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Assessment of ethanol production by pentose-degrading yeasts in an integrated corn-soy bio-refinery and testing of efficacy of chemical preservatives in distillers wet grains

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Assessment of ethanol production by pentose-degrading yeasts in an integrated corn-soy bio-refinery and testing of efficacy of chemical preservative in distillers wet grains

by

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This thesis is dedicated to my parents Vijay Gaonkar and Ashalata Gaonkar for their constant support and for their faith and confidence in me. They have been with me through ups and downs. This thesis is also dedicated to my sister, Vidya Gaonkar and my best friend, Pradnya Shahapurkar, who have been my strength and told me repeatedly that I can do it. Thank you for being there for me even when we were miles apart.

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ABSTRACT

This thesis can be divided into two parts: one was to evaluate the effect of integrating coproducts of enzyme assisted aqueous extraction of oil from soybean oilseeds, namely soy skim and soy insoluble fiber, in corn fermentation when different yeast species are used, and the second was to evaluate the efficacy of different chemical preservatives in increasing the shelf life of distillers wet grains (DWG), which is a co-product of the corn fermentation process and sold as animal feed.

For evaluating the performance of different yeast species in ethanol production in an integrated corn-soy fermentation system, a review of published literature was conducted to understand the enzyme-assisted extraction process (EAEP) of oil from soybean. The review paper discusses in detail the evolution of enzymes based extraction process from aqueous extraction process and compares the process with conventional solvent-based oil extraction. The review indicates that the total time of oil extraction decreased and the oil yield from soybean oilseeds increased by 43-45% when enzymes were added to hexane-based oil extraction process. In aqueous extraction process, where water is used as extraction medium, the oil yield increased from 50% to around 85% when the soybean seeds were crushed using milling, flaking or extrusion. Hydrolytic enzymes were added to breakdown down cellulose and the protein network that captures oil molecules, which increased the oil yield to 98%. Pre-extraction steps such as flaking and extrusion, and enzymes used for extraction and de-emulsification (proteases and cellulases) are important factors that affect the oil and protein yield of EAEP of soybeans.

Once the process and factors related to EAEP was understood, co-fermentation of corn and soy products was carried out while testing the fermentation performance of two

yeasts species (*Pichia stipitis and Candida shehatae*) and comparing it with *Saccharomyces cerevisiae*. Since *S. cerevisiae* cannot assimilate pentose sugars such as xylose into ethanol, *P. stipitis* and *C. shehatae* were added to the system. Baseline data were obtained in synthetic media with 100% glucose, 100% xylose and glucose-xylose mixture. Further, fermentation was carried out for 72 hours in slurry containing just ground corn and water and slurry with ground corn, liquid soy skim and soy insoluble fiber. The performance of the three yeast species was compared on the basis of ethanol yield, ethanol production rate, ethanol final concentration and by-product concentration. It was observed that there was no significant difference at $p<0.05$ between the ethanol yields of the yeasts species and their mixture in corn only slurry, the mixture of *S. cerevisiae* and *C. shehatae* had the highest ethanol yield when compared to others in corn-soy product slurry. Individually, the production rate of *P. stipitis* and *C. shehatae* was slightly lower but when inoculated along with *S. cerevisiae*, the maximum production rate was comparable to *S. cerevisiae*.

Distillers wet grains is a co-product of ethanol production process from corn fermentation. These are unfermented ground corn; rich in fat, protein and few minerals. Addition of DWG to animal feed at 8-10% inclusion rate increased the total feeding value from 100 to 178 according to Klopfenstein et al (2008). Distillers grains are either fed as a wet product or dried to 20-30% moisture content. While the shelf life of dry product is higher and it is easy to transport, wet product is cheaper since no drying cost is involved. Wet product is preferred by livestock producers with farm near an ethanol plant. Since the moisture content is as high as 60%, chemical preservatives are used to increase the shelf life. The aim of the third objective was to test the efficacy of four commercial chemical preservatives and to compare their performance with a new preservative under development.

Lactic acid bacteria, yeast and mold and aerobic heterotrophic cells were enumerated on the day of addition of preservatives up to day 10. Mold-X and Fungiless were very effective in controlling the population of yeasts and mold over the period of ten days. The performance of the new product, SHIELD, increased with increasing concentration and it was observed that the preservative was the most effective in decreasing the population of aerobic heterotrophs.

CHAPTER 1. GENERAL INTRODUCTION AND REVIEW OF LITERATURE

General Introduction

The demand for energy has been increasing with the population and with changing times and climate conditions; demand for an environmentally, socially and politically sustainable energy source is increasing (Brown et al., 2012). One of the reasons the US is moving towards renewable energy is to decrease the dependency on foreign oil and the scientific community of the country has been trying to address this issue with bioenergy for some time now. Bioethanol and biodiesel as few of the prime options of renewable transportation fuels.

Bioethanol is produced from biomass like corn, sugar cane, wood, food waste, miscanthus and other energy crops. Any biomass containing carbohydrates can be used to produce ethanol. Biodiesel, on the other hand, is produced from oil extracted from oilseeds such as soybean, canola and jatropha. While the present method of oil extraction has been in use for a long time, there is a room for improvement.

Solvent-based oil extraction is one of the prevalent extraction method present today with yields greater than 95% (Campbell et al., 2011). Researchers started looking for alternatives when the environmental issues regarding hexane (most commonly used solvent for oil extraction) came into light. One of the alternatives is the aqueous extraction process where water is used as the extraction and separating medium. Further, the role of enzymes in this process was investigated. These processes have shown to achieve yield as high as 99% which has additional advantage of separating protein from these oil seeds. While oil is extracted from soybeans through enzyme-assisted aqueous extraction process, the coproducts of the process are insoluble fiber, consisting of major carbohydrates, and skim which is rich in isolate proteins.

The insoluble fiber fraction can be further treated to hydrolyze the polysaccharides into soluble sugars. Glucose yield of insoluble fiber fraction increased from 2% to 40% when the soybean flakes were extruded and the fiber fraction was pretreated (Karki et al., 2012). Co-fermenting corn with soybean insoluble fraction and skim, which provides a protein source for yeast, has been studied and increased ethanol yield have been observed. Soy skim has also shown to increase the fermentation rate and decrease the overall fermentation time (Yao et al., 2011). While Bakers' yeast, *Saccharomyces cerevisiae* is capable of giving an ethanol yield of over 100 mg/L, it is not very efficient in converting pentose sugars like xylose into ethanol. Yeasts like *Pichia stipitis* and *Candida shehatae* have been investigated in past for corn fermentation and have been shown to efficiently reduce pentose sugars into ethanol.

Once corn is fermented and the ethanol is distilled out, the slurry left behind is called whole stillage. Whole stillage is high in moisture (85-90%) and is further centrifuged to separate the thin stillage out. Approximately half of the thin stillage is sent back to the fermentation system as backset. The wet cake left behind is sold to livestock producers as animal feed. This cake is rich in proteins and fat and can be either sold as distillers wet grains (DWG) or distillers dried grains (DDG). DWG has around 60-65% of moisture in it and is susceptible to spoilage due to high water activity and high nutrient content. Livestock producers generally mix other forages to DWG to reduce the overall moisture content. Chemical preservatives are another way of reducing the spoilage of DWG and extending its shelf life. There are various chemical preservatives in the market. While formaldehyde is very efficient in reducing microbial growth, it is highly flammable and is carcinogenic. Lauric Arginate (LAE) is an antimicrobial majorly used in food products, is derived from

natural components and was approved by FDA in 2005 (Barros-Velazquez., 2016). Organic acids like benzoic acid, propionic acid, potassium sorbate have shown anti-microbial activity.

In this thesis, a review was conducted on the enzyme-assisted extraction process (EAEP) of soybean oil and was compared to hexane-extraction process. The coproducts of EAEP process were fermented along with ground corn and the performance of *Pichia stipitis, Candida shehatae* in converting the mixture of corn and soybean coproducts to ethanol was compared to *Saccharomyces cerevisiae.* Further, the efficacy different chemical preservatives in increasing the shelf life of DWG was tested by enumerating colony forming units of lactic acid bacteria, yeast and mold and aerobic heterotrophs.

Literature Review

Oil extraction- aqueous extraction with enzymes

Aqueous extraction process is an environmental-friendly alternative to hexane extraction as the medium used for extraction is water. In addition, in aqueous extraction process (AEP), protein isolates are extracted along with edible oil. The damage done to protein is negligible and elimination of solvent makes the process cost effective. Adequate treatment of effluent and low oil yield are among few drawbacks of AEP too (Rosenthal et al, 1996).

Addition of hydrolytic enzymes at 3%, extracted from *A. niger*, to AEP of soybeans increased the oil extraction yield to 90% (Fullbrook, 1983). The carbohydrase breaks down the cotyledon cell wall structure and the membranes. Protease hydrolysis the lipophilic proteins and the lipids trapped in the protein network are released.

Inadequate pre-treatments for rupturing the cell wall of oleaginous materials has thought to be the reason of low yield. Flaking and extruding processes are the means to

enhance the oil extraction during EAEP where extruding has shown to increase the extraction by 23% in AEP (Campbell, 2010). Soybeans have dominated the oilseed industry after World War II and in 2014, it represented 59.3% of world's oilseed production (National Oilseed Processors Association, 2015).

Corn-soybean coproducts fermentation

Dry-grind corn has been used in industry for ethanol production for a long time. Around 14.2 billion bushels of corn were grown in US in 2014 and 35% of it was used to produce ethanol. Total of 14.3 billion gallons of ethanol was produced in US in the year 2014, 3% higher than in 2013. Renewable fuel standard (RFS) program ensures that there is a continuous demand for ethanol every year. The volume standard for conventional biofuel has reached its maximum since 2015 (Renewable Fuel Standard Program, 2016).While, bioethanol industry is working towards achieving the RFS goals, issues like indirect land use change impacts have been associated with biofuel production. As engineers and researchers, making the process more efficient is our responsibility. Use of Soybean oil-extraction coproducts have been proved to significantly increase the ethanol production rate and ethanol yield while decreasing the fermentation time(Sekhon et al. 2015).

Apart from the common yeast, other organisms are being researched upon for their fermentation performance. Since the growth rate is higher in bacteria, they have garnered more attention than the conventional *Saccharomyces cerevisiae* for ethanol production(Senthilkumar & Gunasekaran, 2005). Metabolically engineered *Zymomonas mobilis* is seen as efficient replacement of baker's yeast for cellulosic ethanol production. The bacterial metabolic pathway has been proved to be more effective in ethanol fermentation from sugars when compared to the yeast(Lau et al., 2010). Though bacteria

have been studied to have the ability to digest these sugars, they cannot withstand high ethanol concentration making them economically less attractive.

Xylose makes up 1/3 of the total carbohydrate sugars present in lignocellulosic biomass and efficient conversion of xylose will definitely be economically favorable. *S. cerevisiae* cannot efficiently assimilate pentose sugars such as xylose and hence other organisms are researched upon for xylose fermentation. Since yeasts are widely used fermentation organism, they are metabolically engineered to get the desired characteristics(Pereira et al., 2014). Recombinant strain of *S. cerevisiae,* where genes for endoglucanses, cellobiohydrolases and β-glucosidases were inserted in wild type *S. cerevisiae* and resultant strain was able to produce 1.8g/L ethanol (Du et al., 2011). Among other yeast species, *Candida shehatae* and *Pichia stipitis* seemed promising for pentose fermentation. *P. stipitis* CSIR-Y633 strain does not produce Xylitol, byproduct of Xylose fermentation (Preez et al., 1985). Furthermore, this organism does not require any vitamin for xylose fermentation. Though both the organisms are good xylose converters, they have low tolerance for inhibitors and sensitive to ethanol unlike *S. cerevisiae* (Matsushika et al., 2009).

Preservatives of distillers wet grains

Along with protein, distillers grains are a good source of digestible neutral detergent fiber (NDF; 40-45% in DG), which is an indicator of high energy value product. Gross energy of the feed is further classified into digestible energy (DE), metabolisable energy (ME) and net energy for lactation (NE) and theses values for DWG are 1.81 Mcal/lb, 1.63 Mcal/lb and 1.00 Mcal/lb (dry matter) (Schroeder, 2003). Just 30% of distillers wet grains can replace a diet consisting alfalfa and corn silage in 1:1 mixed with corn silage and soybean meal concentrate.

Studies have shown that the feeding value of the diet increased from 100 to 178 just by including 10% of wet distillers grains with solubles into the feed. It also increased the palatability and condition the feedlot diet (Klopfenstein et al., 2008). In fact, it is reported that the energy value of distiller's grains is 120-180% of corn itself (Vander Pol et al., 2006).

There is no significant difference in the nutritive value of the wet and dry grains. Distillers dried grains (DDG) and distillers dried grains with solubles (DDGS) have extended shelf life and can be transported easily to longer distances and hence preferred by livestock producers as well as the ethanol plant. The drying step, though, incurs high cost and energy. Livestock producers with a farm near an ethanol plant generally prefer distillers wet grains (DWG) due to their lower cost. DWG prices are generally lower than usual in summer season as their shelf life is shorter when the temperature is high and air is humid. Livestock producers stock up when the prices are low and store DWG with other forages like alfalfa to preserve it. Storing DWG in 9-12 foot sealed bag can minimize its interaction with atmosphere decreasing the microbial growth rate hence extending its shelf life. These sealed bags add on to the cost which is estimated to be around $5-$8 per ton of DWG (Schroeder, 2003). Another alternative is to add chemical preservative before bagging or storing it in silo bunkers.

References

Campbell, Kerry Alan. (2010). "Protein and oil recoveries from enzyme-assisted aqueous extraction of soybeans and sunflower seed". Graduate Theses and Dissertations. Paper 11172

du Preez, J. C., Bosch, M., & Prior, B. A. (1986). Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: effects of pH, temperature and substrate concentration. *Enzyme and Microbial Technology*. http://doi.org/10.1016/0141-0229(86)90136-5

Du, J., Shao, Z., and Zhao, H. (2011). Engineering microbial factories for synthesis of value-added products. *Journal of Industrial Microbiology and Biotechnology.* 38(8):873-890

Fullbrook, P. D. The use of enzymes in the processing of oilseeds. J. Am. Oil Chem. Sot. 1983, 60,428A-430A

Klopfenstein, T.J., Erickson, G.E., Bremer, V.R. (2008). Use of distillers by-products in the beef cattle feeding industry. *Journal of Animal Science* 86:1223-1231

Lau, M. W., Gunawan, C., Balan, V., & Dale, B. E. (2010). Comparing the fermentation performance of Escherichia coli KO11, *Saccharomyces cerevisiae* 424A(LNH-ST) and Zymomonas mobilis AX101 for cellulosic ethanol production. *Biotechnology for Biofuels*, *3*, 11. http://doi.org/10.1186/1754-6834-3-11

Pereira, F. B., Romaní, A., Ruiz, H. a., Teixeira, J. a., & Domingues, L. (2014). Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. *Bioresource Technology*, *161*, 192–199. http://doi.org/10.1016/j.biortech.2014.03.043

Rosenthal, A., Pyle, D. L., & Niranjan, K. (1998). Simultaneous aqueous extraction of oil and protein from soybean: mechanism for process design. *Food Bioprod. Process.* 76:224-230

Renewables Fuel Association. *2015 US Ethanol Co-product Exports and Imports Statistical Summary.* Retrieved from http://www.ethanolrfa.org/wp-content/uploads/2016/02/2015-U.S.-Distillers-Grains-Trade-Statistics-Summary.pdf

Schroeder, J.W. (2003). Distillers Grains as a Protein and Energy Supplement for Dairy Cattle. *NDSU Extension Service* AS-1241

Sekhon, J. K., Jung, S., Wang, T., Rosentrater, K. a., & Johnson, L. a. (2015). Effect of coproducts of enzyme-assisted aqueous extraction of soybeans on ethanol production in dry-grind corn fermentation. *Bioresource Technology*, *192*, 451–460. http://doi.org/10.1016/j.biortech.2015.05.096

Senthilkumar, V., & Gunasekaran, P. (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. *Journal of Scientific and Industrial Research*, *64*(11), 845–853.

Taheripour, F., Hertel, T., Tyner, W., Beckman, J., & Birur, D. (2010). Biofuels and their by-products: Global economic and environmental implications.

United States Environmental Protection Agency. (2016). Renewable Fuel Standard Program. Retrieved from https://www.epa.gov/renewable-fuel-standard-program/program-overview-renewable-fuel-standard-program

Vander Pol, Kyle J.; Erickson, Galen E.; Greenquist, Matthew A.; Klopfenstein, Terry J.; and Robb, Thomas, "Effect of Corn Processing in Finishing Diets Containing Wet Distillers Grains on Feedlot Performance and Carcass Characteristics of Finishing Steers" (2006). Nebraska Beef Cattle Reports. Paper 119. http://digitalcommons.unl.edu/animalscinbcr/119

Wang, H., Wang, T., Johnson L.A., (2009). Effect of Low-Shear Extrusion on Corn Fermentation and Oil Partition. *Jornal of Agricultural and Food Chemistry*, *57*(6), 2302-2307.

CHAPTER 2. RESEARCH OBJECTIVES

Objectives

This thesis is written in two folds- first to evaluate the performance of pentose degrading yeasts in comparison to Bakers' yeast in an integrated corn-soy fermentation system and to evaluate the effect of chemical preservatives on the shelf life of distillers wet grains (DWG).

Further, the whole study is divided into three clear objectives:

1. To review the enzyme-assisted aqueous extraction process (EAEP) of soybean and compare it with conventional hexane-extraction method;
2. To compare the fermentation performance of *Pichia stipitis* and *Candida shehatae* with *Saccharomyces cerevisiae* in an integrated corn-soy fermentation system; and
3. To evaluate the efficacy of different preservative in increasing the shelf life of distillers wet grains produced in corn ethanol plant

Thesis Organization

Chapter 3, 4 and 5 discusses in detail the research goal stated above. Chapter 3 is an review of literature of enzyme assisted oil extraction process of soybean and its comparison to conventional solvent-extraction process. Chapter 4 is an experimental study where the co-products from EAEP of soybean were added to corn fermentation and the performance of pentose-digesting yeasts namely *Pichia stipitis* and *Candida shehatae* was evaluated in comparison with *Saccharomyces cerevisiae*. Chapter 5 is again an experimental study where the efficacy of various chemical preservative in prolonging the shelf life of distillers wet grains was tested and compared. Chapter 6 is an overall conclusion of the studies conducted as a part of my research and the plans for future work.

CHAPTER 3. OIL EXTRACTION- FROM SOLVENT BASED TO ENZYME-ASSISTED AQUEOUS EXTRACTION PROCESS- A REVIEW

Abstract

Drawbacks of solvent based oil extraction from oilseeds have led to increased usage of aqueous extraction process where water is used as the extraction medium and the basic principle of separation of oil is its immiscibility in water (Aqueous extraction process- AEP). Hydrolytic enzymes like proteases, cellulases can further facilitate AEP by breaking the cell walls of these oilseeds and by breaking the protein network, which releases the oil molecules (Enzyme-assisted aqueous extraction process-EAEP). Physical and enzymatic pretreatment of oilseeds makes extraction of oil and downstream processing easier and faster when compared to solvent-based oil extraction. EAEP can be performed in either single stage or two stages. Two-stage EAEP has proven to be useful and economical when the process is scaled up to pilot plant since the ratio of oil yield to the amount of enzyme used is higher compared to single stage EAEP. The oil, protein and solids yield obtained in pilot plant were consistent with those obtained at laboratory scale. This paper focuses on AEP and EAEP of soybeans and compares the environmental and economic aspect of these processes to solvent based extraction method. It also discusses important factors that affect the oil and protein recovery in EAEP process and de-emulsification of oil. In conclusion, AEP and EAEP appear to be better alternatives despite low yield (AEP) and high operational cost (EAEP) because when compared to solvent-based oil extraction, they are safe and capitally less intensive.

Introduction

The conventional oil extraction methods used in industries are solvent extraction, expeller pressing and hydraulic pressing. The oldest method, Hydraulic pressing, is labor

intensive and the use has diminished over the years. Pressing is now combined with solvent extraction in a two-stage extraction process. When the raw material cannot be processed directly, pre-pressing method helps to prep the material.

Solvent extraction, on other hand, has proven to be excellent when the extraction substrate has low oil content along with high oil content. The commonly used solvent is Hexane, and it is separated from the oil by evaporation and distillation (Rosenthal et al, 1996). Solvent extraction is the most preferred method when edible oil needs to extracted from oilseed because the process is efficient in yielding oil up to 95% or more and recovers 95% of hexane. Despite all the pros of this process, it is known that Hexane is a highly flammable liquid and can pose a health hazard to the workers in the extraction plant. It causes severe damage to the nervous system with chronic as well as low exposure for a long time. This is where the need for a much safer and eco-friendly extraction process arises.

Aqueous extraction process is an environmentally cleaner alternative to hexane extraction as the only medium used for extraction is water. In addition, in aqueous extraction process (AEP), protein isolates are extracted along with edible oil. The damage done to protein is negligible and elimination of solvent makes the process cost effective. Adequate treatment of effluent and low oil yield are among few drawbacks of AEP too (Rosenthal et al, 1996).

Addition of hydrolytic enzymes at 3%, extracted from *A. niger,* to AEP of soybeans increased the oil extraction yield to 90% (Fullbrook, 1983). The carbohydrase breaks down the cotyledon cell wall structure and the membranes. Protease hydrolysis the lipophilic proteins and the lipids are released.

Inadequate pre-treatments for rupturing the cell wall of oleaginous materials has thought to be the reason of low yield. Flaking and extruding processes are the means to enhance the oil extraction during EAEP where extruding has shown to increase the extraction by 23% in AEP (Campbell, 2010). Soybeans have dominated the oilseed industry after World War II and in 2014; it represented 59.3% of world's oilseed production (National Oilseed Processors Association, 2015).

This review paper discusses aqueous and enzyme assisted extraction process of soybeans and compares them with solvent extraction method.

Conventional Oil Extraction Processes

Solvent extraction is a widely used extraction process due to its high efficiency and low cost (Sawada et al, 2014). Once extracted using a solvent (mostly Hexane), the crude oil undergoes degumming (to remove phosphatides and gums), alkali treatment (to remove metallic pro-oxidants, free fatty acids acids), bleaching (to remove soap particles and pigments) and deodorization (to remove off-flavors) (Brekke, 1980) as shown in Figure 3.1.

Studies have been conducted to test if supercritical fluid specifically Supercritical CO_2 (SC-CO_2) could replace hexane. Oil from full-fat flaked was extracted using hexane and SC-CO_2 and the yield obtained was 20% and 19.9% respectively. Along with the yield, the % free fatty acid, peroxide value and unsaponifiables of SC-CO_2 (0.5%, 0.2 and 0.5% respectively) were comparable to hexane too. The phosphorus content of oil decreased by approximately 10 fold and the chromatography refining loss reduced by 3.8% when SC-CO_2 was used (Friedrich and List, 1982). It was also observed that when the pressure at which CO_2 is supplied was increased from 5000psig to 8000psig, the extraction efficiency

increased. At higher pressures, the solubility of oil in CO_2 increased with increasing temperature (from 50˚C to 60˚C at 6000psig) (Friedrich et al., 1982).

Other solvent like isopropanol were studied individually and in combination of hexane. After 3 hours of extraction, oil extraction yield of isopropanol was 28%, hexane 34% and mixed solvent (isopropanol + hexane) ~ 40%. Various levels of ultrasound waves were tested while using hexane and it was observed that increasing intensity, the oil yield increased. This is the result of cavities produced by compression and shearing while ultrasound waves are passed through the flaked soybeans. The yield was more than 45% when ultrasound of intensity 47.6 W/cm^2 was used (Li et al., 2004).

Ethanol was also investigated as a potential solvent. Absolute ethanol could achieve the yield of 21% at 60˚C. Ethanol was diluted with de-ionized water at 6% mass basis and its yield was greater than 20% only at 90˚C. Protein extraction decreased with dilution (Sawada et al., 2014).

Adding Enzymes to the Solvent-based Extraction

Sherba et al. (1972) first investigated enzymatic extraction when they fractionated Soybean using protease as reported by Rosenthal. Fullbrook then worked on aqueous hydrolysis of oilseed followed by addition of solvent and in simultaneous presence of solvent. His results showed that oil extraction was more efficient when the solvent was present during the aqueous hydrolysis.

Extraction of oil with mixture of enzymes (Pectinase, Cellulase and Hemicellulase) was also conducted where the solvent used was petroleum ether (Olsen et al., 1988). Enzymes partially hydrolyze the cell wall and increase the cell permeability. When the canola flakes are autoclaved and moisture adjusted and then subjected to enzyme mixture

along with different kinds of carbohydrase, yield of canola oil and extraction time varies. It is then followed by drying and hexane extraction. Using the enzymes increases the yield by 45% and the time of extraction decreases comparatively. (Sosulski et al., 1988)

To enhance extraction of antioxidant, pectin polysaccharide is processed with enzyme mixture. Enzymes have shown to increase the extraction to 7.4 g/kg from 1.7g/kg of raw material (dry weight) (Gan et al., 2010). For enhanced extraction of lycopene from tomatoes, cellulase and pectinase are used under optimal condition that increased the yield by 2.5 fold. (Choudhari et al., 2007)

Enzymatic treatment along with hexane extraction have been studied for low moisture content Soybean. For soybeans with moisture content between 15-20%, cellulose and Multifect enzymes were added before solvent extraction and they were added simultaneously for soybeans with moisture content lower than 12%. While the total oil extracted from untreated sample (only solvent extraction; no enzymes) was 79.50%, it was 84.85% for 10% moisture soybean that was treated with enzymes and solvent at 0.75 E/S ratio (Dominguez et al., 1995).

Aqueous Extraction Process (AEP)

In this process, oil is extracted from the oilseeds with the help of water using the principle of dissolution of oil in water. The oilseeds are conditioned, ground and oil is extracted by boiling water, which floats on the surface. Oil is removed and dried. Instead of boiling water, oil and protein isolates can be separated using centrifuge too. This prevents protein denaturation caused by high temperatures.

Based on the pH of the extraction medium, protein can be collected from solids as concentrate or from liquid phase as isolates (Figure 3.2).

For extraction of oil from soy flour, temperature, solid-liquid ratio, particle size, agitation speed and pH are deciding factors. It is shown that at pH of 4.5, the oil and protein yield of AEP of soy flour is very low since at that pH, soy protein have very low solubility. The oil and protein yield decreases with increasing particle size too (Rosenthal et al., 1998).

Mechanism of crushing the soybean grains majorly affects the particle size that in turn affects the oil yield. Milling, flaking, milling+flaking (flour from flakes) and extrusion were tested and complete cellular disruption was achieved only in extrusion. Light microscopy of residual matter after 2 hours of aqueous extraction of flour from flakes showed coalesced oil droplets, dissolved proteins and very little residual matter was present in extracellular space. In case of extruded soybean, oil droplets were found in the solid matrix. The oil yield was 75% for flour from flakes and 68% in case of extruded material (Campbell et al., 2009). Combining flaking and extrusion would ensure complete cell disruption and increase the oil yield.

The oil yield of AEP is usually lower when compared to solvent extraction since 100% recovery of oil from skim is not achieved and some oil stays un-extracted in the fiber rich fraction. This problem can be addressed by using subcritical water (water with temperature greater than 100˚C but less than 374˚C at pressure between 1-8 MPa; condensed form) which can be used to extract proteins since the polarity of water decreases at subcritical conditions making the extraction of bioactive compounds easier. Oil yield varied from 38.2% to 83.9% in case of extruded soybean flakes and between 4.2% and 50.2% for un-extruded soybean flakes when the solids to liquid ratio changed between 1:3.3 and 1:10, temperature varied between 66˚C and 234˚C and the extraction time varies between 13 to 47 mins. Temperature affects the solubility and dissolution of solute in solvent. When heated,

the denatured proteins sequester the oil particles and at temperature greater than 150˚C, protein extractability was slightly higher in case of extruded flakes. Conditions that increased the oil and protein yield in extruded flakes and just the flakes were 150˚C, 66˚C, respectively, and 1:11.7 solids-to-liquid ratio for both (Ndlela et al., 2012).

Enzyme-assisted Aqueous Extraction Process (EAEP)

Addition of enzymes like cellulase, pectinase or protease to the aqueous extraction process for enzymatic breakdown of the cell components makes the process enzyme-assisted aqueous extraction process. It has been widely used in past to extract natural pigments, flavors, medicinal compounds, polysaccharides and oils. The advantage is accelerated extraction, enhanced recovery, energy efficient process and it is eco-friendly process as solvent usage is reduced (Puri et al., 2010). Phenolic compounds have been extracted from citrus peel using EAEP. The citrus peel was grounded and pre-treated with Cellulase® MX, Cellulase® CL and Kleerase® AFP (food grade enzymes). After centrifugation and filtration, the filtrate is extracted by evaporation of solvent using rotary evaporator (Li et al., 2006).

Enzymes have been incorporated in the rural extraction processes too. The copra meal was finely milled and slurry with water was prepared. The slurry then was pre-treated with enzymes like protease and pectinase from *Aspergillus niger*, cellulase/hemicellulase from *Trichoderma reseei* and α-amylase from *A. oryzae* and incubated for 6 hours at 37℃. Water floatation technique was used to extract the oil. Extraction Yield reportedly increased by 50% when compared to the controls (Kwaku et al., 1997).

Enzyme mixture depends on the meal that is being used. For peanuts oil, cellulase, protease and α-1,4-galacturonide glucanohydrolase has shown to increase the yield by 6-

10%. Protizyme containing papain, trypsin and chymotrypsin has shown to increase the peanut oil yield from 44% to 92% when the shaking speed was 80 rpm (Sharma et al., 2002).

In the study conducted by Campbell et al. (2009), they compared AEP to EAEP and it was seen that addition of a protease (Protex 7L) increased the oil yield of flour from flakes and extruded material as tabulated in Table 3.1.

It was clear with the light microscopy image of protease hydrolyzed residual matter that most of the protein was dissolved and the material is loose and amorphous. Cellulase was shown to have no effect on oil yield in case of extruded material and this indicates that extrusion process ensures complete cell disruption (Campbell et al., 2009).

In case of study conducted by deMoura et al (2008), soybeans were extruded at around 100℃ and 100 rpm screw speed in a twin-screw extruder. The extruded flakes were added to water to achieve 1:10 solids-to-liquid ratio. Proteases (Protex 6L-alkaline protease- at 0.5 and 1.0% and Protex 7L-neutral protease- at 0.5%) was added at optimum concentration, temperature and pH. The slurry was centrifuged to separate out insolubles after the extraction and the liquid fraction was processed to separate the free oil. A process diagram of the same is displayed in Figure 3.3.

The authors attributed the high oil yield of Protex 6L (Table 3.2) to better selection of soybean variety, extrusion parameters and enzymes. The amount of free oil obtained by Protex 6L (0.5%) was twice of the amount obtained from Protex 7L. It was observed that conditions favoring protein and oil extraction were similar because protein networks and oleosin membrane capture oil particles that are released when the network is broken down. High amount of solids were found in skim fraction in case of 0.5% Protex 6L (58%).

Factors Affecting Oil and Protein Recovery

Pre-extraction steps

Grinding and flaking operation determines the particle size of the oil seed. Smaller the particle size, easier it is for water-soluble particles to separate out. Enzyme dissolution also becomes easier when the size is small. Based on the moisture content of the seeds, they are either ground dry or wet. These operations along with extrusion determine the stability of emulsion. In absence of enzymes, the oil recovery dry pellets of soybean was 50%, which increased to 60% on grinding the pellets to about 2-3mm in diameter and the oil recovery further increased to 75% when the pellets were extruded under water at 100˚C (Lamsal et al., 2006).

EAEP of extruded full-fat soy flakes yielded more oil when compared AEP of full fat soy flours and separatory funnel procedure was used to quantify the oil in each fractions (Lamsal et al., 2007). The free oil obtained by EAEP of extruded flakes was 8 times that of AEP of flour (Table 3.3) indicating extrusion and use of enzymes for extraction has significant effect on the oil yield. The size of oil droplet produced by extruded flakes was 2.25 times larger than droplets produced by AEP of soy flour. Increase in size results in higher terminal velocity and the oil particles rise up, coalesce making more and more free oil available (Lamsal et al., 2007).

Temperature while extrusion plays a major role in protein extraction and in turn oil extraction. High temperature denatures proteins, breaking the protein networks hence releasing the oil trapped. At higher temperatures than 100˚C, frothing was observed which leads to oil sequestering thereby decreasing the oil yield. Temperature of extruder barrel has shown to positively affect the oil and protein yield till 100 ˚C (at 12% moisture content and

100rpm screw speed; oil yield 55%) and negatively affect it at temperatures 120˚C (at 12% moisture content and 100rpm screw speed; oil yield 45%) and higher (Lamsal et al., 2006).

It is clear from the data displayed in Table 3.4 that increase in temperature has negative effect on the oil and protein yield. Moisture content has positive effect on the dependent variables since the diffusion of oil and its release becomes easier. The screw speed did not seem to have much effect on the oil and protein yield since at 14% moisture content and 100˚C temperature, change in rpm did not matter.

Enzymes used for extraction and de-emulsification

Since enzymes have shown to increase the oil and protein yield, their concentration, pH of the slurry when added and temperature is very important. It is not necessary that every enzymes may give good results. Cellulase, for example, have not shown any effect on the oil yield of extruded soy flakes. Proteases, on other hand, can degrade the peptides surrounding oil molecules called oleosin, making oil extraction easier.

Kapchie et al. (2008) compared the effect of enzyme cocktail (Pectinase, Cellulase and Multifect CX3L) at different concentration and total time of application with the control (no enzymes) on oleosomes. Extraction was performed by blending the hydrated soybean flour with buffers in a Waring blender. As the blending time increased, the oil yield in control decreased since it created an emulsion in the upper layer after centrifugation. In case of enzyme-assisted extraction, the oil yield increased with increasing blending time. With increase in enzyme concentration, the total oil yield increased and the oil content in residue decreased (Table 3.5).

When similar studies were carried out at pilot plant scale, after 10 hours of centrifugation, 93% of oil was recovered from the oleosome fraction. The enzyme cocktail

used here was slightly different from the laboratory scale: Pectinase, Multifect CX B and Multifect CX G. Laboratory scale experiments were performed again with the new set of enzymes and the oil yield was found to be 77%. Horizontal decanter centrifuge used in pilot plant in comparison to series benchtop centrifuge used in lab-scale was better at mixing and re-circulating slurry for maximum oil release. Protein content was highest in supernatant after centrifugation (~26%) and the distribution of glycinin and β-conglycinin fractions were studied in supernatant, precipitate (oleosome) and initial soy flour and there was a slight decrease in % of total mass in supernatant of these protein subunits in pilot plant when compared to lab-scale (Towa et al., 2011).

When 0.5% Multifect Protease enzyme was added to soybean flakes, the oil yield increased from 46% to 71% and for extruded flakes, it increased from 56% to 88%. Extrusion on its own increased the yield by 10% (Lamsal et al., 2006).

Protex 6L when added to isolated oleosomes at 0.25% dosage recovered oil lesser (3 hours of hydrolysis and 30 mins of destabilization time; oil yield of 65%) when compared to 2.5% of Protex 6L (3 hours of hydrolysis and 30 mins of destabilization time; oil yield of 85%). The yield increased with increase in hydrolysis and destabilization time. Maximum yield of 90% was reached at 2.5% of Protex 6L, 18 hours of hydrolysis and 3 hours of destabilization time (Towa et al., 2011).

Presence of substance like free fatty acids or phospholipids decreases the quality of oil and it was studied that while the % of free fatty acid of hexane-extracted freeze dried soybean oil was 1.18%, enzyme assisted aqueous extracted soybean oil had only 0.11-0.18% of fatty acids. This was because enzymatic hydrolysis occurred at high temperatures and basic pH at which the free fatty acids would neutralize and precipitate out when centrifuged.

These fatty acids affect the oxidative stability index (OSI) of the oil too. While the OSI of soybean oil extracted by EAEP was lower (12 hours) than the crude soybean oil (27 hours), it was slightly higher when compared hexane extracted soybean oil (9 hours) and almost similar to commercial sold soybean oil (11 hours) (Towa et al., 2011).

When different food grades enzymes (endopeptidase: Multifect Neutral (MN), Bromelain (BR) and exopeptidase: exo. C) were tested for their ability to emulsify soy flour hydrolysates and the emulsification capacity (gram of oil/gram of protein) decreased from 1935 (control-no enzyme) to 1702 for MN, 1456 for BR and 1288 for exo. C (Lamsal et al., 2006).

While reviewing the literature of oil extraction from soybean, it was learnt that two main factors which significantly affect the oil and protein yield of the process where extrusion and addition of enzymes as mentioned above. Extrusion mainly ruptures the cell wall and makes the proteins available for enzymes (proteases) to breakdown and for water to flow and carry the free oil. Cellulases are ineffective when the extraction is preceded by extrusion.

Table 3.6 data shows that the oil yield of 15% moisture soy flakes increases to 90% from 60% when extruded at 100rpm at 100˚C before extraction and 0.5% of proteases is added while extraction. The solubility of isolated proteins increased when the flakes were extruded from 71% to 94% (Jung et al., 2009).

Downstream Processing

Generally, in aqueous extraction process, oil and protein are demulsified and then centrifuged to separate into layers of aqueous and oil phase. Centrifugation can cause creaming at times but it gives complete separation through phase inversion (Rosenthal et al.,

1996). In EAEP of soybean, the free oil+cream fraction undergoes de-emulsification to separate residual skim and to obtain free oil. Centrifugation separates the de-emulsified fraction into three distinct layers- free oil layer, intermediate layer and skim layer (Figure 3.5*: Flow diagram of cream de-emulsification (based on de Moura et al., 2008).*) (Campbell et al., 2010). De-emulsification can be either done physically (freeze-thaw, heating), chemically (pH 4.5) or with enzymes (like Phospholipase C, Protex 6L or enzyme cocktail).

When heating at 95˚C, freeze-thaw, Lysomax/G-zyme cocktail (enzyme cocktail) and phospholipase C were tested for cream de-emulsification against the control and except for heating, every other treatments were very effective in destabilizing the emulsion. While freeze-thawing cause denaturation of soy protein, breaking the emulsion and releasing the oil as free oil, Lysomax and phospholipase breaks the ester bond between the fatty acid groups. Freeze-thaw yielded around 86% of free oil and phospholipase recovered 73% of oil as free oil (Lamsal et al., 2007). Effect of pH was tested on the stability of cream and free oil emulsion. Control was held at pH 8, which is the initial cream pH. Yield of free oil increased with decreasing pH and maximum (100% free oil yield) was reached at pH 4- 4.5 as shown in Figure 3.4.

LysoMax™ was compared to Protex 51FP to test its impact on cream destabilization and it was observed that Protex 51FP yielded 88% of oil at 0.2% level, which was more than twice of what LysoMax ™ achieved at the same concentration (Wu et al., 2009).

According to deMoura et al. (2008), the free oil yield in percentage is calculated as:

$$Free\ Oil\ Yield(\%) = \frac{[Free\ oil(g) + hexane\ washed\ free\ oil(g)]}{[cream(g) \times oil\ content(\%) in\{cream + free\ oil\ fraction\}]} \quad (3.1)$$

Pasteur pipette was used to collect free oil and it was quantifies using hexane following procedure mentioned in Lamsal et al. (2007).

2.5% of Protex 6L (P6L) was used for de-emulsifying cream produced by Protex 6L and Protex 7L extraction of soybeans. Maximum free oil yield for Protex 7L-cream was 91% and for Protex 6L-cream was 100%. When the pH of cream extracted with Protex 6L was set to 4.5, temperature was maintained at 25°C and no agitation was provided, 100% free oil yield was achieved (deMoura et al., 2008).

Cream de-emulsification is an important step in EAEP and enzymes like proteases prove to be efficient it destabilizing the cream and separating out skim from the free oil. Research has been conducted to integrate EAEP and the de-emulsification step which will be discussed in the next section.

Two-stage EAEP and Scale-up

According to J. M. L. N. Moura's referenced paper on scaling up of EAEP, work done in past in EAEP of soybeans resulted in high oil and protein extraction yield when the solids-to-liquid ratios (1:10) is relatively low and extraction is performed in single stage. When scaled up, at same ratio, the water required to too large which leads to large skim production. To enhance the protein and oil extraction, the amount of water has to be reduced without compromising the efficiency of extraction. This is where two-stage counter-current EAEP comes into play. The extruded flakes are processed through two stages of extraction and liquid fraction of second stage is recycled to first. Slurry from first stage is centrifuged to remove insoluble particle. Liquid phase is further separated into skim and cream as shown in Figure 3.6. The insoluble part is fed again into the extractor. Second stage slurry is centrifuged and the liquid phase is recycled to first stage (de Moura et al., 2009).

The oil content in skim and insoluble decreased slightly in two-stage EAEP, which is desirable as it decreases downstream processing efforts. With increase in solid content in two-stage EAEP (solids to liquid ratio 1:5), the amount of water used decreased reducing the oil content in skim fraction and the extraction efficiency improved by 2%.

When scaled up from 0.08 kg extruded flakes to 1 kg extruded flakes, the oil, protein and solids extraction yield of single stage EAEP remained the same. The amount of oil in the skim fraction increased from 14% at laboratory scale to 20% at pilot-plant scale-up. When the two stage EAEP was scaled-up, there was slight increase in all the three extraction yields and the oil content in skim fraction was higher (Table 3.7). The amount of oil in skim was reduced by changing the extraction condition (pH 8.0 for 60 mins) (deMoura et al., 2009).

Concurrent two-stage EAEP was integrated with Cream de-emulsification with three stages in total- two extraction stages and one de-emulsification stage. The enzyme added in de-emulsification step was reused in the 2^{nd} stage of EAEP and the enzyme recovered after the 2^{nd} stage was recycled in the 1^{st} stage of EAEP (Figure *3.7*) (deMoura et al., 2011).

There was slight decrease in the oil and protein yield when compared to two stage EAEP as shown in Table 3.8. Since the first liquid fraction was settled overnight, the weight and viscosity increased making it difficult to separate cream. The separated cream needed enzymes 1.5 times more than usual. When the amount of protease increase, it results in extensive hydrolysis of proteins and there are high chances that new interactions between peptides and oil may have stabilized the emulsion instead of destabilizing it (deMoura et al., 2011).

The amount of enzyme required in this process is very high and the yield obtained is comparatively lower than two-stage EAEP. Although this process was developed to reduce

the overall enzyme use, the amount of enzymes required in de-emulsifying 3rd skim is greater than the amount required in two-stage EAEP.

Solvent Extraction v/s AEP and EAEP

Environmental aspect

Cheng et al. (2016) conducted environmental impact assessment of soybean oil extraction by hexane-based oil extraction method and EAEP. Hexane extraction had an input component environmental impact (EI) value of approximately 39 with hexane contributing 92% to it. EAEP, on the other hand, had an input component impact value of ~10 in a multiplying system. The general environment impact (GEI) value of hexane-based extraction and EAEP were ~3.5 and 1.0, respectively.

The output component EI of EAEP was higher when compared to hexane-based extraction (~42 and 36) with soy skim contributing 76% to the EI of EAEP. Soy skim, co-product of EAEP, is produced in large quantity and as mentioned before, contains around 15-20% of extracted oil. Since the downstream processing of soy skim is difficult, finding a commercial use of it can reduce its impact. Hexane-based extraction still had the highest GEI of output components (~3.4).

At the rate of 1 kg of soybean oil produced, the greenhouse gas (GHG) emissions of EAEP were higher. Pre-treatment of soybean is an essential step in EAEP for higher oil and protein yield and these steps consume electricity three times more than the hexane-based extraction method, which drives up the process's GHG emission (Cheng et al., 2016).

Economic aspect

For the years 2010-2014, the total plant direct cost (TPDC) of hexane-based oil extraction process accounted for ~44-48% of total capital investment. While startup cost was

just ~4%, the working capital accounted for 14.95% of the total investment. In 2015 estimate, TPDC takes up 45% of the total capital cost of EAEP with 13% working capital and 4% start-up capital. In hexane-based extraction, hexane and soybeans are the two raw materials that are used which accounted for 89.75% of total annual operating cost in 2010-2014. At a large scale of EAEP, materials contribute 83-88% of the total operating cost: 60.45% Soybean, 30.68% enzymes and remaining water and ammonium hydroxide. While hexane-based extraction is capital intensive, EAEP has higher operating costs. Hexane recovery rate is as high as 95%, which reduces annual consumption rate and saves on the operating cost. Recycling enzymes can largely improve the economic feasibility of EAEP.

While soybean oil contributed ~33% toward the total revenue of hexane-based oil extraction, it accounts for just 23.84% in EAEP. The economic aspect of EAEP can be improved if the coproducts of the process can be sold for integrated corn-soy fermentation.

Conclusion

This review shows that the basic understanding of the protein-oil matrix in soybeans, the effect of mechanical pre-treatment of soybeans on total oil yield and the process of de-emulsification of cream has improved with the evolution of the aqueous oil extraction process. Addition of proteases helps in breaking down the matrix of denatured proteins of extruded soybeans, resulting in release of oil trapped in the matrix. Further, the oil in cream fraction can be demulsified either enzymatically or with pH adjustment, a process that is less time and energy consuming when compared to degumming process in solvent extraction. While solvent extraction just results in crude oil, protein isolates can be recovered in EAEP of soybeans along with oil. These soy protein isolates contain many bioactive peptides,

which may have therapeutic applications, and Ultrafiltration has been reported to be the best method of protein precipitation and separation.

While the highest reported total oil yield of EAEP has levelled and even exceeded the oil yield of solvent-based extraction process (~99%), the free and demulsified oil amounts up to only 85%. The oil present in skim fraction is regarded as lost due to the difficulty in separation of oil from the fraction and a commercial use of skim fraction need to be identified. The process of addition of the coproducts of EAEP of soybeans to corn fermentation has been investigated at the laboratory and pilot scale. It is reported that the soy skim increases fermentation rate and decreases the amount of water required during fermentation. It also adds value to the distillers wet grains produced after the fermentation. This result needs to be scaled to the commercial level.

References

Brekke. 0. L. Edible oil processing. An introduction. In: Handbook of Soy Oil Processing and Utilization (Erickson, D. R., Ed.). American Soybean Association and American Oil Chemists Soci- isolate-production Properties. J. Food Sci. Technol. (India) ety, St. Louis, 1980

Choudhari, S.M. and Ananthanarayan, L. (2007) Enzyme aided extraction of lycopene from tomato tissues. Food Chem. 102, 77–81

Campbell, Kerry Alan, "Protein and oil recoveries from enzyme-assisted aqueous extraction of soybeans and sunflower seed" (2010). Graduate Theses and Dissertations. Paper 11172

Campbell, K.E., and C.E. Glatz. 2010. Protein Recovery from Enzyme-assisted Aqueous Extraction of Soybean. Biotechnol. Prog. 26(2):488-495.

Campbell, K.E., and C.E. Glatz. 2009. Mechanisms of Aqueous Extraction of Soybean Oil. J. Agric. Food Chem. 57:10904-10912.

Campbell, K.A., C.E. Glatz, L.A. Johnson, S. Jung, J.M.N. de Moura, V. Kapchie, and P. Murphy. 2011. Advances in Aqueous Extraction Processing of Soybeans. J. Am. Oil Chem. Soc. 88:449-465.

Cheng, M., Zhang, W., Rosentrater, K., Sekhon, J., Wang, T., Jung, S. and Johnson, L. (2016). Environmental Impact Analysis of Soybean Oil Production from Expelling, Hexane Extraction and Enzymes Assisted Aqueous Extraction. An ASABE Meeting Presentation. doi 10.13031/aim/20162459781

de Moura, J. M. L. N., Campbell, A. K., Mahfuz, A. A., Jung, A. S., Glatz, A. C. E., & Johnson, A. L. (2008). Enzyme-Assisted Aqueous Extraction of Oil and Protein from Soybeans and Cream De-emulsification. http://doi.org/10.1007/s11746-008-1282-2

de Moura, J.M.L.N., D. Maurer, S Jung, and L.A. Johnson. 2011. Integrated Countercurrent Two-stage Extraction and Cream De-emulsification in Enzyme-assisted Aqueous Extraction of Soybeans. J. Am. Oil Chem. Soc. 88(7):1045-1051.

de Moura, J.M.L.N., D. Maurer, S Jung, and L.A. Johnson. 2011. Pilot-plant Proof-of-concept for Countercurrent Two-stage Enzyme-assisted Aqueous Extraction Processing of Soybeans. J. Am. Oil Chem. Soc. 88(10):1649-1658.

Dominguez, H., Nunez, M.J. and Lema, J.M. (1995). Enzyme-assisted hexane extraction of soya bean oil. *Food Chemistry.* 54:223-231

Friedrich J.P., List G.R. (1982). Characterization of Soybean Oil Extracted by Supercritical Carbon Dioxide and Hexane. *J. Agric. Food Chem.* 30:192-193

Friedrich, J.P., List, G.R. and Heakin, A.J. (1982). Petroleum-Free Extraction of Oil from Soybean with Supercritical CO_2

Fullbrook, P. D. The use of enzymes in the processing of oilseeds. J. Am. Oil Chem. Sot. 1983, 60,428A-430A

Gan, C.Y. and Latiff, A.A. (2010) Extraction of antioxidant pecticpolysaccharide from mangosteen (Garcinia mangostana) rind: optimization using response surface methodology. Carbohydr. Polym. 83, 600–607

Jung, S., B.P. Lamsal, V. Stepien, L.A. Johnson and P.A. Murphy. 2006. Functionality of Soy Proteins Produced by Enzyme-assisted Extraction. J. Am. Oil Chem Soc. 83(1):71-78.

Jung, S., D. Maurer, and L.A. Johnson. 2009. Factors Affecting Emulsion Stability and Quality of Oil Recovered from Enzyme-assisted Aqueous Extraction of Soybeans. Bioresour. Tech. 100(21):5340-5347.

Jung, S., and A. Mahfuz. 2009. Low-temperature Dry Extrusion and High-pressure Processing Prior to Enzyme-assisted Aqueous Extraction of Full-fat Soybean Flakes. Food Chem. 114:947-954.

Kapchie, V., D. Wei, C. Hauck, and P.A. Murphy. 2008. Enzyme-Assisted Aqueous Extraction of Oleosomes from Soybeans (Glycine max). J. Agric. Food Chem. 56:1766-1771.

Karki, B., D. Maurer, and S. Jung. 2011. Efficiency of Pretreatments for Optimal Enzymatic Saccharification of Soybean Insoluble Fractions. Bioresource Technol. 102:6522-6528.

Karki, B., D. Maurer, T.H. Kim, and S. Jung. 2011. Comparison and Optimization of Enzymatic Saccharification of Soybean Fibers Recovered from Aqueous Extractions. Bioresource Technol. 102:1228-1233.

Karki, B., D. Maurer, H. Kim, and S. Jung. 2012. Ethanol Production from Soybean Fiber: A Co-product of Soybean Oil Extraction. J. Am. Oil Chem. Soc. 89:(1)1-9.

Kwaku, Tano-Debrah and Ohta, Y. (1997). Aqueous Extraction of Coconut Oil by an Enzyme-Assisted Process. *Journal of Science of Food and Agriculture.*74(4) :497-502

Lamsal, B.P., C. Reitmeier, P.A. Murphy, and L.A. Johnson. 2006. Enzymatic Hydrolysis of Extruded-expelled Soy Protein and Resulting Functional Properties. J. Am. Oil Chem. Soc. 83(8):732-737.

Lamsal, B.P., P.A. Murphy, and L.A. Johnson. 2006. Flaking and Extrusion as a Mechanical Treatment for Enzyme-assisted Aqueous Extraction of Oil from Soybeans. J. Am. Oil Chem. Soc. 83(11):973-979.

Lamsal, B.P., and L.A. Johnson. 2007. Separating Oil from Aqueous Extraction Fraction of Soybeans. J. Am. Oil Chem. Soc. 85(8):785-792.

Leite, J. M., De Moura, N., De Almeida, A. N. M., & Johnson, A. L. A. (2009). Scale-up of Enzyme-Assisted Aqueous Extraction Processing of Soybeans. http://doi.org/10.1007/s11746-009-1406-3

Li, B. B., Smith, B., & Hossain, M. (2006). Extraction of phenolics from citrus peels II. Enzyme-assisted extraction method. *Separation and Purification Technology*, *48*, 189–196. http://doi.org/10.1016/j.seppur.2005.07.019

Li, H., Pordesimo, L. and Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. *Food Research Journal*. 37(7): 731-738

Nobrega de Moura, J.M.L, K. Campbell, A. Mahfuz, S. Jung, C.E. Glatz, and L.A. Johnson. 2008. Enzyme-assisted Aqueous Extraction of Soybeans and Cream De-emulsification. J. Am. Oil Chem. Soc. 85(10):985-995.

Nobrega de Moura, J.M.L, and L.A. Johnson. 2008. Two-stage Countercurrent Enzyme-assisted Aqueous Extraction of Oil and Protein from Soybeans. J. Am. Oil Chem. Soc. 86(3):283-289.

Nobrega de Moura, J.M.L, N.M. de Almeida, and L.A. Johnson. 2009. Scale-up of Enzyme-assisted Aqueous Extraction of Soybeans. J. Am. Oil Chem. Soc. 86(8):809-815.

Ndlela, S.C., J.M.L.N. de Moura, N. K. Olson, and L.A. Johnson. 2011. Aqueous Extraction of Oil and Protein from Soybeans with Subcritical Water. J. Am. Oil Chem. Soc. (accepted).

Olsen, H. S. (1988). Aqueous enzymatic extraction of oil from seeds. *Food Science And Technology In Industrial Development, 1,* 30-37.

Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. *Trends in Biotechnology*. http://doi.org/10.1016/j.tibtech.2011.06.014

Rosenthal, A., Pyle, D. L., & Niranjan, K. (1996). Aqueous and enzymatic processes for edible oil extraction.*Enzyme and Microbial Technology.* 19(6):402-420

Rosenthal, A., Pyle, D. L., & Niranjan, K. (1998). Simultaneous aqueous extraction of oil and protein from soybean: mechanism for process design. *Food Bioprod. Process.* 76:224-230

Sawada, M.M., Venâncio, L.L., Toda,T.A., & Rodrigues, E.C., (2014). Effects of different alcoholic extraction conditions on soybean oil yield, fatty acid composition and protein solubility of defatted meal. Food Res. Int., 62, 662-670.

Sharma A., Khare, S.K, Gupta, M.N. (2001). Enzyme-assisted aqueous extraction of rice bran oil. *AOCS Journal of the American Oil Chemists' Society, 78,* 949–951.

Sharma A., Khare, S.K, Gupta, M.N. (2002). Enzyme-assisted aqueous extraction of peanut oil. *AOCS Journal of the American Oil Chemists' Society, 79*, 215–218.

Sherba. S. E., Steigerwalt, R. B., Faith, W. T., Smythe Jr., C. V. Soybean fractionation employing a protease. U.S. patent 3640725 A; 1972

Sosulski, K., Sosulski, F.W. and Coxworth, E. (1987). Carbohydrase hydrolysis of canola to enhance oil extraction with hexane.*Journal of the American Oil Chemists' Society.* 65(3):357-361

Towa, L.T., V.N. Kapchie, C.C. Hauck, H. Wang, and P.A. Murphy. 2011. Pilot Plant Recovery of Soybean Oleosome Fractions by an Enzyme-assisted Aqueous Process. J. Am. Oil Chem. Soc. 88:733-741.

Towa, L.T., V.N. Kapchie, G. Wang, C. Hauck, and P.A. Murphy. 2011. Quantity and Quality of Free Oil Recovered from Enzymatically Disrupted Soybean Oleosomes. J. Am. Oil Chem. Soc. 88:1581-1591.

Wu, J., L.A. Johnson, and S. Jung. 2009. De-emulsification of Oil-rich Emulsion from Enzyme-assisted Aqueous Extraction of Extruded Soybean Flakes. Bioresource Tech. 100(2):527-533.

Yao, L, T. Wang, and H. Wang. 2011. Effect of Soy Skim from Soybean Aqueous Processing on the Performance of Corn Ethanol Fermentation. Bioresource Technol. 102(19):8727-9334.

Yao, L., S. Lee, T. Wang, J.M.L.N. de Moura, and L.A. Johnson. 2011. Effects of Fermentation Conditions on Corn-soy Co-fermentation for Fuel Ethanol Production. Bioresource Technol. (accepted).

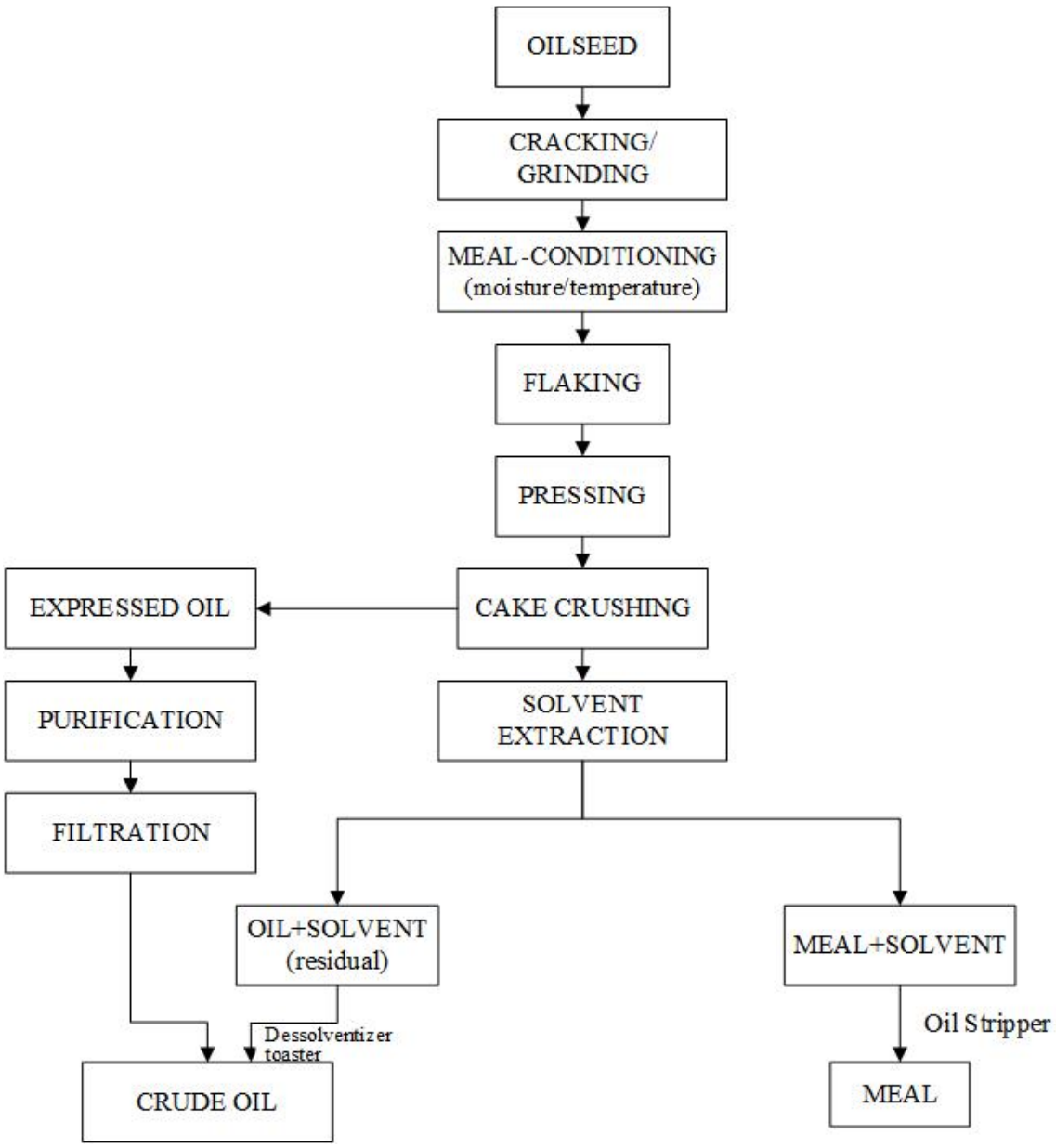

Figure 3.1: Combination of solvent extraction and pressing for extracting oil from an oilseed (based on Rosenthal, 1996).

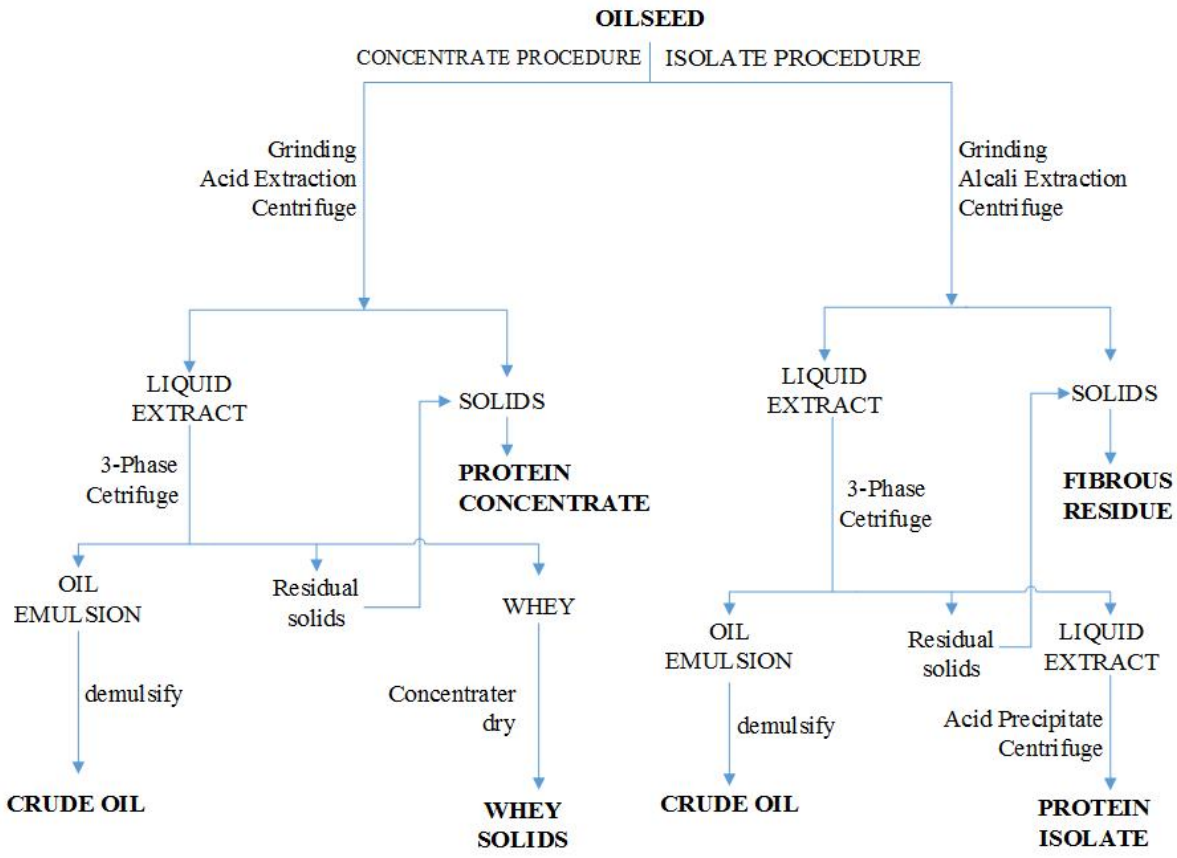

Figure 3.2: Steps involved in the aqueous extraction process (AEP). Two alternatives are provided (based on Rosenthal et al., 1996).

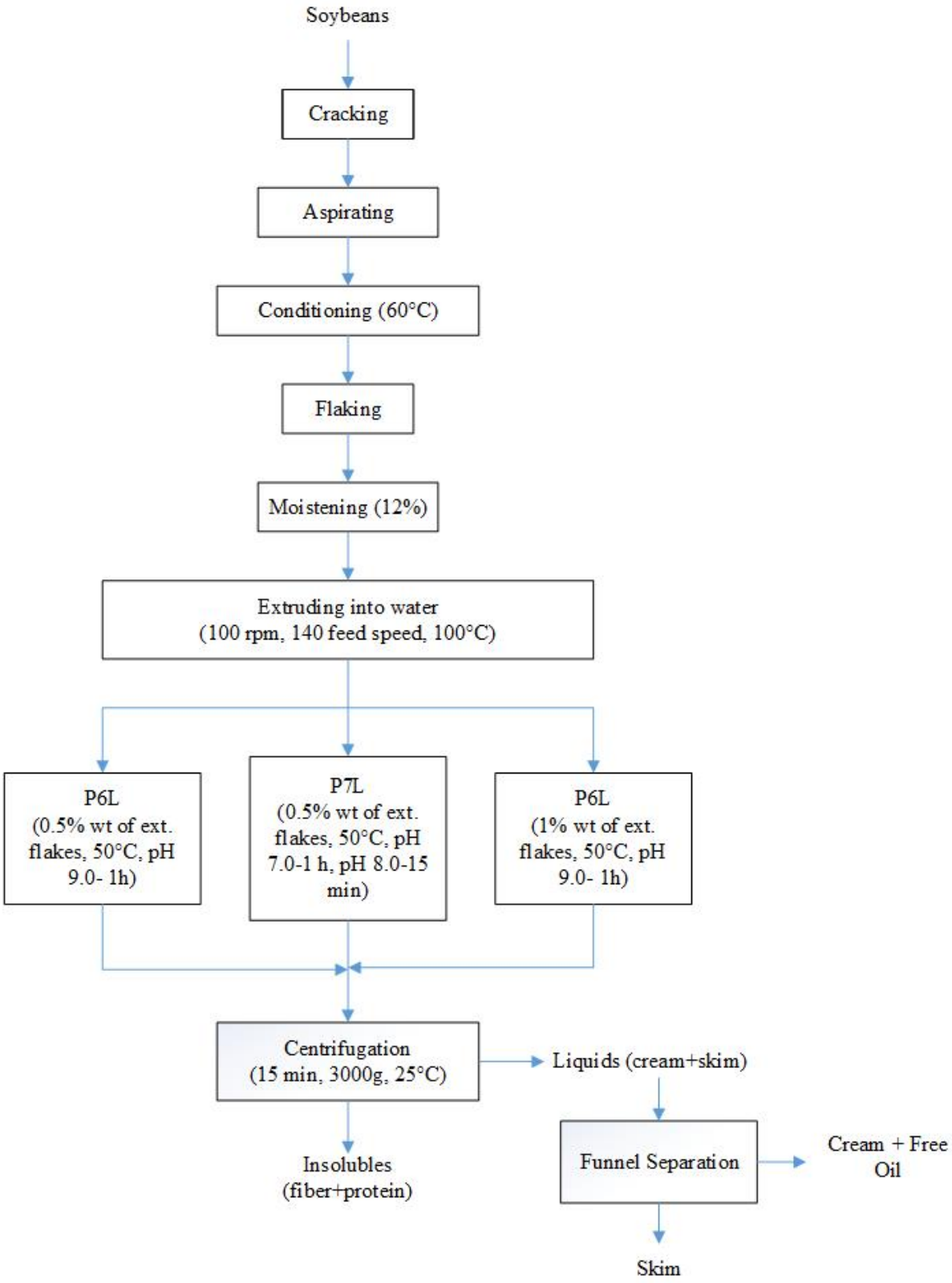

Figure 3.3: Flow chart of the enzyme assisted extraction process of soybean where two different Proteases were compared (Protex 6L and Protex 7L) (based on de Moura et al., 2008).

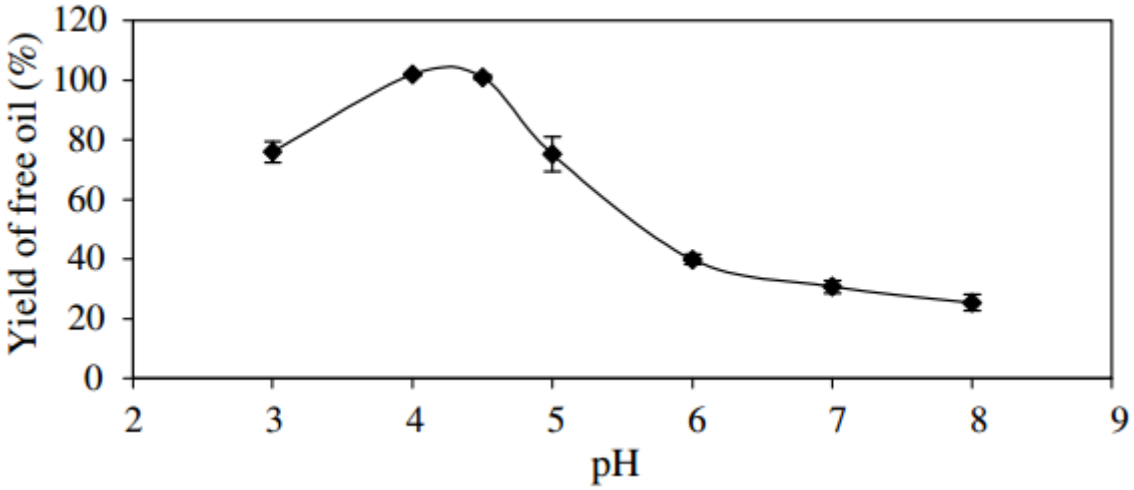

Figure 3.4: Free oil yield as the pH of (cream+free oil) fraction changes (from Wu et al., 2009)

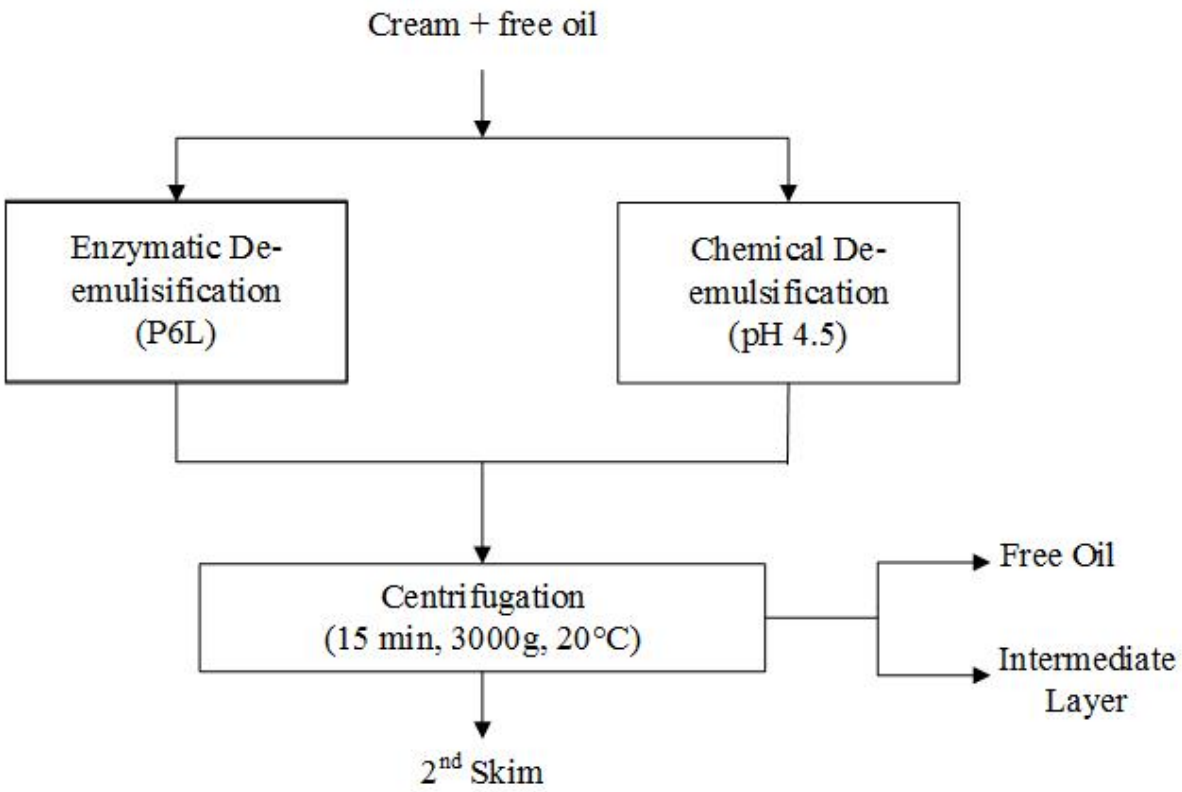

Figure 3.5: Flow diagram of cream de-emulsification (based on de Moura et al., 2008).

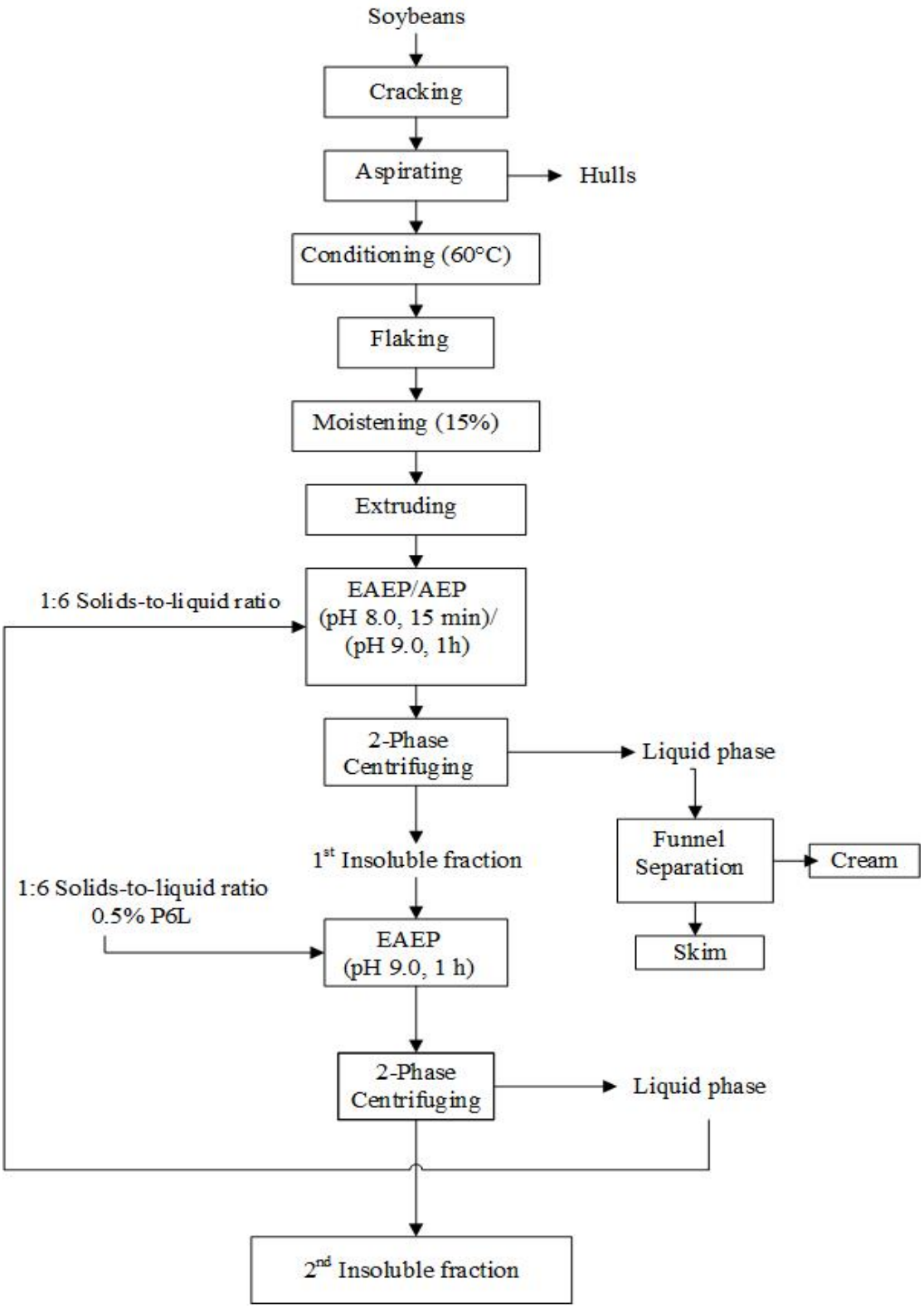

Figure 3.6: Two stage EAEP of Soybean- Flowchart (based on de Moura et al., 2008)

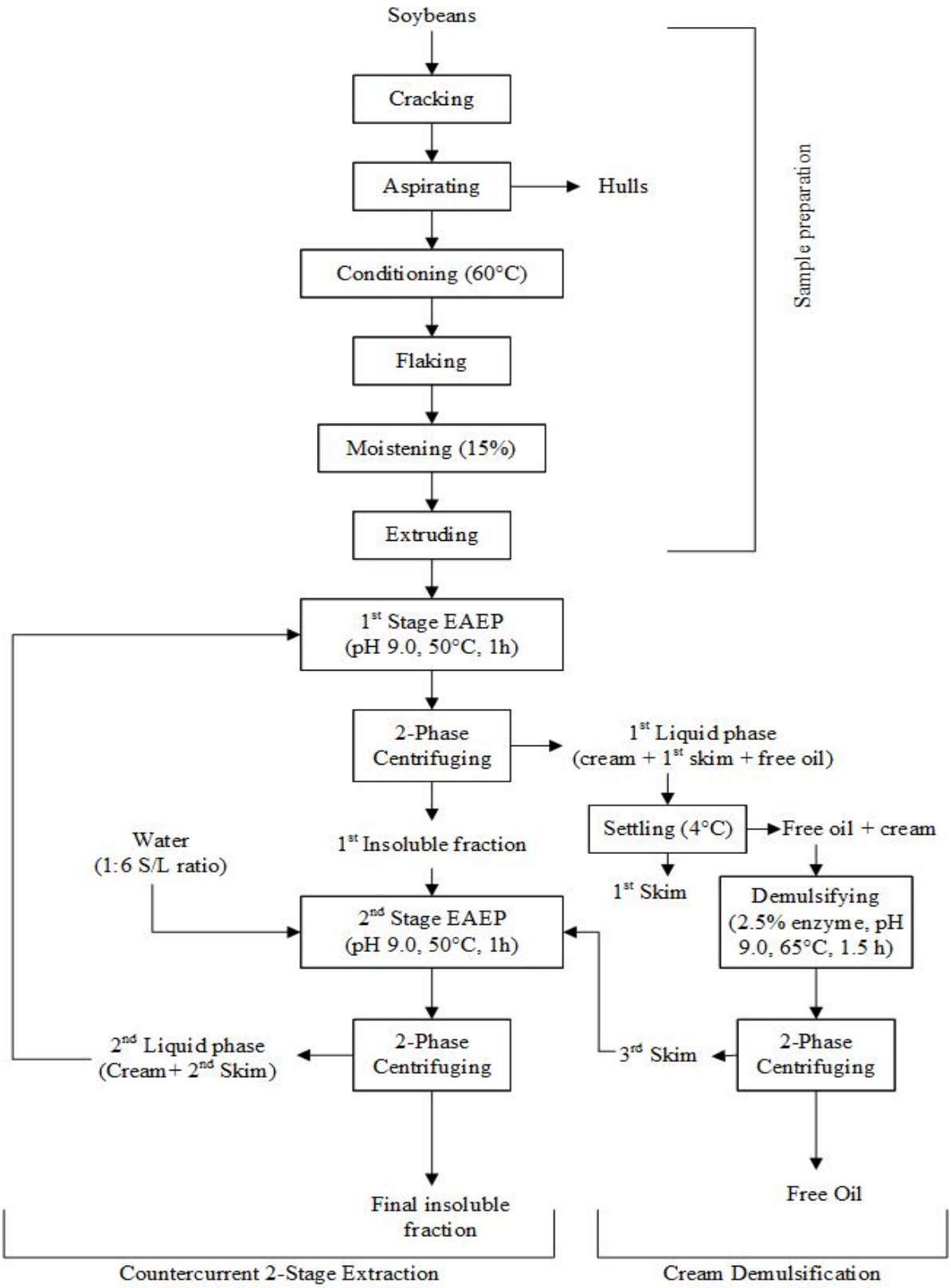

Figure 3.7: Integrated concurrent two-stage enzyme-assisted aqueous extraction process of soybeans and cream de-emulsification (based on deMoura et al., 2011).

Table 3.1: Oil yield (in %) with and without enzymes (Protease- Protex 7L) (Campbell et al., 2009)

Oil Yield (%)	**Soybean Flour from Flakes**	**Soybean Extruded Flakes**
In AEP (without enzymes)	75	68
With Protex 7L	79	88-96

Table 3.2: Oil and protein yield of different enzyme treatments on extruded soybean flakes along with size of peptides produced (deMoura et al., 2008). All the values are rounded-up.

	Protex 6L 0.5%	**Protex 6L 1%**	**Protex 7L**
Oil Yield (%)	96	97	93
Protein Extraction Yield (%)	85	87	73
Molecular weights of peptides yielded	<30 kDa	-	>54.1 kDa
Dry matter extraction yield (%)	77	79	71

Table 3.3: Distribution of oil, protein and solids in various fraction of AEP of soy flour and EAEP extruded flakes (Lamsal et al., 2007). All the values are rounded up.

	Fractions	**Cream**	**Skim**	**Insolubles**	**Free Oil**
Oil Yield (%)	AEP of full-fat soy flour	45	15	35	2
	EAEP of full-fat extruded soy flakes	60	13	13	16
Protein Yield (%)	AEP of full-fat soy flour	2	85	19	-
	EAEP of full-fat extruded soy flakes	1	79	23	-

Table 3.3 continued

Dry Matter (%)	AEP of full-fat soy flour	12	53	34	1
	EAEP of full-fat extruded soy flakes	15	53	30	4

Table 3.4: Effect of temperature of extruder barrel, moisture content of flakes and screw speed on oil and protein yield (Lamsal et al., 2006). All the values are rounded up. Bold formatted numbers have the highest oil and protein yield.

Temperature of Barrel (˚C)	**Moisture content of soybean extruded flakes (%)**	**Screw speed of extruder (rpm)**	**Oil Yield (%)**	**Protein Yield (%)**
100	12	100	53	51
100	12	150	50	54
100	**14**	**100**	**55**	**59**
120	12	100	46	33
100	14	150	55	54
120	14	150	42	35

Table 3.5: Change in oil yield with the enzyme concentration. Enzyme cocktail used here was made of Pectinase, Cellulase and Multifect CX3L (Kapchie et al., 2008). All the values are rounded up.

Oil Yield (%)	**Control (0% enzyme)**	**0.6% enzymes (v/w)**	**1.5% enzymes (v/w)**	**3.0% enzymes (v/w)**
45 secs blending time	36	-	-	36
180 secs blending time (1st extraction)	38	52	59	64
180 secs blending time (2nd extraction)	7	10	14	14
180 secs blending time (3rd extraction)	2	1	8	5
180 secs blending time (4th extraction)	1	1	3	3
Summation of Yield (%) at 180 secs blending	49	57	82	85

Table 3.6: Effects of extrusion and enzymes on oil and protein yield (Jung et al., 2009). All the values are rounded-up to next higher whole number.

Processing prior to extraction	**Extraction process**	**Oil yield (%)**	**Protein yield (%)**
Full fat flakes	Aqueous extraction without enzymes	60	74
Full fat flakes	Aqueous extraction with enzymes (Protex 7L at 0.5% dosage)	60	76
Extruded Flakes	Aqueous extraction without enzymes	68	45
Extruded Flakes	Aqueous extraction with enzymes (Protex 7L at 0.5% dosage)	90	75

Table 3.7: Comparison of single stage EAEP to two-stage EAEP and scale-up of two-stage EAEP in terms of Oil Yield, Protein Yield and Solids Yield (deMoura et al., 2009). All values are in percentage. Bold formatted numbers are higher among respective yields.

	Type of EAEP	**Cream**	**Skim**	**Insoluble**	**Total extracted**
Oil Yield (%)	Standard single stage EAEP	61	14	4	96
	Two Stage EAEP	85	13	2	98
	Two Stage EAEP-Scale Up (pH 8.0, 15 mins)	76	23	1	**99**
	Two Stage EAEP-Scale Up (pH 9.0, 60 mins)	86	12	1	**99**
Protein Yield (%)	Standard single stage EAEP	1	87	13	87
	Two Stage EAEP	0	91	8	92
	Two Stage EAEP-Scale Up (pH 8.0, 15 mins)	7	87	6	94
	Two Stage EAEP-Scale Up (pH 9.0, 60 mins)	9	87	4	**96**
Solids Yield (%)	Standard single stage EAEP	17	56	23	77
	Two Stage EAEP	23	57	20	80
	Two Stage EAEP-Scale Up (pH 8.0, 15 mins)	23	59	17	83
	Two Stage EAEP-Scale Up (pH 9.0, 60 mins)	28	56	16	**84**

Table 3.8: Yields of integrated two-stage EAEP and cream de-emulsification (deMoura et al., 2011).

	Cream	**Skim**	**Insoluble**	**Total extracted**
Oil Yield (%)	64	32	4	96
Protein Yield (%)	8	81	11	89
Solids Yield (%)	21	59	18	81

CHAPTER 4. COMPARING THE FERMENTATION PERFORMANCE OF *CANDIDA SHEHATAE, PICHIA STIPITIS* AND *SACCHAROMYCES CEREVISIAE* IN AN INTEGRATED CORN-SOY FERMENTATION SYSTEM

Abstract

Since xylose is one of the major pentose sugars present in biomass, producing ethanol from biomass is economically favorable when xylose is efficiently consumed during the fermentation by the microbe. *Pichia stipitis* and *Candida shehatae* are two yeasts species known to efficiently degrade pentose sugars. Hence, this study compares their performance (in terms of ethanol yield, ethanol production rate, ethanol concentration and by-product concentration) with industrially used yeast, *Saccharomyces cerevisiae* in an integrated fermentation system where, the coproducts of the enzyme assisted oil extraction process (EAEP) of soybeans are mixed with ground corn. Insoluble fiber (IF) and soy skim are the two co-products of EAEP of soybean oilseeds which have shown to enhance ethanol production rate along with the quality of distillers grains. First, fermentation in synthetic media (YPD) was carried out at different initial sugar concentration (100% Glucose, 100% Xylose and Glucose-Xylose mixture) to obtain baseline data. Further, the corn-only slurry and corn-IF-skim slurry were treated with either (a) *S. cerevisiae* (active dry baker's yeast), (b) wild-type *P. stipitis*, (c) wild-type *C. shehatae*, (d) mixture of *S. cerevisiae* and *P. stipitis* (1:1) or (e) mixture of *S. cerevisiae* and *C. shehatae* (1:1). Additionally, *S. cerevisiae* was added to treatment (b) and (c) after 24 hours of start of fermentation to create treatment (f) and (g), respectively. Yeasts in treatment (d) and (e) completely utilized the sugars in Glucose-only and Xylose-only synthetic media and had the maximum ethanol production rate. Similar results were also observed in Glucose-Xylose media. While there was no significant difference between the ethanol yield of the yeast species or their mixture in corn

only slurry at 95% confidence interval, mixture of *S. cerevisiae* and *C. shehatae* had an ethanol yield 4% greater than *S. cerevisiae* in corn-IF-skim slurry. No improvement in ethanol yield or production rate was observed in treatment (f) and (g). Glycerol (by-product) concentration in treatment (b) and (e) decreased by approximately 40% and 41% for corn-only and 11% and 16% in corn-IF-skim slurry respectively. A 50% decrease in acetic acid (byproduct) concentration was observed in corn-only slurry when *P. stipitis* was added. While there was no significant difference in ethanol yield, concentration and production rate, the concentration of byproducts decreased in presence of *P. stipitis* and *C. shehatae*. Surprisingly, in corn-IF-skim slurry, P. stipites and *C. shehatae* could withstand ethanol concentration of ~120mg/ml.

Keywords: *Corn fermentation, Enzyme-assisted aqueous extraction, Ethanol, Modified yeast strains.*

Introduction

According to Economic Research Service of US Department of Agriculture, the United States produced 14.8 billion gallon of ethanol in 2015 accounting for 8% of nation's total fuel production and the use of ethanol as fuel slashed 106.4 million metric tons of greenhouse gas emissions in 2014. A life cycle analysis study, conducted at Argonne National Laboratory, found that the life cycle greenhouse gas emission could be reduced by 19-48% when gasoline is replaced by corn-ethanol (Wang et al., 2012). In the US, over 95% of all fuel sold contains 10% ethanol. Since one of the major commercial products of biofuel technology is ethanol, efficient fermentation of biomass and complete understanding of fermentation parameters are of utmost importance.

Approximately 90% of corn ethanol is produced from dry-grind corn and 10% from wet milling. In the year 2015, 14.7 billion gallons of corn derived ethanol was produced in US along with 40 million metric tons of high-protein animal feed (Renewable Fuels Association, 2016). Sole dependency on corn for ethanol production makes the ethanol prices and annual ethanol production reliant on corn prices and availability. In addition, there have been disputes regarding diverting croplands to grow corn for ethanol production, which may affect the food supply. Addition of coproducts of enzyme-assisted aqueous soybean oil-extraction to corn fermentation have been shown to significantly increase the ethanol production rate and ethanol yield while decreasing the fermentation time at laboratory scale (Sekhon et al., 2015).

Saccharomyces cerevisiae is widely used to produce value-added product since its growth rate is fast, the organism is well studied and characterized and when it comes to ethanol production, its yield and the ability to withstand high ethanol concentrations are exceptional. Apart from the baker's yeast, other organisms are being researched upon for their fermentation performance. Since the growth rate is high in bacteria, they have garnered more attention than the conventional *Saccharomyces cerevisiae* for ethanol production (Senthilkumar & Gunasekaran, 2005). Metabolically engineered *Zymomonas mobilis* have been tested for cellulosic ethanol production. The bacterial metabolic pathway has been proved to be more effective in ethanol fermentation from sugars when compared to the yeast (Lau et al., 2010). Though bacteria have been studied to have the ability to digest these sugars, they cannot withstand high ethanol concentration making them economically less attractive.

In an integrated corn-soy fermentation, the common yeast is not very efficient in digesting few sugars like Xylose. Xylose makes up 1/3 of the total carbohydrate sugars present in lignocellulosic biomass and efficient conversion of xylose will definitely be economically favorable. Since yeasts are widely used fermentation organism, they are metabolically engineered to get the desired characteristics(Pereira et al., 2014). However, it was observed that most of these yeasts produced low yield ethanol but *Candida shehatae* and *Pichia stipitis* seemed promising for pentose fermentation. In fact, *P. stipitis* CSIR-Y633 strain does not produce Xylitol, byproduct of Xylose fermentation (Preez et al., 1985). Furthermore, this organism does not require any vitamin for xylose fermentation. Though both the organisms are good xylose converters, they have low tolerance for inhibitors and sensitive to ethanol unlike *S. cerevisiae* (Matsushika et al., 2009). Co-culturing these organisms may improve the fermentation performance. *S. cerevisiae* cells have been genetically engineered to integrate xylan degradation genes in its D-xylose utilizing pathway and as a result, *S. cerevisiae* was able to convert birchwood xylan to zeaxanthin, a carotenoid alcohol, with 0.74 mg/L concentration (Sun et al., 2012).

This study is designed to compare the fermentation capability of the *Pichia stipitis* and *Candida shehatae* to *Saccharomyces cerevisiae*, in an integrated corn-soy system, in terms of ethanol yield, ethanol production rate and ethanol concentration. By-product concentration is also measured as one of the comparison factor.

Materials and Methods

Microorganism

Pichia stipitis and *Candida shehatae* plates was obtained from Dr. Zengyi Shao's lab at Iowa State University, Ames, IA which were maintained at 4˚C. Dry *S. cerevisiae* was

procured from Fermentis (Lesaffre Yeast Corp, AL). Bacto™ Yeast Extract and Bacto™ Peptone was procured from Becton, Dickinson and Company, MD and Dextrose was obtained from Sigma Aldrich, MO. For inoculum, cells of the yeast were transferred to 500ml Erlenmeyer flasks containing YPD media (10g Yeast extract, 20 g Peptone, 20g Dextrose in 1L broth) and maintained at 30˚C in an incubator.

Corn and soy coproducts

Yellow dent corn was obtained from Honeyville and ground using Fitz Mill (Model DAS 06, Fitzpatrick Co., Elmhurst, IL). The composition of corn was 89.9% of solids i.e. 5.1% of oil, 8.6% protein, 1.1% ash and 74.6% carbohydrates, all on dry basis. Ground corn was stored at 4˚C.

EAEP of soybeans was performed at pilot plant of Centre for Crop Utilization Research, Iowa State University, Ames, IA and skim and insoluble fiber were obtained as the coproducts of this process. *Table 3.1* shows the proximate analysis of skim and insoluble fiber and the data were obtained from Sekhon et al. 2015 since same skim and insoluble fiber was used for this study.

α-amylase and glucoamylase enzymes were used for liquefaction and Saccharification and were obtained from Novozymes, NC in liquid form. Ammonium sulfate (Nitrogen source) was bought from Fisher Scientific, NJ. Commercial grade *S. cerevisiae* and chlorine dioxide (antibacterial) were obtained from Lincolnway Energy LLC, Ames, IA. Cellulase, which was added to fermentation to hydrolyze soy fiber, was procured from Bio-Cat, VA.

Fermentation medium and conditions

Study of effect of sugars and ethanol on *P. stipitis* and *C. shehatae*

Lab scale fermentation in autoclaved standard media (For 100ml media: 1g Yeast Extract, 2g Peptone and 2g Dextrose; autoclave conditions: 121˚C for 1 hour) was carried out with different sugars (100% Glucose, 100% Xylose and Glucose-Xylose 1:1) with (a) *S. cerevisiae,* (b) *P. stipitis*, (c) *C. shehatae,* (d) mixture of *S. cerevisiae* and *P. stipitis* (1:1) or (e) mixture of *S. cerevisiae* and *C. shehatae* (1:1) at 30˚C for 96h in an incubator shaker at 150rpm. Weights were noted as regular interval and the ethanol yield was calculated by mass loss (%) (Wang et al., 2009). All experiments were performed in duplicates.

Integrated corn-soy fermentation

Liquefaction of corn-only slurry (control) and corn-soy skim-soy insoluble slurry was carried out at 85˚C with α-amylase. The flasks were cooled to room temperature and the pH was adjusted to 4.5 with 3M sulphuric acid. Further, Saccharification and fermentation were performed in Tornado IS6 Overhead Stirring System (Radleys Discovery Technologies, Shire Hill, Saffron Walden, UK) equipped with an anchored stirring shaft and six 250mL round bottom flasks at 30˚C at 150rpm for 72 hours with (a) *S. cerevisiae,* (b) *P. stipitis*, (c) *C. shehatae*, (d) mixture of *S. cerevisiae* and *P. stipitis* (1:1) or (e) mixture of *S. cerevisiae* and *C. shehatae* (1:1).

The soy products were added to coarsely ground corn in the ratio 1.8:1 and the ratio of skim to insoluble fiber was 6.5:1 (Sekhon et al. 2015). Antibacterial (Chlorine Dioxide, 0.03ml), ammonium sulphate (0.08ml of 0.2 g/g solution, nitrogen source), Pectinase (activity 3500 ENDO-PG/g; optimum pH 2-5, temperature 40-65˚C, 0.167g), Cellulase powder (activity 75000CU/g; optimum pH 4-6, temperature 30-70˚C, 0.167g), glucoamylase

(0.167ml) and yeast (0.167g) were added to the bioreactor. Samples before and after the fermentation were collected and ran in HPLC to obtain ethanol concentration. Experiments were performed in duplicates.

The ethanol yield was calculated by mass loss (%) and calculated as gram of ethanol produced per 100 grams of carbohydrates in the flask.

$$Ethanol\ Yield\ (\%) = 100 * \frac{46 * (mass\ lost\ in\ grams)}{44 * (mass\ of\ carbohydrates)} \quad (4.1)$$

Carbohydrates is contributed by insoluble fiber (IF) and skim along with corn in corn-IF-skim slurry and hence the total amount of carbohydrates in the flask was calculated as the sum of carbohydrates in corn, skim and IF.

Another set of experiments were performed where in *P. stipitis*/*C. shehatae* were added to the bioreactor first and *S. cerevisiae* was added after 24 hours of fermentation. The idea behind this experiment was to test if *P. stipitis* and *C. shehatae* would consume Xylose in the first 24 hours and then *S. cerevisiae* would consume other sugars, increasing the final ethanol concentration.

Experimental design and statistical analysis

Baseline data were collected by adding (a) *S. cerevisiae,* (b) *P. stipitis*, (c) *C. shehatae,* (d) mixture of *S. cerevisiae* and *P. stipitis* (1:1) or (e) mixture of *S. cerevisiae* and *C. shehatae* (1:1) to synthetic media containing 100% glucose, 100% xylose and glucose-xylose mixture (1:1) and ethanol yield and production rate was calculated on mass loss basis.

Simultaneous Saccharification and Fermentation (SSF) was carried in corn only slurry and corn-soy skim-soy insoluble fiber slurry with (a) *S. cerevisiae,* (b) *P. stipitis*, (c) *C.*

shehatae, (d) mixture of *S. cerevisiae* and *P. stipitis* (1:1), (e) mixture of *S. cerevisiae* and *C. shehatae* (1:1), (f) addition of *S. cerevisiae* 24 hours after *P. stipitis* or (g) addition of *S. cerevisiae* 24 hours after *C. shehatae.* Ethanol yield, ethanol production rate, ethanol final concentration and by-product concentration (Glycerol, Acetic Acid and Lactic Acid) was calculated.

The ethanol yield data was fit using Monod equation where the specific growth rate of microorganisms was replaced by ethanol yield and the concentration of limiting substrate for growth was replaced with time of fermentation.

$$Ethanol\ Yield\ (\%) = Max.Ethanol\ Yield\frac{t}{Kt+t} \qquad (4.2)$$

Where t is the fermentation time in hours and Kt is the time required for ethanol yield to reach half of the maximum ethanol yield. Data was linearized using Lineweaver-Burk plot.

Generated data were analyzed using two-way analysis of variance in JMP Pro 12 (SAS Institute Inc., ver 12.0.1). Null hypothesis stated that there is no difference in the means of ethanol yield, ethanol production rate and concentration of all the treatments and the means were compared using t-test at $p < 0.05$ significance level.

Results and Discussion

Study of effect of sugars on *P. stipitis* and *C. shehatae*

The ethanol yield was calculated by mass loss formula and it was assumed that the mass loss was due to the production and release of carbon dioxide only. In reality, there was some water evaporation from each flask. This was accounted for by keeping a water control and adjusting the mass values but the amount of water evaporated from each flask was not

exactly the same as water evaporated from the control flask. This is why some of the ethanol yield values are greater than 100% and they were assumed as 100% during interpretation.

Except for treatment (b), ethanol yield reached 100% in every treatment in 100% Glucose media (Figure 4.1). At the end of 72 hours, the ethanol yield of treatment (b) was ~76%. These results are similar to those reported by Gutierrez-Rivera et al where under anaerobic conditions; *P. stipitis* NRRL-Y-7124 could consume only 84.5% of glucose. The fermentation efficiency of *P. stipitis* depends upon the oxygen transfer rate of the culture (Taniguchi M., et al, 1997) and since the conditions were anaerobic throughout the experiment in this paper, the fermentation performance of P.stipitis may have been affected.

Ethanol production picked up at 15^{th} hour of fermentation for all the treatments but the production rate of treatment (b) was lower compared to the others (Figure 4.2). In treatment (d), the higher production rate and yield can be attributed to *S. cerevisiae*. The ethanol production rate of treatment (e) was almost twice of ethanol production rate of treatment (c). It can be concluded that *S. cerevisiae* contributed more towards the production rate in treatment (e) (Table 4.2).

In 100% xylose media, treatment (d) and (e) achieved 100% yield (Figure 4.3). While it is unclear as to which yeast in the mixture in treatment (d) and (e) was responsible for uptake of sugar and production of ethanol, taking into account the ethanol yield of treatment (a), it can be said that *P. stipitis* and *C. shehatae* also contributed in ethanol production in treatment (d) and (e). The ethanol yield of treatment (b) was lower compared to treatment (c) which is in agreement with the results obtained by du Preez et al, 1986, where at the same initial concentration of xylose, ethanol yield of *C. shehatae* was 11% more than *P. stipitis*. The ethanol production rate of treatment (c) is also higher than treatment (b). It can observed

in Figure 4.4 that in 100% xylose media, the ethanol production rate of *S. cerevisiae* in is boosted in presence of *P. stipitis* and *C. shehatae*. duPreez et al (1986), observed that at 37th hour of fermentation, *C. shehatae* produced 37% ethanol yield with initial xylose concentration of 50g/L and *P. stipitis* gave 43% ethanol yield at the end of 48th hour. At 2g/L xylose concentration, in this work, the ethanol yield of treatment (c) (*C. shehatae*) at 37th hour was 58% and that of treatment (b) (*P. stipitis*) at 48th hour was around 30%. The ethanol production rate was similar in all the treatments in 100% xylose media (Table 4.2).

In presence of equal amount of glucose and xylose, treatment (d) and (e) were able to achieve 100% ethanol yield (Figure 4.5). The ethanol yield of treatment (a) was lower than 100% as expected. This is in accordance with the results obtained by Gutierrez-Rivera, B. et al. (2011), where they observed than in 70-30 glucose-xylsoe mixture, under anaerobic conditions, *S. cerevisiae* was able to consume just 33% of xylose and 100% of glucose. Based on literature, it can be suggested that, in 1:1 glucose-xylose mixture, it is very likely that *S. cerevisiae* would have completely converted glucose to ethanol but partially digested xylose. In presence of glucose, the xylose utilization is inactivated in *P. stipitis* and *C. shehatae* (diauxic lag), until the concentration of glucose falls below a level, which differs between organisms. The total yield of treatment (c) is higher than (b) probably because this inactivation is partial in *C. shehatae* (Webb et al., 1990). The yield of treatment (b) exceeded the yield reported by Agbogbo et al., (2006). The ethanol production rate of *S. cerevisiae* almost doubled when *P. stipitis* and *C. shehatae* were added along with it in treatment (d) and (e) (Table 4.2). Co-culturing yeast species with complimentary metabolism have shown faster substrate utilization and increased product formation rate (Chen, J. 2011).

While the mass loss in the fermentation flask is related to ethanol production, another reason for mass loss can be production of xylitol, which produces carbon dioxide in the process too. Gutierrez-Rivera, B. et al. (2011) observed that the xylitol production increased by two fold when *P. stipitis* was co-cultured with *S. cerevisiae* in glucose-xylose mixture. Past studies claim that *S. cerevisiae* are unable to ferment xylose to produce ethanol since in absence of oxygen, *S. cerevisiae* takes up pentose phosphate pathway to metabolize xylose, which is very slow. Slow metabolism prevents accumulation of pyruvate and pyruvate is essential for efficient ethanol production (Kotter et al, 1992). In this study, it was observed that *S. cerevisiae* was able to consume xylose to some extent. It is possible that commercial grade *S. cerevisiae* are capable of converting xylose to xylitol. This may have caused the recorded change in mass of flask.

The parameters of the modified Monod equation are listed in Table 4.6*: Monod equation parameters for all the treatments in 100%glucose, 100% Xylose and Glucose-Xylose mixture media.*. These parameters were used to predict the ethanol yield of treatment (a), (b) and (c).

Corn only fermentation

There was no significant difference observed in the ethanol yield of treatment (a), (b), (c), (d) or (e) in corn-only slurry (Figure 4.9) and it ranged between 53-54%. This indicates that the performance of treatment (b) and (c) is comparable to (a) in presence of various sugars and that they can withstand the ethanol concentration of ~120mg/ml (Table 4.4: Carbohydrate, by-products and product concentration analysis before and after fermentation in CORN ONLY slurry treated with (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* . Values are presented as mean ±

standard deviation and levels connected by same letter are not significantly different at 95% confidence level. (ferm. stands for fermentation)). There is a possibility that *S. cerevisiae* dominated and repressed the growth of *P. stipitis*/*C. shehatae* in treatment (d) and (e) and hence the yields are similar to treatment (a). There is around 10-12% increase in ethanol yield of treatment (a) when compared to the values reports by Sekhon et al (2015). While the process in both cases were the same, the change in yield may be a result of difference in batch of commercial grade dry yeast.

Treatment (b) and (c) were in lag phase for 20 hours from the start of fermentation and the production rate and hence the microbial growth was steeper in treatment (a), (d) and (e) probably due to the presence of *S. cerevisiae* (Figure 4.8).

Table 4.4 shows that the final ethanol concentration, after 72 hours of fermentation, is slightly higher in case of treatment (b) (120.41±0.53mg/ml). The final concentration of glucose, xylose, sucrose, galactose and arabinose in treatment (b) and (c) is lower when compared to (a) indicating that *P. stipitis* and *C. shehatae* degraded every sugar more efficiently than *S. cerevisiae*. When co-cultured in treatment (d) and (e), the final concentration of xylose and arabinose was higher than treatment (a). In general, the final ethanol concentration of treatment (b) and (c) was slightly but not significantly higher than treatment (a) (*P. stipitis*: 120.41±0.53mg/ml; *C. shehatae*: 118.81±2.62 mg/ml and *S. cerevisiae*: 116.13±1.25mg/ml). The concentration of by products is lower too. The final acetic acid concentration was lower in treatment (b) and (c) when compared to (a). Generally, yeasts are highly selective of ethanol when it comes to product formation but studies have shown they may alter their metabolic pathway to produce other products like glycerol (McMillan, 1993). Glycerol is the second common product formed by yeast after ethanol.

This coproduct concentration was found highest in treatment (a) (23.86±1.75 mg/ml) and was least in treatment (b) and (c) (14.20±0.69 mg/ml and 14.07±0.27 mg/ml). Maximum ethanol production rate of all the treatments in corn-only slurry were not significantly different (Table 4.3).

Integrated corn-soy fermentation

Unlike results reported in Sekhon et al. (2015), addition of soy skim and insoluble fiber did not improve the ethanol yield but reduced it slightly (Figure 4.9). This difference was noted because of the difference in calculation of ethanol yield in this paper and in Sekhon et al (2015). Ethanol yield is grams of ethanol produced per 100 grams of carbohydrate present in the slurry. Skim and insoluble fiber also provide carbohydrates (26.0% and 88.6% on dry basis, respectively) besides corn and hence total carbohydrates provided were taken into account while calculating ethanol yield. Ethanol yield in Sekhon et al. (2015) is presented as g of EtOH/100g of dry corn even when soy skim and IF were added to the slurry. Ethanol yield of treatment (a) improved slightly when *C. shehatae* was added to it in treatment (e). Treatment (b) was comparable in terms of ethanol yield with treatment (a). In presence of 34g/L of xylose and 8 g/L of glucose, *P. stipitis* has been reported to produce 37-44% ethanol yield (Agbogbo and Wenger, 2007). In corn, with initial xylose concentration of 12.4g/L and initial glucose concentration of 13.1g/L, *P. stipitis* has an ethanol yield of 48%.

Similar trends like corn-only slurry for ethanol production over time were seen in corn-IF-skim slurry (Figure 4.10). The production entered on log phase at 14^{th} hour and the maximum ethanol yield was reached by treatment (a), (d) and (e) by the end of 46^{th} hour. When compared to the corn-only slurry, the maximum ethanol production rate improved

slightly in corn-IF-skim slurry. Addition of soy skim to the slurry have shown to increase the ethanol production rate (Yao et al., 2011). Soy skim consists of 57.6% protein, which yeasts utilize for their growth in turn increasing the production rate of ethanol.

S. cerevisiae was added to treatment (b) and (c) after 24 hours of fermentation to avoid initial suppression of growth of *P. stipitis* and *C. shehatae* which might have happened in treatment (d) and (e) (Figure 4.11). The ethanol yield obtained were similar to treatment (b) and (c) while there was considerable decrease in ethanol production rate.

There was almost no ethanol production in first 24 hours in case of *P. stipitis* but *C. shehatae*'s ethanol yield was around 20%. Both the production increased exponentially with addition of *S. cerevisiae* (Figure 4.12). Similar results were seen in first 24 hours of treatment (b) and (c).

The final ethanol concentration of treatment where *S. cerevisiae* was added to treatment (c) after 24 hours (123.59±3.61g/L) was comparable to ethanol concentration of treatment (a) (124.82±4.37g/L). The total ethanol concentration in the Corn-IF-skim slurry in treatment (a) increased when compared to corn only slurry by 6.9%. The concentration of ethanol decreased in treatment (b) and (c) when compared to their performance in corn only slurry and this could be due to the osmotic stress on the yeast species in presence of high sugar concentration. The concentration of lactic acid produced by treatment (b) and (c) in corn-soy product slurry was high when compared to corn-only slurry. High amount of lactic acid may have decreased the overall pH of the slurry, which in turn may have affected the growth of *P. stipitis* and *C. shehatae*. Like the corn-only slurry, the acetic acid production and glycerol concentration of the fermentation system reduced in treatment (b) and (c).

Glycerol concentration of treatment (a) was lower when compared to its performance in corn-only slurry.

Overall, there was slight increase in final ethanol concentration of *S. cerevisiae* when soy skim and IF was added to the corn fermentation system. While the performance of *P. stipitis* and *C. shehatae* in corn-IF-skim slurry was not significant, their addition to *S. cerevisiae* reduced the by-product (lactic acid, acetic acid and glycerol) concentration while maintaining the final ethanol concentration

Conclusion

Addition of soy skim increase the ethanol production rate and soy insoluble fiber provided extra source of carbohydrates. Skim also increased the water-to-solids ratio decreasing the viscosity of the slurry resulting in better mixing. While *P. stipitis* and *C. shehatae* prefer aerobic condition, their fermentation performance in anaerobic corn-soy system was comparable to *S. cerevisiae* Aeration can enhance the growth rate of these pentose-degrading organisms in turn increasing the ethanol productivity. While co-culturing, different ratios of *P. stipitis*/*C. shehatae* and *S. cerevisiae* should be investigated in this corn-soy system.

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References

Agbogbo, F., Coward-Kelly, G., Torry-Smith, M. and Wenger, K. (2006). Fermentation of glucose/xylose mixtures using Pichia stipitis. *Process Biochemistry.* 41:2333-2336.

Agbogbo, F. and Wenger, K. (2007). Production of ethanol from corn stover hemicellulose hydrolysate using Pichia stipitis. *Journal of Industrial Microbiology and Biotechnology.* 34(11):723-727

Chen, J. (2011). Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review. *Journal of Industrial Microbiology and Biotechnology.* 38(5):581-97

du Preez, J. C., Bosch, M., & Prior, B. A. (1986). Xylose fermentation by *Candida shehatae* and *Pichia stipitis*: effects of pH, temperature and substrate concentration. *Enzyme and Microbial Technology*. http://doi.org/10.1016/0141-0229(86)90136-5

Gutierrez-Rivera, B. et al. (2011). Conversion efficiency of glucose/xylose mixtures for ethanol production using *Saccharomyces cerevisiae ITV01* and *Pichis stipitis NRRL Y-7124*. *J Chem Technol Biotechnol*.87:263-270

Hickert, L. R., Da Cunha-Pereira, F., De Souza-Cruz, P. B., Rosa, C. A., & Ayub, M. A. Z. (2013). Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate. *Bioresource Technology*. http://doi.org/10.1016/j.biortech.2012.12.135

Kotter, P., Ciriacy, M. (1992). Xylose fermentation by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, 38, 776-783

Karagoz, P., Ozkan, M. (2014). Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture in batch and continuous system. *Bioresource Technology,* 158:286-293

Lau, M. W., Gunawan, C., Balan, V., & Dale, B. E. (2010). Comparing the fermentation performance of Escherichia coli KO11, *Saccharomyces cerevisiae* 424A(LNH-ST) and Zymomonas mobilis AX101 for cellulosic ethanol production. *Biotechnology for Biofuels*, *3*, 11. http://doi.org/10.1186/1754-6834-3-11

McMillan, J. D., Xylose Fermentation to Ethanol: A Review (1993). *National Renewable energy Laboratory/*TP-421-4944.UC Category:246

Pereira, F. B., Romaní, A., Ruiz, H. a., Teixeira, J. a., & Domingues, L. (2014). Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. *Bioresource Technology*, *161*, 192–199. http://doi.org/10.1016/j.biortech.2014.03.043

Renewable Fuels Association. (2016). Ethanol Industry Outlook. Retrieved from http://www.ethanolrfa.org/wp-content/uploads/2016/02/Ethanol-Industry-Outlook-2016.pdf

Sekhon, J. K., Jung, S., Wang, T., Rosentrater, K. a., & Johnson, L. a. (2015). Effect of coproducts of enzyme-assisted aqueous extraction of soybeans on ethanol production in dry-grind corn fermentation. *Bioresource Technology*, *192*, 451–460. http://doi.org/10.1016/j.biortech.2015.05.096

Senthilkumar, V., & Gunasekaran, P. (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. *Journal of Scientific and Industrial Research*, *64*(11), 845–853.

Sun, J., Shao, Z., Zhao, H., Wen, F., Xu, J. and Zhao, H. (2012) Cloning and characterization of a panel of constitutive promoters for applications in pathway engineering in Saccharomyces cerevisiae. *Biotechnology and Bioengineering.* 109(8):2082-2092

Taniguchi, M., Tohma, T., Itaya, T. and Fuji, M. (1997). Ethanol production from a mixture of glucose and xylose by co-culture of *Pichia stipitis* and a respiratory-deficient mutant of *Saccharomyces cerevisiae. J Ferment Bioeng.* 83:364-370

Wang, H., Wang, T., Johnson L.A., (2009). Effect of Low-Shear Extrusion on Corn Fermentation and Oil Partition. *Jornal of Agricultural and Food Chemistry*, *57*(6), 2302-2307.

Wang, M., Han, J., Dunn, J., Cai H. and Elgowainy, A. (2012). Well-to-wheels energy use and greenhouse gas emission of ethanol from corn, sugarcane and cellulosic biomass for US use. *Environmental Research Letters.*7:045905(13pp)

Webb, S.R., Lee, H. (1990). Regulation of D-xylose utilization by hexoses in pentose-fermenting yeasts. *Biotechnol Adv.* 8(4):685-97.

Yao, L., Wang, T. and Wang, H. (2011). Effect of soy skim from soybean aqueous processing on the performance of corn ethanol fermentation. *Bioresource Technology.* 102:9199-9205.

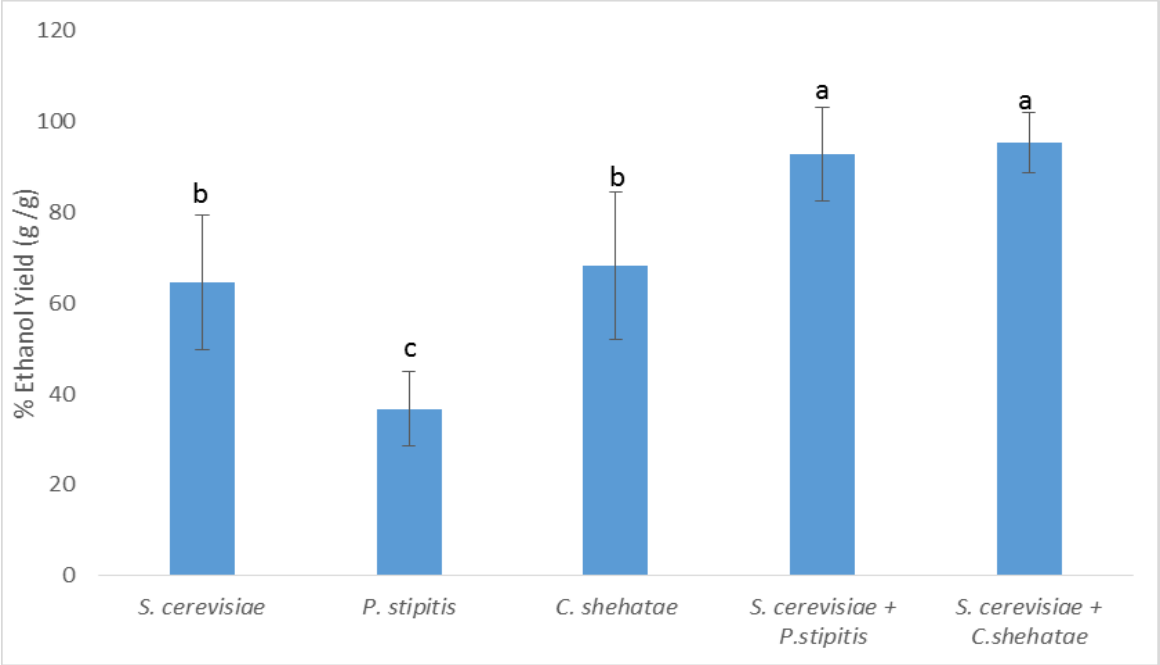

Figure 4.1: Ethanol yield of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in 100% glucose media. The error bars represent standard deviation from the mean values and the bars connected with same letter are not significantly different.

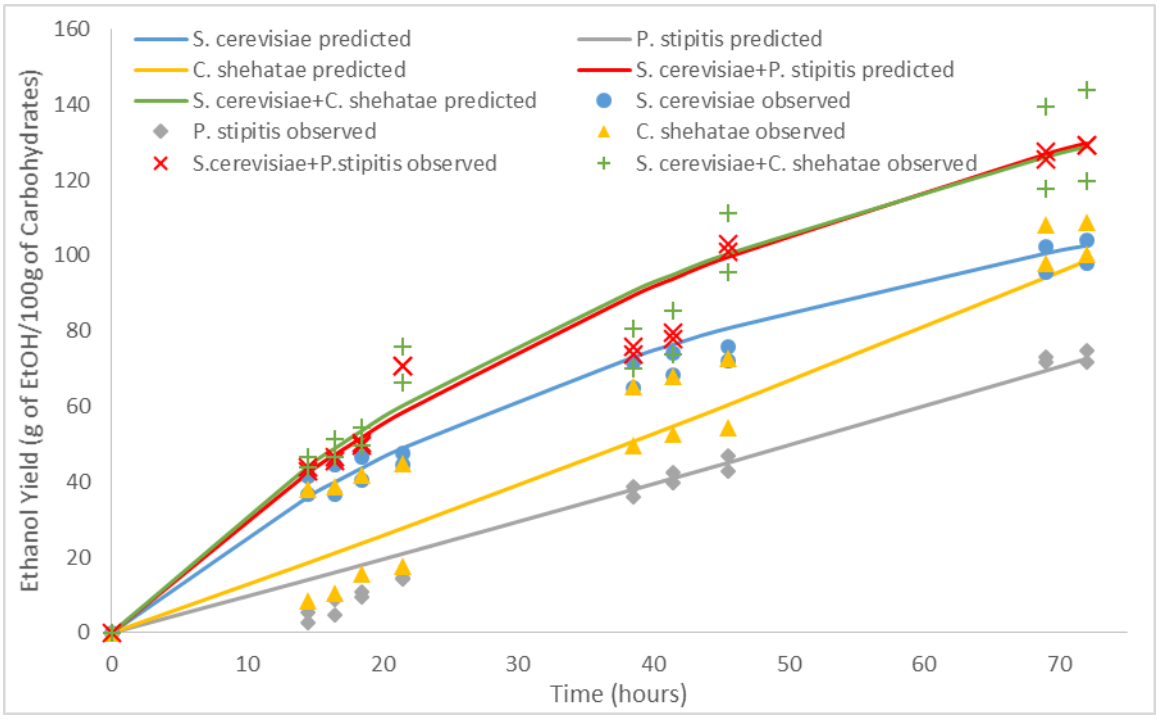

Figure 4.2: Effect of the treatment (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in 100% glucose media on ethanol yield.

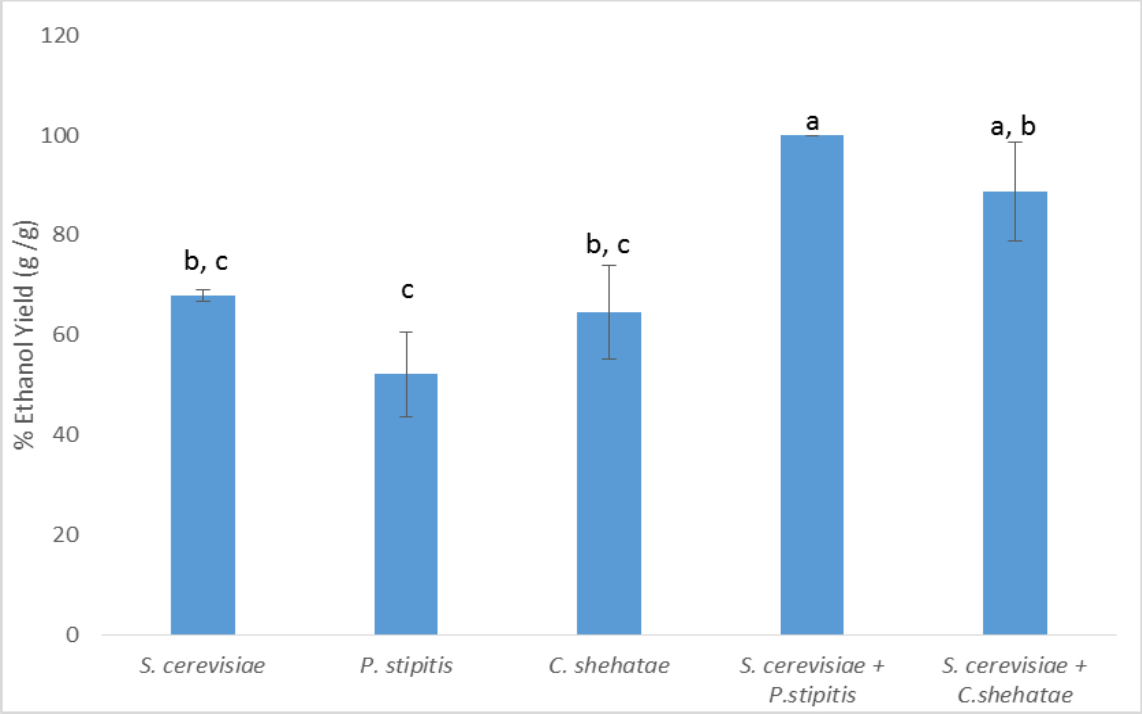

Figure 4.3: Ethanol yield of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in 100% xylose media. The error bars represent standard deviation from the mean values and the bars connected with same letter are not significantly different.

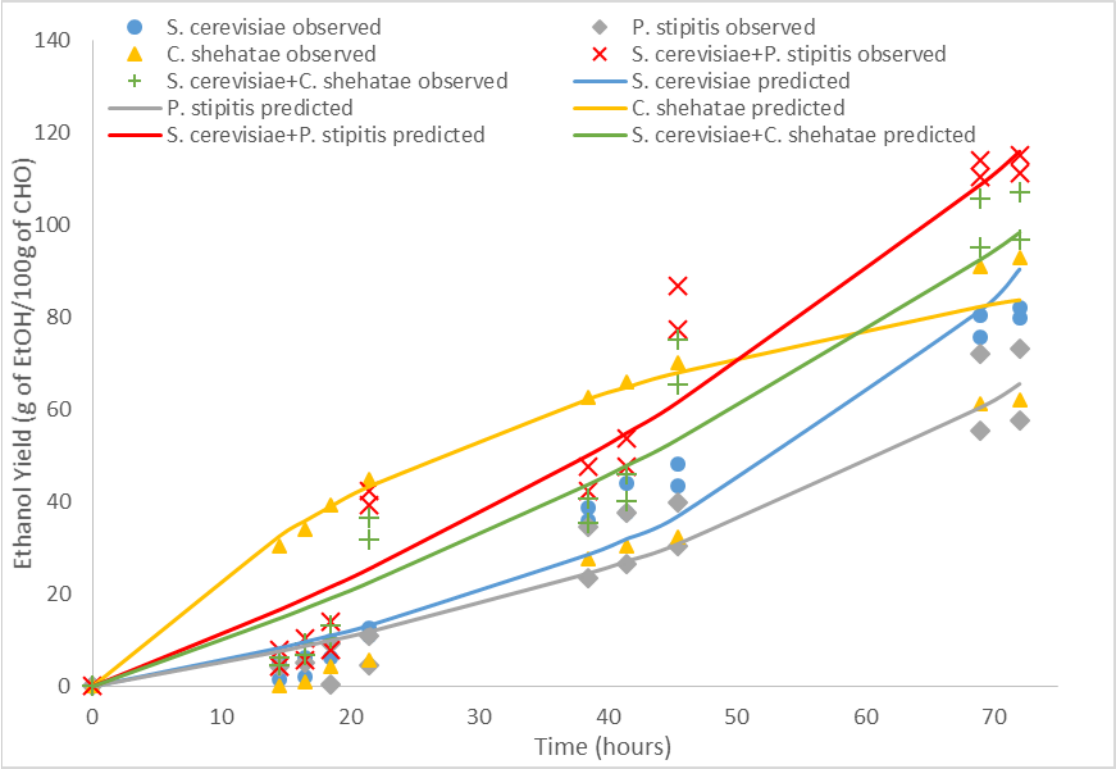

Figure 4.4: Effect of the treatment (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in 100% xylose media on ethanol yield.

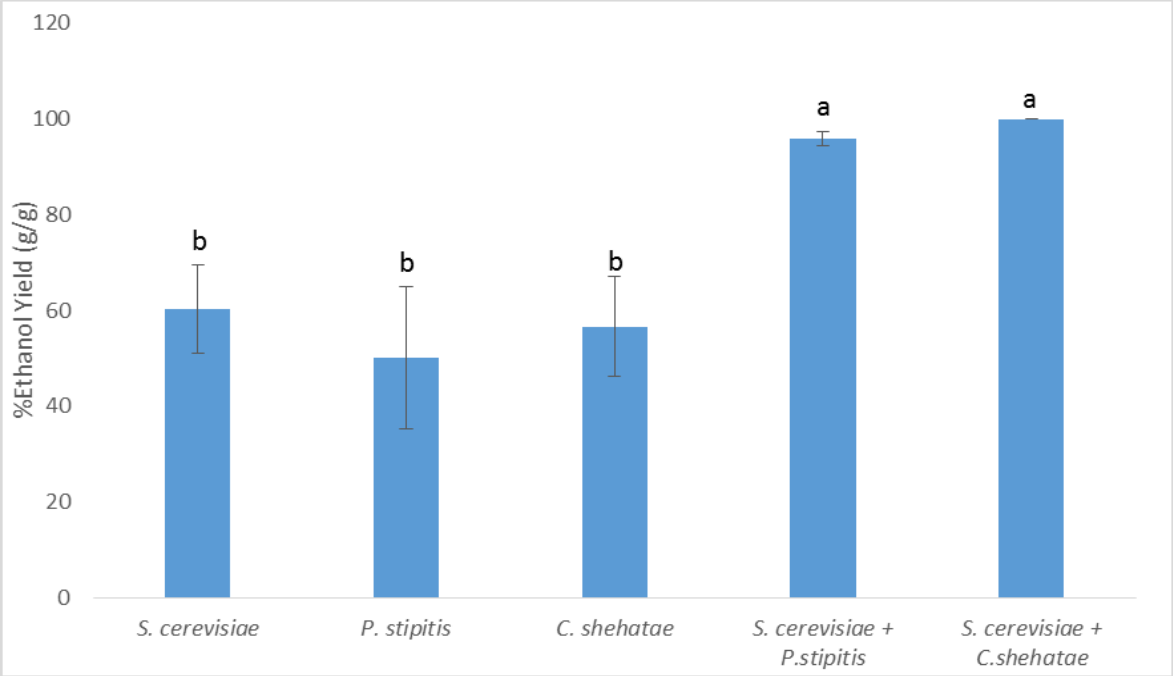

Figure 4.5: Ethanol yield of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae*+*P. stipitis* and (e) *S. cerevisiae*+*C. shehatae* in glucose-xylose (1:1) media. The error bars represent standard deviation from the mean values and the bars connected with same letter are not significantly different.

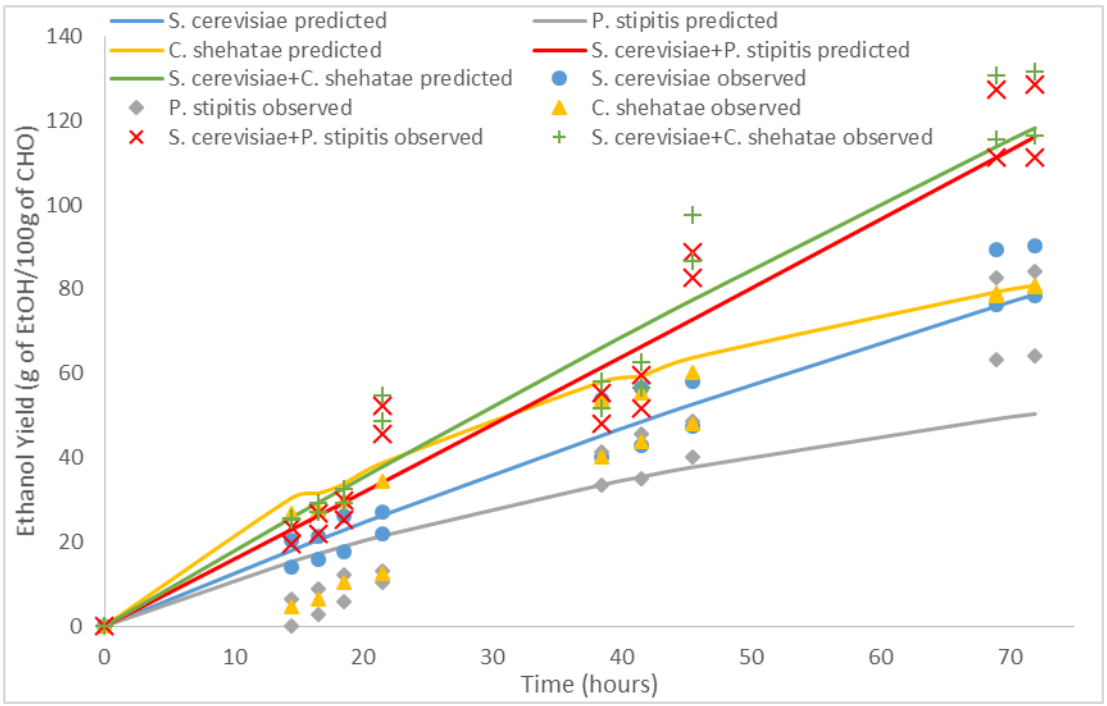

Figure 4.6: Effect of the treatment (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in glucose-xylose (1:1) media on ethanol yield.

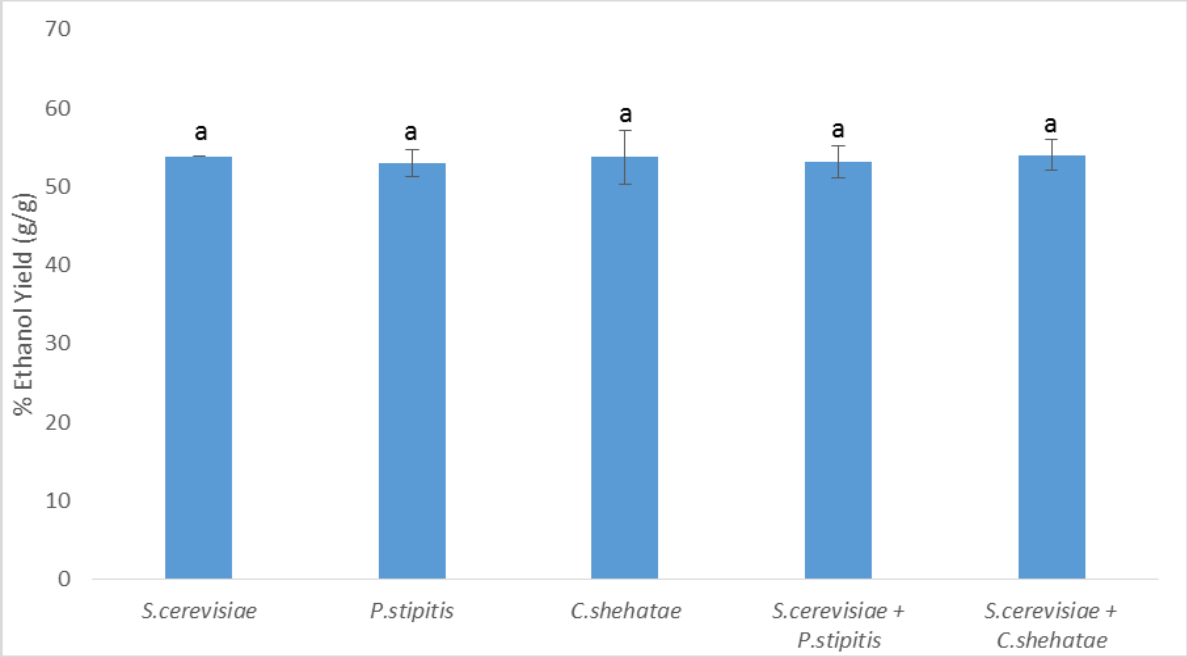

Figure 4.7: Ethanol yield of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in corn-only slurry. The error bars represent standard deviation from the mean values and bars connected with same letters are not significantly different.

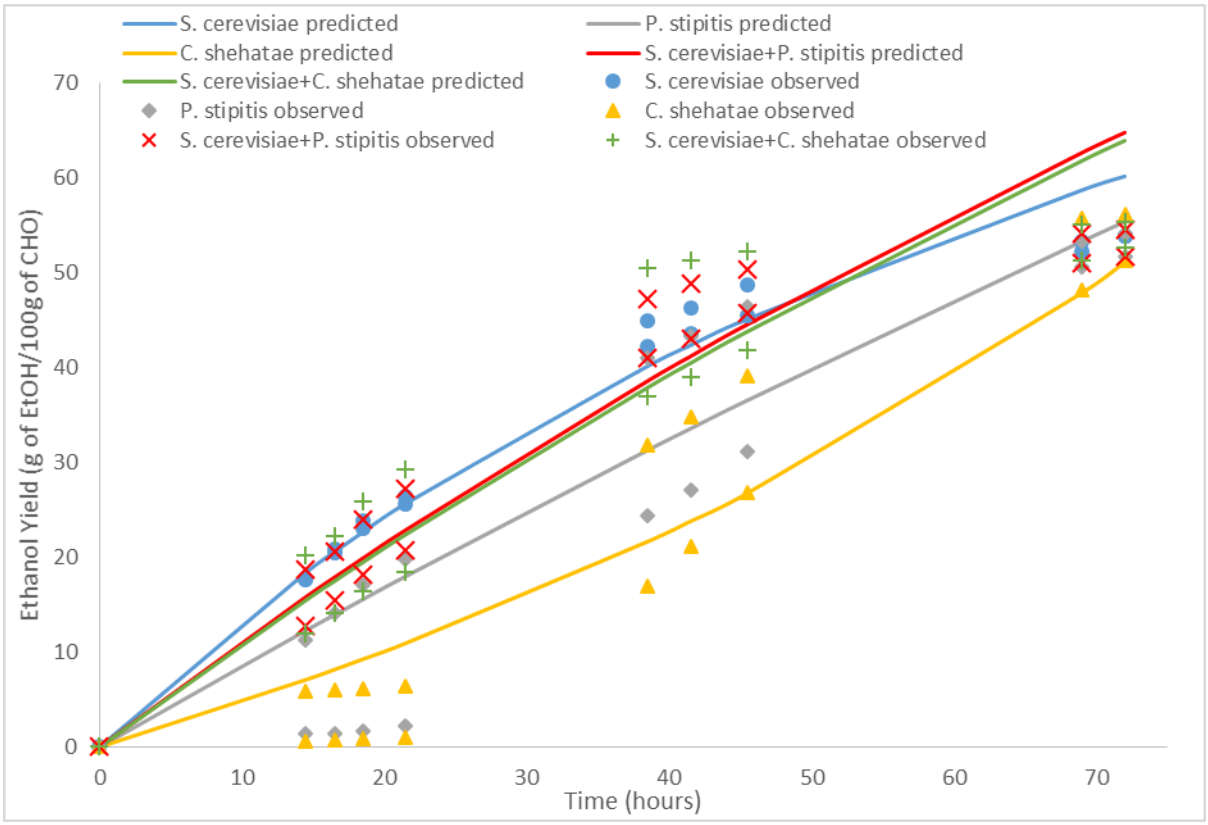

Figure 4.8: Effect of the treatment (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in corn-only slurry on ethanol yield.

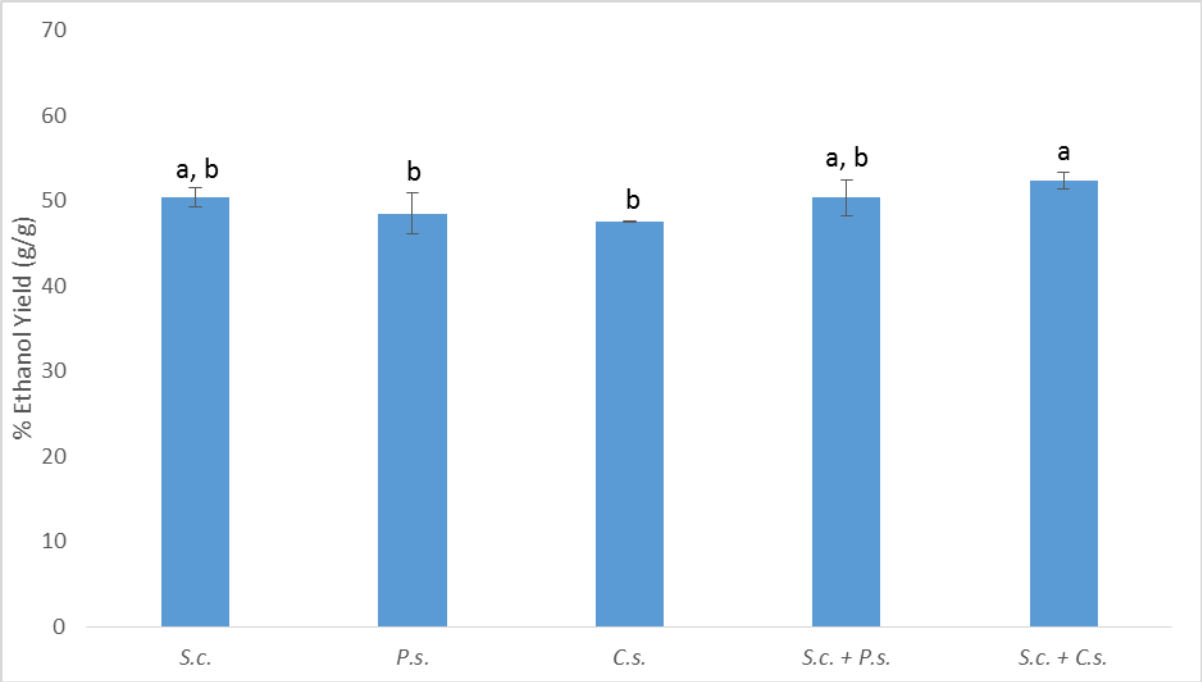

Figure 4.9: Ethanol yield of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in corn-soy skim-soy insoluble fiber slurry. The error bars represent standard deviation from the mean values and bars connected with same letters are not significantly different.

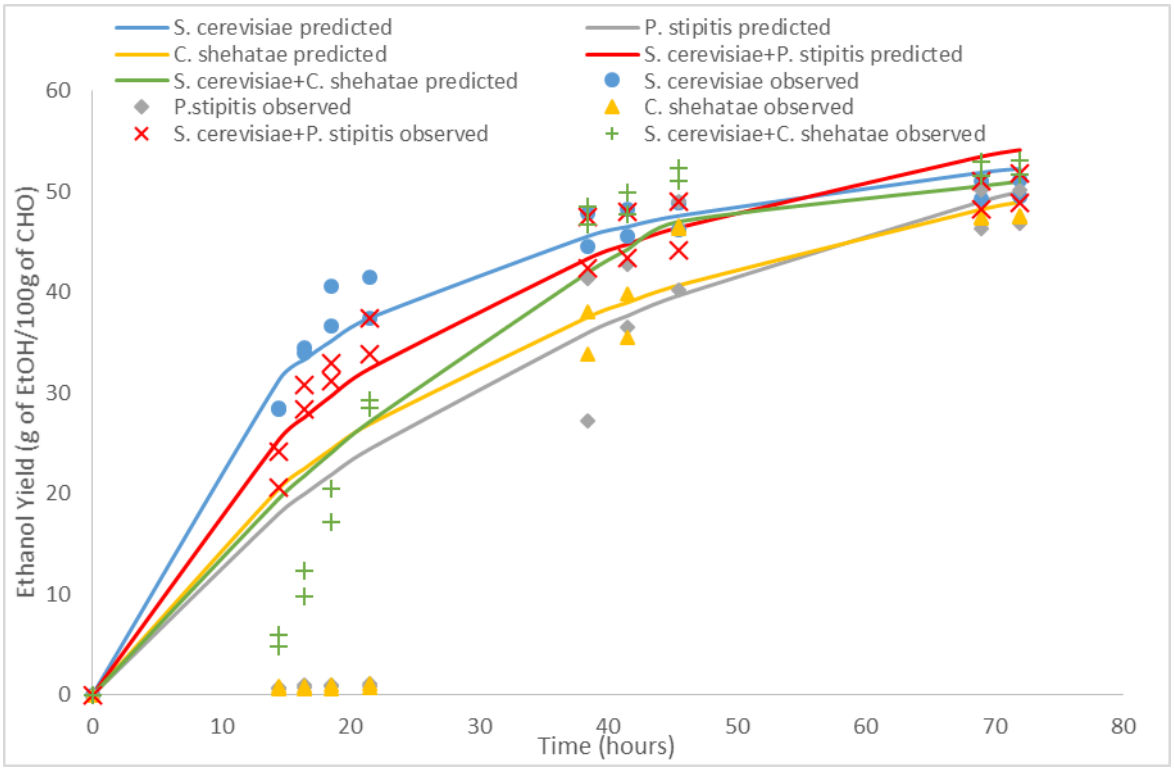

Figure 4.10: Effect of the treatment (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in corn-soy skim-soy insoluble fiber slurry on ethanol yield.

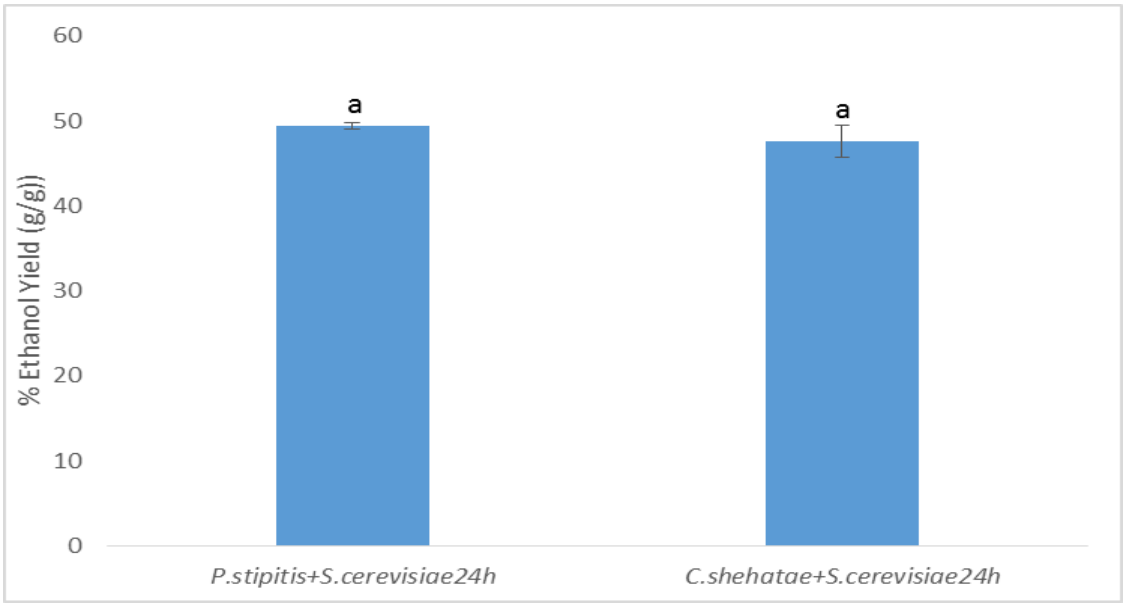

Figure 4.11: Ethanol yield of *P. stipitis* and *S. cerevisiae* (added 24 hours after the fermentation began) and *C. shehatae* and *S. cerevisiae* (added 24 hours after the fermentation began) in corn-soy skim-soy insoluble fiber slurry. The error bars represent standard deviation from the mean values and bars connected with same letters are not significantly different.

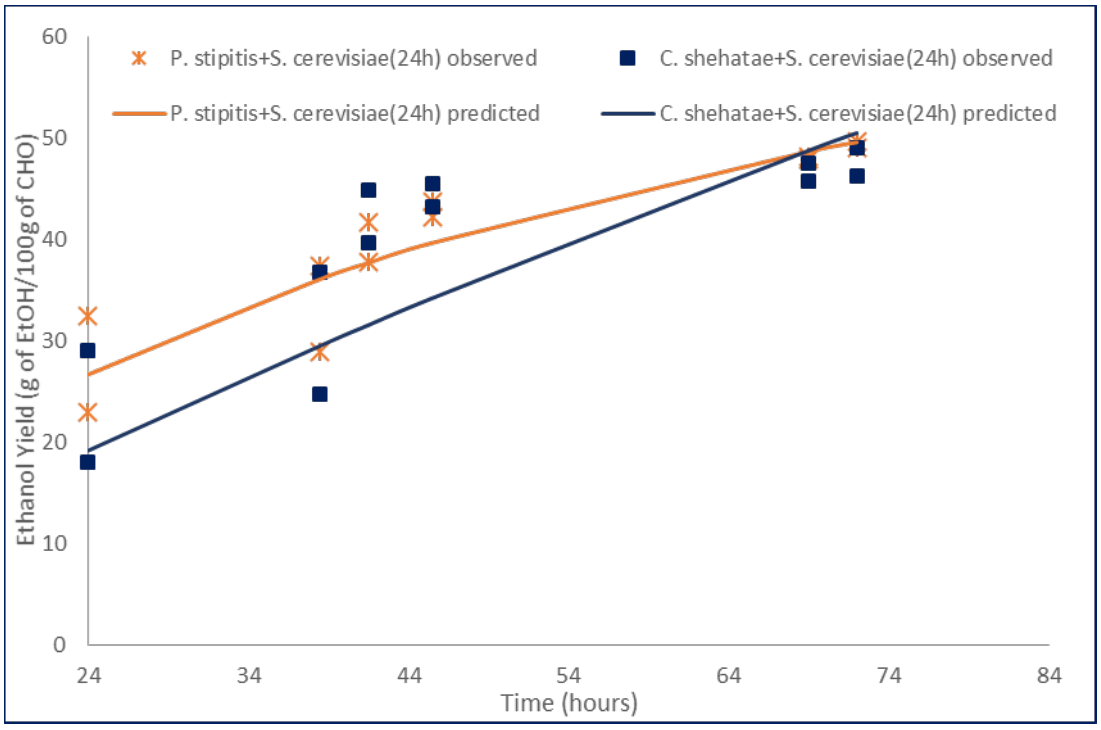

Figure 4.12: Effect of the treatment (a) *P. stipitis* and *S. cerevisiae* (added 24 hours after the fermentation began) and (b) *C. shehatae* and *S. cerevisiae* (added 24 hours after the fermentation began) in corn-soy skim-soy insoluble fiber slurry on ethanol yield.

Table 4.1: Proximate analysis of soybean skim and soybean insoluble fiber (Sekhon et al., 2015)

Composition (db.)	**Solids (%)**	**Oil (%)**	**Protein (%)**	**Ash (%)**	**Carbohydrates (%)**
Skim	9.0	6.3	57.6	10.1	26.0
Insoluble Fiber	15.1	4.7	7.7	4.0	83.6

Table 4.2: Ethanol production rate of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in 100% glucose, 100% xylose and glucose-xylose media. Values are presented as mean ± standard deviation and levels connected by same letter are not significantly different in the given media at 95% confidence level. Numbers that are bold formatted indicate higher ethanol production rate in the particular media.

Media	**Yeast strain**	**Ethanol production rate (g/100g of CHO/h)**
100% Glucose	*S. cerevisiae*	1.89 ± 0.47 b c
	P. stipitis	0.71 ± 0.30 d
	C. shehatae	1.43 ± 0.06 c
	S. cerevisiae+P. stipitis	**2.33 ± 0.56** a b
	S. cerevisiae+C. shehatae	**2.39 ± 0.60** a
100% Xylose	*S. cerevisiae*	0.72 ± 0.37 b c
	P. stipitis	0.52 ± 0.35 c
	C. shehatae	1.10 ± 0.05 a
	S. cerevisiae+P. stipitis	**1.18 ± 0.58** a
	S. cerevisiae+C. shehatae	**1.04 ± 0.47** a b
Glu-Xyl 1:1	*S. cerevisiae*	1.14 ± 0.03 b
	P. stipitis	0.69 ± 0.33 c
	C. shehatae	1.09 ± 0.06 b
	S. cerevisiae+P. stipitis	**1.59 ± 0.30** a
	S. cerevisiae+C. shehatae	**1.73 ± 0.30** a

Table 4.3: Ethanol production rate of (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* in corn-only slurry and corn-soy product slurry. Levels connected by same letter are not significantly different in the given media at 95% confidence level. *S. cerevisiae*(24h) means *S. cerevisiae* was added 24 hours after the fermentation began.

Media	**Yeast strain**	**Ethanol production rate (g/100g of CHO/h)**
Corn Only	*S. cerevisiae*	0.98±0.12a
	P. stipitis	0.62±0.20b
	C. shehatae	0.45±0.28b
	S. cerevisiae+P. stipitis	0.94±0.11a
	S. cerevisiae+C. shehatae	0.94±0.10a
Corn + Soy products (Insoluble fiber and skim)	*S. cerevisiae*	1.42±0.57a
	P. stipitis	0.49±0.43d
	C. shehatae	0.49±0.43d
	S. cerevisiae+P. stipitis	1.27±0.42a b
	S. cerevisiae+C. shehatae	0.94±0.32b c
	P. stipitis + S. cerevisiae (24h)	0.47±0.37d
	C. shehatae + S. cerevisiae (24h)	0.60±0.23c d

Table 4.4: Carbohydrate, by-products and product concentration analysis before and after fermentation in CORN ONLY slurry treated with (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae* . Values are presented as mean ± standard deviation and levels connected by same letter are not significantly different at 95% confidence level. (ferm. stands for fermentation)

Conc. (mg/ml)	**Sucrose**	**Glucose**	**Xylose**	**Galactose**	**Arabinose**	**Lactic Acid**	**Acetic Acid**	**Glycerol**	**Ethanol**
Corn slurry before ferm.	17.1±2.42	13.1±5.63	12.4±2.04 a	8.5±3.67	NA	NA	NA	1.3±0.24	NA
After fermentation									
S. cerevisiae	1.91±1.16 a	2.60±0.84 a	2.21±0.09 b	3.07±0.05 a	2.06±0.09 a	0.56±0.09 a	0.77±0.04 a	23.86±1.75 a	116.13±1.25 a
P. stipitis	0.92±0.42 a	1.47±0.67 a	1.92±0.50 b	2.92±0.26 a	1.83±0.48 a	0.86±0.25 a	0.38±0.02 c	14.20±0.69 b	120.41±0.53 a
C. shehatae	0.86±0.30 a	1.60±0.85 a	2.14±1.12 b	3.04±0.59 a	1.93±0.82 a	0.75±0.11 a	0.50±0.05 b c	14.07±0.27 b	118.81±2.62 a
S. cerevisiae + P. stipitis	1.70±0.65 a	1.28±0.38 a	2.35±0.61 b	3.14±0.32 a	2.17±0.54 a	0.57±0.11 a	0.44±0.17 c	16.53±0.75 b	117.95±1.94 a
S. cerevisiae + C. shehatae	2.12±0.01 a	1.00±0.00 a	2.42±0.34 b	3.18±0.18 a	2.35±0.20 a	1.63±1.62 a	0.73±0.11 a b	19.92±5.96 a b	114.95±3.32 a

Table 4.5: Carbohydrate, by-products and product concentration analysis before and after fermentation in CORN+SOY PRODUCTS slurry treated with (a) *S. cerevisiae*, (b) *P. stipitis*, (c) *C. shehatae*, (d) *S. cerevisiae+P. stipitis* and (e) *S. cerevisiae+C. shehatae*. Values are presented as mean ± standard deviation and levels connected by same letter are not significantly different at 95% confidence level. (ferm. stands for fermentation)

Conc. (mg/ml)	**Sucrose**	**Glucose**	**Xylose**	**Galactose**	**Arabinose**	**Lactic Acid**	**Acetic Acid**	**Glycerol**	**Ethanol**
Corn + soy Prod slurry before ferm.	33.74±0.56	12.62±0.24	19.38±1.01 a	12.13±0.15	NA	0.97±0.02	0.66±0.04	1.45±0.07	NA
After fermentation									
S. cerevisiae	4.63±0.12 a	1.83±1.04 a	0.83±0.03 b	2.27±0.12 a	2.82±0.02 a	4.68±0.05 a	0.78±0.34 a	15.48±1.48 a	124.82±4.37 a
P. stipitis	2.97±0.01 a b	1.69±0.93 a	0.67±0.01 d	3.03±1.10 a	3.26±1.77 a	5.52±1.19 a	0.49±0.00 a	13.76±1.30 a b	116.16±2.31 a b
C. shehatae	2.84±0.16 a b	1.87±1.19 a	0.61±0.02 e	3.04±1.16 a	3.60±1.94 a	6.70±1.69 a	0.43±0.18 a	12.92±0.85 a b	109.66±4.66 b
S. cerevisiae + P. stipitis	4.21±0.48 a	2.02±1.22 a	0.61±0.01 e	2.29±0.10 a	2.23±0.54 a	2.87±3.22 a	0.68±0.10 a	13.99±1.90 a b	122.96±5.34 a
S. cerevisiae + C. shehatae	4.00±1.61 a	1.48±0.58 a	0.60±0.01 e	2.95±1.04 a	3.87±1.39 a	2.54±2.62 a	0.64±0.03 a	13.24±0.74 a b	116.40±2.27 a b
P. stipitis+ *S. cerevisiae* (24h)	1.98±1.17 b	3.06±0.56 a	0.83±0.03 b c	3.77±0.14 a	3.99±1.16 a	2.53±1.73 a	0.66±0.7 a	14.79±0.34 a b	122.21±2.48 a
C. shehatae + S. cerevisiae (24h)	2.04±0.98 b	2.12±0.63 a	0.82±0.04 c	3.72±0.22 a	4.28±1.45 a	2.85±2.24 a	0.61±0.1 a	12.69±0.93 b	123.59±3.61 a

Table 4.6: Monod equation parameters for all the treatments in 100%glucose, 100% Xylose and Glucose-Xylose mixture media.

Substrate	**Treatment**	**Maximum Ethanol Yield (µm, %)**	**Time (Km, hours)**	**Coefficient of determination (R^2)**
100% Glucose	*S. cerevisiae*	192.31	62.92	0.9262
	P. stipitis	-1428.57	-1487.57	0.9898
	C. shehatae	-1111.11	-882.778	0.471
	S. cerevisiae+P. stipitis	270.27	77.97	0.9301
	S. cerevisiae+C. shehatae	250	67.75	0.9079
100% Xylose	*S. cerevisiae*	-60.97	-120.57	0.904
	P. stipitis	-70.92	-149.87	0.9465
	C. shehatae	138.89	47.39	0.1443
	S. cerevisiae+P. stipitis	-227.27	-213.11	0.6956
	S. cerevisiae+C. shehatae	-227.27	-238.36	0.7328
Glucose-Xylose (1:1)	*S. cerevisiae*	526.32	409.11	0.903
	P. stipitis	119.05	98.07	0.2314
	C. shehatae	454.54	383.18	0.4255
	S. cerevisiae+P. stipitis	-5000	-3169.5	0.8878
	S. cerevisiae+ C. shehatae	1250	688.63	0.9252

Table 4.7: Monod equation parameters for all the treatments in corn-only and corn-IF-skim slurry.

Substrate	**Treatment**	**Maximum Ethanol Yield (μm, %)**	**Time (Km, hours)**	**Coefficient of determination (R^2)**
Corn-only slurry	*S. cerevisiae*	140.84	96.42	0.9859
	P. stipitis	476.19	546.85	0.9354
	C. shehatae	-94.34	-193.15	0.6139
	S. cerevisiae+P. stipitis	294.12	254.68	0.8806
	S. cerevisiae+C. shehatae	303.03	269.06	0.7893
Corn-IF-skim slurry	*S. cerevisiae*	62.89	14.62	0.8838
	P. stipitis	90.09	57.81	0.4412
	C. shehatae	75.19	38.57	0.6514
	S. cerevisiae+P. stipitis	75.75	28.75	0.8687
	S. cerevisiae+C. shehatae	136.99	87.07	0.8757
	P. stipitis+ S.cerevisiae(24h)	86.96	54.3	0.7368
	C. shehatae +S.cerevisiae(24h)	277.78	324.08	0.8396

CHAPTER 5. TESTING THE EFFICACY OF PRESERVATIVES IN INCREASING THE SHELF LIFE OF DISTILLERS WET GRAINS

Abstract

With increasing concerns regarding the carbon footprint of corn-ethanol production plants, there is a need to increase the ethanol yield and to find a market for the coproducts. One of the coproducts of dry-grind ethanol production is distillers wet grains (DWG), which is rich in proteins, fiber and fats and is subsequently used as animal feed. Since it has over 60% moisture content, it has a shelf life of 5-6 days. There are chemical preservatives, mostly containing organic acids, currently available for reducing bacterial and fungal growth in DWG and to increase its shelf life. The objective of this study was to test and compare the efficacy of various chemical preservatives by quantifying the microbial growth in DWG in a 10-day period. Four existing preservatives and multiple concentrations of a new preservative were evaluated. Colony forming units (CFU) of lactic acid bacteria, yeast and mold, and anaerobic bacteria were counted on agar media specific to each microbe. Measurements were taken on day 0, 2, 4, 6, 8 and 10 and the number of CFU of each microbe on day 10 determined the efficacy of preservatives. The LAB population on day 10 was significantly low in two existing preservatives (Fungiless and formaldehyde-based). The population of yeasts and mold in Fungiless was 99.94% less than that of control. The new preservative was successful in inhibiting the growth of aerobic heterotrophs, even at the lowest concentration level, when compared to existing preservatives.

Keywords: Ethanol, Corn, Distiller's Wet Grains, Preservatives, Flow cytometer, Bacteria, Fungi

Introduction

The primary coproduct of the process of production of ethanol from dry-grind corn is distillers grains (DG). These are unfermented, coarse grain particles, which are separated from the liquid fraction, called thin stillage, by centrifugation. The wet cake is either dried at the ethanol plant to produce distillers dried grains (DDG) or sold wet as animal feed to livestock producers (distillers wet grains, DWG). The thin stillage is further concentrated to solubles which is either added to the distiller wet or dried grains to produce distillers wet/dried grains with solubles (DWGS and DDGS) or sold as concentrated dried solubles (CDS). Since carbohydrates from corn kernels is converted into ethanol during fermentation, what's left behind are the unfermented kernels with high amount of proteins, fats, minerals and fibers which are fed to animals (Rosentrater, 2011).

Along with protein, distillers grains are a good source of digestible neutral detergent fiber (NDF; 40-45% in DG), which is an indicator of high-energy value product. Gross energy of the feed is further classified into digestible energy (DE), metabolisable energy (ME) and net energy for lactation (NE) and theses values for DWG are 1.81 Mcal/lb, 1.63 Mcal/lb and 1.00 Mcal/lb (dry matter) (Schroeder, 2003). Just 30% of distillers wet grains can replace a diet consisting alfalfa and corn silage in 1:1 mixed with corn silage and soybean meal concentrate (Table 5.1).

Studies have shown that the feeding value of the diet increased from 100 to 178 just by including 10% of distillers wet grains with solubles into the feed. It also increased the palatability and condition of the feedlot diet (Klopfenstein et al., 2008).

There is no significant difference in the nutritive value of the wet and dry grains. Distillers dried grains (DDG) and distillers dried grains with solubles (DDGS) have extended

shelf life and can be transported easily to longer distances. The drying step, though, incurs high cost and energy. Livestock producers with a farm near an ethanol plant generally prefer distillers wet grains (DWG) due to their lower cost. DWG prices are generally lower than usual in summer season as their shelf life is shorter when the temperature is high and air is humid. Livestock producers stock up when the prices are low and store DWG with other forages like alfalfa to preserve it. Storing DWG in 9-12 foot sealed bag can minimize its interaction with atmosphere decreasing the microbial growth rate hence extending its shelf life. These sealed bags add on to the cost, which is estimated to be around $5-$8 per ton of DWG (Schroeder, 2003). Another alternative is to add chemical preservative before bagging or storing it in silo bunkers.

The wet cake, which comes out of the plant, has minimum microbial content as the temperature and pH changes drastically before, during and after fermentation and the heat treatment kills undesirable microbes. DWG comes out at around 80°C and the pH is usually lower than 4, condition at which only few yeasts and molds can survive. Spoilage can begin when DWG is exposed to the processing equipment or while it is being transported or at the storage (Kung, L. Jr.). Due to its high nutrient and moisture content, DWG is susceptible to microbial growth once it comes out of the plant. At 1% treatment level, Sorbic acid have shown to extend the storage time of DG. After 21 days of storage, around 0.4 grams of dry matter was lost to spoilage by microorganism when 1% sorbic acid was added. For fungal cells, weak organic acids act as uncoupling agents. Potassium sorbate could extend the shelf life of DG to 9 days only at the same level of application. Ammonia is another preservative but a critical concentration level must be achieved for it to be effective. When ammonium hydroxide was added at 1%, dry matter loss reached to 0.4 g within a week. Carbon dioxide

can also act as preservative but it has to be stored in an airtight container with DG which would again incur high cost. It was observed that adding preservatives at lower levels resulted in more spoilage than not adding then at all (Nofsinger et al., 1983). A mixture of various organic acids, which can stay un-dissociated at pH below 4, can also be used at once and has shown to be effective than just one active ingredient. Kemin Industries tested a particular combination of organic acid and it was found that just using 6 lbs of that preservative increased the shelf life from 7 to 20 days and using 18 lbs ensured that no molds were visible until 27 days (Kung et al., 2003). *Salmonella* in animal feed has been a problem for a long time since it indirectly affects humans when they consume meat coming from the animal, which was fed Salmonella, affected feed. Propionic acid at pH 6.8 added at 10% level can cause one log reduction of *Salmonella* after 7 days of incubation. It was observed that in oilseed meals like soybean meal, which has moisture content as high as 13%, less than 1% application of formaldehyde resulted in greater than 2 log reduction of *Salmonella* (Wales et al., 2010).

Lactic acid bacteria (LAB) are considered probiotic to animal health due to their ability to inhibit the growth of spoilage microorganism in feed. Weak organic acids such as lactic acid and acetic acid, hydrogen peroxide and reuterin are few of the antimicrobial substances produced by LAB (Brashears et al., 2005). LAB also facilitate the conservation of free amino acids and labile proteins in forages (D'Mello, 2001).

Yeast and Mold growth in distiller's wet grains is highly undesirable; the toxins, like Aflatoxin, Fumonisins, Citrinin produced by them, can cause digestive and reproductive problems in animals and they can compromises their immune function. If grown

considerably, they can increase the pH level resulting in conditions favorable for growth of other spoilage organism (Sperber and Doyle, ed.)

Aerobic heterotrophic bacteria such as Staphylococcus aureus produce toxins that can cause mastitis in cows and bumblefoot disease in chickens. It can also produce toxins, which damage the cell membranes of red and white blood cells (Otto, 2015). Along with animals, these bacteria can cause deadly disease in human too.

Development of microbial population in DWG with moisture content of 53-54% (wb) has been studied and it was observed that the total number (bacterial and fungi) increased from $4*10^5$ colony forming units (CFU) per gram of dry mass of DWG to 10^9 CFU/g of DWG. Lactic acid bacteria (LAB), aerobic heterotrophs (bacteria and fungi) and yeasts and mold population was enumerated on Day 0 to Day 10 and exponential increase was observed over time. While the LAB number stayed at $5X10^5$ CFU/ g of dry DWG on Day 10, aerobic heterotrophs and yeast and mold was found to be greater than 10^8 CFU/g of dry DWG (Lehman and Rosentrater., 2007).

In this study, various commercially available chemical preservative of animal feed were tested and compared with a preservative at developmental stage based on the microbial population of LAB, aerobic heterotrophs and yeasts and mold at the end of Day 10.

Materials and Methods

Distillers wet grains and sampling

DWG was collected from Golden Grain Energy, Mason City, Iowa in summer of 2016. It was transported in a clean 5-gallon airtight bucket and stored in -20°C until used. Chemical preservatives were added to 300 g of DWG and kept in an open plastic container in the lab with room temperature between 22-24°C. Samples were taken on Day 0, 2, 4, 6, 8 and

10 and the entire content of the container were mixed thoroughly before sampling (Lehman and Rosentrater, 2007). Water lost due to evaporated was noted and corrected every day.

Chemical preservatives

Mold-X liquid 65 is a mold inhibiting buffered liquid manufactured by Agresearch Inc., Illinois. This preservative is combination of various organic acids like Propionic Acid, Acetic Acid, Benzoic Acid, Phosphoric Acid and Propylene Glycol (product information sheet). The recommended usage level is 1-3 lbs per ton of animal feed. Experiments for this paper were carried out as 2 lbs of Mold-X per ton of DWG.

Fungiless is yellow-colored fungicide marketed by Vedeqsa, Spain and has two active ingredients- Natamycin and ethyl N-lauroyl-L-arginate monohydrochloride (LAE). The combination results in reduction of amount of each component required to prevent the growth of yeasts and molds. Fungiless was applied to DWG at the rate of 2 g of Fungiless to 1 kg of DWG and the concentration of Natamycin and LAE is 833.3 ppm and 210 ppm at this level of dosage.

Silo-King is used for grain and forage treatment and manufactured by Agri-King, Inc., Illinois. While Silo-King is widely researched and used for silage and forage, due to the presence of organic acids, it was tested for DWG too. The active ingredients of this preservative are potassium sorbate and butylated hydroxytoluene. The application rate for silage and haylage with moisture content between 60-70% is 0.2-0.5 lbs per ton and for this study, 0.5 lbs per ton of DWG was used.

Formaldehyde based preservative is a mixture of Formaldehyde (mixed with a solvent-methanol), Propionic Acid and D-Limonene. The application level of this liquid

preservative is 2 kg per metric ton of DWG. Formaldehyde usually breaks down into formic acid and carbon monoxide.

The sample to be tested is identified as Hydri-Maize Defender L-310, also known as SHIELD. It will be referred as SHIELD henceforth. This chemical preservative is developed by Hydrite Chemical Co. and consists of LAE as the active ingredient at 10% level.

Cell counting by Agar plating

Collected samples were diluted serially in 1X Phosphate buffered saline and plated in duplicates at 10^{-1} to 10^{-6} dilutions. Three agars were used: Oxoid^{TM} deMan-Rogosa-Sharpe agar (MRS) for Lactic Acid Bacteria, Oxoid^{TM} Di-cholran Rose Bengal Chloramphenicol agar (DRBC) for Yeasts and Molds and Oxoid^{TM} Plate Count Agar (PCA) for Aerobic Heterotrophs (Lehman and Rosentrater, 2007). All the agars were procured from Thermo Fisher Scientific, MA. 100mg/L of Chloramphenicol was added to DRBC before autoclaving. MRS plates were maintained at 37°C for 2 days, DRBC and PCA plates were maintained at 23°C for 7 days and 2 days, respectively. Cells were enumerated and expressed in Colony forming units (CFU) per gram of DWG (Lehman et al., 2007).

Experimental design and statistical analysis

Distillers wet grains were treated in 10 different ways (a) Control, (b) 100 ppm SHIELD, (c) 150 ppm SHIELD, (d) 200 ppm SHIELD, (e) 3% (w/w) SHIELD, (f) 5% (w/w) SHIELD, (g) Mold-X, (h) Fungiless, (i) Silo-King and (j) Formaldehyde-based preservative. Samples were collected on Day 0, 2, 4, 6, 8 and 10 from each treatment and the number of colony forming units of lactic acid bacteria, yeasts and mold and aerobic heterotrophs were enumerated. The independent variables were the type of preservative and time and the dependent variable was the CFU/g of DWG.

CFU data of each microbe for each preservative were analyzed and the means were compared using t-test at $p<0.05$ in JMP Pro 12 (SAS Institute Inc., ver 12.0.1). Null hypothesis stated that there was no difference between the means of CFU/g of DWG of lactic acid bacteria or aerobic heterotrophs or yeasts and mold in different preservatives over time.

Results and Discussions

Based on the data of Control, it was observed that the shelf life of procured DWG was 5 days. There was an exponential increase in CFU/g of DWG of LAB, yeasts and molds and aerobic heterotrophs after day 5 in control. Figure 5.1 shows the growth of lactic acid bacteria from day 0 to day 10 in every treatment. Growth of LAB was greater than control in treatment (b), (c), (d) and (i) on Day 6 and by Day 10, they were equal to the control. There was no noticeable growth in treatment (f), (h) and (j) until Day 10. The lowest CFU/g of DWG of LAB on day 10 was in treatment (h) and (j) with no significant difference between the two at 95% confidence level. Treatment (h) contains natamycin and LAE. Natamycin is poly-unsaturated organic compound produced by *Streptomyces natalensis* during fermentation and it is used as an antibiotic. While it is mostly used against yeasts and mold, it has no antibacterial activity (Stark and Tan, 2003). LAE is a stable antimicrobial food preservative and is known to act on the cell membrane of gram positive and negative bacteria and yeast and mold inhibiting their growth (Kawamura and Whitehouse, 2008). At very low minimum bactericidal concentration, LAE has been shown to create larger inhibition zone for bacteria such as *Listeria monocytogenes* when compared to salts of organic acids namely sodium citrate, sodium lactate or sodium diacetate. Since natamycin has no antibacterial properties, LAE in treatment (h) must be the major cause in inhibited growth of LAB. In treatment (j), formaldehyde is the main active ingredient with antimicrobial activity.

Formaldehyde's action of denaturing the cell membrane proteins and the nucleic acid of bacteria and fungi inhibits their growth, which was observed in the Figure 5.1.

Figure 5.2 compares the performance of various concentration of the new preservative, SHIELD, from day 6 to 10, time after the typical shelf life of DWG. It is evident that as the concentration increases, the efficiency of the preservative in inhibiting the growth of LAB increases. Since SHIELD contains 10% LAE, the antimicrobial activity increases with the concentration of LAE.

Figure 5.3 shows the change in CFU of yeasts and molds per gram of DWG when treated with treatment (a) to (j). Similar to LAB, the yeasts and mold grow exponentially over time in control. CFU of yeasts and mold were the least in treatment (h) by a factor of 10^3 in comparison with other treatments. Natamycin is an efficient antifungal agent, which binds to a sterol present in the cell membranes of fungi, without which they cannot survive. This specific binding blocks the growth of fungi without permeating the membrane (Welscher et al., 2007). The power equation line of treatment (a), (b), (c) and (d) indicates accelerated growth of yeast and mold after day 9. It was expected that the presence of potassium sorbate in treatment (i) would inhibit yeast and mold growth but the growth in treatment (i) was higher than that of control. It has been observed that while potassium sorbate is moderately effective in inhibit microbial growth, at 0.25% level, dry matter loss was higher than the control. The loss decreased with increase in the preservative level (Nofsinger et al., 1983). Treatment (g) had moderate performance in inhibiting the growth of yeast and mold. Treatment (g) is a mixture of various organic acids and due to their ability to penetrate bacterial cell wall disrupting the homeostasis, organic acids are used in food and feed preservation. Weak organic acids like Benzoic acid, present in treatment (g), are shown

to inhibit the growth of yeasts such as *Saccharomyces cerevisiae* by blocking their membrane trafficking pathways (Hazan et al., 2004).

The performance of different SHIELD concentration in comparison with control is illustrated in Figure 5.4. There is no significant difference between the performance of treatment (b) and (c). Increasing the preservative concentration by 50-100 ppm did not show significant difference in final CFU/g of DWG data. Treatment (f) was the most effective among all and its yeast and mold population on day 10 was only 40% of the population in control sample.

Besides agar plate counting, one obvious indicator of mold growth is the moldy smell. After day 6, moldy smell was prominent in treatment (a), (b), (c), (d) and (i). Due to the mold growth, the DWG particles started sticking together and mixing it uniformly before sampling became challenging.

Figure 5.5 shows the growth of aerobic heterotrophs over time in DWG in treatment (a) to (j). The most common aerobic heterotroph observed on the PCA plate was *Staphylococcus aureus.* While the growth of aerobic heterotrophs was highest in control, the exponential equation line of treatment (g), (h) and (i) showed accelerated increase from day 6. One of the active ingredient in treatment (g) was Propionic acid, which is industrially used to inhibit the growth of bacteria and mold. It was expected that propionic acid would inhibit the growth of *S. aureus* but its presence in treatment (g) and (j) was less effective than presence of LAE in treatment (b) to (f). Nofsinger et al. (1983) obtained similar results, where increase in the level of propionic acid increased the dry matter loss.

Treatment (h) proved to be the least effective amongst all, even in presence of potassium sorbate. The amount of active ingredient is as important as its mode of action in

inhibiting the microbial growth. The day 10 CFU of LAB and yeasts and mold in treatment (h) was higher than the day 10 data of control. The small quantity of preservative may have selectively inhibited specific microbes resulting in growth of others hence application of smaller quantity of preservatives may have adverse effect than not adding it at all.

Conclusion

Distillers wet grains are a value added coproduct of corn-ethanol production process. Since their nutritive value and energy content is high, they are fed to farm animals along with other forages. At 60% moisture level, spoilage microorganisms such as yeast and mold and aerobic heterotrophs can grow quickly. Chemical preservatives containing weak organic acids, esters and bacterial fermentation products like natamycin have shown to inhibit the growth of microbes increasing the shelf life of DWG. The shelf life of DWG can be increased to 10 days when strong preservatives like Fungiless and Formaldehyde-based are added to it. Use of formaldehyde may not be advisable since it is carcinogenic in nature.

With just 10% active ingredient and at 5% (w/w) application level, the new preservative was able to decrease the yeast and mold population by 60% and aerobic heterotroph population by 94% when compared to control on day 10. The new preservative has the potential to match the performance of its competitors when used at appropriate concentration and addition of organic acids would possibly enhance its efficiency.

References

Brashears, M.M, Amezquita, A., Jaroni, D. (2005). Lactic Acid Bacteria and Their Uses in Animal Feeding to Improve Food Safety. *Advances in Food and Nutrition Research.* 50:1-31

D'Mello, J.P.F. (2001). Microbiology of Animal Feeds. *Food and Agriculture Organization of the United Nations.* Retrieved from http://www.fao.org/docrep/article/agrippa/556_en.htm

Kawamura, Y., Whitehouse, B. (2008). Ethyl Lauroyl Arginate-Chemical and Technical Assessment. *Food and Agriculture Organization of the United Nations.* Retrieved from http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/69/Ethyl_Lauroyl_arginate.pdf

Klopfenstein, T.J., Erickson, G.E., Bremer, V.R. (2008). Use of distillers by-products in the beef cattle feeding industry. *Journal of Animal Science* 86:1223-1231

Kung, L. Jr. Managing High Moisture Co-product Feeds for Improved Aerobic Stability. University of Delaware. Retrieved at http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.348.1643&rep=rep1&type=pdf

Kung, L., Jr., M. R. Stokes, C. J. Lin. (2003). Silage Additives. In Silage Science and Technology. Eds. Buxton, Muck, and Harrison. American Society of Agronomy. Madison, WI. Pp 305-360.

Lehman, R.M., Rosentrater, K.A. (2007). Microbial development in distillers wet grains produced during fuel ethanol production from corn (Zea mays). *Can. J. Microbiol.*53(9):1046-52

Nofsinger, G.W., VanCauwenberge, J.E., Bothast, R.J., Kwolek, W.F. (1983). An evaluation of Chemical Methods to Extend the Allowable Storage Time of Wet Distillers Grains. *J. Agric. Food Chem.* 31:276-279

Otto, M. (2015), *Staphylococcus aureus* toxins. *Current Opinion in Microbiology.* 17:32-37

Rosentrater, K.A., Liu, K. (2011). Distillers Grains Production, Properties, and Utilization. Florida:CRC Press

Schroeder, J.W. (2003). Distillers Grains as a Protein and Energy Supplement for Dairy Cattle. *NDSU Extension Service* AS-1241

Sperber, W.H., Doyle, M.P. (Eds.) (2009). Compendium of the Microbiological Spoilage of Foods and Beverages. New York, Springer.

Stark, J., Tan, H.S. (2003). Natamycin. In Russell, N.J., Gould, G.W. (Eds.), *Food Preservatives* (179-182). Springer US.

Wales, A.D., Allen, V.M., Davies, R.H. (2010). Chemical Treatment of Animal Feed and Water for the Control of *Salmonella. Foodborne Pathogens and Disease* 7(1):3-15

Welscher, Y. M. et al. (2007). Natamycin Blocks Fungal Growth by Binding Specifically to Ergosterol without Permeabilizing the Membrane. *The Journal of Biological Chemistry.* 283:6393-6401

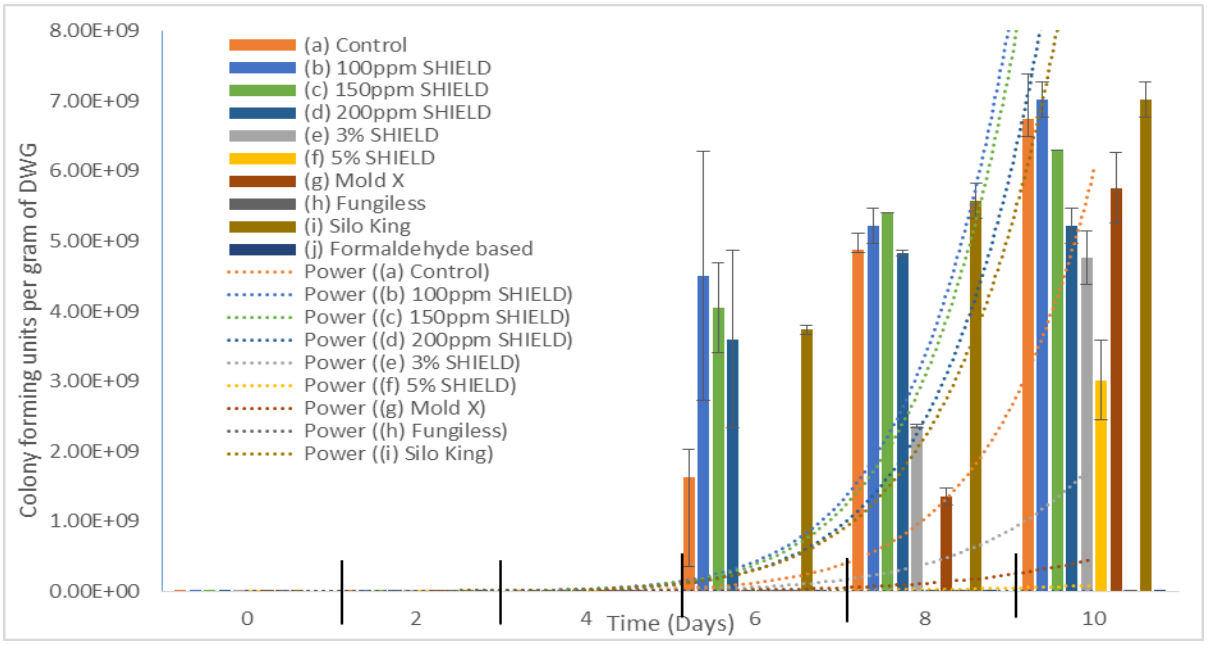

Figure 5.1: Growth of lactic acid bacteria over time when different chemical preservatives were added to distillers wet grains (60% moisture content on wet basis) at room temperature. Error bars represent standard deviation from mean. Dotted lines represent the best fit. Refer Table 5.3 for significant values.

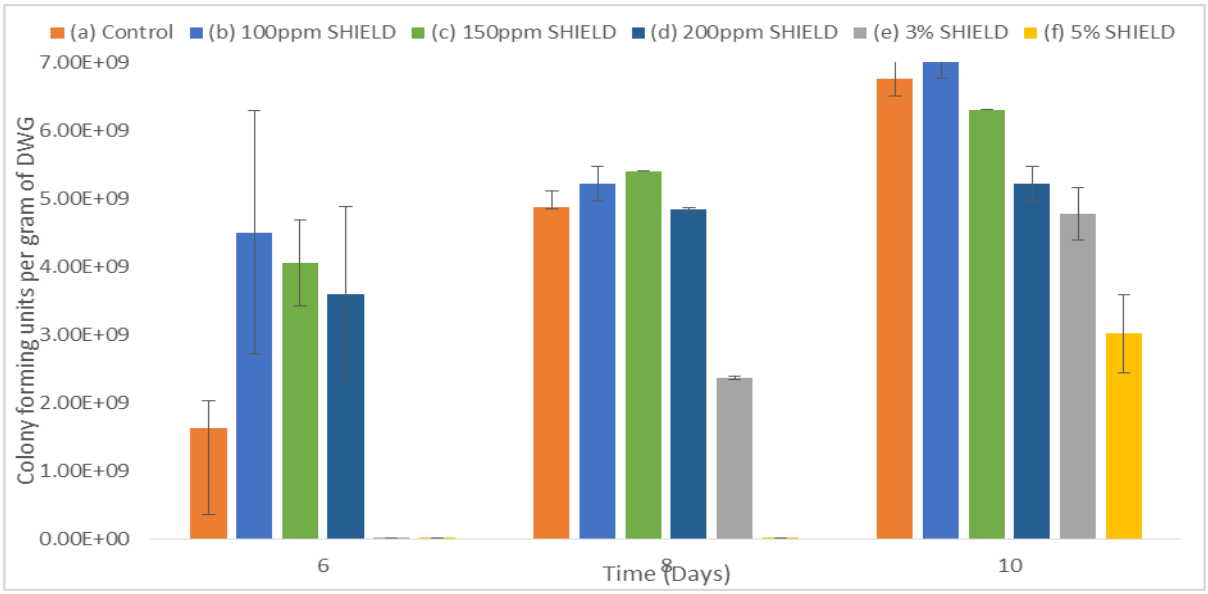

Figure 5.2: CFU per gram of DWG of lactic acid bacteria from day 6 to day 10 when the new preservative, SHIELD, was added to distillers wet grain at various concentration (treatment (b) to (f)): 100 ppm, 150 ppm, 200 ppm, 3% and 5% (w/w) levels. Error bars represent standard deviation from mean.

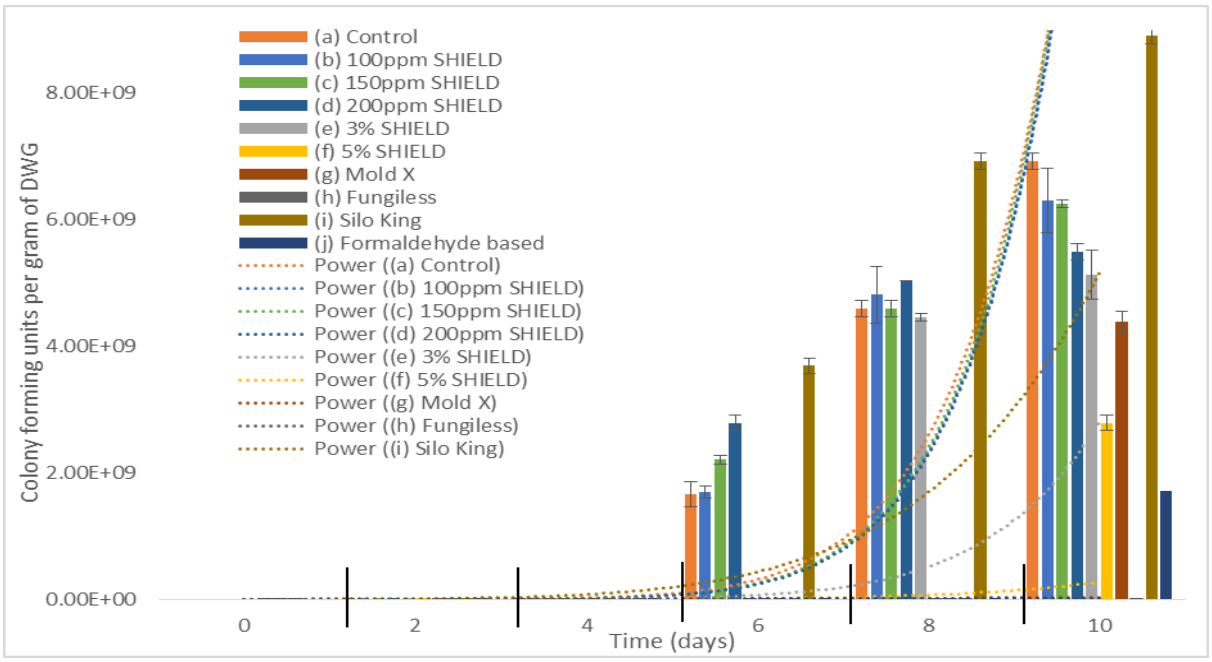

Figure 5.3: Growth of yeasts and molds over time when different chemical preservatives were added to distillers wet grains (60% moisture content on wet basis) at room temperature. Error bars represent standard deviation from mean. Dotted lines represent the best fit. Refer Table 5.4 for significant values.

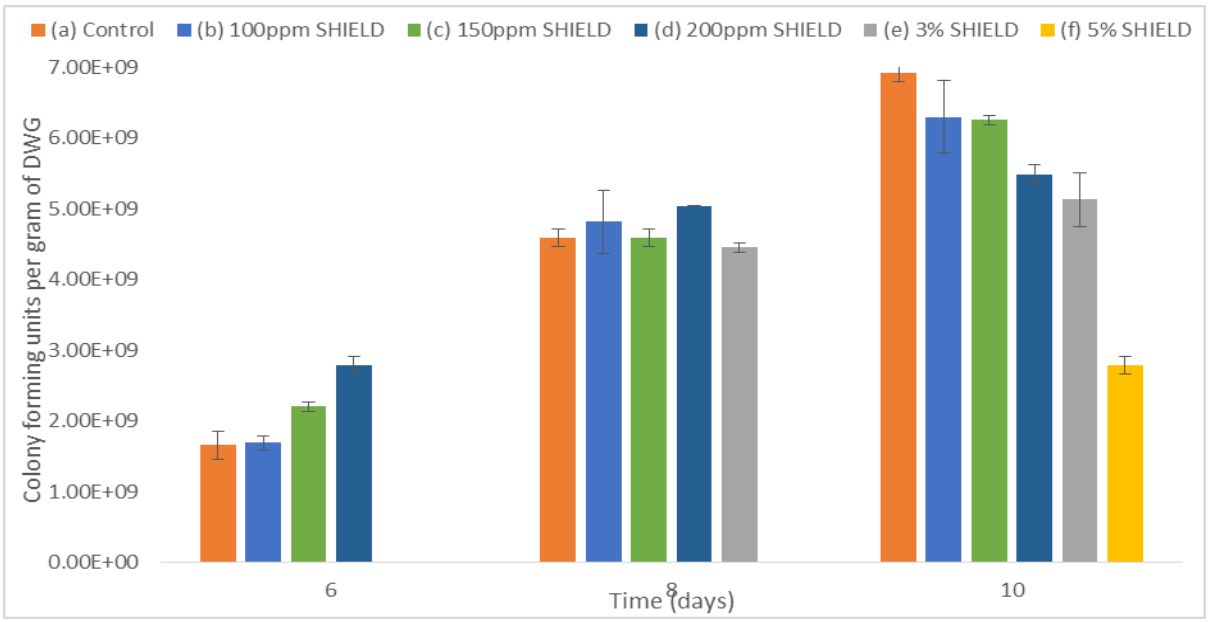

Figure 5.4: CFU per gram of DWG of yeasts and mold from day 6 to day 10 when the new preservative, SHIELD, was added to distillers wet grain at various concentration (treatment (b) to (f)): 100 ppm, 150 ppm, 200 ppm, 3% and 5% (w/w) levels. Error bars represent standard deviation from mean.

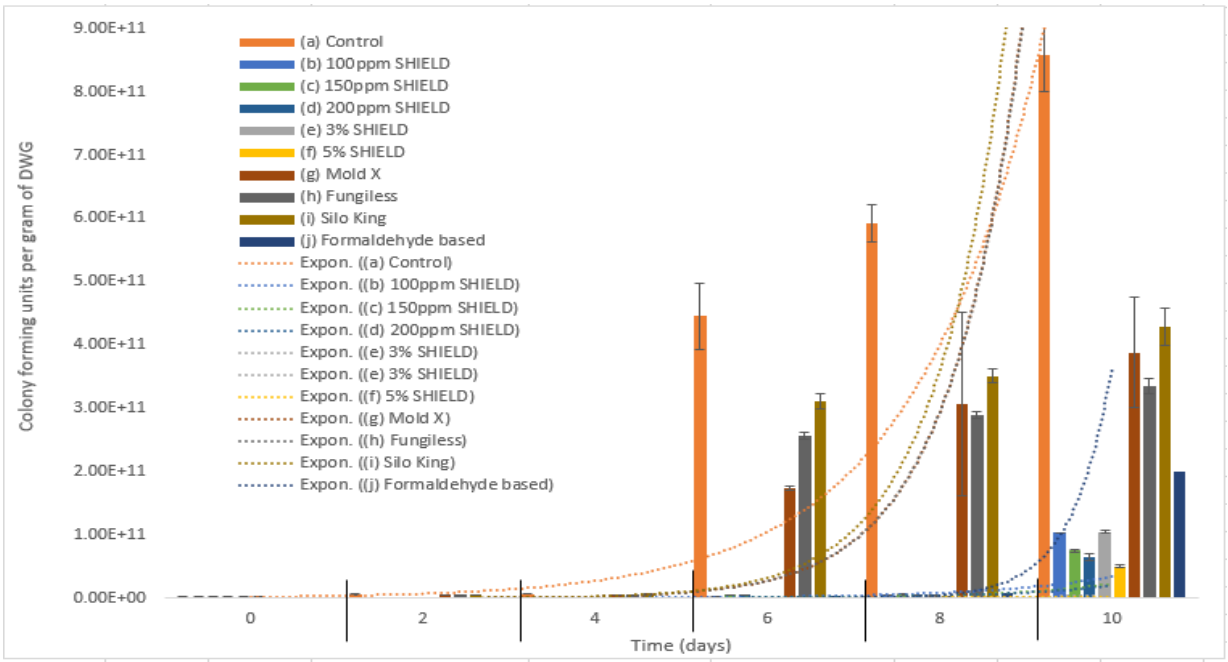

Figure 5.5: Growth of aerobic heterotrophs over time when different chemical preservatives were added to distillers wet grains (60% moisture content on wet basis) at room temperature. Error bars represent standard deviation from mean. Dotted lines represent the best fit. Refer Table 5.5 for significant values.

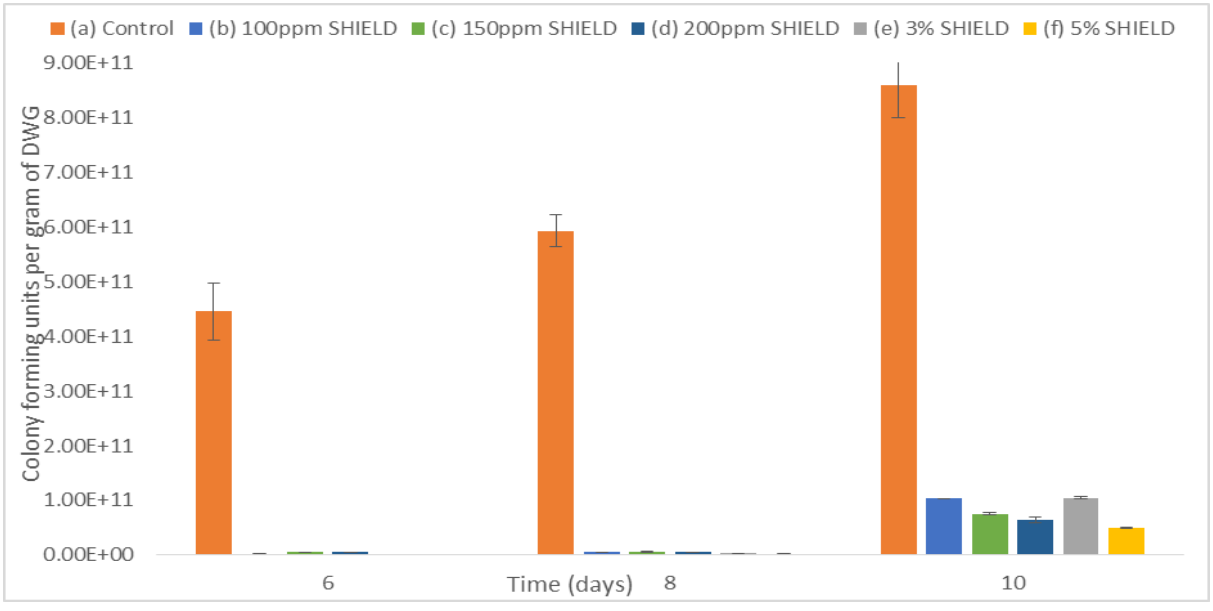

Figure 5.6: CFU per gram of DWG of aerobic heterotrophs from day 6 to day 10 when the new preservative, SHIELD, was added to distillers wet grain at various concentration (treatment (b) to (f)): 100 ppm, 150 ppm, 200 ppm, 3% and 5% (w/w) levels. Error bars represent standard deviation from mean.

Table 5.1: Nutrient level required in dairy diet, standard diet and 30% DWG (Schroeder, 2003).

Nutrients	**Requirement for dairy farm animals**	**Standard Diet (50% alfalfa+50%corn silage+ soybean meal and corn concentrate)**	**30% distillers wet grains**
Crude protein	18.0	18.0	18.0
Rumen un-degradable protein	6.3	6.3	6.3
Calcium	0.66	0.66	0.66
Phosphorus	0.38	0.40	0.50

Table 5.2: Colony forming units of lactic acid bacteria, aerobic heterotrophs and yeasts and mold on day 10 in presence of different preservatives. Values are presented as mean ± standard deviation and levels connected by same letter as not significantly different.

Preservative Type	**Concentration of active ingredients (ppm)**	**CFU of Lactic Acid Bacteria/g of DWG**	**CFU of Yeasts and Molds/g of DWG**	**CFU of Aerobic Heterotrophs/g of DWG**
Control (no preservative)	-	6.75E+09 ± 6.36E+08 a	6.93E+09 ± 1.27E+08 b	8.58E+11 ± 5.78E+10 a
SHIELD	100	7.02E+09 ± 2.55E+08 a	6.30E+09 ± 5.09E+08 c	1.03E+11 ± 6.36E+08 c d
	150	6.30E+09 ± 0.00E+00 a b	6.26E+09 ± 6.36E+07 c	7.52E+10 ± 2.42E+09 d
	200	5.22E+09 ± 2.55E+08 c d	5.49E+09 ± 1.27E+08 d	6.50E+10 ± 4.84E+09 d
	2913 (3% w/w)	4.77E+09 ± 3.82E+08 d	5.13E+09 ± 3.82E+08 e	1.04E+11 ± 2.23E+09 c
	4762 (5% w/w)	3.02E+09 ± 5.73E+08 e	2.79E+09 ± 1.27E+08 g	5.00E+10 ± 1.91E+09 d
Mold-X	999	5.76E+09 ± 5.09E+08 b c	4.38E+09 ± 1.65E+08 f	3.88E+11 ± 8.67E+10 b
Fungiless	1996	4.69E+06 ± 1.78E+05 f	4.35E+06 ± 2.29E+05 i	3.35E+11 ± 1.16E+10 b
Silo King	350	7.02E+09 ± 2.55E+08 a	8.91E+09 ± 1.27E+08 a	4.29E+11 ± 2.89E+10 b
Formaldehyde-based preservative	1996	7.05E+04 ± 2.13E+04 f	1.71E+09 ± 1.16E+10 h	2.00E+11 ± 5.78E+09 c

Table 5.3: Colony forming units (CFU) per gram of distillers wet grains of lactic acid bacteria in presence of chemical preservatives over time. Levels connected by same letter are not significantly different. Uppercase letters are associated with difference in CFU/g of DWG according to preservatives and lowercase are associated with difference in CFU/g of DWG according to time.

Preservative		**Time (in days)**		**CFU/ g of DWG**
Control	A B	0	c	1.62E+03
		2	c	2.20E+04
		4	c	7.71E+04
		8	b	4.88E+09
		10	a	6.75E+09
100 ppm SHIELD	A	2	c	4.52E+05
		4	c	4.75E+06
		8	b	5.22E+09
		10	a	7.02E+09
150 ppm SHIELD	A B	2	c	2.71E+05
		4	c	4.65E+06
		8	b	5.40E+09
		10	a	6.30E+09
200 ppm SHIELD	A B	2	c	2.18E+05
		4	c	2.78E+06
		8	b	4.83E+09
		10	a	5.22E+09
3% SHIELD (w/w)	A B	2	c	3.83E+04
		4	c	3.19E+06
		8	b	2.36E+09
		10	a	4.77E+09
5% SHIELD (w/w)	A B	2	c	1.89E+04
		4	c	6.36E+04
		8	b	5.77E+06
		10	a	3.02E+09
Mold-X	A B	2	c	7.84E+03
		4	c	2.97E+04
		8	b	1.35E+09
		10	a	5.76E+09
Fungiless	B	2	c	4.68E+03
		4	c	2.07E+04
		8	b	1.72E+06
		10	a	4.69E+06
Silo King	A	2	c	3.08E+04
		4	c	3.47E+06
		8	b	5.58E+09
		10	a	7.02E+09

Table 5.3 continued

Formaldehyde-based	B	2	c	2.79E+03
		4	c	1.17E+03
		8	b	3.03E+04
		10	a	7.05E+04

Table 5.4: Colony forming units (CFU) per gram of distillers wet grains of yeasts and mold in presence of chemical preservatives over time. Levels connected by same letter are not significantly different. Uppercase letters are associated with difference in CFU/g of DWG according to preservatives and lowercase are associated with difference in CFU/g of DWG according to time.

Preservative		**Time (in days)**		**CFU/ g of DWG**
Control	A B	0	c	5.40E+02
		2	c	1.07E+06
		4	c	2.06E+06
		8	b	4.59E+09
		10	a	6.93E+09
100 ppm SHIELD	A	2	c	6.34E+04
		4	c	4.04E+06
		8	b	4.82E+09
		10	a	6.30E+09
150 ppm SHIELD	A B	2	c	5.08E+04
		4	c	3.93E+06
		8	b	4.59E+09
		10	a	6.26E+09
200 ppm SHIELD	A B	2	c	2.99E+04
		4	c	3.03E+06
		8	b	5.04E+09
		10	a	5.49E+09
3% SHIELD (w/w)	A B	2	c	2.90E+04
		4	c	3.21E+06
		8	b	4.46E+09
		10	a	5.13E+09
5% SHIELD (w/w)	A B	2	c	1.59E+04
		4	c	4.52E+06
		8	b	7.17E+06
		10	a	2.79E+09
Mold-X	A B	2	c	6.24E+03
		4	c	2.93E+04
		8	b	7.79E+06
		10	a	4.38E+09

Table 5.4 continued

Fungiless	B	2	c	2.05E+03
		4	c	1.77E+04
		8	b	4.45E+06
		10	a	4.35E+06
Silo King	A	2	c	4.99E+04
		4	c	5.40E+06
		8	b	6.93E+09
		10	a	8.91E+09
Formaldehyde-based	B	2	c	3.06E+03
		4	c	3.67E+03
		8	b	3.25E+06
		10	a	1.71E+09

Table 5.5: Colony forming units (CFU) per gram of distillers wet grains of aerobic heterotrophs in presence of chemical preservatives over time. Levels connected by same letter are not significantly different. Uppercase letters are associated with difference in CFU/g of DWG according to preservatives and lowercase are associated with difference in CFU/g of DWG according to time.

Preservative		**Time (in days)**		**CFU/ g of DWG**
Control	A	0	c	2.70E+09
		2	c	5.85E+09
		4	c	6.66E+09
		8	a b	5.92E+11
		10	a	8.58E+11
100 ppm SHIELD	B	2	c	2.70E+05
		4	c	1.32E+09
		8	a b	5.31E+09
		10	a	1.03E+11
150 ppm SHIELD	B	2	c	3.76E+04
		4	c	5.08E+06
		8	a b	6.03E+09
		10	a	7.52E+10
200 ppm SHIELD	B	2	c	5.43E+04
		4	c	4.37E+06
		8	a b	5.58E+09
		10	a	6.50E+10
3% SHIELD (w/w)	B	2	c	4.98E+04
		4	c	3.79E+06
		8	a b	3.74E+09
		10	a	1.04E+11

Table 5.5 continued

5% SHIELD (w/w)	B	2	c	3.42E+04
		4	c	1.65E+06
		8	a b	2.79E+09
		10	a	5.00E+10
Mold-X	A B	2	c	5.58E+09
		4	c	5.85E+09
		8	a b	3.06E+11
		10	a	3.88E+11
Fungiless	A B	2	c	5.54E+09
		4	c	5.40E+09
		8	a b	2.90E+11
		10	a	3.35E+11
Silo King	A B	2	c	5.63E+09
		4	c	5.94E+09
		8	a b	3.51E+11
		10	a	4.29E+11
Formaldehyde-based	B	2	c	5.88E+03
		4	c	5.79E+03
		8	a b	7.20E+09
		10	a	2.00E+11

Table 5.6: Coefficient of determination and the equation of growth of lactic acid bacteria in distillers wet grains over time. ‘x’ is the time variable.

Preservatives	**R^2**	**Equation**
Control	0.8418	$182.43\ x^{9.66}$
100 ppm of SHIELD	0.9481	$1108.10\ x^{9.27}$
150 ppm of SHIELD	0.9466	$927.38\ x^{9.32}$
200 ppm of SHIELD	0.9368	$819.48\ x^{9.26}$
3% SHIELD (w/w)	0.9225	$416.21\ x^{8.54}$
5% SHIELD (w/w)	0.8150	$305.25\ x^{6.98}$
Mold-X	0.7345	$361.21\ x^{7.85}$
Fungiless	0.7484	$1729.00\ x^{3.74}$
Silo King	0.8800	$1301.60\ x^{8.91}$
Formaldehyde-based	0.8062	$351.16\ x^{2.69}$

Table 5.7: Coefficient of determination and the equation of growth of yeast and mold in distillers wet grains over time. ‘x’ is the time variable.

Preservatives	**R^2**	**Equation**
Control	0.9531	$598.84\ x^{9.50}$
100 ppm of SHIELD	0.9610	$240.19\ x^{9.99}$
150 ppm of SHIELD	0.9543	$222.84\ x^{10.06}$
200 ppm of SHIELD	0.9393	$179.13\ x^{10.17}$
3% SHIELD (w/w)	0.9316	$160.02\ x^{9.31}$
5% SHIELD (w/w)	0.8988	$268.53\ x^{7.71}$
Mold-X	0.2851	$31642.00\ x^{3.84}$
Fungiless	0.0382	$62833.00\ x^{0.99}$
Silo King	0.6307	$89092.00\ x^{6.12}$
Formaldehyde-based	0.6686	$46.68\ x^{6.97}$

Table 5.8: Coefficient of determination and the equation of growth of aerobic heterotrophs in distillers wet grains over time. ‘x’ is the time variable.

Preservatives	**R^2**	**Equation**
Control	0.8683	$5.0*10^{8}.e^{1.34x}$
100 ppm of SHIELD	0.3593	$8.0*10^{6}.e^{1.39x}$
150 ppm of SHIELD	0.3378	$8.5*10^{5}\ .e^{1.70x}$
200 ppm of SHIELD	0.3326	$1.0*10^{6}.e^{1.65x}$
3% SHIELD (w/w)	0.2663	$5.5*10^{5}.e^{1.50x}$
5% SHIELD (w/w)	0.2375	$4.8*10^{5}.e^{1.42x}$
Mold-X	0.7534	$2.0*10^{6}.e^{2.36x}$
Fungiless	0.7400	$2.0*10^{6}.e^{2.35x}$
Silo King	0.7485	$2.0*10^{6}.e^{2.40x}$
Formaldehyde-based	0.8739	$2.2\ .e^{4.31x}$

CHAPTER 6. GENERAL CONCLUSION AND FUTURE WORK

General Conclusion

Aqueous extraction process and enzyme assisted aqueous extraction process have few advantages over solvent-based oil extraction process. Environmental aspect, economics and efficiency of the AEP and EAEP of soybeans have been discussed in this thesis. Since, the yield is the major issue in AEP and EAEP, more work needs to be directed towards solving this problem. The downstream processing of both the aqueous methods can be made more economical by recycling the water and enzymes. Important parameters mentioned in the thesis such as pre-treatment steps, extraction enzymes and de-emulsification enzymes should be taken into consideration while optimizing the process. Scaling up single stage EAEP as well as the two stage results in similar oil, protein and solids extraction yield as in laboratory. Steady-state oil extraction can be achieved in second trial when enzymes is added in the first stage of extraction.

It was observed that not only could *S. cerevisiae* digest xylose to some extent, *P. stipitis* and *C. shehatae* were able to digest glucose and xylose and withstand ethanol concentration similar to *S. cerevisiae*. Integrating soy products to corn fermentation gave value to the skim fraction of EAEP of soybeans, which contained a part of oil extracted from the soybeans. While insoluble fiber provided additional carbohydrates, the overall ethanol yield did not improve much. In presence of *P. stipitis* and *C. shehatae*, the by-product concentration decreased. Role of these pentose-degrading yeast species in integrated corn-soy fermentation system can further be investigated.

The high moisture distillers wet grains are susceptible to microbial growth and chemical preservatives can be added to decrease spoilage and to increase the storage time of this animal feed. Along with esters and organic acids, fermentation products of some bacteria

have shown antimicrobial activity, which is taken advantage of in chemical preservatives. DWG, when produced, is at high temperature and at low pH, providing unfavorable conditions for growth of spoilage microorganisms. It is often sold to livestock producers within a day of production and the chances of contamination are high while transportation and storage. Preservatives are added before storing DWG in silo bunkers and forages like alfalfa can be added to reduce the overall moisture content. It is very important to note that adding appropriate amount of preservative is the key. Addition of too little preservative can be detrimental than not adding it at all.

Future Work

For future work, different ratio of combination of *P. stipitis* or *C. shehatae* and *S. cerevisiae* could be investigated in corn-soy bio-refinery. The insoluble fiber and soy skim could be pre-treated before adding it to ground corn.

The efficacy of the new preservative can be improved by combining it with mixture of organic acids. Mixing dry forage such as alfalfa to DWG before adding the preservative can decrease the overall moisture content. This is how livestock producers usually store high moisture products.