to 1980's, global wild caught fishmeal production was under 5 Mt per year, but after 1985 production increased to ~6 or 7 Mt per year, except for El Niño years of 1987 and 1998 which had a significant decrease in production (Deutsch et al., 2007). Corresponding with increased fishmeal production, there was a significant increase in fishmeal use by the aquaculture industry, which ranged from 10 to 45% from 1988 to 2002 (Barroso et al., 2014). By 2008, 57% of fishmeal was being used in aquaculture production (Tveterås & Tveterås, 2010). Since then demand for fishmeal has continued to increase and hence the price of fishmeal has also increased substantially. Figure 1.1 displays the growth of the aquaculture industry vs the wild capture industry. The sharp increase in the amount of aquaculture production compared to the wild caught industry started after 1980.

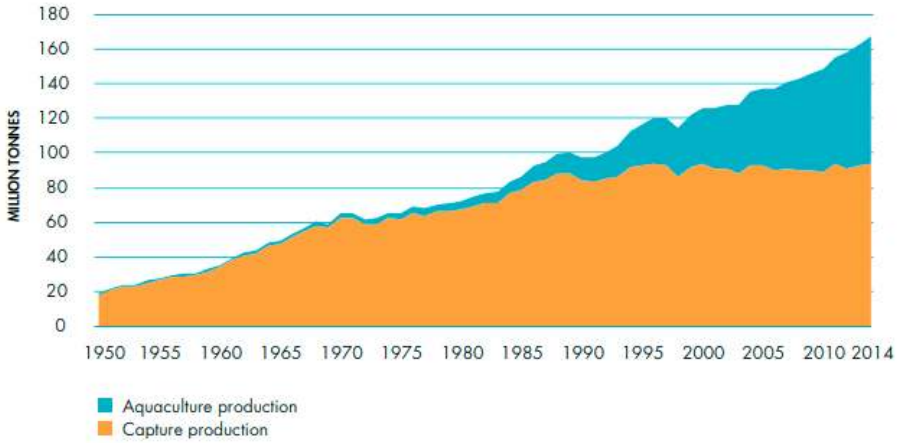

Figure 1.1: Growth of the aquaculture industry versus capture fishing

taken from Ababouch (2016)

1.2 Alternatives to fishmeal

1.2.1 Need for alternatives

There are several reasons why alternatives to fishmeal are needed. Although the major consumer of fishmeal was not always aquaculture, the proportion of fishmeal in aquaculture feeds is much higher than the average 2-3% content in poultry and pork diets (Naylor et al., 2000). Due to the increasing demand for fish as a source of animal protein, the global aquaculture industry is expanding rapidly. In 2010 fish demand was twice as large as poultry and three times bigger than cattle (Bene et al., 2015). This rapid growth has resulted in progressively higher use of fishmeal causing a rise in prices

Another issue that has arisen is the avoidance of intra species recycling (ISR). ISR is when a species is feed back into its species. This practice is avoided to prevent the spread of disease or environmental contaminate (Tacon & Metian, 2008). Although it is not regulated for aquaculture, it is regulated in livestock to prevent the spread of transmissible diseases such as mad cow, it is highly considered for the health of the fish (Turchini, Torstensen, & Ng, 2009). A third reason that needs to be considered is the lack of wild caught fish. Currently, species harvested for fishmeal production are harvest at or above sustainable levels (A. Oliva-Teles, Enes, & Peres, 2015).

1.2.2 Cost

Feed cost in aquaculture accounts for 30 - 60% of the production cost (Shipton & Hasan, 2013). This is due to the rise in fish meal prices over the past several decades (Naylor et al., 2000). Although the price continued to increase, the inclusion levels were left unaffected until 2005. After 2006, the price of fishmeal rose from $400-900 per

metric ton, to over $1500 per metric ton (Hardy, 2010). This price surge was due to the El Niño of the 2006 season. Currently the market for fishmeal is continuing to grow. An increase in seafood consumption is requiring more fish be produced which in return requires more fishmeal for feed (Abhishek, 2019).

1.2.2.1 Lack of sustainability

Eighty percent of all fish stocks are characterized as fully exploited or overexploited (FAO, 2009). This has been caused by the rapid and long-term growth in aquaculture production, which has caused increasing demand for fishmeal and corresponding increase in fishmeal prices.

unbalanced ratio of fish meal in: fish meal out. On average, 1.9 kg of fishmeal is fed for every kilogram of fishmeal produced. There is a wide variation of what ratio is needed for each of the species. Catfish, milkfish, and common carp require less input than produced versus the carnivorous species that may require 2.5 to 5 kg of feed for the 1 kg produced (Naylor et al., 2000).

The aquaculture industry saw a growth of over 115-fold production in tons from 1950 to 2006 which equates to an 8.5% increase per year (Tacon & Metian, 2008). For the industry to be sustainable the feed input also needs to grow at a similar rate (Tacon & Metian, 2008).

1.2.3 Algae

In ancient times, the Aztecs of Mexico used various algae species as sources of food due to their availability (Suman, Nupur, Anuradha, & Pradeep, 2015). For years

microalgae have been utilized as a feed in aquaculture (Patil, Källqvist, Olsen, Vogt, & Gislerød, 2007). Current estimates are that 30% of the worlds algal production is sold for feeds (E. W. Becker, 2007). Today, microalgae are recognized as a safe feed ingredient with a high nutrient value, and are included in diets of larval crustaceans, rotifers, and brine shrimp (Patil et al., 2007; Perez-Velazquez, Gatlin, González-Félix, & García-Ortega, 2018).

1.2.3.1 Advantages

One advantage of algal biomass as a fishmeal replacement is it's a low cost, since it is a by-product of the biofuel industry which allows for a lower cost (Mussgnug, Klassen, Schlüter, & Kruse, 2010). Microalgae could also be produced in arid lands, allowing for year- round production (Kovač, Simeunović, Babić, Mišan, & Milovanović, 2013). However, evaporation can cause an issue in open air ponds (Wen & Johnson, 2009). Microalgae are known to produce several beneficial compound that can increase the overall nutrition of the feed (Yaakob, Ali, Zainal, Mohamad, & Takriff, 2014).

1.2.3.1.1 Protein

Algae proteins are highly comparable to high protein meals of oilseed and cereal grains (E. W. Becker, 2007; Nasseri, Rasoul-Amini, Morowvat, & Ghasemi, 2011). The high protein content is connected to the ability of the algae to fix nitrogen under certain circumstances as shown in Table 1.3 (E. W. Becker, 2007). Algae contain lower levels of nucleic acids than other single cell protein sources (Ravindra, 2000), thus limiting the production of uric acids occurs during nucleic acid metabolism.

Table 1.3: Composition of different algae (% db)

Alga	Protein	Carbohydrate	Lipids
Anabaena cylindrica	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Arthrospira maxima	60-71	13-16	6-7
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridum cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp	6-20	33-64	11-21
Spirulina platensis	46-63	8-14	4-9
Synechococcus sp.	63	15	11

Taken from E. W. Becker, 2007

1.2.3.1.2 Vitamins and minerals

Some genera of algae offer high concentrations of vitamins and minerals. The genus *Spirulina* contains 10 time more β-carotene than most carrots and higher levels of vitamin B12 when compared to all fresh plants sources (Mohammed & Mohd, 2011), However, vitamin B12 can be unstable under certain drying conditions (W. Becker, 2004). Other vitamins that are present in microalgae include vitamins K and isomers of vitamin E (tocopherols) (W. Becker, 2004). Additional microalgae species should be assess for their potential to supply vitamins and minerals for feed purposes (Shields & Lupatsch, 2012).

1.2.3.1.3 Omega-3 fatty acid composition

In the natural food chain, fish consume algae that containe fatty acid chains (Kovač et al., 2013). The natural ability of the microalgae to produce omega-3 fatty acids such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)

directly aids in the health of the fish (Shields & Lupatsch, 2012). Fish fed higher levels of *Scenedesmus* microalgae were higher in the long chain omega-3 fatty acids (EPA and DHA) as compared to the control feed (Gong et al., 2019). Some species under nitrogen deprivation showed an increase in lipid content to compensate (W. Becker, 2004). However the use of algal lipids cannot compete with conventional sources of fish oils (W. Becker, 2004).

1.2.3.2 Disadvantages/limitations

When feeding microalgae, diets with live microalgae tend to result in higher fish growth rates and lower mortalities when compared to fish fed with non-living microalgae (Ponis, Robert, & Parisi, 2003). Due to the high heat required to produce some types of pelleted feeds, it may be necessary to incorporate the microalgae after pelleting. Another concern with microalgae is the high production cost to grow microalgae (Olsen & Hasan, 2012). Yarnold, Karan, Oey, and Hankamer (2019) reported the price of *Spirulina* or *Chlorella* microalgae meals were 10 to 15 times more expensive than fishmeal between September and December of 2018.

1.2.3.2.1 Amino acid balance

The essential amino acids tryptophan, methionine, and histidine were found to be low 16 microalgae species evaluated (MR Brown, 1991). These microalgae were also low in the non-essential amino acids cystine and ornithine (MR Brown, 1991). It is important to note that the sulfur containing amino acids are among the lowest concentrations in microalgae (W. Becker, 2004).

1.2.3.2.2 Anti-nutritional factors

Currently, no known ANF's can be found within the microalgae biomass. However, some studies have shown that a low level of intake and inflammation of the gastrointestinal tract could be the result of an unknown ANF (Perez-Velazquez et al., 2018).

1.2.3.2.3 Low digestibility

Due to the cellulosic structure of the microalgae, digestibility can be low unless the cell is ruptured to allow for access to the nutrients (Ravindra, 2000). Disruption may be caused by sonic vibration or enzymatic digestion (E. W. Becker, 2007). A decrease in digestibility of the dry matter, lipid, and energy was observed when inclusion of *Scenedesmus sp.* in Atlantic salmon (*Salmo salar)* diets was increased (Gong et al., 2019). However, authors reported no difference in protein digestibility at lower inclusion levels. Additionally it was reported that overall digestibility is dependent upon the microalgae species (Gong et al., 2019).

1.2.3.3 Single celled protein

In 1968, Mateles and Tannebaum coined the phrase "single-cell protein" or SCP to refer to cells of microorganisms grown in large quantities and then dried for the use as a protein source for humans or animals. Microorganisms that can be used as SCP can be placed into four categories: bacteria, yeast, fungi and algae (Kuhad, Singh, Tripathi,

Saxena, & Eriksson, 1997; Richard Mateles & SE Tannenbaum, 1968; Suman et al., 2015).

1.2.3.3.1 Advantages

With the relative ease of growth and fast reproduction of microbes, SCP started to emerge as a top contender for non-conventional protein sources (Olsen & Hasan, 2012). The use of low-cost substrates or industrial waste products as media for production SCP has improved its cost competiveness (Suman et al., 2015).

Yeast are commonly thought of as the most important microorganism for SCP production (Tacon, 1995). Yeast strains used for SCP are believed to have an immunostimulatory property according to Anderson, Siwicki, and Rumsey (1995). Marine yeast have stood out for aquaculture feed because of their high content of essential amino acids (Nasseri et al., 2011). An additional benefit of some types of SCP is the production hydrolytic enzymes that improve the digestibility of other feed components (Ravindra, 2000).

1.2.3.3.2 Composition

Single cell protein is composed of 60-82% protein on dry basis (Suman et al., 2015). The fungus, *Kluvyeromyces. fragilis*, was cultured on whole whey yielded a high crude protein content that was high in sulfur containing amino acids, but low in ash (Gálvez, Ramírez, & García-Garibay, 1990). Bacterial SCP also offers a high crude protein content, however the high nucleic acid content (15-16%) may be a negative factor, as upon metabolism in the fish results in uric acid (Ravindra, 2000). Fungal SCP

contains low concentrations of methionine and cysteine (Ebrahim & Abou-Seif, 2008; Suman et al., 2015), but bacteria tend to be rich in the essential amino acid methionine (Ravindra, 2000).

Single cell protein enriched feed can supply B-complex vitamins and minerals along with several other components that encourage disease resistance in marine animals (Zhenming Chi et al., 2006). Yeast represent one of the richest sources of vitamins B12 (MR Brown et al., 1996; Ebrahim & Abou-Seif, 2008) along with thiamine, riboflavin, biotin, niacin and many other nutrients (Ravindra, 2000).

1.2.3.3.3 Disadvantages/limitations

Although all strains used for SCP production are thoroughly screened prior to use, the potential of contamination by a pathogenic microorganism is a continuous risk (Kuhad et al., 1997). Other disadvantages that arise from the use of microorganisms include the colors and flavors present may be unpalatable. Furthermore, consumption of foreign proteins may result in unfavorable immune responses (Adedayo, Ajiboye, Akintunde, & Odaibo, 2011; Suman et al., 2015).

1.2.3.3.4 Anti-nutritional factors

The production of mycotoxins by certain fungi can preclude their use for SCP (Ravindra, 2000). Ochratoxin A (OTA) is an important metabolite produced by certain species of fungi. OTA can cause a reduction in weight gain, low survival, and tissue lesions to the liver and kidneys of fish (El-Sayed, Khalil, & Saad, 2009). Aflatoxins are also known to damage the liver in various ways (Ravindra, 2000).

1.2.3.3.5 Low digestibility

The cell wall represents up to 10% of a microbial cell's dry matter, and can reduce digestibility (Kuhad et al., 1997). The cell wall is designed to keep native structures inside while preventing foreign invaders outside (RI Mateles & SE Tannenbaum, 1968). Although the digestibility is an issue, the cell wall components of yeast such as glucans and mannans, have an added benefit of stimulating an immune response (Kuhad et al., 1997; Portnoy, Williams, & Barnes, 2016).

1.2.4 Insect larvae

1.2.4.1 Advantages

Naturally found in the diets of fish, insects and their larvae make for an easy transition for an alternative to fish meal. They are rich in amino acids, vitamins, and minerals (Van Huis, 2013). They do not require much for land, energy, or water because they grow in organic waste or manure piles (Oonincx & De Boer, 2012). An added advantage is the reduction of the nitrogen and phosphorus waste (Diener, Zurbrügg, & Tockner, 2009; Newton et al., 2005; Van Huis, 2013). Lastly, the price of insect larvae varies greatly between species, but maggot meal was competitive with fishmeal. The price is still much higher when compared to soymeal, but would require lower amounts due to the nutritional composition (Veldkamp & Bosch, 2015).

1.2.4.1.1 Minerals and nutrients

Collection of edible insects for consumption has been conducted for millennia (Rumpold & Schlüter, 2013). This is driven by the high nutrient values they contain. For example, 100g of caterpillars (*C. forda* and *B. alcinoe*) can provide almost 100% of the daily vitamin requirements for humans while three silkworm pupae can be as nutrient rich as one chicken egg (Rumpold & Schlüter, 2013).

1.2.4.1.2 Protein titer

The same 100g of caterpillars as discussed above can provide 76% of daily protein required for humans, furthermore the silkworm pupae contain up to 50% protein (Rumpold & Schlüter, 2013). Other insects' protein content may vary from 50 to 82% on dry basis (Rumpold & Schlüter, 2013).

1.2.4.2 Disadvantages/limitations

1.2.4.2.1 Amino acid balance

Generally the amino acid composition is dependent upon the taxon to which the insect belongs (Henry, Gasco, Piccolo, & Fountoulaki, 2015). However trends do appear, with some insects containing high contents of phenylalanine and tyrosine, but low levels of methionine (Rumpold & Schlüter, 2013).

1.2.4.2.2 Anti-nutritional factors

Plants consumed by the insects may have an effect on the components of the insect larvae which may include unpalatable characteristics (Finke, 2002). According to

Sánchez-Muros, Barroso, and Manzano-Agugliaro (2014) medium to large sized chitin particles can induce asthma and allergies in humans, but smaller sized particles can induce an anti-inflammatory effect.

1.2.4.2.3 Low digestibility

Chitin, a polysaccharide present in the exoskeleton of arthropods, is a crude fiber that is indigestible by most monogastric animals (Sánchez-Muros et al., 2014). Interference by chitin can limit the usability of the insect protein, (Longvah, Mangthya, & Ramulu, 2011), but several studies have shown that some fish have the capability to produce a endogenous chitinase to break down the chitin in the feed (Sánchez-Muros et al., 2014). Nevertheless, it has been reported that chitin can stimulate a beneficial immune response in fish (Sánchez-Muros et al., 2014).

1.2.4.2.4 Lack of critical elements such as omega 3 fatty acids

Reducing the fishmeal content of an aquafeed by 9% via replacement with black soldier fly larvae reduce the natural fish oil content of the feed by 5% (St-Hilaire et al., 2007) One way to counter act this reduction is to grow the insect prepupae in manure enriched with fish offal (entrails) (St-Hilaire et al., 2007). The insects can absorb poly-unsaturated fatty acids (PUFA) from their growth medium and incorporate it into the insect cell mass (Sealey et al., 2011). It is important to note that insect lipid levels can be higher than fishmeal and soymeal, but are extremely variable (Henry et al., 2015).

1.2.5 Oilseeds and oilseed meals

Several different plant-based meals have been evaluated for their potential to replace fishmeal. One major contender for a partial replacement is soybean meal (Gibbons & Brown, 2016). A few requirements for the use of the plant-based meals is a low fiber and ANF components, while having a high protein content with an acceptable amino acid profile, high digestibility, and a favorable palatability (Naylor et al., 2009). Other oilseed such as canola and sunflower seed (Aslaksen et al., 2007), and cereal grain byproducts such as corn distillers' grains and barley fractions (ML Brown, Schaeffer, Rosentrater, Barnes, & Muthukumarappan, 2012; Jabeen, Salim, & Akhtar, 2004)

1.2.5.1 Advantages

1.2.5.1.1 Omega 3 fatty acids

Terrestrial oilseeds known to contain omega-3 fatty acids include: Sunflower, linseed, canola, soybean, olive, and palm oils. However, vegetable oils do not contain long chain omega-3 fatty acids such as EPA and DHA needed in fish nutrition (Naylor et al., 2009). However, replacement with 75% vegetable oil while maintaining long chain fatty acid requirements did not hinder fish health or growth (Naylor et al., 2009)

1.2.5.2 Disadvantages/limitations

Detrimental factors for the use of plant proteins as fishmeal replacers include the plant protein having low digestibility, deficiency of several essential amino acids, palatability, or the presence of ANFs (Daniel, 2018).

1.2.5.2.1 Anti-nutritional factors

Several different ANF's are present in meals but are dependent upon the oilseed (Soetan & Oyewole, 2009). They can be divided into four broad groups: 1) affecting protein utilization and digestion; 2) affecting mineral utilization; 3) anti-vitamins; 4) other miscellaneous compounds (Francis, Makkar, & Becker, 2001). Soybean meal is known to contain all four groups with protease inhibitors, saponins, antivitamins and many more (Francis, Makkar, and Becker 2001). Other potential replacements for fishmeal include canola or rapeseed meal (Hardy, 2010). Canola contains a high amount of glucosinolates which when hydrolyzed release isothiocyanate, nitrile, and thiocyanate that can affect thyroid function (Gatlin III et al., 2007). Phytate or phytic acid are found in most meals, providing phosphorus storage system (Cao et al., 2007). Phytates chelate with mineral ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, or Fe^{3+}) or complex with proteins and vitamins rendering them unusable by the animal eating the feed (Francis, Makkar, and Becker 2001).

1.2.5.2.2 Protein titer

According to Olsen and Hasan (2012), plant proteins have been a primary choice in the replacement of fishmeal for aquaculture diets. However, the protein composition is not always favorable to carnivorous fish species (Hardy, 2010). The protein efficiency ratio (PER) of soybean meal is relatively low when compared to fishmeal (1.6 and 3.4 respectively) (Friedman, 1996). Soybean meal is considered highly antigenic due to the presence of what which this causes intestinal enteritis (Drew, Borgeson, & Thiessen, 2007)

1.2.5.2.3 Amino acid balance

Some current feed ingredients, such as soybean protein concentrate, wheat, or corn gluten meals, require supplementation with essential amino acids (Olsen & Hasan, 2012). Corn protein is highly digestible to fish, but due to the lysine deficiency supplementation is required (Hardy, 2010). Supplementation of methionine greatly improves the protein efficiency ratio (Drew et al., 2007). Treatment to remove the enzyme that cleaves glucosinolates requires degrees exceeding 110°C, that can affect the amino acid digestibility. (Bell, 1984).

1.2.5.2.4 Low digestibility

The lignocellulosic components of the plant-based meals limit the digestibility of the feed for non-ruminant animals (MR Bedford, 1995). Lignocellulose is the main support system of plant cell walls and is composed of lignin, cellulose, and hemicellulose as shown in Figure 1.2 (P. Kumar, Barrett, Delwiche, & Stroeve, 2009). Cellulose is a linear glucose polymer joined by β-1,4-glycosidic bonds (Bledzki & Gassan, 1999). Through hydrogen and vander Waals bonds, the cellulose is packed tightly together making it resistant to hydrolysis (Kumar et al. 2009). Hemicellulose components vary in composition between species (Nevell & Zeronian, 1985), and provide for branching that strengthens cell wall (Bledzki & Gassan, 1999). Lignin is a large cross-linked structure composed of phenolic monomers. It provides support and protection to the cell wall microbial attack (Pérez, Munoz-Dorado, De la Rubia, & Martinez, 2002). Grasses typically have a lower content of lignin compared to softwoods (Kumar et al. 2009).

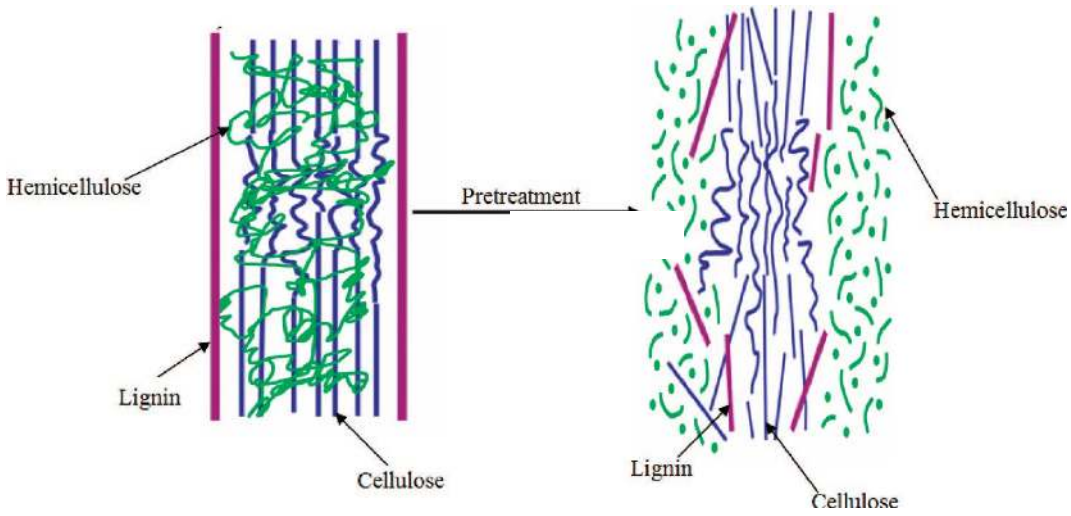

Figure 1.2: Lignocellulose structure

taken from Kumar et al. 2009

While the plant needs all these components to survive, they greatly affects digestability by animals. The nutrients stored with in the cells are encapuslated by lignocellulose which prevents digestion and absorbsion by monogastric animals. However, the use of early vegetation aid in digestiblity due to low lignocellulose componets (George & Bell, 2001).

1.3 Distillers dried grains with solubles (DDGS)

DDGS has been an alternative to fish meal since the late 1940's. Inclusion levels of the DDGS were low; several plant-based material were mixed together with the beef liver or spleen (Phillips, 1949). This had led to the incorporation of more DDGS in alternative fish meal (Lim & Yildirim-Aksoy, 2008). Benefits of including DDGS in fish meal indicate that a 5.7% reduction in feeding cost per kg of fish produced Diógenes et al. 2019).

1.3.1 Production

DDGS is a corn-based co-product from the ethanol industry (Liu & Rosentrater, 2012). Several co-products are available from the ethanol industry, but the most common co-product is DDGS which contains distillers grains with +70% of the solubles condensed from post fermentation (Stein, 2008).

Once corn is dried in the field and harvested, it is transported to an ethanol plant. After cleaning the corn by removing the broken kernels, fines, and foreign material, the corn is ground to reduce the particle size which increases surface area for later saccharification and fermentation (Rosentrater, Ileleji, & Johnston, 2012). Subsequently, the ground corn flour is transferred to the cooking step. This is conducted by mixing the corn flour with water to form a 30% solids slurry. Next, the slurry is pH adjusted to 5.5 – 6, and an α-amylase is added to being breaking up the starch molecules, while the slurry is heated to 120 to 140℃. This temperature increase begins to gelatinize the starch allowing for better saccharification. The slurry is then cooled to ~80℃. More α-amylase is added along with nutrients need for fermentation (Rosentrater et al., 2012).

The α-amylase is used to break the α-1,4 glucosidic linkages, but after cooking a gluco-amylase is added to cleave the α-1,6 glucosidic bond to which starts the saccharification process (Ingledew, Kelsall, Austin, & Kluhspies, 2009). This reaction is conducted at 55 to 65℃ and 4.0 -4.5 pH which is optimal conditions for the enzyme. The slurry is then cooled to 30℃ for the fermentation process to being. Fermentation is conducted at ~30℃ and pH of ~4.0 with *Saccharomyces cerevisiae.* Using the simple sugars produced during the saccharification, the yeast converts glucose into ethanol, CO_2, and heat as secondary products (Rosentrater et al., 2012).

After fermentation and distillation of the ethanol, the unfermentable portion of the slurry is considered the whole stillage. At a solid content of between 5 and 15%, the whole stillage is centrifuged to separate the solids (wet cake) and the liquid portions (thin stillage). Thin stillage contains mostly water with the soluble solids from the whole stillage. The thin stillage is condensed by removing the water creating a highly viscous liquid called syrup. The syrup can be mixed with the wet cake and dried. This final product is known as dried distillers' grains with solubles. The wet cake can be sold prior to drying but is not stable for long. A third option available at some facilities is dried distillers' grains which are the DDGS without the addition of solubles prior to drying (Rosentrater et al., 2012).

1.3.2 Composition

After saccharification and fermentation, roughly a third of the corn remains in the wet cake (Liu, 2012). An increase is seen in protein, oil, and ash, as fermentation has concentrated the non-starch fractions as shown in Table 1.4. The starch has been drastically reduced along with the total carbohydrates. These number however, vary greatly between different batches, production facility, and year (Belyea, Rausch, & Tumbleson, 2004). Other components that are specifically important in DDGS is the total phosphorus content at 2 to 3 times more than the original corn (Widyaratne & Zijlstra, 2007).

Table 1.4: Proximate composition of corn DDGS

	Corn*	DDGS⸸
Protein	10.2	31.4
Oil	4.6	10.9
Starch	69.5	5.3
Ash	1.3	4.6
Total CHO	83.9	52.1

CHO= carbohydrate
*(Liu and Rosentrater 2012)
⸸(Belyea et al., 2004)

1.3.2.1 Protein and amino acid composition

A three-fold increase is typically seen in the conversion from corn to DDGS. However, an improvement of the quality of the protein regarding amino acid composition is not seen. This effect is because fermentation process does not add any new proteins or amino acids, little to no change is found in the product (Liu, 2012). Several essential amino acids are required due to animals' inability to produced them. Caused by the drying process above 300˚C, the amino acid availability is diminished (Lumpkins & Batal, 2005). One of the biggest limiting amino acids is L-lysine (Pfefferle, Möckel, Bathe, & Marx, 2003). DDGS is deficient in lysine, methionine and cystine as shown in Table 1.5 At low inclusion rates of DDGS the lack of these amino acids can be compensated for (Welker, Lim, Barrows, & Liu, 2014). When the inclusion of DDGS is increased the difficulty becomes the insufficiency. To compensate for the low lysine, the addition of crystalline lysine can improve the amino acid profile (Diógenes et al., 2019).

Table 1.5: Amino acid composition of Corn DDGS and Soybean Meal

Amino acid	Corn DDGS	Soybean Meal
Arginine	1.19	4.09
Histidine	0.68	1.48
Isoleucine	1.09	2.95
Lysine	0.87	3.43
Methionine	0.55	0.80
Phenylalanine	1.30	3.07
Threonine	1.0	2.27
Tryptophan	0.22	0.80
Valine	1.45	3.07

Values are expressed as percent dry basis (Taken form Lim, Li, and Klesius (2011))

1.3.2.2 Minerals

Within the highly variable composition of DDGS the mineral composition can vary as well. This can have a major impact of the animals during consumption. Too much sulfur in the diet can lead to thymine deficiencies (Niles, Morgan, Edwards, & Lalman, 2002). High nitrogen levels have been associated with increased odor from manure, and high phosphorus levels cause land and waterway pollution (Spiehs & Varel, 2009). The phosphorus content of DDGS can be 2 to 3 times higher than the original corn (Widyaratne & Zijlstra, 2007). The means of pH adjustment during saccharification and fermentation can lead to a high sulfur, sodium, and calcium content (Liu, 2012). This high variation is limiting the inclusion of the DDGS in feed.

1.3.2.3 Fiber and digestibility

Fiber portion of the meal is composed mainly of lignin, cellulose, and hemicellulose. These can be broken down in to three categories of fiber analysis: neutral detergent fiber, acid detergent fiber, and acid detergent lignin. These components are what form the cell wall of the corn and are not easily broken down. Because of the

removal of most of the starch this fiber fraction becomes concentrated. The high level of structural fiber that is found in DDGS limits its digestibility (Liu, 2012; Welker et al., 2014).

1.3.3 Cost

According to the Renewable Fuels Association 2019 Ethanol Industry Outlook, the average cost per bushel of corn in 2018 was $3.35 US dollars. However, the same bushel could sell for $5.19 after ethanol fermentation with the collection of ethanol, distillers' grains, and the corn distillers' oils. As of June 5th, 2019, the cost of a metric ton of DDGS was $140.24 while at the same time fishmeal cost ~$1500/ton and soymeal at $356/ton (Council, 2019; Mundi, 2019).

1.3.4 Current supply and uses

In 2018, the US produced 60.8 billion L (16.06 billion gal) of corn ethanol which equates to 56% of the world production (Association, 2019b). With that 23,627,456 metric tons of DDGS was produced (Association, 2018). A large portion of this was used for livestock feed due to the economics of DDGS verse soybean meal (Swiatkiewicz, Swiatkiewicz, Arczewska-Wlosek, & Jozefiak, 2016).

1.3.5 Limitations as a fish meal replacer

Several essential amino acids are needed for the fish. The essential amino acids are not produced by the fish itself so it needs to have them supplied by the nutrition (Enes, Panserat, Kaushik, & Oliva-Teles, 2009). One of these such amino acid is lysine, but due

to the production process of the DDGS the lysine get eliminated making it a limiting essential amino acid for the fish (Lim & Yildirim-Aksoy, 2008). This is especially important for carnivorous fish species which have specific amino acid requirements as well as poor digestibility (Naylor et al., 2000). However, unlike many other alternative fish feed substrate DDGS does not contain anti-nutritional factors such as trypsin inhibitors and glucosinolates commonly found in soy and canola meals respectively (J. Becker & Wittmann, 2012).

1.3.5.1 Protein and amino acid concentrations

The highest concentration of protein found in DDGS is ~34%, as compared to fishmeal at ~65% protein (ML Brown et al., 2012). This difference causes more DDGS to be added which reduces the amount of other ingredients that can be added to obtain proper nutrition ratios. The drying process of the DDGS causes the digestibility of lysine to decrease which also affects the digestibility of the protein (Magalhães et al., 2015). Furthermore, the inclusion of DDGS in aquaculture diets has been shown to decrease the livers ability to absorb the peptide chains due to the limited digestibility (Diógenes et al., 2019).

1.3.5.2 Lack of omega 3 fatty acids

As previously mentioned, terrestrial crops are lacking in long chain fatty acids such as EPA and DHA required for use in fish diets. With the original grain being corn based DDGS is lacking in the omega 3 fatty acids. Although the digestibility of the lipids from DDGS (82-89%; Magalhães et al. 2015) falls within recommended ranges for some

species (Rainbow Trout, 79-89%; Cheng and Hardy, 2004), the fish digestive system struggles to process vegetable oils the same way it processes fish oil resulting in lower digestibility due to the shorter and highly varied fatty acid chain composition (Magalhães et al., 2015). In carnivorous fish this can decrease absorption of protein and lower growth performance (Santigosa et al., 2011).

1.3.5.3 High fiber and low digestibility

Fiber in is often correlated with better digestion. However, there are different types of fiber such as soluble and insoluble. The cell wall components are considered insoluble and can encapsulate nutrients preventing the absorption in the small intestine (Buxton & Redfearn, 1997). After ethanol fermentation, the insoluble fibers are concentrated at almost three times higher than the original grain. Because of this, inclusion of DDGS in diets of carnivorous fish is limited. Species of cold water and marine aquaculture can tolerate slightly higher levels (Diógenes et al., 2019).

1.4 Approaches to improve DDGS

1.4.1 Pretreatment

Pretreatment is used to break up the lignocellulosic structure by physical or chemical means as shown in Figure 1.2 (Ezeji & Blaschek, 2008). Effective pretreatment includes improvement of sugar ability to be hydrolyzed, avoid loss of carbohydrate and formation of inhibitory compounds for hydrolysis while being cost effective (Sun & Cheng, 2002).

1.4.1.1 Dilute acid pretreatment

Dilute acid pretreatment is one of the most studied pretreatments for enhancing biomass digestibility. The dilute acid causes the breakage of the rigid lignocellulosic structures. Typically, a strong acid such as hydrochloric, nitric or sulfuric acid are used. The sulfuric acid has been shown to have the best success (Mosier et al., 2005).

Originally, the use of sulfuric acid on cellulosic material had been conducted to manufacture furfural, a high value renewable organic compound (Anthonia & Philip, 2015; Mosier et al., 2005). The process hydrolyzes the hemicellulose to pentoses such as xylose and arabinose can be further processed into the furfural (Anthonia & Philip, 2015). After the hemicellulose is removed the digestibility of the cellulose is improved (Mosier et al., 2005). Therefore, the process provides two positive products. Lower loading rates of solids have been shown to be preferred for hydrolysis of the hemicellulose, but an increased acid concentration decreases the time needed for the hydrolysis to take place (P. Kumar et al., 2009).

Inhibitors produced using this method tend to inhibit fermentation (Ezeji & Blaschek, 2008) or enzymatic hydrolysis (Chatzifragkou et al., 2015). If all furfural is not removed it negatively impacts fermentation by microorganism (Palmqvist, Almeida, & Hahn-Hägerdal, 1999). Regulation of temperature has been shown to aid in the degradation of sugars to the unwanted products (Noureddini & Byun, 2010). Dilute acid requires special precautions to be taken to limit the risk of corrosion or environmental damage (Mosier et al., 2005). It also requires a second step of neutralization prior to fermentations which increases the cost of the process (Agbor, Cicek, Sparling, Berlin, & Levin, 2011).

1.4.1.2 Ammonia-fiber expansion (AFEX) pretreatment

AFEX pretreatment uses heated biomass and dilute ammonia. The ammonia reacts with the lignin to break the lignin-carbohydrate bond (Mosier et al., 2005). When the temperature and pressure is increased the cellulose swells, then the pressure is quickly dropped which decrystallizes the cellulose (Sun & Cheng, 2002). This also causes the ligand structure to be altered which allows for its susceptibility (Agbor et al., 2011). After this, the ammonia can be recovered (Bals, Dale, & Balan, 2006). Moderate conditions for processing limit the formation of inhibitors for microbial and enzymatic treatments (Dale, Henk, & Shiang, 1984). Finally, the process does not require further grinding to improve efficiency (MT Holtzapple, Jun, Ashok, Patibandla, & Dale, 1990).

AFEX is unable to solubilize hemicellulose efficiently as compared to other pretreatments (Sun & Cheng, 2002) this in return requires that addition of enzyme hydrolysis step with a hemicellulase such as a xylanase (Chatzifragkou et al., 2015). A second problem with the AFEX system is the use of ammonium which is recovered after treatment to be reused and avoid the potential ecological effects. This is done by heating the meal to high temperatures (200°C) which vaporizes the ammonia removing it from the pretreated meal. The vaporized ammonia is then condensed and reused for future pretreatments (Mark Holtzapple, Davison, & Stuart, 1992).

Pretreatment with ammonia can be used on a wide range of substrates including wheat, barley and rice straw, switchgrass, aspen chips, and bagasse (Sun & Cheng, 2002). According to Dien et al. 2008, the released sugars are fermentable suggesting that there are no negative inhibitors created during the process. AFEX treatment has that added

benefit of the yield only decreasing slightly at higher moisture content which reduces the cost of drying (Bals et al., 2006).

1.4.1.3 Liquid Hot water treatment

The liquid hot water pretreatment keeps water at a liquid state at higher temperature by use of pressure (Mosier et al., 2005). This process forces the partial dissolving of the biomass. The process uses the hot water to weaken the hydrogen bonds which causes the breakdown of the material (Chatzifragkou et al., 2015). The production of a hydronium ion from the hot water acts to catalyze glyosidic bonds. The cleavage of an O-acetyl group and uronic acid substitution on glucuronoarabinoxylan allows for further catalyzation of hemicellulose into mono- and oligosaccharides (Chatzifragkou et al., 2015; Mosier et al., 2005). The liquid hot water treatment is a favorable choice due to the lack of chemicals and catalyst required.

The high volume of water, energy input, and the requirement of an enzyme hydrolysis post-treatment limits the effectiveness of the treatment by itself (Chatzifragkou et al., 2015). The liquid hot water pretreatment does not saccharify xylan sugars, instead hemicellulase are required for improved processing of DDGS (Ximenes et al., 2007).

Wang, Yuan, Wang, Cui, and Dai (2012) found that the use of liquid hot water pretreatment at a temperature over 200°C broke down acid soluble lignin. They suspect that the high temperature was able liberate acids from the solids facilitating the degradation (Antal Jr, 1996).

1.4.1.4 Steam explosion

Steam explosion uses the physics of releasing steam pressure at high temperatures to break down the lignocellulosic components of the meal (P. Kumar et al., 2009). This breaks down the hemicellulose and lignin exposing the cellulose. After the cellulose is exposed an enzymatic hydrolysis can hydrolyze the cellulose in to cellobiose for fermentation (Sun & Cheng, 2002). The process can also be optimized for hemicellulose reduction by holding the pressure for a longer hold time and at lower temperature (Wright, 1988). Currently steam explosion pretreatment is most effectively used for the pretreatment of hardwoods and agricultural residue (Sun & Cheng, 2002).

The energy requirements for steam explosion are greatly reduced when compare to the other mechanical methods (P. Kumar et al., 2009). Other advantages include the lack of need for recycling due to no harsh chemical, this also benefits the environment (Sun & Cheng, 2002). The high recovery rate of hemicellulose and lignin in a usable for hydrolysis adds to its benefits (Chandra et al., 2007).

The process of steam explosion cannot always complete disruption of the lignocellulose matrix. This can also generate inhibitory compounds that can prevent enzymatic hydrolysis or microorganism's growth for subsequent fermentations such as acetic and formic acid production (Chandra et al., 2007). The biomass then needs to wash to remove these compounds, with the removal of the inhibitory compounds the hemicellulose is removed by solubilization. This water wash removes up to 25% of the original mass make the process less economically efficient (Sun & Cheng, 2002).

1.4.1.5 Dry fractionation

Dry fractionation of corn DDGS is the process of reducing particle size then separating components (Liu, 2009; Srinivasan, To, & Columbus, 2009). Particle size reduction can be accomplished by either milling or grinding to smaller particles for easier separation. Following the grinding process, the DDGS is separated based on size, shape, and density. The first process is sieving which separate particles based upon size. The second and third techniques for separation commonly used are winnowing and air classifying; both use the difference in density to separate particles. Fiber particles are separated based upon their lower density which would allow them to be carried further. Often these methods are combined to improve results (Liu, 2009; Welker et al., 2014). A technique of first grinding, then sieving the DDGS by size then air classifying helps to reduce fiber while increasing protein. The air classifying can also be used to help enrich the product for protein, oil, carbohydrate or ash depending upon the desired traits of the meal (Liu, 2009). Due to this processing a more controlled and consistent quality product can be achieved.

1.4.1.6 Wet fractionation

Wet fractionation has shown to be effective in increasing the protein content while decreasing the ADF content of the DDGS. The process involves soaking the corn for a short time before milling and ethanol fermentation. This process has been shown to recover endosperm, pericarp and/or germ fiber (Welker et al., 2014). By preventing the fiber from entering the fermentation process a more efficient outcome is gained. However, the use of the wet fractionated DDGS has only been seen in small scale and has

not been applied in fish feed trial (Singh et al., 2005; Welker et al., 2014). Instead a modified procedure post fermentation has been shown to improve DDGS quality for fish trials. DDGS is first mixed with water for 60 min then filtered through a 10-µM screen. Autoclaving is used to precipitate the proteins which can be collected using centrifugation and dried for addition to meals creating wet dried distiller's grains with soluble- protein concentrate (WDDGS-PC). Several positives were found such as increase crude protein levels, reduced structural fiber levels, as well as energy digestibility increase. It was also found that up to 300g/kg of the WDDGS-PC could be added to the diets without negative effects on the growth of the fish (Reveco, Collins, Randal, & Drew, 2012; Welker et al., 2014).

1.4.1.7 Extrusion

High-temperature, short time cooking combined with pressure, and mechanical shear are the basic principles behind extrusion (Stone, Hardy, Barrows, & Cheng, 2005). The final product is achieved through the use of heat and shearing when forced through a barrel (Kokini, Chang, & Lai, 1992). After exiting the barrel through a die, the product expands due to the loss of pressure and moisture evaporation (ML Brown et al., 2012). Other effect of extrusion may include reduction of microbial counts, gelatinization of starches, and denaturation of proteins due to the heat (Harper, 1989). Two different types of extruders are available, single screw and twin-screw. A single screw extruder is best used for low fat/ processed materials, while a twin-screw extruder is more equipped to handle the high fat/ raw materials. The fat content can reduce the shear strength

preventing the shear energy from being trans formed into heat (Castells, Marín, Sanchis, & Ramos, 2005)

There are several benefits to the used of extrusion in food production. The continuous movement of the material encourages high output while the use of relatively dry materials such as grains and flours increase energy efficiency (ML Brown et al., 2012). The high temperature can improve the texture and flavor of the food, while the temperature can also allow for the use of unconventional ingredient and reduce mycotoxins levels. Finally, the extrusion process deters high production of waste water due to low moisture, and high temperature evaporations (Castells et al., 2005). When properly done, the nutrient composition can be preserved while the feeding value is enhanced (Kannadhason, Rosentrater, Muthukumarappan, & Brown, 2010). Through all this, the structural fiber is solubilized which leads to an increase in the digestible energy. If the process is improperly done several issues could arise. Incorrect heating or pressure could lead to Maillard reactions between the protein and fiber leaving the fiber inaccessible, the mechanical force has the possibility for the loss of vitamins and amino acids (Ilo & Berghofer, 2003).

1.4.2 Enzymatic

The use of enzymes for the purpose of breaking undesirable components of plant-based biomass has been widely studied. One of the leading field in the use of enzymatic hydrolysis is the biofuels industry (García-Aparicio et al., 2007; Wright, 1988). In order to improve the efficiency of the ethanol production the degradation of lignocellulose has considered to be significant importance (Sun & Cheng, 2002). Several studies have

shown that the use of accessory enzymes, such as xylanases, β-galactosidase, or phytase, can help to improve the yield by limiting cellulase inhibitors (Hu, Arantes, Pribowo, & Saddler, 2013; Hu, Arantes, & Saddler, 2011; Luangthongkam, Fang, Noomhorm, & Lamsal, 2015).

1.4.2.1 Commercial enzymes

Industrial use of enzyme has become a widely used feature to improve digestibility of feed in a targeted approach. Currently, most enzyme used for industrial application use hydrolysis to degrade various fractions of the substrate (Kirk, Borchert, & Fuglsang, 2002). Several enzymes are commercially available for DDGS and all approved for the use in feed (Table 1.6Table 1.6). The enzyme can be utilized as a form of pretreatment or mixed with the feed for use in the digestive system. When enzymes were originally used for the degradation of cellulose, costs could exceed $2000 for one mg of purified cellulase (Walker & Wilson, 1991). With the advancements in genetic engineering microbes and targeted selection, today the cost is slightly reduced; however; enzyme cost is still considered to be one of the expensive process variables. Therefore, several studies have been conducted in assessing the effect of various enzyme dosages and types in degrading the lignocellulosic structure during the cellulosic ethanol production (Dien et al., 2008; Pryor, Karki, & Nahar, 2012). Some studies have studied how the extent of hydrolysis is related to the reaction time and enzyme dosages. Sattler, Esterbauer, Glatter, and Steiner (1989) reported that extension of hydrolysis at fixed time can be increased with increase in enzyme dosages.

Table 1.6: Commercially available enzymes

Commercially available enzyme	Enzymes contained	Source
Agri-King REAP (Agri-King)	Beta-glucanase, cellulase, and protease	(Świątkiewicz & Koreleski, 2006)
Allzyme (Alltech)	Protease, amylase, xylanase, beta-glucanase, pectinase, cellulase, and phytase	(Świątkiewicz & Koreleski, 2006)
Beta-glucosidase (Novozyme)	Beta-glucosidase	(Bals et al., 2006)
Easyzyme Mixer 1 (ADM)	Galactomannanase, beta-glucanase, xylanase,	(Jones et al., 2010)
Enspira (Enzyvia)	NSPase	(Campasino et al., 2015)
Hemicell (CehmGen Corp.)	Beta-mannase	(Jones et al., 2010; Świątkiewicz & Koreleski, 2006)
Multifect® CX (DuPont)	Cellulase	(Lamsal, Pathirapong, & Rakshit, 2012)
Multifect® GA10L (DuPont)	Glycol-amylase	(Lamsal et al., 2012)
Multifect® P-3000 (DuPont)	Endo-1,4-beta-xylanase, XylA, XylB, XylC, XylD	(Pedersen et al., 2015)
Multifect® Pectinase FE (DuPont)	Pectinase	(Lamsal et al., 2012)
Multifect® Xylanase (DuPont)	Xylanase	(Bals et al., 2006)
Natugrain TS (BASF)	Endo-1,4-beta-xylanase and endo- 1,4-beta-glucanase	(Diógenes et al., 2019)
Natuphos (BASF	Phytase	(Cao et al., 2007)
Protext 89L (DuPont)	Protease	(Lamsal et al., 2012)
Prozyme (Danisco)	Xylanase	(Jones et al., 2010)
Ronozyme P (Novozymes)	Microbial phytase	(Cheng & Hardy, 2004)
Spezyme CP (Genencor)	Cellulase	(Bals et al., 2006)
Stargen (Genencor)	Amylase	(Bals et al., 2006)
Viscozyme L (Novozymes)	Hemicellulase, arabinose, cellulase, β-glucanase, xylanase	(E. Baldwin, Karki, Iten, & Gibbons, 2018)
Ronozyme VP (DSM)	Multizyme complex, carbohydrase	(E. Baldwin et al., 2018)
Superzyme L (Canadian Biostems Inc.)	Multienzyme complex, carbohyderase,	(E. Baldwin et al., 2018)

1.4.2.2 Enzymatic hydrolysis pretreatment

In order to conduct an enzymatic pretreatment parameters of dosage loading must be established. The two most common dosage loading forms are activity unit/g of solid or mg of protein/g of solid. Use of the activity unit/g of solid quantifies activity of one enzyme, such as filter paper units for cellulase enzymes. Determination of protein for the protein/g of solid can be done by either BCA (bicinchonicic acid) kit or by nitrogen determination (Berlin, Maximenko, Gilkes, & Saddler, 2007; Krishnan et al., 2010). This method accounts for all proteins within the enzyme complex.

1.4.2.3 Use of phytase

Phytic acid is an organic form of phosphate composed of a 6-carbon ring with phosphates attached to each of the 6 carbons as seen in Figure 1.3. Due to phytic acids ability to hold on to six different phosphates in one molecule makes this one of the major forms of storage phosphorus in plants (Raboy, 1997). Phytases can cleave one of the phosphates off the phytic acid transforming it into myo-inositol (phosphate$_n$) and phosphoric acid.

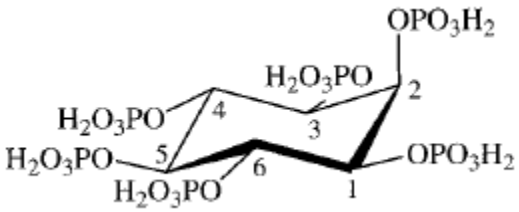

Figure 1.3: Phytic acid structure

take from Wyss et al. (1999)

Phytates (phytic acid salts) reduce the digestibility of proteins, starch and lipids. This is done by phytates complexing with the molecules making them less soluble or

resist proteolysis (Selle, Ravindran, Caldwell, & Bryden, 2000). Other enzymes can also be affected by the phytic acid, such as amylase and trypsin, which has been shown to inhibit there functions (Harland & Morris, 1995). By using the phytase these negative reactions could be limited. According to Papatryphon and Soares (2001), the addition of phytase increases the apparent digestibility of protein. Only about half of the DDGS phosphorus is in the form of phytate. This is because the yeast hydrolyze phytate during the ethanol fermentation process (Welker et al., 2014).

Using phytase can cause a reduction in the digestibility of the phosphorus (Yáñez, Beltranena, Cervantes, & Zijlstra, 2011). A limited effect has been shown for use of phytase just as an additive to the DDGS (Rojas, Liu, & Stein, 2013). This is due to the different processing factors that go into the production of the feed. A high temperature can be used in the extrusion of pellets for feed which can denature the phytase (Selle et al., 2000). The long-term storage of the feed with the phytase included can reduce the successfulness of the enzyme. Also, the environmental conditions of the animal's digestive system can decrease the productivity in of the enzyme.

Phytic acid and phytate are significant causes of water pollution because of excretion of undigested phytic acid in water systems (Turner, Papházy, Haygarth, & McKelvie, 2002). Phytase aids in the adoption of phosphorus release in to the environment standards (Kirk et al., 2002). Studies have also shown that supplementation of phytase could decrease the levels of trace mineral addition needed in the feed without affecting the nutrient retention or the body composition of fish because of the freeing of bound calcium and magnesium (Cheng & Hardy, 2004; V. Kumar, Sinha, Makkar, & Becker, 2010). Luangthongkam et al. (2015) found that using a phytase in combination

with a mixture of hemicellulases and a protease improved the efficiency of ethanol production. This reaction could be due to the inability of the phytic acid to bind with ions needed for the hemicellulose hydrolysis (Hruby, 2012).

1.4.2.4 Use of cellulase and hemicellulase

Cellulose, xylose, and lignin's make up the majority of the corn cell wall (Kulkarni, Shendye, & Rao, 1999). These are collectively termed non-starch polysaccharides (NSP). Non-starch polysaccharide enzymes (NSPase) are the second most effective enzyme used to enhance the nutritional quality of DDGS (MR Bedford & Cowieson, 2012). These enzymes include cellulases, xylanases, beta-glucanases etc. The NSP content contributes to the viscosity of the feed and the hydration properties, this in return influences intestinal transit time along with the bulking properties and the microbiota activity of the gut (Knudsen, Jensen, & Hansen, 1993). These properties of the DDGS make it hard for the digestion of the plant by some monogastric animals (Campasino et al., 2015).

The use of NSPase has been shown to improve the growth performance in low energy diets while increasing the nutrient digestibility in DDGS incorporated diets (Campasino et al., 2015). Xylanases has demonstrated an increase in the gut microbiota while stimulating intestinal enzyme activities of carp species (Jiang et al., 2014). It is also important to note that the use of a xylanase has no effect on the energy and amino acid digestibility of the DDGS (Yáñez et al., 2011).

Several factors of the use of the enzyme need to be evaluated prior to use. Such as the use of a pretreatment on the DDGS can limit the ability of the xylose release using the

a xylanase (Dien et al., 2008). Other aspect of the use of the enzyme hydrolysis are the reaction conditions such as the pH, environmental temperature, as well as the substrate to be hydrolyzed (Sun & Cheng, 2002). Due to the lignocellulosic structure being degraded, the lignin can act as an inhibitor during the cellulase reaction (Chandra et al., 2007).

The microbial flora present in the intestinal tract of the monogastric animals can ferment the NSPs only partially, which adversely affects the nutrient availability in the monogastrics, primarily young animals(Swiatkiewicz et al., 2016). Diógenes et al. (2019) found that inclusion of a NSPase reduced the cost of feeding by 16.6% per kg of fish produced. When this is combined with replacing DDGS (5.7% reduction/kg) equates to ~21.4% reduction in cost per kg. This can be attributed to the higher efficiency of the feed requiring less feed.

1.4.2.5 Use of pectinase

In many fruits, pectin is a component of the neutral detergent fiber, however pectin is not a component of corn neutral detergent fiber, but a pectinase has shown to improve the quantity of the NDF (Dien et al., 2008). This is due to the pectinases ability to break down the matrix joining the cell wall, but not the cell wall itself (M. Jackson, 2010). Addition of a pectinase and feruloyl esterase has been shown to increase the release of xylose and arabinose from the xylan (Dien et al., 2008). And up to 90% of the theoretical value after AFEX pretreatment has been seen (Bals et al., 2006).

1.4.3 Microbial

1.4.3.1 Probiotic support

Inclusion of probiotics in food has seen an increasing trend (Shields & Lupatsch, 2012). Probiotics are defined by the FAO/WHO as "live microorganisms which administered in adequate amounts confer a health benefit on the host" (Araya et al., 2002). Abd El-Hack, Mahgoub, Alagawany, and Ashour (2017) determined that a dietary inclusion of 100g/kg of DDGS and the probiotic *Bacillus substillis* increase the performance of laying hens while lowering harmful emissions of nitrogen and phosphorus. In order for a bacterial species to be considered for probiotic it must not harm the host, survive harsh conditions of the digestive system, reproduce within the gut of the host, and include no virulent or antibiotic resistant genes (Tan, Chan, Lee, & Goh, 2016).

The modification of DDGS with probiotic bacteria has been shown to lower phytic acid content (Lamsal et al., 2012). Supplementation with *B. subtillus* produced a decrease of 14.4 and 5.14% for nitrogen and phosphorus respectively in manure excretion in laying hens (Abd El-Hack et al., 2017). Other studies have shown the use of probiotics can reduced the need for amino acid and crude protein supplementation, which in return reduces the feed costs (Zaghari, Zahroojian, Riahi, & Parhizkar, 2015).

Some of the identified species of bacteria that have been identified include *Streptomyces* (Tan et al., 2016). The species had been known to produce terpenoid compounds that give an earthy or musty taste when consumed which reduces palatability (Auffret et al., 2011)

Typical probiotics are a combination of many different bacteria and fungi (Alagawany et al., 2018). Through this a multitude of mechanism are present to aid in the digestion and immune systems of the animal. Depending on the species organic acids or antibacterial substances maybe produced, host T cell stimulation, or blocking the adhesion of pathogens to the intestinal epithelial are a few ways that the probiotic can support the animal (Alagawany et al., 2018).

1.4.3.2 Fungal

Unlike the physical and chemical pretreatments mentioned previously the use of microbial pretreatment is more environmentally friendly because of the lack of chemicals and waste (Mood et al., 2013). In general, three classes of fungi have been identified in the use of degrading lignocellulose: white-, soft-, and brown rot (Shi, Sharma-Shivappa, Chinn, & Howell, 2009). To be used in feed the species must comply with the FDA Generally Recognized As Safe (GRAS) list (Administration, 1997). Some of the filamentous Ascomycota species that comply with the GRAS status include: *Aspergillus* spp, *Fusarium* spp., and *Neurospora* spp. (Ferreira, Mahboubi, Lennartsson, & Taherzadeh, 2016). Marine yeast and brewers' yeast also show potential due to their palatability and high protein content (Z. Chi, Liu, Lu, Jiang, & Chi, 2016; A Oliva-Teles & Goncalves, 2001)

After addition of the fungi, the organism survives by feeding off the unwanted components. The production of the secondary metabolites such as enzymes add the benefit of digesting the fibers (Capolupo & Faraco, 2016). The degradation of fibers frees sugars allowing for the continued survival of the fungi. Using biotechnological

advances, increased production and efficiency of the enzymes has been achieved (Capolupo & Faraco, 2016). In addition to the enzyme production, the lack of chemicals helps to create an eco-friendly product.

A limit number of species of fungi has been approved for the use in feed. Proper selection of the most adequate species for each new meal becomes difficult (Capolupo & Faraco, 2016). Other limitation that occur is the slow growth rate with the need for sterile setting. This takes a lot of time and space for desired outcome (Capolupo & Faraco, 2016). Lastly, the time needed for complete degradation takes days to weeks making it not always economically efficient (Mood et al., 2013)

As discussed earlier, plant-based meals are strong contenders for the replacement of fishmeal in fish diets, presuming nutritional improvement be made. SCP is one way to improve the nutritional quality of plant-based meals. Kasiga and Brown (2019) showed that the use of aerobically converted carinata meal with *A. pullulans* was able to replace 25% of the fishmeal before feed consumption was diminished. However, the author did note that the limitation of lysine in the replacement meal could have caused the reduction in feed consumption.

1.4.3.3 Co-culturing

In order to continuously improve the nutrient quality of single cell protein the application of co-culturing is used. When two or more species are applied such as *T. reesei* and *A. niger* a synergy occurs in the production of lignocellulosic enzymes (Ximenes et al., 2007). When applied individually *A. niger* had a higher xylan saccharification (64%) than *T. reesei* (48%), while T. *reesei* had a higher cellulose

saccharification (84%) compared to 77% with *A. niger*. However, when added together an expected result of 99% glucose from cellulose and 71% xylose were yielded (Ximenes et al., 2007).

Others have found *T. reesei* and *K. marxianus* grown on beet pulp, which has a poor amino acid profile normally, had an essential amino acid content comparable to soymeal and the FAO reference composition (Ghanem, 1992). Co-culturing of *A. flavus* with other microorganisms has been shown to reduce the fungi production of the mycotoxin aflatoxin (Ravindra, 2000). Another benefit of co-culturing is the improved biomass yields when compared to their monoculture counterparts (Tesfaw & Assefa, 2014).

1.4.4 Combinations

With the long list of plant-based biomass substrates available for feed or biofuel use, a multitude of combinations are available. The use of physical or chemical pretreatment and enzymatic hydrolysis is a popular form of degrading the biomass (Suman et al., 2015). A more increasing trend is the use of pretreatment prior to SCP.

Chapter 2 -Introduction

Currently, the projected world population is 9 billion by the year 2050 (Bene et al., 2015). With this continuing population growth an increase demand for nutrient rich feed source follows. To feed the growing population creative and alternative feed stuffs are needed. Fish is widely known to be an important source of protein as well as long chain fatty acids that aid in adolescent development and adult health (Larsen, Eilertsen, & Elvevoll, 2011). In 2010, the use of fish as a protein source was two times higher than poultry and three times higher than cattle (Bene et al., 2015).

Due to unsustainable practices many fish stocks are over-exploited or depleted (Bene et al., 2015). This finite supply of wild caught fish has led to rapid growth of the aquaculture industry as an alternative (Tidwell & Allan, 2001). The rapid expansion of the industry has resulted in an increasing need for feed ingredients. The primary protein source being fishmeal, which is the by-product left over from fish processing (bones, blood, trimmings, etc.) (Stevens et al., 2018). With a high protein content and favorable amino acid profile it is widely used for its growth performance (Tidwell & Allan, 2001). However, this need for fishmeal has expedited the collapse of the fisheries stock as typically wild fish is used to feed farmed stock (Naylor et al., 2000).

In order to correct these unsustainable practices, alternatives to wild caught fishmeal are required. Several alternatives such as soymeal, insect meal, or algae have been suggested (Cummins et al., 2017; Shields & Lupatsch, 2012; Voorhees et al., 2019). However, all of these have deficiencies. Insect meal contains a high content of chitin that is difficult for the fish to digest (Barroso et al., 2014); when algae is dried and ground it becomes very expensive (Borowitzka, 1997). Soymeal is higher in protein than most

plant-based meals, but it contains trypsin protease which is classified as an anti-nutritional factor that is harmful to the animals digestive system (Soetan & Oyewole, 2009). Many other alternative plant-based meals such as canola meal, sorghum hominy, carinata meal have been evaluated for their potential application as protein ingredients (Jason R Croat, Berhow, Karki, Muthukumarappan, & Gibbons, 2016; Kasiga & Brown, 2019; J. D. Zahler, Karki, & Gibbons, 2018).

Single cell protein (SCP) is another viable option in the replacement of fishmeal. Derived from microorganisms, SCP is a low cost and quickly produced feed (Suman et al., 2015). Many added bonuses such as natural sources of vitamins and minerals as well as immune boosting cellular components support the high protein content (Ebrahim & Abou-Seif, 2008; Portnoy et al., 2016; Suman et al., 2015). Unfortunately, a lack of omega-3 fatty acids and low digestibility of the cell wall hinder the stand alone use (Richard Mateles & SE Tannenbaum, 1968).

As a co-product of the ethanol industry, corn distillers' grains offer a low-cost option for fishmeal replacement. Depending on the processes being used for processing the corn grains by ethanol industries, wide range of DDGS is available in the market. Based on the level of processing, distillers' grains can be dried down to remove excess water or left wet depending on customer preference. The liquid fraction left over after distillation can be dried to create the solubles fraction that is typically added back to the meal to create distillers' grains with solubles (Liu & Rosentrater, 2012). An added benefit of the distillers' grains is the low content of anti-nutritional factors as compared to other agricultural commodities such as oilseed meals. The fermentation with yeast and the high

heat used during processing helps in reducing several anti-nutritional factors that the corn contains ("Anti-nutitional factors within feed ingredients,").

Unfortunately, the usage of DDGS in feed is limited. The relatively low protein content and high fiber as compared to soymeal detract from its value (Diógenes et al., 2019). The drying step of the process helps to inactivate most of the anti-nutritional factors that affect the animal, but the drying process also degrades the essential amino acid lysine making it a limiting factor for feed (Liu, 2012). The low lysine can limit growth performance in animals (Lumpkins & Batal, 2005). By increasing the protein content and decreasing the fiber content the usability of the distillers' grains can be improved.

Through the combination of both single cell protein and plant-based meals, hopefully an alternative feed can be produced. Currently, Gibbons and Brown (2016) have successfully been able to produce a commercially available microbially enhanced soy-based high protein product for the use in fish feed. Utilizing the same principles, it is hoped that the same can be done for distillers' grains.

In order to improve the nutritional quality of the distillers' grains several treatments were attempted. First a fungal fermentation utilizing four types of fungi at a varied solid loading rate was tested. All fungal strains used are generally recognized as safe (GRAS) from the FDA and is known to improve the nutritional quality of other meals. The use of multiple solid loading rates during fermentation helped to address the economic aspect of large-scale production. Typically, at lower solid loading rate more drastic differences are seen in protein and fiber values, but economics of processing does

not match. A higher solid loading rate does not see as beneficial of values on the content but is more economically efficient. This work is discussed in detail in chapter III.

Second, an enzyme saccharifications with cellulase, xylanase, phytase, and pectinase was tested. Enzyme saccharification is done to hydrolyze various unwanted components. In this case, cellulose, hemicellulose, and phytic acids were targeted. As the major component of the cellular structure of the corn, cellulose is high in content and not easily digested by monogastric animals who lack the ability to produce a cellulase. Hemicellulose that surrounds the cellulose can prevent the cellulose from being degraded, but if a hemicellulase is added hydrolysis of the cellulose can be enhanced. Although DDGS is not high in phytic acid content, the trace amounts can affect the animal and pollute the water. Therefore, our study focused on identifying the optimal enzyme dosages and enzyme types by conducting series of experiments using enzymes individually and in combination. These results can be found in chapter IV.

Based upon the previously mentioned work, down selecting for use with extruded DDGS was tested using four fungal fermentations at higher solid loading rates and six enzymatic hydrolysis treatments separately. Finally, the combined use of *N. crassa* and enzyme mixture was tested on unextruded and extruded DDGS. All these results can be found in chapter V.

Chapter 3 – Fungal Fermentation of DDGS at varied solid loading rates

Abstract

With the increasing demand for renewable energy, corn ethanol had been developed to fill the need. As a result of corn ethanol production, dried distillers' grains with solubles (DDGS) has become an inexpensive feed for several animal species. However, due to the presence of high amount of fibers and low protein content of DDGS, it's use in the animal diet is limited to less than 30% on dry basis. In this study, fungal metabolic process was used to determine if nutritional composition of DDGS could be improved by increasing the protein levels and reducing the fibers and phytic acid content in the DDGS. Four different fungal strains [*T. reesei* (NRRL-3653), *N. crassa* (NRRL-2332), *R. oligosporus* (NRRL-2710), and *A. pullulans* (NRRL-Y- 2311-1)] at three solid loading rates (SLR) (5%, 10%, and 20%) were tested. The protein titers ranged from 35 to 37% irrespective of the microbes used. As expected, protein titers were slightly decreasing with the increase in SLR. The crude fiber content was increased as compared to that of the un-inoculated control due to the concentration effect. Whereas phytic acid level was decreased in the fermented DDGS as compared to that of the control. Among all the strains evaluated, the *N. crassa* at 20% SLR and *A. pullulans* at all levels of SLRs were effective in reducing the level of fibers and phytic acid respectively. The study showed that fungal fermentation at higher SLRs (20%) can be conducted without having any adverse effect on the overall composition of DDGS.

3.1 Introduction:

The ever-growing demand for renewable energy sources has led to corn ethanol becoming one of the most important alternatives to gasoline (Liu, 2011). This increase in ethanol production has also resulted in an increase production of distillers' grains, which have become a major feed additive for many different livestock species (Magalhães et al., 2015). Dried distillers' grains with solubles (DDGS) is primarily composed of cellulosic fibers (cellulose, hemicellulose), proteins, residual oil, and essential minerals (Belyea et al., 2004). Despite being nutrient rich byproduct its usage in the animal feed is limited to only ~30% (Stein, 2008). The monogastric animals such as pig, poultry, and fish lack the fiber degrading enzymes necessary for digesting the fiber present in DDGS (Choct, 2015). Another factor limiting the use of DDGS for animal diet, is the presence of phytic acid. Phytic acid is an organic salt containing a 6-carbon ring with phosphates attached to each carbon, this structure leaves the phosphate in an unusable form (Yao et al., 2012). Although phytic acid is present in DDGS at low concentrations, it reduces the nutrient availability in animal diet (Cheng & Hardy, 2004). Additionally, due to the lack of phytase enzyme in the monogastric animals, phytic acid is not fully digested, hence its excreted in the environment (Fox & Tao, 1989). The high amount of residual phosphorus in the water system leads to the water pollution causing algae blooms (Cheng & Hardy, 2004; Ketola & Harland, 1993).

Fungal fermentation has been used for centuries for many different processes (Pandey, Soccol, & Larroche, 2008). Currently, several investigators are using fungal fermentation to enhance the value of agricultural byproducts such as canola meal, soybean meal, wheat straw, food waste, and industrial waste water (J. R. Croat et al.,

2017; Gibbons & Brown, 2016; Pensupa, Jin, Kokolski, Archer, & Du, 2013; Pleissner, Kwan, & Lin, 2014; J. Zahler, Karki, Isaac, & Gibbons, 2018). Byproducts of agricultural processing tend to have variable composition; therefore, it is critical to optimize processing conditions for each feedstock. Two important factors are the microorganism and solid loading rate used in the process. A screening process can be used to select the best performing microorganism for a specific feedstock (Sandhya, Sumantha, Szakacs, & Pandey, 2005; Subramaniyam & Vimala, 2012). Similarly, one should also determine the optimum solid loading rate for the process to be commercially viable. Solid loading rate is the ratio of substrate to water in the conversion process. Higher solid loading rates can minimize production costs, but may adversely impact microbial performance (Sandhya et al., 2005).

In this study the effect of four fungi on DDGS was accessed; crossed with three different solid loading rates for the concentration of protein, fiber, and phytic acid. Combinations of fungi and SLR were tested daily for 120-hour trials. The goal is to be able to determine the optimum loading rate along with the optimum fungal strain for the most efficient product. Using biological methods such as microbes which produce enzymes, DDGS's fiber and phytic acid content should be reduced. In reducing negative factors of the meal, efficiency of the animals' digestion should improve helping the overall nutrition of the animal.

3.2 Materials and Methodology:

3.2.1 Distillers dried grains with solubles (DDGS):

DDGS was obtained from the Dakota Ethanol plant (Wentworth, SD). The DDGS samples were then stored in a plastic container at room temperature until use.

3.2.2 Submerged fermentation:

3.2.2.1. Microorganisms tested: T. reesei (NRRL-3653) (pH 5), *N. crassa* (NRRL-2332) (pH 5), *R. oligosporus* (NRRL-2710) (pH 5), and *A. pullulans* (NRRL- 2311-1) (pH 3). All strains were obtained from the culture collection at USDA ARS.

3.2.2.2. Inoculum preparation: Cultures on PDA plates were transferred to GYE and incubated 48 h at 150 rpm and 30°C in a shaker (New Brunswick Scientific Excella, E24 rotary shaker, Edison, NJ, USA)

3.2.2.3. DDGS slurry preparation: Dried DDGS (from Dakota Ethanol Wentworth, SD) was ground through a 1 mm screen. The ground DDGS was mixed with DI water to achieve 5%, 10%, and 20% w/v solid loading rate (SLR) on a dry matter basis, and 100 ml volumes were placed in 250 ml flasks. The pH was adjusted with 3.6M H_2SO_4 to the optimal level for each fungus. Flasks were foam stoppered and autoclaved at 121°C for 20 min.

3.2.2.4. Inoculation and incubation: Eighteen flasks were inoculated with 1 ml of fungal broth for each fungal strain. Flasks were incubated at 30°C and 150 rpm for 120 h.

3.2.2.5. Sampling: At 0, 24, 48, 72, 96, and 120 h, three random flasks were withdrawn from the shaker for each fungus. The complete flask contents were quantitatively transferred into 250 ml centrifuge tubes, which were then centrifuged for 10 min at 20°C and 4,000xg.

3.2.2.6. Sample processing: The supernatant was checked for pH, volume measured, and then dried to a constant weight in an 80°C oven. These solids were subjected to protein analysis.

The solids were dried to a constant weight in an 80°C oven before being subjected to protein, fiber, and phytic acid analysis.

3.2.3 Analytical assays:

3.2.3.1.Protein determination using a LECO FP-528 instrument (LECO Corp., St. Joseph, MI, USA). This method uses the Dumas Method according to the official method approved by AOAC international. A sample is started by weighing out 0.25 ± 0.001g. The sample is then dropped in to a 900˚C furnace causing the sample to combust releasing the nitric oxide from the amino acids which is then pushed through a reduction tube filled with copper. The reduction then produced the nitrogen gas which is then sent to a gas chromatograph where the nitrogen content is quantified. The conversion factor of 6.25 is used. Finally, proteins are displayed using dry basis after moisture correction

(Sweeney & Rexroad, 1987). Pellet fraction samples were run in duplicate for each of the replicates. Supernatant fractions were run individually for each of the replicates.

3.2.3.2. Phytic acid analysis using Megazyme kit. Analysis begins with an extraction on one gram of solid using a 0.66M HCl stirring for overnight, after neutralization with 0.75M NaOH, a phytase and alkaline phosphatase with supplied buffers are added to cleave the phosphorus from the phytic acid and myo-inositol phosphate. Incubation for the individual enzymes is conducted at 37˚C for 10 and 15 minutes respectively. The enzymatic reactions are stopped by the addition of 50% w/v trichloroacetic acid. The freed phosphorus is then used in a color determination with ammonium molybdate and sulfuric acid/ ascorbic acid mixed at a 1 to 5 ratio. Samples are then incubated for one hour at 37˚C before reactions are read on a microplate reader at 655nm (Megazyme, 2017). Replicates were run individually.

3.2.3.4. Fiber Analysis was conducted at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia. Due to limited quantity of solids in the 5% all combinations were pooled for analysis and conducted in duplicate. Crude fiber was conducted according to the AOAC official method 978.10 2006. Neutral detergent fiber (NDF) test for the cellulose, lignin, and hemicellulose in the sample while acid detergent fiber (ADF) test for cellulose and lignin. Analysis were conducted according to JAOAC v. 56, 1352-1356, 1973 for NDF and AOAC official method 973.18 (A-D), 2006 for ADF.

3.2.4 Statistical analysis:

Data were analyzed using a combination of Microsoft Excel® and R studio 3.5.0 (Vienna, Austria) with downloadable packages.

3.3 Results and Discussion:

Four fungal strains were grown on three different solid loading rates under submerged incubation conditions. Each replicate was done in 250 ml Erlenmeyer flasks while being shaken. These trials were meant to aid in down-selecting for optimal fungus and solid loading rate. Total protein, fiber (crude, NDF, and ADF), and phytic acid content was tested for each of the trials.

3.3.1 Proximate composition of the DDGS

Proximate composition of the DDGS prior to any experimental test are listed in Table 3.1. DDGS is moderately low in crude protein at 29.98%, with higher crude fiber at 7.8%. The NDF and ADF were 24.21% and 10.85% respectively. Crude fat was 10.89% with ash at 4.43%. Overall composition of the DDGS is very similar to that of reported in the literature by (Belyea et al., 2004)

Table 3.1 Proximate analysis of Dakota Ethanol (Wentworth, SD) DDGS

Component	Percentage (%) (db)
Dry Matter	89.80
Moisture	10.20
Crude Protein	29.98
Crude Fiber	7.80
NDF	24.21
ADF	10.85
Crude Fat	10.89
Ash	4.43

3.3.2. Protein content of the DDGS after fungal fermentation

Figure 3.1 represents the protein content of the uninoculated control. Since two fractions were obtained from the process, line and bar diagram respectively represents the protein titers of the liquid and solid fractions. The protein titers of the solid fractions are increased due to removal of the soluble components presents, primarily small carbohydrates. During the submerged fermentation, mixing the solids in water followed by the autoclaving and further mixing for several days helps in concentrating the protein and fibers in the solids.

In each of the sampling times the 5% SLR had significantly higher content. This is because high degree of mixing can be achieved at lower SLRs. A lower ratio of solids to liquids will result in more kinetic energy removing the solubles. Therefore the 5% and the 20% were significantly different at each time point. Protein content of the supernatant protein stayed relatively steady. The small particles unable to hold in the pellet account for the protein content of the supernatant.

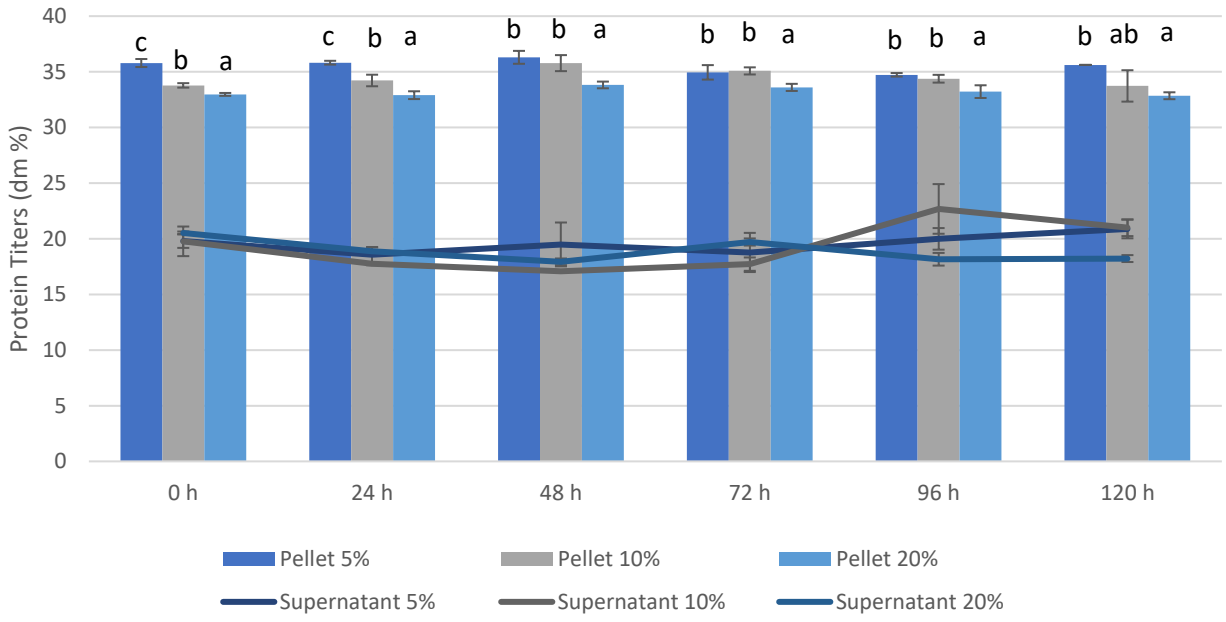

Figure 3.1 Mean values for uninoculated protein titer at each sampling point.

Groups of bars for each sampling point with same letter above are not significantly different at p<0.05.

T. reesei inoculated flasks are shown in Figure 3.2. The same solubilization effect can be seen in the 0h samples when compared to the 0h control in Figure 3.1. However, by the 24h the protein was beginning to appear in the supernatant. This increase in protein content of the liquid fraction is attributed to the metabolization of the proteins by the fungal strains. The decrease in the 5% is most likely due to freeing of protein into the supernatant. This is reciprocated in the supernatant protein. This trend continued until the 72h samples. In the 48h protein the 5% becomes the lowest, although not statistically significant, but at 72h the difference between 5% 10 and 20% becomes significant. At 96h there is no difference but there is an increase from 0h to 96h protein in the 20% pellet. Finally, at 120h the 5% was lowest with the highest supernatant protein, followed by the 20%. Whereas the 10% have the highest protein.

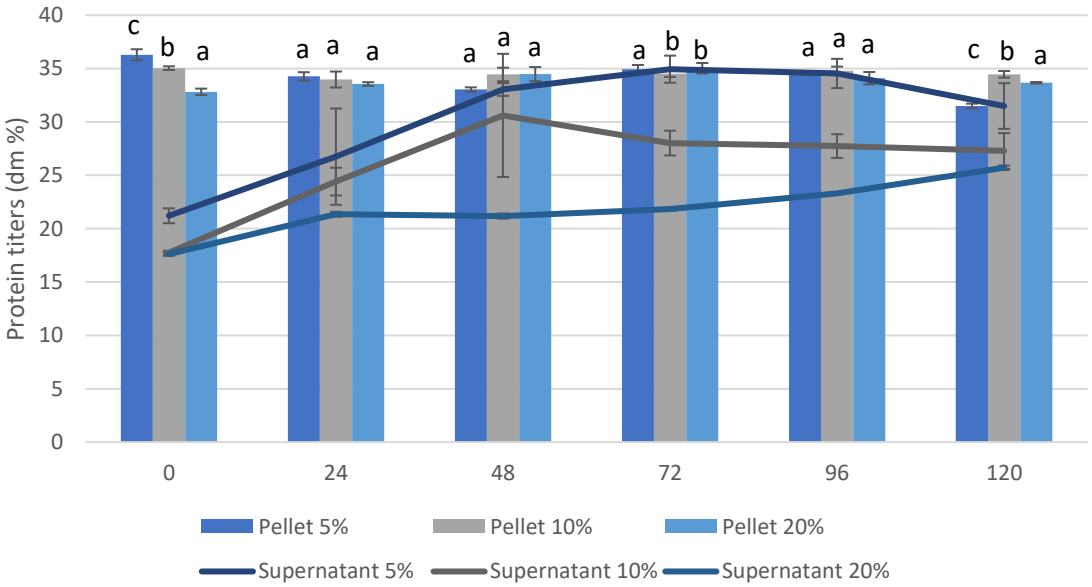

Figure 3.2: Mean values for *T. reesei* protein titers at each sampling point.

Groups of bars for each sampling point with same letter above are not significantly different at p<0.05.

Figure 3.3 shows the pellet and supernatant protein of the *N. crassa* trials. The solubilization effect is seen in the 0h and 24h samples with the 20% being the lowest at those times. After 48h the protein begins to increase in the 20% while the 5 and 10% decrease. At 120h, the 5% decreased more making it the lowest protein content; 10% began to increase, but 20% was the highest. For the supernatant the 0h points were more spread out than any other species; this could be due to sticky supernatant composition. The values begin to group together at 24h but diverge at 48h. Lowest protein content for all samples after 48h was the 20% which reinforces the solubilization effect. The 10% showed a steady increase in protein up to 120h which ended at a higher content than the pellet. This is due to some of the fungus not being held in the pellet after centrifugation, the mycelium of the fungi did not pellet well which resulted in it being removed with the supernatant.

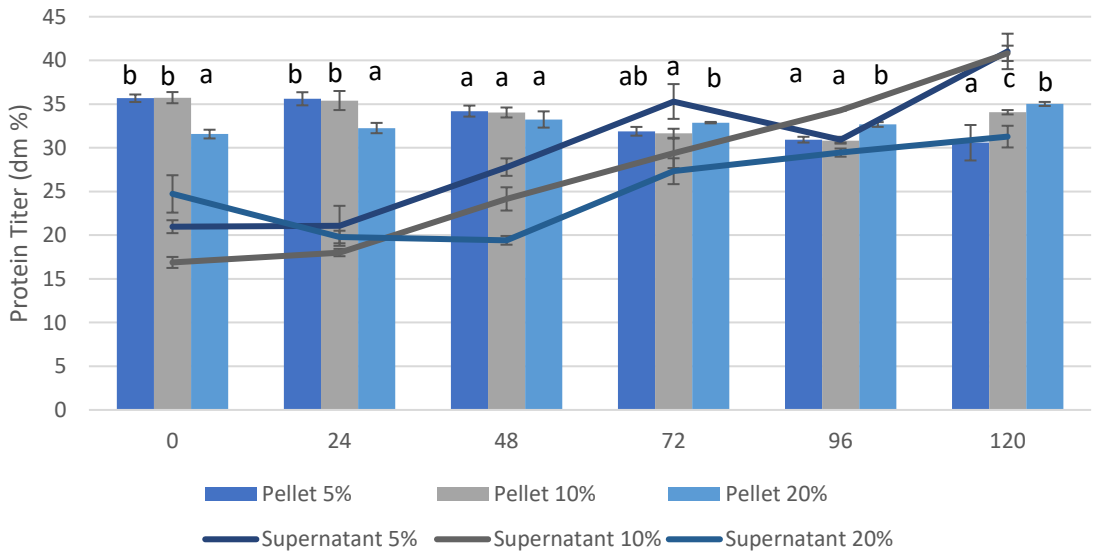

Figure 3.3: Mean values for *N. crassa* protein titers at each sampling point.

Groups of bars for each sampling point with same letter above are not significantly different at $p<0.05$.

R. oligosporus trials are shown in Figure 3.4. The solubilization effect is seen in the 0h and 24h samples. At 48h, the 5 and 10% decreased from the 0h while the 20% was increasing. The same trend follows in the 72h. At 96 hour all samples were equal independent of SLR. In 120h, the 5% was higher than the 96 h, but lower than the 0h, 10 and 20% increase in comparison to every hour. The supernatant was well grouped together at the 0h but began to split by the 24h samples. The lower the SLR the higher the protein level in the supernatant. This could be due to the fungus freeing protein.

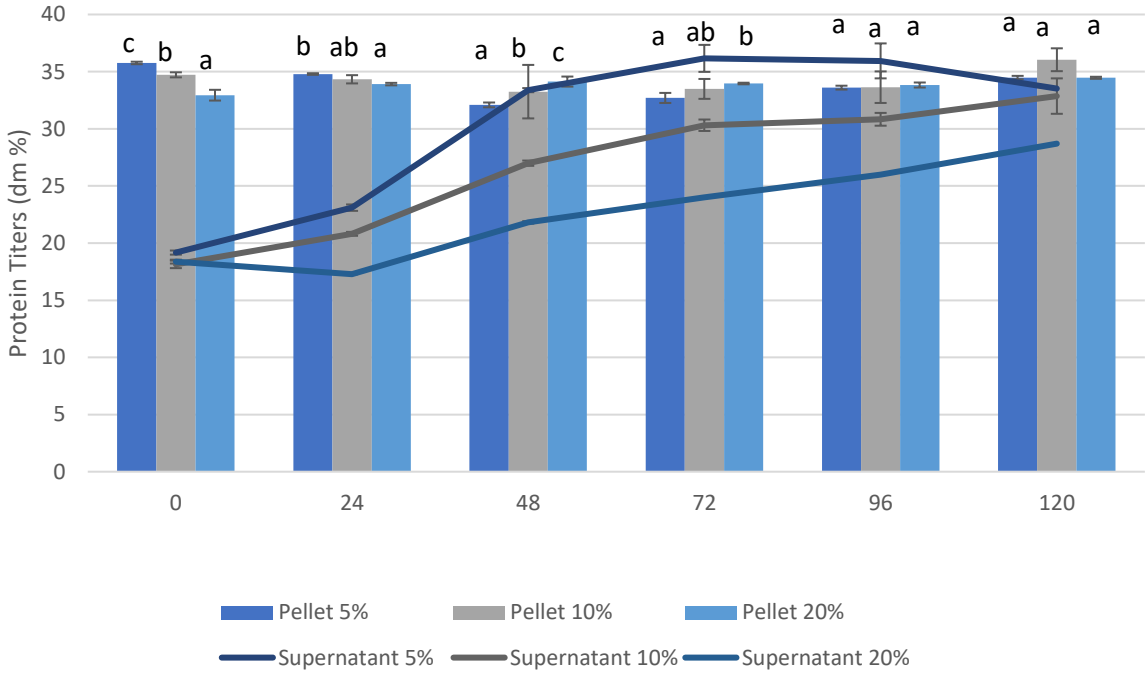

Figure 3.4: Mean values for *R. oligosporus* protein titers at each sampling point.

Groups of bars for each sampling point with same letter above are not significantly different at $p<0.05$.

Flask inoculated with *A. pullulans* 2311 are shown in Figure 3.5. The solubilization effect can be seen in the 0h, 24h, and 48h pellet protein. However, at 72h the 5% protein content dropped while the 10% stayed the same and the 20% increased.

The trend continued at 96h and 120h with the 5% decreasing and the 10 and 20% staying the same.

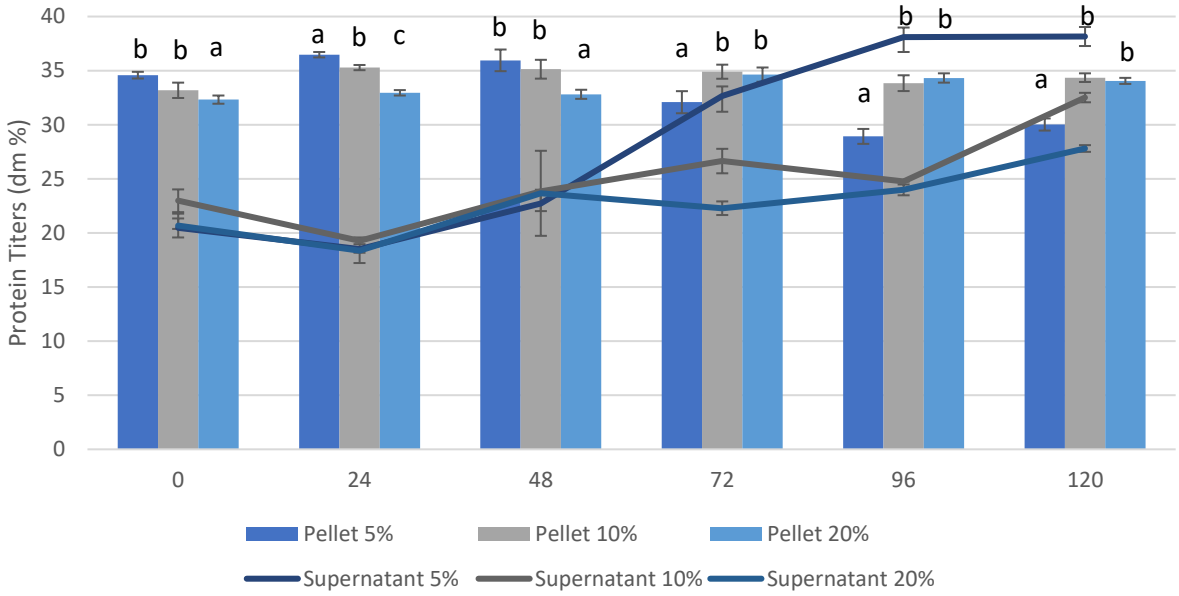

Figure 3.5: Mean values for *A. pullulans 2311* protein titers at each sampling point.

Groups of bars for each sampling point with same letter above are not significantly different at $p<0.05$.

3.3.3 Phytic acid content of the DDGS after fungal fermentation

Figure 3.6 is showing the mean values of the phytic acid contents in the fermented solids after 120 h for each of the fungal/SLR combinations. Inoculation groupings with the same letters above each bar show no significance with an $\alpha< 0.05$. For the uninoculated control, increase in SLR increased the phytic acid levels This possibly could be due to the washing effect, where maximal solubilization could occur at lower SLR as compared to the high SLR resulting into the low levels of phytic acid in solids. Although fungal fermentation was not effective in completely reducing the phytic acid levels there was some degree of degradation due to fungal fermentation. Among the fungal strains tested, *N. crassa* and *A. pullulans* were found to be most effective in reducing the phytic

acid content as compared to *T. reesei* and *R. oligosporus*. *N. crassa* and *A. pullulans 2311* showed no significant difference in phytic acid levels. According to Johnson and Tate (1970), *N. crassa* has been known to produce phytase which would result in the reduction of phytic acid irrespective of the solid loading rate. When comparing the *A. pullulans* to the *N. crassa* a similar patterned occurred which may suggest a phytase production in the *A. pullulans* strain. *T. reesei* showed increasing levels of phytic acid respective of the solid loading rate at the 120h time point. Currently, there is no research stating *T. reesei* produces a phytase which would result in this build up. *R. oligosporus* was significantly different at each loading rate. *R. oligosporus* however is known to produce a phytase according to Fardiaz and Markakis (1981). They showed a 60-65% hydrolysis of the phytic acid into inositol and inorganic phosphorus at 72h of fermentation. A significant increase in the amount of inositol is seen between the uninoculated and the *R. oligosporus* peaks after fermentation.

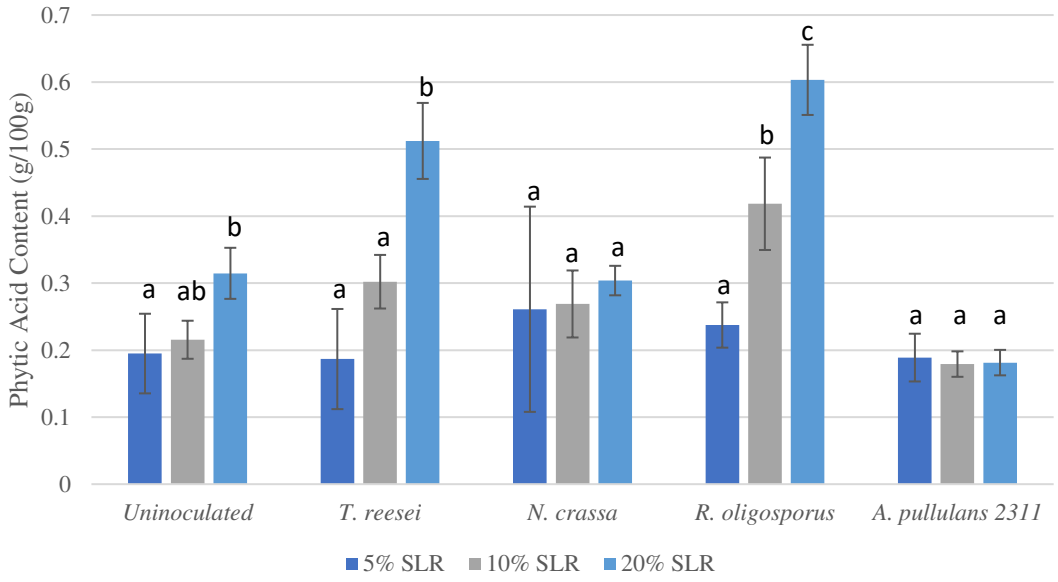

Figure 3.6: Mean values of phytic acid content at 120 h of incubation

Groups of bars for each sampling point with same letter above are not significantly different at $p<0.05$.

One explanation for the difference between these samples and the Fradiaz and Markakis may be the detection of the inositol in the sample. If the *R. oligosporus* is breaking down the phytic acid into myo-inositol we may be seeing this in our grain. The Megazyme assay is designed to test for phytic acid, but also can detect the amount of *myo*-inositol. In order to test this hypothesis. The samples were rerun using the alkaline phosphatase in both the free and the total tube as suggested on the kits FAQs link (Megazyme, 2016). This would allow for only the phytic acid to be detected in the sample. When the samples were rerun according to manufacture recommendations the 5% SLR showed no phytic acid in the sample. The 10 and 20% SLR showed 0.1609±0.05 and 0.0593±0.03 phytic acid respectively. The difference between the original value and the new value would equal the *myo*-inositol in the sample.

Although a lower phytic acid level is seen in the *R. oligosporus* samples, alternative forms of phosphorus storage do not limit concerns of water pollution (Turner et al., 2002). When down selecting for fungal strains to limit eutrophication selecting a strain able to process many forms of phosphate storage is important.

3.3.4 Change in fiber composition due to fungal fermentation

The crude fiber, NDF, and ADF after 120 h fermentation are shown in Table 3.2. The initial SLR content had significant impact on the crude fiber and NDF content of 120 h fermented solids. For example, the crude fiber content of A. pullulans treated DDGS at 5% SLR was 13.47 whereas at 20% SLR it was 9.11% (Table 3.2). This was likely due to the solubilization effect seen in the protein and phytic acid analysis. Whereas, with ADF

content there was no significant correlation observed between the SLR and ADF content of fermented DDGS.

Due to the necessity for the fungi to survive, smaller components of the slurry are broken down first which results in the concentration of the fiber components in some cases. This is seen in the crude fiber comparisons of corresponding SLR of uninoculated to each of the fungi. This correlates to what have been previously published in fungal fermentation of meals (J. R. Croat et al., 2017). All crude fiber increased significantly except for the *N. crassa* at all three solid loading rates. Furthermore, an intra species significance for *N. crassa* and *R. oligosporus* at three different SLR can be seen, while the other two species and uninoculated control were only different at the 5% SLR. This shows that the SLR can affect the ability of the fungi to concentrate or degrade the crude fiber components. Concentration effect is least at high SLRs.

Depending on what enzyme the fungi can produce, the different components of the DDGS will be degraded. The hemicellulase producing fungi will have lower NDF concentration whereas the cellulase producers will have lower NDF and ADF concentrations (2018). It is known that *T. reesei* is a strong producer of the cellulase enzyme (Nevalainen, Suominen, & Taimisto, 1994) however it was not significantly different from the control in the NDF or ADF fibers for all SLRs; suggesting no or minimal production of cellulases by the *T. reesei* on DDGS.

N. crassa was not significantly different at the varied SLR in the NDF concentration, but it was significantly different when the respective SLR are compared to the uninoculated control. According to Znameroski et. al, (2012) most of the species of *N. crassa* produce a hemicellulose that is upregulated when grown in the presence of

lignocellulosic material such as DDGS (Znameroski et al., 2012). This would cause an insignificant difference independent from the hemicellulose concentration but allow for the difference after fermentation. A significant difference is also seen within varied SLR in the ADF concentration, but not with the respective SLR uninoculated control. Most likely explanation for this is the *N. crassa* is producing a cellulase, which causes the difference with in the varied SLR, however with the fermentation a concentration effect of all the materials is happening. The least soluble fractions are being left behind which would include the cellulose and the lignin structure. It is important to note that crude fiber, NDF, and ADF of the 20% *N. crassa* fermentation are statistically the lowest concentration of each respective form.

R. oligosporus was the only fungi to have an intra species significance at the varied SLRs with NDF concentrations but is not significantly different from the uninoculated control. In fact, the 5% is higher in concentration than the 5% control. This would suggest a concentration effect again. Absolutely, no significance was seen in the ADF *R. oligosporus* fermentation in comparison to the varied solid loading rate or fermentation by another fungi. *R. oligosporus* species are known to produce several enzymes, but hemicellulase and cellulases have not been observed.

The *A. pullulans* strain used within this study is known to produce several hemicellulases such as xylananses (Christov, Myburgh, O'Neill, Van Tonder, & Prior, 1999) and arabinosidase (Myburgh, Prior, & Kilian, 1991). No significant difference was seen at the varied SLR within the species for the NDF fractions. This suggest a production of the hemicellulase is independent of the hemicellulose concentration. T.D Leathers saw an overproduction of xylanase in the Y-2311-1 strain of *A. pullulans*

(Leathers, 1986) which is consistent with the results. Furthermore, a significant reduction in the hemicellulose between the uninoculated and the *A. pullulans* fermented DDGS is seen. This is more evident of the production of hemicellulases by *A. pullulans*. One reason for the over production of xylanases could be to free the sugars from the xylose to be utilized for the production of pullulans (Duan, Chi, Wang, & Wang, 2008).

The 5 and 20% SLR were significantly different in the ADF concentration within the species, with the 5% being lower in concentration. This could be the result of the cellulose being freed during the hemicellulose hydrolysis which would result in the cellulose being washed away with the supernatant removal. A concentration of the non-soluble cellulose would account for the higher content in the higher SLRs. Several studies have lacked evidence of the natural production of a cellulase by *A. pullulans* and this strain in particular (Zhenming Chi et al., 2009). This is also further supported by the significant difference in the ADF concentration of the uninoculated vs the *A. pullulans* fermented DDGS.

Table 3.2: Fiber content of combination after 120h of submerged fungal fermentation.

	SLR	Crude Fiber	NDF	ADF
Control	5	9.37 ± 0.05 [b, DE]	39.24 ± 0.00 [a, JK]	19.75 ± 0.00 [a, ABCDE]
Control	10	8.52 ± 0.25 [a, AB]	39.30 ± 0.54 [a, GHI]	19.45 ± 1.14 [a, ABC]
Control	20	8.12 ± 0.11 [a, A]	38.56 ± 1.22 [a, DEFGHI]	19.22 ± 0.06 [a, ABCD]
T. reesei	5	12.92 ± 0.36 [b, FG]	41.97 ± 0.38 [b, IK]	24.66 ± 0.21 [b, DEF]
T. reesei	10	9.44 ± 0.24 [a, CDE]	35.77 ± 0.09 [a, CGHJ]	23.48 ± 0.20 [b, BCDEF]
T. reesei	20	8.76 ± 0.12 [a, BCD]	37.35 ± 0.93 [a, DEFGH]	20.65 ±0.66 [a, CDEF]
N. crassa	5	11.42 ± 0.06 [c, EFG]	36.52 ± 2.14 [a, BCFGH]	17.84 ± 0.44 [ab, AB]
N. crassa	10	9.85 ± 0.13 [b, BCD]	34.16 ± 1.13 [a, ADE]	15.22 ± 0.32 [a, A]
N. crassa	20	7.80 ± 0.16 [a, ABC]	30.37 ± 2.33 [a, A]	19.82 ± 1.29 [b, A]
R. oligosporus	5	13.03 ± 0.72 [c, G]	42.15 ± 0.15 [c, HIJK]	22.60 ± 1.43 [a, ABCDEF]
R. oligosporus	10	10.80 ± 0.14 [b, DEF]	35.72 ± 0.12 [b, ABCDEFG]	19.66 ± 0.40 [a, ABCDEF]
R. oligosporus	20	8.84 ± 0.08 [a, BCE]	33.68 ± 0.19 [a, ABCDEFG]	19.66 ± 0.24 [a, ABCDEF]
A. pullulans	5	13.47 ± 0.21 [b, G]	38.97 ± 4.57 [a, EGH]	22.08 ± 0.98 [a, F]
A. pullulans	10	9.80 ± 0.06 [a, DE]	31.81 ± 1.11 [a, ABDF]	23.46 ± 0.17 [ab, DEF]
A. pullulans	20	9.11 ± 0.03 [a, BCD]	33.37 ± 0.38 [a, ABC]	25.13 ± 0.18 [b, EF]

Mean value of fiber percentage sharing same superscript letter within the column are not significantly different at $\alpha < 0.05$. Lower case letters are comparing effect between solid loading rate for same fungi, whereas, the capital letters are comparing all treatments for respective fiber analysis.

3.4 Conclusion

Fungal fermentation when performed at optimal solid loading rates can help in fine tuning the overall process for maximum yield and efficiency. In this study, fungal fermentations using four strains, each at three levels of solid loading rates resulted in the solid fractions with concentrated level of proteins and fibers. However, maximum solubilization effect was observed at the low SLRs, irrespective of the fungal strains used. Effectiveness of fungal strains in improving the overall composition of the DDGS varied depending on the parameter tested. For example, all strains performed equally on protein levels; while maximal fiber and phytic reduction was achieved with *N. crass* and

A. pullulans respectively. The results obtained indicated that further fiber degradation could be achieved by pretreating the DDGS prior to fungal fermentation and/or co-culturing the DDGS with two or three different fungal strains may improve the degree of fiber reduction.

Chapter 4 – Evaluating efficiency of commercial enzymes in improving the composition of DDGS for feed application

Abstract

The goal of this study was to determine the optimal dosage of enzyme to degrade the various fractions of the distillers' grain such as cellulose, hemicellulose and phytic acid. Utilizing a submerged state condition, enzymes were added to the DDGS to hydrolyze targets. Trials were conducted using 10% solid loading rate for the ground meal with BIO-CAT cellulase, xylanase, phytase, and pectinase enzymes. Although only phytase is designed to target phytic acid, all four enzymes had some effect on the level of phytic acid, showing that by degrading fibers, the phytic acid can be removed. Utilizing the synergistic effect of the xylanase to the cellulase aided in the reduction of fibers. However, when hydrolysis was extended for too long, a solubilization effect was seen that increased the insoluble fibers.

4.1 Introduction

Corn dried distillers' grain with solubles (DDGS) has become a feed component in several animal's diets. As a by-product of the plant-based ethanol industry, certain unwanted residues are a part of the feed (Lim & Yildirim-Aksoy, 2008). Some of these include, a high fiber content, lack of essential amino acid, and lower protein levels. The high fiber content can reduce digestibility and prevent availability of essential nutrients when included in the animal diet (Stein, 2008). Several different methods have been developed in an attempt to reduce the level of unwanted residues. Some studies have previously reported the use of physical, chemical and biological pretreatment methods to

enhance the nutritional profile of DDGS (E. L. Baldwin, 2017; Bals et al., 2006; Dien et al., 2008; Nghiem, Montanti, & Kim, 2016). Among these pretreatment methods, adding enzyme directly in to the feed the digestion process is commonly used practice by the animal feed industry (Diógenes et al., 2019; Ndou et al., 2015; Tsai et al., 2017). However, this process is limited by the several factors and therefore, not completely effective. For example, following factors may influence the enzymatic hydrolysis process; i) if the enzymes were to be added into a pelletized form, the heat from the extrusion process may inactivate the enzymes; ii) secondly, enzyme activity may be reduced during the extended feed storage period; iii) animal digestive system may not be appropriate to achieve the optimal enzyme activity (pH, temperature) (Eeckhout & De Paepe, 1994; Yang, Beauchemin, & Rode, 1999) .

Cellulose and xylan are two major components of the DDGS. Since the yeast used during corn-ethanol fermentation lacks cellulases enzyme complex, thus these components are concentrated in the DDGS (Liu, 2011; Zijlstra, Owusu-Asiedu, & Simmins, 2010). Using a cellulase and xylanase for hydrolysis prior to feed can help to reduce the level of these components (Zijlstra et al., 2010). Other studies have shown that the inclusion of a pectinase can help solubilize other residual components (Dien et al., 2008). Also included in this experiment is a phytase. Although it is known that the yeast reduces the amount of phytic acid in the meal, the further reduction can help in reducing water eutrophication by phosphorus due to excretion by animals (Andlid, Veide, & Sandberg, 2004; Turner et al., 2002). Li and Robinson found that the cost associated with the addition of a phytase compared to the supplementation with inorganic phosphorus were almost equal (1997)

Currently, several feeds utilize the enzyme during the digestion process of the animal however several inefficiencies have been identified. Typically feed is prepared with the enzyme premixed, but due to the instability of the enzymes at high temperatures the process of addition becomes complex and costly (Velmurugu Ravindran & Son, 2011). Environmental conditions such as optimal temperature and pH of the digestive system can result in varied efficiencies of the enzyme among animals (Hardy, 2000).

To minimize the loss of activity or inefficiency, enzymes are being used as a type of pretreatment. Some methods have looked at individual enzymes while others look at the combinations. Berlin et al. (2005), saw a heavy dependence on the amount of cellulose broken down based upon the addition of a β-glucosidase, which also contained xylanase help to improve the cellulase hydrolytic ability on soft and hard wood with steam pretreatment. Others look at the effect of adding the enzyme at the same time versus adding at staggered times (Hu et al., 2011). They saw that when a cellulase and a xylanase were added at the same time to pretreated corn stover a synergistic effect that amplified the breakdown of the cellulose within the substrate. A third consideration of enzyme pretreatment is the dosage rate at which the enzymes are used. Although a higher dosage of enzyme will produce a higher yield, an economical approach must also be evaluated (E. Baldwin et al., 2018).

In this study different enzymes types (cellulase, xylanase, pectinase, and phytases) were tested for their ability to reduce unwanted components of the meal. The enzymes were first test individually at different dosage rates followed by in combination. Testing dosages will help to determine the most efficient yet economical method for

improvement. Through this we hope to gain insight into the ways to improve the DDGS prior to feed applications.

4.2 Methodology:

4.2.1 Feedstock:

DDGS was received from Dakota Ethanol (Wentworth, SD) and was ground through a 1 mm screen using Knife mill (Retsch; Haan, Germany). The ground DDGS was then stored in air-tight bucket at room temperature until use.

4.2.2 Enzymes:

The enzymes used in this study Cellulase 200,000 FPU/g (pH 5.0), Pectinase 1,000 PG/g (Ph 3.5), Phytase 1,000 FTU/g (pH 5.5), and Xylanase 100,000 XU/g (pH 5) were purchased from BIO-CAT (Troy, VA). All enzymes were received as a powder and sealed to prevent contamination by moisture and air. The protein content of the enzymes as tested using a LECO (FP-528 instrument, LECO Corp., St. Joseph, MI, USA) were 42.52%, 1.75%, 1.67%, and 8.86% on dry basis respectively for cellulase, pectinase, phytase, and xylanase. The enzymes were loaded based on the protein content of the enzymes per dry gram of biomass (mg protein/dry gram of DDGS). All the enzymes were stored at room temperature.

Table 4.1 Enzyme utilized

Enzyme	Activity	Optimal pH	Protein Content (%, db)
Cellulase	200,000 FPU/g	5	42.52
Xylanase	100,000 XU/g	5	8.86
Pectinase	1,000 PG/g	3.5	1.75
Phytase	1,000 FTU/g	5.5	1.67

4.2.3 Enzymatic saccharification of DDGS slurry:

4.2.3a Enzyme saccharification using individual enzymes

For the saccharification trials, 250 ml Erlenmeyer flask with 100 ml working volume and 10% solid loading rate (SLR) on a dry matter basis was used. The pH was adjusted with 3.6M H_2SO_4 and 10M NaOH to the optimal level for ach enzyme. Flasks were foam stoppered and autoclaved at 121°C for 20 min. The enzymatic saccharification was conducted at various enzyme levels (0.5, 0.75, 1, and 2 mg of protein/g of solids). Enzyme dosages are based on the total protein content of the enzymes. Since the BIO-CAT enzymes are in the powder form, calculated amount of the enzymes was first dissolved into the sterilized water and 1 ml of each was added to the DDGS slurry using sterile pipette. Flasks were incubated at 55°C and 200 rpm for up to 24 h. At 0, 2, 4, 6, 8, 20, and 24 h, three random flasks were withdrawn from the shaker for each sampling point. The complete flask contents were quantitatively transferred into 250 ml centrifuge bottles, which were then centrifuged for 10 min at 20°C and 10,000 rpm.

4.2.3b Enzyme saccharification using enzyme combinations

As mentioned in the section 2.3a; series of saccharification trials were conducted using the enzyme mixtures. Where two or more enzymes were added at different dosages. The list of the enzyme combinations used in this study is presented in Table 4.2.

Table 4.2 Enzyme combinations with corresponding denotation (mg of protein/ g of Solid)

	Cellulase dosage	Xylanase dosage	Phytase dosage	Pectinase dosage
A	1	1	-	-
B	1	1	1	-
C	0.5	1	-	-
D	0.5	0.5	0.5	-
E	0.5	1	0.5	-
F	0.5	0.5	0.5	0.5
G	1	1	1	1
H	-	1	1	1
I	-	0.5	0.5	0.5
J	1	-	-	1
K	0.5	-	-	0.5
L	1	-	1	-
M	0.5	-	0.5	-
N	0.5	0.5	-	-

4.2.4 Sample processing:

The supernatant volume was measured, 10 ml for HPLC sample removed, and then dried to a constant weight in an 80°C oven. Samples were boiled for 10 min to ensure inactivation of enzyme. Tubes were then transferred for centrifugation at 10, 000 x g for 10 min and frozen. After freezing, samples were thawed and centrifuged again at 10,000 x g for 10min, then transferred to a 2 ml microcentrifuge tube and refrozen. Samples were rethawed and filtered through a 0.2-micron syringe filter and stored frozen until analysis

The solids were dried to a constant weight in an 80°C oven. The solids were then subjected to fiber and phytic acid analysis.

4.2.5 Analytical Assays:

4.2.5a) Phytic acid analysis using Megazyme kit.

Analysis begins with an extraction using 0.66M HCl and one gram of sample stirring overnight. Neutralization with 0.75M NaOH is used after the overnight spin. Two simultaneous reaction were conducted labeled a free and total phosphorus with the difference being the addition of enzymes to the total phosphorus reaction. Phytase and alkaline phosphatase with supplied buffers are added separately to cleave the phosphorus from the phytic acid and myo-inositol phosphate. Incubation in a water bath for the individual enzymes is conducted at 37˚C for 10 and 15 minutes respectively. Trichloroacetic acid 50% w/v is added to stop the enzymatic reaction. Quantification of the phosphorus is done by colorimetric determination with ascorbic acid/sulfuric acid and ammonium molybdate in a 5 to 1 ratio. One ml of sample is mixed with 0.5 ml of color reagent and incubate for one hour at 37˚C, then read at 655nm on microplate reader (Megazyme, 2017). Replicates for each combination at 24 hours were ran individually.

4.2.5b: Fiber analysis

Fiber analysis was conducted by the University of Missouri Columbia Agricultural Experiment Station Chemical Laboratory (Columbia, MO) using standard protocol. Fiber analysis included, crude fiber, acid detergent fiber (ADF) and neutral detergent fiber (NDF).

4.2.5c: Carbohydrate analysis using HPLC

HPLC analysis was used to measure residual sugars. Analysis was conducted on an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) on a HPLC system (Agilent Technologies, Santa Calara, CA) equipped with refractive index detector (Model G1362A). Deionized water (18.2Ωm) was used as mobile phase at flow rate of 0.6 ml/min and column temperature of 85 °C. Sugars such as cellobiose, glucose, xylose, galactose, arabinose, and mannose were quantified using HPLC system.

4.2.6 Statistical analysis:

Statistical analysis was conducted using a combination of Microsoft Excel® and R studio 3.5.0 (Vienna, Austria) with downloadable packages.

4.3 Results and Discussion:

DDGS was treated with one or more enzymes either individually (16 trials) or in combination (14 trials) to test their effectiveness. All trials were conducted in 250 ml Erlenmeyer flasks while being shaken at optimal temperatures for optimal enzyme activity. Trials were designed in order to aid in identification of optimal dosage levels and enzyme synergy. Fiber (crude, NDF, ADF), phytic acid, and total sugars were tested for each of individual dosages and combinations.

Proximate analysis was conducted prior to experimental trials as shown in Table 4.3. The meal was moderately low protein (29.98%) and high crude fiber (7.8%) when compared to soymeal (52.6% and 3.86% respectively) (Long & Gibbons, 2012). NDF of 24.21%

and ADF of 10.85% was present; while a crude fat and ash were found to be 10.89% and 4.43% respectively.

Table 4.3 Proximate analysis of DDGS

Component	Percentage (%) (db)
Dry Matter	89.80
Moisture	10.20
Crude Protein	29.98
Crude Fiber	7.80
NDF	24.21
ADF	10.85
Crude Fat	10.89
Ash	4.43

4.3.1 Individual enzyme effect on phytic acid and fiber

Cellulose is a major component of the cellular structure. Addition of cellulase can break down the β-1,4 linkages of the cellulose. As cellulose is a tightly packed structure that can hold on to extra cellular components such as phytic acid (Steadman, Burgoon, Lewis, Edwardson, & Obendorf, 2001). When the cellulose is degraded the phytic acid is released which can lower its content. At higher dosages of cellulase, a significant reduction of phytic acid is seen when compared to the control as shown in Figure 4.1A. Figure 4.1B displays the fiber results for the four dosages of cellulase at 4 and 24 h of saccharification. Crude fiber utilizes the Weende method to determine its value. The moisture, ash, protein, and fat are first subtracted from the cell contents leaving behind the carbohydrate portion. The carbohydrate fraction is determined by extraction that removes the free sugars, starch, protein, and saccharides using an acid and base. This extraction also removes up to 80% of the hemicellulose and 50 to 90% of the lignin. This value then becomes underestimated as to the total indigestible fiber (Foss, 2018). Crude fiber showed little difference between the 4 and 24 h sampling. The crude fiber value is

mostly consisting of cellulose. Because the cellulase is degrading most of the cellulose prior to the 4 h time point the values do not differ. However, when compared to the control, a reduction in crude fiber was seen for all dosages.

NDF and ADF are calculated using the Van Soest method. The extraction then begins with the use of an amylase to remove starch, sodium lauryl sulfate (detergent) to remove protein, trietheylene glycol removed non-fiberous soluble material, and EDTA dissolves the pectin; the reaction is kept at a neutral pH to prevent hemicellulose hydrolysis. The remaining fraction is classified as the NDF fraction which contains hemicellulose, cellulose, and lignin. In order to determine the ADF content a acidic detergent is added which hydrolyzes the hemicellulose leaving behind the cellulose and lignin content (Foss, 2018). This explains the higher NDF content as compared to the ADF. As all dosages show, an increase in content from 4 to 24 h of saccharification occurred. The likely explanation for this phenomenon is the solubilization of the soluble fraction. After four hours the enzymes had degraded all that they could, however the continuous shaking of the flasks allowed for better mixing which allowed for the solubilization effect to occur in much the same manner seen in the fungal fermentation trials.

Figure 4.2A shows the phytic acid values at 24 h of saccharification with xylanase. A significant decrease was seen in all dosages when compared to the control. As discussed earlier, phytic acid is held within the lignocellulosic structure of the cell. This in part contains the hemicellulose which would be degraded by the xylanase. During degradation of xylan the phytic acid was most likely released into the supernatant for removal.

The fiber result for xylanase hydrolysis are shown in Figure 4.2B. No significant difference is seen within the crude fiber values for an of the dosages, however they are higher for xylanase than cellulase due to the methods fraction selection as outlined above. An insignificant decrease between the 4 and 24 h NDF values. The xylanase had a lower activity which would result in a lower hydrolysis rate. Therefore, at 4 h of saccharification, the enzymatic digestion was incomplete, very little additional time could have been needed to complete the digestion. The ADF value did not show much difference independent of the dosage due to the lack of targeted fraction in the value.

Pectinase results for phytic acid and fiber analysis are shown in

Figure 4.3. Figure 4.3A displays the phytic acid results. Again, a significant decrease in phytic acid levels is seen from the control. Two possibilities could be occurring. The pectinase is freeing the phytic acid from the cellular components or, the continuous shaking is solubilizing the phytic acid. Based upon the fiber results for the pectinase dosages showing the solubilization effect from 4 to 24h the later possible.

The NDF values shown in figure 4.3B are much lower than those of the xylanase trials. An insignificant decrease is seen in the NDF concentration at four hours of saccharification. According to Dien et al. (2008) An increase in xylose release was found with increasing pectinase and feruloyl esterase dosages. As xylose is classified as a hemicellulose monosaccharide which would fit with the observations of Dien et al. (2008).

As the primary target of phytase, phytic acid was significantly reduced at all dosages of addition (Figure 4.4A). However, it is unknown if the phytic acid was being degraded or was being solubilized. In order to test this the supernatant could have been ran using the same kit. No significant changes were seen within the fiber results due to the lack of fiber degrading enzymes (Figure 4.4B).

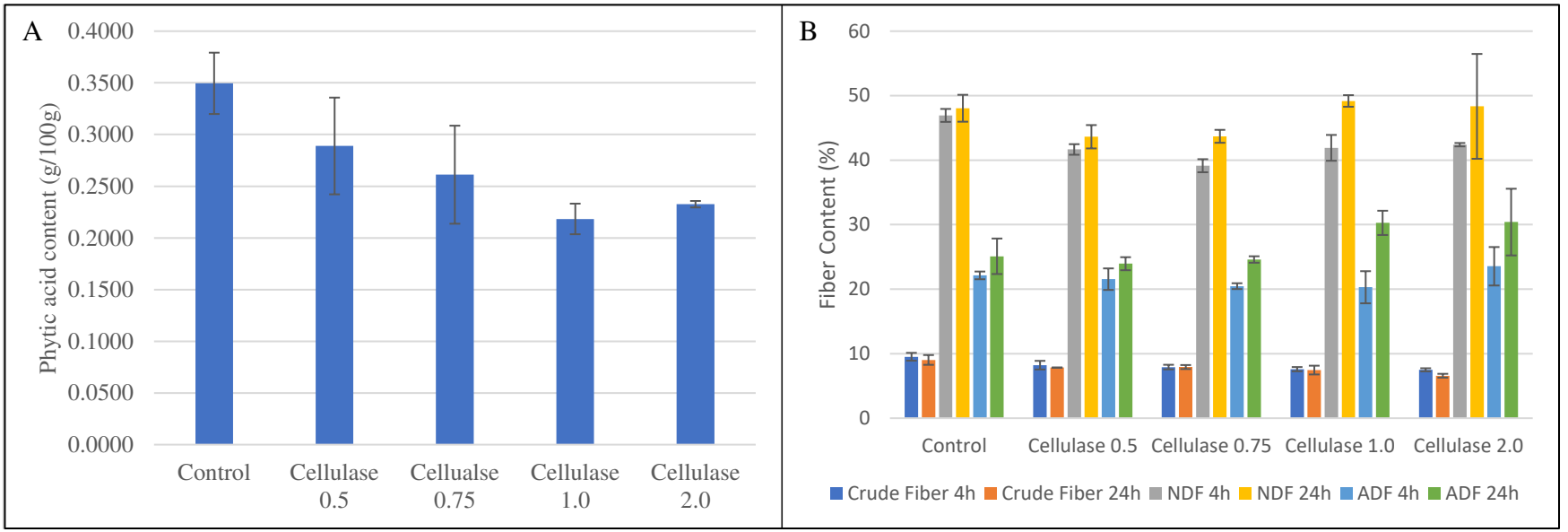

Figure 4.1 A:Mean values of phytic acid results for cellulase dosage trials after 24 h of saccharification. B: Mean values of crude fiber, NDF, and ADF at 4 and 24 h of saccharification with cellulase.

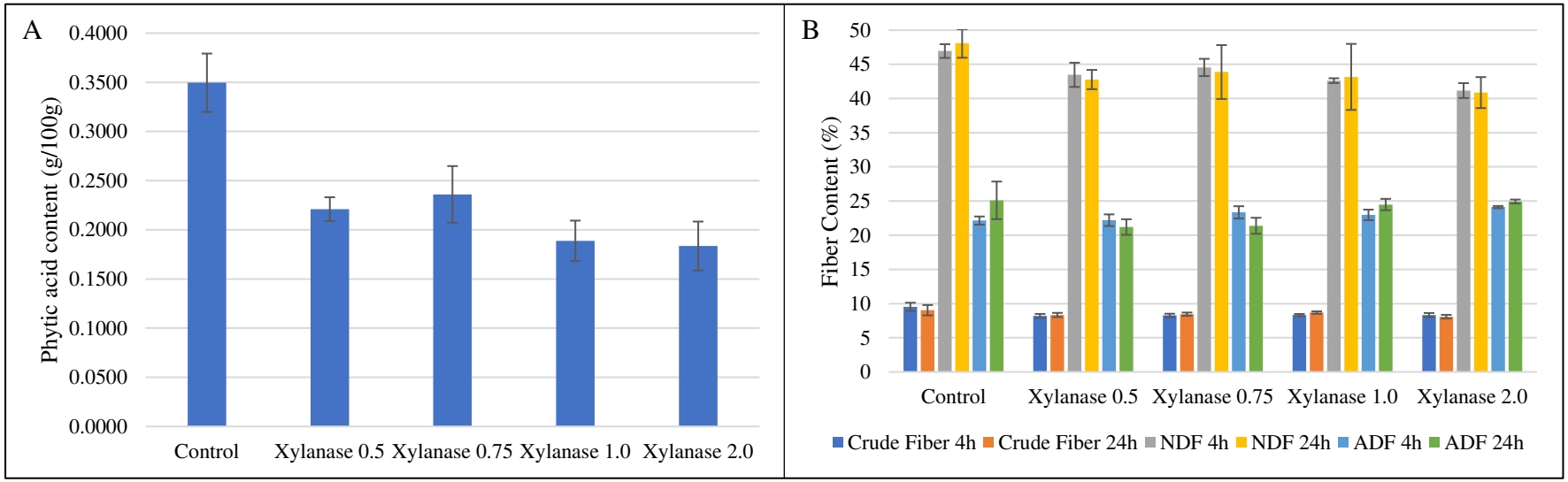

Figure 4.2 A: Mean values of phytic acid results for xylanase dosage trials after 24 h of saccharification. B: Mean values of crude fiber, NDF, and ADF.

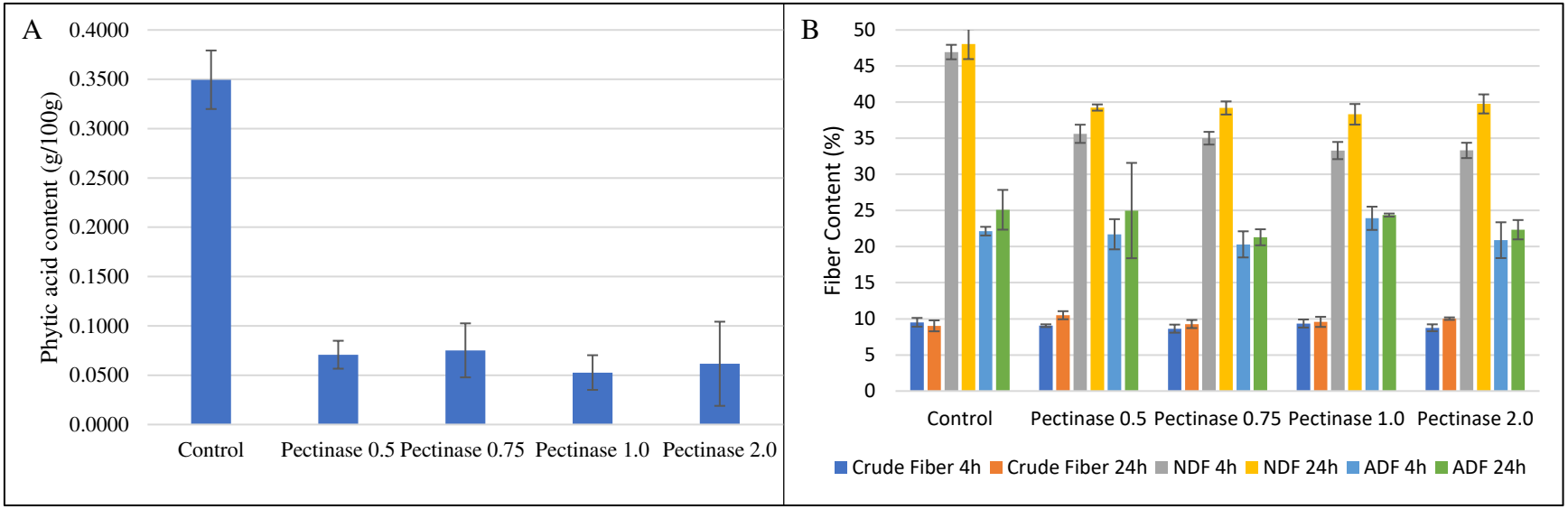

Figure 4.3 A: Mean values of phytic acid results for pectinase dosage trials after 24 h of saccharification. B: Mean values of crude fiber, NDF, and ADF at 4 and 24 h of saccharification with pectinase

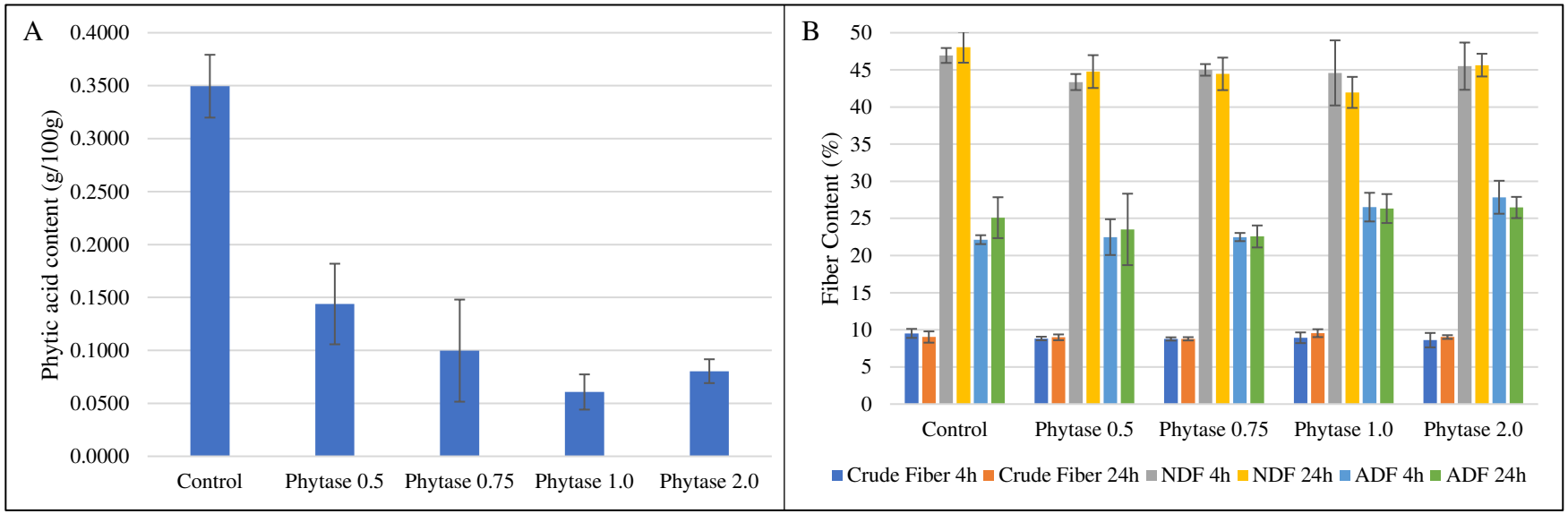

Figure 4.4 : Mean values of phytic acid results for phytase dosage trials after 24 h of saccharification. B: Mean values of crude fiber, NDF, and ADF at 4 and 24 h of saccharification with phytase.

4.3.2 Combined enzyme effect on phytic acid and fiber

Fourteen combination were developed based on literature and the values received from the previous 16 trials with individual enzymes. In the individual enzyme test, all but cellulase at 0.5 mg/g and 0.75 mg/g were significantly different from the control. Under the combinations, only one of the 14 combinations (C- 0.5 mg/g cellulase + 1 mg/g of xylanase) was not significantly different from the control. Three other combinations were significantly different from both the control and the rest of the combinations (A- 1mg/g cellulase + 1mg/g xylanase, D- 0.5 mg/g cellulase + 0.5 mg/g xylanase + 0.5 mg/g phytase, and N – 0.5 mg/g cellulase + 0.5 mg/g xylanase). It should not go unnoticed that all three combinations had equal concentrations of cellulase and xylanase. Also, of the three, one (D) did have the lowest concentration of phytase, this could be attributed the low degradation of fiber that will be discussed later.

Of the other 10 combination that were conducted only two were significantly different from each other (J- 1 mg/g cellulase + 1 mg/ pectinase, and L- 1 mg/g cellulase + 1 mg/g phytase). This suggest the phytase had the added effect of degrading the phytic acid when the pectinase could not.

Eight other combination that were tested were significantly lower than the control but not significantly different from each other (B, E, F, G, H, I , K, and M). Of these K (0.5 mg/g cellulase + 0.5 mg/g pectinase) was the only one to not contain a phytase. It is strange that the higher dosages of both did not significantly decrease the phytic acid content.

According to Hu et al. (2013), there is a synergistic effect that a hemicellulase can have on the effect of a cellulase. The same can be said for pectinase on hemicellulose

(Dien et al., 2008). At first glance Figures 4.5 B-D some treads do appear among the combination and fiber types. For instance, the lowest combination for each of the three fiber types is combination G (1 mg/g cellulase + 1 mg/g xylanase + 1 mg/g phytase + 1 mg/g pectinase). This probably is due to the presence of the high amount of the enzymes in the combination. The highest values in crude fiber and NDF content was combination D which was one of the higher values in phytic acid as well. As mentioned earlier, the phytic acid is trapped within the lignocellulosic structure, so if the lignocellulose is not being degraded the phytic acid cannot be reached by the phytase for degradation or be solubilized after being freed which prevents the decrease of the phytic acid content in the meal.

The control crude fiber at was one of the highest when compared to the combinations except for combinations H and I which did not contain cellulase. The control NDF was also the highest overall, control ADF however, was not significantly different from any of trials. Most likely this is due to the use of primarily hemicellulases. As expected, the lower the enzyme dosage the lower the fiber degradation is. This is demonstrated when comparing F and G in all three fiber components. The G had a higher enzyme dosage which reduced fiber more effectively.

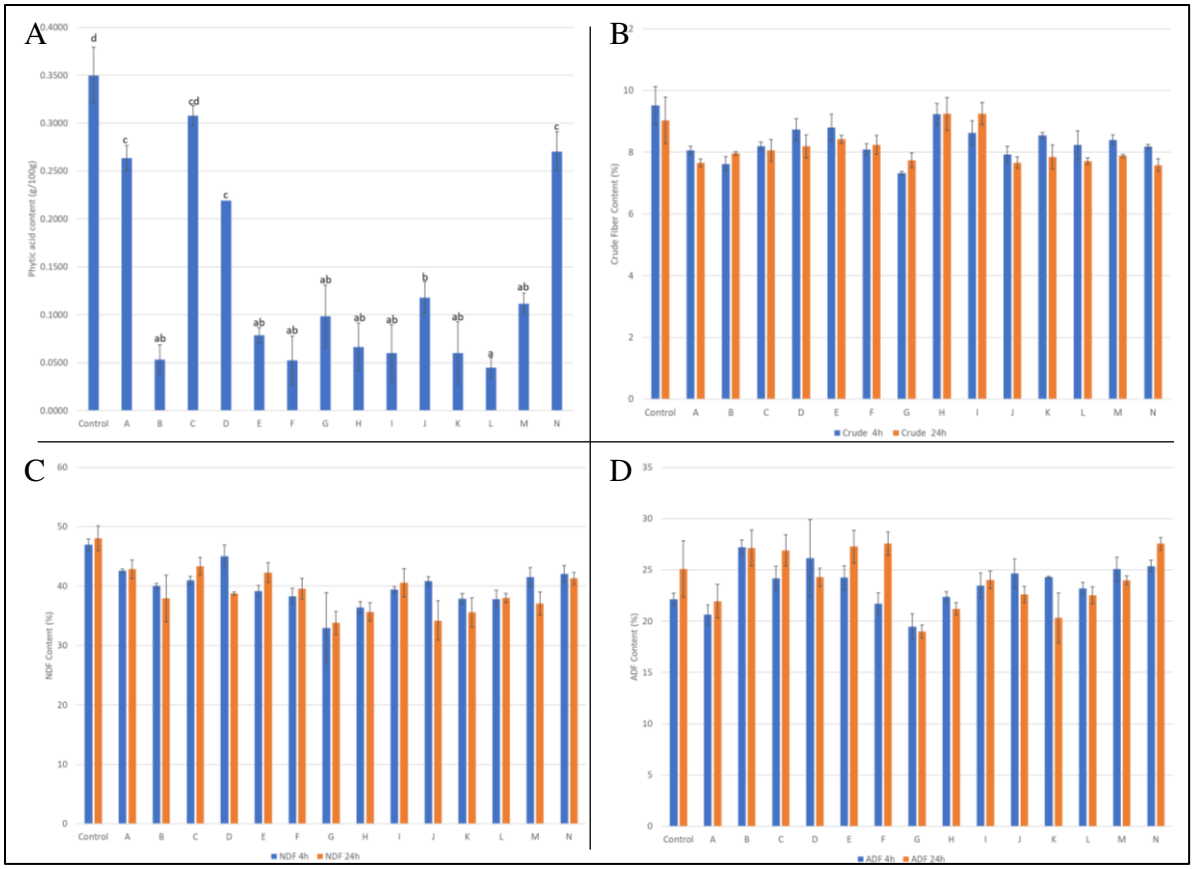

Figure 4.5 A: Mean values of phytic acid content for combinations at 24 h of saccharification. B: Mean values of crude fiber for the combination at 4 and 24 h. C: Mean value of NDF for the combination at 4 and 24 h. D: Mean value of ADF for the combinations at 4 and 24 h.

Bars with same letter above show no significance within the treatment compared to the control at $p < 0.05$. All letters correspond to combination in Table 4.2.

4.3.3. Sugar profile of the enzyme hydrolyzed DDGS

4.3.3a: Individual enzyme effect on the DDGS

Though samples were tested for different time periods, the glucose and total sugar data of the DDGS showed maximal effect at 4 hours, hence; only 4 hours data is discussed in this study. Sugars that are presented in the total sugar include glucose, cellobiose, galactose,

arabinose, xylose, and mannose. The control sample had only negligible amount of glucose present whereas total sugar concentration of 3.6 g/L was detected after 4 h of hydrolysis (without enzymes). The enzyme saccharification with four different class of enzymes (cellulase, pectinase, xylanase and phytase) resulted in increase in the glucose as and total sugar concentration as compared to that of the control (Table 4.4). There was an increase in glucose concentration with the increase in enzyme dosage from 0.5 to 1 mg/g dry DDGS. However, subsequent increase in enzyme dosage to 2 mg/g did not have significant impact in terms of glucose release, except for the phytase enzyme where big boost in glucose release was achieved with the increase in phytase dosage from 1 to 2 mg/ dry gram. Among the enzymes that were tested, phytase was found to be the most effective with the maximal amount of glucose release even at the lower level. The study by Baldwin et al, (2018) reported similar results where increase in Viscozyme level from 0.75 to 1 mg/dry gram DDGS did not have any significant effect on glucose release of pretreated DDGS. Additionally, the glucose and total sugar results as obtained on this study are comparable with the glucose reported for the enzymatic hydrolysis of pretreated DDGS by Baldwin et al., (2018) indicating BIO-CAT enzymes used in this study are more efficient than the other commercial enzymes that were used previously.

Table 4.4: Glucose and total sugar concentration of DDGS pretreated with four different enzymes at four different level of dosages

Enzyme dosages (mg protein/dry gram of DDGS)	Glucose (g/L)	Total sugars (g/L)
Cellulase		
0.5	1.88±0.17	7.22±1.93
0.75	1.80±0.06	8.48±0.11
1.0	2.88±0.53	10.79±0.55
2.0	1.48±0.35	9.34±0.85
Xylanase		
0.5	0.32±0.07	3.08±0.11
0.75	1.24±0.05	7.78±0.98
1.0	2.99±0.35	7.23±0.26
2.0	3.30±0.16	6.73±0.28
Pectinase		
0.5	1.11±0.11	5.50±0.21
0.75	2.61±0.05	5.97±0.91
1.0	3.35±0.52	7.92±0.45
2.0	3.77±0.63	7.61±0.68
Phytase		
0.5	2.91±0.42	6.37±1.06
0.75	3.53±0.23	7.64±0.42
1.0	4.28±0.30	8.45±0.40
2.0	10.17±0.29	17.43±0.50

4.3.3b: Effect of enzymes on DDGS when used in combination

Several studies have found that enzymes when used in the combination can have synergistic effect and hence can be used to maximize the process efficiency. Hence in this study, four enzymes were tested at different combinations where dosages were also varied. The glucose and total sugar results obtained for the enzyme combination are presented in the Table 4.5.

Table 4.5: Glucose and total sugar concentration of the DDGS treated with different enzymes combination at various level of dosages

	Cellulase dosage	Xylanase dosage	Phytase dosage	Pectinase dosage	Glucose (g/L)	Total sugars (g/L)
A	1	1	-	-	4.04±0.18	6.06±0.34
B	1	1	1	-	5.14±0.79	10.97±2.74
C	0.5	1	-	-	1.60±0.26	8.91±0.90
D	0.5	0.5	0.5	-	3.59±0.39	8.67±0.71
E	0.5	1	0.5	-	6.54±0.22	14.31±0.76
F	0.5	0.5	0.5	0.5	8.48±1.06	16.34±0.74
G	1	1	1	1	9.89±0.40	16.80±1.20
H	-	1	1	1	11.81±1.31	20.46±0.91
I	-	0.5	0.5	0.5	7.92±0.79	15.66±1.33
J	1	-	-	1	9.56±0.79	17.53±1.36
K	0.5	-	-	0.5	6.28±0.27	13.25±0.40
L	1	-	1	-	9.58±0.99	17.78±1.97
M	0.5	-	0.5	-	7.77±0.46	17.01±0.92
N	1	0.5	-	-	4.47±0.29	9.07±0.33

With the enzyme combinations that were tested, the maximal glucose release of 11.81 g/L and minimal glucose release of 1.6 g/L was obtained with the enzyme mixture of H and C respectively. The enzyme mixture that had the maximal glucose release contained the xylanase, phytase and pectinase at level of 1 mg/g and no cellulase was added in the mixture. Alternatively, enzyme mixture containing the cellulase at 0.5 and xylanase at 1 mg/g dry DDGS resulted in the low glucose content. The results showed that when all four enzymes were added in a mixture, irrespective of the dosage (0.5 vs 1 mg/dry gram) level, there was no change in the glucose and total sugar release. But in the absence of the cellulase, significant difference in the glucose release was observed with the change in enzyme dosages (comparing H and I). Similarly, the combinations, where cellulase was supplemented with pectinase at 0.5 mg/g (K) showed significant amount of the glucose and total sugar release comparable to other enzyme combinations (presented

in the Table 4.5) indicating effectiveness of the enzyme mixture can vary widely. Results also indicated that there is possibility to trade-off between the enzyme dosages and enzyme types when applied in the mixture.

4.4 Conclusion

The enzyme treatment of the DDGS using different enzyme types (Cellulase, pectinase, phytase and xylanases) improved overall DDGS composition. Irrespective of the enzyme types, sugar yields were not improved beyond 1 mg/g dosages when treated individually. Results showed that enzyme cocktails when used in the mixture has potential to have synergistic effect and high efficiency could be achieved even using the lower dosages. However, further research needs to be conducted to determine the enzyme cocktail that would help in achieving the complete fiber degradation. Mild pretreatment prior to enzyme treatment would probably lead to the further reduction of the fibers.

Chapter 5 – Extruded DDGS treated with fungal fermentation and enzymatic hydrolysis

Abstract

Experimentation with standard DDGS showed positive results after fungal fermentation and enzymatic hydrolysis. In order to improve the fiber degradation, the addition of extrusion prior to fermentation and saccharification was tested. When it was determined that this had a greater effect the combination of both fungal fermentation and enzymatic saccharification was combined as a treatment for standard and extruded DDGS. The submerged trials at a 15% SLR were evaluated for improvement in protein, fiber, phytic acid, and residual sugar content. After fungal fermentation, *N. crassa* at 48 h of fermentation showed the greatest improvement in protein content. However, as compared to standard DDGS, extruded DDGS showed less degradation of fiber after fungal fermentation. When the combined enzymatic hydrolysis and fungal fermentation , maximum protein yields were seen at 24h with extruded DDGS and simultaneous addition of enzyme. These trials also showed improved fiber reduction.

5.1 Introduction:

Distillers dried grains with solubles (DDGS), a corn ethanol industry by-product is feed to livestock. However, under processing condition for ethanol fermentation some nutritional components are lost while others are concentrated. Part of the concentrates include the cellulosic structure that forms the cell wall (Liu, 2012). The cellulose is

difficult for many animals to break down making DDGS inefficient in digestibility (Stein, 2008), hence DDGS inclusion level is limited to < 30% in animal diets.

Extrusion has been used for decades in food production; however, it was not until the 1980's that the use of extrusion as a pretreatment became popular. During extrusion pretreatment feedstocks are subjected to simultaneous heating, mixing and shear forces which results physical and chemical changes (Karunanithy et al., 2013). Advantages to the use of extrusion as a pretreatment include: continuous process, adaptability for feed stock, no sugar degradation, and low cost (Zheng & Rehmann, 2014). Little to no loss of solids happens because of the lack of post treatment recovery (Mood et al., 2013).

In addition to extrusion pretreatment, the use of biological conversion is promising. Biological pretreatment is considered environmentally safe and low energy process (A. K. Kumar & Sharma, 2017). As shown in the fungal fermentation and enzyme hydrolysis chapters the use of microbes to alter the composition of DDGS is possible. In the past the use of biological pretreatment in large scale has been avoided due to the prolonged treatment and expensive vessels (Mood et al., 2013).

However, it has been shown that the combination of biological pretreatments and physical/chemical pretreatments aids in the process (A. K. Kumar & Sharma, 2017; Mood et al., 2013). J. R. Croat et al. (2017) found that the protein increase while lowering the NDF content for hexane extracted and cold press canola was best treated with extrusion/ *T. reesei* and extrusion/ *F. venenatum* respectively. This suggest that the use of extrusion does not produce inhibitors for secondary fermentations.

This study aims to look at the individual and combined effect of enzyme saccharification and fungal fermentation in three separate experiments. The first is

utilizing fungal fermentation of extruded DDGS, the second uses enzyme saccharification on the extruded DDGS, and the third after a down selection of parts 1 and 2 are combined to look at its effect on the extruded DDGS.

5.2 Methodology:

5.2.1 Feedstock

DDGS was collected from Dakota Ethanol (Wentworth, SD). Solids were ground through a 1mm screen and stored in an air-tight bucket at room temperature until use.

5.2.2 Extrusion Pretreatment

Four kg of DDGS (moisture content 12%, dry basis) was extruded using a Brabender Plasti-Corder model PL2000 (South Hackensack, NJ) The extruder had 3 temperature zones set to 90, 90 and 100°C respectively. A screw with a compression ratio of 3:1 and barrel ratio of 20:1 was set to 100 rpm. Finally, the extruded DDGS was pushed through a die with a 3 mm opening. Extruded DDGS was spread on to trays and left to cool and dry over night before being stored in an air-tight bucket at room temperature until use.

5.2.3 Microorganisms Used

T. reesei (NRRL-3653) (pH 5), *N. crassa* (NRRL-2332) (pH 5), *R. oligosporus* (NRRL-2710) (pH 5), and *A. pullulans* (NRRL- 2311-1) (pH 3). All strains were obtained from the culture collection at USDA ARS and stored at 4°C and re-cultured every 30 days on to potato dextrose agar (PDA, BD Difco)

5.2.4 Enzyme used

Enzymes purchased from BIO-CAT (Troy, VA) are shown in Table 5.1. All enzymes were received as powder and sealed to prevent contamination. Enzyme protein content amounts were determined by using a LECO FP-528 instrument (LECO Corp., St. Joseph, MI, USA. The values obtained was then used to determine the amount of enzyme needed to achieve the desired level of enzyme loading.

Table 5.1 Enzymes utilized

	Activity	Optimal pH	Protein content (%, dry basis)
Cellulase	200,000 FPU/g	5	42.52
Pectinase	1,000 PG/g	3.5	1.75
Phytase	1,000 FTU/g	5.5	1.67
Xylanase	100,000 XU/g	5	8.86

5.2.5 Inoculum Preparation:

Cultures on PDA plates were transferred to 5% glucose 0.5% yeast extract (GYE) and incubated for 48 h at 150 rpm and 30°C in a shaker (New Brunswick Scientific Excella, E24 rotary shaker, Hauppauge, NY, USA).

5.2.6 Fermentation of Extruded DDGS

Extruded DDGS was subjected to the fungal fermentation using 100 ml working volume in 250 ml Erlenmeyer flasks and 15% solid loading rate (SLR) on dry matter basis. Flasks were pH adjusted to optimal pH for fungi or enzyme using 3.6M H_2SO_4 and 10M NaOH. Samples were foam stoppered and autoclaved at 121°C for 20min. Eighteen flasks were inoculated with 1 ml of 48 h fungal broth for each fungal strain. All flasks

were incubated at 30℃ and 150 rpm for 120 h. Samples were collected at 0, 24, 48, 72, 96, and 120 h. Three flasks were drawn at random from the shaker, at each time point and respective fungus. The complete content of each flask was transferred to a 250 ml centrifuge bottle and spun for 10 min at 10,000 rpm and 20℃. The solids and liquid sample weights were recorded, and oven dried at 80 ℃ prior to grinding and storing at room temperature.

5.2.7 Enzyme Saccharification of Extruded DDGS:

For each trial twenty-one flasks were prepared with extruded DDGS. Each 250ml flask was prepared with 100ml working volume and 15% SLR on dry basis. Flasks were pH'ed using 3.6M H_2SO_4 and 10M NaOH, to 4.5 to best accommodate mixed enzymes. Next, each flask was foam stoppered, covered with foil, and autoclaved. After autoclaving, flasks were left to cool to room temperature. When ready, all flasks were treated with the proper dosage and incubated at 55 ℃ and 200 rpm for up to 24h. At 0, 2, 4, 6, 8, 20, and 24 h, three flasks were drawn at random from the shaker for each time point. After bringing to 80 ℃ on a hot plate to inactivate enzyme, flasks were quantitatively transferred to 250 ml centrifuge bottles, which were then centrifuged for 10 min at 20 ℃ and 10,000 rpm.

5.2.7.1 Treatments Used:

Optimal treatments were down selected from the enzyme study and shown in Table 5.2. Prior to addition to flasks, enzymes were dissolved into sterile water. After

water had been added, solutions were mixed by vortex and left to sit for an hour to fully dissolve and defoam.

Table 5.2 Enzyme dosages with combination abbreviation (mg of protein/ g of solid)

	Cellulase	Xylanase	Phytase	Pectinase
A	1	1	1	1
B	0.5	0.5	0.5	0.5
C	1	1	1	-
D	1	-	-	1
E	0.5	-	-	0.5
F	-	1	1	1

5.2.8 Combined Enzyme and Fungal Treatment

A saccharification utilizing combination A (Table 5.2) for four hours prior to inoculation with *N. crassa* and simultaneous inoculation and saccharification was tested on both untreated DDGS and extruded DDGS. Flasks were prepared using a 15 % w/v SLR and pH adjusted to 4.5 followed by autoclaving. Samples with the saccharification prior to inoculation was sampled at time of enzyme addition (-4 h), time of fungal addition (0 h), 24, 48, 72, 96, and 120 h of incubation for a total of 21 flasks prepared. Samples without prior saccharification were sampled at 0, 24, 48, 72, 96 and 120 h for a total of 18 flasks prepared.

5.2.9 Sample Processing:

5.2.9a Protein

Protein analysis was conducted on a LECO FP-528 instrument (LECO Corp., St. Joseph, MI, USA) for the fungal fermentation trials. This method utilizes the Dumas method according to the official method approved by AOAC international. Samples of 0.25 ± 0.001g are weighed out then transferred to the instrument. The instrument drops

the sample into a 900℃-furnace causing the sample to combust releasing the nitric oxide from the amino acids. The nitric oxide is moved to a copper reduction tube where the nitric oxide is reduced to nitrogen gas. The nitrogen gas is then read by a gas chromatography where the nitrogen content is quantified. The conversion factor of 6.25 is used to display as is protein content (Sweeney & Rexroad, 1987). Finally, moisture correction is used to present protein on dry basis. All fungal fermentation pellets and supernatants were run individually for each replicate.

5.2.9b Phytic acid analysis

Phytic acid analysis was conducted using the Megazyme Phytic acid/Total phosphorus kit for all 120h fungal screen samples and 24h enzyme saccharification. Analysis being with one gram of solids being mixed with 0.66 HCl stirring overnight for extraction. Using 0.75 NaOH the sample is neutralized prior to testing for free and total phosphorus values. The free phosphorus value is determined by not adding the enzymes, while the total value requires the addition of a phytase and alkaline phosphatase. The kit comes with prepared phytase and alkaline phosphatase along with the buffers for the reaction. The reaction is conducted at 37℃ with phytase for 10 min and alkaline phosphatase for 15 min. The enzyme reaction is stopped by the addition of trichloroacetic acid 50% w/v. Phosphorus is quantified using a color metric determination. Ascorbic acid/sulfuric acid and ammonium molybdate mixed in a 5:1 ratio creates the color reagent. One ml of the sample is mixed with the color reagent and incubated for 1 hour at 37℃. Reaction are read on a microplate reader at 655 nm (Megazyme, 2017). Replicates for each sample were run individually.

5.2.9c Fiber analysis

Fiber analysis was conducted by the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia. Fungal samples at 120h, and 4 and 24h enzyme saccharification samples were analyzed for crude fiber, neutral detergent fiber (NDF), and acid detergent fiber (ADF). Crude fiber was tested according to AOAC official method 978.10 2006. NDF was conducted according to JAOAC v. 56, 1352-1356, 1973, while ADF was tested using AOAC 973.18 (A-D) 2006 official method.

5.2.9 Statistical Analysis

Data were analyzed using a combination of Microsoft Excel® and R studio 3.5.0 (Vienna, Austria) with downloadable packages.

5.3 Results and Discussion

Utilization of extrusion pretreatment followed by enzymatic and fungal treatment replicates were done in 250 ml Erlenmeyer flasks while being shaken. Trial were designed to down selected for optimal fungal and enzyme treatment. Trials conducted with fungi were tested for protein at all time points, and fiber (crude, NDF, ADF) and phytic acid content at 120 h replicates. Enzyme hydrolysis trials were analyzed, and fiber and phytic acid at 4 and 24 h.

5.3.1 Proximate analysis

Proximate analysis was conducted on the DDGS before and after extrusion as shown in Table 5.3. There was no significant change in the proximate composition of the

DDGS after extrusion pretreatment. There was only slight increase in ADF. Phytic acid was also reduced due to the heat sensitivity of the compound.

Table 5.3 Proximate composition of DDGS before and after extrusion

	Protein (%)	Crude (%)	NDF (%)	ADF (%)	Phytic Acid (g/100g)
DE DDGS	29.98	7.8	24.21	9.75	0.58
Extruded Pellets	30.88	6.6	23.94	11.38	0.52

All values on dry basis except phytic acid

5.3.2 Fungal Fermentation

5.3.2.1 Protein

Protein for the fungal fermentations can be seen in Figure 5.1. At 0h the values were not normally distributed which is why they do not contain values above the set of bars. However, it should be noticed that these values are higher than the extruded DDGS, which is due to the solubilization effect seen in prior testing. The control protein held steady throughout the 120 h of incubation. This is confirmed in the supernatant values shown in Figure 5.2

The most effective fungi to increase protein was *N. crassa* at 48 h by reaching over 40% on dry basis. After 48h the protein decreased in the pellet but increased in the supernatant. This could be due to the isoelectric point of the protein within the sample. As the protein in the pellet decreased the pH of the samples increased. At 48 h of incubation the pH was 4.85 while 72 and 96 h were 5.23 and finishing at 5.44 at 120 h.

Following *N. crassa, A. pullulans 2311* was not significantly different at 96 and 120 h of fermentation. It was not significantly different after 48 h when compared to longer fermentation with *A. pullulans.* Due to the consistency of the supernatant, the 48 and 72 h samples were unavailable, but the protein did not show much difference between hour 24 and 96. No contrast was seen between the control, *T. reesei*, and *R. oligosporus* protein within the pellet.

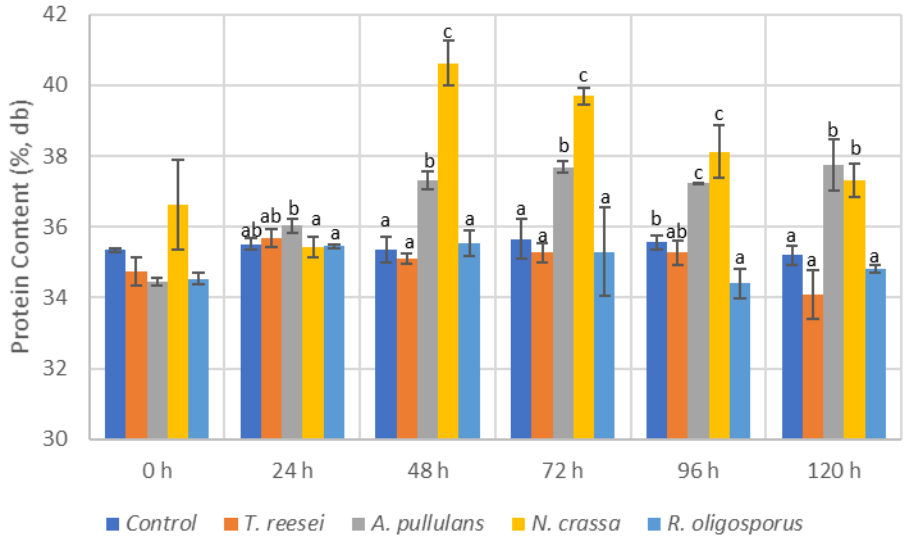

Figure 5.1 Mean values of protein content in pellet after fungal fermentation

Groups of bars with same letter above show no significance when compared to the control at $p < 0.05$. All values on dry basis

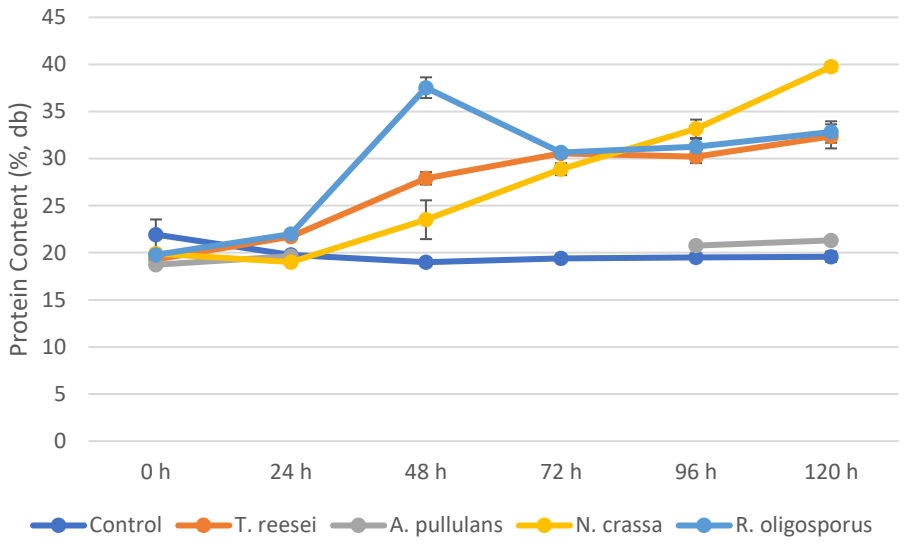

Figure 5.2 Supernatant protein content after fungal fermentation

All values on dry basis

5.3.2.2 Crude fiber, NDF, and ADF

Fungal fermentation of extruded DDGS fibers after 120 h of fermentation are shown in Table 5.4 . The first important observation is the increase in content under all three forms of fiber analysis from the extruded pellets to the uninoculated control samples. The increase is due to the solubilization effect, that was observed in chapter 3, as the soluble portion of the meal was removed with the supernatant. This effect was amplified using fungal fermentation when the fungi hydrolyze components that concentrate in the insoluble fraction such as the fiber.

Crude fiber is determined using the Weende method which removes the soluble fraction leaving behind the cellulose and other insoluble components(Foss, 2018).

T. reesei is known for producing many carbohydrase's' that can degrade the fibrous fraction (Nevalainen et al., 1994). If a cellulase is being produced both the NDF

and ADF will be significantly lower than the corresponding control value which is shown in data. The insignificance of the *T. reesei* crude fiber value also indicates fibers were being degraded and not concentrated while the combined protein of the supernatant and the pellet were increasing.

A. pullulans produces many hemicellulases (Christov et al., 1999; Myburgh et al., 1991). The significant difference of NDF of the *A. pullulans* fermented solids supports this. A lack of cellulase production was seen in the unextruded DDGS and is concurrent with the extruded fermentation demonstrated by the significantly higher crude fiber and the insignificance of the ADF.

Unlike the standard DDGS, the extruded DDGS when fermented with *N. crassa* did not have the largest decrease in fiber concentration. In fact, it was not significantly different from control in the ADF or the NDF value. It did have the highest increase in the crude fiber. This in part may be due to the extrusion of the DDGS which has the effect of altering the digestibility of the fiber for the fungi. The drastic increase in protein may have also caused the concentration of the fibers.

Table 5.4 Fiber content of extruded DDGS with 120h fungal fermentation

	Crude Fiber	NDF	ADF
Uninoculated	8.67 ± 0.12 ^{a}	43.16 ± 4.27 ^{b}	23.02 ± 1.09 ^{bc}
T. reesei	8.94 ± 0.33 ^{a}	32.15 ± 0.70 ^{a}	18.03 ± 2.42 ^{a}
A. pullulans	9.87 ± 0.20 ^{bc}	35.09 ± 0.65 ^{a}	19.06 ± 2.43 ^{ab}
N. crassa	10.56 ± 0.29 ^{c}	41.08 ± 0.81 ^{b}	24.30 ± 1.13 ^{c}
R. oligosporus	9.35 ± 0.57 ^{ab}	35.11 ± 0.80 ^{a}	23.19 ± 1.12 ^{bc}

Mean value of fiber percentage sharing same superscript letter within the column are not significantly different at $p < 0.05$. All value on dry basis

5.3.2.3 Phytic acid

After processing, standard DDGS and extruded DDGS phytic acid values did not change (Chapter 3). When comparing to standard DDGS *R. oligosporus* also had the highest value which was determined as the phytase produced by the fungi degrading the phytic acid into myo-inositol phosphate which is detected by the Megazyme kit.

As discussed in Chapter 4, lignocellulose material of the meal can hold the phytic acid, but when the lignocellulose is degraded the phytic acid is released. This can be seen in the *T. reesei* sample. With the lowest NDF and ADF values the fiber released the phytic acid allowing it to be removed with the supernatant. As *N. crassa* and *A. pullulans* were nearly equal in the standard DDGS fermentation trials the values which is concurrent with the results of the extruded DDGS.

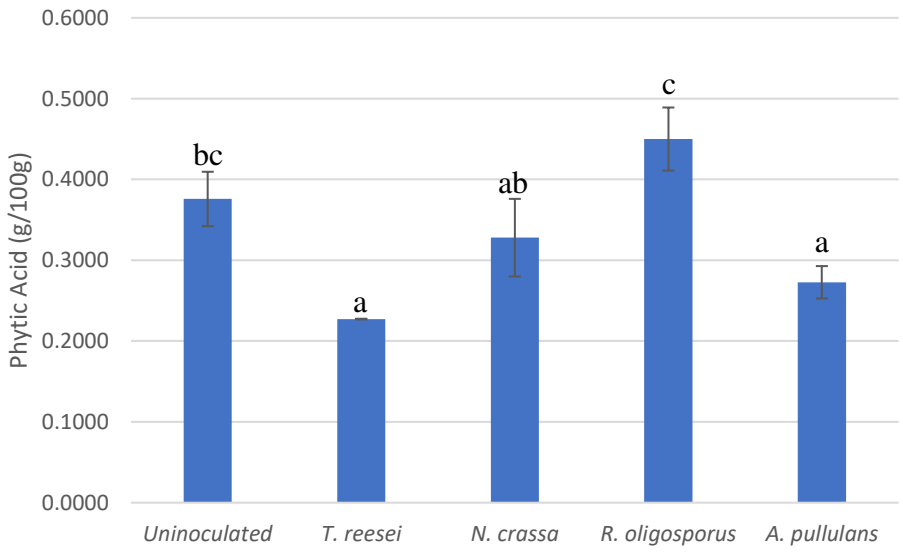

Figure 5.3 Phytic acid at 120 h fermentation.

5.3.3 Enzyme hydrolysis

5.3.3.1 Crude fiber, NDF and ADF

After extrusion the crude fiber and NDF content were reduced. However, after enzymatic treatment NDF was mostly unaffected at 4 and 24h when compared to the control as seen in Figure 5.4. Combination A (1mg/g of cellulase, xylanase, phytase, and pectinase) was able to reduce crude fiber which compares with the results from the standard DDGS studies. Higher NDF and ADF concentrations for all combinations were seen for the extruded DDGS than with the same treatments for the standard DDGS. According to de Vries et al. (2013) extrusion and enzyme hydrolysis had little effect on the NSP. However, better NDF values were seen for the extruded DDGS after fungal fermentation. This may suggest that the ability of the fungi to produce several enzymes may be better treatment of NSP.

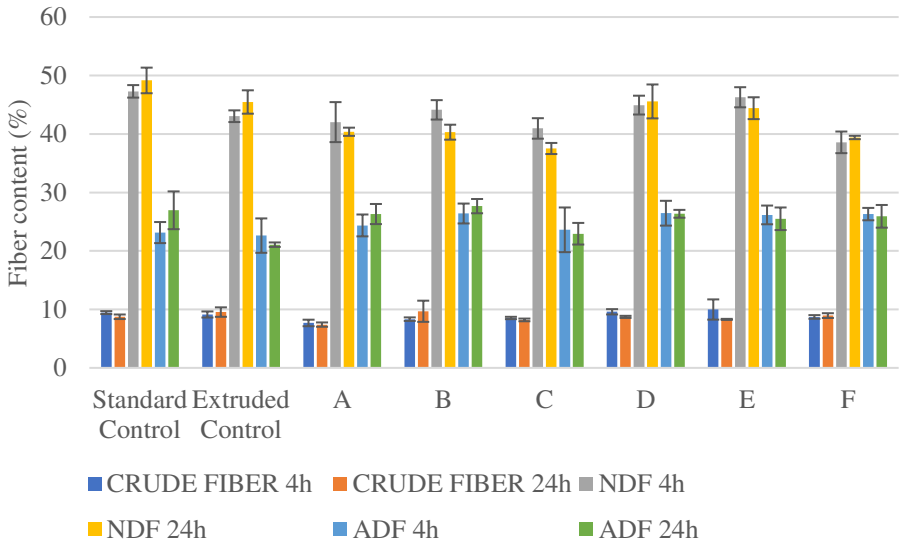

Figure 5.4 Enzymatic hydrolysis of extruded DDGS

All values on dry basis

5.3.3.2 Phytic acid

Phytic acid was significantly reduced after enzymatic hydrolysis as shown in Figure 5.5. Most likely this is due to the use of the phytase during hydrolysis which was included in combination A, B, C, and F. Combinations D and E did not contain phytase which explain the insignificant values. All trials except for F (1mg/g of xylanase, phytase, and pectinase) showed significant decrease in phytic acid from 4 to 24h. This is due to the solubilization effect, that as mixing time increased more phytic acid was solubilized, or the passive diffusion of the phytic acid when in phytate salt form (Perlas & Gibson, 2002).

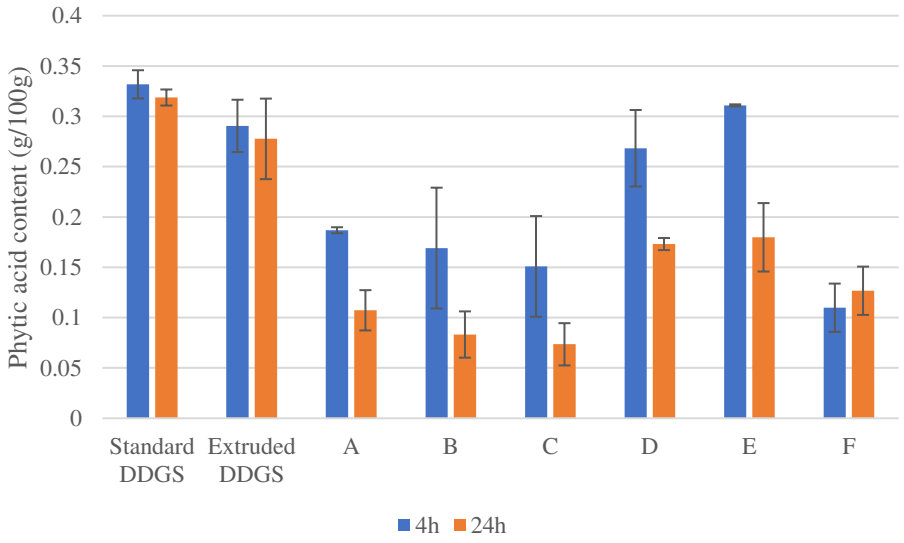

Figure 5.5 Phytic acid content of extruded DDGS after enzymatic hydrolysis

5.3.4 Combined fungal fermentation and enzyme hydrolysis

When discussed combined fermentation and hydrolysis, a saccharification prior inoculation was questioned. Based upon the prior studies, two types of trials were

designed: 4 h saccharification prior to inoculation and addition of enzyme and fungi at the same time. This was then tested on both standard DDGS and extruded DDGS.

5.3.4.1 Protein

Protein content after enzyme hydrolysis and fungal fermentation can be seen in Figure 5.6. In the sequential addition of the enzyme and fungi, 0 h and all time points are considered from the addition of the fungi. Some difference was seen at the 0 h sampling time due to the prior hydrolysis of the samples. An increase in all protein content was seen from 0 to 24 h of incubation due to the growth of the *N. crassa*. However, protein content decrease with the following hours due to the increase in the supernatant protein content as shown in Figure 5.7. This decrease congruent with the other results shown in Figure 5.1 and Figure 5.2; however these may have peaked faster due to the addition of the enzyme.

The most effective treatment was the extruded DDGS using the simultaneous addition of fungi and enzyme at 24 h. *Neurospora crassa* species are a known producer of an extracellular protease, specifically, when grown on a high protein content medium to utilize the amino acids from the macromolecules (Drucker, 1972). The protease activity responsible for the increase of protein in the supernatant, because of the smaller peptide chains it produced during the proteolytic action. Drucker (1972) also found that the inclusion of 2% D-cellobiose was able to increase growth and protease activity of the *N. crassa*. Due to the high content of cellobiose in the DDGS also aids in the conversion of the DDGS by *N. crassa*.

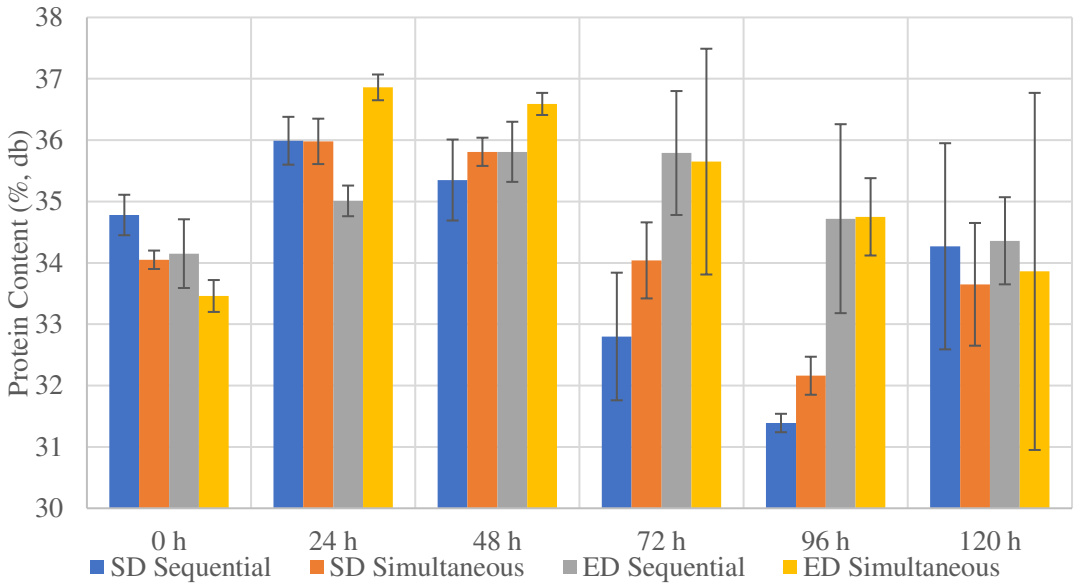

Figure 5.6 Protein content of pellet after enzymatic hydrolysis and fungal fermentation

SD = Standard DDGS, ED= Extruded DDGS, Sequential = 4 h saccharification followed by inoculation of *N. crassa,* Simultaneous = enzyme and fungi added at the same time. All values on dry basis

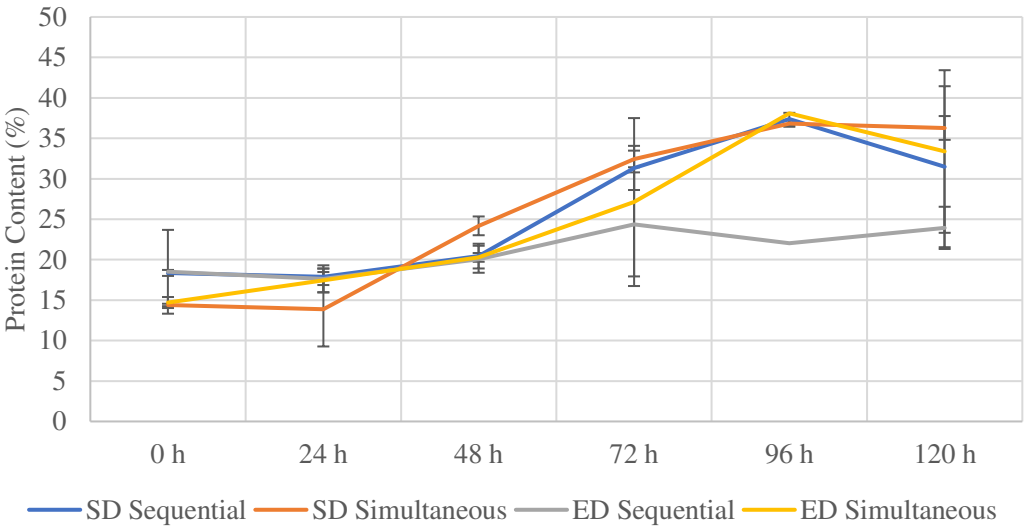

Figure 5.7 Protein content of supernatant after enzymatic hydrolysis and fungal fermentation

All values on dry basis.

5.3.4.2 Crude Fiber, NDF and ADF

Fiber values were lowered at 120 h by using a combination of enzyme hydrolysis and fungal fermentation when compared to the respective controls (standard or extruded DDGS) and to *N. crassa* treated DDGS. During the lag phase of growth, the fungi is not producing lignocellulosic enzymes such as cellulase or xylanase. The addition of the supplemental enzyme during this phase helps increase hydrolysis of the lignocellulosic components of the DDGS until the *N. crassa* can start to produce secondary enzymes such as the cellulase, endoxylanase, or β-glucosidase.

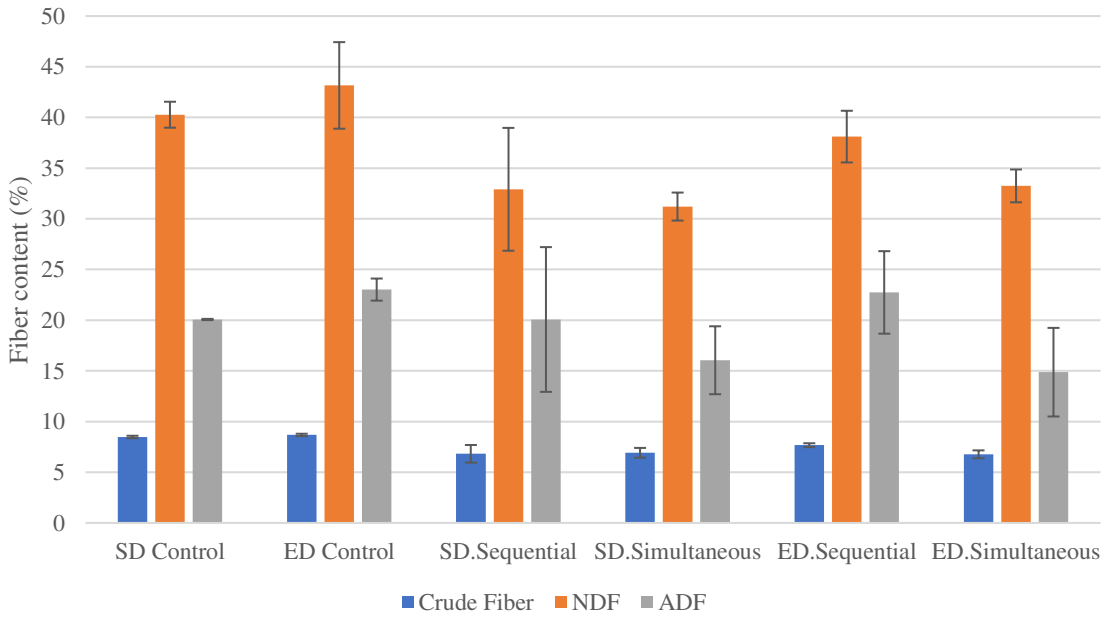

Figure 5.8 Fiber content of DDGS at 120h after enzymatic hydrolysis and fungal fermentation

SD = Standard DDGS, ED= Extruded DDGS, Sequential = 4 h saccharification followed by inoculation of *N. crassa,* Simultaneous = enzyme and fungi added at the same time. All values on dry basis.

5.3.4.3 Phytic acid

Phytic acid was reduced in all combinations of enzyme hydrolysis and fungal fermentation, but the more beneficial combination was through the use of sequential addition of the enzyme and fungus as seen in Figure 5.9 irrespective of extrusion pretreatment. A statistical significance is seen between the extruded simultaneous and extruded sequential trials. It is not understood why they phenomenon occurs.

In previous studies, it was questioned whether the phytic acid was being washed into the supernatant or degraded by the phytase included in the enzymatic hydrolysis. To address this, the same phytic acid assay was tested on the supernatant from the 120 h fermentation. The results are displayed in Figure 5.9 which show very little if any phytic acid in the supernatant. This implies that the phytic acid is being degraded by phytase during the enzymatic hydrolysis.

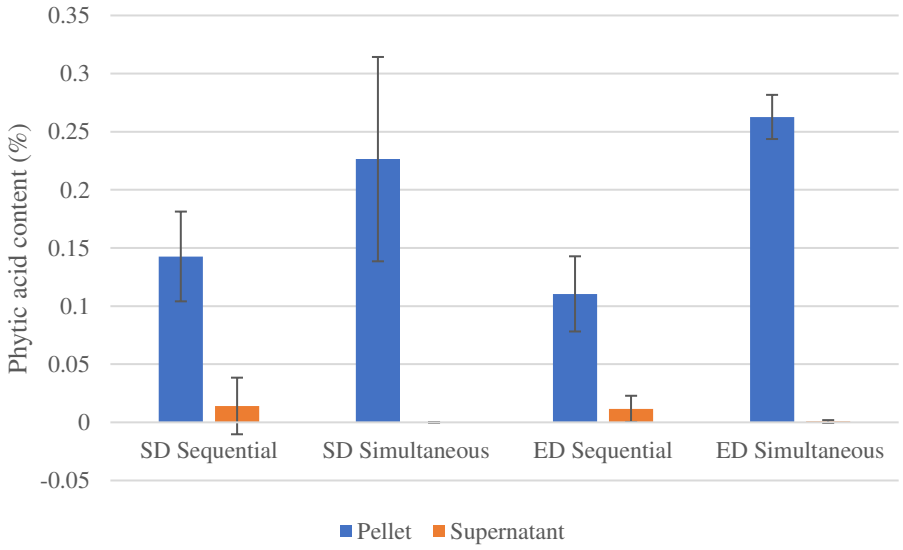

Figure 5.9 Phytic acid content of DDGS pellet and supernatant after enzymatic hydrolysis and 120 h fungal fermentation

5.4 Conclusion

The use of extrusion does improve the basic composition of the DDGS. This can further be improved through enzymatic hydrolysis or fungal fermentation. The combination of extrusion pretreatment, enzymatic hydrolysis, and fungal fermentation can help to further reduce fiber content of DDGS. This can be linked to the extended fermentation than hydrolysis or fermentation could do alone. Maximum protein content can be reached sooner due to the increased access of nutrients by fungi allowed by the extrusion pretreatment and enzymatic hydrolysis. Further research should be done to find minimum time requirement for maximum fiber reduction.

Chapter 6 -Summary and Conclusion

After the congressional act that mandated an increased production of renewable energy sources, the production of distiller' grains as co-products followed (Congress, 2007). Currently, DDGS is utilized in many livestock feeds due to its palatability and availability (Stein, 2008). DDGS is generally composed of 28.7-31.6% protein, 8.3-9.7% crude fiber, 36.7-49.1% NDF, and 13.8-18.5% ADF (Spiehs, Whitney, & Shurson, 2002).

The main limitations of dried distillers' grains are the low protein and high fiber content. The high fiber prevents monogastric consumption due to reduce growth rates and digestive stress (Stein, 2008). The added inefficiencies of the protein and essential amino acids reduces digestibility. Several studies have been done to improve the fiber content by using physical and chemical pretreatments or by adding carbohydrase enzymes to the feed (Noureddini, Byun, & Yu, 2009; Tsai et al., 2017). With all this, the inclusion of DDGS in animal diet is still low. In order to address these challenges, this study used fungal fermentation, enzymatic hydrolysis, and extrusion pretreatment individually and in combinations to treat the DDGS.

Fungal fermentation utilizes the natural production of enzymes to degrade fiber and increase the protein content. This allows for a low-input method of improving the feedstock, easy scale up, and material handling. However, this process takes considerable amount of time and space to obtain preferred results which ultimately increases the cost (Subramaniyam & Vimala, 2012). Fungal fermentation when performed at optimal solid loading rates can help in fine tuning the overall process for maximum yield and efficiency. Submerged state fermentation with different fungal strains enhanced the nutritional profile of the DDGS (Chapter III). However, maximum solubilization effect

was observed at the low SLRs, irrespective of the fungal strains used. Effectiveness of fungal strains in improving the overall composition of the DDGS varied depending on the parameter tested. For example, all strains performed equally on protein levels; while maximal fiber and phytic reduction was achieved with *N. crass* and *A. pullulans* respectively.

N. crassa (NRRL-2332) at a solid loading rate of 20% degraded the fiber the best of all strains at resulting with the 7.8% crude fiber, 30.4% NDF and 19.8% ADF. Also seen was solubilization effect, whereby the process of removing the liquid fraction removed soluble portions such as free sugars and phytic acid. At lower SLR the meal had higher protein but also higher fiber, alternatively the higher the SLR the lower the fiber and protein.

Enzymatic hydrolysis utilizes enzymes to degrade targeted fractions. Typically, the use of commercially available, pre-isolated enzymes is used. This method may be preferred over the fungal fermentation due to the ready active enzyme that can cut down on production time (Shi et al., 2009). As compared to mixing the enzymes with the feed, this processes also ensures that proper conditions for the activity of the enzyme can be met. When fed directly, pH and temperature of the animal's digestive system can effect efficiency (V. Ravindran, 2013).

Enzyme hydrolysis was conducted with a commercially available cellulase, xylanase, phytase, and pectinase at dosages between 0.5 to 2 mg of protein/ g of solid individually and combined (Chapter IV). The most effective treatment was with the use of 1 mg of protein/ g of cellulase, xylanase, phytase, and pectinase on DDGS. Through synergistic effects of the enzymes this was achieved. The cellulase with the accessory

enzymes was able to degrade the most cellulose because the xylanase and pectinase degraded the hemicellulose surrounding the cellulose. The decrease in phytic acid is also due to the synergistic effect because the degradation of the cellulose released the phytic acid and allowed it to be removed with the liquid portion.

Based upon this work, down selection was used to evaluate the effect of fungal fermentation and enzymatic hydrolysis on extruded DDGS (Chapter V). Several studies have shown that to increase access for the biological treatment, a physical or chemical pretreatment is essential. (Kim, Yu, Han, Choi, & Chung, 2011; Zhong, Lau, Balan, Dale, & Yuan, 2009). Following extrusion fermentation and hydrolysis were conducted at a higher SLR based upon results from Chapter III. From Chapter IV, six of the most effective enzyme treatments were selected. And finally, the combination of fungal fermentation, enzymatic hydrolysis, and extrusion were tested (Chapter V).

Fungal fermentation with extruded DDGS utilizing *N. crassa* had the greatest increase in protein content at 48 h of incubation. Enzymatic hydrolysis using 1 mg/g of cellulase, xylanase, phytase and pectinase yielded the most effective treatment in reduction of fiber, but the combination of 1 mg/g of cellulase, xylanase, and phytase resulted in the best reduction of phytic acid in the meal. When extrusion pretreatment, enzymatic hydrolysis, and fungal fermentation were combined in a sequential or simultaneous addition, the use of extrusion and simultaneous addition at 24 h yielded the highest protein content. However, the used of sequential addition was more effective in reducing the phytic acid content in standard and extruded DDGS. All fibers were reduced irrespective of extrusion or enzyme and fungal addition.

Fungal fermentation of DDGS in combination with pretreatments (enzymes and extrusion) has potential to improve the nutritional profile of the DDGS. Therefore, future research could be focused into multiple directions. For example, more small laboratory scale studies should be conducted in exploring the effect of co-fermenting the DDGS using two or more fungi. Since, our findings have suggested that different fungal strains have different ability in terms of fiber and phytic acid reduction; co-fermenting could be beneficial for the overall process. Co-fermentation of the DDGS could be carried out using solids and submerged state fermentation. Whereas further research could also be geared towards upscaling and improving lysine content. In order to attempt feeding trials, production within a larger vessel with better condition control such as aeration, agitation, and temperature is needed. This would hopefully result in better mass balance and more efficiency. Improving the lysine content of the DDGS would also aid it in increased usage. This could be done by simply supplementing the meal or finding a natural process to enhance the amino acid content. Finally, converted DDGS should be then tested to determine palatability, growth performance, and digestibility in fish feeding trials.

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