FERMENTATION OF DRIED DISTILLERS GRAINS WITH SOLUBLES: SCALABILITY AND PHYSICAL PROPERTIES ANALYSIS

by

JONATHAN WILSON

B.S., Kansas State University, 2009
M.S., Kansas State University, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Whole stillage and thin stillage from the ethanol production process were evaluated as substrate sources for the production of β-carotenes using *Sporobolomyces Roseus* (ATCC 28988). This product has the potential to be used as a novel feed ingredient for poultry, swine, or cattle diets. β-carotenes have been supplemented in animal diets to improve animal health, enhance meat color and quality and increase vitamin A concentrations in milk and meat. Microbial fermentations involving growth and product kinetics were performed in 500 mL baffled shake flasks and in a 5 L fermentation bioreactor.

Media optimization was conducted in shake flasks to evaluate two carbon sources: glucose and glycerol, and two nitrogen sources: ammonium sulfate and urea. Final β-carotene concentration of 272.57±4.34 μg β-carotene/g biomass was found to be highest for the whole stillage, with 10 g/L added glucose and 10 g/L nitrogen added through ammonium sulfate supplementation. Glycerol addition yielded no significant increase ($P<.05$) in β-carotene yield, while urea addition significantly decreased ($P<.05$) the final β-carotene concentrations. The resulting fermented product can be blended with regular feed using either whole stillage as a dry feed ingredient or thin stillage as a liquid feed additive.

The fermentation of whole stillage significantly influenced the physical and flow properties of the material. Even though there was a significant decrease ($P<0.05$) in bulk density and increase ($P<0.05$) in tapped density between DDGS and fermented whole stillage, there was a less pronounced difference between the whole stillage and fermented whole stillage. The fermentation of whole stillage significantly influenced the physical and flow properties of the material. This showed that the fermentation process and resulting nutritional profile had a significant effect on the resulting fermented whole stillage.

A 50 L bioreactor was specifically designed to evaluate the scalability of the process and to perform subsequent feed production trails. Pilot scale feed pelleting runs were conducted and the resultant product was put in environmental chambers to determine if β-carotene concentration was reduced as a result of storage. There was a significant decrease ($P<0.05$) in β-carotene levels after pelleting and after 28 d of storage at elevated temperature and humidity. These decreases were consistent with previous research.

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Major Professor
Dr. Praveen Vadlani

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Dedication

To my wife and daughters, without whom, I would be nothing.

Chapter 1 - Literature Review

Carotenoid Usage

Carotenoids are organic pigments that play a major role in the protection of plants against photo-oxidative processes (Chew, 1993). A number of studies have shown that an increased consumption of a diet containing carotenoids is correlated with a decreased risk for several degenerative disorders, including various types of cancer, cardiovascular, or ophthalmological diseases (Mayne, 1996). They also act as color enhancers, as they are commonly used to modify the color of carcass meats to make them appear more desirable to the consumer (Ruiz et al., 2001).

Carotenoids can be produced by a variety by red yeasts. Typical product concentrations range from 50 to 350 μg/g dry weight in strains of *R. glutinis* (Park et al., 2007), *Phaffia rhodozyma*, and *Sporobolomyces roseus* (Nanjundaswamy, 2010). Carotenoids are generally localized in lipid droplets in red yeasts and other fungi, while the reddish-pink color of *Sporobolomyces roseus* is due to the presence of three carotenoid pigments ß-carotene, torulene, and torularhodin (Vandamme, 1992). Carotenoids are responsible for many of the red, orange, and yellow hues of plant leaves and fruits, as well as the colors of some birds, fish, and crustaceans (Frengova and Beshkova, 2009). Only plants, bacteria, fungi, and algae can synthesize carotenoids, but many animals incorporate them from their diet (Mayne, 1996).

The red yeasts *Phaffia, Rhodotorula* and *Sporobolomyces* have been identified to produce large amounts of carotenoids, and have been the main subjects for genetic modification from their wild forms to increase product yields. Some of these yeasts can produce the two most valuable carotenoids, ß-carotene and astaxanthin, thereby providing an alternative to chemical

synthesis (Vandamme, 1992). These yeast cultures can also thrive on low cost substrates harvested from underutilized product streams, such as distillers grains (Nanjundaswamy, 2010).

Major sources of naturally occurring β-carotene include green leafy vegetables, as well as orange and yellow fruits. However, the bioavailability of β-carotene from green leafy vegetables, such as spinach, is thought to be low (Castenmiller et al., 1999). Factors other than the type of food containing the β-carotene are thought to be important in its bioavailability. These include cooking, grinding, and the presence of dietary fat, all of which improve the bioavailability (Rock et al., 1997).

The vitamin A activity by a number of pigmenting carotenoids used in animal feed has been studied intensively (Chew, 1993; Emin et al., 2012; Jintasataporn et al., 2012). Carotenoid molecules that are converted into vitamin A tend to lose their pigmenting properties (Olson, 1989). However, there is no fixed ratio between the deposition rate of individual carotenoids for pigmenting efficiency and their vitamin A activity. Only 10% of the 600 carotenoids are known to have pro-vitamin A activity in mammals (Rock, 1997). β-carotene, α-carotene and β–crytoxanthin are the major pro-vitamin A carotenoids, with β-carotene generally regarded as the most important of the three because it is more easily converted to vitamin A (Olson, 1989).

The unique structural differences between carotenoids determine their potential biological functions (Britton, 1995). Figure 1.1 shows a simplified pathway of how the yeast strain converts acetyle CoA in β-carotenes. Most carotenoids can be derived from a 40-carbon basal structure, which includes a system of conjugated double bonds. The central chain may carry cyclic end-groups, which can be substituted with oxygen-containing functional groups (Stahl, 2003).

Based on their composition, carotenoids are divided in two classes: (1) carotenes containing only carbon/hydrogen atoms and (2) xanthophylls, which carry at least one oxygen atom. The pattern of conjugated double bonds in the polyene backbone of carotenoids determines their light-absorbing properties and influences the antioxidant activity of carotenoids (During et al., 2002). Carotenoids tend to isomerize and form a mixture of mono- and poly- cis-isomers in addition to the all-trans form. The all-trans form of carotenoids is commonly found in natural environments (Stahl et al., 1993).

Carotenoids, which are fat-soluble, follow the same intestinal absorption path as dietary fat. As a consequence, carotenoids tend to accumulate in fatty tissues within animals. Due to their varying lipophilic properties between carotenoid varieties, there is a difference in the efficiency of absorption and transportation within the animal, based on the type (Wellenreiter et al., 1969). Release from the food matrix and dissolution in the lipid phase are important initial steps in the digestion process. Carotenoids are thought to be absorbed by the small intestinal mucosa via passive diffusion (During et al., 2002). Fatty acid esters of xanthophylls are cleaved in the lumen of the small intestine prior to uptake by the mucosa. Carotenoids are taken up by the mucosa of the small intestine, and packaged into triacylglycerol-rich chylomicrons. β-carotene and other provitamin A carotenoids are partly converted to vitamin A in the intestinal mucosa. Both carotenoids and retinyl esters are incorporated into chylomicrons, and secreted into the lymph duct for transport to the liver (Parker, 1996).

β-Carotene is the most biologically active, provitamin A carotenoid in nature. However, the efficiency of conversion of β-carotene to vitamin A differs between different species. For example, 1 mg of β-carotene is equivalent to 500 IU of vitamin A in pigs, with lower conversion efficiencies observed with higher β-carotene intake (Wellenreiter et al., 1969). These conversion

efficiencies are three times lower than those for poultry (Chew, 1993). In addition, there are wide variations among different species in the ability to absorb β-carotene intact and the quantities present. For example, carotenoids are detected in much lower quantities in the digestive tracts of swine than they are in ruminants or humans (Chew et al., 1984). Species differences in β-carotene conversion to vitamin A and absorption of β-carotene are due to differences in the quantity and activity of conversion enzymes, presence or absence of transport proteins, and other factors (Chew, 1993).

Not only do β-carotenes act as a precursor of vitamin A and thus an essential component of the diet of humans and animals, but they also play an important physiological role as protective antioxidants (Krinsky, 1994). Within the yeast cells, they function to help preserve the lipids used for cell maintenance during time of dormancy.

By fermenting different product streams from the distillers' grains process, there is a potential for a value-added, customizable feed product to be created. In swine diets, about 10% dried distillers grains with solubles (DDGS) is normally used, although higher amounts of up to 50% can be used depending on the stage of growth (Shurson and Noll, 2005). By enhancing the vitamin A concentration of DDGS, there is an opportunity to decrease the levels of supplementation in the overall diet and add value to the feed ingredient.

The composition of carotenoids differs qualitatively and quantitatively based on the source, recovery method, and method of inclusion into a diet. Efficacy is also highly dependent on factors such as processing and storage conditions (Rodriguez-Amaya, 1999). Micronutrients, such as carotenoids and vitamins, are more likely to be damaged by the feed manufacturing process than the macro ingredients (Rickman, 2007). Generally, micro ingredients contain very concentrated levels of nutritional components, which also suggests that they are generally the

most expensive on a per pound basis (Rickman, 2007). Vitamins and carotenoids are sensitive organic compounds that can be denatured by water, oxygen, trace minerals, heat and other factors (McGinnis, 1986).

The feed manufacturing process involves water, heat, and mechanical stresses, all of which can negatively impact the vitamin and carotenoid stability. Feed processing is a versatile, but high temperature process that may alter the nutritional value of macro ingredients as well (Fahrenholz, 2012). In some instances, this effect is positive for the digestibility levels of the diet and for processing considerations. For instance, the gelatinization of corn starch not only increases the bioavailibity of the nutrients in the diet, but it also increases feed quality due to its pellet binding ability (Fahrenholz, 2012).

A research study conducted by Emin et. al. (2012) observed effects of extrusion on the stability of β-carotene. In this study, they used an oil-based solution as a carrier for β-carotene due to its lipophilic nature, and processed it under varying pre-conditioner retention times (300,500, and 800) and different temperatures (135-170°C). As expected, the shorter time the β-carotene was exposed to the extreme processing conditions, the less degradation occurred. The results showed that at the lowest rpm (300) and the highest rpm (800) negatively impacted β-carotene levels. The 500 rpm shaft speed had the least effect on β-carotene levels. However, temperature did not effect β-carotene concentration or cis-isomers.

Additional processing can sometimes have a beneficial effect on sensitive feed ingredients. Increased steam conditioning can lead to a decrease in mechanical friction during pelleting, as determined by lower temperature rise across the pellet die, decreased electrical energy consumption, and an improvement in pellet quality (Fahrenholz, 2012). Steam energy requirements and electrical energy requirements both increased with temperature due to the

higher steam requirement. This higher energy requirement is offset by improvements in throughput and pellet durability. This is fairly dependent on factors such as diet formulation and the types of processing equipment used (Skoch et al., 1981).

Jintasataporn et al. (2012) examined the effect of diet pelleting on synthetic β-carotene addition. They examined stability of formulated carotenoid diets during feed pelleting and measured the efficacy of β-carotene under different storage conditions. Two treatments with and without butylated hydroxytoluene (BHT) were evaluated for total carotenoid loss during feed processing. The completed dietary carotenoids were stored in an aluminum foil bag and kept under different storage conditions at 26 to 28°C and 4°C. The results showed that the diet pelleting process did not significantly affect ($P<0.05$) the carotenoid content of the diets, and pellets stored at 4°C contained the highest levels of β-carotene post storage. Feed for this trial was processed using a HOBRAT mincer, a bench-top pelleting machine that does not accurately represent the processing conditions that commercially produced feed undergoes, because there is no steam conditioning process. The only heat the pellets were subjected to was due to pellet die friction. It is possible that the β-carotene in the diet was subjected to lower levels of heat than it would be subjected to in commercial operations.

Dried Distillers Grains with Solubles (DDGS)

Ethanol has had a huge impact on U.S. economy over the past few years. It helps to create a cleaner burning fuel and provide an alternative marketplace for farmers to sell their grains. There are opportunities which exist within the corn ethanol production process that can yield additional revenue. Many of these opportunities exist within the production of dried distillers grains with solubles (DDGS), which is a byproduct resulting from the fermentation of cereal grains for the production of alcohol for beverage or fuel (Renewable Fuels Association,

2015). They are a valuable source of energy, protein, water-soluble vitamins, and minerals for animals. Various methods are used in the preparation of DDGS and variables such as grinding and drying methods can play a significant role in the resulting composition and quality of the DDGS (Knott et al., 2004).

Distillers grains have the reputation for providing a low quality pellet when it is the major ingredient in the pellet structure (Rosentrater, 2007). The advantage of using DDGS is that it offers a high protein, readily available ingredient which can be utilized by all production animals. Higher inclusion levels in diets have the potential to lower feed prices while providing the industry with an additional quality feed ingredient (Fahrenholz, 2012).

Before the ethanol boom in the early 2000s, DDGS were produced primarily from the beverage industry and were utilized by nearby farms in wet form. Even ethanol production from cereal grains for the purpose of energy is not a new concept. When Henry Ford designed the first vehicles, he originally envisioned them running off ethanol (Kovarik, 1998). The reason ethanol has become so important in the last decade is because of rising fuel prices and the dependency on imported fuel to sustain our energy needs. Corn-based ethanol became one of the proposed solutions to the problem, and soon became federally mandated. Not only did this start to consume much of our feedstock resources, but it also drove up the ingredient prices (Renewable Fuels Association, 2015). Still in this case, the problem also created opportunities for new feed ingredients.

Two major co-products from the corn to ethanol production process are the CO_2 and DDGS, which contain all the major nutrients of corn minus the starch, which is consumed during the ethanol production. In general, this separation is a three way split consisting of equal parts

ethanol, CO_2 and DDGS. With the removal of starch, other nutritional components are compounded during the corn to ethanol conversion process.

There are numerous reasons for feed mills to be interested in incorporating DDGS into their diet formulation. On average, DDGS have a protein content of 26% (Knott et al., 2004). The high protein content makes DDGS an ideal ingredient for ruminant diets as well as monogastric diets (Behnke, 2007). The protein is particularly beneficial to ruminants because it is a bypass protein (Ganesan et al., 2007). Its high protein makes DDGS a good substitute for soybean meal and can also help to offset the amount of corn being taken out of the feed production stream by the ethanol industry.

DDGS has poor flow properties during storage and transportation (Behnke, 2007). It has also been reported that this valuable product will not pellet when used as a large portion of the diet. Recent research conducted at Kansas State University has shown that DDGS can be included in up to 30% of the complete diet formulation and still demonstrate acceptable pellet quality (Fahrenholz, 2008).

Challenges with transporting DDGS, particularly over long distances, include not maximizing vessel payload due to the product's low product bulk density, and bridging problems resulting in unloading difficulties (Ganesan et al., 2005). Pelleting has long been recognized as a way in which the bulk density and the flowability of a product can be improved, and this has been applied extensively to DDGS in the past decade.

Rosentrater and Kongar (2009) reviewed the cost factor of implementing pelleting technology within the ethanol processing facility to improve transportation and flow ability of DDGS. They reported that with increasing DDGS production rates, the cost of pelleting is significantly reduced because of the economies of scales. For instance, the cost to pellet 100

ton/d was $14.07/ton/y, whereas at a heightened scale of 1000 ton/d, the cost to pellet was reduced to $3.95/ton/y. The option of including a pellet mill is appealing to the ethanol industry because the cost to pellet is minimal in large scale production facilities compared to the overall cost of manufacturing.

According to Skoch et al. (1983), "Advantages of pelleting include decreased segregation, increased bulk density, reduced dustiness and improved handling and transportation of finished feeds. However, rising energy and equipment costs are making it increasingly difficult to justify the pelleting of formula feeds." If energy prices were a consideration in the 1980's, then they certainly are an important factor in today's market.

The quality of pellets made using DDGS are generally poor and lack the structural integrity of high quality pellets. Pellet quality can be defined by the ability of pellets to be subjected to handling and transportation without generating excessive fines (Behnke, 2005). Factors that influence pellet quality are the pellet die, steam conditioning, drying, grinding, and product formulation (Behnke, 2005).

Compression of the mash through the pellet die supplies the resistive force by which pellets are pressed and formed. This causes the cylindrical shape of pellets as well as an increase in bulk density. The amount of friction can be increased by slowing the production rate, thereby increasing retention time in the die, or by increasing the length of the die. The drawback to these two methods is they cause lower throughput and increased energy consumption, respectively (Fahrenholz, 2012). The ability to produce a quality pellet is balanced by the amount of money a producer is willing to spend on the means to achieve it.

Steam conditioning is used to add moisture and heat to the mash prior to pelleting. This combination causes a "liquid bridge" between particles and this bond is exacerbated by the

friction caused in the die. The temperature the mash is heated to determines how strong this bond will be (Behnke, 2005). The higher the heat and moisture the stronger the bond, but the amount of steam that can be added is limited by the initial moisture content and by interactions with the formulation. After steam conditioning and pelleting the product must then be dried to increase shelf life through moisture removal (Skoch et al., 1983).

Particle size of the incoming mash also has a large impact on the resulting pellet quality. There is still some debate amongst experts, but it is generally accepted that smaller particle sizes expose more surface area for particle to bond to each other during pelleting. Particle shape also has an influence on flowability and pelleting. Particle shape for distiller's grains can be likened to that of soybean meal, in that the bran particles are flat, plate-like structures that have a tendency to stack and compress, causing product bridging (Behnke, 2007). In addition, Ileleji (2007) found that distiller's grains flow is impacted by the granulometric properties of the mash, which include particle size and shape, particle distribution, particle density and bulk density. These properties can be influence by processing procedures during the ethanol production as well as conditioning steps taken in the feed mill.

Of all the factors influencing pellet quality, formulation is the most important. Formulation refers to protein, carbohydrate, fat, fiber and ash content of the material. Increased levels of protein and fiber are known to increase pellet quality (Behnke, 2007). Fat levels also have a positive effect on pellet quality up to a point. When fat is added to the diet in levels greater than 1% of the entire diet, pellet quality suffers (Behnke, 2001). While the high fat content of DDGS is regarded as positive from an animal nutrition standpoint, it is a detrimental feature in regards to flowability and storage. Knott (et al., 2004) took distillers grains samples

from sixteen ethanol plants. They found an average fat content of 10.06%. The high fat content reduces friction in the die and results in low pellet quality.

Along with formulation, the problem of nutrient variability among ethanol plants is persistent. In addition to the high fat content Knott observed in the ethanol plants, a CV of 7 was also found. The lowest fat content was 9.2% compared to a high of 11.55%. This kind of variation can also be found in the protein and fiber content of DDGS among different plants. This results in difficulties in the pelleting process due to a non-uniform product and variables that need to be adjusted in order for efficient production.

Outside of rejecting loads which fall outside of purchasing specifications, feed mills cannot control characteristics of DDGS such as the fat content. These are issues that need to be resolved in the plants by standardizing drying processes and the levels of added solubles. Looking beyond the ethanol production process, there are some issues that we can resolve at the tail end of the ethanol plants to make shipping and processing much more efficient by the inclusion of a pellet mill.

Research using finishing pigs has shown that DDGS can be fed in diets up to 30% before performance is affected (DeDecker et al., 2005). However, DeDecker also reported inconsistent responses to added fat fed with dietary DDGS. Because of the high oil content of DDGS, interactions with diets containing added fat and DDGS might be present (Spiehs et al., 2002). This could potentially pose a problem since added fat is used to increase the energy content of diets.

In addition to growth performance, studies have also been conducted to examine how the level of DDGS might affect feed preference. Hastad et al. (2005) conducted a series of experiments to determine if growing pigs showed preference to diets containing a specific level

and source of DDGS. Pigs were fed diets with and without DDGS (30% inclusion) and from DDGS which were manufactured in two different plants. They found that feed intake was lower for each of the diets containing DDGS compared to the control diet. In a second experiment, diets were fed containing 0%, 10%, 20%, or 30% DDGS, and there was a linear decrease ($p<0.01$) in average daily feed intake (ADFI) as the level of DDGS in the diet increased. These experiments demonstrate a preference for feeds not containing DDGS and indicate that there is a palatability issue with high levels of inclusion.

It is possible that through a value added fermentation process, DDGS could be made into a more palatable feedstock as well as the amount of total fat in the feedstock. There is an opportunity to substitute current DDGS inclusion levels in animal diets with a modified DDGS product which can provide the additional nutritional benefits already associated with added synthetic β-carotene.

Good Manufacturing Practices in Fermentation

Biomaterial manufacturing facilities in the United States are regulated by the Food and Drug Administration (FDA). Specifically, they are governed by the Food, Drug, and Cosmetic Act of 1938 and the amendments of 1962 and 1976. The Act and subsequent amendments give the FDA the power to regulate the processes for feed ingredient manufacturing. The Act addresses the requirements of manufacturing facilities in the Code of Federal Regulations, Title 21, Part 210 and 211, referred to as Current Good Manufacturing Processes (CGMPs).

β-carotenes are covered under ingredients listed as generally regarded as safe (GRAS), but still require appropriate labeling so they can be identified in finished feed (CFR 21, 2012). To determine adequate levels for inclusion, the most appropriate method would be to put β-carotenes in terms relative to vitamin A inclusion. Vitamin inclusion is generally calculated

based on the appropriate International Unit (IU) for the specific vitamin. The mass or volume that constitutes one IU varies based on which substance is being measured. For example, 1 mg of β-carotene is equivalent to 500 IU of vitamin A in pigs (NRC, 2012).

The targeted level of β-carotenes in the finished modified DDGS product will likely be based on the projected inclusion rate in the specific diet, the age of the animal and the species of animal it is being fed. β-carotene levels can be controlled by the total amount of available substrate and the total fermentation time. Provided the ethanol plant on the front end is providing a fairly consistent product, the level of β-carotene can be control by just the active time in the fermenter.

The manufacturing process for modified DDGS could either use the same centrifuge and dryer system as the existing DDGS production line or require the installation of new equipment. Plants would most likely want to retain the ability to manufacture unmodified DDGS and while reusing equipment could save money, this could also cause problems with cross-contamination. In the proposed model, a retrofit would be added to existing ethanol plants to include a separate centrifuge and dryer. This will not only reduce the chances for cross-contamination but also reduce cleanout time and labor required.

After fermentation, the stillage will be centrifuged and drum dried to a moisture level of <12% (w.b.). Once appropriate de-activation temperatures and water activity levels are determined, protocols may be set in place for critical limits that the product must meet before exiting the manufacturing facility.

After the drying process, the product will be sent to a series of load-out bins. The finished product will either be sacked off in individual bags, bulk sacks, or sent to a bulk load-out system and be directly loaded into trucks depending on the customer base in the surrounding

area. Bagged and bulk product will be subject to labeling requirements which include concentration, storage instructions and appropriate usage. It is unlikely that plants would run unmodified DDGS through a bagging, because of the low throughput of bagging lines, system so there should not be any chance for cross-contamination, but they will most likely share some downspouts from the bulk load-out.

Storage and Transportation of DDGS

The nutritional composition of the modified DDGS can differ greatly from traditional DDGS because of the energy required for the additional fermentation. Nanjundasamy and Vadlani (2010) showed that the nutritional composition of DDGS fermented with *Sporobolomyces roseus* was negatively affected by the fermentation. They noted a decreased level of crude protein (27.77% to 17.75%) and an unchanged level of lysine and tryptophan in the fermented product. There is a possibility that the nutritional composition of the modified DDGS was artificially higher than the commercial DDGS because not all the glycerol was consumed by the microbes; in addition the portions of the stillage which *Sporobolomyces Roseus* consumes for the production of β-carotene.

During the fermentation process, yeast cells are reproducing and generating β-carotene intracellularly. The yeast will first consume the residual simple sugars from the ethanol process as it is the easiest energy source for them to convert toward growth and product development. After all the residual glucose has been consumed, the yeast will consume non protein nitrogen, other protein sources, and fatty acids for growth. While not as efficient as glucose, it still provides them with an adequate energy source. As a result of this process, the nutritional profile and chemical composition of the resulting product is changed significantly (Nanjundasamy, 2010).

Since the yeast will consume a portion of the lipids present for the production of β-carotenes, the modified DDGS could exhibit flow properties similar to those of de-oiled DDGS. This could expand the types of conveying equipment which could be used and allow the product to be stored in bins unsuitable for regular DDGS due to bridging problems. DDGS contains widely variable levels of fat (between 3 and 13%) based on the method of concentration for solids or the up-front fractionation of corn (Kingsley et al., 2010).

Most of the oil in DDGS is contained in the solubles which are added in after the slurry is dried. Oil may be present in the solubles in four different forms: 1. oil in water emulsion; 2. oil droplets attached to hydrophobic proteins; 3. oil bodies in endosperm and germ particles; and 4. oil bodies from broken cellular structures (Majoni et al., 2010). It is unclear at this time if the *Sporobolomyces roseus* will consume oil from a specific form if it is indiscriminate.

Depending on the nutritional profile of the modified DDGS, there may be a need to add the solubles after the β-carotene fermentation has been completed so the overall energy level of the ingredient is still relatively high. Notably if a flowability problem that arises with the finished product related to the fat levels, numerous steps can be taken to mitigate the impact on overall product quality and perception.

Certain diet processing methods, such as extrusion, can require a post-grind after all ingredients are mixed to ensure there are no large particles that could disrupt the screw flighting. Extrusion processes tend to post coat fat onto the finished product because high fat levels can lead to a decreasing in milling efficiency (Briggs, 1999). A decreased level of fat in the modified DDGS might be advantageous in a process such as this.

Srichuwong (2011) measured the total residual sugar (TRS) in commercial DDGS. The TRS consisted of free glucose, oligosaccharides and residual starch. The commercial

manufacturer's DDGS contained 15.8%, w/w TRS. These residual sugars are easily utilized by a variety of microbes and can lead to mold growth and the resulting spoilage of the product very easily. Water activity level, storage temperature, and storage method all play a very important role in determining storage stability, but by decreasing the TRS level of the finished product, mold and microbial growth could be slowed due to the lack of an easily digestible substrate.

Lastly, the modified DDGS pose some interesting mechanical processing and storage considerations. Physical factors like temperature, aeration, pH and light have been shown to have a significant effect on β-carotenes (Frengova et al., 2009). Aeration is not something that can be controlled in most storage scenarios for processing plants, and it is unlikely that pH will be much of a cause for concern as the finished product has a pH close to neutral. This leaves temperature and light exposure as the primary factors that could harm the β-carotenes in the modified DDGS.

Foremost on the list is the sensitivity to temperature. This could cause a risk to β-carotene efficacy as storage facilities generally do not have temperature. It is unlikely that the temperatures in a normal storage bin would reach high enough to compromise β-carotenes, but in hot and humid regions, such as the southeastern portion of the United States there is a potential for condensation to develop in the bins and lead to hot spots. In most cases, this is going to be isolated to bins which have product stored at high moisture levels. Depending on where these bins are located within the cluster, however, they could put off radiant heat and affect nearby bins. Long-term high temperature exposure could denature β-carotene in the right conditions.

While not a storage or transportation concern, there is a significant temperature increase when grinding feed products. This is more of a cause for concern than a process like pelleting or extrusion because the temperatures are not monitored in most grinding operations. If plants have

a need to post-grind ingredients after mixing for uniformity purposes, they should be especially cautious in the flow rate of the mill, since a higher flow rate can lead to increased grinding temperatures.

Light exposure can also lead to the oxidation and degradation of β-carotenes (Henry et al., 1998). It is very unlikely that a high-value product would be stored in a concrete bunk that is exposed to the elements, but it is an important limitation that should be noted.

A moisture content of less than 12% is recommended for feed products because this level minimizes transportation costs and is microbiologically stable (Beuchat, 1981). Water activity refers to the amount of unbound water available for use by microorganisms and chemical agents, and is subsequently a measure of a material's susceptibility to spoilage and mold growth. Products with no free water (aW =0.0) are not at risk for spoilage, while products with free surface water (aW =1.0) are at a very high risk for product spoilage. Products have a reduced chance for bacterial growth below water activities of approximately 0.9, mold growth below approximately 0.7 to 0.8, and yeast growth below 0.7 (Barbosa-Canovas, 1996).

Rosentrater et al. (2006) measured the water activity in DDGS from six different plants. Even though the samples studied had low levels of water available for microorganism growth, with water activity values between 0.53 and 0.63, it appears that they may be prone to spoilage problems due to potential moisture migration (because of the high moisture content levels) when they are stored in bulk. It is important to note that water activity and moisture content are two separate measurements. The results reported by Rosentrater et al (2006) are presented in the Table 1.1. as presented by the authors.

The average moisture content is high enough to present a potential storage issue even though the water activity levels are below the risk levels for microbial growth. Moisture

contents at these high levels can cause problems during storage and unloading such as bridging or the generation of hot spots and rapid mold growth (Higgins and Brinkhaus, 1999).

Mold growth and the by-products which it produces are perhaps the primary cause for concern in DDGS. Different species of *Aspergillus* and *Fusarium* are capable of producing aflatoxin and vomitoxin, respectively (Xie, 2006). DDGS are already red flagged for the potential to contain high levels of aflatoxins, due to the compounding effects of the ethanol process, so further contamination is particularly problematic. Xie (2006) also found that *Aspergillus Niger* produced high levels of citric acid in wet distillers' grains. Citric acid will alter the flavor of the feed, alter the pH and could provide a growth medium for other types of mold growth.

Microflora can decrease grain value through nutritional changes, physical damage, feed palatability or the production of toxins (Higgins & Brinkhaus, 1999). Currently a variety of organic acids and salt derivatives are used as anti-microbial agents in animal feed. For example, propionic acid inhibits the growth of fungi and prevents production of mycotoxins. Use of propionic, formic, sorbic and lactic acids in feedstuffs has been reported to reduce the growth of fungi, yeast and bacteria (Paster et al., 1988).

Magan et al. (1988) found that storage fungi, especially Aspergillus spp. and Penicillium, are able to grow at low water activities (aW = 0.70 to 0.75) enabling them to initiate grain spoilage. The ability of storage fungi to germinate, grow and sporulate in stored grain is dependent on the availability of water in the substrate, temperature and the intergranular gas composition. Since DDGS go through a grinding process before fermentation, there should be very little intergranular gas composition since the original endosperm matrix has already been ruptured.

In a study by Kingsley et al. (2010), it was concluded that product variability of DDGS was primarily due to the levels of thin stillage added during the drying process. The whole stillage is made up of primarily the fiber, protein and ash residuals from the ethanol process while the thin stillage contains primarily fats and other solubles. Increasing CDS levels in the drying process resulted in increasingly darker colored DDGS with reduced levels of protein, ADF and NDF, while ash, oil, residual sugars and glycerol contents in DDGS increased. Additionally, the true and bulk density, particle size and particle size distribution increased with increasing CDS levels. They concluded that the best way for controlling the product consistency in DDGS lies in adding a consistent level of CDS to WDG during the drying process.

Figures and Tables

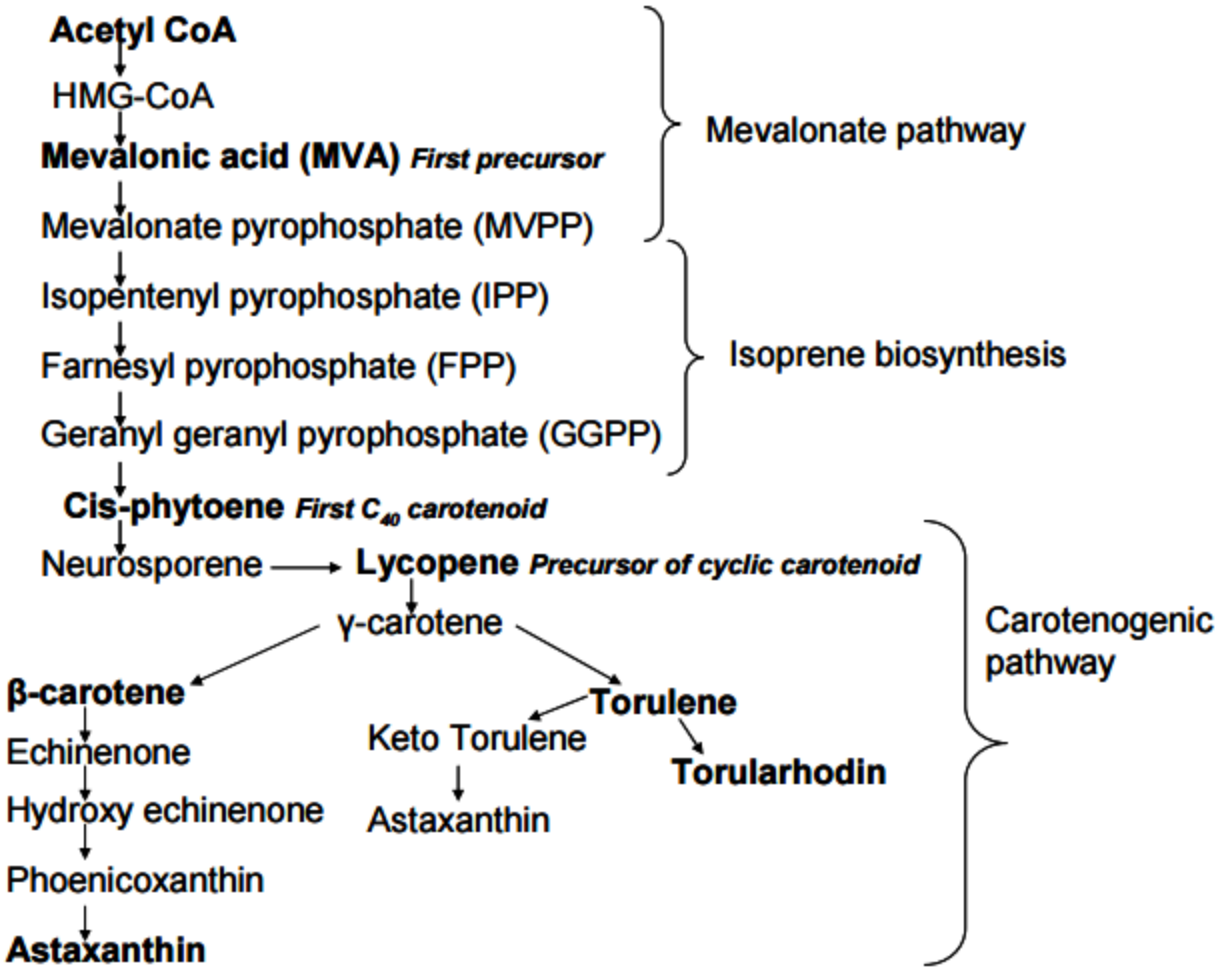

Figure 1.1 Carotenoid Production in *S. roseus* (Source Nanjundaswamy, 2010)

Table 1.1 Nutritional Analysis of DDGS From Various Plants (Source: Rosentrater et al., 2006)

Physical Property	Plant	Observations	Minimum	Maximum	Mean	Standard Deviation
Moisture (% d.b.)		72	13.20	21.20	14.7	1.5
	1	16	13.90	14.80	14.5	0.2
	2	4	13.20	13.40	13.3	0.1
	3	4	14.30	14.70	14.5	0.1
	4	16	13.80	15.00	14.3	0.3
	5	16	13.60	21.20	16	2.1
	6	16	14.20	14.90	14.6	0.2
Compressibility Index		72	0.53	0.63	0.55	0.02
	1	16	0.54	0.56	0.55	0.01
	2	4	0.62	0.63	0.63	0.01
	3	4	0.54	0.55	0.55	0.01
	4	16	0.54	0.55	0.54	0.00
	5	16	0.54	0.57	0.55	0.01
	6	16	0.53	0.56	0.54	0.01

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Chapter 2 - Objectives

The objectives of the research described in chapter three are: (1) to determine if *Sporobolomyces Roseus* can produce β-Carotenes by utilizing residual sugars from whole stillage, and (2) to determine if protein and fat levels could be maintained by supplementing the fermentation with various source of non-protein nitrogen.

The objective of the research described in chapter four is to determine if the production process could be scaled to a 50 L vessel and produce an equivalent or better product, both nutritionally and in regards to handling/processing variables, compared to lab scale.

The objective of the research described in chapter five is to evaluate the physical properties of the resulting product in regards to bulk bin storage and transportation.

The objectives of the research described in chapter six are (1) to determine if the β-carotene concentration is affected by the pelleting process after the modified whole stillage is included into a complete feed diet and (2) to determine if there was a relationship between storage stability and the final β-carotene concentration of the complete diet.

Chapter 3 - Benchtop Optimization of Modified DDGS Fermentation and Analysis

Introduction

Distillers dried grains with solubles (DDGS) is a byproduct resulting from the fermentation of cereal grains for the production of alcohol for beverage or fuel. DDGS is a valuable source of energy, protein, water-soluble vitamins, and minerals for animals. Various methods are used in the preparation of DDGS, and variables such as grinding and drying methods can play a significant role in the resulting composition and quality of the DDGS.

Research using finishing pigs has shown that DDGS can be fed in diets up to 30% before performance is affected (DeDecker et al., 2005). However, DeDecker also reported inconsistent responses to added fat fed with dietary DDGS. Because of the high oil content of DDGS, interactions with diets containing added fat and DDGS might be present (Spiehs et al., 2002). This could potentially pose a problem since added fat is used to increase the energy content of diets.

In addition to growth performance, studies have also been conducted to examine how the level of DDGS might affect feed preference. Hastad et al. (2005) conducted a series of experiments to determine if growing pigs showed preference to diets containing a specific level and source of DDGS. Pigs were fed diets with and without DDGS (30% inclusion) and from DDGS manufactured in two different plants. They found that feed intake was lower ($p<0.05$) for each of the diets containing DDGS compared to the control diet. In a second experiment, diets were fed containing 0%, 10%, 20% or 30% DDGS and there was a linear decrease ($p<0.05$) in average daily feed intake as the level of DDGS in the diet increased. These experiments

demonstrate a preference for feeds not containing DDGS and indicate there might be a palatability issue.

It is possible that through a value added process, DDGS could be made into a more palatable feedstock as well as modify the fatty acid profile of the feedstock. β-carotene is a product of yeast fermentation that provides enhanced health effects when consumed in adequate quantities. β-carotenes also have been shown to reduce the risk of negative health diseases such as cancer and heart health issues (Awad & Fink, 2000; Emin et. al., 2012). Carotenoids are also bright red in color and will directly influence the color of the diet and add a pinkish tint to the resulting color of the animal meat (Chew, 1995).

Nanjundaswamy and Vadlani (2010) showed that whole stillage could be fermented by *Sporobolomyces Roseus* to produce β-carotenes which can provide numerous health benefits to swine such as antioxidant addition to the diet, fertility enhancement (Palozza and Krinsky, 1992) and pro vitamin A activity (Rock, 1997). Whole stillage was used because it is a precursor to DDGS and would eliminate the need for a drying step before fermentation. Glycerol was used as a carbon source in their experiments. Its availability being justified due to rapid growth in bio-diesel production. However, the anticipated growth from the bio-diesel industry did not occur and there is a need to determine alternative carbon sources for this fermentation to stay cost effective. Also, the addition of glycerol influenced the nutritional profile of the modified DDGS as not all of the added oil was consumed by the yeast.

The goals of this study were to (1) evaluate the production curve of *Sporobolomyces Roseus* and (2) ferment whole stillage and thin stillage slurries with *Sporobolomyces Roseus* and optimize the overall fermentation time and final β-carotene concentration and (3) evaluate the nutritional composition of the product.

Materials and Methods

Culture Information

Lyophilized cultures of *Sporobolomyces Roseus* (ATCC 28988) were obtained from American Type Culture Collection (ATCC, Manassas, VA), revived on yeast malt agar (YMA) and incubated at 18°C for 10 d. Cultures were then inoculated into yeast malt broth (YMB) and incubated at 18°C on an orbital shaker at 180 rpm for 72 h. A 10% volume/volume inoculation was used for all fermentations.

β-Carotene Production in Test Media

Synthetic media: Modified medium composition of Kusdiyantini et al. (1998) was used. The media contained 1% yeast extract, 1% soy peptone, 2% glucose, 0.1% KH2PO4, 0.05% MgSO4, 0.05% MnSO4 and 0.05% ZnSO4 and pH was adjusted to 6.0 before sterilization. 100ml of respective media in 500ml flasks were sterilized at 121°C for 15min and fermentation was performed in triplicate.

Whole stillage and thin stillage media: A liter of the fermentation medium contained 25% (w/v) whole stillage, 0.1% KH2PO4, 0.05% MgSO4, 0.05% MnSO4 and 0.05% ZnSO4. pH was again adjusted to 6.0. 100ml of each media were placed in 500ml baffled flasks and sterilized at 121°C for 15min and fermentation. Fermentations were performed in triplicate.

Stillage Fermentation and Carbon Addition

A loopful of cells from incubated petri dishes was inoculated into sterile 100 ml yeast media broth in 500 ml flasks, replicated in triplicate. Flasks were incubated at 18°C, 180 rpm for 72 h. Development of red color in *Sporobolomyces Roseus* flasks indicated good fungal growth. A 10% (v/v) inoculum was used for monoculture fermentation.

Whole stillage and thin stillage media were prepared the same as in the production curve experiment. Fermentation was conducted using a 500 ml baffled flasks; the entire fermentation broth was harvested on day 4 and freeze dried. After freeze drying, samples were blended using a coffee blender. Samples were stored at –20 °C until further analyses.

Stillage Fermentation and Carbon Addition

A loopful of cells from incubated petri dishes was inoculated into sterile 100 ml yeast media broth in 500 ml flasks, replicated in triplicate. Flasks were incubated at 18°C, 180 rpm for 72 h. Development of red color in *Sporobolomyces Roseus* flasks indicated good fungal growth. A 10% (v/v) inoculum was used for monoculture fermentation.

Whole stillage and thin stillage media were prepared using same mixture as the 10g/L added glucose in the previous experiement. Fermentation was conducted using a 500 ml baffled flasks; the entire fermentation broth was harvested on day 4 and freeze dried. After freeze drying, samples were blended using a coffee blender. Samples were stored at –20 °C until further analyses.

Nutritional Composition

A large amount of solid material was required for the various assays to be performed so the highest β-carotene yielding treatment was scaled up using a 5 Liter B Braun Biostat Fermenter (Bohemia, New York). 4.5 liters of the medium contained 25% (w/v) whole stillage, 10 g/L glucose, 10g/L nitrogen (ammonium sulfate), 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% $MnSO_4$ and 0.05% $ZnSO_4$ and pH was adjusted to 6.0 before sterilization. Media was sterilized at 121°C for 30min. The entire fermentation broth was harvested on day 7, split into five bottles and freeze dried for five days. After freeze drying, samples were pooled and blended using a coffee grinder. Samples were stored at –20 °C until further analyses.

Crude fat, crude protein, crude fiber, and ash were measured for the optimal carbon/nitrogen blend of fermented whole stillage. About 80 g of each from each replication were sent to Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO) for total fatty acid profile (AOAC Official Method 996.06 AOCS Official Method 72Ce 2-66, AOAC Official Method 965.49, AOAC Official Method 969.33), crude fat (acid hydrolysis, AOAC Official Method 954.02, 2006) and protein (Kjeldahl method, AOAC Official Method 984.13 (A-D), 2006). Estimation of % phosphorus, potassium, sulfur, and crude fiber were also conducted.

Chemical Analysis of β-Carotene

A known quantity of freeze dried sample was weighed into a mortar, 0.2 g of acid washed sand (40-100 micron size) was added and carotenoids extracted by grinding the mixture in dichloromethane solvent. Samples were centrifuged at 5000 rpm for 5 min and supernatant filtered into 1.5 ml HPLC vials using 0.2 µm filters.

High performance liquid chromatography (HPLC) was used for quantification of carotenoids. β-carotene standards were obtained from Sigma Aldrich (St Louis, MO). A Shimadzu HPLC equipped with LC-20AB pump, SIL-20AC auto sampler, SPD-M20A PDA detector and CTO-20A column oven was used. Phenomenex Prodigy C18 column (150 mm x 1.6 mm) along with a C18 guard column was used for the separation of carotenoids. Acetonitrile and methanol (80:20) was used as the mobile phase. Flow rate was maintained at 2.0 ml/min and the column was operated at 40°C. 20 µl of the sample was injected using the autosampler. HPLC data was acquired using Lab Solutions software. Carotenoid yield was expressed as mg/kg of complete feed instead of yield per gram of yeast dry weight as the separation of cells from the complete diet is impossible after mixing.

Statistical Analysis for Experiment One

Data were analyzed using the GLIMMIX procedures of SAS (Cary, NC, v. 9.4). Fixed effects included the main effect of substrate type (synthetic, thin stillage, or whole stillage), and time served as a repeated measure. Differences were considered significant if $P < 0.05$ and trends if $0.05 < P < 0.10$.

Statistical Analysis for Experiment Two

Data were analyzed using the GLIMMIX procedures of SAS (Cary, NC, v. 9.4). Fixed effects included the main effects of carbon source (glucose vs. glycerol), carbon level (low vs. high), and stillage type (thin vs. whole), as well as all significant interactions. Differences were considered significant if $P < 0.05$ and trends if $0.05 < P < 0.10$.

Statistical Analysis for Experiment Three

Data were analyzed using the GLIMMIX procedures of SAS (Cary, NC, v. 9.4). Fixed effects included the main effects of nitrogen source (urea vs. ammonium sulfate), nitrogen level (0, 5, or 10), and stillage type (thin vs. whole), as well as all significant interactions. Differences were considered significant if $P < 0.05$ and trends if $0.05 < P < 0.10$.

Results and Discussion

β-Carotene Production in Test Media

The β-carotene content of the samples were measured every 12 h over a 122 h time span. It is important to note that the β-carotene production was increasing even though cell growth attained stationary phase, which indicates mixed-growth association. The experiments were terminated at 122 h since nutrients are exhausted as determined from previous research completed (Nanjundaswamy, 2010). Figure 2.1 illustrates the product curves of Sporobolomyces Rosues in synthetic media, whole stillage and thin stillage, respectively. It shows that both the

whole stillage and thin stillage yielded significantly higher amounts of β-carotene than the synthetic media from 84 h onward (P<0.05). LS Means and standard error of the mean were 37.22±.2301 ug/g for synthetic media, 40.06±.2301 ug/g for thin stillage, and 50.08±.2301 ug/g for whole stillage. Whole stillage finished with the highest concentration of β-carotene at 167.72 μg β-carotene/mg biomass compared to 115.50 ug/g for the synthetic media and 131.49 ug/g thin stillage. β-carotene yields in whole stillage were 45% higher than synthetic media and 28% higher than thing stillage, both of which were significant differences (P<0.05). This is most likely due to the overall amounts of available sugar left over from the ethanol fermentation in whole stillage, whereas the nutritional composition of thin stillage is mostly made up of various fats and oils with a very low percentage of solids. *Sporobolomyces roseus* is capable of using fatty acids for the creation of β-carotene, but does so at a slower rate than simple sugars, and hence explains the presence of lower amount of β-carotene in thin stillage than whole stillage.

Carbon/Nitrogen Addition

Due to the long lag time present in all the experiments that used the three media types, there is a need to supplement the media in order to reduce the fermentation time and increase production efficiency. Previous research indicated that glycerol could be supplemented into the media and was successful in both reducing lag time and increasing the overall β-carotene yields (Nanjundaswamy and Vadlani, 2010). However, adding high levels of glycerol to the whole stillage yields a higher amount of crude fat to the dried feed product. DDGS already has flowability issues, and increasing the fat content by 5% or more could further exacerbate the problem. Therefore, an experiment was designed to measure difference in fermentation efficiencies when different levels of glucose or glycerol we supplemented into whole stillage or thin stillage.

Glycerol was supplemented at 0%, 5% or 7.7%, which is consistent with the values used by (Nanjundaswamy, 2010). Glucose, chosen as alternate substrate due to its low price and ease of availability, was supplemented at 0g/L, 5g/L or 10g/L. Table 2.1 shows a comparison between the two stillage types, two carbon sources, the two level of supplementation, and a control sample. Whole stillage yielded significantly higher values in final β-carotene concentration than thin stillage of the same type and level of carbon supplementation ($P<0.05$). There was also a upward trend in β-carotene concentration when either glycerol or glucose was increased in both stillage types. Overall, glucose at 10 g/L addition yielded the highest amount of β-carotene in the whole stillage at 177.06 ug/mg. This combination was used in experiments that involved nitrogen supplementation.

Each main effect (carbon source, carbon level, and stillage type) had a significant effect ($P<0.05$) on the levels of β-carotene. In addition, carbon source × carbon level (Table 3.2), carbon level × stillage type (Table 3.3), and carbon source × stillage type (Table 3.4) were all significant interactions ($P<0.05$). There was not a significant three way interaction.

Urea and ammonium sulfate were chosen as nitrogen (N) sources. It is important to note that the amount of nitrogen added to the media remains constant between urea and ammonium sulfate. By weight, urea contains 46% N and ammonium sulfate contains 21% N. For example at 5g/L N added, 10.87 g urea/L is added or 23.81 g of ammonium sulfate/L is added. This non-protein nitrogen addition helps in reducing the lag time of the fermentation and to limit the amount of protein that the *Sporobolomyces roseus* will consume in the slurry, since it is more easily digestion by the yeast.

Table 3.5 shows a comparison between the two stillage types, two nitrogen sources and the two level of supplementation. Again whole stillage yielded significantly higher values in

final β-carotene concentration than thin stillage of the same type and level of nitrogen supplementation ($P<0.05$). This is likely due to the higher levels of solubilized glucose present in whole stillage versus the higher levels of fats present in thin stillage. Urea was shown to have a negative effect on β-carotene concentration at both the 5 g/L and 10g/L, while ammonium sulfate had a positive effect on both the 5g/L and 10/L for both stillage types.

Each main effect (nitrogen level, nitrogen source, and stillage type) had a significant effect ($P<0.05$) on the levels of β-carotene. In addition, nitrogen level × nitrogen source (Table 3.6), nitrogen source × stillage type (Table 3.7), and nitrogen level × stillage type (Table 3.8) were all significant interactions ($P<0.05$). There was not a significant three way interaction.

Overall, the whole stillage supplemented with 10g/L ammonium sulfate performed the best with a β-carotene concentration of 272.57 μg β-carotene/ g biomass.

Nutritional Profile

Table 3.9 shows the nutritional profile of DDGS, dried whole stillage and fermented whole stillage. There was a significant decrease in both crude protein and crude fat from dried whole stillage to fermented whole stillage; 36.57% to 24.97% and 15.37% to 5.85%, respectively. This drastic of a drop in protein was unexpected due to the fact NPN was supplemented, but it is likely the yeast consumed the protein present in whole stillage first. The same event took place for the fat content; however, this is actually beneficial in regards to handling and transportation of the product. High amounts of fat can cause both rancidity and bridging issues during storage and handling.

In addition to modifying the nutritional profile, the fermentation also had a qualitative impact on the color appearance and the smell of the resulting product. Figure 2.2 shows a very significant change in color from the golden yellow of whole stillage to the pinkish red of

fermented whole stillage. The new product also had a very sweet odor, compared to the alcoholic smell of whole stillage or DDGS.

Conclusions

In conclusion, final β-carotene concentration was found to be highest at 272.57±4.34 µg β-carotene/g biomass for the whole stillage, at 10g/L glucose addition, and at 10 g/L nitrogen addition through ammonium sulfate. Glycerol addition yielded no significant increase ($P<0.05$) in β-carotene yield compared to glucose addition. Urea addition significantly decreased ($P<0.05$) the final β-carotene concentrations compared to both the control and ammonium sulfate addition. The resulting fermented product can be effectively blended with regular feed to generate a premium nutritionally enhanced feed product using either whole stillage as a dry feed ingredient or thin stillage as a liquid feed additive.

Figures and Tables

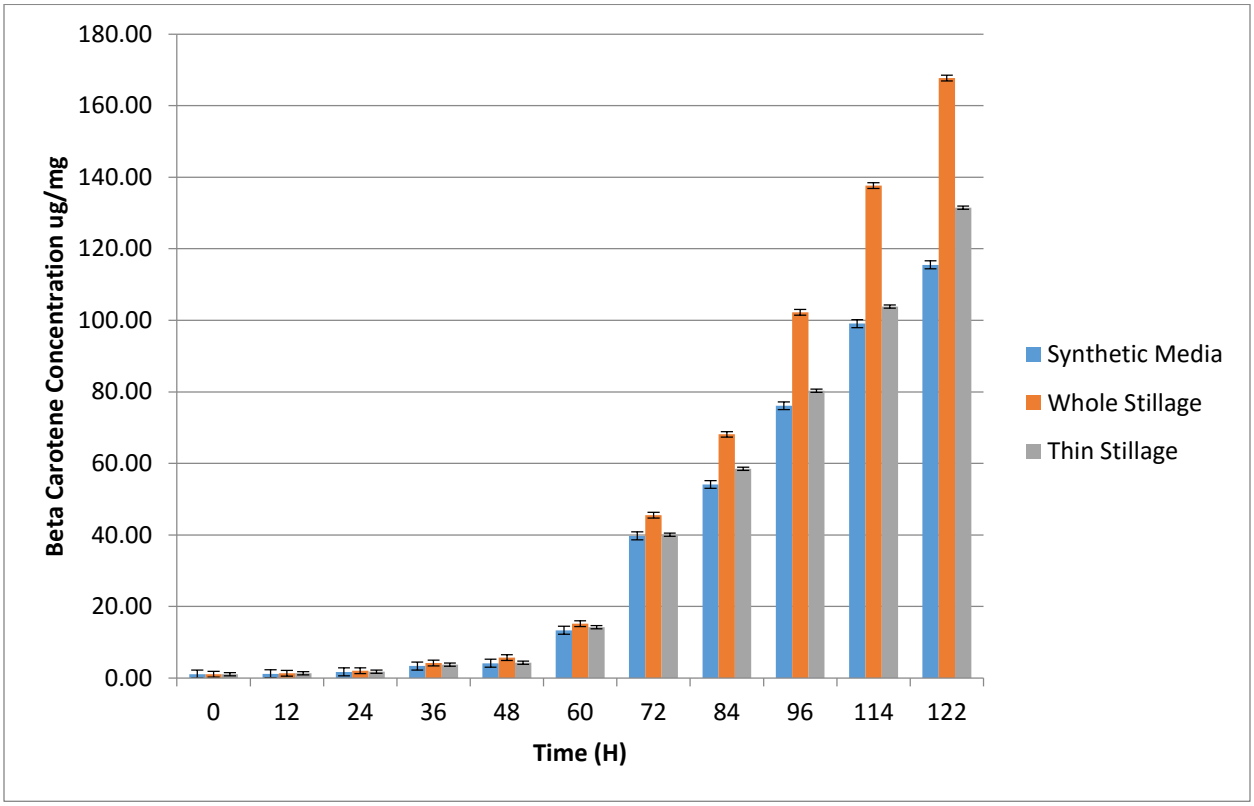

Figure 3.1 β-Carotene Production in Whole Stillage, Thin Stillage and Synthetic Media

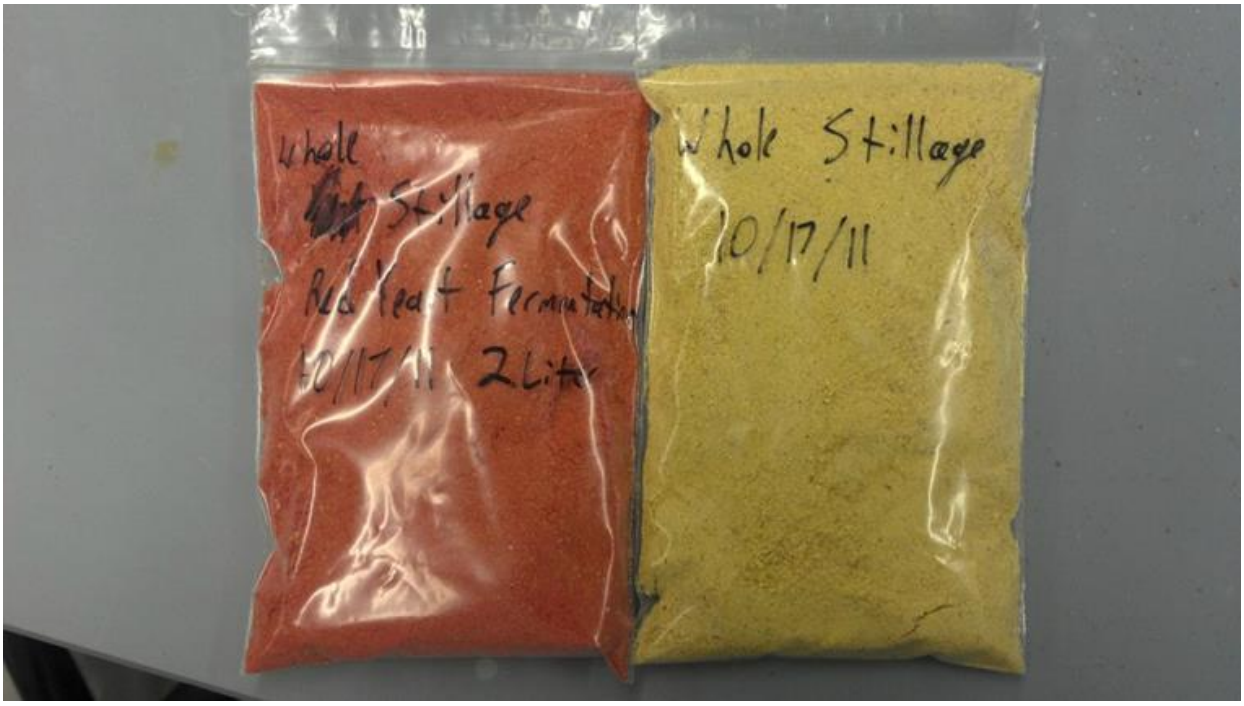

Figure 3.2 Visual Comparison of Modified Whole Stillage and Regular Whole Stillage

Table 3.1 β-Carotene Production When Glucose or Glycerol is Supplemented in Stillage

Media	Added Glucose g/L	Added Glycerol % Total Volume	μg β Carotene/ g freeze dried stillage
Thin Stillage	0.00	0.00	88.41±1.08[a]
	0.00	5.00	92.67±0.65[a]
	0.00	7.70	94.90±0.71[a]
	5.00	0.00	123.09±1.17[b]
	10.00	0.00	134.53±1.66[b]
Whole Stillage	0.00	0.00	113.35±2.47[b]
	0.00	5.00	123.53±1.53[b]
	0.00	7.70	133.59±0.71[b]
	5.00	0.00	157.74±1.47[c]
	10.00	0.00	177.06±0.83[d]

Values in this table represent the average(N=2)

Differing superscripts in a given column denotes a significant difference (P<0.05)

Table 3.2 LS Means for Carbon Source × Carbon Level

Carbon Source	Carbon Level	LS Means Estimate	SEM
Glucose	High	155.8^{a}	0.7935
Glucose	Low	140.42^{b}	0.7935
Glycerol	High	114.25^{c}	0.7935
Glycerol	Low	108.1^{d}	0.7935

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.3 LS Means for Carbon Level × Stillage Type

Carbon Level	Stillage Type	LS Means Estimate	SEM
High	Whole	155.33[a]	1.1222
Low	Whole	140.64[b]	1.1222
High	Thin	114.72[c]	1.1222
Low	Thin	107.88[d]	1.1222

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.4 LS Means for Carbon Source × Stillage Type

Carbon Level	Stillage Type	LS Means Estimate	SEM
Glucose	Whole	167.40[a]	1.1222
Glucose	Thin	128.81[b]	1.1222
Glycerol	Whole	128.56[b]	1.1222
Glycerol	Thin	93.79[c]	1.1222

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.5 β-Carotene Levels When Urea or Ammonium Sulfate is Supplemented in Stillage

Media	Nitrogen g/L (Urea)	Nitrogen g/L (Ammonium Sulfate)	μg β Carotene/ g freeze dried stillage
Thin Stillage	0.00	0.00	131.69±0.69[a]
	0.00	5.00	133.66±3.52[a]
	0.00	10.00	131.45±1.20[a]
	5.00	0.00	64.58±1.93[b]
	10.00	0.00	23.22±1.46[c]
Whole Stillage	0.00	0.00	166.98±0.94[d]
	0.00	5.00	211.57±0.62[e]
	0.00	10.00	272.57±4.34[f]
	5.00	0.00	144.32±1.96[a]
	10.00	0.00	115.59±0.75[g]

Values in this table represent the average(N=2)

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.6 LS Means for Nitrogen Level × Nitrogen Source

Nitrogen Level	Nitrogen Source	LS Means Estimate	SEM
High	Urea	69.41[a]	1.1222
Low	Urea	104.45[b]	1.1222
High	Ammonium Sulfate	202.01[c]	1.1222
Low	Ammonium Sulfate	172.61[d]	1.1222

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.7 LS Means for Nitrogen Source × Stillage Type

Nitrogen Source	Stillage Type	LS Means Estimate	SEM
Urea	Thin	43.90[a]	1.1222
Urea	Whole	129.96[b]	1.1222
Ammonium Sulfate	Thin	132.55[c]	1.1222
Ammonium Sulfate	Whole	242.07[d]	1.1222

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 3.8 LS Means for Nitrogen Level × Stillage Type

Nitrogen Source	Stillage Type	LS Means Estimate	SEM
High	Thin	77.34[a]	1.1222
Low	Thin	99.12[b]	1.1222
High	Whole	194.08[c]	1.1222
Low	Whole	177.95[d]	1.1222

Differing superscripts in a given column denotes a significant difference (P<0.05)

Table 3.9 Comparison of Nutritional Composition Among Feed Types

Nutritional Components	Feed Type		
	DDGS	Whole Stillage	Fermented Whole Stillage
Crude Protein, %	34.97[a]	36.57[b]	24.97[c]
Crude Fat, %	11.74[a]	15.37[b]	5.85[c]
Crude Fiber, %	6.25[a]	8.17[b]	14.57[c]
Ash, %	5.42[a]	2.91[b]	5.77[a]
Lys, %	0.99[a]	1.48[b]	1.23[c]
Met, %	0.60[a]	0.77[b]	0.44[c]
Thr, %	1.25[a]	1.40[b]	1.03[c]
Trp, %	0.30[ab]	0.39[a]	0.24[b]

Values in this table represent the average (N=2)

Differing superscripts in a given row denotes a significant difference (P<0.05)

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Chapter 4 - Scale-Up Fermentation and Processing

Introduction

The purpose of a fermentation vessel is to provide a contained, sanitary environment where the fermentation media can be controlled homogenously. A high degree of flexibility, safety, reliability, and compliance to regulatory factors are key factors for production equipment. The most common style of fermentation vessel is the stirred-tank reactor, which will typically operate via internal mechanical agitation.

Fermentation scale up is aimed at the manufacture of larger product quantities, as required, with consistent specific yield and product quality. The performance of fermentation vessels depends on several key aspects of their design and operation, such as reactor configuration, reactor size, mode of operation, and processing conditions inside of the reactor. The final reactor design will be a reflection of all these process requirements (Doran, 1995).

Oxygen or air is supplied by a sparger, which is typically either a ring with a series of holes or a single orifice located beneath the agitator. Perforated rings generally lead to greater gas dispersion, due to decreased bubble size, than a single orifice, but they also require greater maintenance and cleaning due to an increased chance of plugging or fouling in high-solid fermentation media. Actually gas dispersion is the function of the agitator and not the sparger itself. Gas inlets are generally located under the lowest impellor which will provide rapid gas diffusion within a viscous liquid. This action will also lead to a longer residence time of the gas in the liquid slurry, before it eventually rises to the headspace of the fermenter and is expelled or captured (Van't Riet and Tramper, 1991).

The degree of gas dispersion and solid/liquid mixing is heavily influenced on impellor design. The main types of agitators used in fermentation are disk, marine, paddle and rushton

turbine impellors. Marine and paddle impellors are generally used in fermentation that are sensitive to shearing forces generated by the centrifugal forces of mixing, while disk and rushton turbine impellors offer a high degree of mixing for a comparatively lower energy requirement (Schuler and Kargi, 2002).

Figure 4.1 shows the layout of a rushton turbine impellor. These impellors normally contain 4-8 blades designed to stir a fermentation slurry in a radial direction. They are the predominate impellors used in bench-top fermenters. For most microbial fermentations, impellor diameter to operating tank diameter should be between 0.35 and 0.45 (Lydersen et al., 1994). The fermentation slurry will exert more torque on the impellors when either the viscosity of the liquid or the dimensions of the impellor paddles are increased. When either of these things occur the shaft diameter will need to be increased, heavier duty-bearing will need to be installed and the gearbox might need to be modified.

Sieblist (et al., 2011) presented a diagram which illustrates how fluid flow occurred during mixing with rushton turbine impellors. Figure 4.2 shows a baffled fermenter with three rushton turbine impellors installed. Baffles help to minimize fluid swirling and vortexing. Full baffling of a vessel requires four baffles, placed equidistally from one another, that each have a width of at least 1/10 the total tank diameter. A lack of baffling decreases power output, mixing quality and oxygen transfer. Because of this power drop, the slurry could experience ebbs and flows in terms of its total viscosity due to solids settling and becoming suspended. This change in resistance can cause instability in the slurry fluid properties resulting in equipment damage (Gill et al., 2008).

The choice of fermenter construction materials should be based on the sensitivity of the organism, the corrosiveness of the initial broth and the product characteristics and the total cost

of the material with respect to the expected lifetime of the vessel. In addition to these main points the vessel will also need to be sterilized and cleaned in between runs. Decontamination chemicals will generally contain corrosives and special consideration will need to be given in that regard. Lab scale equipment is normally constructed from glass and sometimes different plastic polymers. The benefit of plastics is the decreased initial cost, but the lifecycle of the equipment will be decreased due to repeated sterilization (Shuler and Kargi, 2002).

Pilot scale equipment will almost never be constructed from glass due to overall construction costs and durability concerns. Similarly, plastics cannot be used due to vessel rigidity and sterilizing issues. Therefore, metal is the primary construction material used in large scale fermenter construction. A wide variety of microbial cells are sensitive to ferrous materials and there is also a concern over rust contamination of the broth and breakdown of materials from cleaning and use (Lyderson et al., 1994). As a consequence, stainless steel and aluminum as potential building materials.

Aluminum offers the advantage of being very lightweight and resistant to chemical breakdown, but it still experiences oxidation and it greatly increases the cost of the equipment. A variety of stainless steel types are available for construction. The two most common types of stainless steel used are 304 and 316 stainless steel. 304 stainless steel is the most common grade and is the classic 18/8 (18% chromium, 8% nickel) stainless steel. Outside of the US it is commonly known as "A2 stainless steel", in accordance with ISO 3506. 316 stainless steel is the second most common grade (after 304) and is used for food and the medical industry. Alloy addition of molybdenum prevents specific forms of corrosion. It is also known as marine grade stainless steel due to its increased resistance to chloride corrosion compared to type 304 (Herting et al., 2007).

Construction material and overall vessel design also need to take into account how the vessel will be sterilized and cleaned. Pilot scale equipment is large enough that it is typically clean-in-place equipment and will require chemical sterilization and scrubbing instead of autoclave sterilization, which is how laboratory scale equipment is normally sterilized. Constructing a vessel with a removable top plate and removable attachments will greatly increase the efficiency and ease of cleaning. Interior and exterior surfaces should be smooth and free of pitting or crevices to avoid material buildup and contamination issues. To accomplish this, vessels should have joints which are welded instead of bolted and sealed. The drain of the vessel is also a region where material could build up. The lower portions of the fermenter should be angled so product will flow freely once the fermentation is completed (Schmidt, 2005).

Most liquid fermentations are carried out using pure cultures and under aseptic processing conditions. Keeping the bioreactor free of unwanted organisms is a key component for product purity and process efficiency. Industrial fermenters are designed to be sterilized with steam under pressure. The vessel should have a minimum number of ports, crevices, connections and attachments to ensure proper sterilization can take place. The flow in and out of a fermenter should be controlled with the use of pumps or valves, which will also need to be capable of enduring sterilization between runs (Cameron, 1987).

The overall vessel dimensions are very important from both a fluid dynamics and optimal microbial growth condition point of view. Figure 4.3 illustrates the various dimensions which need to be taken into account for designing a vessel. Fermenters should maintain a height to diameter ratio of 3 to 1. During scale up if this ratio remains constant, then the surface to volume ratio will decrease. This change will decrease the contribution of surface aeration and will need to be accounted for by additional gas sparging.

It is most important to recognize that the conditions present in a scaled up reactor can never be exactly duplicated from that of a bench top reactor, even if the geometrical similarities are maintained. For example, increasing the dimensions of a vessel by a factor of 5 will increase the volume by 125 fold, if the 3 to 1 height/diameter ratio is maintained (Doran, 1995). This change in volume will affect variables such as oxygen transfer rate, shear at impellor tip, and constant power unit. These changes can alter the metabolic response and growth curve of the microbes. In some cases robust cultures can be resistant to these changes. Common scale up rules are the maintenance of constant power to volume ratios, constant tip speed, a constant oxygen transfer rate, and the maintenance of a constant substrate and product level. It has been shown that mixing time can be correlated to reactor volume according to different variables such as mixing time, impellor placement, and impellor type (Shuler, 2002).

By determining the relationship between certain variables the fermentation conditions can be scaled to within an acceptable degree of operation. The exact placement of impellors and variables such as tip speed will need to be altered based on the specific media and size of the vessel.

Materials and Methods

General

Lyophilized cultures of *Sporobolomyces Roseus* (ATCC 28988) were obtained from American Type Culture Collection (ATCC, Manassas, VA), revived on yeast malt agar (YMA) and incubated at 18°C for 10 d. Cultures were then inoculated into yeast malt broth (YMB) and incubated at 18°C on an orbital shaker at 180 rpm for 72 h. A 10% volume/volume inoculation was used for all fermentations.

Fermenter Construction

It was necessary to construct a vessel capable of producing a large quantity of modified DDGS for subsequent physical properties analysis and for the complete feed production runs. A 45 L fermentation vessel for this study was designed in our laboratory and constructed by Advanced Manufacturing Institute (AMI, Kansas State University, Manhattan, KS). The vessel was manufactured with 316 stainless steel, to ensure longevity of the reactor and a decreased chance of any chemicals reacting with the material.

One of the most commonly used criteria in aerobic fermentations is constant K_La (volumetric oxygen transfer coefficient). The supply of oxygen to the growing cells is usually the limiting operation in industrial bioprocesses. This is the case when the oxygen demand is high (fast growing microorganisms at high cell concentrations). The rationale of constant K_La is to ensure a certain mass transfer capability that can cope with the oxygen demand of the culture. However Ju and Chase (1992) showed that it is not the transfer capability (k_La), but the oxygen transfer rate (OTR), which is the product of K_La and the mass transfer potential (C*)CL). The equations to establish a constant OTR while scaling up were presented by Diaz and Acevedo (1999).

Variables listed in the following calculations are as follows:

$_{1,2}$= Benchtop, Pilot

a,b = Exponents

C_1,C_2 =Constants

C*= Equilibrium dissolved oxygen concentration (kg/m^3)

C_L= Dissolved oxygen concentration (kg/m^3)

D=Impellor diameter

F= Volumetric air flow (m^3/s)

H= Henry's law constant (Pa*m^3/kg)

K_1-K_6= Constants

K_La= Volumetric oxygen transfer coefficient

N= Agitation speed

OTR= Oxygen transfer rate

P= Ungassed agitation power (W)

P_g= Gassed agitation power (W)

P= Oxygen partial pressure (Pa)

V= Fermentation Liquid Volume (L)

V_s= Gas velocity (m/s)

Diaz and Acevedo (1999) presented scale up based on previous work (Ju and Chase, 1992; Eisele, 1976; Michel and Miller, 1962). Based on the work of Ju and Chase (1992), the following scale-up strategy will be considered:

i. Geometric similarity:

$$D_2/D_1 = (V_2/V_1)^{1/3} \ldots \quad (1)\dagger$$

ii. Constant impeller tip speed:

$$\frac{N_2}{N_1} = \left(\frac{D_1}{D_2}\right) = (V_1/V_2)^{1/3} \ldots \quad (2)\dagger$$

iii. Agitation power per unit volume as a function of volume (Einsele, 1976): ……

$$\frac{(P_g/V)_2}{(P_g/V)_1} = (V_1/V_2)^{0.37} \quad (3)\dagger$$

iv. Constant Oxygen Transfer Rate (OTR). Considering that the OTR equals $k_La(C^*)C_L$), and C^*=p/H, an equation of the form:

$$K_L a = K_1 (\frac{P_g}{V})^a v_s^b \ldots \quad (4)\dagger$$

the following equation results for constant oxygen transfer rate: …

$$\left(\frac{P_g}{V}\right)_1^a v_{s2}^b \left(\frac{p_1}{H} - C_{L1}\right) = \left(\frac{P_g}{V}\right)_2^a v_{s2}^b \left(\frac{p_2}{H} - C_{L2}\right)\dagger :$$

It has been observed that the yield or the productivity of many fermentation processes is dependent on the dissolved oxygen concentration, so an optimal response will be obtained at a defined constant value of C_L. So if the optimal value of C_L, as determined in the pilot equipment, is to be used in the industrial bioreactor: …

$$\frac{p_2 - HC_L}{p_1 - HC_L} = \frac{(P_g/V)_1}{(P_g/V)_2}^a (\frac{v_{s1}}{v_{s2}})^b = k_L a_1 / k_L a_2 \ldots \quad (5)\dagger$$

Equation (5) tells us that in order to have constant OTR, the mass transfer coefficient must be different at each scale.

The Michel and Miller equation (1962) is considered in our work, which is given as:

$$P_g = C_1 (\frac{P^2 N D^3}{F^{0.56}})^{0.45} \ldots \quad (6)\dagger$$

in which, under turbulent flow, P is:

$$P = K^2 N^3 D^5 \ldots \quad (7)\dagger$$

Combining equations (6) and (7), and using the relation $V = K_3 D^3$:

$$\ldots \frac{P_g}{V} = C_2 N^{3.15} v_s^{-0.252} D^{2.346} \quad (8)\dagger$$

Replacing Equations (1), (3) and (8) in Eq. (5): …

$$\ldots \quad \frac{p_2 - HC_L}{p_1 - HC_L} = (N_1/N_2)^{12.5b} (V_2/V_1)^{0.37a - 0.40b} \quad (9)\dagger$$

If the condition of constant tip speed is included in (Eq. (2)), then: …

$$\frac{p_2 - HC_L}{p_1 - HC_L} = \left(\frac{V_2}{V_1}\right)^{0.37a - 0.40b} \quad (10)$$

Equations (9) and (10) show that in order to obtain the same OTR in both sizes, the oxygen partial pressures must be adjusted. Since it is simpler and less expensive to manipulate the total pressure or the gas composition of the pilot fermenter, it will be assumed that p_1 will be varied and p_2 kept constant.

The modified DDGS fermentation process was scaled from 5 L (0.005 m^3) to 50 L (0.05 m^3). This translates to a V2/V1 ratio of 10. From Equations (1) to (3): D2/D1= 2.154; N2/N1=0.4641; $\frac{(P_g/V)_2}{(P_g/V)_1}$ =0.4266.

Fukuda et al. (1968) presented the following equation (valid for Newtonian broths) and sizes up to 40 m^3:

$$K_L a = K_4 \left(\frac{P_g}{V}\right)^{0.56} v_s^{0.07} N^{0.7} \ldots \quad (11)\dagger$$

Using equations (6) and (7), equation (11) is rearranged as:

$$K_L a = K_5 \left(\frac{P_g}{V}\right)^{0.78} v_s^{0.76} \quad (12)\dagger$$

The exponents of equation (12) and the value of V1/V2 allow the evaluation of the right hand side of equation (10): …

$$\frac{p_2 - HC_L}{p_1 - HC_L} = 10^{-.0154} = 0.97$$

If the assumption of Ju and Chase (1992) is made (C_L=0), then:

$$p_2/p_1 = 0.97$$

So if the bench-top fermenter is run at atmospheric pressure (1.013×10^5 Pa) with regular air, the pilot unit should be operated with air at 1.03 atm total pressure. Figure 4.4 shows that for Newtonian broths the scale-up ratio (V2/V1) has little effect on P1 when CL =0. The effect of the scale-up ratio at different dissolved oxygen concentrations on P1 is shown in Figure 4.5. The effect is only moderate and it can be observed that it decreases with increasing oxygen concentrations.

Figure 4.6 shows an inside view of the fermenter that was constructed. The cylindrical fermenter design measured 66 cm × 30.5 cm (height x diameter), internally. It was fitted with a bearing guided, central shaft (1.25 cm diameter) that was equipped with two rushton turbine impellors (4.5 cm × 3.4 cm × 11.5 cm, Length × Height × Diameter). A removable baffle, which featured 4 connecting bars measuring 38 cm × 3 cm (height × width) was used to increase mixing efficiency.

The removable head plate was fitted with a large rubber O-ring to ensure an airtight seal and features 6 ports that allow for the addition of an air sparger, a pH probe, a sampling port, a dissolved oxygen probe, an inoculum injection port and a temperature probe. The air sparger is mounted on a support bracket, which places the sparger head directly beneath the lowest impeller in order to ensure the air being injected is properly dispersed.

Figure 4.8 shows the fermenter system with the motor and the motor controller installed. The fermenter was powered by a top mounted, slip drive, 120 V, 1 HP electric motor. RPM was controlled via variable speed drive and held at a constant 350 RPM throughout the trial.

45 L Fermentation Experiments, Processing and Drying

Whole stillage media: 40 L of the fermentation medium contained 25% (v/v) whole stillage, 10 g/L glucose, 10g/L nitrogen (ammonium sulfate), 0.1% KH2PO4, 0.05% MgSO4,

0.05% MnSO4 and 0.05% ZnSO4. pH was again adjusted to 6.0. Due to sterilization concerns, the remaining volume was filled with steam water instead of room temperature water. The slurry was allowed to reach room temperature and then 0.25 ppm Virginiamycin was added. After 1 hour, inoculum (10% v/v) was pumped through sterilized tubing via peristaltic pump. Sterile air was injected at 1.5 VVM during the entire course of the fermentation.

The slurry was harvested after 122 h of fermentation and transferred into 5 gallon buckets. The slurry was split into 500 mL containers and immediately centrifuged using a Sorvall RC4 floor model centrifuge (Thermo Fisher Scientific, Waltham, MA) at 1800 RPM for 10 minutes. Product was collected and frozen until the entire slurry was centrifuged.

Once the entire run was centrifuged, product was thawed and oven dried in a Fisher Isotemp 500 Oven (Thermo Fisher Scientific, Waltham, MA). A trial drying run was conducted with the first fermentation run to establish a baseline drying time and temperature. Product was dried at 140^{o} C and moisture content was checked at one hour intervals with a rapid moisture meter until a moisture content of 12% (w.b.) was reached. A drying time of 6.5 hours was determined to be sufficient at lowering the moisture content to 12% (w.b.). All subsequent runs were dried for 6.5 hours and then moisture content was checked to verify that it was within 0.25% of the initial run. After all the runs were completed, product was homogenized and combined into one uniform lot. β-carotene levels were determined by HPLC using the same method as outlined in Chapter 3.

Results and Discussion

A total of 14 fermentation runs were completed in order to generate enough product for the physical properties analysis and the subsequent feed manufacturing. After centrifugation and drying, modified DDGS from all runs were combined into a conglomerate to eliminate any

variability between runs that might have occurred and to generate enough product for the subsequent experiments. Samples were taken from that lot and tested for β-carotene levels in order for the final feed inclusion levels to be calculated. β-carotene levels were determined after all runs were completed and samples were taken from the a composite sample of all runs. Average β-carotene were found to be 286.60±8.64 ug β-carotene/ g feed, which was slightly higher than the value obtained from the shake flask study, which was 272.57±4.34 ug β-carotene/ g feed.

While the scope of this project only focuses on the scale up from bench-top production to pilot production, in order for a modified DDGS product to become a feasible option for the feed industry, the production process will need to be streamlined into the existing production process for distillers' grains. Five of the main concerns related to scalability are new/upgraded equipment for processing, contamination of pure DDGS stream, product quality and screening, profit margin considerations and market research to identify the main outlets for sale.

The largest barrier to the production of modified DDGS is the need to modify existing ethanol plants with additional equipment so both regular and modified DDGS can be produced in order to satisfy the market demands. A schematic of the potential equipment that will need to be installed alongside an existing DDGS operation is shown in Figure 4.9, which shows the addition of a seed tank for inoculum generation, additional fermentation vessels, and a second centrifuge and drum dryer so both product streams can be manufactured at the same time. This process could be simplified by using the same centrifuge/drying system that the existing process utilizes; however, it is unlikely feasible due to the production schedule of commercial manufacturing plants.

The main drawback that plants will have is related to finding available space to install this new equipment in their plants. Floor plans and flow sheets will need to be examined in each ethanol plant this process will be integrated into to determine if there is adequate room to install the machinery without hindering the existing process. There will also be an up-front cost of equipment purchase and installation that will need to be considered.

Load-out equipment will also play a factor in the scale up. Plants will need to consider their sales outlet for modified DDGS and determine if the bulk load-out will be sufficient or if various forms of bags should be utilized (i.e. 50 pound sacks, 1 ton super-sack, ect.). If a bulk load-out system cannot be utilized, additional consideration needs to be made for the increase in man-hours in order to package and ship the product.

As previously stated, the process could be simplified by using the same centrifuge and dryer as the existing stream, but this increases the chances for product contamination. β-carotenes are listed as generally regarded as safe (GRAS), but still require appropriate labeling (CFR 21, 2012). If a plant has a HAACP plan on file, the centrifuge and dryer system would be changed to critical control points with additional cleanout requirements and a requirement for the testing of β-carotenes after a run change.

While this could mitigate the cost of the initial investment; however, more work and time are required and might not be the best option if the plant has space available for the additional equipment. However, the bulk-load out system will most likely be used for both modified and unmodified DDGS.

Most plants will have load-out systems designed uniquely for their operation, but it is safe to assume that most will have a series of bins with some form of conveying equipment

which use a central load out system with a specific number of downspouts depending on the truck capacity. This is another point for potential cross contamination.

If adequate bin space is available and the bin design allows for it, modified DDGS should be stored only in specified bins to avoid mixing the two product streams. There will still need to be a cleanout protocol to flush any part of the load-out system that will be handling both product streams. This "flushed" product could be recycled back into the initial β-carotene fermentation to avoid waste.

If plants have a HAACP plan on file they will need to include record-keeping protocols at all critical control points. This would not only ensure that cross contamination is not occurring but also provide a screening measure for quality control and to verify β-carotene levels for labeling requirements.

UV-spectroscopy is a quick and easy method for detecting the presence of β-carotenes. It is not as accurate as an HPLC analysis, but it will produce a low-cost high-frequency method of verification that can be kept on file for all identified critical control points. This will ensure that if there is a deviation in product consistency it will be identified quickly to minimize product loss.

Lastly, plants will need to consider conducting market research to identify potential outlets for their product and the desired profit margin. These variables are dependent on each other and are restricted by shipping available based on the plants geographic location and the proximity of the plant to commercial farms.

Profit margin will be closely linked to the amount of retrofitting required to produce modified DDGS and the desired method of shipping. There are typically more costs associated with sack load-out than bulk-load out due to the extra equipment required, price of bags,

additional labor and possibly modifying receiving bays to deal with additional truck traffic. Bulk load-out is capable of shipping larger quantities of product because it is not restricted as harshly by the man-hours per ton and will produce an increased profit margin. This is completely dependent on the buyers and the relative shipping distance.

Commercial feed mill operations will generally have access to bulk receiving systems and will have a series of bins dedicated to specific products. This should be the main target for potential consumers of modified DDGS as the shipping costs will be lower and the potential for demand would be much greater than if this product were used as a feed supplement and sold in sacks.

While commercial feed mills will provide more quantity demand for the product, there could be a chance for a higher price point if the modified DDGS were marketed as a premium feed supplement to be fed alongside a complete diet or blended in. This would be a product marketed more towards hobby farmers and small operations possibly targeting the organic food market. If this were the case, consumers might be willing to pay a higher price and it would greatly improve the profit margins. This would require a very aggressive marketing strategy, however, to distribute information to organic producers and has the risk of being a niche market that could collapse easily.

Conclusions

A scale-up strategy consists of identifying key stress factors and parameters that influence product yield and quality. These factors are balanced to ensure appropriate process control for optimum mixing and reaction conditions. Common scale up rules are the need for a constant oxygen/mass transfer coefficient, constant impellor tip speed, combination of fermentation time and the Reynolds number and maintaining the product curve.

The final fermenter a factor of the laboratory design and the process requirements needed at the pilot scale. It should be noted that the fermenter is part of a process and not a perfectly contained system. The fermenter designed was successful in generating enough product for a pilot scale feed production trial and to complete physical properties analysis for the modified DDGS.

Figures and Tables

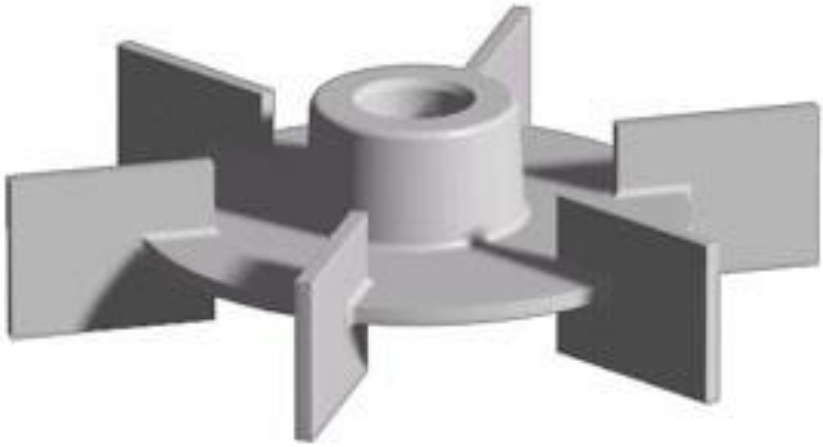

Figure 4.1 Rushton Turbine

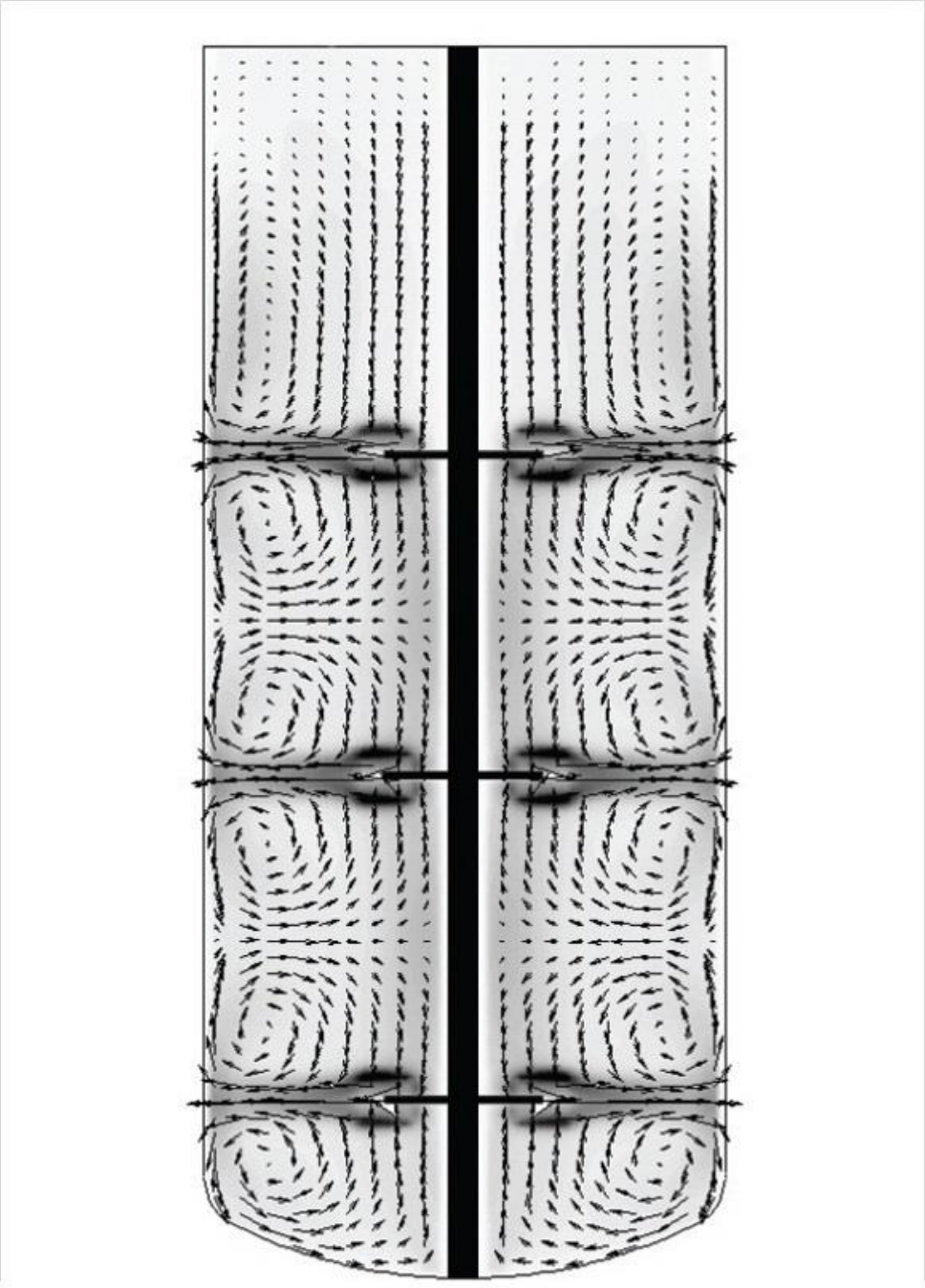

Figure 4.2 Rushton Turbine Fluid Flow (Source: Sieblist et al., 2011)

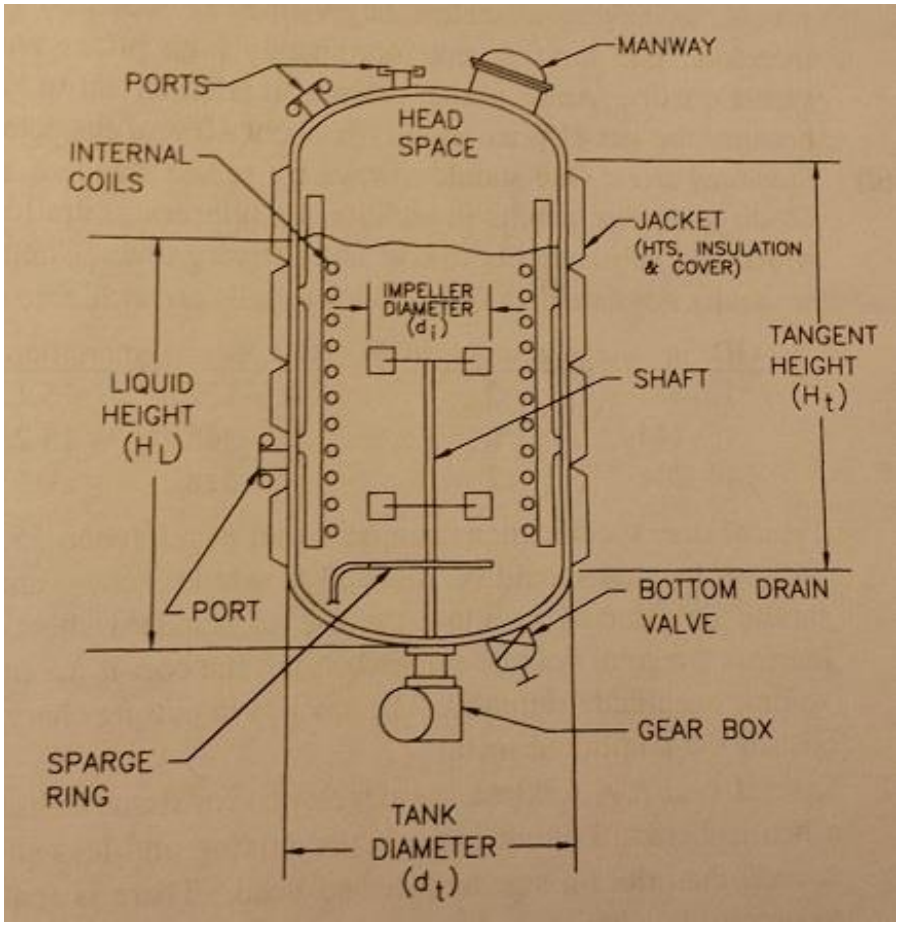

Figure 4.3 Fermenter Vessel Design (Source: Shuler, 2002)

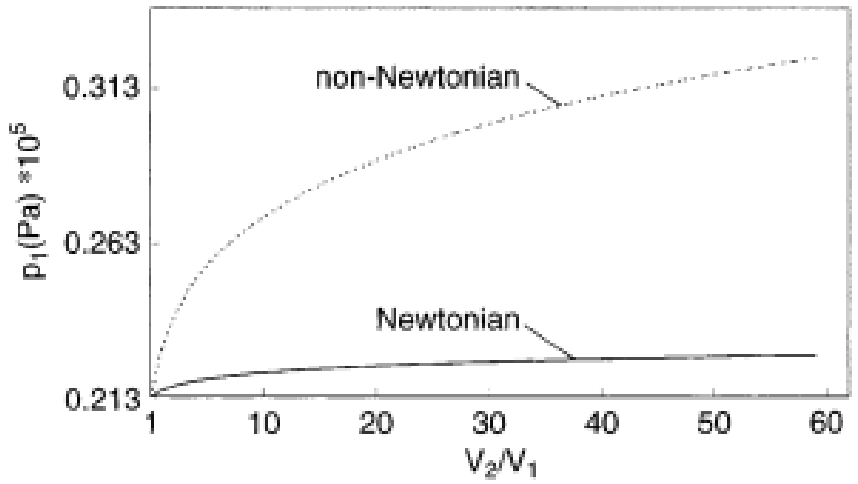

Figure 4.4 Effect of scale-up ratio (V2/V1) on the pilot bioreactor oxygen partial pressure in order to maintain constant OTR in Newtonian and non-Newtonian fermentations for the case when CL =0 (Source Diaz and Acevedo, 1999)

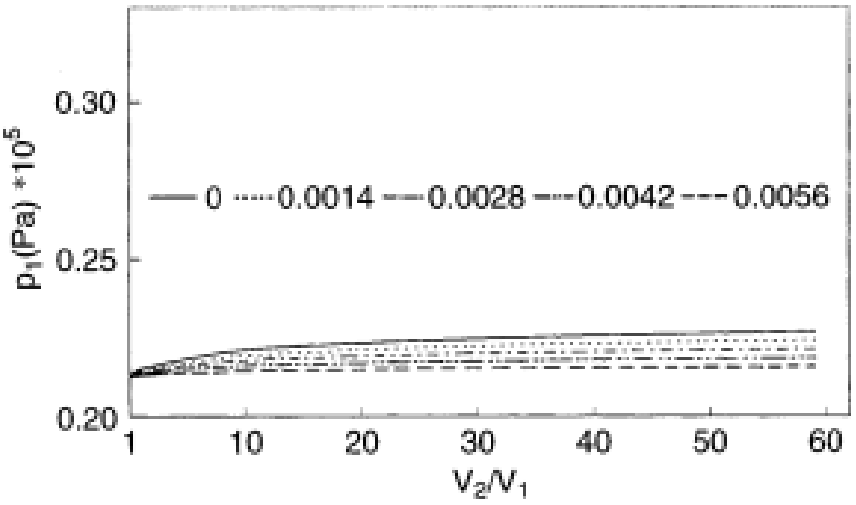

Figure 4.5 Effect of the scale-up ratio at dissolved oxygen concentrations of 0.0, 0.0014, 0.0028 and 0.0056 kg/m^3 on the pilot bioreactor oxygen partial pressure in order to maintain constant OTR in Newtonian fermentations (Source Diaz and Acevedo, 1999)

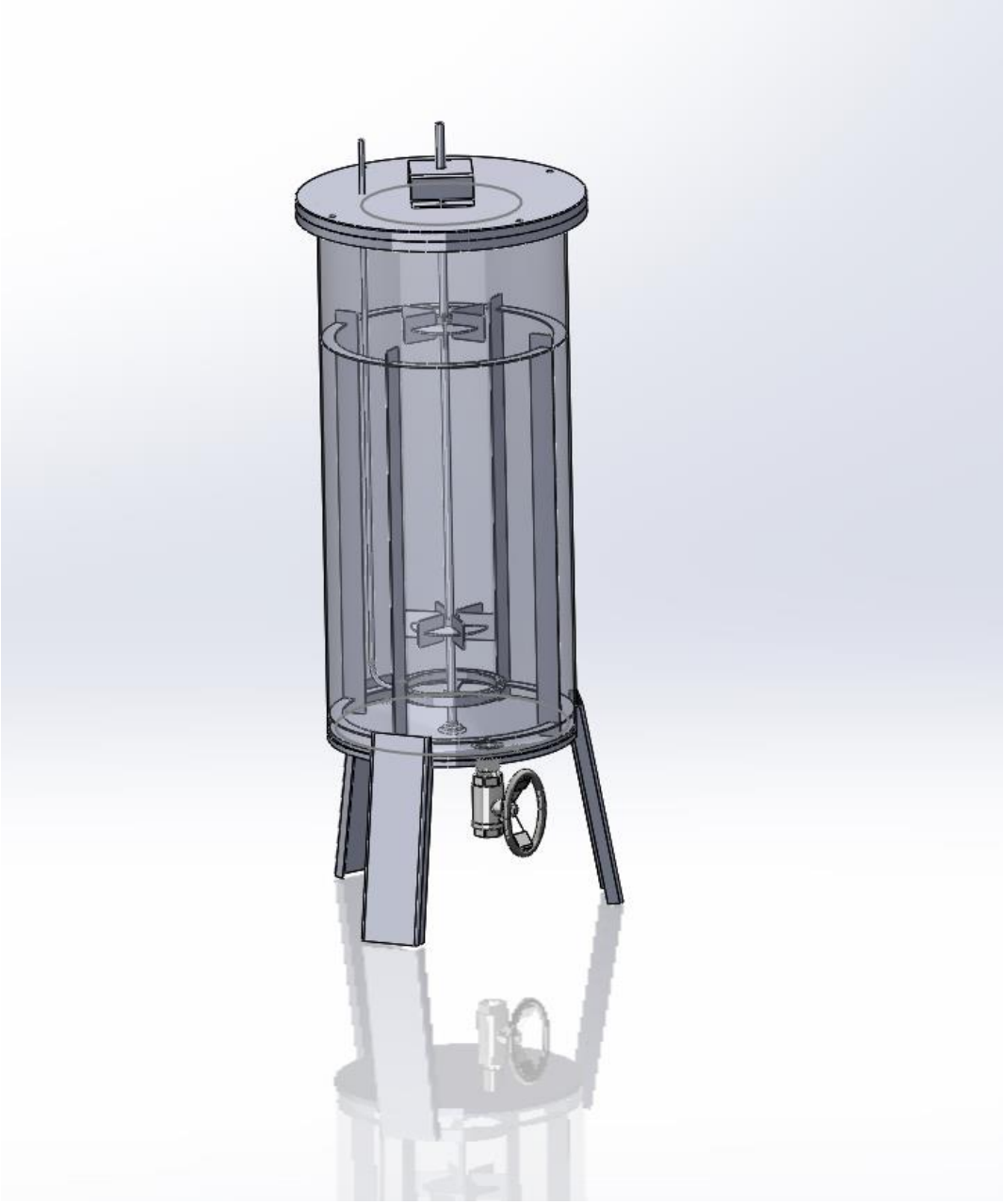

Figure 4.6 45 L Fermenter Mock-Up

Figure 4.7 45 L Fermenter Top Plate

Figure 4.8 45 L Fermenter

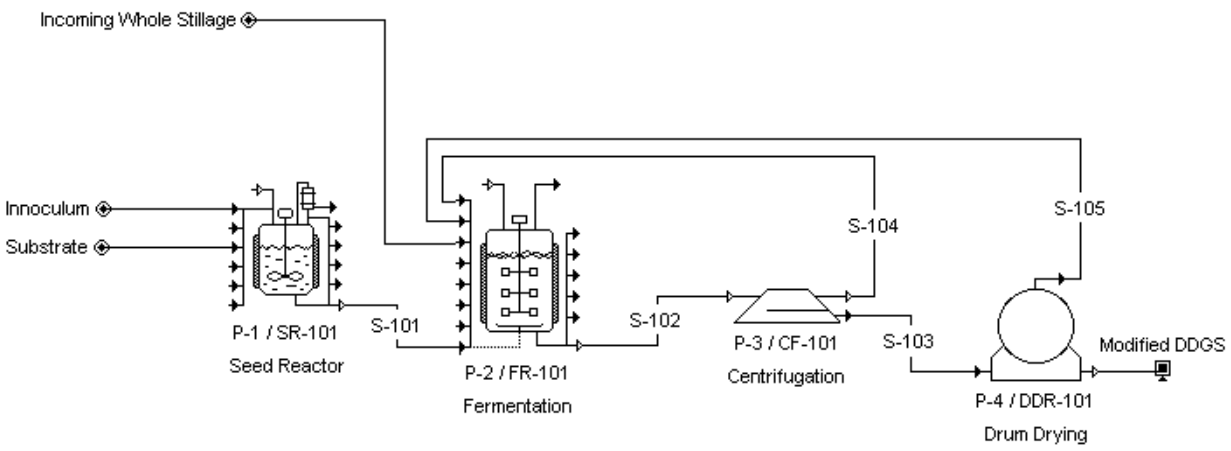

Figure 4.9 Integration of Modified DDGS Production Line

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Chapter 5 - Physical and Flow Property Analysis

Introduction

The fermentation of DDGS by *Sporobolomyces Roseus* has been shown to modify the physical, chemical and the processing qualities of the finished product (Nanjundaswamy, 2010). This data is only available for samples from bench-top scale experiments, but some of the general characteristics of the finished product can give some insight to the handling and storage of modified DDGS in a commercial setting.

Quantifying the physical properties is important because of their large influence on the storage and flow behavior of DDGS, in addition to various environmental conditions. Some of the properties that effect flowability are particle size, particle interaction, temperature, and vibrations. The flowability of DDGS under various pressures and conditions is important in handling operations such as storage in silos or hoppers, and transportation through railcars and trucks. It is necessary to determine and quantify the differences between commercially-available DDGS, dried whole stillage and fermented dried whole stillage.

Flow can be described as the relative movement of a bulk of particles among neighboring particles or along a wall surface (Harnby, 1987). Flow characteristics are important in bulk material handling and processing because of their impact on conveying, blending, and packaging. Reliable flow is necessary to create a consistent product that can be used in a variety of manufacturing processes in feed mills.

The bulk density of dried spent yeast is between 180 g/L and 210 g/L compared to a bulk density of 480 g/L for DDGS (TAPCA Inc., 2012). While it is difficult to estimate the total amount of yeast cells present in the modified DDGS based on the results of bench-top trials,

there is a very real possibility that the bulk density of the modified DDGS will be lowered due to the presence of the dried yeast cells.

Bulk density has been shown to have a significant effect on the flowability of a granular product. As a general rule, products with a low bulk density will exhibit more flow problems, such as clumping or bridging, that products with a higher bulk density (Harnby, 1987). It should be noted that other factors such as temperature, relative humidly and product composition also play crucial roles in particulate material flow.

Particle size and shape of DDGS was studied by Ileleji et al. (2008). In their study, only a sample of DDGS from an old generation ethanol plant was used. Particle size distribution of the DDGS sample was categorized according to U.S. sieves 4 – 12 (Group 1), 16 – 30 (Group 2), and 40 – 140 (Group 3). Their results showed that DDGS particles shape and sizes were related to particle structural components (fiber, germ and or agglomerates). They suggest that spherical particles of DDGS are the agglomeration of individual heterogeneous particles of corn kernel components (germ, fiber, tip cap and other solids) that are shaped into spherical balls and cemented firmly together with the aid of the condensed solubles and drying.

One of the most drastic changes that occurs in the modified DDGS is a decrease in oil content after fermentation. DDGS contains widely variable levels of fat (between 3 and 13%) based on the method of concentration for solids or the up-front fractionation of corn. Ganesan et al. (2007) showed that removing oil from DDGS did improve flowability slightly. Differences were observed for compression behavior and other indicators of flowability such as compressibility, angle of repose and dispersibility.

The goals of this study were to measure and compare the physical and flow variables relating to bulk bin storage and handling between commercial DDGS, dried whole stillage and fermented dried whole stillage.

Materials and Methods

Physical Properties

Samples

Unmodified DDGS, Dried Whole Stillage and Modified DDGS were used in this study. The unmodified DDGS were obtained from MGP Ingredients (Atchison, Kansas) and they came from the same production stream that the whole stillage, both dried and fermented, were obtained from. After fermentation and drying to <12% (w.b.), the product was placed in a cold storage room (<0°C) until this trial was begun. The unmodified DDGS were used to establish a baseline since the drying process used in the laboratory setting was significantly different than what is typically used in industry.

Particle Size

The particle size and size distribution of DDGS, dried whole stillage and fermented whole stillage were analyzed to determine the geometric mean particle size (dgw) and geometric standard deviation (sgw) as described in ASAE S319.4 (ASAE, 2009):

$$d_{gw} = \log^{-1}\left[\frac{\sum_{i=1}^{n}\left(W_i \log \overline{d_i}\right)}{\sum_{i=1}^{n} W_i}\right] \quad (1)$$

$$S_{\log} = \left[\frac{\sum_{i=1}^{n} W_i\left(\log \overline{d_i} - \log d_{gw}\right)^2}{\sum_{i=1}^{n} W_i}\right]^{1/2} \quad (2)$$

$$S_{gw} = \frac{1}{2} d_{gw} \left[\log^{-1} S_{\log} - \left(\log^{-1} S_{\log} \right)^{-1} \right] \quad (3)$$

where

d_{gw} = geometric mean diameter or median size of particles by mass (mm)

$S_{\log}$ = geometric standard deviation of log-normal distribution by mass in ten-based logarithm (dimensionless)

S_{gw} = geometric standard deviation of particle diameter by mass (mm)

W_i = mass on i^{th} sieve (g)

n = number of sieves plus one pan

$\overline{d_i}$ = $(d_i \times d_{i+1})^{1/2}$

d_i = nominal sieve aperture size of the i^{th} sieve (mm)

d_{i+1} = nominal sieve aperture size of the i^{th}+1 sieve (mm).

A ro-tap sieve shaker (Tyler Ro-Tap Model B, Mentor, OH) with thirteen screens and sieving aids were used. The test was run for ten minutes, and a silica flow aid (Huber, Atlanta, Georgia) was used as a dispersion agent. The flow aid was added at a level of 0.5%, or 0.5 grams added to the 100 gram sample. The silica was added to the sample prior to analysis and then shaken to fully mix the dispersion agent with the sample. After the test was completed, the mass on each screen was recorded to obtain both the average particle size (dgw) and standard deviation (sgw).

Bulk Density

The bulk density was measured by using a Winchester cup (Seedburo equipment Company, Chicago, IL, USA.). A standardized pint cup was set under a closed funnel, the

samples were poured through and once the cup was full the entire funnel was released into the cup. Excess sample was scraped off using a wooden scrapper and the cup was weighed using a balance (sensitivity: 0.001g; Mettler- 30 Toledo, Heightstown, NJ, USA). The bulk density was then calculated from the weight and volume of the samples.

Tapped Density

Tapped density was measured using an auto-tap instrument (AT 6-1-110-60, Quantachrome Instruments, FL, USA) according to ASTM Standard B527-6 (ASTM, 1985). The samples were filled in a volumetric cylinder (250 ml) and the cylinder was tapped 250 times. After tapping, the change in volume of sample was measured and the tapped density was then calculated from the volume of sample after tapping and the weight.

True Density

True density of the samples was measured using a gas pycnometer (AccuPyc II 1340, Micromeritics, Norcross, GA, USA). Helium gas was used to fill the chamber containing samples to determine the particle volume and the true density was calculated from the weight and the solid particle volume.

Flow Indicators

Compressibility Index and Hausner Ratio

Compressibility Index (CI) and Hausner Ratio (HR) indicate the cohesiveness and compaction mechanism that occurs during handling of particulate materials due to vibration or tapping. CI and HR were calculated from the bulk and tapped density using the following equations (Kingsly et al., 2010):

$$Compressibility Index = 100\left[\frac{\rho T - \rho B}{\rho T}\right]$$

$$HausnersRatio = \left[\frac{\rho T}{\rho B} \right]$$

where ρB is the bulk density (kg/m3) and ρT is the tapped density (kg/m3).

Angle of Repose

A fixed diameter (0.09 m) plate was set under a funnel which was held at a height (0.1 m) above the plate and the samples were poured to maintain a natural flow on the plate. After pouring the samples, the height of the cone was measured and the resulting angle of repose was measured.

Basic Flow Energy

The FT4 Powder Rheometer (FT4, Freeman Technologies, Gloucestershire, UK) was used to evaluate the flow properties in terms of energy required to make flow occur. The FT4 powder rheometer system consists of a vertical glass sample container (120 mm height; 50 mm internal diameter) and a rotating blade (48 mm diameter; 10 mm height), which navigates through the sample up and down, and either in clockwise or counterclockwise direction. The FT4 standard dynamic test cycle includes preconditioning, conditioning cycle, and test cycle. Preconditioning cycle mixes the sample to make the particle dispersion uniform before energy measurement.

The energy required to establish a specific flow pattern for a precise volume of materials is called the basic flowability energy. SI evaluates the effect of flow on the bulk physical changes on powders and solids. BFE is used to evaluate the flow properties of the granular material under free surface conditions. The flow energy is calculated from the counterclockwise motion of blade (23.5 mm diameter) as it moves through the vessel top to the bottom. The instrument conducts eleven test cycles to calculate BFE. The first seven test cycles were

performed at a blade tip speed of 100 mm/s to examine the effect of segregation on the bulk particles during flow. For subsequent tests (test 8 to 11), the blade tip speed was gradually reduced from 100 mm/s to 70, 40 and 10 mm/s to evaluate the sensitivity of the particles to different flow rates. From the 11 test cycle results, the flow parameters were calculated (Leturia et al., 2014).

Aeration

Bulk DDGS is very porous and has intergranular spaces filled with air. The presence/absence of air or the porosity affects the bulk flow properties. For aeration measurement, the samples were placed in a 160 ml vessel (50 mm inner diameter) glass vessel with a porous base that was connected to an air flow 33 controller. The flow of samples was simulated by moving the blade in an axial helical path through the test sample with a blade tip speed of 100 mm/s. In the second test cycle, the blade moved along a downward helical path, (-10° at the sample blade tip speed) but in the opposite direction, to impose compaction, thereby forcing the sample to flow around the blade. Progressively, from test 1 to 6, the air flow rate was increased from 0 mm/s to 10 mm/s at 2 mm/s increment. At each condition, the flow energy was recorded by the instrument the aeration ratio was calculated.

Compressibility

The compressibility reflects the particle density change during compaction, which is the decrease in volume of the packed bed of particles under normal stress (Turki and Fatah, 2008). Samples were placed in a 50 ml cylindrical vessel and using a vented piston normal stress from 0.5 to 15 kPa (0.5, 1, 2, 4, 6, 8, 10, 12, and 15 kPa) was applied to consolidate the samples. Each normal stress was maintained for about 25 s. The force applied on the sample and the compressibility as a percentage change in volume was recorded.

Permeability

Permeability is part of the proportionality constant in Darcy's law which relates discharge (flow rate) and fluid physical properties (e.g. viscosity), to a pressure gradient applied to the porous media. The FT4 measures the pressure drop across the powder bed while the applied normal pressure is varied and the air velocity through the aeration base was maintained constant at 2 mm/s (Leturia et al., 2014). The sample was compressed using a piston with stainless steel mesh end that allowed air to pass through during compression. The air flow velocity was kept constant at 2 mm/s and the resistance to air flow was measured as air pressure drop.

Shear Stress and Wall Friction

The relationship between normal stress and shear stress are plotted to obtain experimental yield locus that represents the failure during shearing of the bulk solids. In free flowing powders, the yield locus follows a straight line that passes through the origin (Peleg, 1978) and its slope defines the angle of internal friction. For cohesive powders, however, the experimental yield locus is generally non-linear at different consolidation stresses (Thomson, 1997; de Jong et al., 1999). The shear test determines the flow function (FF), the effective angle of internal friction, and the angle of wall friction (ϕw). Flow function represents the strength of the consolidated sample that must be surpassed to begin flow.

Statistical Analysis

The physical and flow property measurements were performed in triplicates. Statistical analysis was conducted using SAS (SAS Institute Inc., Cary, NC, USA). Comparisons were made using Tukey-Kramer test at $P \leq 0.05$. Individual responses based on the specific test were initially analyzed to determine if significant differences existed between treatments.

Results and Discussion

Physical properties

Particle Size

The average particle size of DDGS, whole stillage and fermented whole stillage are presented in Table 5.2. All particle sizes were significantly different from one another ($P<0.05$) with the DDGS having the largest particle size and the fermented whole stillage having the smallest particle size. This supports the hypothesis that the fermentation of whole stillage reduces the particle size by the addition of yeast cells, which are very small in particle size.

Particle size is one of the processing factors linked to pellet durability. It is typical for a single cereal grain, usually corn, to make up a majority portion of a pelleted swine diet (Fahrenholz, 2012). The corn is ground prior to diet mixing and is generally performed via hammermill. Whole corn is also ground before ethanol fermentation for enzymatic breakdown of starch and subsequent fermentation. The resulting DDGS will have a decreased particle size as a result of this process. This eliminates the need to grind DDGS before diet inclusion but exposes feed production plants to product variability based on the processing parameters of the ethanol plants.

The small particle size and wider distribution of the whole stillage and fermented whole stillage caused a significantly higher angle of repose. This is an indicator of poor flow and a decrease in overall bin capacity.

Angle of Repose

Carr (1965) suggested that angle of repose below 30° indicate good flowability, 30°-45° some cohesiveness, 45°-55° true cohesiveness, and >55° very high cohesiveness with very

limited flowability. Both whole stillage and fermented whole stillage would be classified as very cohesive materials while DDGS would be classified as somewhat cohesive.

Densities

Both the bulk density and tapped density of the whole stillage and fermented whole stillage were significantly less than that of the DDGS ($P<0.05$). This decrease in bulk density between DDGS and whole stillage could be attributed to the differing particle sizes and nutritional composition between the three ingredients.

During handling or transportation, the particle arrangement of bulk solids shifts significantly due to vibration and tends to rearrange itself based on particle size distribution. When there is reduced friction between the particles, the particles rearrange and thus tapping results in tighter packing conditions. This is generally regarded as a negative effect when dealing with ground feed ingredients since it results in bridging and product flow issues. The tapped density for fermented whole stillage was significantly different ($P<0.05$) than both commercial DDGS and whole stillage. This could indicate that the presence of yeast cells are increasing the amount of potential compaction occurring and could have a significant impact on the resulting grain characteristics.

There were no observed differences in true density ($P>0.10$) and this was expected since true density does not account for the void space between particles and the true density of the individual ground particles is relatively similar.

Porosity

Porosity is the fraction of a porous medium that is void space (John, 2010). The porosity of whole stillage and fermented whole stillage were significantly greater than that of DDGS (Table 5.2). Again this is most likely due to the particle size distribution and drying processes.

Flow indicators

Compressibility Index and Hausner Ratio

Because the interactions between individual particles influence the bulking properties of a solid and are also shown to interfere with granular material flow, a comparison of the bulk and tapped densities gives a measure of flow characteristics. Hausner Ratio and Compressibility Index for the samples are shown in Table 5.2. Table 5.1 shows the classification of powders based on Hausner Ratio and Compressibility Index (Eben, 2008). All three products showed a Hausner Ratio of above 1.6, which would be classified as having poor flow characteristics. This is most likely do to the wide particle size distribution. The Compressibility Index for DDGS was 19.1, which correlates to passable on the index. Whole stillage and fermented whole still were 43.36 and 43.92, respectively, which correlates to poor flow. This could be due to a combination of factors such as differences in the drying processes and differences in particle size distribution and overall size.

Dynamic flow properties

Flow Properties

Table 5.4 shows the comparisons of various flow properties between feed types. Basic flow energy corresponds to the stabilized flow energy that represents the energy needed to begin moving bulk particulate ingredients. The basic flow energy for DDGS was significantly higher than both whole stillage and fermented whole stillage ($P<0.05$). With a higher particle size and low compressibility, DDGS has greater forces between particles that requires a greater amount of energy to begin product movement.

Air flow and its effect on flow properties is an important factor to consider when moving and processing bulk solids. Aeration ratios which are close to 1 indicate that a material is not sensitive to flow and also that they do not segregate during flow. DDGS was the only product to have an aeration ratio between these values at .98 with whole stillage being 1.15 and fermented whole stillage at 1.31. There was a significant difference between aeration ratios of DDGS and fermented whole stillage ($P<0.05$) indicating the possibility of segregation of product during transportation. In addition, unloading of whole stillage and fermented whole stillage would be difficult due to their low aeration ratio.

Compressibility is an important factor and can indicate flow problems that might potentially occur during bulk bin storage and unloading from overhead bins. Fermented whole stillage had the highest compressibility at 14.5%. This is most likely due to the wide particle size distribution and the tendency of small particles to fill voids between the larger particles and pack tighter. This can lead to product bridging inside storage bins or in piles stored in concrete pads.

Permeability is a measurement of the relative ease with which a fluid can pass through a bulk granular material. Packing and compression during storage results in a change in the density of the grain mass, which in turn results in a change in porosity. Air permeability through the bulk solids is important for flow through hoppers and during unloading from any storage structures. Higher pressure loss (low permeability) would indicate flow challenges due to denser packing of particles. Under pressurized conditions, due to particle compaction and reduction in the void spaces between particles, the DDGS demonstrated the lowest permeability among ingredients.

Wall friction values provide important information on how bulk solids will flow against the sidewall of a bin or storage container. Increased resistance between the ingredient and the sidewall can lead to segregation and irregular flow patterns. Table 5.4 shows that wall friction was highest for DDGS and lowest for whole stillage. There was a significant difference between each ingredient ($P<0.05$). The wall friction for fermented whole stillage was greater than whole stillage which indicates greater adhesion between the particle and the wall material.

Shear Properties

As previously stated granular solids are subjected to consolidation stresses during storage, which results in changes in density and increased forces between the individual particles. Shear properties measure the flowability of powder under stress. At normal stress, higher shear stress corresponds to higher angle of internal friction and wall friction angle.

Figure 5.1 shows that by fitting Mohr stress circles to the yield locus identifies the Major Principle Stress (Sigma 1) and Unconfined Yield Strength (Sigma c), and the ratio of the former to the latter quantifies the Flow Function, FF. Flow Function is a parameter commonly used to rank flowability, with values below 4 denoting poor flow and above 10, good flow. Based on the FF classification by Jenike (1964), DDGS would be classified as intermediate flow quality while dried whole stillage and fermented dried whole stillage would be classified as good. The higher the flow function value, the easier the bulk solid can flow from bins and silos.

Table 5.5 shows that DDGS demonstrated lower unconfined yield strength and flow function than both whole stillage and fermented whole stillage. It also shows a significant decrease in flow function and angle of internal friction due to the fermentation of whole stillage ($P<0.05$).

Conclusions

Physical and flow properties of DDGS, dried whole stillage and dried fermented whole stillage were investigated. Establishing parameters of the physical properties is necessary for design of handling equipment and to understand the behavior of how these ingredients will interact with various processing equipment and bins during handling and storage. The fermentation of whole stillage significantly influenced the physical and flow properties of the material. Even though there was a significant decrease in bulk density and increase in tapped density between DDGS and fermented whole stillage, there was either a much less impact or no impact at all between the whole stillage and fermented whole stillage. This indicates that the fault lies in the fact that whole stillage and fermented whole stillage was processed manually for this study. If product were to go through a commercial separation and drying process then there would be very little influence on the physical differences between DDGS and modified DDGS other than particle size and angle of repose. This is due to the addition of yeast cells and the modified nutritional profile of the product.

Figures and Tables

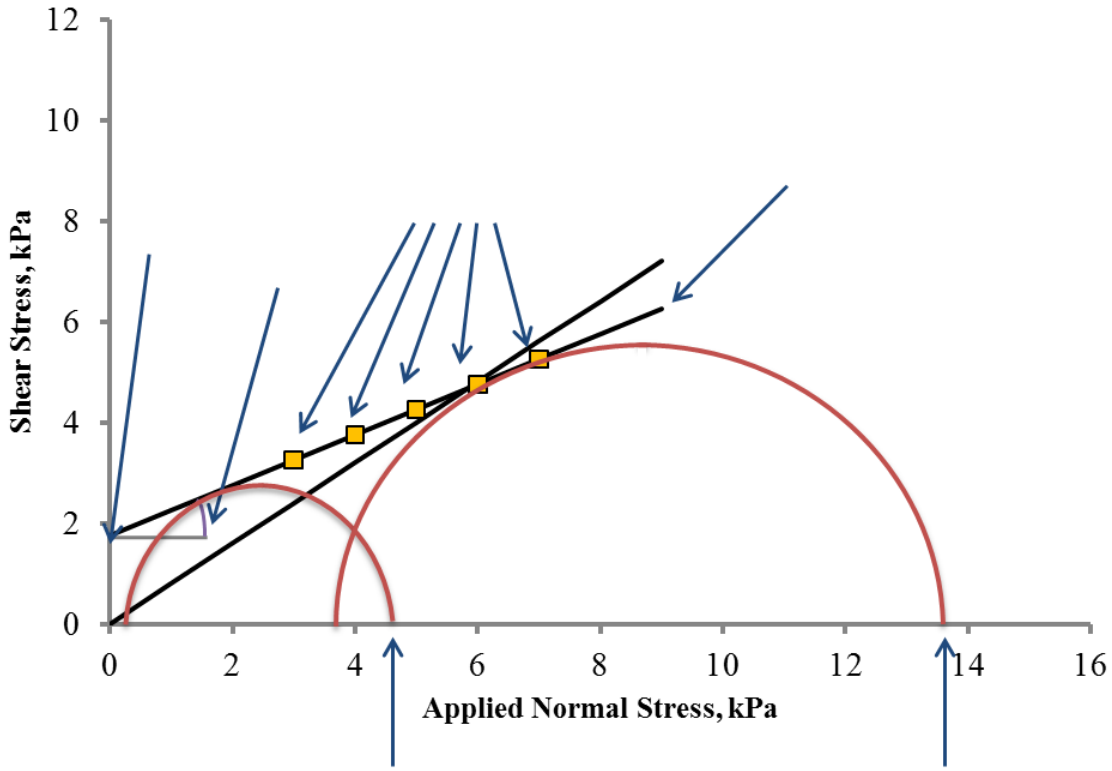

Figure 5.1 Major Principle Stress and Unconfined Yield Strength (Source: Freemantech, 2015)

Table 5.1 Comparison of Densities Among Feed Types

Ingredient	Bulk Density kg/m^3	Tapped Density kg/m^3	True Density kg/m^3
DDGS	306.51 ± 0.65^a	382.72 ± 3.56^a	1324.12 ± 0.72^a
Whole Stillage	235.99 ± 0.43^b	416.66 ± 1.64^b	1327.78 ± 0.65^a
Fermented Whole Stillage	237.9 ± 0.62^b	424.35 ± 1.45^c	1328.13 ± 0.80^a

Values in this table represent the average(N=2)
Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 5.2 Comparison of Bulk Physical Properties Among Feed Types

Physical Properties	Compressibility Index	Hausner Ratio	Porosity %	Particle Size μm	Angle of Repose, °
DDGS	19.91 ± 0.07^{a}	$1.85\pm0.02\text{x}10^{-2\ a}$	76.85 ± 0.08^{a}	787 ± 5.80^{a}	43.6 ± 1.2^{a}
Whole Stillage	43.36 ± 0.13^{b}	$1.77\pm0.11\text{x}10^{-2\ b}$	82.23 ± 0.05^{b}	576 ± 4.26^{b}	57.9 ± 0.9^{b}
Fermented Whole Stillage	43.92 ± 0.14^{b}	$1.78\pm0.14\text{x}10^{-2\ b}$	82.09 ± 0.07^{b}	495 ± 3.27^{c}	64.7 ± 1.4^{a}

Values in this table represent the average (N=2)

Differing superscripts in a given column denotes a significant difference ($P<0.05$)

Table 5.3 Hausner Ratio and Compressibility Index (Source: Eben, 2008)

Flow Indicator	Excellent	Good	Fair	Passable	Poor	Very Poor	Cohesive
Hausner's Ratio	1.00-1.11	1.12-1.18	1.19-1.25	1.26-1.34	1.35-1.45	1.46-1.59	>1.60
Compressibility Index	≤10	42323.00	16-20	21-25	26-31	32-37	>38

Table 5.4 Dynamic Flow Properties of Feed Types

Ingredient	Basic Flow Energy, MJ	Aeration Ratio	Compressibility, %	Permeability, mBar	Wall Friction, kPa
MGPI DDGS	1296.33±11.05[a]	0.98±0.08[a]	9.04±0.15[a]	0.84±0.02[a]	33.13±0.17[a]
Whole Stillage	568.67±32.57[b]	1.15±0.02[ab]	9.32±0.12[a]	1.17±0.01[b]	19.87±0.03[b]
Fermented Whole Stillage	546.33±7.36[b]	1.31±0.02[b]	14.50±0.17[b]	1.37±0.01[c]	23.97±1.30[c]

Values in this table represent the average (N=2)

Differing superscripts in a given column denotes a significant difference (P<0.05)

Table 5.5 Shear properties of Feed Ingredients

Ingredient	UYS, kPa	Cohesion	Flow Function	Angle of Internal Friction°
MGPI DDGS	4.78±0.16[a]	1.13±0.03[a]	7.00±0.20[a]	39.25±0.20[a]
Whole Stillage	3.40±0.05[b]	0.73±0.01[b]	10.02±0.07[b]	42.95±0.04[b]
Fermented Whole Stillage	3.47±0.02[b]	0.84±0.02[ab]	9.20±0.04[c]	40.00±0.65[a]

Values in this table represent the average (N=2)

Differing superscripts in a given column denotes a significant difference (P<0.05)

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Chapter 6 - Complete Diet Processing and Storage Stability

Introduction

Pelleting of animal feeds can improve both animal performance and material handling (Behnke, 2001). DDGS are the result of removing the starch for ethanol production, and as such, the concentrating of protein, fat, fiber and ash in DDGS is approximately three times the amount of the raw cereal grain (Spiehs, 2002). Combined with their relatively low cost, it makes DDGS a very attractive ingredient in least-cost formulation.

While this study has confirmed that *Sporobolomyces roseus* is capable of fermenting DDGS and producing β-carotenes, it is unclear how they will withstand processing in a complete feed. The sensitivity of β-carotene to temperature is of particular interest, because of the high temperatures and pressures that feed mash undergoes during pelleting. The majority of the heat is the result of steam conditioning and friction from the pellet die (Behnke, 2001).

Both temperature and amount of time the feed mash is conditioned must be considered. Gilpin et al. (2002) found that increasing retention time in two separate conditioners by slowing the rotation using a VFD led to significantly improved pellet durability. Briggs et al. (1999) also found that an increase in retention time improved pellet durability, which also lead to a decrease in pellet fines directly after the pellet mill and after pellet cooling. This equates to less feed being recycled through the pellet mill and being subjected to multiple pre-conditioning steps.

One reason why DDGS can only be used as a small percentage of pelleted diets is that the starch, which would normally gelatinize and act as a binder, has been removed for ethanol production. Normally, this could be partially offset by increasing the retention time which could compromise the β-carotene because of its temperature sensitivity.

Not only can the steam conditioner transfer heat and energy to the feed, but the pellet die will also transfer heat because of the wall friction. Multiple factors such as metal type, hole design, hole pattern and the number of holes can all have an effect on heat transfer, but the predominant characteristic for heat transfer is the pellet die length to diameter ratio (Fahrenholz, 2012). A larger L:D ratio means that the die is thicker, which will typically increase pellet durability due to friction and die retention time.

Materials and Methods

Pelleting

A total of three diet formulations were each run at two different retention times (30 s and 60 s) and performed in triplicate. The diet is presented in table 6.1. The diet was split up into 100 kg batches. 20 kg of the complete diet was replaced with either 20 kg DDGS, 10 kg DDGS/ 10 kg fermented DDGS or 20 kg fermented DDGS. This resulted in treatments A, B and C which had a total β-Carotene concentration of 0 grams per ton, 25 grams per tonne or 50 grams per tonne, respectively. Run order was blocked by retention time and randomized. This was done to ensure the pellet mill could be operated continuously and reduce the chance of a die plug due to overfilling. Treatments were performed in triplicate for a total of 18 runs.

The diets were formulated based on the nutrient requirements for nursery swine 11-23 kg. Pellets were manufactured on a CPM ECO-R30 pellet mill (Crawfordsville, IN) equipped with a 3.97 mm x 31.75 mm pellet die. A throughput of 135 kg/hr was used for all runs. Retention times (30 s and 60 s) were set using a variable frequency drive and the same temperature of 80°C was used for all runs. The mash feeder screw, which feeds the steam conditioner, was also set to a constant RPM for all treatments using a variable frequency drive.

Pellets were collected immediately upon discharge from the pellet mill and placed into cooling trays at a depth of approximately 4 cm. Pellets were then cooled with ambient air in a locally constructed batch cooler. When pellets reached a temperature within 2.8°C of the ambient temperature, as measured by an infrared temperature gun (Fisher Scientific, 15-077-968), they were removed from the cooler and sifted through a U.S. #6 Sieve. This was done in order to simulate similar fines removal that would occur during a commercial run.

Moisture Content

Moistures were determined for the mash and cooled pellets via AACC International Method 44-19.0, Air Oven Drying at 135°C. Triplicate samples were collected at the mixer and pelleted samples were taken after they were cooled. Samples were immediately frozen, and were kept in the freezer until moisture analysis was conducted.

Storage Stability

Pelleted and mash samples of each treatment were collected into single layer paper bags and sewn closed. Samples were split into 2 bags and stored either in 21°C/45% humidity (ambient temperature/humidity) or 30°C/70% humidity to simulate multiple storage conditions the feed might be subjected to. Samples were left in these storage conditions for 28 d and samples were either kept on the same storage rack or in the same environmental chamber.. After the cycles were complete they were removed and immediately analyzed for β-carotene concentration and moisture content.

Statistical Analysis

Data were analyzed using the GLIMMIX procedures of SAS (Cary, NC, v. 9.4). Fixed effects included the main effects of added beta-carotene concentration (0, 25, or 50 g/tonne), diet form (mash, pelleted with a 30 s conditioning time, or pelleted with a 60 s conditioning time),

and storage conditions (none, ambient temperature, or accelerated temperature, as well as all significant interactions. Additionally, orthogonal contrasts were used to evaluate mash vs. pellet (including both 30 and 60 s conditioning times) and no storage vs. 29 d storage (including both ambient and accelerated storage conditions). Differences were considered significant if $P < 0.05$ and trends if $0.05 < P < 0.10$.

Results and Discussion

General

Feed manufactures have the ability in most instances to control the moisture content of the feed leaving their facility, but they have much less or no control over how and where the feed is stored once it leaves there facility. An integrated swine facility, for example, would utilize the feed manufactured within a few days or even that day, where as a commercial mill might bag feed and store it in an open warehouse, which may or may not have a ventilation system, before it goes to a retail facility. There is a need to establish what effect moisture content and storage time has on the efficacy of β-carotene.

Furthermore, there are still a fair number of operations that choose not to pellet feed. While this can increase the amount of feed wasted and decrease the nutritional performance of the swine, it eliminates the majority of processing costs and is sometimes more favorable to the bottom line of an operation. There was a need to evaluate both unpelleted, mash feed and feed pelleted at multiple conditioning times to determine what kind of downstream effect processing will have on final β-carotene concentration.

Moisture Content

There was a significant difference ($P<0.05$) in the moisture contents between the different forms of feed produced (mash, 30 s retention time, and 60 s retention time). There was

no significant difference (P=0.0619) between the different amount of added β-carotene, however there was a significant interaction (P<0.05) between feed form and added β-carotene.

Storage (before storage, 28 d storage at ambient, and 28 d storage at elevated conditions) was found to have a significant effect on moisture content. There was an average increase difference of 2.17% (w.b.) between the mash samples stored at ambient and elevated conditions. This would indicate the mash feed had a tendency to attract moisture at a much faster rate than the pellets. There was also a significant interaction (P<0.05) between the amount of added β-carotene and the type of storage

Qualitative analysis of the mash feed stored under elevated conditions showed signs of mold growth, an off smell and an increase in the temperature of the feed bed. There was little visible difference among the pelleted samples for both storage conditions. Paster (1984) showed a decrease in Colony Forming Units/g feed after diets had gone through pelleting, which is likely why there was no visible growth present. Further analysis will need to be conducted to determine what effect β-carotene might have on the presence of mold in feed.

β-Carotene Concentration

As expected, pelleting had a negative effect on β-carotene content (Table 6.3). Among the 30 s retention time there was a decrease from 24.84 g/tonne to 19.64 g/tonne and a 50.13 g/tonne concentration to 39.10 among treatments B and C respectively. In the 60 second retention time there was a decrease (P<0.05) from 24.84 g/tonne to 19.38 g/tonne and a 50.13 g/tonne concentration to 37.10 among treatments B and C respectively. This translated to an average of 21% decrease in β-carotene concentration for the 30 s retention time and a 23.5% for the 60 s retention time due to pelleting.

Among the 30 s retention time stored at ambient temperature for 28 d, there was a decrease from 24.30 g/tonne to 18.64 g/tonne and a 48.96 g/tonne concentration to 37.14 among treatments B and C, respectively (Table 6.3). In the 60 sec retention time there was a decrease from 24.30 g/tonne to 17.53 g/tonne and a 48.96 g/tonne concentration to 35.23 among treatments B and C, respectively. This translated to an average of 21% decrease in β-carotene concentration for the 30 s retention time and a 23.5% for the 60 s retention time due to pelleting.

The treatments stored in the environmental chamber showed a significant decrease ($P<0.05$) in β-carotene concentration from the samples stored at ambient. There was a 20% decrease in concentration from samples in the ambient environment to those stored in the environmental chamber for the 30 s retention time and a 17% decrease ($P<0.05$) for the 60 s retention time. Once again, there was a decrease from mash concentrations to pelleted concentrations.

Each main effects (feed form, level of β-carotene, and type of storage) had a significant effect ($P<0.05$) on the levels of β-carotene. In addition, feed form × level of β-carotene, feed form × type of storage, level of β-carotene × type of storage, and , feed form × level of β-carotene × type of storage were all significant interaction ($P<0.05$).

The major causes of carotenoid destruction during processing and storing of dietary carotenoids is enzymatic or non-enzymatic oxidation. Thus, carotenoids are susceptible to the loss of provitamin A activity through oxidation during processing (Dutta et al., 2005). Studies have shown reported values of carotenoids averaged 86% of the initial concentration after undergoing processing or storage (Jintasatapornv and Yuangsoi, 2012; Salunkhe et al., 1991), which is consistent with the results found in this study. They both concluded that total

carotenoid stability in the final product was mostly dependent on processing temperature and overall storage time.

Jintasatapornv and Yuangsoi (2012) did not utilize any conditioning step before pelleting. However, moisture was added to the mash before pelleting and the feed was still subjected to heat due to friction from the pellet die, but not as much heat as the diet would be exposed to had a conditioning step been added.

Conclusions

This study found that while there was a decrease in the overall β-carotene concentration of the diets both after pelleting for all retention times and concentrations and again after storage in elevated conditions, the concentration losses were consistent with previous studies which used bench-top scale processing equipment, which might not accurately reflect all the potential degradation points this ingredient might encounter during commercial processing.

The value-added ingredient inclusion outlined in this study has many advantages: 1) the use of modified ddgs in existing diets requires operational changes and physical behaves very similarly to traditional ddgs, 2) use of a readily available substrate for the production of modified ddgs means it could generate additional revenue streams for the corn wet-milling industry, 3) β-carotene does not require any expensive extraction/purification costs as it is already in a form that can be included directly into the diet, 4) has good shelf life after pelleting or as a mash at room temperature, and 5) provides ‘natural’ carotenoids.

Figures and Tables

Table 6.1 Diet Formulations

Ingredient	Control Diet	25 g/tonne β-Carotene	50 g/tonne β-Carotene
Ground Corn	41.30%	41.30%	41.30%
Soybean Meal	33.80%	33.80%	33.80%
Commercial DDGS	20.00%	10.00%	0.00%
Modified DDGS	0.00%	10.00%	20.00%
Choice White Grease	1.50%	1.50%	1.50%
Mono-Calcium Phosphate	1.15%	1.15%	1.15%
Limestone	0.95%	0.95%	0.95%
Salt	0.35%	0.35%	0.35%
L-Lysine 78.8%	0.30%	0.30%	0.30%
DL-Methionine	0.13%	0.13%	0.13%
L-Threonine	0.12%	0.12%	0.12%
Swine Trace Mineral KSU	0.15%	0.15%	0.15%
Swine Vitamin KSU	0.25%	0.25%	0.25%

Table 6.2 Moisture Content of Feed Before and After Storage

		Treatment A	Treatment B	Treatment C	SEM
Initial	Mash	$12.7\pm0.32^{i,j}$	$12.1\pm0.15^{l,m}$	$12.3\pm0.27^{l,k}$	0.1286
	30 s	11.8 ± 0.25^{n}	$12.5\pm0.53^{k,j}$	$12.8\pm0.48^{i,j}$	0.1286
	60 s	11.8 ± 0.25^{n}	$12.6\pm0.41^{k,j}$	$11.9\pm0.11^{m,n}$	0.1286
Ambient	Mash	$12.9\pm0.25^{i,h}$	$12.3\pm0.38^{l,k}$	$12.2\pm0.24^{l,k}$	0.1286
	30 s	$12.1\pm0.10^{l,m,n}$	12.6 ± 0.36^{j}	11.9 ± 0.16^{n}	0.1286
	60 s	$12.4\pm0.59^{k,j}$	$12.7\pm0.40^{i,h,j}$	13.0 ± 0.43^{h}	0.1286
70% H/30° C	Mash	14.7 ± 0.15^{b}	14.3 ± 0.08^{c}	14.9 ± 0.63^{a}	0.1286
	30 s	$13.4\pm0.17^{g,f}$	$13.6\pm0.20^{e,f}$	$13.7\pm0.28^{e,d}$	0.1286
	60 s	$13.5\pm0.10^{e,f}$	13.8 ± 0.09^{d}	13.3 ± 0.51^{g}	0.1286

Values in this table represent the average(N=2) moisture wet basis %

Differing superscripts in this table denotes a significant difference ($P<0.05$)

Table 6.3 β-Carotene Levels In Complete Feed Before and After Storage

		Treatment A	Treatment B	Treatment C	SEM
Initial	Mash	0.02 ± 0.001^{p}	24.84 ± 0.77^{i}	50.13 ± 0.96^{a}	0.221
	30 s	0.01 ± 0.002^{p}	19.64 ± 1.20^{l}	39.10 ± 1.54^{d}	0.221
	60 s	0.03 ± 0.003^{p}	19.38 ± 1.14^{m}	37.1 ± 1.62^{e}	0.221
Ambient	Mash	0.05 ± 0.003^{p}	24.30 ± 0.90^{j}	48.96 ± 0.88^{b}	0.221
	30 s	0.03 ± 0.002^{p}	18.64 ± 0.89^{n}	37.14 ± 1.03^{e}	0.221
	60 s	0.01 ± 0.001^{p}	17.53 ± 0.28^{o}	35.23 ± 0.82^{f}	0.221
70% H/30° C	Mash	0.03 ± 0.004^{p}	22.30 ± 0.67^{k}	46.38 ± 1.41^{c}	0.221
	30 s	0.02 ± 0.003^{p}	17.17 ± 1.02^{o}	29.71 ± 1.27^{g}	0.221
	60 s	0.02 ± 0.004^{p}	17.39 ± 0.82^{o}	29.24 ± 1.09^{h}	0.221

Values in this table represent the average(N=2) β-carotene content expressed in µg/g

Differing superscripts in this table denotes a significant difference ($P<0.05$)

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Chapter 7 - General Conclusions and Future Research

Potential Benefits of Fermented DDGS

Carotenoids have traditionally been classified as high value feed additive, but by co-producing them with a widely available feed ingredient, the overall cost of production and inclusion into diets can be mitigated.

This study establishes that (1) *Sporobolomyces roseus* can produce β-Carotenes by utilizing residual sugars from whole stillage, (2) protein and fat levels of the modified feed ingredient were decreased compared to unfermented whole stillage even though the fermentation was supplemented with various sources of non-protein nitrogen, (3) the production process could be scaled to a 50 L vessel and produce an equivalent product, both nutritionally and in regards to handling/processing variables, than a shake flask fermentation, (4) the physical properties of the resulting fermented ingredient in regards to bulk bin storage and transportation were measured and found to be very similar to that of dried whole stillage, and (5) β-Carotene concentration is affected by the pelleting process after the modified whole stillage is included into a complete feed diet, specifically there was a decrease in efficacy due to retention time.

Animal feed ingredients can be produced to included natural sources of β-carotenes. Benefits include decreased on-hand products for the feed mills, advantages for product storage stability and longevity, generation of a value-added product which could lead to higher revenue streams for ethanol facilities.

Future Research

Due to the decrease in protein and fat in modified DDGS, there is still a need to balance the nutritional profile of the modified DDGS to bring it closer to that of standard DDGS. This could still be accomplished by a variety of ways, but most notably, there could be a way to either

co-fermented ethanol and β-carotenes simultaneously or to stop the ethanol fermentation prematurely in order to increase the levels of residual sugars in DDGS. The obvious downside to this process would be a decreased throughput in ethanol production. It would be necessary to conduct a cost analysis of the process to see if any financial benefits could be realized.

While the scale up from laboratory to pilot scale reaction was successful in producing modified DDGS of extremely close quality and nutritional properties, there is still a need to evaluate the changes that might occur during large scale production of modified DDGS. Because of the increased volume of the production scale tank it is impossible to duplicate the same shear force, mixing time and product distribution that occurred in the pilot scale.

Large scale bioreactors are typically thermally sterilized with steam or hot water. It is difficult to predict the time and level of exposure that would need to be maintained in order to adequately sterilize production equipment or if additional steps, such as chemical cleanout, would be required. Plant sanitation schedules will be dramatically altered by the addition of this process and will need to be integrated in such a way so that the production schedule can be maintained.

Because whole stillage would likely be added directly after the distillation step, the slurry would already come into the fermentation vessel at a very high temperature (>85^{o}C). *Sporobolomyces roseus* has an optimal fermentation temperature of 28^{o}C to 34^{o}C so additional time will need to be taken for the mixture to cool or a temperature control line will need to be added to the fermentation vessel.

During the research conducted in Chapter 6, it was noted that mold growth occurred in some of the treatments with lower levels of β-carotene present. Preliminary studies suggest that the inclusion of β-carotenes in these diets are working to inhibit mold growth. Further research

is needed to classify the fate at which these molds are growing, what species of molds are growing and different factors which

Now that it has been demonstrated that *Sporobolomyces roseus* fermentation of whole stillage is capable of producing a feed with increased β-carotene content, consideration must be given to the nutritional aspects and its effect on swine growth. Specifically, a feeding trial will need to be conducted to measure the effects that different levels of modified DDGS has on swine average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G).

In addition to a standard feeding trial, it would also be advantageous to conduct an ileal digestibility trial. Information such as total tract digestibility and protein digestibility could be derived from such a study in order to obtain any significant nutritional differences between pigs fed standard DDGS and modified DDGS.