

Algae Biofuel Triacylglyceride Transesterification Optimization

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Abstract

Algae biofuels may hold the key to solving the problem of fossil fuel consumption by being comparable in content, renewable, and carbon-neutral. Many biofuel researchers and corporations have undertaken to increase the production rate or capacity of triacylglycerides (TAG), the fat precursor to biodiesel fuel produced by algae, in algae cultures and published articles documenting their findings. This research is devoted to evaluating effect of water that may be present in samples on the conversion efficiency of TAG into fatty acid methyl esters (FAME), commonly referred to as biodiesel. Therefore, that efficiency was studied to find the water content which optimizes the yield and determine if further drying of algae was necessary as an additional step in sample preparation. The results showed that the water content typically present in lyophilized algae samples is not sufficient to appreciably inhibit the reaction efficiency and necessitate extensive drying as a sample preparation step prior to transesterification.

Introduction

Worldview Implications

The Bible is infallible and must be understood as such. Everything the Bible says is true (John 17:17) by nature of being the inspired word (2 Timothy 3:16) of the God who cannot lie (Hebrews 6:18). However, the Bible does not mention everything. God has revealed Himself through both special (Hebrews 1:1-2) and general revelation (Psalm 19:1-4). This means God's nature can be found, although not completely, in studying His creation (Romans 1:20). In fact, this verse says that enough is revealed about God in His creation—His power and divinity—that all people are expected to conclude that He exists and are thus accountable to Him.

The above perspective, the mindset of the scientific community during the scientific revolution and the enlightenment,¹ is in part responsible for the fastest progression and accumulation of scientific knowledge the world had seen up until that time. Unfortunately, because the laws of nature appear to be self-evident, studying these laws led to the belief in a removed Creator who set laws in place and has ceased to interfere (hence the uprising of deism in the 1700-1800s). Therefore, Christians must not become consumed by the study of general revelation at the expense of the study of special revelation—both Christ, the incarnate Word of God (John 1:1-18), and the Scriptures, the written word of God (Hebrews 4:12), by which Christ is revealed to us (John 5:39, 46).

¹ Francis A. Schaeffer. *How Should We Then Live?: The Rise and Decline of Western Thought and Culture*. Place of Publication Not Identified: Crossway Books, 2005.

Special revelation provides countless examples of God suspending the natural laws He has set in place to intervene supernaturally (Exd 7:10-12, Num 17:1, Lev 10:1, 2, Jos 6:6-20, Jdg 15:19, 1Sa 5:1-12, 2Sa 5:23-25, 1Ki 13:4, 5, 2Ki 4:2-7, 2Ch 26:16-21, Dan 3:19-27, Jn 2:1-10 to name a few). He still reserves the right to do so as Lord and Creator of the universe, but usually works within the laws that He set up. Moreover, He put them in place for a reason—they work.

Remaining cognizant that God is fully able to intervene and justified in doing so should He choose, scientific experimentation is done. In Genesis 1:26-31, God gave man dominion over all creation to be His representative and to glorify Him. Because God gave this authority, the responsibility to be good stewards falls on the shoulders of mankind as well. This study has been done to find the best conditions—the ones which produce the highest yield—under which the triacylglycerides (fats) of algae should be trans-esterified into fatty acid methyl esters to make the best use of what He has provided. These compounds can then be used as biofuels which may hold the key to more responsible fuel acquisition and better stewardship of this planet.

Purpose

Much effort and research has been dedicated to finding a sustainable, green alternative because of concern about rising greenhouse gas levels and fossil fuel reserve depletion. While solar and wind energy are prime examples, they lack the robustness of chemical potential energy, like that of hydrocarbons and their derivatives, in energy

content and stability² while also requiring large investments of rare metals.³ Therefore, a carbon-neutral hydrocarbon alternative has been of great interest, and this search has uncovered algae.

Hydrocarbon Fuel History

In the 1800s, whale oil was the primary source of fuel. Whales were harvested, and the blubber was boiled down to an oil that was then burned for fuel. An oil is a viscous liquid at standard temperatures that is neutral and nonpolar. Whale oil was mostly composed of unsaturated triacylglycerols⁴ which typically were wax esters—primarily a derivative of oleic acid,⁵ a fatty acid containing 18 carbons and one double bond.

After whale oil, petroleum became the primary source of energy for America. The word “petroleum” is a compound loan word where *petra* means rock (as in the word “petrified” [turned into rock] and the name “Peter” [small stone]) and “oleum” means oil (as in “linoleum”); when it is translated, “petroleum” means “rock oil.”⁶ This fuel is oil that comes from rock (or at least the crust of the earth)—as opposed to whales.

² Travis Fisher, and Alex Fitzsimmons. "Big Wind's Dirty Little Secret: Toxic Lakes and Radioactive Waste." IER. March 05, 2014. Accessed February 19, 2018.

³ David Ferris, E&E reporter Energywire: Monday, January 12, 2015. "Technology: 5 years after crisis, U.S. remains dependent on China's rare earth elements." Technology: 5 years after crisis, U.S. remains dependent on China's rare earth elements -- Monday, January 12, 2015. Accessed February 19, 2018.

⁴ M. M. Chakrabarty, *Chemistry and Technology of Oils and Fats*. New Delhi: Allied, 2003. Print.

⁵ Nestor R. Bottino, "The Composition of Marine-oil Triglycerides as Determined by Silver Ion-thin-layer Chromatography." *Journal of Lipid Research* 12 (1971): 24-30. Print.

⁶ William Arndt et al., [*A Greek-English Lexicon of the New Testament and Other Early Christian Literature*](#) (Chicago: University of Chicago Press, 2000), 809.

Petroleum, like whale oil, is a mixture of many types of hydrocarbons, but contains even more variation in its composition.

Neither whale oil nor petroleum are renewable resources. While all fossil fuel deposits on earth contain the equivalent of 800 billion tons of oil,⁷ the most accessible reserves are used first leaving only the oil that is hard to reach and purify. As this continues, oil becomes increasingly difficult to obtain and refine. Because it is not a renewable resource, even these will eventually run out. Therefore, new, preferably renewable or “green,” energy sources are necessary.

One energy source that has also been studied is ethanol production from crops such as corn. At many fuel depots, there are labels that indicate the gasoline may contain up to 10% ethanol. Ethanol is a common hydrocarbon with a substantial energy content, sparking interest into its uses as a supplemental energy source. In 2008, there was a global food crisis that was partially blamed on the use of corn and other foods as biofuels.⁸ This led to a push for algae research because it did not cut into a food source or land potentially used to grow food.

Oil, whether from whales, rocks, or crops, has been used as a fuel because it is high in energy. This energy comes from the oxidative potential in the bonds of the hydrocarbons.⁹ All life uses this chemical potential to store energy. For example, burning

⁷ "The World Factbook: WORLD." Central Intelligence Agency. January 23, 2018. Accessed February 04, 2018.

⁸ "Energy from Algae - National Research Council Canada." *Government of Canada. National Research Council Canada*. Government of Canada. National Research Council Canada, 09 Aug. 2012. Web. 28 Feb. 2017.

⁹ David L. Nelson, Michael M. Cox, and Albert L. Lehninger. *Lehninger Principles of Biochemistry*. 5th ed. New York: W.H. Freeman, 2008. Print.

wood is a chemical reaction that oxidizes the carbon and hydrogen stored in sugars and other compounds. Moreover, the human body uses saturated triacylglycerols, or triglycerides (commonly known as fats), to store energy. These fat molecules are anabolized, stored, and then metabolized.

Algae undergo this production and degradation of fats which is why they have been studied as a possible replacement fuel source. Research has been done on algae for over sixty years.¹⁰ In the 1950s, algae was proposed as a source of methane and small-scale research was done from the 1970s until the 1990s. Due to recent environmental concerns of CO₂ emissions, increasing demand for fuel, limited resources, and other matters, the US is becoming more interested in biofuels.¹¹

Algae Background

The name algae is a broad term that covers a range of aquatic life from complex, multicellular organisms, to unicellular microalgae.¹² The diversity is exemplified even by the food sources of algae: some species are heterotrophic (cannot produce their own energy and must consume other living things, like both herbivores and carnivores), while others are autotrophic (can produce their own energy so they do not consume other life), and still others are mixotrophic (depend on both photosynthesis and consuming organic

¹⁰"Algae Basics - History of Algae as Fuel." *Algae Basics - History of Algae as Fuel*. N.p., n.d. Web. 28 Feb. 2017.

¹¹"Ibid.

¹²A. Tartar; Boucias, D. G.; Becnel, J. J.; Adams, B. J. (2003). "Comparison of plastid 16S rRNA (rrn 16) genes from *Helicosporidium* spp.: evidence supporting the reclassification of *Helicosporidia* as green algae (Chlorophyta)". *International Journal of Systematic and Evolutionary Microbiology*. **53** (Pt 6): 1719–1723.

material).¹³ Researchers have benefitted from the diversity of algae because each of the 100,000 strains has unique traits that can be exploited.¹⁴

Most algae are photosynthetic, which means it produces its own energy by harnessing radiation from the sun. When this energy is captured, it is usually stored in the form of glucose, a monosaccharide known as the universal sugar. The sugar is then broken down into two pyruvates and functionalized by Coenzyme A into Acetyl-CoA. In catabolic reactions, specifically cellular respiration, these molecules enter the Citric Acid Cycle and then the Electron Transport Chain. Here, they release a large amount of energy for the cell to use.

In anabolic reactions, many two-carbon acetyl groups are linked together to form fatty acids with an even number of carbons. These fatty acids are then grouped together by glycerol molecules into triacylglycerol molecules (lipids) and stored. Lipids, while hydrophobic and not ideal for a watery cell living in an aqueous environment, have a much higher energy content than sugars and are used for long term energy storage. When the algae cells need energy, they can break down the fats into fatty acids. These are then broken into acetyl groups and metabolized normally.¹⁵

¹³F. Figueroa-Martinez; Nedelcu, A. M.; Smith, D. R.; Reyes-Prieto, A. (2015). "[When the lights go out: the evolutionary fate of free-living colorless green algae](#)". *New Phytologist*. **206** (3): 972-982. doi:10.1111/nph.13279. Archived from the original on 10 December 2015.

¹⁴"Algal Biofuels." *Algal Biofuels* / Department of Energy. Energy.gov Office of Energy Efficiency & Renewable Energy, n.d. Web. 28 Feb. 2017.

¹⁵ David L. Nelson, Michael M. Cox, and Albert L. Lehninger. *Lehninger Principles of Biochemistry*. 5th ed. New York: W.H. Freeman, 2008. Print, 652.

If the algae are restricted and not given an essential nutrient like silicon,¹⁶ nitrogen, or phosphorus, they are no longer able to divide and multiply. As a result, the cells reach maturity and are forced to begin storing the energy they acquire in the form of oil.¹⁷ Unfortunately, algae are not alone in the biosphere, and containing large amounts of stored energy makes them prime targets for predation. Many fish, frog, crab, and aquatic insect species feed on algae. Therefore, the high energy content desired for fuel draws predators which put the profit margin in jeopardy.¹⁸

If, however, the algae are harvested before metabolizing the fat or succumbing to predation, its triacylglycerides (TAG) could be substituted as a fuel. This is the purpose of researching algae for biofuels. Algae are grown and fed with essential nutrients. After the algae culture has grown and filled its holding container, the algae are transferred to larger reservoirs where they continue to convert sunlight into high energy molecules. Then the algae are stressed, by starvation of an essential nutrient, and begin to produce fats to store energy. As time continues and the algae are left in this state, some hardy species will accumulate much triacylglycerol, but others will become brittle and begin to use the fats or lyse—spilling their content into the environment, making harvesting

¹⁶ Emily M. Trentacoste, et. al. "Metabolic Engineering of Lipid Catabolism Increases Microalgal Lipid Accumulation without Compromising Growth." *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 03 Dec. 2013. Web. 28 Feb. 2017.

¹⁷ "Public Genomes, Algae Fuel, Mystery of the Gakkel Ridge,..." *PBS*. Public Broadcasting Service, 18 Aug. 2009. Web. 28 Feb. 2017.

¹⁸ D. Rego, L.m. Redondo, V. Geraldes, L. Costa, J. Navalho, and M.t. Pereira. "Control of predators in industrial scale microalgae cultures with Pulsed Electric Fields." *Bioelectrochemistry* 103 (June 2015): 60-64. Accessed February 19, 2018. doi:10.1016/j.bioelechem.2014.08.004.

impossible. Therefore, the timing must be right to allow the algae to produce maximum triacylglycerols without waiting so long as to allow for consumption of these fats.

When the algae have grown and produced fats, they are ready to be harvested. The algae culture is filtered, dried and prepared for the transesterification process. In this process, the fat molecules, or TAG react with methanol. This uses three molecules of methanol but produces three molecules of fatty acid methyl esters and one molecule of glycerin for every TAG molecule. The glycerin is a valuable byproduct that can be sold to be used in many products, like soap.¹⁹ The other product, fatty acid methyl ester (FAME), is the chemical name of the final product—biodiesel. “Biodiesel is the only alternative fuel to have fully completed the health effects testing requirements of the 1990 Clean Air Act Amendments.”²⁰

Importance of Algae Biofuels

Upon first thought, algae biofuel seems infeasible: the idea sounds almost too good to be true. However, with the current push for clean energy and the promising preliminary results, algae may become the next source of energy—a renewable resource with the power to sustain the needs of the world.

¹⁹ "Algae Fuels - Biodiesel." *Algae Fuels - All About Algae*. N.p., n.d. Web. 28 Feb. 2017.

²⁰ "Biodiesel Basics." *Biodiesel Basics - Biodiesel.org*. Federal Equipment Company, n.d. Web. 28 Feb. 2017, 9.

Figure 1²¹ depicts the need for a replacement fuel. According to the U.S. Energy Information Administration, the “total U.S. petroleum and other liquid fuels consumption is forecast in the STEO [short-term energy outlook] to average 20.3 million barrels per day (b/d) in 2018.”²² These numbers cannot be sustained on petroleum.

U.S. crude oil and liquid fuels production



Figure 1--This graph depicts the consumption of various fuels by the United States and its expected growth into the next few years. Because crude oil, natural gas, and fuel ethanol are not viable, biodiesel must take over when they expire

Fossil fuels are limited, and reserves are quickly depleting. The fact that the world, not only the United States, has such a dependence on fossil fuel demands that the question of what will happen when fuel sources run out needs to be answered. According to the Central Intelligence Agency (CIA) factbook, at the current rates, oil will run out in 2051, gas about 10 years later and coal in still another 20 years.²³

²¹ "U.S. Energy Information Administration - EIA - Independent Statistics and Analysis." Short-Term Energy Outlook - U.S. Energy Information Administration (EIA). January 9, 2018. Accessed February 04, 2018, 2.

²² Ibid.

²³ "The World Factbook: WORLD." Central Intelligence Agency. January 23, 2018. Accessed February 04, 2018.

Figure 2²⁴ does not account for growth in consumption or a switched dependence. The rates, unless a new fuel source is found, of consumption of each will continue to grow. Moreover, when oil runs out, gas and coal consumption will rise to take its place. Again, when gas is depleted, coal production will rise. This will accelerate the coming extinction of fossil fuels even more.

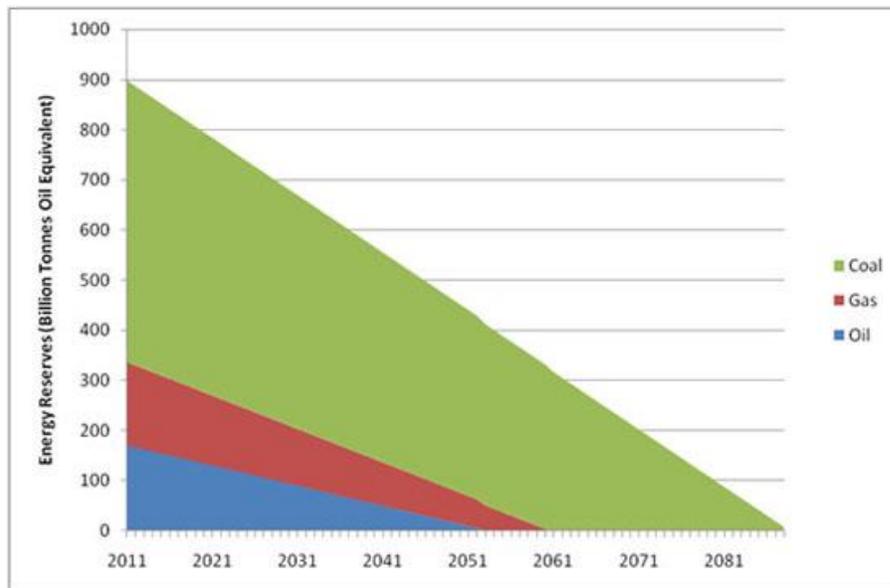


Figure 2--Forecasted usage of fossil fuels to their extinction.

This is alarming as one is left wondering what will happen if fossil fuel consumption continues at its current rate, or even at its current acceleration. There are other problems with fossil fuels that necessitate a replacement fuel, like climate change and pollution, and these only underscore the need to find additional fuel sources.

Fossil fuel is not the only energy source, but it is the dominant one, and no current alternative can replace it. Nuclear is promising but produces radioactive waste and has not grown to its potential because of a questionable public image, and solar or wind could

²⁴ "The End of Fossil Fuels." Ecotricity. Accessed 1.23.2018, 6.

expand but require rare earth metals that are not abundant enough to sustain the world's needs.²⁵ However, there is significant investment into this field and companies are growing industrial quantities of biofuel, as shown in figure 3.²⁶



Figure 3-- This picture shows the industrial growth of algae for the use as biofuel.

Imagine, however, a biological “solar panel.” Instead of producing an electrical current, it would store the energy as diesel fuel—or as TAG which can be converted to diesel. This biological solar panel could be grown in industrial vats or allowed to bask in the sunlight and convert visible light into chemical potential energy in swampy, unproductive land. This is the goal of algae biofuel.

When the “solar panel,” truly an algae culture, is provided necessary nutrients (naturally occurring in the ocean), it will grow and reproduce exponentially. As

²⁵ "Are Wind Turbine Rare Earth Minerals Too Costly for Environment?" Newshour. Accessed March 12, 2018.

²⁶ Marry-Ann Muffoletto, "Algae biofuel can help meet world energy demand, researchers say." Phys.org - News and Articles on Science and Technology. May 26, 2016. Accessed February 04, 2018, 1.

photosynthetic eukaryotes, algae create sugar from sunlight through photosynthesis but can be manipulated into mass-producing lipids. If an essential nutrient, such as nitrogen, phosphorus, or silicon, is withheld, algal cells will no longer be able to multiply, and will hoard energy and store it as fat. These lipids are the precursors to biodiesel fuel. The fat-laden biofuel source can then be collected and harvested for its TAG, which would be transesterified into biodiesel fuel—instead of producing an electrical current.

Research Objective

Algae have been studied as a potential biofuel source. In this process, fatty acids, mostly found in (TAG) are converted into fatty acid methyl esters (FAME). Because water competes with methanol for its place in this reaction, further research was done on the conversion process in the presence of differing concentrations of water. The focus of this research was to determine if the residual moisture content, ranging from 1-10%, in lyophilized and filtered algae samples would inhibit the transesterification reaction efficiency.

Experiments, on commercially available triglyceride standards, were conducted to convert glyceryl tristate (C18:0 TAG) into methyl stearate (C18:0 FA). Glyceryl tristate, selected because many types of algae analyzed in our lab produce C18:0, was studied instead of using whole algae samples, to more fully characterize its reaction. Whole algae samples have many other compounds that make the process more complicated, so for this study it was not used.

Liberty University Algae Research

The algae biofuel research team at Liberty University has been involved in multiple projects. Besides transesterification efficiency, some students have studied the residual fatty acids in the post-transesterified algae biomass. In this project, the infrared spectra of algae samples, like that of figure 4²⁷, were studied to developing a screening technique to quantify the TAG and FAME content in lyophilized (freeze-dried) algae samples. Another purpose of the study was to use the quantities of fats in the samples to determine if how much of the TAG was converted to TAG during the transesterification

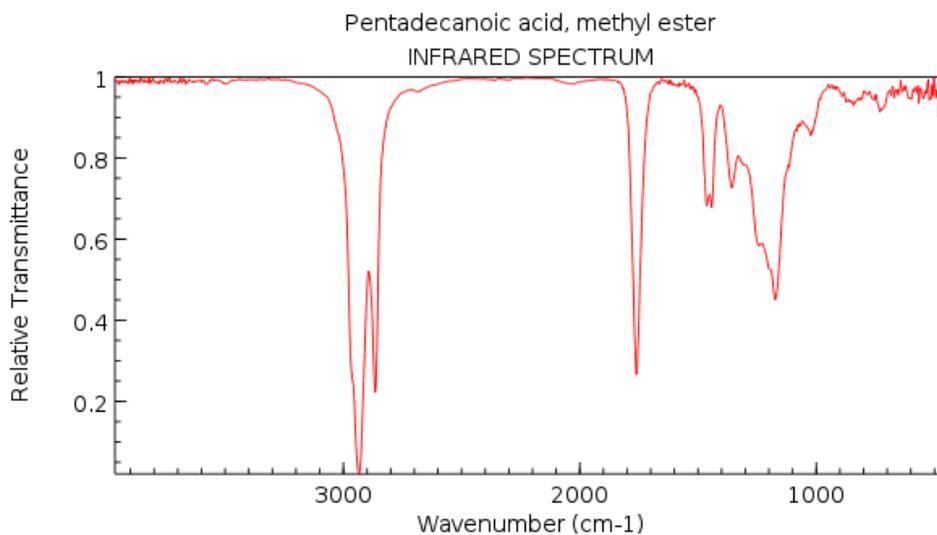


Figure 4—This is an IR spectrum of a fatty acid methyl ester, pentadecanoic acid, the internal standard used in studying lipid content.

process. Therefore, algae samples are tested before and after transesterification to observe the change in peaks and hopefully detect any remaining lipid content—whether fatty acid, FAME, or TAG.

²⁷ "NIST Chemistry WebBook, SRD 69." Pentadecanoic acid, methyl ester. Accessed February 04, 2018, IR Spectrum, Gas Phase Spectrum, Additional Data, 1.

Another project being studied, which is also related to the residual biomass of algae samples, is the determination of the amino acid composition of algal cells so that the protein content of defatted biomass may be used as a supplement in animal feed. If the algae cells are harvested solely for their lipid content, there is substantial bio-waste. Reducing bio-waste would alleviate pressure on the agricultural industries and augment the monetary yield of the algae crop. Moreover, if algae can be used for animal feed, less of the available food must go to sustain livestock and the concerns of the 2008 food crisis, namely that necessary food and valuable farm land was irresponsibly dedicated to fuel production, will be appeased.

On the other hand, many species of fish survive primarily on algae. There is the potential to grow algae and fish symbiotically.²⁸ If algae and fish, or bacteria,²⁹ are grown together, though separated to prevent fish from consuming the algae, one will aerate the water as it photosynthesizes and the other will fertilize the water with necessary nutrients.³⁰ When the algae crop is harvested, its organic material may be reintroduced into the synthetic environment and feed the fish, perpetuating the cycle.

²⁸ Penina Graubart, and Lauren Blanchard. "Green Energy: Algae-Based Biofuel Could Power Cars, Airplanes." Israeli Innovation News - Environment. January 04, 2016. Accessed April 04, 2018.

²⁹ Yan Zhou. "Improving Algal Biofuel Production Through Nutrient Recycling and Characterization of Photosynthetic Anomalies in Mutant Algae Species." 2010. Accessed April 3, 2018.

³⁰ "National Algal Biofuels Technology Review." Bioenergy Technologies Office. June 2016. Accessed April 3, 2018.

However, this ideal situation depends on the viability of algae providing sustenance to herbivores. To study this possibility, the protein content of algae must be

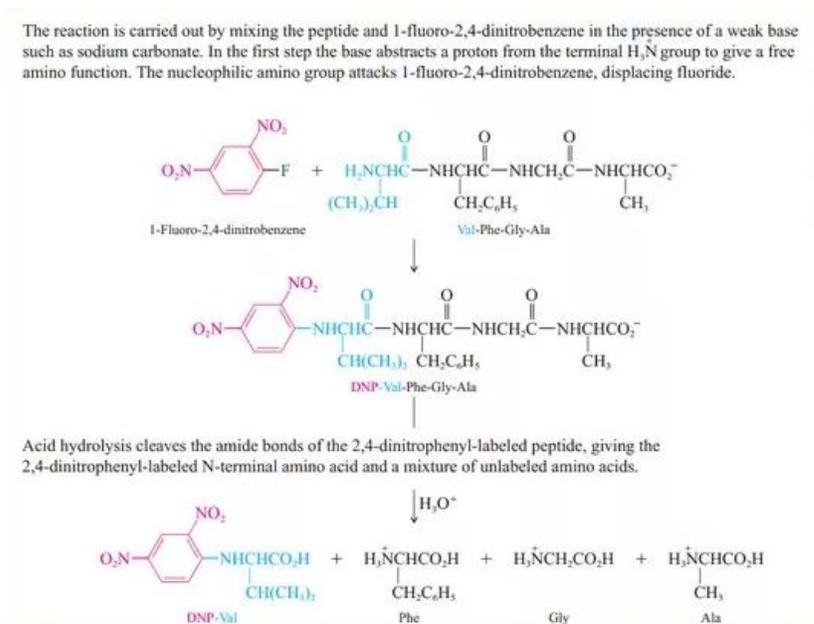


Figure 5-- This depicts the derivatization for identification and quantification of the N-terminal amino acid using 1-fluoro-2,4-dinitrobenzene (DNFB).

quantified. The research team is working on creating standards of amino acids derivatized by dinitrofluorobenzene. This compound binds to the terminal amino acid of any protein it encounters and removes it.

Figure 5³¹ depicts the derivatization of proteins with Sanger's reagent. Given sufficient DNFB in a protein-containing sample, every protein in the sample will be broken down into its respective amino acids. These samples and standards are analyzed by High Performance Liquid Chromatography Diode Array Detector (HPLC/DAD). Once the content of each amino acid is quantified for a specific strain of algae, that data

³¹ Francis A. Carey, and Robert M. Giuliano. *Organic Chemistry*. 4th ed. New York: McGraw-Hill, 2000, 1103.

can be compared to known essential amino acids of livestock to determine which animals the algae could feed.

Additional Algae Research

Because algae efficiently capture CO₂ from the air to produce TAG, they are useful in wastewater treatment, yield high oil and biomass, and do not require agricultural land, they have drawn attention as a renewable energy source.³² “Photosynthetic cyanobacteria are able to produce roughly 100 times the amount of clean fuel per acre compared with other biofuel crops, and because their survival needs are simple—sunlight, water, CO₂ and a few nutrients—they do not require arable land to be taken out of food production.”³³ Because algae use naturally occurring sunlight to eliminate carbon dioxide from the air, it has been suggested that they be grown in close proximity to powerplants to reduce the net emissions.³⁴

As stated above, there are over 100,000 strains of algae, all with differing qualities. If the use of algae biofuels is to be successful, the strain that produces the most lipid content in the shortest time must be identified and possibly genetically engineered to produce even more. However, the selection process is not this simple because some strains of algae become brittle when ready for harvest and cannot undergo filtration—part

³²"Algae, Cyanobacteria and Aquatic Plants for Production of Biofuels." *Algae, Cyanobacteria and Microbiological Production of Biofuels*. European Biofuels Technology Platform, n.d. Web. 28 Feb. 2017

³³ Richard Harth, and Joe Caspermeyer. "Benchtop biofuels: fine-tuning growth conditions helps cyanobacteria flourish." *Biodesign Institute Science*, June 29, 2010. Accessed February 3, 2018.

³⁴"Algae, Cyanobacteria and Aquatic Plants for Production of Biofuels." *Algae, Cyanobacteria and Microbiological Production of Biofuels*. European Biofuels Technology Platform, n.d. Web. 28 Feb. 2017

of the reaping process—without rupturing and spilling the cytosol and lipids into the environment. Pacific Northwest Laboratory has developed a climate-simulating laboratory system and is attempting to find the most productive strain.³⁵

As research continues, more breakthroughs are being made. At Arizona State University, for example, researchers are attempting to design a strain of algae that will excrete oil instead of storing it in the cells. This would mean that as the algae produce oil, it would accumulate on the surface of the water. Then, it could be harvested without lysing the algae cells, therefore increasing the efficiency to essentially infinite. If this is possible, the algae can continue to grow and multiply, and its oil production rate would increase exponentially.³⁶ Yet even here, there will be a small amount of water in and around the excreted fat vesicles. Therefore, finding the sample preparation conditions which optimize the transesterification yield is still necessary for accurate and precise sample analysis.

At a company called Solazyme, a group of researchers have accelerated algae's production of multiple oils. This research enables scientists to "tailor the oil profiles by carbon chain and saturation."³⁷ Unfortunately, water is still present in the cells and their environment. Therefore, its inhibition must be studied to find the best conditions for transesterification.

³⁵ Frances White. "Biofuel Matchmaker: Finding the Perfect Algae for Renewable Energy." *News - Biofuel Matchmaker: Finding the Perfect Algae for Renewable Energy*. Pacific Northwest National Laboratory, 13 Jan. 2017. Web. 28 Feb. 2017.

³⁶ Richard Harth, and Joe Caspermeyer. "Benchtop biofuels: fine-tuning growth conditions helps cyanobacteria flourish." *Biodesign Institute Science*, June 29, 2010. Accessed February 3, 2018.

³⁷ James Ayre. "Breakthrough in Microalgae Oil Production, Accelerated Ability To Produce Variety Of Different Oils." *Transport*. Clean Technica, n.d. Web. 28 Feb. 2017.

In a similar article, Trentacoste et al have engineered two random algae strains of the species *Thalassiosira pseudonana* (1A6 and 1B1) by knockdown of lipase T. *pseudonana* genome version 3 protein ID 264297 (Thaps3_264297) that produced 2.4 and 3.3 times the lipid content, respectively, of wild-type-algae (the algae with natural characteristics found under natural conditions, as opposed to mutants created in labs)³⁸ when allowed to grow naturally.³⁹ Conversely, the cultures produced 4.2 and 3.2 times the lipid content, respectively, of wild-type-algae when starved of silicon for 40 hours without compromised growth rates. Silicon was chosen because a lack of this essential material induced a cessation of the cell cycle—typically 80% remain in G1 phase of mitosis.⁴⁰ Therefore, the algae were unable to multiply and had a defective lipase enzyme, rendering it unable to metabolize the TAG it produces. This is significant because the decreased growth rate would severely offset the lipid production increase.

Obviously, there is substantial research being done on the strains of algae that produce the oil, but a significant amount is dedicated to farming practices.⁴¹ This research is making algae energy more readily available. In fact, a company called Global Algae

³⁸ Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U (2009) Algal lipid bodies: Stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot Cell* **8**(12):1856–1868.

³⁹ Emily M. Trentacoste, et. al. "Metabolic Engineering of Lipid Catabolism Increases Microalgal Lipid Accumulation without Compromising Growth." *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 03 Dec. 2013. Web. 28 Feb. 2017.

⁴⁰ M. Hildebrand, Frigeri LG, Davis A (2007) Synchronized growth of *Thalassiosira pseudonana* (Bacillariophyceae) provides novel insights into cell-wall synthesis processes in relation to the cell cycle. *J Phycol* **43**(4):730–740.

⁴¹ "Energy 101: Algae-to-Fuel." *Energy.gov*. Office of Energy Efficiency & Renewable Energy, n.d. Web. 28 Feb. 2017.

Innovations has shown a 15-fold increase in cost reduction and two-fold increase in algae productivity.⁴² Because algae oil is becoming easier to acquire and petroleum is becoming more difficult to acquire, their Energy Return on Investment (EROI) are nearing—and presumed to cross.⁴³

Each of the previously described studies focused on bolstering the oil content—by finding algae that produce more oil, making algae that excrete oil, engineering algae to produce oil faster or only the oils of interest—but the transesterification process has not been the primary concentration. Once established, the procedure seems to have remained unaltered or augmented.

Experimental

Instruments and Analysis

Instruments Used

Gas chromatography/mass spectrometry (GC/MS) is an analytical instrument. It combines gas chromatography and mass spectrometry. Gas chromatography separates the different components in a sample and quantitatively measures each compound. It contains a long tube, the 30-meter long by 250-micron wide column, in a very sensitive oven (accurate to 0.1 degrees C). Helium is pumped through the column at a rate of 1 mL/min. The sample is introduced and carried through the column by the helium. At the end of the column is a transducer and a mass spectrometer. Mass spectrometry breaks the separated—and thus pure—components to identify them. High energy (70 eV) electrons

⁴² "What We've Achieved." Global Algae Innovations. Accessed April 09, 2018.

⁴³ "New Study: Algae Biofuel Cuts CO2 50% vs. Petroleum." *Algae Industry Magazine*. N.p., 21 Sept. 2013. Web. 28 Feb. 2017.

bombard the various compounds as they elute from the column and produce positively charged ions from each compound.⁴⁴ Then the ions are guided by four charged poles, which filter out particles that are too heavy and too light, to the detector. The quantity of results at each mass is recorded. The retention time, peak height/area, and mass spectrum can be used together to identify the compound.⁴⁵

The name implies that the sample runs through the GC as a gas. A liquid sample is drawn out of a GC vial by the tower dispenser and injected into the inlet. This is about 10-15 degrees C higher than the sample boiling point and induces vaporization. The increasing pressure from vaporization causes the sample to be pushed down into the column. From there, it is carried through the column in the helium. The vaporized molecules will adhere to the wall of the column and stop. From there it will desorb and return to the helium mobile phase.⁴⁶ The longer it is on the stationary phase, the longer the retention time. Different molecules adsorb and desorb at different rates, which causes different retention times for each chemical.

The MS must be kept in a vacuum. Gas particles will interact with and join the analyte as the MS records its spectra. This would render it useless, but it is kept at 10^{-5} – 10^{-6} torr—low enough to make environmental noise negligible. MS is not perfect, but it does have very little noise. It has very high resolution.

⁴⁴ Ibid.

⁴⁵ Douglas A. Skoog, Stanley R. Crouch, and F. James. Holler. *Principles of Instrumental Analysis*. 6th ed. Belmont, CA: Thomson Brooks/Cole, 2007.

⁴⁶ Ibid.

Analysis Techniques

A modified version of the NREL⁴⁷ GC/MS sample preparation method was used for the identification and quantification of fatty acid methyl esters derived from the lipids found in algae samples and triglyceride standards. All the lipids are transesterified, including the fatty acids in the phospholipid bilayer. These FAMES are produced by the acid and heat catalyzed, methanolytic transesterification “using sulfuric acid in methanol reagent. This procedure accounts for the total fatty acid content in the biomass and represents the biofuel potential of an algae culture.”⁴⁸

Reaction Type and Mechanism

Esterification Vs. Transesterification

Esterification is the process of making an ester. An ester is a combination of an ether and an aldehyde. An ether is a substance which uses an oxygen atom to bridge two carbon atoms while an aldehyde is where an oxygen is double bonded to a terminal carbon. Therefore, an ester is an ether where one of the carbons attached to the bridging oxygen is also double bonded to another oxygen.⁴⁹ Another way to think about it is like an organic acid—in which a primary carbon is double bonded to an oxygen and bonded to an alcohol—where the acidic proton is replaced with an organic group.

⁴⁷ Stephanie Van Wychen, Kelsey Ramirez, and Lieve M.L. Laurens. "Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in Situ Transesterification." Laboratory Analytical Procedure (LAP). December 29, 2015. Accessed April 10, 2018.

⁴⁸ Todd Allen, “GC/MS/FID Lipid Content from Algae Samples.” SOP 01, 2.0. January 23, 2016

⁴⁹ Neil D. Jespersen, James E. Brady, Alison Hyslop, and James E. Brady. *Chemistry: The Molecular Nature of Matter*. 6th ed. Hoboken, NJ: Wiley, 2012.

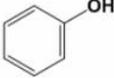
Name	Functional Group	IUPAC Ending
Alcohols	$R-OH$	-ol
Phenols		phenol
Ethers	$R-O-R'$	ether
Aldehydes	$R-\overset{O}{\parallel}-H$	-al
Ketones	$R-\overset{O}{\parallel}-R'$	-one
Carboxylic Acids	$R-\overset{O}{\parallel}-OH$	-oic acid
Esters	$R-\overset{O}{\parallel}-O-R'$	-ate

Figure 6-- functional groups related to esters.

A transesterification reaction, on the other hand, is a reaction that starts with an ester and an alcohol and ends with a different ester and different alcohol. Just as water can act as a nucleophile on an ester and create an alcohol and an organic acid, an alcohol can replace another alcohol in an ester. Common functional groups are depicted in figure 6.⁵⁰

Transesterification in this Experiment

For this reaction, a transesterification reaction was performed. The substrate was an ester (TAG) and the product was also an ester. Therefore, the simplest reaction is substituting the glycerol with methanol—precisely what was done. Water could have been used to break the TAG into fatty acids and glycerol. Afterward, the fatty acids

⁵⁰ "CH105: Chapter 9 - Organic Compounds of Oxygen." Chemistry. Accessed April 03, 2018, 9.1, 1.

would have to be esterified with methanol. However, because esterification reactions are thermodynamically unfavorable, this reaction would have lower efficiency and was not performed.

Transesterification Mechanism

The reaction used to convert TAG into FAME is classified as a transesterification reaction. It involves rupturing the algal cells to expose the triacylglycerides. Then methanol is used to replace the glycerol. The mechanism of this reaction can be broken into two steps. First, sulfuric acid is used to catalyze the reaction. The hydrogen donated by the acid has, by the electromagnetic force, lower potential energy near, and thus is forced towards, one of the lone pairs of electrons on the oxygen that is double bonded to the acid carbon. This gives the oxygen a positive charge, but because oxygen is very electronegative, it is an unstable intermediate. Therefore, the electronegative oxygen pulls the electrons from the double bond. This produces a strong partial positive charge on the acidic carbon. On the methanol, the oxygen has a partial negative. These partial charges are attracted to each other. Second, the methanol oxygen donates electrons to the acidic carbon and forces it to relinquish its grasp of the electrons on the aldehyde making it an alcohol. This leaves the methanol oxygen with a positive charge. Therefore, it releases the hydrogen, preserving the acidic conditions, and an unstable intermediate is formed with the acidic carbon bonded to three oxygens.⁵¹

⁵¹ Jim Clark. "Transesterification." Chemistry. January 08, 2018. Accessed April 04, 2018.

Next, the released proton finds the glycerol oxygen and protonates it, making the bond very unstable. This leaves a large partial positive on the acidic carbon. In response, the alcohol is deprotonated and uses its lone pair to form a double bond with the acidic carbon. This process eliminates the glycerol. In this reaction, the hydrogen ion acting as a catalyst is preserved and the ester bond to the glycerol is traded for an ester bond to the methanol. However, the glycerol still has two other fatty acid groups attached to it, so this reaction proceeds twice more with them to produce three fatty acid methyl esters and one free glycerol.⁵²

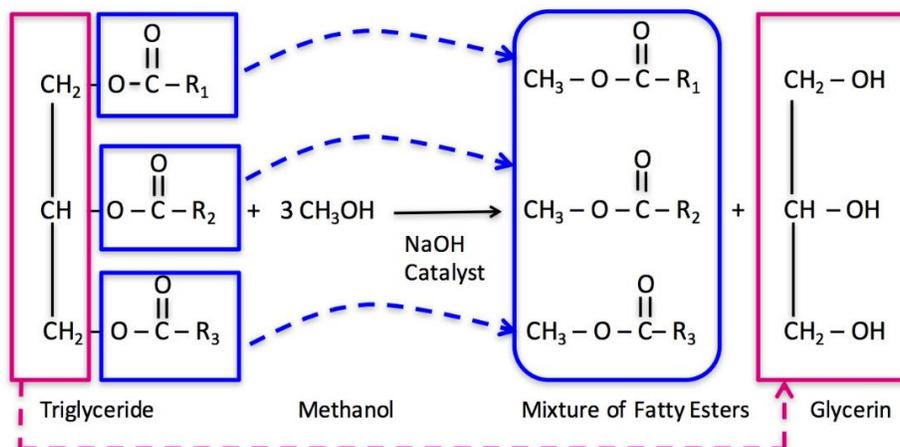


Figure 7-- This is a reaction diagram of the base catalyzed transesterification reaction. Here, instead of the proton facilitating the reaction and stabilizing the intermediate, methanol is deprotonated and made a better nucleophile.

The reaction depicted in figure 7⁵³ is kinetically controlled, but thermodynamically unfavorable. Therefore, the sulfuric acid catalyst is necessary but insufficient to produce FAME. Because the reaction is nonspontaneous, it must be forced to proceed by heating the vials. The rate of the reaction is controlled and bolstered by

⁵² L. G. Wade, and Jan William. Simek. *Organic Chemistry*. 9th ed. Glenview, IL: Pearson, 2017.

⁵³ "9.2 The Reaction of Biodiesel: Transesterification." *Energy and Geo-Environmental Engineering*. Accessed February 04, 2018, paragraph 2.

increasing the concentration of the TAG, methanol, sulfuric acid, and the temperature. Therefore, for research on this topic, where the TAG is the limiting reagent, the best conditions for this reaction have methanol in excess with sufficient acid and heat.

Purpose of Transesterification

Algae produce TAG. Despite being high in energy, these large molecules have high melting points and low boiling points. This makes them impractical for fuel—combustion takes place in the gas phase and would not be attainable at the relatively low temperatures found in engines. Therefore, the molecules must be broken. When the triacylglycerides undergo hydrolysis, fatty acids are created, along with glycerol.

Fatty acids would potentially be good fuel molecules because they have lower melting points, higher vapor pressure and contain about the same energy per unit mass, but they are far more corrosive. The acidic head would damage the engine and thus, they are not suitable for fuel.

However, if the corrosivity of the acidic head of fatty acids is mitigated, the compound would be a prime fuel. Instead of hydrolysis, if TAG is trans-esterified, the acidic hydrogen would be replaced with a benign alkyl group. The melting point would still be lower than that of the TAG precursor, and the vapor pressure would be high enough for use in a diesel engine.

Method

Analyte of Interest

For this project, the esterification efficiency of C18:0 TAG was studied because it is a naturally occurring triglyceride of average size—therefore its properties will

hopefully be representative of all TAG found in algae. Also, methanol was used in the alkyl substitution because it has the highest efficiency due to the least steric inhibition and is the least expensive alcohol, so it is used in industrial biofuel reactions.

Independent Variable

The condition that was varied was the water content in the methanol. Water is present in every sample of algae because algae are aquatic. But even in dried samples, there is water in the cells. The water in the sample engages in hydrolysis and competes with the transesterification process for TAG to form fatty acids. Therefore, water potentially contributes to decreased efficiency. A drying agent was added to some samples to adsorb any water while 1-1000 μL water was added to other samples to quantify the competition and inhibition. This range was chosen, because algae samples may have up to 10% by mass water, which would be at most 50 μL . Therefore, up to 20 times the maximum was tested. The samples were then trans-esterified under the same method described above.

Experimental Design

General Design

The focus of this project is concerned with the transesterification efficiency. Water is present in algae samples—both in the liquid environment and within the cells themselves. Therefore, the transesterification efficiency of the C18:0 triglyceride standard in the presence of different amounts of water was studied. The conversion of the standard C18:0 triglyceride to FAME was performed outside the natural algae matrix so

as to better characterize the transesterification reaction in its simplest environment and, thus, the nature of the reaction while all other variables are held constant or eliminated.

A known, constant concentration of C18:0 triglyceride, an accurately weighed amount of C15:0 fatty acid, and different amounts of water were reacted with acidified methanol in separate reaction vessels at a temperature of 99 °C for 30 minutes.

Fatty acid methyl esters, produced from the transesterification of the C18:0 TAG, were extracted into a known amount hexane. An internal standard of C15:0 FA was added, and the solution was analyzed by GC/MS. The peak area for C15:0 was calculated and normalized to the concentration in the sample. Then, the peak area of the C18:0 was multiplied by the same normalization constant to calculate the concentration in the sample. The actual yield was compared to the theoretical yield and graphed to show the change of percent yield as the concentration of water changed.

Experimental Specifics

In these experiments, the triacylglyceride C18:0 (NU-CHEK PREP, INC. Tristearin, >99%, LOT T-160-JY10-Y) was dissolved in toluene (Aldrich. CAT#17-996-5, >99.3%, CAS# 108-88-3, Lot# HA 01862 EA). Then, 50 μL ⁵⁴ of the 2.521 mg/mL TAG solution (quantitatively weighed⁵⁵ by difference) was reacted with 3 mL solution of MeOH with 4% Sulfuric Acid. Then the reaction vial containing these two reactants is heated in a ThermoMixer,⁵⁶ an instrument that heats reaction vials to a desired

⁵⁴ 130 μg or 0.13 mg C18. This amount is equivalent to the amount typically produced by algae.

⁵⁵ Analytical Balance: MTAL104, SN 1229020128

⁵⁶ Eppendorf ThermoMixer C, Serial number: 5382DM605404

temperature (99 degrees Celsius for this experiment) and shakes at a desired speed (600 RPM) for thirty minutes.

Reacting in a ThermoMixer overcame the kinetic barrier, and allowed the system enclosed in the reaction vial to reach thermodynamic equilibrium, which highly favored products (FAME) over reactants (TAG and methanol). After samples were cooled for 30 minutes, 3 mL of hexane was added to the system which was vortex mixed for 1 minute to extract the FAME from the methanol. Next, the vials were centrifuged. All this was done to simulate the sample preparation process performed on algae samples which must be centrifuged to collect the biomass to the bottom of the centrifuge tube and purify the hexane layer of biological contaminants.

A small (450 μ L) sample of the hexane layer was taken and put in a Gas Chromatography/Mass Spectrometry vial. An internal standard (50 μ L) of a 2.5697 mg/mL solution of C13:0/C19:0 FAME was added to normalize the peak area and calculate the concentration of the C18:0 based of the GC/MS results, and thus the efficiency of the transesterification reaction.

Results and Discussion

Results

The results indicated that the drying agent did not increase the esterification efficiency, but that high volumes of water decreased the efficiency. When no water was added to the samples, the esterification efficiency was essentially 100%. In fact, the

samples with a drying agent did not have increased efficiency. Therefore, if the samples are relatively dry, there is no need for a desiccant.

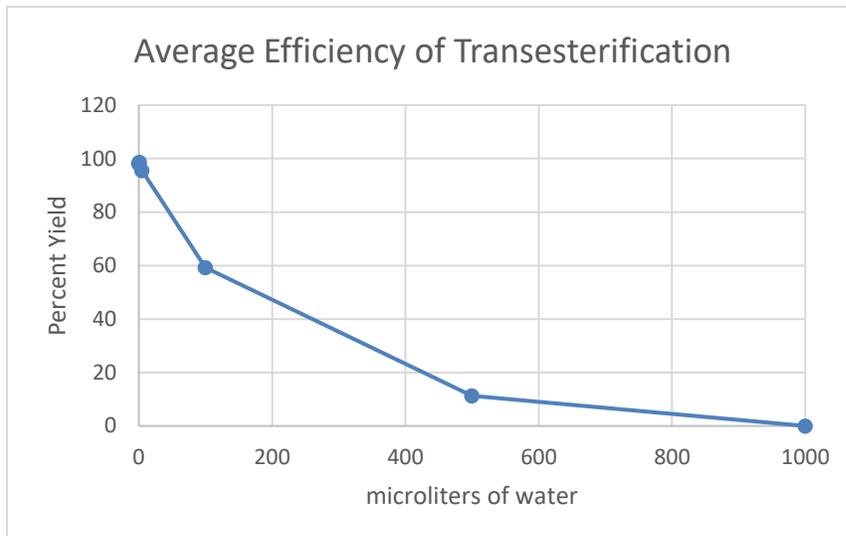


Figure 8-- Efficiency of transesterification in the presence of varying amounts of water.

For samples with water, the transesterification efficiency was around 60% when 100 μL , or 0.100 mL of water was added. This is a molar ratio of 38,000 moles of water to 1 mole of C18:0. Graphing the results provided an exponential decay plot that reached 10% at 5 mL and essentially 0% at 10 mL of water.

This plot follows the general equation $y=ab^x$ where $0 < b < 1$. Calculations have not been done to determine the equation, nor have enough data points been recorded to have confidence in such an equation were it to be computed. However, since the plot does seem to follow this formula, a range of acceptable efficiencies can be determined. Less than 10 μL of residual water may be present for highest efficiency, but algae samples must not contain more than 0.100 mL (100 μL) of water for transesterification to have an

appreciable efficiency. Furthermore, the regression is quite linear for points within the first 100 μL of water added. The linear equation and R^2 values are recorded on the chart.

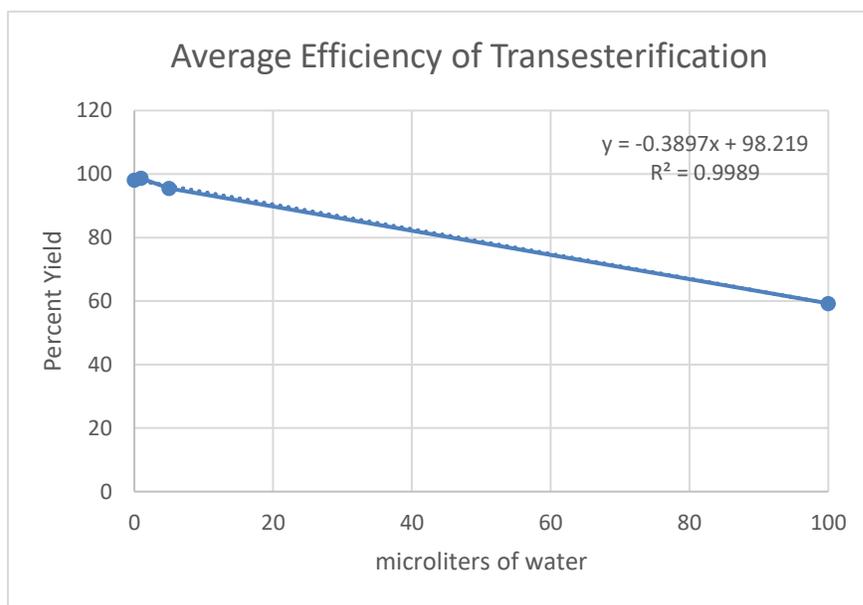


Figure 9-- Data that is within 100 μL of water. The R^2 value indicates that this plot is strongly linear, and the equation closely approximates the true rate of change of efficiency—therefore, within the linear-dynamic range of the method and instrument.

One important note is that there was an outlier data point not included in this plot. The samples contained approximately 5 μL of water, but the average efficiency was over 100 %. It did not seem reasonable to include that data, but it is necessary to repeat the experiment to determine if there was human error that affected all the replicates—i.e. a miscalculation or incorrect recording of the mass of TAG originally in the samples. Moreover, including the point in the plot decreased the R^2 value to roughly .9, a severe drop, indicating an error.

Discussion

The results indicate water does have an adverse effect of the efficiency. Best results, 95% efficiency, may be found with under 10 μL of water present. Because TAG was the limiting reagent, and it was in the presence of an excess of methanol, by Le

Chatelier's principal, equilibrium should strongly favor FAME despite the reaction being thermodynamically unfavorable.

In the presence of water, because the hydrolysis reaction is thermodynamically favorable, equilibrium should favor the production of fatty acids. In fact, it did. The results showed that water acted as a stronger nucleophile, and was more successful, per capita, than the methanol. However, because filtered algae samples rarely have more than 50 μL of water,⁵⁷ and lyophilized samples have even less, 80% efficiency can be expected. This indicates there is sufficient methanol to compensate for being out competed by water for TAG.

Conclusion and Recommendation for Future Work

The research presented suggests, based on triacylglyceride standard experimentation, that water content up to 2 μL in a 20 mg algae sample will not present a significant source of inhibition. However, there is still much research to be done—especially rerunning the problematic samples with 5 μL of water, obtaining more points of data for C18:0, and doing similar tests on other triacylglycerides. Also, more research must be done on dried, whole algae samples to confirm that this holds true in the presence of all other compounds in cells.

⁵⁷ Previous experimentation on old algae filters yielded measurements of approximately 25-50 mg water lost upon drying.

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