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Evaluation of Parameters Affecting the Performance of Lipase Transesterification Using Cottonseed Oil

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EVALUATION OF PARAMETERS AFFECTING THE PERFORMANCE OF LIPASE TRANSESTERIFICATION IN COTTONSEED OIL

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Accepted by:
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Dr. Caye Drapcho

ABSTRACT

Eversa Transform® was used with crude and glandless cottonseed oil to evaluate the key parameters in its performance. Eversa is one of the newer products produced by Novozymes A/S (Copenhagen, Denmark) and has attracted much industry use. Cottonseed oil is a fringe oil in the biodiesel production market that was not tested with Eversa Transform® to our knowledge. The goal of this research was to see which dominant parameters were the most influential on the performance of Eversa. After careful review of literature, temperature, enzyme amount and water amount were the most consistently manipulated variables to produce more optimal results. The analysis was conducted using gas chromatography method of ASTM D6584 to estimate the total conversion and the induction time to measure oxidative stability was conducted using EN15751. The conversion averaged to 98 % wt. across all samples. The enzyme dosage was observed to not be a factor in the enzyme's ability to convert while water dosage and temperature were determined to be significant. ANOVA analyses show no indication of interaction between any variables. Induction periods of the glandless and heavy pigment (crude) cottonseed oils had distinct differences, but there was no indication that there was a difference between the two oils in conversion. A SuperPro Designer simulation up was simulated using 2% wt. Eversa to examine its potential for scale up and assumptions that washing steps were accurately estimated. Based on the output stream results of the kinetic model, the assumptions accurately represented what the data previously recorded.

DEDICATION

To Kendal Simmons for showing me what dreams are made of and how to not fade in the pursuit of them.

To my mother, Sandra Anderson, for nurturing me and throwing money at this dream when there was no substance to it.

To my father, Stanley Anderson, getting me interested in science by watching science fiction and listening to your army stories.

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David Thorton and Ning Zhang: Both of you were invaluable teachers to me because of your knowledge of lab safety procedures and the biodiesel process in general. My first day entering the lab, I felt as if the information was sand pouring into a sieve, but the practical wealth of knowledge you two shared made the adjustment easier.

Dr. Yi Zheng: There were many things about enzymes and biodiesel production that I never imagined that I would consider as factors in the investigation. Thanks to your knowledge on how enzymes perform I could create a more detailed perspective on the performance of Eversa.

Dr. Caye Drapcho: Thank you for your encouragement and insights on the field. I learned so much about the world of academia from our conversations and I never considered myself part of Biosystems Engineering until I heard you talk so highly of the discipline.

Dr. Oliver Myers: Thank you for offering me an opportunity to work in your lab while I was suspended. It was difficult work and the rewards were few, but before I demonstrated that I had the ability to do anything, I had to demonstrate that I was willing to do everything.

Dr. Bryan Moser: While I only known you for a short four months, I have appreciated every bit of work you have contributed to the production of this thesis. Your analysis of the samples was invaluable. We never would have gotten here without you.

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Barbara Smith: You are the example that every graduate assistant should follow. You would do anything for your students and professors; Clemson needs more people like you.

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Chapter One

Introduction

Biodiesel is a biorenewable derived fuel from processed organic oils and fats. The structure of the fuel resembles that of petroleum diesel proven as a capable replacement in nearly every application. Biodiesel has received increased attention as more affirmative policies, such as blending credits and subsidies, are given to producers and researchers (de Gorter and Just, 2009; Szulczyk and McCarl, 2010). Formerly ethanol was the renewable fuel of choice, but its low energy density and supply factors are current weaknesses. Biodiesel is much more energy dense and can also be manufactured from waste crops and oils without directly without interfering with food supplies using current infrastructure (Banković-Ilić et al., 2012). The fuel is a mixture of fatty acids bonded with an alkyl group. Traditionally manufactured biodiesel uses a chemically catalyzed reaction to breakdown triglycerides and esterify them to create a diesel-like molecule.

The proposition of replacing petrodiesel with an organic substitute could potentially bring about a major shift in the way human activity influences the planet because biodiesel can be carbon neutral. Several different feedstocks used for biodiesel production are proven as successful alternatives. Some major differences between petrodiesel and biodiesel include lower emissions from combustion, low sulfur content, biodegradable and has superior viscosity. On the other hand, the biodegradable nature of biodiesel may lead to a shorter life span. Other potential drawbacks include lower energy density and an increase NO_x emissions (Shahid and Jamal, 2011), the relative inability to flow at cooler temperatures (Joshi et al., 2011; Knothe, 2007; Tang et al., 2008) and the creation of a byproduct, glycerol, as the result of the esterification of the alcohol acceptor. Conventional biodiesel production is dependent on alkaline catalysts because of its high conversion speeds at low temperatures in the range of room temperature to around 50 °C (Atadashi et al., 2013). One of the unintentional products that can form however, is the production of soaps and excess water from free fatty acids in the feedstocks, which limits the types of oils used with alkaline catalysts (Musa, 2016; Shahid and Jamal, 2011). While other biodiesel production methods are used like the use of acid catalysts or reacting at supercritical conditions with methanol to achieve high yield, the higher temperatures make these method less appealing, even without the risk of soap production (Lotero et al., 2005; Shahid and Jamal, 2011; Yaakob et al., 2013). Enzymes, although with slightly higher catalytic costs, perform well at lower temperatures. Enzyme reactions are not

as fast as alkaline catalysts, with many users taking a full 24 h to see results, but are versatile in their use in both high and low quality oils (Gog et al., 2012).

Contemporary evidence suggests that the properties of a hydrolyzed blend of oils could help tune the properties of the final product. This combination of blending and the use of interesterification may enhance the performance of biodiesel. The goal of this research is to evaluate the use of this enzyme to convert triglycerides to biodiesel. Strictly looking at conversion can answer many questions about the enzyme's ability to perform and cottonseed's ability to be used as a biodiesel feedstock. This study comes down to what are the best conditions to produce enzymatically transesterified cottonseed biodiesel:

- What are the significant parameters that affect the performance of Eversa in cottonseed oil?
- Is there any difference between glandless and crude cottonseed oil in their ability to be converted to biodiesel?

Chapter Two

Literature Review

Biodiesel Properties and Additives

Biodiesel is an organic product that suffers from decomposition more readily than its petroleum-derived cousin. This thermal stability is examined in part by the oxidative measures. The oxidative stability index (OSI) and American Society of Testing and Measurements (ASTM) methods are the top methods of choice to determine the oxidative stability. A detailed thermos-gravimetric analysis with differential thermal analysis (TGA/DTA) method is a more analytical alternative to measure of degradation. Figure 1 displays a schematic of the Rancimat test. The oil sample is heated while air is applied to the sample. When the oil sample oxidizes it will release secondary oxidation products resulting in an electrical response (Jain and Sharma, 2010). This tool determines the induction period of the biodiesel, which measures its ability to resistant to degradation. Different feedstocks have different levels of resistance to oxidation. This stems from each individual methyl ester decaying at different temperatures. The presence of double bonds in the biodiesel was determined to be one of the factors in the biodiesel with the more unsaturated esters having lower OSI values (Knothe, 2007).

Variance in biodiesel properties does occur naturally based on the composition of the methyl esters (or more precisely, triglyceride composition). One can use the number of double bonds to predict trends in the oxidative stability as well as the energy density of the fuel based on triglyceride composition, as demonstrated above with double bonds (Przybylski, 2011). Another example would be the cold flow points when using the use of animal fats as the biodiesel feedstock. Methyl esters produced with animal fats have higher cold flow points—plug flow, cloud point and pour point— making them less useful in cooler climates (Joshi, Moser, Toler, Smith, et al., 2010; Joshi et al., 2011). Work by Joshi demonstrated the effect of blending biodiesel and alcohol to decrease the cloud point, pour point and cold filter plugging points postreaction (Joshi, Moser, Toler, Smith, et al., 2010). . Another attempt was made by using ethyl levulinate to reduce the cold flow temperatures while also increasing the oxidative stability of the oil-based biodiesel, but not for the animal fat derived fuels. Both attempts saw significant success in the attempt but required quantities as high as 20% by volume to achieve the largest effect.

More direct means of controlling the properties of biodiesel is done using additives. Flow properties and fuel stability are often the most frequently tracked when additives are discussed. Cold flow properties are crucial factors for biodiesel performance as it places a lower temperature limit on the fuel. ASTM requires that the cold flow points are reported, but do not have a limit for what would constitute a good biodiesel. Studies have shown that all cold flow points typically near or just above the freezing point of water, higher than petrodiesel for engine operation (ASTM D6751-15c, 2010). Stability additives are also studied at large in both organic and chemical origins. The organic additive tocopherol, in its various isomers, is noted for its miscibility within biodiesel but outperformed by synthetic additives (Dunn, 2005). Halting the primary and secondary oxidation reactions is the goal when maintaining stability and synthetic agents perform at higher levels (Jain and Sharma, 2010). Gossypol is another natural antioxidant found in cottonseed oil. The effectiveness of the pigment to slow the oxidation process is demonstrated with Rancimat oxidative stability testing (Joshi, Moser, Shah, et al., 2010).

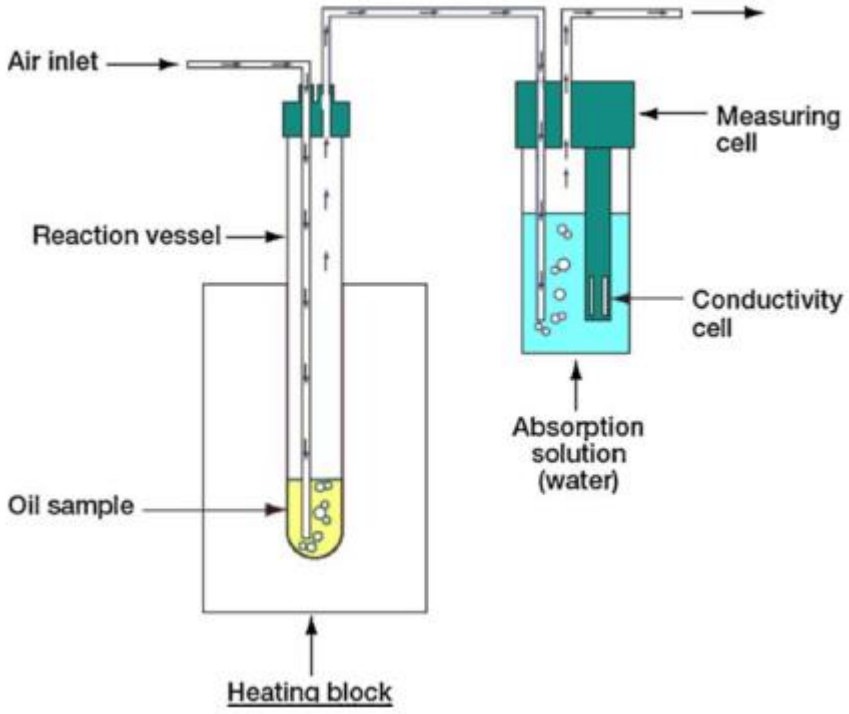


Figure 1: Schematic of the Rancimat Method (Jain and Sharma, 2011)

Biodiesel Feedstocks

Many organic oils have been studied for their potential to convert fatty acid into esters. Each organic oil has its own fatty acid composition, but the process to hydrolyze these fatty acids works similarly across feedstocks. This process is generally known as transesterification and works using a process described in Figure 1. The triglycerides are broken down into di- and monoglycerides and release a fatty acid in the process. That acid combines with an acyl acceptor to create a fatty acid ester. An acyl acceptor takes on the acyl component of the fatty acid and creates a byproduct along with the biodiesel. Oils that have garnered the most attention are the waste vegetable oils, palm, rapeseed and cottonseed oils (Dos Santos Corrêa et al., 2011; Lee et al., 2011; Liu et al., 2010; Lopresto et al., 2015; Rashid and Anwar, 2008; Royon et al., 2007). Dominant factors in the usage of an oil are its availability and price. The properties present within oils can be deterministic in the type of reaction that is used to hydrolyze the oil. These properties include the amount of free fatty acids, which form soaps and ruin the biodiesel post reaction (Lotero et al., 2005). Every oil has a unique chemical profile that varies the properties such as viscosity, higher heating value, and oxidative stability. Few studies have been conducted on cottonseed oil to thoroughly examine the potential of it to be used as a fuel. The quantities are limited and it primarily been used a protein stock for animals (Cai et al., 2009; Farfán et al., 2013).

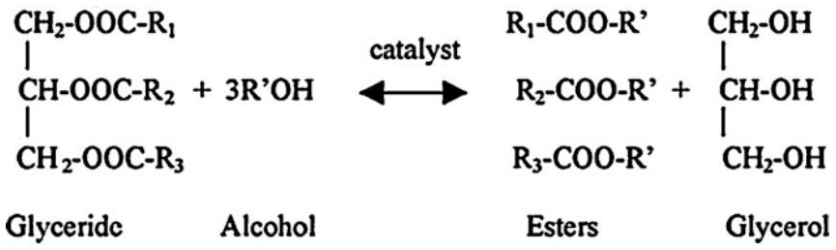


Figure 2: Transesterification chemical reaction (Shahid and Jamal, 2011)**.**

Large quantities of biodiesel are made using waste cooking oil and food crops like canola oil. Canola oil has several features that make it a desirable fuel feedstock. Tocopherol is a natural antioxidant present within the oil and the amount of triglycerides present within the oil are in high amounts (Przybylski, 2011). Soybean oil is another popular choice for feedstock, third most popular globally and the primary choice in the United States (Souza et al., 2017). Soybeans and other oil crops are also used as food creating the potential for a conflict of resources (Souza et

al., 2017; Szulczyk and McCarl, 2010). This looming conflict and a desire for lower costs, a market has favored waste cooking oil over fresh vegetable oil for production of biofuels. Waste vegetable oil utilized for production of biodiesel accomplished with several different catalyzing techniques and different alcohol configurations. Lopresto et al. were among many investigators who covered the transesterification of waste vegetable oil to produce biodiesel (Lopresto et al., 2015). Lopresto et al used an enzyme catalyst and determined at 5% wt/wt enzyme to oil, there was not an observed difference in the yields between the waste vegetable oil and fresh vegetable oil. They demonstrated that the enzyme catalysts can degrade over several uses. The quality of the oil was less of an issue if the enzyme was properly loaded. A peak biodiesel ester yield of 46.32 % wt. was observed using fresh enzyme. Waste vegetable oil had only a marginal decrease in ester concentration. This indicated that the enzymatic process handled lower quality oils under similar conditions (Lopresto et al., 2015).

Waste cooking oil has an inherent risk stemming from its higher free fatty acid content using traditional alkali transesterification processes. Heavily fried oils had fewer triglycerides because of thermolysis, the thermal breakdown of the triglyceride, and hydrolysis, the process of reacting with water (Lopresto et al., 2015; Okullo et al., 2013). Excess water is also a quality that cooking oils because of contact with food, and sustained heating results in highly degraded oil. Lower quality oils typically make inferior and nonstandard fuels (Hama and Kondo, 2013). The biodiesel that was produced with waste cooking oil in Chheteri, Watts and Islam's study on the waste biodiesel showed the fuel they produced did not meet specifications failing the ASTM D6584, with a total glycerin content of 0.566 % wt. The problem of soap production (Figure 3) also halted the amount of biodiesel produced at higher levels, while taking a half hour to react completely (Chhetri et al., 2008). Soap production also makes it more difficult to reuse catalysts by consuming the metallic ions too quickly. Countermeasures have been implemented to circumvent problems with high acid oils to prevent soap production using base catalysts. Acid-catalyzed reactions are often done first to the oil to catalyze the free fatty acid to an acceptable level before being traditionally transesterified using a much faster base catalyst or an enzyme that works at lower temperatures (Canakci and Gerpen, 2001; Kumar Tiwari et al., 2007; Lotero et al., 2005; Ridha et al., 2014).

Challenges from used cooking oil create a market for non-edible vegetable oils for biodiesel. Some of the non-edible vegetable oils that are produced stem from the plant seeds of cash crops, like tobacco and rubber. Using the seeds and in some cases the husk of these plants, ranging from 10-75 % wt (Banković-Ilić et al., 2012). While not

as widely used, cottonseed oil has attracted some attention as a biodiesel feedstock. In its glandless form, the oil used as a food product for animal feed and to help diversify the cooking oil supply (Farfán et al., 2013). Presently, Cottonseed oil features a useful natural antioxidant pigment to improve the life of the fuel called gossypol. For food purposes, this antioxidant is not productive as it is toxic and linked to infertility in mammals (Wang et al., 2009). Several studies in food research have focused on the removal of the antioxidant, which is why the glandless form has attracted so much attention (Kuk et al., 2005; Kuk and Tetlow, 2005; Ribeiro et al., 2009).

Cottonseed oil was researched in a food role through creating a plant species that did not produce the gossypol gland (Cai et al., 2009). Motivated by the need to sell the oil, cotton producers developed a cottonseed oil that did not have the gossypol toxins. Successful breeding developed a low gossypol-content cottonseed, but these populations are more susceptible to insects and other pests because the toxic antioxidant is not present. Specific glands located in the plant were determined to produce gossypol. Gossypol, hemisgossypolone and heliocides were produced in the leaves via biosynthesis in those glands (Cai et al., 2009). Gossypol present in cottonseed biodiesel will increase the life of the fuel was demonstrated by Joshi et al by testing the oxidative stability of the biodiesel (Joshi et al., n.d.). At gossypol quantities of 250 ppm, the biodiesel stability is able to meet ASTM standards (Joshi, Moser, Shah, et al., 2010). Previous studies have demonstrated interests in blends with more traditional feedstocks to reduce the overall cost, but still take advantage of the properties present within the fuels (Joshi et al., n.d.; Lepak et al., n.d.). Cottonseed oil, from a United States economic perspective, is a more expensive vegetable fuel and is produced in far fewer quantities than some other oils such as soy and canola oil, but does not compete with the food market (US Department of Agriculture, 2016).

Cottonseed oil is an alternative product from the cotton plant used primarily as a source of protein for animal feed (using a modified form), an infertility compound and natural pesticide (Wang et al., 2009; M. Zhou et al., 2013). One of the weaknesses of the organic fuel is that the lifetime due to its propensity to oxidize over time making the fuel unusable. Gossypol, as a natural additive will slow this process down and observed to enhance ASTM oxidation tests by more than twice their recommended quality (Joshi, Moser, Shah, et al., 2010). Shankar, Pentapati and Prasad using a homogenous alkali catalyst and multi-walled carbon nanotubes (Shankar et al., 2016). The final analysis used both Fourier Transform infrared spectrometry. The analysis used a half factorial model and contour plots. The interaction matrices determined that the ideal alcohol ratio is 6:1 while catalyst amounts varied due to other factors at

a significant ($p<.05$) levels. Optimal yield was 96 % wt. and a quartic regression was generated to predict the properties of the biodiesel (Shankar et al., 2016). Test results met the ASTM standards for both catalysts indicating that the fuel was acceptable for blending into petrol diesel samples for improved diesel emissions.

Catalysts and Processing Techniques

The primary method of biodiesel production is accomplished through the process of transesterification. Transesterification uses an alcohol, typically ethanol or methanol, as the acyl acceptor. Transesterification is the most traditional method of producing biodiesel and is the primary method used for the various biodiesel mixes that are commercially available. Esterification is a catalyst-assisted reaction that uses three main methods: acid catalysts, alkaline catalyst, both heterogeneous and homogenous, and bio catalysts (Atadashi et al., 2013). Acid catalysts have a distinct advantage over the more conventional alkaline catalyst because they are capable of handling oils with more free fatty acids as well as higher water content (Lotero et al., 2005; Zabeti et al., 2009). The failure of alkaline catalyst when used with feedstocks of low purity is due to increased processing cost (Atadashi et al., 2013) due to high amounts of free fatty acids present that cause the creation of soap during the transesterification reaction. The disadvantages of the acid catalysts, however, are the need for high temperatures and pressures for an effective reaction. The reaction rate of an acid catalyzed reaction is cited as being 4000 times slower than an alkaline based reaction (Lotero et al., 2005; Shahid and Jamal, 2011). This slow process mitigated using higher temperature reactions, but require much more expensive equipment for operation. One example was cited for the temperature of the 240 °C at 70 bar showing ester conversion at 90% efficiency in 15 minutes. At temperatures of 77 °C to 117 °C the conversion times occur over a range of 20 hours to 3 hours respectfully (Lotero et al., 2005).

Alkaline catalysts are the dominant method of conventional production using transesterification. Alkaline catalyzed biodiesel reactions occur at lower temperatures and methanol ratios, typically in the range of 60 °C and molar ratios of methanol in the range of 3:1 to 9:1 experiencing yields greater than 90% (Hoda, 2010; Joshi et al., 2012). Rashid and Anwar evaluated biodiesel property at different molar ratios, catalyst amounts, and temperatures. The yields under various runs they came out with a clear winner with a 95 % wt. conversion, at 600 rpm, 65 °C, 1 % KOH and a 6:1 alcohol to oil molar ratio (Rashid and Anwar, 2008). High conversion rates have been observed in optimized biodiesel production conditions. Using response surface methodologies Jeong and Park determined that the amount of alkaline catalyst is the most significant factor in the production of the biodiesel (Jeong and Park, 2009).

Through their methods, used KOH and methanol to reach yields of 92% in 40 minutes. Despite the advantages, using alkaline catalysts to facilitate conversion is still difficult because of soap production.

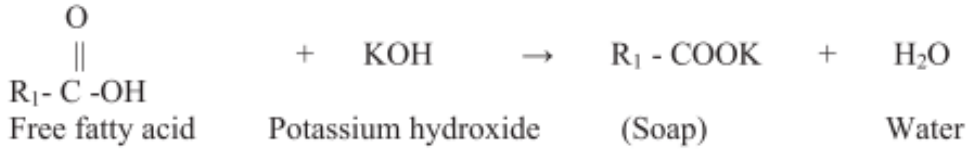


Figure 3: The production of soap in biodiesel reaction (Atadashi et al., 2013)**.**

This creates a conflict for biodiesel producers to desire new catalysts that do not have soap production issues, while having the high amount of conversion, under reasonable temperatures. Enzymes have been the tool of choice to catalyze biodiesel reaction. Shahid and Jamal discussed dominant factors for increased biodiesel production that included enzyme catalysts and thermodynamic properties used to enhance yield and feedstocks (Shahid and Jamal, 2011). The enzymes that were covered included the immobilized Novozym 435 and a few others. Enzymes have the benefit of lower temperature requirements and ability to process any feedstock (Shahid and Jamal, 2011). More ambitious approaches either used radio waves to enhance the kinetic rate and supercritical fluids which prompted reaction without the need for a catalyst. This method is infrequently used because of the high thermodynamic demand of supercritical conditions.

Enzymatic Biodiesel Production

Enzymes are divided into two main classes: intercellular and extracellular. The enzymes that are used in lipid reactions are known as lipases. Extracellular lipases are more costly and are harder to recover, so the whole cell catalysts use is more frequent when the cells can be used inside the reaction (Fukuda et al., 2008; Gog et al., 2012). Two additional subcategories of extracellular enzymes are immobilized, which produce a heterogenous mixture, and free or liquid, which a homogenous mixture. An intracellular enzyme is already immobilized and does not require any additional processing. Immobilization does make an enzyme easier to separate because of phase difference, similar to a whole-cell catalyst, but immobilized extracellular enzymes do not require reactor conditions to be suited to support a living organism (Lam et al., 2010). Hama reviewed enzymatic biodiesel production extensively covering preferred feedstocks and enzyme, reactor design and cost management. Key takeaways from this investigation the importance of glycerol removal from the reactor. The glycerol is believed to inhibit enzyme active sites (Robles-Medina et al.,

2009). As with the chemical catalysts, transesterification was the primary method discussed for biodiesel production and the relationship between methanol and inhibition were listed as hazards to production (Hama and Kondo, 2013).

Some immobilized lipase preparations include enzymes attached to a porous support material. Tan et al conducted a rough comparison for enzymatic production and conventional chemical methods (Nie et al., 2006). Transesterification was the only method explored and the factors that were demonstrated to affect the process quality included the presence of alcohols, water content and type of feedstock. Methanol may inhibit enzyme reactions as well regardless of the catalyst (Ridha et al., 2014). A sulfuric acid catalyst was used to pretreat a feedstock and the amount of methanol had an optimum point before decreasing the amount of fatty acid converted based on acid value measurements (Ridha et al., 2014). This was also seen across several other samples using enzymes, with a peak amount before falling as methanol molar ratios exceeded a certain value (Canakci and Gerpen, 2001; Musa, 2016). The other inhibitor identified in the system was accumulated glycerol during the reaction (Tan et al., 2010). Even with challenges that face enzymatic biodiesel there are clear winners with lipases being the dominant enzymes produced for biocatalysts. The advantages of immobilized enzymes include the improved recovery and reusability for continuous processes. The number of enzyme candidates continue to grow, but the ones receiving the most attention are the microbial-sourced enzymes. They require fewer resources to produce effectively with the process for enzymatic production being far simpler than the chemical process making it a much more attractive option for reactor design and temperature control (Christopher et al., 2014).

Whole-cell biocatalysts have also been active participants in the research for biodiesel production. Researchers determined that the inefficiencies coming from not using a pure enzyme did not rule out using the whole-cell. The advantage of using the whole cell was the ease of reuse through maintaining the life of the organism. From the behavior of the whole cell biocatalyst and enzyme produced from *Rhizopus oryzae*, an ideal temperature of around 30 °C, a 1:3 molar oil:solvent ratio and a lipase:oil ratio of higher than 10 % wt. was determined (Bharathiraja et al., 2014). Methanol was declared the best acyl acceptor of the solvents used in this study. Denaturing was an issue beyond molar ratios of 1:3 for this enzyme. Kabasakal and Caglar sought to reduce problems with enzymatic biodiesel production stemming from the methanol denaturation. The project used both the slower paced interesterification, using methyl acetate, while reducing the faster, but inhibitory reaction that is associated with methanol with transesterification (Kabasakal and Caglar, 2010). The combination provided a "best of both worlds" solution that

reacted at a speed closer to transesterification and with reduced transesterification. The blends also allowed a single phase because of dissolved glycerol existing in the fluid due to the presence of methyl acetate.

Dos Santo Carrera et al observed the behavior of the enzyme and what features worked against it in this reaction. The analysis concluded that denaturation from high alcohol content and decline in performance from reuse are inherent problem (Dos Santos Corrêa et al., 2011). Initially, ethanol reacted faster across the various alcohol weights giving it the appearance of the better acyl acceptor. Over time and with stepwise as opposed to single addition of the methanol showed that methanol was better for high conversion rates. Enzyme weight percentage was also an important factor in the product yield. Novozym 435 produced the best results among other commonly tested Novozyme immobilized enzymes Lipozyme RM-IM, and Lipozyme TL-IM based on conversion (Dos Santos Corrêa et al., 2011). Another immobilized lipase called Candida sp. 99-125 on a cotton membrane catalyzed a continuous flow reactor design that resulted in a greater reaction rate for this immobilized form. The conversion ratio reached 96% and significant optimization variables considered were temperature, water content, flow rate and solvent type. The immobilized enzyme needed a solvent to support the function of the enzyme by preventing water inhibition. The acyl acceptor was methanol, which meant there was a threat of denaturation that slowed the reaction in higher amounts without stepwise approaches (Nie et al., 2006).

Other physical factors that can affect enzyme actvitiy are the substrate components themselves. Lower quality oils are not only higher in free fatty acids, but also phosphorous containing lipids. The factors influencing the enzymes used in Cesarini's group to hydrolyze feedstocks for biodiesel measured the impact of phospholipases on the yield of oil used in this study. The liquid enzyme used for transesterification was Callera Trans L. The phospholipases act as additives to degum the unrefined or crude oil. These additives increased the yield of the Callera Trans L catalyzed biodiesel by 11 % from a combined process and nearly three percent in an impure oil with phospholipids, both using methanol as an acyl acceptor. The removal of the phospholipids results in biodiesel that meets phosphorus standards as well as increases the value of the esters obtained (Cesarini et al., 2014).

Free lipases usage is less frequent than immobilized enzyme due to reaction feasibility, both in terms of halting the enzyme-substrate interaction and enzyme recycling (Christopher et al., 2014). Liquid enzyme is less costly, and if reuse is not desired, would be an effective means of production (Nordblad et al., 2014). A mathematical kinetic model simulated to action Callera transform as a catalyst and used relevant knowledge about the enzyme to simulate

the biodiesel yield and calculate rates for all the various reactions. The reaction period lasted 24 hours and the water content ranged from 5-7 % wt. Enzyme weight varied, but ranged between 0.1 to 0.5 % wt. of oil. Methanol was added in a stoichiometric rate slowly to avoid the assumed competitive inhibition. This model was optimized and resulted in a maximum yield of 95% (Price et al., 2014). Based on residual shape the model was an accurate depiction of the process indicating a high probability inhibition of methanol is competitive. The model is however only valid under low concentrations of methanol. Gunvachai et al. had a unique approach to analyze the solubility of the oil as a measure of the methyl ester production. The reaction proceeded at temperatures of 293, 313 and 333 K before the conversion calculations. Following the reaction sequence, the solubility model that predicted the yield and data required several assumptions for proper analysis. This process explains the nature of the reaction as one that accelerates as due to solubility, therefore resulting in increased molecular interactions. The analytic model turned out to be a consistent fit to the oil reaction data, but did not consider the production of glycerol to inhibit the reaction (Gunvachai et al., 2007).

Quantification of Biodiesel in Reaction

Gas chromatography (GC) is the leading method for biodiesel analysis because of its speed and high accuracy. Liquid chromatography and mass spectrometry are alternatives methods and complimentary approaches to determining biodiesel quality. Typically for GC methods for biodiesel involve the use of a split injection method, a capillary column on the scale of 30 m and uses flame ionization detection to identify the split volatiles (Carvalho et al., 2012; Farfán et al., 2013; Tang et al., 2008). Biodiesel quality testing used the ASTM D6584 GC method for identifying free and total glycerin (Okullo et al., 2013). The analyses were conducted with a GC 3400cx gas chromatograph (Varian Star) equipped with a split/splitless injector and flame ionization detector (FID). A capillary fused silica column SPTM 2380 (30 m × 0.25 mm × 0.2 μm film thickness) with matrix active group stabilized poly (90% biscyanopropyl/10% cyanopropylphenyl siloxane) phase was operated under programmed temperature conditions: 140-240 ºC at 5 ºC/min in 30 min (detector and injector temperatures of 260 °C), injection volume and mode of 0.4 μL and split (100:1), and nitrogen gas as carrier gas (20 cm/min). The desired amount (ca. 20 mg) of samples was dissolved in 2 mL in hexane before injection (Carvalho et al., 2012). The fatty acid methyl ester (FAME) composition of each biodiesel used a PerkinElmer Clarus 500 GC–MS with a split automatic injector, and a Rtx-WAX (Restek, Bellefonte, PA) column (length: 60 m; ID: 0.25 mm, coating: 0.25 lm) for verification. The column was held at 120 °C for 1 min and then ramped to 240 °C at 20 °C/ min, and it was then held at 240 °C for 13 min. The transfer line be- tween GC and MS was kept at 240 °C. Helium (99.9999%, Cryogenic Gases, Detroit, MI) used as the carrier

gas with a flow rate of 1.5 mL/min. Total Ion Count (TIC) was used for the quantification of each component (Tang et al., 2008).

The limits of gas chromatography are the possibility for certain compounds to not evaporate. High performance liquid chromatography (HPLC) should be used due to its high boiling point. Several different investigators analyzed biodiesel using the HPLC as opposed to GC to examine if it is a viable alternative. When tested side by side Carvalho et al. determined that the two methods compare consistently (Carvalho et al., 2012). Solvents and/or heating can remove the gossypol from biodiesel (Kuk et al., 2005; A. Nomeir, 1982). The mobile phase was an important factor in the success of the chromatography. The selection of the mobile phase is dependent on the stability of the compounds within the oil. An important component in the cottonseed oil is gossypol and understanding its chemical stability in different solvents for HPLC is critical. Nomeir and Abou-Donia studied gossypol for its stability in various HPLC solvents. The compound is not very stable in methanol at temperatures of 37 °C making it a difficult choice for a solvent (A. A. Nomeir and Abou-Donia, 1985). The solvent best at maintaining the integrity of gossypol, was acetonitrile, which could be a potential mobile phase because it is miscible with triglycerides (A. Nomeir, 1982). Royon et al. used Novozym to transesterify cottonseed oil. The resulting biodiesel was created using an optimized solvent to prevent methanol denaturation thus keeping the enzyme active. The method that was used to evaluate the triglyceride conversion was an HPLC analysis with a C18 Phenomenex column. The mobile phase for this investigation was a mixture of methanol and hexane, varied in concentration across time (Royon et al., 2007). The method was used to evaluate the amount of methyl esters, monoglycerides, diglycerides, triglycerides, and free fatty acids.

Joshi et al. used methanol and ethanol ratios to see what the yields and ester makeup would be using the HPLC. As expected, the higher methanol to ethanol mixes performed better under nearly every circumstance (Joshi, Moser, Toler, and Walker, 2010). The conversion rate was confirmed by the HPLC to analyze the mixture of esters presented. The machine used a mixture of acetonitrile and dichloromethane (DCM), a chemical no longer used in current facilities (Joshi, Moser, Toler, and Walker, 2010). Among those is the work by Shang et al who demonstrated that fatty acid profiles may be characterized by HPLC. This study used pure acetonitrile for its mobile phase, but demonstrated that there are limitations to using the HPLC alone, because the overlapping of methyl oleate and the methyl palmitate chromatographic peaks. A mathematical relation developed from the calibration curves of the

individual compounds using both ultraviolet (UV) and refractive index (RID) detection methods to set a predictive relation between the two (Shang et al., 2012). Fedosov, Fernandes and Firdaus used HPLC methods to analyze the lipase catalyzed rapeseed oil biodiesel to evaluate the use of HPLC with evaporative light scattering detection (ELSD) for quantifying biodiesel yield (Fedosov et al., 2014). The elution of the compounds was tuned by the manipulation of two solvents, whose concentrations were controlled by a time program. Once the team was confident in the retention times of the biodiesel, they used the binary system to elute the components of the biodiesel mixture that was not directly soluble. The calibration curves indicated the esters did not decay and the biodiesel yield was able to be modeled well over the 24 hr reaction period (Fedosov et al., 2014). Based on these analyses, there would be evidence that either HPLC or GC would be useful in the quantification of biodiesel, but the speed of the GC makes it more desirable despite the potential of the gossypol components not evaporating.

Chapter Three

Experiment

Introduction

Biodiesel has become a strong contender in the world of alternative renewable fuels. The ability to use agricultural and certain waste products for production of biofuel has garnered some government subsidy opportunities (Marchetti, 2012; Wu et al., 2012). Optimistic production models show that the fuel will experience sufficient growth under contemporary assumptions of rising petrodiesel prices and competitive subsidies (Szulczyk and McCarl, 2010). A high potential exists for the fuel to make an impact in the global market with vegetable oils like rapeseed oil and soybean remaining strong candidates for improved production (Souza et al., 2017). Biodiesel is produced by esterifying triglycerides and creating three biodiesel molecules with the use of another compound, known as an acyl acceptor. The most dominant process used for biodiesel production comes from transesterification using methanol as the acyl acceptor (Szczęsna Antczak et al., 2009). A catalyst is required a catalyst for the reaction to occur in a hourly timeframe using methanol at subcritical conditions. The catalyst can be either an acid or base existing in either a in a homogenous or heterogeneous mixture depending whether an adsorption technique is used to ease separation. Heterogeneity typically comes at an increased cost for the catalyst, but allows for the simple reuse of the chemical catalyst through the use of physical separation techniques like filtering and decanting (Atadashi et al., 2013).

Alkaline catalysts (e.g., NaOH and KOH) are the most common because they function at a lower temperature, but are not appropriate for use in oils with high amount of free fatty acids (FFAs) due to excessive production of soaps and need of high alkali loading (Christopher et al., 2014). Typically, an acid catalyst (e.g., H_2SO_4) is used first to directly esterify the high acid oils portion, at slower speeds albeit, to make the oil acceptable for use with an alkaline catalyst (Lotero et al., 2005). The weakness of alkaline catalysts comes from the presence of free fatty acids where the catalyst reacts with the free fatty acid producing soap and water. Both products contaminate the engine making the fuel unusable. To combat this, only oils low in free fatty acids can be used for transesterification (Atadashi et al., 2013).

A consistently successful method to avoid soap production is through the use of enzymatic instead of chemical catalysts (Christopher et al., 2014; Robles-Medina et al., 2009; Szczęsna Antczak et al., 2009). The enzyme class used in the biodiesel production are called lipases and are produced in a variety of ways from either fungal or bacterial sources. The enzymes replace the chemical catalysts and use their active sites to breakdown triglyceride structures. Enzymes have demonstrated good performance using a variety of oils and even a variety of processing techniques (Dos Santos Corrêa et al., 2011; Nie et al., 2006; Price et al., 2014; Raita et al., 2011; Remonatto et al., 2016; Royon et al., 2007). Several successful enzyme products include Novozym 435 (immobilized *Candida antarctica*), Candida sp. 99-125, and Callera Trans L. While it is well known that excessive methanol causes inhibition of lipase activity, an optimum value for methanol that has not been identified in the literature. Some literature dealing with liquid lipases did not use the species currently used at the industrial scale. While more literature exists for immobilized biocatalysts, the preferred amount of methanol is still unclear as several research groups have found conversion rates above 90% at different methanol molar concentrations (Du et al., 2004; Nie et al., 2006; Royon et al., 2007).

Concerns over methanol inhibition spurred study into using feeding apparatuses to control the methanol consumption. One such study examined using a feeding structure to limit this inhibition effect and could get conversion ratios of 90.8 % wt. (Price et al., 2014). Methanol inhibition occurs at different levels depending on the reaction configuration. Price et al. used a controlled feeding apparatus to keep the molar ratio low throughout the entire process, but supplied the reaction with enough acyl acceptor to continue moving the reaction forward. Several other studies were conducted to determine the amount of methanol that inhibits the reaction (Musa, 2016; Tan et al., 2010; Verma et al., 2016) and reported that an the optimal molar ratio for methanol:oil was 6:1. Rashid and Anwar used ratios higher than 6:1 and noticed diminished returns at ratios of 9:1 and above (Rashid and Anwar, 2008). Methods to navigate around methanol inhibition have included the use of solvents. A pilot system was established to avoid inhibition from methanol using *Candida* sp. 99-125. With three additions of methanol, the biodiesel conversion was 92% (Nie et al., 2006). In addition, to the petroleum ether solvent, a hydrocyclone was used to separate the glycerol after every step wise addition. This was important to reduce potential inhibition due to mass transfer limitations on the immobilized enzyme.

Another potential source of inhibition includes impurities within the oils. FFA is not considered a problem for enzyme catalysis, because they do not produce soap. Feedstock mixtures of more than FFA 50 % wt. were shown to cause inhibition in one study using Eversa Transform® (Liu et al., 2010). In addition, natural compounds such as antioxidants present in the feedstock oils could be potential sources of inhibition as well, but no study was found that considered this. The effect of an additive was analyzed by conducting a transesterification reaction using crude cottonseed oil as the feedstock. Remonatto et al. observed a 97 % conversion rate for the oil, but also observed inhibition with higher amounts of waste oil where at 25 % FFA content showed a reduction in conversion of more than 20 % (Remonatto et al., 2016). Immobilized enzymes with acid-catalyzed configuration produce biodiesel at a conversion rate of more than 93 % for tallow oils. If this is the case, the assumption that acids universally inhibit lipases would be unsupported (Bhatti et al., 2008).

Water aids lipases in reaction and overall conversion by enlarging the interfacial surface between the lipase and the oil (Price et al., 2014). This means that water spreads the enzyme out making interaction more accessible to triglycerides in the mixture. Other experiments varied water content from 0.5 to 15 % wt., using both liquid and immobilized enzymes (Cesarini et al., 2013; Nordblad et al., 2014; Price et al., 2014) and demonstrated while the effect was infrequently tested for water content was consistently monitored. When a Callera Tranform enzyme was used, the biodiesel yields reached around 96.3 % and 95.6 % wt. with the water concentration of 3 and 5 % wt. ., respectively (Cesarini et al., 2013). For immobilized lipases, the amount of water is noted to have an influence on both the catalytic activity and stability of the lipases (Tan et al., 2010).

Cottonseed oil, a relatively minor vegetable oil is of great interest due to its natural antioxidant composition, mainly from the compound gossypol observed to enhance the life of biodiesel from which it was derived (Moser, 2012). A major concern of producers is enhancing the life of biodiesel to increase its selling capacity. Many researchers add antioxidants in the post production phase, but with gossypol being naturally present it may become a strong contender in the market. Since the crop cannot be used for food because gossypol is toxic, cottonseed oil containing gossypol does not directly compete with food crops (De Sousa et al., 2014; Dunn, 2005; Wang et al., 2009). Glandless cottonseed oil has a visual appearance similar to other vegetable oils not containing gossypol and may be used in food. This oil can be compared to measure the effect of gossypol for conversion in terms of possible inhibition.

Eversa Transform® is one of the most recent liquid lipases that Novozymes Inc. has released. Although several optimal parameters are provided in its recommended user handbook, this enzyme has not been studied in the biodiesel production from cottonseed oil to our knowledge. The purpose of this investigation is to determine how influential the parameters of methanol:oil ratio, water content and enzyme content are to the conversion of biodiesel from cottonseed oil. This examination will also provide data that determines how effective Eversa Transform® at converting fringe feedstocks as opposed to the recommended waste cooking and pure soybean oils. This research will examine the quality of Eversa as an enzymatic catalyst and determine what factors affect the ability of the enzyme to convert triglycerides into esters.

Materials and Methods

Materials

Methanol was purchased from Fisher Scientific. Glandless cottonseed oil and heavy pigment (crude) cottonseed oil were obtained from Cotton Inc. (Cary, North Carolina, United States) affiliate suppliers. The initial acid values for the crude pigment and the glandless oils were 0.32 mg KOH/g and 0.05 mg KOH/g, respectively. The water content of the oils was both below 0.1%. A lipase, Eversa Tranform® was kindly supplied by Novozymes North America, Inc. (Franklinton, North Carolina, United States) for catalyzing biodiesel production from cottonseed oils.

Experimental Design

Table 1 below presents the samples that were used in the experimental level of the design. The glandless cottonseed oil was treated with a full 2x3x3 factorial design varying water dosage, enzyme dosage and temperature respectively. The crude or heavy pigment cottonseed oil was treated with a 2x3 full factorial design varying only water and enzyme content. Both oils of 100 mL each were placed into 250-mL Erlenmeyer flasks with a prescribed amount of enzyme, water and methanol:oil ratio (see Table 1). The reaction occurred in a horizontal shaker at 250 rpm for 24 hours at 25, 35, or 45 °C. After the reaction time was complete, the flasks were removed from the shaker and the fuel was washed with distilled water, before waiting another 24 hours for the fuel to separate. The washing removed the enzyme, excess glycerol and thus halted the reaction. Once washed, the samples were transferred to 50-mL tubes for the analysis of fatty acid methyl esters (FAME) at the United States Department of Agriculture (USDA) National Center for Agricultural Utilization Research (Peoria, Illinois, United States).

The conversion of glycerin was calculated using the ASTM D6584 method (ASTM International, West Conshohocken, PA). The sample was analyzed by a gas chromatography, after silyating with N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA). Calibration was achieved using two internal standards and four reference materials. The column operation was conducted using helium as a carrier gas with a flow rate of 3 mL/min. Column temperature was as follows: 50 °C for 1 min, followed by increases of 15 °C /min to 180 °C, 7 °C /min to 230 °C, and 10 °C /min to 380 °C, kept for 8 min.

The differences in the two oils was evaluated by using3x3 full factorial blocks, modifying water and enzyme dosage. The two different types of cottonseed oil were compared using the mean mass conversion to determine the difference in the enzyme performance based on the oil differences. This principle in practice may apply to used vegetable oils or high FFA cottonseed oil to see if high amounts of FFA is limited with Eversa as well. The induction time is a measure of oxidative stability using the Rancimat method and is useful for determining the effect of antioxidants present in the oil. Other additives featured included synthetic additives placed in biodiesel to determine oxidative stability (Dunn, 2005). The synthetic antioxidants recorded high and effective improvements to the induction time even at smaller quantities of 100 ppm (J. Zhou et al., 2017). The induction time was the mean of two or three measurements depending on the samples tested.

Data Analysis

Data was analyzed and graphed using JMP Pro 12 Statistical Software. All statistical tests were conducted at a significance level of $\alpha=0.05$. All variance was assumed to be the same for all samples and the blocking effect for all samples was masked within temperature. No variance was assumed for the differences in water quality or lipase units between samples.

Results and Discussion

Dry Run Studies

Since the agreement on the methanol molar ratio was not consistent among existing publications, experiments were needed to determine the proper ratio for our case. A dry run, samples that were not heavily analyzed, was initially conducted to confirm the need for evaluating methanol to oil molar ratio as a reaction parameter for further study. Three levels of methanol molar ratios were used 6:1, 9:1 and 12:1. Based on the prevalence of a glycerol layer, all samples reacted, but there was a visible difference between them. There was a distinct visual decrease in the amount

of glycerol present indicating the reaction was at a lower conversion (Figure 1). This observation was also seen in literature and further supported, even in this case that the 6:1 methanol:oil molar ratio was a sufficient molar ratio to use (Musa, 2016; Rashid and Anwar, 2008). This result supported that the 6:1 molar ratio was a reasonable value that less methanol would be needed for study.

To compare the capability of keeping antioxidant gossypol in the biodiesel product between alkaline and enzyme catalysts, KOH and lipase were used respectively for transesterification of both glandless and crude cottonseed oils. With KOH being a catalyst, the crude cottonseed oil, while initially dark, ended up not maintaining its dark color in the biodiesel layer once the glycerol layer separated. However, this observation was not seen in the enzymatic biodiesel samples with crude cottonseed oil. The biodiesel layer was somehow able to maintain a large amount of dark pigment most likely associated with gossypol (depicted to the right in Figure 4). Perhaps this was the result of an extended reaction time or the presence of water in the system, but may give major advantage to a biodiesel producer due to the presence and effectiveness of the antioxidant gossypol (A. Nomeir, 1982; Wang et al., 2009).

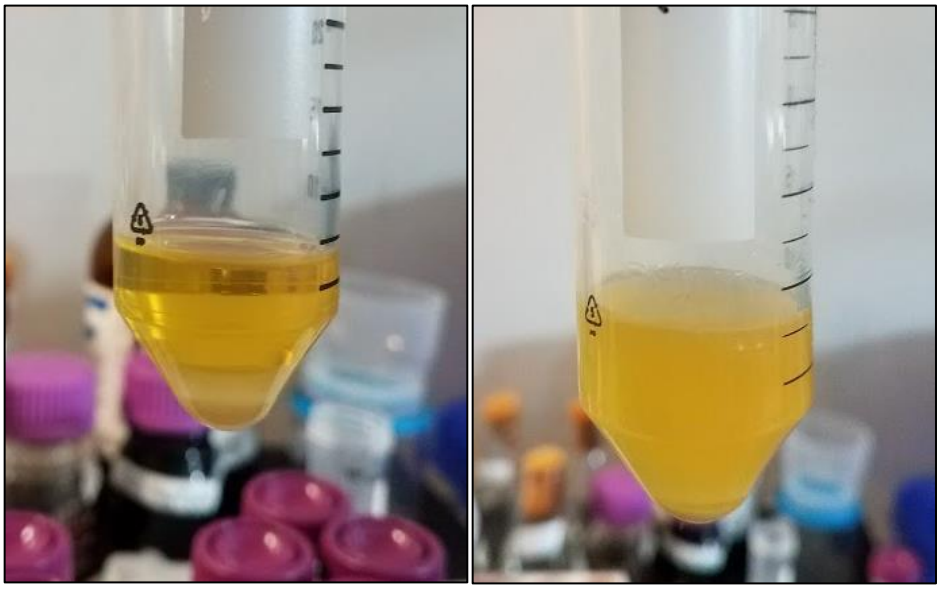


Figure 4: To the left is an image of the glandless oil reacting with a 6:1 methanol molar ratio, with 3 % wt. water content and 5 % wt. enzyme content. The right image is one with a 12:1 methanol molar ratio.

Figure 5. The crude cottonseed oil biodiesel color comparison for the alkaline catalyzed biodiesel and the enzymatically catalyzed biodiesel.

Conversion was calculated using the ASTM D6584 method for the calculation of free and total glycerin. The method is not specifically suited for the calculation of the conversion however, based on the accuracy in which it quantifies the non-ester components of the mixture a legitimate estimate of conversion could be calculated. Error would arise only due to trace amounts of FFAs that were removed with washing and natural compounds in the fuel that are not water soluble (ex. Gossypol). The conversion percentage was calculated on a mass basis in the following manner:

$$X = \frac{\Sigma\, ME}{\Sigma\,(ME+TG+DG+MG+G)} = 1 - \Sigma\,(ME + TG + DG + MG + G) \quad (1)$$

where X=Conversion (% wt.), ME=Methyl esters (% wt.) TG=Triglycerides (% wt.) DG=Diglycerides (% wt.), MG=Monoglycerides (% wt.) and G=Glycerol (% wt.).

Effect of lipase content on conversion

The ANOVA result presented in Table 2 measures the effect of the enzyme loading on the yield of the biodiesel. The enzyme loading is insignificant for the overall biodiesel yield. This was not a consistent within other studies. There was an optimal loading for Bharathiraja et al (Bharathiraja et al., 2014), using a whole cell catalyst of

Rhizopus oryzae as well as Macerias et al (Maceiras et al., 2009) using Novozym 435 discovered an optimal enzyme loading at 10 % wt.. Interestingly, the study done by Macerias et al. used a methanol molar ratio as high as 25:1 (Maceiras et al., 2009). The differences in the conversion could be sources from resiliency to inhibition due to the immobilization process, based on other research. Liquid lipase, Callera Transform with similar systems had conversion of more than 90 % wt.. The mean yield observed for all samples was 98 % wt., which was consistent with what other researchers observed (Maceiras et al., 2009).

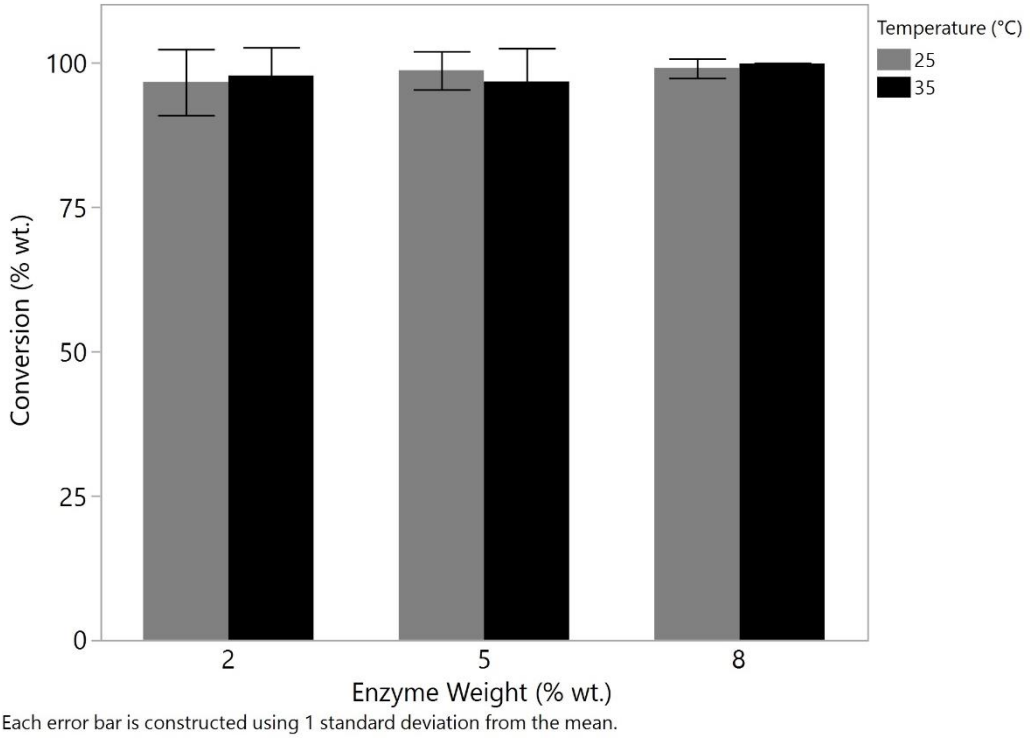


Figure 6: A graph of the conversion of the biodiesel based on enzyme weight, split by temperature. Across enzyme weights, temperature effects are not small, but have larger variances.

Observing a difference in the standard deviations between the enzyme weights across temperature, testing if the variances were different was desirable. While there was not a significant difference based on the ANOVA test in Table 2, Table 3 offered some possibility to the variances being different. The Bartlett and Levene tests both suggest that the variances are not equal, but the distribution of the conversion variable is not verified to be distributed normally. This makes the Levene test more useful in this case. When more extreme screening screen of the mean is applied in calculation, the Levene test becomes the Brown-Forsythe test. The evidence of the different variances is no longer significant which may indicate the larger variances was from an outlier effect. Using a Welch's test to determine if a

difference in the means exists when variances were allowed to differ, the level of p-value was lower and still insignificant.

Table 2: ANOVA analyzing the effect of enzyme wt. on the conversion of the biodiesel

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Ratio*	*Prob > F*
Enzyme Weight (% wt.)	2	60.1850	30.0925	1.8263	0.1700
Error	59	972.1775	16.4776		
C. Total	61	1032.3625			

Table 3: Tests for differences in variances from the effect of water.

Test	*F Ratio*	*DF Num*	*DF Den*	*Prob > F*
O'Brien [.5]	2.1454	2	59	0.1261
Brown-Forsythe	1.8116	2	59	0.1724
Levene	7.5733	2	59	0.0012
Bartlett	17.7829	2	.	<.0001

Table 4: The Welch's Test for the effect of enzyme weight was nearly significant when considering that the variances may not be equal.

F Ratio	*DF Num*	*DF Den*	*Prob > F*
2.9612	2	27.393	0.0685

Effect of water content on conversion

Water content had the strongest effect of any variable. This was consistent with the evidence of interfacial surface area increasing the reaction area. It is important to note that the glandless cottonseed oil did not react at 1 % wt. water content. This would naturally suggest that the amount of water needs to be higher for a more complete reaction. For several studies using immobilized lipases, the biodiesel conversion rate was above 90%, but not all of them had the same amounts of water present. For instance, Cesarini et al. used 3 to 5 % wt. water and Price et al. used 5 – 7 % wt. water, with conversions of 96 % and 90.8 %, respectively (Cesarini et al., 2013; Price et al., 2014). An important note is that the glandless cottonseed oil samples reacted with 1 % wt. water did not react. This would indicate a difference in consistency in the enzyme performance with different quantities of water used. The difference in the

variance was confirmed with the O'Brien, Bartlett, Brown-Forsythe and Levene ($p < 0.0001$). A Welch's test supported the hypothesis that there was a difference in the means ($p < .0022$) considering the different variances.

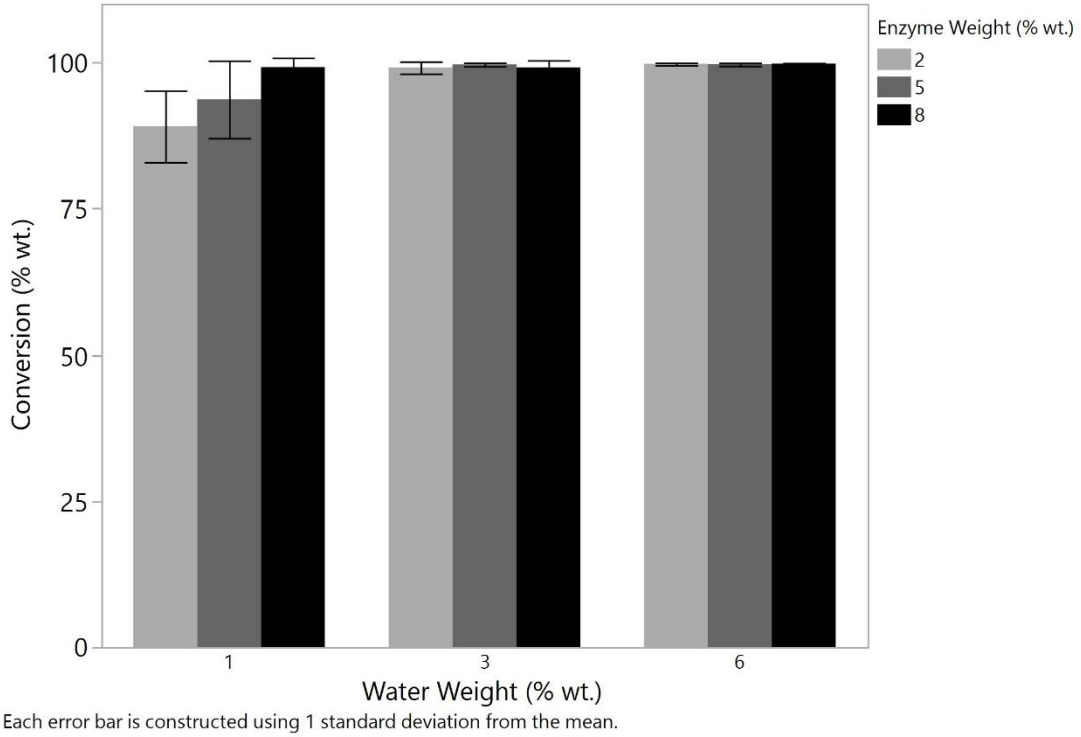


Figure 7: Biodiesel conversion with varied water weight categorized by enzyme weight

Effect of oil and temperature on conversion

Antioxidants have not been studied as potential inhibitors for lipases in biodiesel production. All oils have other compounds in them in trace amounts and screening for the additives is a challenge especially at that low level of concentration. For synthetic antioxidants, they are often added in post reaction steps, so there is little opportunity for inhibition to occur. Using both crude and glandless cottonseed oils allows the conversion rates to be compared. The crude cottonseed oil was only reacted at 35°C and based on the results of the ANOVA ($p < 0.0706$) there is no reason to believe that additional compounds mixed in the oil had any impact on the conversion of the biodiesel (Table 3). The temperatures tested were 25 and 35 °C and there was no significant response from the conversion ($p < 0.9174$).

Table 3: ANOVA observing the effect of oil species on conversion

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Ratio*
Model	1	55.1714	55.1714	3.3875
Error	60	977.1911	16.2865	Prob > F
Total	61	1032.3625		0.0706

Effect of parameter interactions on conversion

Effect of enzyme loading, water content and temperature are considered possible sources of differences within the groups. Table 4 shows temperature as the only significant parameter among the three parameters tested while the interaction between temperature and water content is the only significant interaction. The effects of water and enzyme weight were not significant parameters alone and seem to only have significant interactions with temperature. The chemistry of the reaction depends on temperature and this reflects that the enzyme is listed as becoming unstable at higher temperatures and the result of lower reaction temperatures producing less efficient chemical reactions (Novozymes Bioenergy, 2015). Water and temperature was not expected to have a strong interaction, due to the small temperature range that was used. Additionally, there was no evidence that higher water amount is indicative of a higher yield. Higher temperature water may enter the vapor state more readily, possibly preventing sufficient interfacial area (Colucci et al., 2005; Price et al., 2014).

Table 4: The full factorial model ANOVA for conversion without and with screening. Both models had a significant interaction with the screen model having a larger F-ratio, indicated the model fits better.

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Ratio*
Model	17	682.5695	40.1511	5.0506
Error	44	349.7930	7.9498	Prob > F
C. Total	61	1032.3625		<0.0001*

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Ratio*
Model	8	578.2983	72.2873	8.4376
Error	53	454.0642	8.5672	Prob > F
C. Total	61	1032.3625		<0.0001*

Due to the increase in interfacial area caused by water present in the system, a positive interaction was expected from an increase in water and the total amount of enzyme. Water and enzyme interaction was the third most significant effect in the model. Other interactions were not significant as the inclusion of temperature was insignificant.

The final statistical model was depicted in equation (2). For the two temperatures, the difference in the conversion is reduced and there appears to be stability in conversion for both 25 and 35 °C. Eversa is rated to perform best at 35 °C by Novozymes® in the Eversa Transform® Handbook (Novozymes Bioenergy, 2015) and there was not strong temperature difference for the majority of the data. There was a slight drop in the conversion for the 1 % wt. water content, but this effect was not significant (Figure 6). In Figure 7, the stability of the 8 % wt. enzyme is clearly displayed. This contributed to the lack of effect that enzyme had on conversion and shows that the increased presence of water almost eliminates the differences that occur because of enzyme dosages. Based on the findings and interaction studies we can conclude that if Eversa is placed in an environment where there is at least 6 % wt. water, it will convert cottonseed oil at a high rate. Other measures do not have as much of an effect in this regard.

Table 5: Effect summary of the full factorial model for conversion, with and without screening. The first model had an $r^2 = 0.661$ while the second had an $r^2 = 0.560$.

Source	*LogWorth*	*PValue*
Water Weight (% wt.)	4.785	0.00002
Enzyme Weight (% wt.)	4.021	0.00010
*Enzyme Weight (% wt.)*Water Weight (% wt.)*	2.360	0.00437
*Enzyme Weight (% wt.)*Temperature (°C)*	2.161	0.00691
*Enzyme Weight (% wt.)*Water Weight (% wt.)*Temperature (°C)*	0.923	0.11933
Temperature (°C)	0.671	0.21350
*Water Weight (% wt.)*Temperature (°C)*	0.331	0.46614

Source	*LogWorth*	*PValue*
Water Weight (% wt.)	6.361	0.00000
Enzyme Weight (% wt.)	5.745	0.00000
*Enzyme Weight (% wt.)*Water Weight (% wt.)*	3.360	0.00044

$$Y = \mu + E + W + EW + \varepsilon \quad (2)$$

where, Y = Output Variable, E = Enzyme Effect, W = Water Effect, μ = true mean, ε = error

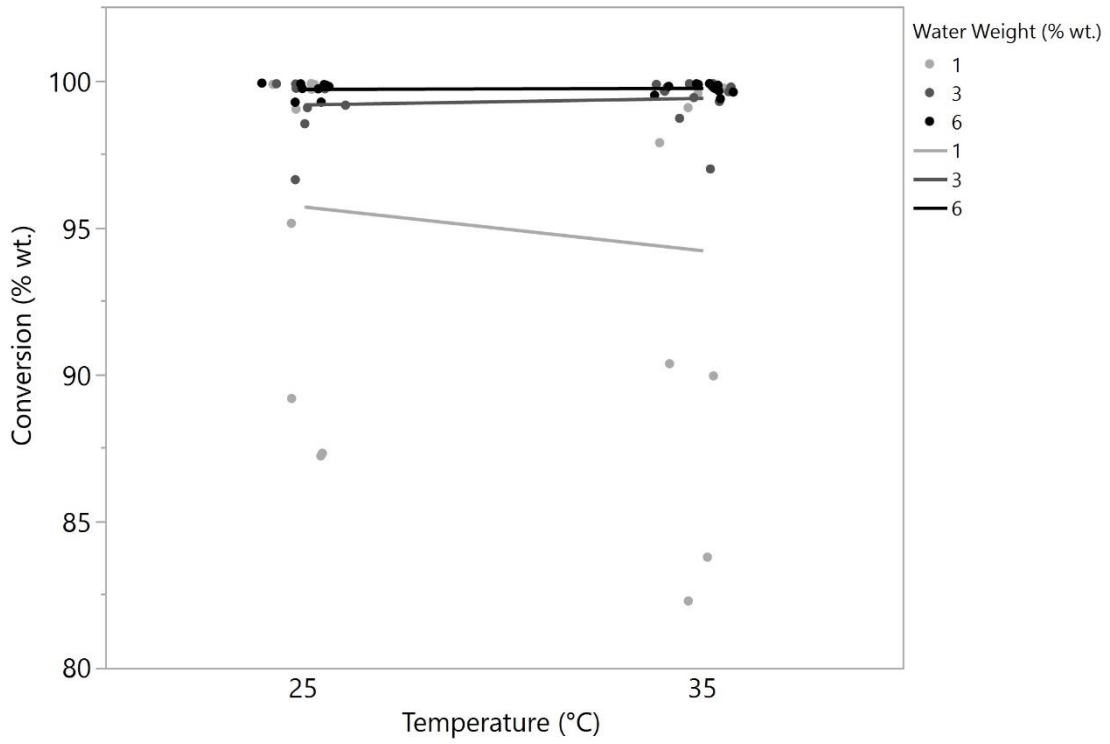


Figure 8: Conversion with special attention special attention to the interaction across enzyme and water.

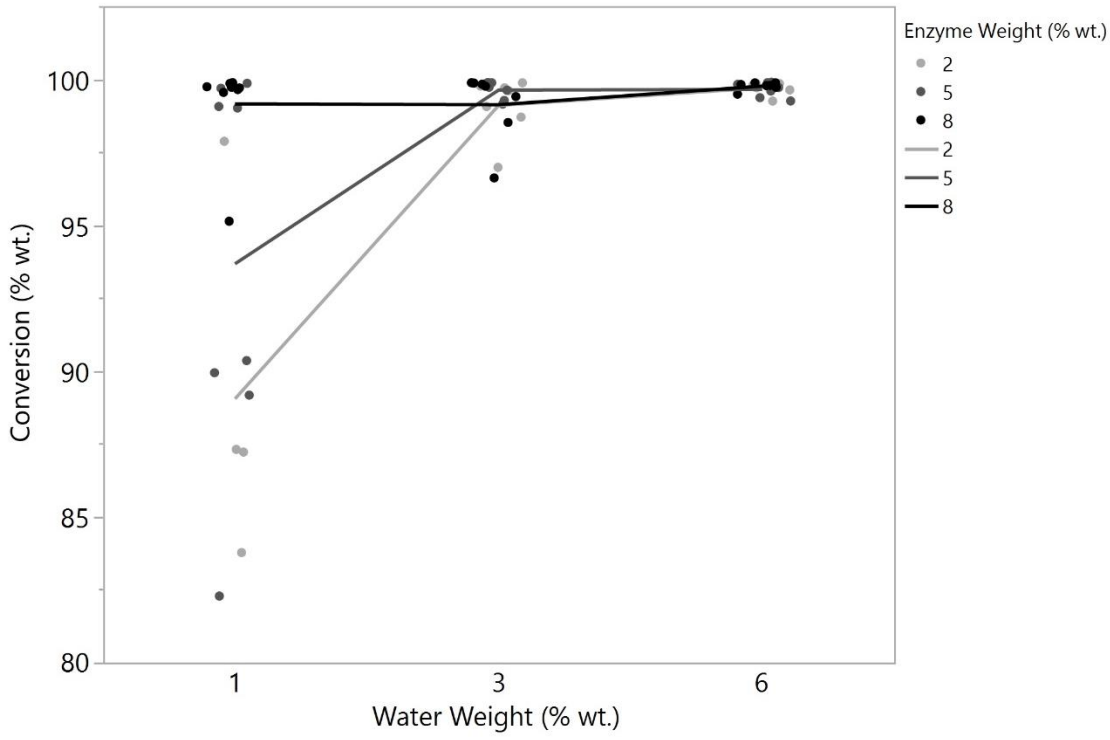


Figure 9: Conversion with special attention special attention to the interaction across enzyme and water. Dots represent each sample taken while lines connect the means across different water amounts.

Oxidative Stability

Gossypol, as expected, had a significant effect ($p < 0.0001,\ F = 581.60$) on the induction time based on the comparison between the two oils. While there was a clear distinction between the two oil sources, there was also significant ($p < 0.0250$) effect of water weight on induction time for crude (gossypol containing) cottonseed oil (Figure 8). The enzyme effect on the induction time was at a near significant level of $p < 0.0581$. There are no explanations as to why this could have occurred to our knowledge. For the glandless oil, there were not any significant effects on the induction time. The main difference between the oils is the concentration of gossypol and other pigments. A possible side reaction facilitated by the enzyme could have occurred that we did not anticipate or possibly a function of time-based interactions with the enzyme and the entire biodiesel mixture together. Further studies to test time effects and reaction rates are recommended.

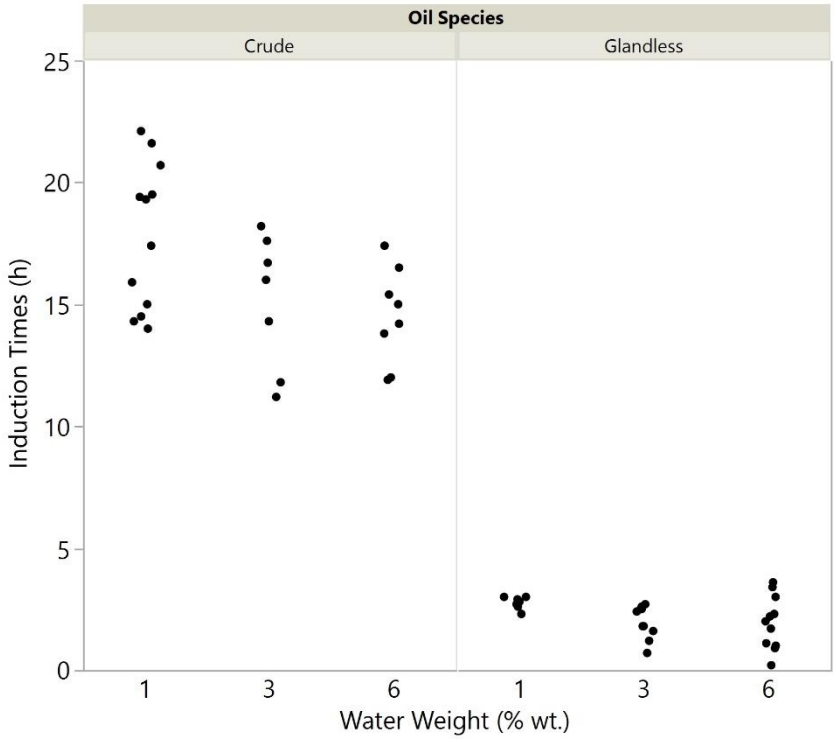


Figure 10: The effect of water weight on the induction time of biodiesel, with the crude oil to the left and the glandless cottonseed oil to the right.

The induction time for the glandless cottonseed oil biodiesel was very low. ASTM D6584 uses the EN 15751 method and has a limit of oxidative stability index, (OSI) of OSI=3.0. Based on the results for glandless oil, the mean

induction time was about half of the OSI limit measured at 1.44, which does not meet the ASTM Standard. This was not the case for the biodiesel derived from crude cottonseed oil where the mean OSI was 17.0, far exceeding the standard. This value compares well against other OSI values calculated with various other antioxidants, that also exceed the standard at concentrations of 250 ppm (Table 6). Moser reported gossypol outperformed the tocopherol and BHT additives in higher grade oils, but was inferior to both additives across longer shelf periods and in lower concentration in waste cooking oil methyl esters (Moser, 2012).

Table 6: Rancimat induction times for this study and several others

Additive	*Induction Time (h)*	*Concentration (ppm)*	*Source*
Gossypol	17.0	n.c.	This study
Tert-butylhydroquinone	22.30	250	(J. Zhou et al., 2017)
Butylated hydroxyanisole	7.24	250	(J. Zhou et al., 2017)
Butylated hydroxytoluene	6.27	250	(J. Zhou et al., 2017)
Pyrogallol	20.07	250	(J. Zhou et al., 2017)
Propyl gallate	15.10	250	(J. Zhou et al., 2017)
Butylated hydroxyanisole	6.7	250	(Moser, 2012)
γ-Tocopherol	5.9	250	(Moser, 2012)
Gossypol	7.2	250	(Moser, 2012)

n.c. – not calculated

Scale-up Study

A scale-up model was tested using SuperPro Desginer® (Intelligen Inc, Scotch Plains, New Jersey, United States). The model used a batch vessel and was scaled up to 1 kmol of soybean oil and 6 kmol of methanol to produce a reaction. The reaction occurred over a 24-hour period and was heated to 35 °C to reflect the conditions of the original reaction. The conversion of the reaction was assumed to be 98 % and the reaction mixture was later decanted before being washed. Water was assumed to remove 90 % of all non-ester and triglycerides with the remainder represented in the output stream. The process diagram in Figure 10 begins with two feed lines (S-101 and S-102) go into the vented reactor where the transesterification process is calculated in one step. Water and Eversa are added to the feedline S-101 and methanol is fed through S-102. Once that step is conducted, product moves from S-103 to the decanting process and splits into streams S-104, waste, and S-105 product stream. Washing occurs with water stemming from an S-107 line and the final product is received in the S-108 line.

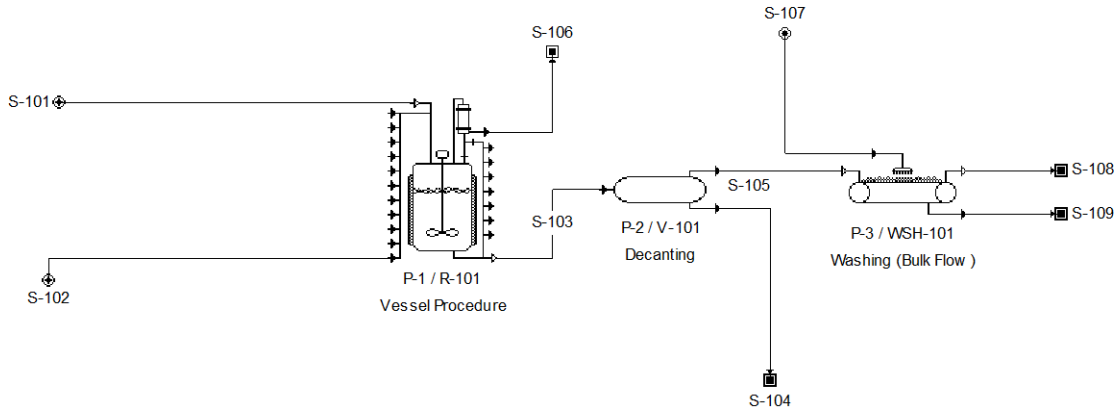


Figure 11: The schematic of the biodiesel production system. Features two inlet feeds for the oil and enzyme while methanol and water come through another inlet to control the reaction speed and balance soluble components with each other.

From the scale-up study, the quantities observed in the output stream was similar to what was observed in the small scale (Table 7). The simulation converted 98 % wt. of Water solubility in biodiesel was confirmed to be insignificant. Fatty acids and enzymes are assumed to be more soluble in water than oil and removed into the washing stream. Soybean oil itself was the only compound in the database that was closest to the cottonseed oil that was in use. The simulation that was examined assumed a 2% enzyme loading. This is consistent with the observations we seen in the small scale indicating that the estimates for water removal and decanting were reasonable. The means for the calculations do not reflected a centrifuge stage.

Table 7: The stoichiometric analysis of the biodiesel conversion final results out of the output stream (S-108)

COMPOUND	KG/BATCH	WT%
BIODIESEL	854.92	97.89
GLYCEROL	0.18	0.02
METHANOL	1.80	0.21
SOYBEAN OIL	16.50	1.89

$$K_{eq} = \Pi\, C_i^{\alpha_i} \tag{3}$$

C_i = concentration ratio, α_i = rate exponent and K_{eq} = equilibrium constant

A second analysis was conducted to examine how the chemical equilibrium would show the optimal point of the reaction. The rate equation used is listed above (3). The function equates the equilibrium constant for the reaction with the product of the concentrations of reactants with the power of the stoichiometric component. This analysis was more powerful than the stoichiometric analysis because it did not rely on the conversion seen from experimentation.

The equilibrium analysis of the simulation showed that there was an even greater conversion that was observed for the stoichiometric case for lower equilibrium constants. The chemical model used was the equilibrium model for reaction rate. The equilibrium constant was adjusted to different values in Table 8 and the biodiesel fraction was recorded. The model predicted 99 % wt. for lower equilibrium values under K_{eq}=1.6. Compared to the results of the small-scale study, equilibrium conversion was within two percent. Although this is a scaled-up model, it indicates that the small-scale reaction reaches near equilibrium under the same reactions conditions.

Table 8: Conversion of the biodiesel in the equilibrium reactor configuration

EQUILIBRIUM CONSTANT	CONVERSION (% WT.)
0.05	99.75
0.10	99.71
0.20	99.65
0.40	99.53
0.80	99.27
1.60	24.01
3.20	29.64
6.40	37.20
12.80	48.43

Conclusion

Eversa Transform® was demonstrated to catalyze biodiesel production from crude cottonseed oil in high conversion. Using a methanol to oil molar ratio of 6:1, a mean conversion of 98% across all samples was obtained and was remarkably close to previous publications utilizing other catalysts. Water content was the most dominant factor of the reaction parameters investigated regarding the ability of the lipase to produce biodiesel from cottonseed oil. Water content also had interaction effect with the enzyme amount, leaving temperature as an insignificant variable for conversion. There was not a distinguishable difference between the two temperatures indicating Eversa is effective in suboptimal conditions. All other factors did not have enough significance to recommend one amount over the other. In the future, research should focus on using a wider variety of oils to see if any changes in the performance of Eversa across oils. Detailing how the reaction kinetics are influenced by these parameters will also yield valuable insight in the production of biodiesel from Eversa. Eversa converted the oil with no discernable difference between the glandless and crude cottonseed oil. Crude cottonseed oil having no adverse effects with the performance of Eversa further proved this as a robust catalytic solution.

Chapter Four

Future Research

The results of the study revealed that the suspected parameters of temperature, water and enzyme weight, had varied degrees of influence. Only water weight and enzyme weight (both $p < 0.0001$) proved to be the most influential parameters. Due to the small sample size of this investigation a second study that includes the use of more samples to create a stronger effect size for all conclusions drawn. Further study on the crude biodiesel is necessary samples is needed since only the transesterified glandless oil was fully explored. The ideal temperature for Eversa to operate was confirmed and there did not appear to be any evidence to counter Novozymes recommendation as a conservative rating.

The analysis of the acyl ratio would also be a critical point of interest because although the dry run confirmed that there was no reason to deviate from the 6:1 ratio, while there is not a definitive literature value. Based on the investigation presented here, using a different acyl ratio may prove to be advantageous, but only in a novel sense. A mean conversion of 98 % reduces the need for any additional optimization studies without first examining if temperature and water weight can be even more influential if reaction kinetics are considered. Most contemporary research did not use liquid enzyme and due to that insufficiency in the data this gives reason to explore them further. Since the trials were mostly successful overall, the most likely question concerning conversion is the speed thus requiring kinetic studies for further research. The acyl ratio, the enzyme weight and even the oils can be factors in the rate that the conversion needs occurs.

Overall, a lack of specific information on the enzyme makes it difficult to draw conclusions on the performance in terms of reaction speed, enzyme activity and its ability to be used. With limited resources, it is inefficient and perhaps insufficient to rely solely on the compiled research without having a strong computer model to determine what the best concentration of various substances, thermochemical properties and reaction parameters for a reaction. Determining the best parameter beforehand will lead to much better performance of the enzyme and the conversion while allowing the focus to be only on the effectiveness of the catalyst and not the efficacy of the oil.

Appendices

Appendix A: Table of biodiesel conversion

Sample ID	*Enzyme Weight (% wt.)*	*Water Weight (% wt.)*	*Temperature (°C)*	*Oil Type*	*Conversion (% wt.)*	*Induction Time (h)*
1	2	3	25	Glandless	99.06	0.7
2	5	3	25	Glandless	99.14	1.2
3	8	3	25	Glandless	96.61	*
4	2	6	25	Glandless	99.25	0.9
5	5	6	25	Glandless	99.25	1.0
6	8	6	25	Glandless	99.81	1.1
7	2	3	35	Glandless	98.70	1.8
8	5	3	35	Glandless	99.62	1.8
9	8	3	35	Glandless	99.76	1.6
10	2	6	35	Glandless	99.63	1.7
11	5	6	35	Glandless	99.59	*
12	8	6	35	Glandless	99.72	2.0
13	2	3	35	Crude	96.97	18.2
14	5	3	35	Crude	99.87	16.7
15	8	3	35	Crude	99.40	16.0
16	2	6	35	Crude	99.85	17.4
17	5	6	35	Crude	99.36	*
18	8	6	35	Crude	99.48	16.5
19	2	1	35	Crude	97.87	17.4
20	5	1	35	Crude	82.28	19.4
21	8	1	35	Crude	99.64	14.0
22	2	1	35	Crude	83.77	21.6
23	5	1	35	Crude	99.06	14.3
24	8	1	35	Crude	99.74	15.0
25	2	1	25	Crude	87.31	20.7
26	5	1	25	Crude	89.17	19.5
27	8	1	25	Crude	95.13	19.3
28	2	1	25	Crude	87.21	22.1
29	5	1	25	Crude	99.01	14.5
30	8	1	25	Crude	99.70	15.9
31	2	3	35	Crude	99.60	*
32	5	3	35	Crude	99.27	*
33	8	3	35	Crude	99.82	11.2
34	2	3	25	Crude	99.70	17.6
35	5	3	25	Crude	99.72	11.8
36	8	3	25	Crude	99.86	14.3

37	2	6	35	Crude	99.80	15.4
38	5	6	35	Crude	99.82	13.8
39	8	6	35	Crude	99.78	15.0
40	2	6	25	Crude	99.70	12.0
41	5	6	25	Crude	99.71	11.9
42	8	6	25	Crude	99.78	14.2
43	2	1	35	Glandless	**	**
44	5	1	35	Glandless	89.93	2.3
45	8	1	35	Glandless	99.72	3.0
46	2	1	35	Glandless	**	**
47	5	1	35	Glandless	90.35	2.6
48	8	1	35	Glandless	99.54	2.7
49	2	1	25	Glandless	**	**
50	5	1	25	Glandless	99.69	2.7
51	8	1	25	Glandless	99.85	2.8
52	2	1	25	Glandless	**	**
53	5	1	25	Glandless	99.86	2.9
54	8	1	25	Glandless	99.88	3.0
55	2	3	35	Glandless	99.76	2.5
56	5	3	35	Glandless	99.85	*
57	8	3	35	Glandless	99.88	2.6
58	2	3	25	Glandless	99.87	2.7
59	5	3	25	Glandless	99.88	2.4
60	8	3	25	Glandless	98.51	2.5
61	2	6	35	Glandless	99.82	2.2
62	5	6	35	Glandless	99.88	3.0
63	8	6	35	Glandless	99.87	3.4
64	2	6	25	Glandless	99.85	3.6
65	5	6	25	Glandless	99.90	0.2
66	8	6	25	Glandless	99.87	2.3

*- Sample was lost in testing

**-Reaction did not occur, samples were not tested

Appendix B: Statistical Model Tables in JMP

Response Conversion (% wt.)

Effect Summary

Source	LogWorth	PValue
Water Weight (% wt.)	4.785	0.00002
Enzyme Weight (% wt.)	4.021	0.00010
Enzyme Weight (% wt.)*Water Weight (% wt.)	2.360	0.00437
Enzyme Weight (% wt.)*Temperature (°C)	2.161	0.00691
Enzyme Weight (% wt.)*Water Weight (% wt.)*Temperature (°C)	0.923	0.11933
Temperature (°C)	0.671	0.21350 ^
Water Weight (% wt.)*Temperature (°C)	0.331	0.46614 ^

Summary of Fit

RSquare	0.661172
RSquare Adj	0.530262
Root Mean Square Error	2.819546
Mean of Response	98.02066
Observations (or Sum Wgts)	62

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	17	682.5695	40.1511	5.0506
Error	44	349.7930	7.9498	Prob > F
C. Total	61	1032.3625		<.0001*

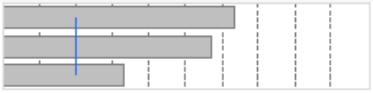
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	87.26	1.99372	43.77	<.0001*
Enzyme Weight (% wt.)[5-2]	9.66875	2.441799	3.96	0.0003*
Enzyme Weight (% wt.)[8-5]	1.71	1.99372	0.86	0.3957
Water Weight (% wt.)[3-1]	12.284333	2.573882	4.77	<.0001*
Water Weight (% wt.)[6-3]	0.054	2.30215	0.02	0.9814
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[3-1]	-9.630417	3.355931	-2.87	0.0063*
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[6-3]	-0.017667	3.255732	-0.01	0.9957
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[3-1]	-2.963333	3.045458	-0.97	0.3359
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[6-3]	1.4543333	3.255732	0.45	0.6573
Temperature (°C)[35-25]	3.559	2.819546	1.26	0.2135
Enzyme Weight (% wt.)[5-2]*Temperature (°C)[35-25]	-10.08175	3.453225	-2.92	0.0055*
Enzyme Weight (% wt.)[8-5]*Temperature (°C)[35-25]	7.5435	2.819546	2.68	0.0104*
Water Weight (% wt.)[3-1]*Temperature (°C)[35-25]	-4.344333	3.547851	-1.22	0.2273
Water Weight (% wt.)[6-3]*Temperature (°C)[35-25]	0.96175	3.045458	0.32	0.7536
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[3-1]*Temperature (°C)[35-25]	10.937417	4.6043	2.38	0.0219*
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[6-3]*Temperature (°C)[35-25]	-0.988583	4.306928	-0.23	0.8195
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[3-1]*Temperature (°C)[35-25]	-6.229167	4.15026	-1.50	0.1405
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[6-3]*Temperature (°C)[35-25]	-1.463583	4.306928	-0.34	0.7356

Response Conversion (% wt.)

Effect Summary

Source	LogWorth	PValue
Water Weight (% wt.)	6.361	0.00000
Enzyme Weight (% wt.)	5.745	0.00000
Enzyme Weight (% wt.)*Water Weight (% wt.)	3.360	0.00044



Summary of Fit

RSquare	0.56017
RSquare Adj	0.49378
Root Mean Square Error	2.926986
Mean of Response	98.02066
Observations (or Sum Wgts)	62

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	578.2983	72.2873	8.4376
Error	53	454.0642	8.5672	Prob > F
C. Total	61	1032.3625		<.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	89.0395	1.463493	60.84	<.0001*
Enzyme Weight (% wt.)[5-2]	4.627875	1.792406	2.58	0.0126*
Enzyme Weight (% wt.)[8-5]	5.48175	1.463493	3.75	0.0004*
Water Weight (% wt.)[3-1]	10.056071	1.834586	5.48	<.0001*
Water Weight (% wt.)[6-3]	0.6035714	1.56454	0.39	0.7012
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[3-1]	-4.100589	2.379181	-1.72	0.0906
Enzyme Weight (% wt.)[5-2]*Water Weight (% wt.)[6-3]	-0.582571	2.212594	-0.26	0.7933
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[3-1]	-5.984036	2.142335	-2.79	0.0072*
Enzyme Weight (% wt.)[8-5]*Water Weight (% wt.)[6-3]	0.618	2.212594	0.28	0.7811

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Enzyme Weight (% wt.)	2	2	293.95346	17.1556	<.0001*
Water Weight (% wt.)	2	2	335.05124	19.5542	<.0001*
Enzyme Weight (% wt.)*Water Weight (% wt.)	4	4	207.36627	6.0511	0.0004*

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