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Bradley D. Wahlen
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1. IMPROVING THE YIELD OF BIODIESEL FROM MICROALGAE AND OTHER LIPIDS. 2. STUDIES OF THE WAX ESTER BIOSYNTHETIC PATHWAY AND POTENTIAL BIOTECHNOLOGICAL APPLICATION

by

Bradley D. Wahlen

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biochemistry

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UTAH STATE UNIVERSITY
Logan, Utah

2012

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ABSTRACT

1. Improving the Yield of Biodiesel from Microalgae and Other Lipids. 2. Studies of the Wax Ester Biosynthetic Pathway and Potential Biotechnological Application

by

Bradley D. Wahlen, Doctor of Philosophy

Utah State University, 2012

Major Professor: Dr. Lance C. Seefeldt
Department: Chemistry and Biochemistry

The production of biofuels and oleochemicals from renewable sources offers an opportunity to reduce our dependence on fossil fuels. The work contained in this dissertation has focused on developing and improving methods for the production of biodiesel from non-traditional feedstocks and understanding biosynthetic pathways that result in the production of oleochemicals and fuels.

Pure vegetable oil can account for 70-80% of the total cost of biodiesel production. Many low-cost oils contain high amounts of free fatty acids, which are unsuitable for base-catalyzed transesterification. Herein an approach is described that efficiently accomplishes the simultaneous esterification and transesterification of both free fatty acids and triglycerides found in low-cost oils. The approach utilizes an acid catalyst and longer-chain alcohols to improve biodiesel yields from oils high in free fatty acids.

Microalgae are a promising biodiesel feedstock, due to its high lipid productivity

and its ability to be cultivated using resources, land and water, unsuitable for agriculture. As part of this work, reaction conditions were optimized for the direct (or *in situ)* transesterification of algal biomass to biodiesel. This approach accomplishes the simultaneous extraction and conversion of the total lipids from microalgae and results in increased yields compared to extraction followed by conversion. The use of this process to effectively produce biodiesel from wet algal biomass is also discussed.

Wax esters are a class of oleochemicals that can be used for a wide range of applications in diverse industries. The chemical composition of native wax esters from the bacterium *Marinobacter aquaeolei* was determined. It was found that including small alcohols in the growth medium resulted in the *in vivo* formation of esters similar to biodiesel. All of the proteins involved in the wax ester biosynthetic pathway are not known. The cloning, purification, and characterization of a putative fatty aldehyde reductase from *M. aquaeolei*, believed to be involved in the production of wax esters, is reported. Finally, the expression of a *ws/dgat* (wax ester synthase) gene from *M. aquaeolei* in the cyanobacterium *Synechocystis* sp. PCC 6803 is discussed as an approach to producing biodiesel *in vivo* from sunlight and CO_2.

(251 pages)

PUBLIC ABSTRACT

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The production of biofuels and oleochemicals from renewable sources offers an opportunity to reduce our dependence on fossil fuels. The work contained in this dissertation has focused on developing and improving methods for the production of biodiesel from sources other than vegetable oil and understanding the biology of microbes that could be harnessed to produce fuels and other important chemicals.

Pure vegetable oil can account for 70-80% of the total cost of biodiesel production. Many low-cost oils contain compounds, which interfere with biodiesel production by traditional methods. Herein an approach is described that can produce biodiesel efficiently from both pure vegetable oil and low value quality oils. The approach takes advantage of different chemistry and alcohols such as butanol to accomplish to produce biodiesel from low quality oils.

Microalgae are a promising source of oil for biodiesel production, due to their rapid growth rate and high lipid content lipid. Because microalgae can be cultivated on

marginal land with wastewater or saline water, it does not compete with agriculture for land and water resources. As part of this work, an approach was developed to produce biodiesel directly from microalgae without first extracting the oil, combining what is traditionally two steps into one.

Wax esters are oily chemicals, naturally produced by a small group of bacteria, which can be used for a wide range of applications in many industries. Work described in this dissertation identifies the basic chemical composition of these naturally occurring oils and how it can be manipulated to produce wax esters of interest. In addition, studies were undertaken to understand the biological process that leads to the formation of wax esters. This understanding will make possible the production of wax esters industrially.

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Bradley D. Wahlen

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ABBREVIATIONS

ppm	Parts per million
DOE	Department of Energy
USDA	United States Department of Agriculture
US	United States
TAG	Triacylglycerol (triglyceride)
FAME	Fatty acid methyl ester
DAG	Diacylglycerol (diglyceride)
MAG	Monoacylglycerol (monoglyceride)
FFA	Free fatty acid
Mpa	Megapascals
THF	Tetrahydrofuran
ASTM	American Society for Testing and Materials
EN	European standards
GC/MS	Gas chromatography/mass spectrometry
NO_x	Mono-nitrogen oxide
CN	Cetane number
FID	Flame ionization detector
NMR	Nuclear magnetic resonance
FT-Raman	Fourier transform-Raman spectroscopy
bbl	Barrels
EPA	Environmental Protection Agency

CDW	Cellular dry weight
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
CoA	Coenzyme A
WS/DGAT	Wax ester synthase/diacylglycerol acyltransferase
FAR	Fatty acyl-CoA reductase
WS	Wax ester synthase
DGAT	Diacylglycerol acyltransferase
His	Histidine
FAEE	Fatty acid ethyl ester
NF	National formulary
MHz	Megahertz
USTAR	Utah Science, Technology, and Research Initiative
PTFE	Polytetrafluoroethylene
GC	Gas chromatography
PTV	Programmable temperature vaporizer
MS	Mass spectrometer
ID	Internal diameter
PHA	Polyhydroxyalkanoate
ATCC	American Type Culture Collection
NIST	National Institute of Standards and Technology
LB	Luria-Bertani media
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
pH	$-\log[H^+]$

TLC	Thin layer chromatography
kDa	kilodalton
MgATP	Magnesium adenosine triphosphate
FALDR	Fatty aldehyde reductase
DNA	Deoxyribonucleic acid
BSA	Bovine serum albumin
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
PCR	Polymerase chain reaction
MBP	Maltose binding protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
EDTA	Ethylenediaminetetraacetic acid
NADH	Nicotinamide adenine dinucleotide (reduced)
MOPS	3-(N-Morpholino)propanesulfonic acid
MES	2-(N-Morpholino)ethanesulfonic acid
TAPS	[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
BLAST	Basic local alignment search tool
ORF	Open reading frame
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfontate hydrate
SDS	Sodium dodecyl sulfate

PAGE	polyacrylamide gel electrophoresis
K_m	Michaelis constant
DMSO	Dimethyl sulfoxide
MCADH	Medium chain alcohol dehydrogenase
BLOSUM	Blocks of amino acid substitution matrix
PCC	Pasteur Culture Collection
OD	Optical density
UV	Ultraviolet
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
TNB^{2-}	5-thio-2-nitrobenzoic acid

CHAPTER 1

INTRODUCTION

Society obtains most of its energy from fossil fuels, using coal and natural gas to generate electricity, and petroleum products to fuel our transportation needs. The use of fossil fuels cannot continue indefinitely. Its continued use presents political, environmental, and sustainability concerns. Some of the largest deposits of petroleum are found in politically unstable regions of the world. Dependence on fuel from these regions of the world constitutes a significant national security risk. Fossil fuels are also a finite resource; eventually our demand for fossil fuels will exceed our capacity to extract it from the earth, ultimately consuming it entirely. The combustion of fossil fuels releases CO_2, a powerful greenhouse gas, into the atmosphere, leading to increased global temperatures, rising seawater levels and decreasing the pH of the oceans. Concentrations of the gas have increased from the pre-industrial age level of ~280 ppm in 1750 to 379 ppm in 2005. These concerns highlight the need to develop renewable fuels that can be produced in quantities sufficient to meet society's needs without contributing to atmospheric CO_2 levels.

Currently 87% of our energy is supplied by fossil fuels (coal 22%, natural gas 26%, and petroleum 39%, **Figure 1-1**) (*1*). Carbon-neutral fuels, fuels with no net release of CO_2, accounted for only 13% of the energy consumed. Nuclear energy, at 8% of the total, was the largest contributor of carbon-neutral energy. Other carbon-neutral resources that currently contribute to the energy supply include hydroelectric, geothermal, wind, biomass, and solar photovoltaic, which collectively only contribute 4% to the energy consumed today. The most promising renewable energy resource is by far

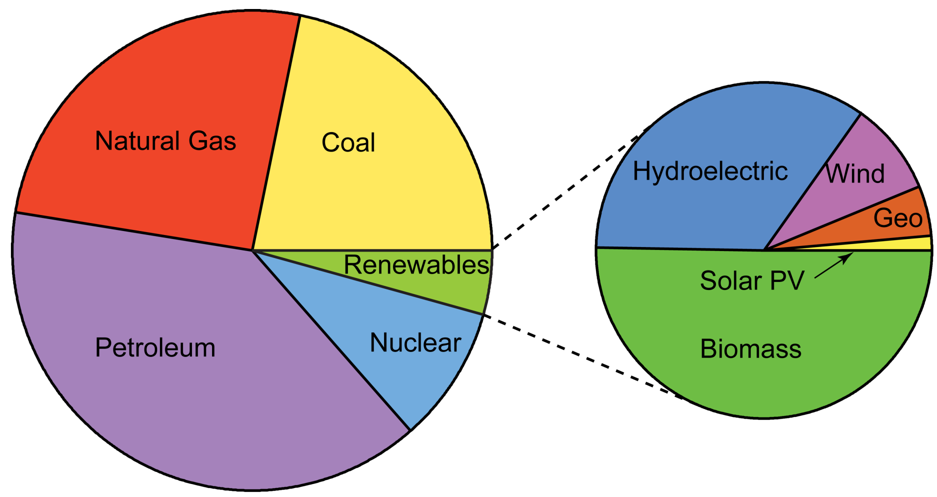

Figure 1-1. Energy consumption by fuel source. Information available at www.eia.gov.

the sun. No other resource comes close to matching the potential of the sun to provide sufficient renewable energy. More energy from sunlight strikes the earth in one hour (4.3 x 10^{20} J) than is consumed on the planet in an entire year (4.1 x 10^{20} J) (*2*). Despite this tremendous resource, only a small fraction of our energy (< 2%, mostly biomass) is derived from the sun. The challenge of utilizing solar energy is a question of its capture, storage, and conversion to a form capable of doing work. Two differing technologies exist to convert solar energy into electricity. Solar thermal, as its name implies, uses solar energy to concentrate heat, which then can be used to generate steam that drives a turbine to produce electricity. A second technology, photovoltaic cells, uses semiconducting materials to convert solar energy to an electric current to produce electricity directly. Both technologies are promising and society would benefit from a wider adoption of this technology. While both solar thermal and photovoltaics are important for the generation of electricity, the diurnal nature of sunlight and the low

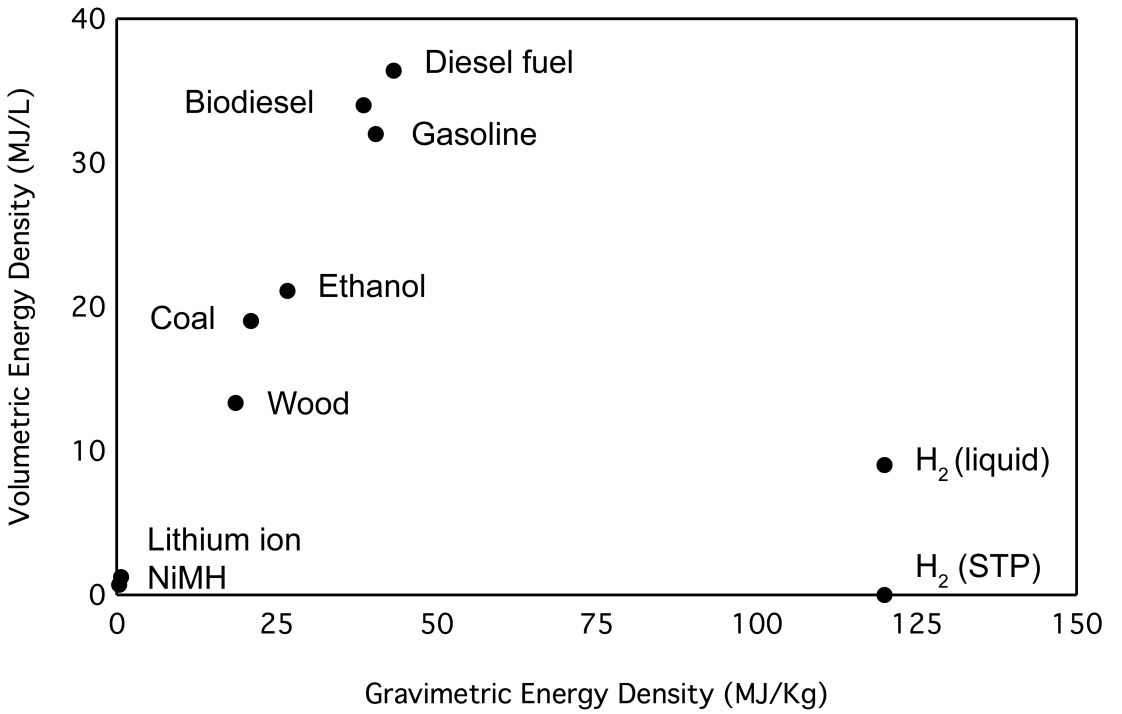

Figure 1-2. Energy density of fuels. The volumetric density (MJ L^{-1}) of select fuels is plotted against their gravimetric density (MJ kg^{-1}). Adopted from reference 3. NiMH (nickel-metal hydride) and lithium ion refer to battery technology.

energy density of batteries limit their potential impact on the transportation sector.

Fuel for the transportation sector has specific requirements that have led to petroleum products being used almost exclusively, supplying 97% of transportation energy (*1*). Energy for transportation, by nature, must be highly portable and contain a lot of energy in a small amount of space. **Figure 1-2** shows a graph which plots the volumetric energy density (MJ L^{-1}) of fuels against their gravimetric energy density (MJ kg^{-1}) (*3*). The renewable approaches to produce energy that have been discussed up to this point have all focused on converting a form of energy to electricity. An electrically

powered automobile would require batteries to store energy for release when traveling. Batteries, however, have the lowest energy density of the fuels plotted on the graph (**Figure 1-2**) both in terms of gravimetric and volumetric energy density. Because of the low energy density of batteries, electric cars would be required to carry heavy batteries that take up a lot of space in order to travel substantial distances. Another potential energy carrier, H_2, has the highest energy density by weight of any fuel, however, at standard temperatures and pressure it has the lowest volumetric yield. Significant advances in H_2 storage technology would need to occur to make H_2 a relevant energy carrier for transportation. The limit to the volumetric density that H_2 could attain is 9 MJ L^{-1}, the volumetric energy density of liquid H_2. This is equivalent to one fourth the volumetric energy density of hydrocarbons. Hydrocarbons, such as diesel fuel and gasoline, have high volumetric energy densities, allowing a vehicle to travel long distances, while requiring minimal volumes of fuel. It is likely that renewable fuels will need to have similar energy densities in order for them to be widely adopted as transportation fuels. Chapters 2 and 3, summarize research seeking to improve the availability of biodiesel by improving conversion technologies of waste vegetable oils and algae, an emerging biodiesel feedstock. Biodiesel is a promising renewable fuel with volumetric and gravimetric energy densities (MJ L^{-1} and MJ g^{-1}) comparable to diesel fuel.

The production of renewable fuel from biomass has been the subject of intense discussion in the political and scientific communities and has captured the attention of the general public (*4*, *5*). This level of discussion is needed if we are to gain energy independence as most petroleum (85%) is used for fuel. In comparison, very little

attention has been given to the production of renewable organic chemicals. A much smaller but significant 13% of crude oil is used in the production of non-fuel chemicals (*6*). Completely replacing petroleum, will require a renewable source of chemicals to produce a wide range of products. The chemical industry synthesizes many compounds from platform chemicals, such as ethylene, propylene, and benzene. From these chemicals, many other chemicals can be produced (*7*). From renewable biomass, analagous chemicals, such as glycerol, 2,5-furandicarboxylic acid, and succinic acid, can be obtained and used for the production of diverse chemicals (*7*).

Biomass can be transformed by chemical (e.g. hydrogenation) or physical treatment (pyrolysis) and through fermentation to make useful products (*8*). Rather than using biomass to produce commodity chemicals, specialty chemicals can be produced directly through the use of metabolic engineering and fermentation technology (*9*). There are examples of products that cannot be produced by chemical modification, either technically or economically, that are produced biologically. Examples of chemicals produced by fermentation at industrial scale include glutamic acid (~1.7 billion kg/year), citric acid (~1.6 billion kg/year), and lysine (~850 million kg/year) (*6*). Genencor and DuPont successfully developed a cost-effective fermentative route to 1,3-propanediol, which is not readily available from petrochemical feedstocks (*6*).

An acyltransferase found in *Acinetobacter* has significant biotechnological potential (*10*). Naturally this enzyme catalyzes the transfer of a fatty acyl group to a fatty alcohol to form wax esters (*11*). This enzyme is very unspecific as it accepts a wide range of molecules as acyl acceptor (*12-14*). This promiscuity is an advantage as it could be used to perform an acyltransferase reaction in the production of a variety of

compounds. Wax esters by themselves are valuable products that can be used in pharmaceuticals, personal care products, and as lubricants. Chapters 4 - 6 discuss the production of wax esters in the marine bacterium *Marinobacter aquaeolei*. Later in the introduction a review of wax esters and their biosynthetic pathway will be given.

Biomass has the potential to lessen our dependence on fossil fuels for energy, while providing a resource for renewable organic chemicals. Recently, US government agencies have developed plans to achieve the ambitious goal to produce 36 billion gallons of bio-based transportation fuels by 2022, increased from the current production of 10.75 billion gallons annually (*15*). A joint report by DOE and USDA determined that US agricultural and forest sources could provide a billion tons of renewable biomass per year of lignocellulosic biomass (*6*). In recent years, progress has been made in obtaining renewable energy from the sun in the form of biomass. The research contained in this dissertation aims to contribute to the future of sustainable energy.

Biodiesel, Renewable Replacement for Petroleum Diesel

Biodiesel is an often-discussed replacement to petrodiesel (*16*, *17*). It has the advantage of being completely compatible with the internal combustion engine and the well-established petroleum diesel fuel distribution network. Biodiesel can be mixed at any ratio with conventional petroleum diesel resulting in a blended fuel (*18*). A common blend sold commercially is a 20% biodiesel and 80% petrodiesel fuel termed B20. “B” stands for biodiesel and “20” refers to the percentage of the total fuel that is made up of biodiesel. Biodiesel is often described as a “drop-in” replacement fuel, because it is completely compatible with petrodiesel, the infrastructure that serves its distribution, and the engine that is designed for its combustion. A detailed discussion of what biodiesel is, how it is made, the advantages that its use provides, and challenges that impede its

widespread adoption is presented here.

Biodiesel is a renewable fuel because its primary feedstock, triacylglyceride (TAG), can be made by photoautotrophic organisms, such as plants and microalgae, using only sunlight, CO_2, and water or by heterotrophic organisms that can effectively utilize waste carbon. Biodiesel is produced by forming fatty acid alkyl esters from the TAG molecules that compose vegetable oil (e.g. soybean oil) or animal fat and a short chain alcohol (e.g. methanol) in the presence of a catalyst by a transesterification reaction. When methanol is used as the alcohol, fatty acid methyl esters (FAMEs) are produced. The general equation for the reaction that forms biodiesel is listed according to **Figure 1-3**. The reaction requires at least 3 moles of alcohol to react with 1 mole of TAG to produce 3 moles of fatty acid alkyl ester. Because the transesterification reaction is an equilibrium reaction, inclusion of alcohol in excess of the stoichiometric amount is generally necessary to drive the reaction to completion (*19*). In practice the amount of alcohol required is highly dependent on the catalyst type and the alcohol used. The volume of alcohol, the type and concentration of the catalyst, the temperature of the reaction and the time of reaction are all common variables that affect the rate of reaction

Figure 1-3. General transesterification reaction diagram. The group R can represent any long chain fatty acid. Here it is depicted as a palmitic acid (C16:0). R1 represents an alkyl group. For biodiesel this is typically either ethanol or methanol.

and the level of completion achieved. By far the most common catalyst used is a base catalyst such as sodium hydroxide or sodium methoxide. Acid and enzyme (lipase) catalyzed and uncatalyzed supercritical methanol biodiesel production methods are not commonly used in the commercial production of biodiesel despite offering some unique advantages. A description of the advantages and disadvantages to each method of production will follow.

Base-catalyzed biodiesel production

The base-catalyzed method for the production of biodiesel offers a number of advantages. It makes use of low cost catalysts such as sodium methoxide or uses sodium or potassium hydroxide to generate the reactive methoxide anion. The methoxide anion serves as a nucleophile to attack the carbonyl carbon of a fatty acid bound to glycerol (**Figure 1-4**). By reacting with the fatty acid glycerol ester the methoxide anion displaces glycerol forming a new fatty acid methyl ester bond and a diglyceride (DAG) molecule. The transesterification reaction is repeated with another methoxide anion and the DAG molecule from the first reaction to yield a second molecule of FAME and monoglyceride (MAG). The conversion of TAG to FAME is completed when MAG reacts with a

Figure 1-4. Alkali catalyzed biodiesel reaction mechanism. Reaction with triglyceride to produce fatty acid methyl ester and diglyceride. R represents a fatty acid chain.

methoxide anion to yield FAME and glycerol. After each transesterification step the methoxide anion is regenerated when the leaving glycerol molecule abstracts a proton from a molecule of the solvent methanol.

The alkali catalyzed method proceeds rapidly at moderate temperatures and atmospheric pressure. Freedman *et al*. reported that in as little as 1 min of reaction 80% of the TAG from soybean and sunflower oils is converted to FAME (*20*). After 1 hour of reaction the percentage of FAME had reached 98% (*20*). The rate of reaction increases with increasing temperature. Alternate heating methods have been found to increase the reaction rate. Using microwave heating Leadbeater *et al*. was able to obtain 98% of soybean oil converted to FAME within 1 min using a base-catalyzed method (*21*). The authors attributed the short completion time to the efficient heating of microwave energy. The authors extended their findings to the preparation of biodiesel in a continuous-flow reactor using microwave heating (*22*). The base-catalyzed method was able to efficiently produce biodiesel that was 99% FAME at a rate of 7.2 L/min in a 4 L vessel (*22*). The base-catalyzed method for producing biodiesel from vegetable oil is robust enough that many biodiesel enthusiasts are capable of producing their own biodiesel in their garages using supplies from their local home store and waste vegetable oil.

The production of biodiesel by base-catalysis does have some significant limitations despite its benefits of rapid and efficient reaction. Although base-catalyzed transesterification is by far the method of choice to produce biodiesel commercially, it is limited in the diversity of vegetable oil feedstocks that it can accommodate (*23*). Feedstocks that contain FFA (free fatty acid), reduce the yield obtained and the quality of the biodiesel produced (*24*). FFA within the vegetable oil reacts with the base to form the carboxylate of the fatty acid (*i.e*. soap) and in the process deactivates the catalyst. Soap promotes the formation of emulsions with glycerol preventing its separation thus reducing the quality of the biodiesel and the overall yield of FAME. Because the base-

catalyzed method cannot directly tolerate FFA some low cost feedstocks that contain a significant amount of FFA cannot be utilized. Vegetable oils containing a significant quantity of FFA are underutilized resources for biodiesel production and include used vegetable oils and many inedible oils such as rice bran oil, mahua (*Madhuca indica*) oil, and jatropha (*Jatropha curcas*) oil (*25-27*). Unrefined vegetable oils such as soybean, peanut, and sunflower oils are found to have 0.8%, 3.3%, and 0.8% FFA, respectively, and cannot be directly used for biodiesel production (*20*). Freedman *et al*. reported that the biodiesel yields obtained from reactions utilizing these unrefined oils were markedly reduced from reactions with the corresponding refined oils (*20*). The refined vegetable oils differ from the crude vegetable oil in their FFA content. Freedman *et al*. concluded that oils containing greater than 0.5% are unsuitable as feedstock for base-catalyzed biodiesel production (*20*). This conclusion has been supported by many other independent studies (*28*, *29*).

The requirement of a refined vegetable oil feedstock contributes significantly to the overall cost of biodiesel production (*30*). Haas *et al*. estimated that the cost of the oil feedstock constitutes 88% of the cost of biodiesel production (*31*). In order to make biodiesel more economical, lower grade feedstock needs to be utilized. Typically lower cost oils contain high levels of free fatty acid. Waste vegetable oils containing less than 15% FFA is known as yellow grease and sells for half of the cost of soybean oil (*28*). Researchers in tropical countries such as India have investigated the use of inedible oils from trees such as the mahua tree, the rubber tree and *Jatropha curcas* (*32-34*). The oils from these trees have no competing food value and grow readily throughout the developing world. Each of these oils contain high levels of FFA (19%, 17%, and 14% respectively) (*32-34*) and cannot be utilized by the base-catalyzed method. The development of methods that successfully produce biodiesel in the presence of FFA has

been a research priority in the field. Research in this area has focused on three promising technologies capable of tolerating high levels of free fatty acids to produce biodiesel. These technologies include the enzyme lipase, supercritical methanol, and the use of acid catalysts in the production of biodiesel. A short description of each technology and their inherent advantages and disadvantages will follow.

Lipase catalyzed biodiesel production

The enzyme lipase is found throughout the biological kingdom from bacteria to higher eukaryotes such as plants and vertebrates. Lipases catalyze both the hydrolysis of triglycerides and the synthesis of triglycerides from long chain fatty acids and glycerol (*35*). Microbial lipases are the most important biocatayst for biotechnological applications (*36*). Enzymes of this class are included in consumer products such as detergents, and are used in industrial processes such as food and paper production. The commercial use of lipases is a billion dollar business (*36*). Because lipases are functional in organic solvents, they have tremendous biotechnological potential (*37*). This is particularly important to the usefulness of lipases in the production of biodiesel. In the presence of a short chain alcohol and vegetable oil, lipases catalyze the methanolysis of triglyceride, to form FAME. Lipases will also effectively catalyze the esterification of FFA with methanol (*38*). The biodiesel produced by this method is highly pure and the glycerol byproduct is readily removed (*39*). In addition, the enzyme can be immobilized within a polymer to enable the reuse of the enzyme (*39*). Enzyme catalyzed biodiesel production does not need extensive downstream processing such as the neutralization of a mineral catalyst or extensive water washing steps (*40*). The drawback is that the high cost of the lipase production currently prohibits its use in the biodiesel industry (*40*).

Supercritical methanol biodiesel production

By heating methanol past its critical point (239°C and 8.09 Mpa) in the presence of vegetable oil, Saka *et al*. discovered that TAG could be converted to FAME within 240 s without requiring a catalyst (*41*). The authors found that the lack of a catalyst reduced the cost of materials for production and greatly improved purification, significantly reducing waste water generated relative to traditional means of biodiesel production. The traditional method of biodiesel production using an alkaline catalyst is very sensitive to the presence of water in the reaction. High water content favors the formation of soap instead of the ester product by hydrolysis of fatty acyl glyceride molecules. Water content in excess of 5% (*42*) is very common to waste vegetable oil, further complicating the production of biodiesel from this waste product. Kusdiana *et al*. *i*nvestigated the effect of water content of up to 36% in the supercritical conversion of rapeseed oil (canola oil) to biodiesel (*43*). Complete conversion of the rapeseed TAG to FAME by supercritical methanol was observed for all water contents tested. For comparison the authors examined the effect of water on the production of biodiesel by base- and acid-catalyzed reactions. They found that water content of 4% significantly affected the yield of FAME in both cases (*43*).

A significant limitation of the commercial, alkali-catalyzed process for the production of biodiesel is its inability to accommodate feedstock oil with a FFA content greater than 1%. The supercritical process was found to effectively complete the esterification of FFA while simultaneously accomplishing the transesterification of TAG. Kusdiana *et al*. conducted detailed studies of the esterification of oleic acid and the effect that increasing water content had on FAME yield. The authors demonstrated that complete conversion of oleic acid to methyl oleate can be achieved by supercritical methanol reaction, with water having a negligible effect on ester yields (*43*). Warabi *et al*. demonstrated that, in addition to methanol, ethanol, 1-propanol, 1-butanol, and 1-

octanol can effectively be used as the alcohol in the supercritical process to effect the esterification of FFA and the transesterification of TAG (*44*). It was determined in this study that each alcohol was capable of esterifying oleic acid at a faster rate than the transesterification of TAG, indicating that the supercritical process is even more effective at converting FFA to biodiesel than it is at converting TAG (*44*).

The supercritical methanol process for biodiesel production is certainly capable of accomodating low cost feedstock oil unsuitable for the alkali-catalyzed process with its insensitivity to both FFA and water content. However, the high temperatures of the process cause undesired side reactions to occur, especially with oils high in unsaturated fatty acids (*45*). The extreme temperatures and pressures required by this process would also significantly increase the cost of building and operating plants designed to utilize supercritical methanol.

Acid-catalyzed biodiesel production

A fourth method of biodiesel production, that has been mentioned several times in the preceeding text, utilizes an acid to catalyze the transesterification of vegetable oil. Like the base-catalyzed method, a mole of TAG is reacted sequentially with 3 moles of an alcohol, such as methanol, to produce 3 moles of FAME. A key difference between the base-catalyzed method and the acid-catalyzed method is that the former is incapable of producing FAME from FFA, while the acid catalyzed method can catalyze the esterification of FFA. **Figure 1-5** depicts the reaction mechanism of the acid catalyzed transesterification of TAG or the esterification of FFA. The variables of time, temperature, alcohol amount, and the catalyst type and concentration are important to the conversion of vegetable oil by an acid-catalyzed method.

The identity of the acid used is important as experiments conducted with sulfuric, hydrochloric, nitric, formic and acetic acid resulted only in significant biodiesel

Figure 1-5. Acid catalyzed transesterification/esterification reaction mechanism.

The reaction mechanism demonstrates why an acid catalyzed biodiesel production method can produce FAME from either FFA or TAG. “R” represents a long alkyl chain typically 15 to 17 carbons in length found in either a FFA or TAG molecule. In the case of a FFA “R1” represents a hydrogen atom. For a TAG molecule “R1” represents a diacylglycerol molecule.

accumulation occurring for the reactions containing sulfuric acid (*46*). Crabbe *et al*. examined the effect of increasing the catalyst (acid) concentration from 1% to 5% and reported that ester yields increased from 52% to 82%, respectively (*47*). Canakci and Van Gerpen reported similar results when varying the concentration of sulfuric acid in transesterfication reactions (*23*).

Yields for the transesterification of soybean oil with methanol have been reported to be highly influenced by the ratio of methanol to oil. Canakci and Van Gerpen studied the effect that varying the molar ratio of methanol:oil had on FAME yield by conducting experiments with methanol:oil molar ratios of 3.3:1, 3.9:1, 6:1, 20:1, and 30:1 (*23*). They determined that high methanol:oil molar ratios were necessary to achieve satisfactory yields of FAME as a 30:1 ratio resulted in 98.4% of TAG converted while the lowest ratio (3.3:1) resulted in a modest FAME yield of 77% (*23*). These results are consistent with the results obtained from other studies (*20*). The high methanol:oil molar ratio required by the acid-catalyzed method to achieve high yield of FAME contrasts sharply with 6:1 methanol:oil molar ratio required by the base-catalyzed method (*48*).

When FFA are esterified with methanol water is displaced. Because the reaction is an equilibrium reaction, water can in turn hydrolyze the methyl ester displacing methanol and reforming the FFA. As the reaction progresses this will become more and more pronounced until FAME will no longer be produced and equilibrium is reached. The effect of water and FFA content of the oil on the yield of FAME was examined, to determine how water already present and water formed during the reaction might affect the biodiesel yield. Canakci and Van Gerpen found that as little as 0.1% (w/w% of vegetable oil) water (distilled) added back to the vegetable reduced the ester yield (*23*). Inclusion of 5% water reduced ester conversion from 95.6% to 5.6% (*23*).

A significant obstacle to the commercial adoption of acid-catalyzed biodiesel production is the amount of time required to complete the conversion of vegetable oil to biodiesel. Freedman *et al*. reported that a methanol:oil molar ratio of 30:1 requires 69 hours to complete the conversion, and others have reported similar times (*48*). This is significantly longer than the short times required by the base-catalyzed method (see base-catalyzed biodiesel production section above). Freedman *et al*. found that by using a

longer chain alcohol, such as 1-butanol, higher reaction temperatures (boiling point 117°C) could be used without increasing the pressure within the reaction vessel. When the authors heated 1-butanol in a transesterification reaction with soybean oil, complete conversion was attained within 3 hours instead of the 69 hrs required when methanol is used as the alcohol reactant (*48*). The authors explained this increased rate of reaction on the increased temperature attainable by using longer chain alcohols. This is a reasonable explanation as others have noted that the rate at which the transesterification reaction proceeds increases with increasing temperature when methanol was included as the reacting alcohol (*47*). There is, however, an additional explanation. The increased rate of reaction observed with longer chain alcohols could be explained by the increased miscibility of vegetable oil and the longer chain alcohols, allowing the reaction to occur throughout the sample and not just at the interface between the methanol and oil phases. This possibility was not explored by Freedman *et al*. Boocock *et al*. examined the reaction rate of the base-catalyzed methanolysis and butanolysis of soybean oil and found that methanolysis proceeded at a rate 15 times slower than did butanolysis (*49*). The authors examined whether the rate of methanolysis could be increased by adding a co-solvent such as tetrahydrofuran to the reaction. When 1.25 volumes of THF per volume of methanol was added to the base-catalyzed reaction a single phase system was formed in which methanolysis was observed occur as rapidly as did butanolysis without the addition of a co-solvent (*49*). Guan *et al*. observed that the mass transfer resistance typical of methanol and vegetable oil transesterification reactions could be eliminated by the addition of a co-solvent (*50*). Dimethyl ether was added to the methanol and oil transesterification reaction generating a single phase, greatly improving the reaction rate (*50*). Additionally, the authors were able to reduce the methanol:oil molar ratio needed to obtain 100 % conversion from 30:1 reported by Freedman *et al*. to 6:1 in the presence of

dimethyl ether (*50*). These experiments demonstrate that the rate at which biodiesel can be produced can be increased significantly by conducting the transesterification reaction in a single phase.

Commercially, acid-catalyzed biodiesel production has not gained broad acceptance, despite the potential of this method to produce biodiesel from low cost feedstock oils, high in FFA. The drawbacks of high methanol:oil molar ratios and time required to reach complete conversion have limited its applicability. In 2001 Canakci *et al*. developed a novel two-step process to produce biodiesel from sources of oil high in FFA that combined the benefits of FFA esterification imparted by an acidic catalyst with the ability of the alkaline catalyst to rapidly convert TAG to biodiesel (*28*). The concept of the process is to esterify the FFA using an acid catalyzed process reducing the FFA content to levels suitable for alkaline transesterification. With the FFA levels reduced, the remaining TAG can then be converted to FAME by the rapid base-catalyzed method. Although effective at utilizing feedstock oil high in FFA, the two-step process requires excessive volumes of methanol and catalyst. Chapter 2 investigates an approach to effectively convert oils high in FFA without the drawbacks associated with the two step process. This approach seeks to enhance the rate of FAME formation from both FFA and TAG in a single step without requiring excessive volumes of alcohol or catalyst.

Biodiesel Quality

The quality of biodiesel is important to the proper performance of the fuel in a diesel engine. ASTM D6751(American Society for Testing and Materials) and EN 14214 are two standards that are used to certify the quality of biodiesel. ASTM D6751 is commonly used in the US and Canada to evaluate the quality of biodiesel, while EN 14214 is a European standard. The standards specified by ASTM D6751 are listed in **Table 1-1**. A discussion of key specifications found in this standard can be found in the following paragraphs.

Table 1-1. Specifications for biodiesel (B100) – ASTM D6751

Property	ASTM Method	Limits	Units
Calcium and magnesium, combined	EN 14538	5 max	ppm (μg g^{-1})
Flash point	D 93	130 min	ºC
Methanol content	EN 14110	0.2 max	% mass
Water and sediment	D 2709	0.05 max	% vol.
Kinematic viscosity, 40ºC	D 445	1.9 – 6.0	$mm^2 s^{-1}$
Sulfated ash	D 874	0.02 max	% mass
Sulfur			
S 15 grade	D 5453	0.0015 max	% mass (ppm)
S 500 grade	D 5453	0.05 max	% mass (ppm)
Copper strip corrosion	D 130	No. 3 max	
Cetane	D 613	47 min	
Cloud point	D 2500	Report	ºC
Carbon residue 100% sample	D 4530	0.05 max	% mass
Acid number	D 664	0.50 max	mg KOH g^{-1}
Free glycerin	D 6584	0.020 max	% mass
Total glycerin	D 6584	0.240 max	% mass
Phosphorus content	D 4951	0.001 max	% mass
Distillation, T90 AET	D 1160	360 max	ºC
Sodium and potassium, combined	EN 14538	5 max	ppm
Oxidation stability	EN 14112	3 min	hours
Cold soak filtration	Annex to D6751	360 max	s
For use in temperatures below -12ºC	Annex to D6751	200 max	s

Characteristics of biodiesel affecting quality and performance

Petrodiesel is a collection of many different compounds that collectively give the fuel its characteristics. Petrodiesel contains a mixture of straight and branched hydrocarbons as well as aromatics. The composition of petrodiesel was found by GC/MS to contain a mixture of carbons ranging from nonane (C9) to tetracosane (C23) with tetradecane (C14) being the most prominent constituent. However, the composition of petrodiesel is likely to vary based on the oil source and different blends throughout the year. Biodiesel on the other hand contains primarily straight chain fatty acid alkyl esters that vary only in carbon chain length and the number and position of double bonds

present in the carbon chain. The carbon chain lengths are almost always even numbered with chains of 16 and 18 carbons constituting the majority of all fatty acids found in oilseeds. The relative concentrations of individual fatty acids within biodiesel heavily influence the properties of the fuel. The use of biodiesel as a petrodiesel substitute offers some advantages such as reduction of most exhaust emmissions, biodegradability, improved lubricity, and can be produced domestically (*16*, *17*). Some disadvantages of substituting biodiesel in place of petrodiesel include susceptibility to oxidation, poor performance in cold weather and higher NO_x emissions (*51*). An examination of how the fatty acid composition of the biodiesel feedstock can influence each of these parameters is presented.

Cetane number is a common descriptor used to describe the quality of a diesel fuel in a manner analogous to the octane rating of gasoline. Cetane number (CN) is a dimensionless value that describes the time required for ignition to occur once a diesel fuel is introduced into the combustion chamber. The CN of hexadecane (C16), an optimal diesel fuel, has been arbitrarily set to 100 and a 2,2,4,4,6,8,8-heptamethylnonane, a poor diesel fuel is arbitrarily set to 15 (*52*). All other fuels are compared to these two standards. The CN of petrodiesel has been experimentally determined to be 46 (*51*, *53*). Knothe *et al*. determined the CN of several neat FAME (*54*). They found that CN increased with saturation and chain length of the fatty acid while unsaturated fatty acids were found to have lower CN. Methyl stearate (C18:0) was found to have a CN of 101 and methyl linoleate (C18:2) recording a value of 38 (*54*).

The dependence of CN on the degree of saturation and chain length of the FAME is significant as studies of the combustion of individual FAME have determined that NO_x emissions increase with increasing number of unsaturated bonds (*51*, *55*). Others have reported that NO_x emissions increase with decreasing CN (*52*, *53*, *56*). Soybean oil typically contains a fatty acid profile that is 25% oleic acid (C18:1) and 50% linoleic acid

(C18:2). Tat *et al*. report the emissions testing of a biodiesel produced from a high oleic acid soybean variety containing 85% oleic acid and 4.3% linoleic acid. The combustion of high oleic acid biodiesel produced significantly less NO_x (*55*). Ladommatos *et al*. recorded the NO_x emissions of petrodiesel with differing amounts of cetane improver additive included. They observed that as the CN increased the NO_x emissions decreased (*56*). It should be noted that the increased NO_x emissions reported for biodiesel fuel relative to petrodiesel is very minimal. Knothe *et al*. reported that the NO_x emissions recorded for the neat FAME methyl palmitate (C16:0) and methyl laurate (C12:0) were actually decreased relative to petrodiesel (*51*). These studies collectively indicate that NO_x emissions from engines using biodiesel could be reduced by preparing biodiesel from fuels with less unsaturated fatty acids.

A significant problem of using biodiesel is its relatively high melting point. Two measurements, cloud point and pour point, describe the suitability of a biodiesel fuel for use in colder climates. Cloud point is a measurement of the temperature at which crystallization begins to occur in biodiesel. A second measurement, pour point, describes the temperature at which biodiesel ceases to flow. As with other properties of biodiesel the cloud and pour points are directly related to the fatty acid composition of the fuel (*54*). This is apparent when comparing the cloud point of biodiesel fuels produced from different feedstock oils, which exhibit a general trend of decreasing cloud point with an increasing proportion of unsaturated fatty acids. Saturated fatty acids exhibit the highest melting points. Methyl stearate and methyl palmitate exhibit melting points of 39°C and 30°C respectively. Unsaturated FAME have the lowest melting temperature. The melting temperature decreases with increasing number of double bonds as can be seen in methyl oleate (C18:1, -20°C), methyl linoleate (C18:2, -35°C), and methyl linolenate (C18:3, -52°C). The melting temperature also decreases with decreasing chain length.

The mono-unsaturated FAME methyl oleate (C18:1), methyl palmitoleate (C16:1), and methyl myristoleate (C14:1) exhibit excellent cold flow properties with melting temperatures of -20ºC, -34ºC, and -52ºC, respectively (*54*). The cold temperature properties of biodiesel can also be improved by using longer chain alcohols in the transesterification process. Lee *et al*. reported that the cloud point decreases with increasing chain length of the alcohol and for increased branching of the alcohol (*57*, *58*).

Because the properties of biodiesel depend entirely on the chemical composition of its constituent FAME, it is implied that the performance of biodiesel could be improved by selecting a feedstock oil enriched for a particular fatty acid. As an example of such an approach, the poor cold temperature properties of biodiesel could be solved by selecting a soybean plant that has a high content of linolenic acid. However, by solving the problem of cold temperature performance by increasing the concentration of linolenic acid, the CN and NO_x emissions would be negatively affected. A bigger problem however would result in a fuel with low oxidative stability (*54*). The double bonds of unsaturated fatty acids are highly susceptible to reaction with oxygen. The oxidation could cause polymerization of the FAME, resulting in the formation of insoluble material ideally suited for plugging the fuel delivery system (*59*).

The ideal FAME to be used as biodiesel should have a low melting point, be resistant to oxidation, and must maintain the minimum CN value of 47 specified for biodiesel by ASTM D6751. Methyl oleate, the fatty acid methyl ester of the monounsaturated oleic acid (C18:1), has a favorable CN of 56 and is resistant to oxidation. Its melting point (-20ºC), while much improved over soybean biodiesel, is still not low enough for extremely cold winters (*54*). A better choice would be methyl palmitoleate (C16:1) because of its low melting point of -34ºC that would withstand very cold winters (*54*). Palmitoleic acid is not a common fatty acid to oils from oilseeds. It is

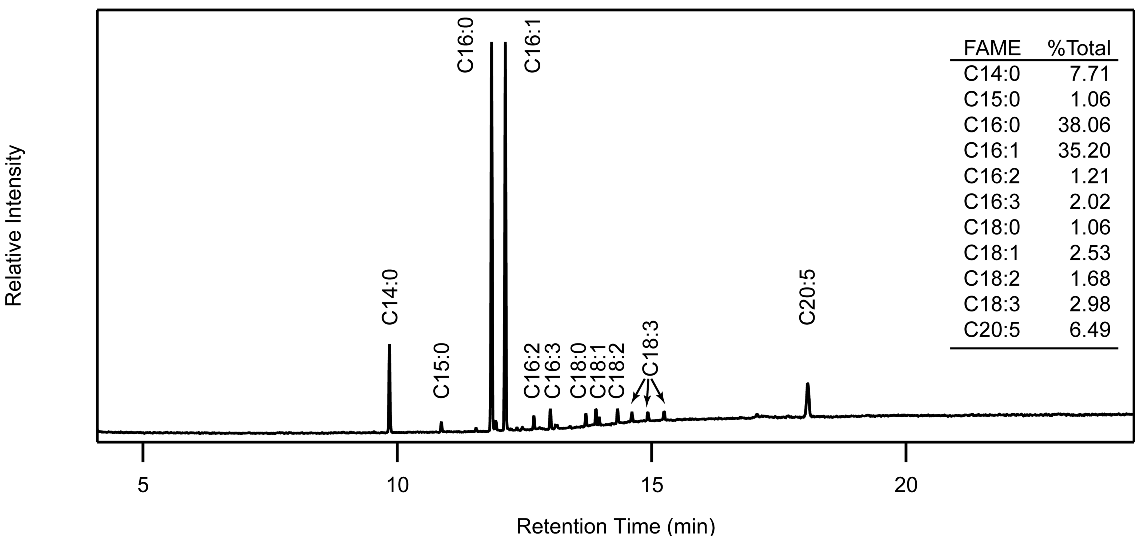

Figure 1-6. GC/MS trace of the fatty acid profile of the diatom, *Chaetoceros gracilis*. The identity of methyl esters are indicated by CX:Y notation where X is the number of carbons present in the fatty acid chain and Y is the number of double bonds. The relative percentage of each fatty acid is listed to the right. Data was collected on a Mass spectrometer (GCMS-QP2010S, Shimadzu Scientific) equipped with a stabilwax column (30 m, 0.25 mm ID, and 0.10 μmol film thickness).

however a major constituent of oils obtained from microalgae such as diatoms as can be seen in the lipid profile of *Chaetoceros gracilis* shown in **Figure 1-6.** In summary, the major technical challenges associated with biodiesel can be alleviated by choosing carefully feedstock oils enriched in monounsaturated fatty acids, particularly palmitoleic acid.

Methods for biodiesel analysis

The quality of biodiesel is a serious concern to the biodiesel industry. As a product of transesterification, glycerol is produced as a byproduct. To obtain high quality biodiesel glycerol must be removed as its presence in the fuel can lead to deposits within

the engine. The glycerol is not very soluble in FAME and because it is more dense quickly forms a lower phase and can be removed. Washing steps with water are typically required to remove all of the free glycerol from the biodiesel. Glycerol can also remain in the biodiesel as mono-, di-, or triglcyerides due to incomplete transesterification reactions. This has a negative effect on the viscosity and cold temperature performance of the fuel as well as issues with deposits forming within the engine. Several methods exist to evaluate the completion of the transesterification reaction and to quantify the amount of free and total glycerol remaining in the biodiesel, the most common of which being gas chromatography.

Gas chromatography utilizes a coating on a glass capillary and the variation of temperature to separate compounds based on their boiling points and their interaction with the coating of the interior of the glass capillary. Peaks of individual compounds detected by the detector, typically flame ionization detector (FID), are integrated to determine their areas. To quantify a peak the detector must first be calibrated with a pure compound similar to the compound of interest. This is done by injecting samples of known concentration and plotting their area against the concentration of the analyte, producing a straight line. The equation of this line is then used to determine the concentration of the unknown peak, assuming a similar detector response. ASTM has published a method (D 6584) for determining the amount of free and total glycerol (includes glycerol bound in mono-, di- and triglyceride) remaining in the fuel (*60*). The method successfully separates each of the compounds that could be present in a transesterification reaction. An example of a chromatogram that results from the analysis of a biodiesel transesterification reaction is shown in **Figure 1-7.**

Additional analytical techniques have been used to determine the extent of the biodiesel reaction but are either not widely used or are not as sensitive to minor components as gas chromatography. ^{1}H NMR can determine the level of completion of a

transesterification reaction by determining the ratio of integrated signals exclusive to FAME to those that are common to both FAME and acylglycerides (*61*, *62*). This has been done for both methyl esters and ethyl esters (*63*). Chapter 2 describes in detail this method and how it was adapted to determine the percentage conversion of TAG to fatty acid butyl esters. FT-Raman and near infrared spectroscopies have also been succesfully used to monitor the progression of a transesterification reaction (*18*, *63*). Although

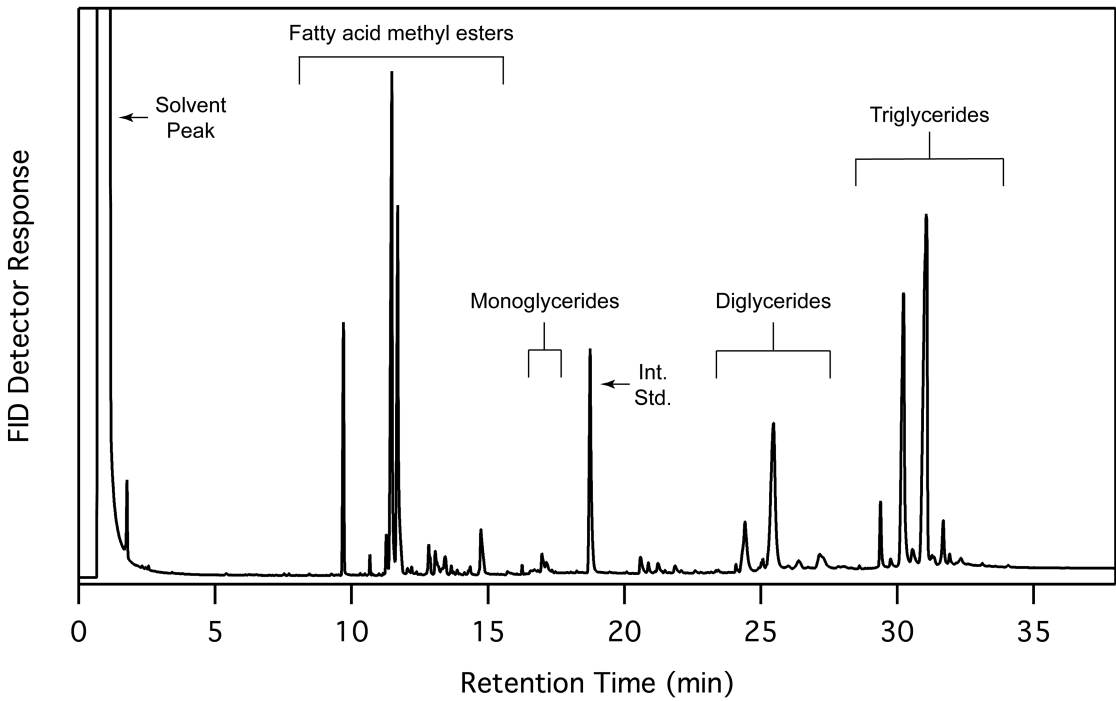

Figure 1-7. GC chromatogram of an algal oil transesterification reaction in progress. A sample of the transesterification reaction of algal oil from the diatom *C. gracilis* was analyzed on a gas chromatograph (GCMS-QP2010S, Shimadzu Scientific) equipped with an FID detector. Samples were injected onto an RTX-Biodiesel column (Restek) and subjected to the following temperature program: 60°C for 1 min, 10°C min^{-1} to 370°C, hold for 10 min.

alternatives exist, gas chromatography remains the most often used method for determining the amount of free and bound glycerol in biodiesel. Chapter 3 describes the utilization of gas chromatography as a tool to aide in the development of an optimal procedure for producing biodiesel from algae.

Biodiesel Potential in the United States

The technical merits of biodiesel and the benefits associated with its use in place of petrodiesel have been discussed. Various methods of the production of biodiesel and the technical challenges related to each have been reviewed. Analytical techniques used to evaluate the level of biodiesel conversion and to determine properties of the fuel have been outlined. What has not been discussed is whether enough biodiesel can be effectively produced to eliminate the need for petrodiesel and empower countries particularly the United States to become energy self-sufficient. Technically, biodiesel has the potential to replace petrodiesel entirely; a discussion of whether that can be done practically follows.

The United States consumed 1.33 billion barrels (bbl)(42 gallons per barrel) of petrodiesel in 2009, down from an all time high of 1.53 billion bbl in 2007 (*1*). Petrodiesel is a major product of crude oil refining; 27% of crude oil consumed in the United States is used in the production of petrodiesel (*1*). Replacing petrodiesel with a

Table 1-2. Biodiesel potential in US from oilseed crops

Oilseed crop	Acres harvested in US (2009)[a]	Oilseed yield (lbs per acre)[a]	Oil content of oilseed	Oil yield (gal per acre)	Total potential biodiesel yield (gal)	Percent of US diesel consumption[c]
Soybean	76,372,000	2,640	18 %	60	4.6×10^{9}	8.4 %
Sunflower	1,653,500	1,563	40 %	67	1.1×10^{8}	0.22 %
Safflower	165,500	1,462	38 %	74	1.2×10^{7}	0.020%
Canola	900	1,700	40 %	74	6.7×10^{4}	1.2×10^{-4} %

[a]Data obtained from the National Agricultural Statistics Service-USDA www.nass.usda.gov, accessed on 11/5/2010

[c]1.3 billion bbl of petroleum diesel consumed in US for year 2009, data obtained from the Energy Information Administration. www.eia.doe.gov, accessed on 11/5/2010.

renewable fuel such as biodiesel will require a lot of land. **Table 1-2** lists common seed oil crops grown in the United States, the acreage grown, and the amount of biodiesel that could be produced from the oil. The most planted oilseed crop in the United States is, by far, soybeans. Cultivated land is in excess of 76 million acres, resulting in 4.60 billion gallons of vegetable oil (*64*). While the size of the soybean oil crop is astounding, dedicating it all to biodiesel production could only meet 8.4% of petrolium diesel demand (*1*). Based on the oil yield of soybeans it is apparent that biodiesel produced from this crop could not supply the demand for petroleum diesel. At present only a small portion of soybean oil is used to produce biodiesel, as soybean oil is a significant component of the human diet

The economic and evironmental consequences that can potentially occur as a result of growing energy crops on arable soil in place of food crops has been the source of much debate. In the case of soybean oil production, only a fraction could effectively be diverted from food consumption to biodiesel production without seriously impacting food prices and supplies. In addition to potential impacts on food prices and supplies, recent high profile studies have questioned the potential of biofuels to achieve their goal to reduce greenhouse gas emissions by displacing the use of fossil fuels (*65*, *66*). These two studies explored the consequences of land use change associated with increased demand for biofuels. Land use change occurs when land that is currently not in agricultural food production is cleared and put into production. Searchinger *et al*. contended that changing land currently being used for food production to the production of a biofuel crop will cause land elsewhere in the world to be cleared to make up for the loss in food production (*65*). This is termed indirect land use change. Such an action results in both the release of sequestered carbon from the cleared land and the potential of future carbon sequestration that would have continued had the land been left unchanged. The second study explored the consequence of directly clearing land not currently in agricultural

production for the cultivation of biofuel crops (*66*). The authors of the second study arrived at a similar conclusion as did the authors of the first; clearing land for biofuel production results in a greater amount of greenhouse gas emissions than is saved by the production of biofuel crops.

Microalgae: Alternative Biodiesel Feedstock

The low potential of oilseed crops to meet the demand for petroleum diesel combined with the potential impact that this approach may have on both food markets and on greenhouse gas emissions has underscored the need for an alternative feedstock for the production of biodiesel. The ideal biodiesel feedstock would have a much higher oil productivity (gallons of oil $acre^{-1}$ day^{-1}) than oilseed crops and could be cultivated on marginal lands unsuitable for agricultural use. The utilization of waste-oil to produce biodiesel would have a positive effect on greenhouse gas emissions and would not impact food. Chapter 2 details the optimization of reaction conditions to convert waste oil to biodiesel. Biodiesel from waste vegetable oil is a good use of a resource that would otherwise require disposal. However, due to the limited quantity of waste vegetable oil, biodiesel produced from this resource will not be a significant factor in displacing fossil fuel use.

Microalgae (photoautotrophic microorganisms) have been investigated as a potential feedstock for biodiesel production (*67*, *68*). A significant obstacle faced by those hoping to increase the cultivation of energy crops worldwide is the lack of fresh water for irrigation. Unlike terrestial plants, many species of microalgae can thrive in water with salinity several times that of the ocean. This characteristic enables the cultivation of microalgae in many arid areas of the world using seawater or water from saline aquifers. Wastewater has also been looked at as not only a potential source of water for microalgal growth but also as a source of phosphorus and nitrogen, both important nutrients (*69*). In wastewater phosphorus and nitrogen are considered to be contaminants and are regulated

by the EPA. When wastewater containing significant concentrations of these two contaminants is released into rivers and lakes, the blooms of algae that occur lead to the eutrophication of the surrounding water and aquatic life is endangered. The use of wastewater as a medium for microalgal culture has two potential benefits, first the cost of algal culture is reduced by decreasing the input requirement of essential nutrients and secondly the water is remediated when the growing algae take up nitrogen and phosphorus from the surrounding water. Utilizing wastewater for microalgal cultivation has the potential to yield both algal biomass for biofuel production and clean water.

Microalgae offer a number of attractive benefits as a biodiesel feedstock relative to terrestial plants. Microalgae, like plants, utilize sunlight, water, CO_2, and nutrients like nitrogen and phosphorus to grow. Microalgae are more efficient at solar energy conversion than are terrestial plants (*68*). Sunlight conversion efficiencies of 3-9% have been reported for microalgae (*68*) compared to theoretical maximums of 2.4% and 3.7% (*70*) reported for C3 and C4 crops respectively based on the calculated solar radiation intercepted by the leaf canopy across a full growing season. Chisti reports that microalgae, assuming a lipid content equal to 30% of the cellular dry weight (CDW), has the potential to produce 6,275 US gallons acre^{-1} year^{-1} (*71*). This is an extremely large number compared to the 48-60 US gallons acre^{-1} year^{-1} productivity of soybeans.

Caution must be taken, however, when considering a productivity claim for microalgae, as very limited amount of growth data is available for large scale microalgal cultivation. Recently many exaggerated productivity claims have been made. In the same report, Chisti claims a high yield potential of 14,635 US gallons acre^{-1} year^{-1} for microalgae based on an extreme lipid content of 70% CDW (*71*). This is unrealistic. The maximal lipid content observed in our laboratory is ~40% CDW. Assuming an organism was capable of achieving such an extreme lipid content, it would do so at the expense of biomass accumulation. Chisti, and many others, do not account for the trade off between

lipid content and growth rate. Both processes cannot occur simultaneously at high rates. Williams and Laurens report that a negative relationship exists between growth rate and lipid productivity (*72*). More realistic numbers can be attained by referring to the few larges-scale outdoor demonstrations of microalgal cultivation. The US Department of Energy sponsored a research program, referred to as the aquatic species program, that conducted outdoor demonstrations of microalgal cultivation. Yields of 94 metric tons acre^{-1} year^{-1} were obtained (*73*). At a lipid content of 30% CDW, this would correspond to an annual production of 1,432 US gallons of biodiesel acre^{-1} year^{-1}. This number, 30 times the productivity of soybeans, is a reasonable expectation for microalgal biodiesel productivity.

Strain selection is an important factor to the success of biodiesel production from microalgae and is an active component of algal biofuels research. Many strains have been identified that achieve a high oil content, accumulating neutral lipids such as TAG. The ideal strain will maintain a rapid growth rate without sacrificing lipid production. This is a difficult challenge. Many strains have demonstrated significant TAG production only after an external stress has been applied, slowing the growth rate significantly (*67*). The most common way to stress microalgae is to starve the culture for a specific nutrient required for growth. This is achieved most often by limiting nitrogen available to the culture or removing it altogether, resulting in an increased lipid content (*74*). Limitation of both phosphorus and silicate (diatoms) have been reported to induce lipid production as well (*75-77*). Inducing lipid production by starving the culture of a key nutrient required for growth has, as a consequence, a corresponding decrease in the rate of growth. This can result in lower lipid yields despite the higher lipid content. Griffiths et al. reviewed growth data for phototrophic microorganisms available in the literature and found that the species that were observed to have the best lipid productivity rates were not always the species with the highest lipid content (*78*).

The quality of microalgal lipids is also important in selecting strains for the production of biodiesel. As is the case for biodiesel produced from oilseeds, the fatty acid profile of the microalgal species determines the properties of the biodiesel produced from its oil. This fatty acid profile varies considerably from one species to another. Some general trends have observed among green algae and diatoms. Green algae have longer chain fatty acids (18 carbons or more) with a greater degree of unsaturation than occurs in diatoms, which have shorter (predominantly 16 carbons), saturated fatty acids.

Table 1-3. Fatty acid composition of select algal species.

Fatty Acid[a]	*Neochloris oleoabundans*	*Chaetoceros gracilis*	*Amphora coffeaformis*	BA117[c]	*Tetraselmis suecica*	*Chlorella* sp.
C14:0	—	8.91	3.7	—	—	—
C15:0	—	1.07	0.72	—	—	—
C16:0	15.26	42.23	23.28	11.5	25.33	24.44
C16:1	2.05	35.06	24.84	2.02	2.78	3.6
C16:2	2.74	—	1.18	6.91	0.4	4.2
C16:3	2.61	—	0.94	10.29	1.33	6.5
C16:4	—	—	—	—	6	—
i-C17:0	—	—	—	—	—	1.77
C17:0	—	—	—	—	—	4.2
C18:0	2.03	1.77	0.7	1.51	0.91	1.41
C18:1	44.25	—	2.57	35.76	34.03	16.56
C18:2	23.69	0.97	5.95	13.07	6.53	22.88
C18:3	7.37	1.03	2.82	18.93	12.61	14.05
C18:4	—	—	—	—	4.28	—
C20:?[b]	—	0.95	—	—	—	—
C20:3	—	0.25	0.31	—	—	—
C20:4	—	0.53	6.16	—	1.38	—
C20:5	—	7.17	25.93	—	4.44	—
C24:0	—	—	0.88	—	—	—

[a]Individual Fatty acid methyl esters (FAME) prepared from whole algae cells were identified by GC-MS. FAME are reported as C (carbon) #(number of carbon atoms present in fatty acid) :# (number of unsaturated bonds in fatty acid.
[b]Degree of unsaturation of this fatty acid is uncertain.
[c]BA117 was isolated from the Willard Bay area of Great Salt Lake.

Table 1-3 lists the relative fatty acid composition of several micoalgal species that have been determined in the Seefeldt laboratory. Careful control of culture conditions will need to be maintained as variables such as salt concentration and temperature can alter the relative concentrations of individual fatty acids.

The general composition of algal lipids is not unlike lipids obtained from terrestial plants. The lipids contain TAG, FFA, and sterols among other lipid materials. Conversion of these lipids can simply be accomplished by one of the many methods used to convert the lipids obtained from oilseeds. This is evidenced by published reports of biodiesel production from algae using conventional base-catalyzed transesterification of the extracted lipids (*79-81*). The biggest difference between the production of biodiesel from algae and oilseeds is the method of extraction required to obtain the oil. Oilseeds can be pressed to extract the oil mechanically. This would be a poor method for oil extraction from microalgae. The oil is separated into individual cells that are approximately 6 μm in diameter. Simple extraction with hexanes is not universally effective for algal species. Experiments conducted to determine the best method for lipid extraction have determined that a cell disruption technique is necessary in addition to solvent extraction (*82*). The best combination of cell disruption technique and solvent for maximal lipid extraction varies from one species to another (*82*, *83*). This poses a problem to the industrial production of biodiesel, requiring plants to change extraction methods depending upon the microalgal strain being used. Microalgae also differ from oilseeds in that the microalgal cell contains both the photosynthetic machinery contained in thylakoid membranes and the lipid containing vesicles, whereas oilseeds do not perform photosynthesis and do not have these membranes. Extraction of microalgae with only a neutral solvent leaves the fatty acids contained within the membrane behind. On an analytical scale mixtures of chloroform and methanol are capable of extracting both polar membrane lipids and neutral lipids, but are impractical on an industrial scale due to

the toxic nature of chloroform (*84*, *85*). Chapter 3 of this dissertation addresses the investigation of using alcohol directly with algal biomass to both extract and convert the total cellular lipids of microalgae. Efficient extraction of microalgal lipids remains an obstacle to the production of biodiesel from microalgae.

Wax Esters

Wax esters are composed of fatty acids in ester linkage with fatty alcohols. They are highly valued and are commonly used as lubricants, ingredients in cosmetics and pharmaceuticals, food additives, and for their general utility in various industrial applications. Wax esters have a variety of functions in nature and can be found in both prokaryotes and eukaryotes. They are a primary component of the spermaceti fluid found in the spermaceti organ of the sperm whale. Sperm whales were prized for this valuable fluid, motivating intense hunting of sperm whales. Sperm whales served as the source for wax esters until the worldwide ban on sperm whale harvesting. Wax esters are also a primary component of the plant cuticle, where, along with other lipid components, serve to seal the surface of the plant, protecting it against excessive water loss (*86*). Some wax esters can be harvested commercially from the cuticle of a limited number of plants, most notably the carnauba palm (*Copernicia* ceriferia) (*87*). The jojoba plant (*Simmondsia chinensis*), a shrub native to the desert southwest, curiously accumulates wax esters in its seeds instead of the typical TAG found in other plants. Jojoba cultivation has been explored as a possible commercial source of wax esters. However, challenges related to its cultivation have limited the availability of this oil (*88-90*). Because of the small number of commercial sources of wax esters, their further industrial adoption is limited both by the low supply, which restricts their use to high value applications, and the low

diversity of their chemical structure. A sound understanding of the enzymes involved in the production of wax esters would lay the foundation for their use to commercially produce wax esters with a desired chemical structure and properties.

Wax ester accumulation among bacteria has been frequently reported for the genera *Acinetobacter*, and less frequently for the genera *Marinobacter, Moraxella, Micrococcus, Rhodococcus, and Alcanivorax*. Wax ester accumulation is induced when these organisms are cultured under low nitrogen conditions (*91*). The stored wax esters are thought to confer a survival advantage to the organism, allowing them to take advantage of nutrients quickly as they become available. Many of of these genera are marine organisms known to grow on *n*-alkanes, and are important to the degradation of hydrocarbons that can, at times, contaminate the worlds' oceans. Studies have suggested that populations of these bacteria can rapidly increase from below detection in pristine waters to comprise up to 91% of the total bacterial community in waters containing hydrocarbon contaminants (*92*).

The composition of the wax esters accumulated in these organisms has been observed to depend heavily upon the substrates that they grow on. Isoprenoid wax esters have been reported to accumulate in various hydrocarbon degrading microbes when grown on compounds such as squalene, phytol, and phytane (*93-97*). Growth of *Rhodococcus opacus* PD630 on phenyldecane in nitrogen limiting conditions resulted in the accumulation of phenyldecyl phenyldecanoate (*98*). The reasons for such a wide diversity of possible wax ester structures have to do with the substrate promiscuity of an enzyme in the biosynthetic pathway, which will be discussed further later in this section. Growth on an organic acid, however, results in a wax ester composition similar to the

fatty acid composition of the cellular membrane of the organism (*91*). These two results indicate that there are two separate pathways for the preparation of substrates for wax ester synthesis. One pathway involves the oxidation of the terminal carbon of alkanes resulting in the formation of a fatty alcohol, which can be further oxidized to the level of a fatty acid. It is apparent that wax esters can also be produced *de novo* from fatty acid biosynthesis. Environmental factors have been shown to affect the composition of wax esters from *Acinetobacter* and *Micrococcus* (*99*, *100*). The composition of wax esters obtained from the marine bacterium *Marinobacter aquaeolei* VT8 and growth conditions that affect their structure are discussed in Chapter 4.

In prokaryotes the biosynthesis of wax esters from fatty acyl-CoA has been proposed (**Figure 1-8**) to involve three steps and three separate enzymes. The first step involves the reduction of a fatty acyl-CoA (or an acyl carrier protein bound fatty acid) to

Figure 1-8. Proposed wax ester biosynthetic pathway for *Marinobacter aquaeolei* VT8. Wax ester biosynthetic pathway beginning with fatty acyl-CoA as the substrate. *Fatty aldehyde reductase has not previously reported from a wax ester accumulating bacterium.

the corresponding fatty aldehyde utilizing NADPH as the reductant (*101*). The fatty aldehyde is proposed to next be reduced to the level of an alcohol. An enzyme capable of catalyzing this reduction has recently been reported from the wax ester accumulating bacterium *Marinobacter aquaeolei* VT8 (*102*). The description of this enzyme can be found in Chapter 5. The final step of wax ester biosynthesis is catalyzed by a bifunctional enzyme possessing wax ester synthesis/diacylglycerol acyltransferase (WS/DGAT) activity (*11*). This enzyme utilizes fatty acyl-CoA as the acyl donor and either an alcohol (wax ester synthesis) or a diacylglycerol molecule (TAG synthesis) as the acyl acceptor.

The enzymes involved in the substrate reduction for wax ester synthesis in bacteria differ from those in plants. The jojoba plant synthesizes wax esters from very long-chain fatty acids and fatty alcohols. The fatty alcohols are generated from fatty acids by a single enzyme, Fatty acyl-CoA reductase (FAR), in an NADPH dependent fashion (*103*). This protein exhibited maximal activity toward very long-chain fatty acyl-CoA molecules (C24:1) and nearly no activity with palmitoyl-CoA (C16:0) (*104*). Reiser and Somerville identified a gene in *Acinetobacter* that when disrupted eliminated wax ester production. Complementing the mutants with the gene restored function. The protein product of the gene exhibited fatty acyl-CoA reductase activity reducing them to the corresponding fatty aldehyde in an NADPH fashion (*101*). The enzyme was most active with C16:0 fatty acyl-CoA molecules and had no activity with the very long-chain fatty acid C24:1. No fatty alcohol product was observed in the activity assays. The bacterial pathway for wax ester production relies on two separate enzymes to produce the fatty alcohol substrate for the wax ester synthase, whereas the plant pathway has a single

enzyme that catalyzes a four electron reduction of the fatty acid to produce the fatty alcohol substrate.

A novel dual function enzyme was identified in *Acinetobacter calcoaceticus* (*11*). The enzyme possesses both acyl-CoA:fatty alcohol acyl transferase activity (wax ester synthase, WS) as well as acyl-CoA:diacylglycerol acyltransferase (DGAT) activity. The WS/DGAT enzyme is responsible for wax ester and TAG production in bacteria. The enzyme has since been discovered in *Arabidopsis thaliana* and in petunia petals where the enzyme functions in the production of wax esters for the cuticle layer of the plant (*105*, *106*). WS/DGAT was found to be a promiscuous enzyme, utilizing a wide range of substrates as acyl acceptors in acyl transferase reactions (*12-14*). Straight chain alcohols ranging in size from ethanol to triacontanol (C30) were accepted as substrates with oleyl alchol being preferred. In addition to linear alcohols, mono- and diglyceride were used as substrates as were cyclic and aromatic alcohols (*10*). The ws/dgat enzyme prefers palmitoyl-CoA as the acyl-CoA substrate but will accept acyl-CoA molecules ranging in size from two carbons to twenty. Among WS/DGAT proteins a highly conserved HHXXXDG motif is present which was suspected to be involved in catalysis (*11*). This assumption was based on the importance of the same motif to catalysis in other acyltransferase proteins. Substitution of the first His residue from the WS/DGAT protein from *Acinetobacter* with leucine resulted in wax ester synthase activity that was 1% of wild-type. Substitution of the second His residue with leucine nearly resulted in the complete loss of activity. No significant activity was observed for the WS/DGAT protein with doubly substituted His residues. Substitutions of the conserved aspartate and glycine residues did not significantly alter the enzyme activity (*107*).

Wax esters can be produced synthetically from fatty acids and fatty alcohols in the presence of an acid catalyst or using the enzyme lipase (*36*). While both systems are effective at producing wax esters both require fatty alcohols as substrates. While lipases are the preferred enzyme for lipid modification, the use of WS/DGAT enzymes offer some advantages. These enzymes have broad substrate specificity that would allow their use to be tailor made for an application of interest. Lipid modifications can be made *in vivo* from inexpensive, renewable materials such as sugar (*10*). The WS/DGAT enzyme has been used to produce jojoba oil-like wax esters by co-expressing the WS/DGAT enzyme with the FAR from jojoba (*108*). However, this approach did not result in high yield of the jojoba-like wax esters. The authors attributed this to the low activity of the FAR enzyme toward C18 and C16 fatty acid chains. Identification of the bacterial enzymes that catalyze the same reaction will be essential to future efforts to produce wax esters commercially using modern biotechnological approaches. The WS/DGAT enzyme was also used to produce biodiesel (fatty acid ethyl ester, FAEE) *in vivo*, which the authors termed “microdiesel” (*109*). Microdiesel was produced by heterologously expressing, in *E. coli*, the *ws/dgat* gene from *Acinetobacter baylyi* along with the genes encoding pyruvate decarboxylase and alcohol dehydrogenase from the ethanol-producing bacterium *Zymomonas mobilis*. When grown on sugars alone no FAEE was observed. The authors concluded that *E. coli* was unable to provide significant fatty acid substrate to the WS/DGAT enzyme and, as a solution, provided oleic acid to the growth medium, resulting in significant FAEE production.

Enzymes from the wax ester biosynthetic pathway have tremendous potential to produce useful chemicals renewably. These enzymes catalyze many of the same

reactions that lipases catalyze. The benefit of WS/DGAT enzymes, is that they can carry out their application *in vivo* significantly reducing the cost of producing a similar chemical using a lipase. The WS/DGAT *in vivo* approach does not require expensive enzyme purification systems or expensive chemical materials. It would appear the only limit to producing an ester of chosen chemical composition is engineering the microorganism to overproduce the subsrates. Chapter 6 discusses an approach taken to improve *in vivo* biodiesel production using WS/DGAT enzymes from *M. aquaeolei*.

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CHAPTER 2

SYNTHESIS OF BIODIESEL FROM MIXED FEEDSTOCKS AND LONGER CHAIN ALCOHOLS USING AN ACID CATALYZED METHOD[1]

ABSTRACT

Biodiesel is typically synthesized from triacylglycerides derived from seed oils (e.g., soybean) and an alcohol (e.g., methanol) with base catalysis, yielding the fatty acid methyl ester, biodiesel. Alternative oil feedstocks (e.g., used cooking oil, rice bran oil, and algae) often have significant quantities of free fatty acids, which greatly complicate the synthesis of biodiesel using the base/methanol method. Here, we have explored a wide range of reaction conditions that optimize biodiesel production from mixed feedstocks containing high free fatty acids. To rapidly survey conditions, a microwave heated reaction was used to accelerate the reaction and the product was quantified by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Conditions were determined that allowed rapid and high yield conversion of oil feedstocks containing significant concentrations of free fatty acids into biodiesel using an acid catalyzed reaction with longer chain alcohols (such as n-butanol) at a slight molar excess. The conditions were replicated in a traditional heating method, where biodiesel yields greater than 98% were achieved in less than 40 min. Key properties of the resulting butyl-diesel were determined including cetane, pour point, and viscosity. The information presented should be valuable for the large scale production of biodiesel from mixed feedstocks that are difficult to utilize by the base/methanol method.

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INTRODUCTION

Biodiesel is an alternative fuel that can be used in place of petroleum-derived diesel with no modifications to the existing transportation system. Since biodiesel can be produced from biologically-derived oils (e.g., plant or animal) and alcohols (e.g., methanol), many benefits to its use have been discussed including less reliance on imported oil, lower pollution emissions, and lower net CO_2 production.[1,2] Biodiesel is composed of alkyl esters of fatty acids. These fatty acid esters are typically synthesized by the transesterification of triacylglycerides from seed oils (e.g., soybean) with a short chain alcohol (e.g., methanol) using either a base or acid catalyst (**Figure 2-1**). In the typical commercial process, biodiesel is synthesized from vegetable oil and methanol utilizing a base catalyst such as sodium hydroxide or potassium hydroxide. This method results in a high yield of biodiesel in a relatively short time. The use of this method, however, is limited to relatively clean vegetable oils containing very low concentrations of free fatty acids (less than 0.5%).[3] The presence of free fatty acids above this quantity in the oils results in the formation of soaps and emulsions, which complicate the washing of the biodiesel and removal of the glycerol. Given that many potential feedstocks for biodiesel production contain relatively high quantities of free fatty acids (**Table 2-1**), various methods have been reported for dealing with such mixed feedstocks.

One such approach is to use an acid catalyst such as sulfuric acid (H_2SO_4) or hydrochloric acid (HCl) instead of a base catalyst. One advantage of an acid catalyst is that free fatty acids and the fatty acid portion of the triacylglyceride are simultaneously converted into alkyl esters (biodiesel). However, it has been reported that the acid catalyzed method requires much longer reaction times to achieve substantial conversion

triacylglyceride: $R-CH_2CO-CH_2$ / $R-CH_2CO-CH$ / $R-CH_2CO-CH_2$ (arrows 1, 3)

$CH_3CH_2CH_2CH_2OH$ n-butanol — $R-CH_2COCH_2CH_2CH_2CH_3$ butyl ester (arrows 3, 2)

$CH_3CH_2CH_2OH$ n-propanol — $R-CH_2COCH_2CH_2CH_3$ propyl ester

CH_3CH_2OH ethanol — $R-CH_2COCH_2CH_3$ ethyl ester

CH_3OH methanol — $R-CH_2COCH_3$ methyl ester (arrow 4)

Figure 2-1. Compounds relevant to this study. Arrows indicate protons examined by ^{1}H- NMR spectroscopy as follows (with chemical shifts in parts per million). 1- glycerol protons (4.10-4.35 ppm); 2- alkoxy protons (quartet for ethyl ester, triplet for all others at 3.9-4.1 ppm) of esters formed from alcohols with two or more carbons; 3- α-methylene protons (triplet at 2.2-2.3 ppm) present in both triacylglycerides and fatty acid alkyl esters; and 4- methoxy protons (singlet at 3.6 ppm) present only in the fatty acid methyl esters.

to biodiesel.[4,5] It has also been reported that a large (30:1) molar excess of alcohol to triacylglyceride is needed to achieve high yields of biodiesel.[5,6] A very recent report investigated the production of biodiesel via acid catalysis using 1-butanol under a variety of conditions. We have performed a more detailed characterization of the specific parameters related to the production of biodiesel using the acid catalysis method as they relate to the size of the alcohol utilized, and further investigated how this technique can be applied to more diverse mixed feedstocks high in free fatty acids.[7]

Here, we have examined a wide range of reaction conditions in order to optimize

Table 2-1. Free fatty acid content of oils from different sources

Potential Biodiesel Feedstock	% Free Fatty Acid[a]	Reference
Jatropha curcas seed oil	15	19, 21
Rubber seed oil	17	20
Yellow grease	< 15	3
Brown grease	> 15	3
Crude palm oil	7	19
Used cooking oil	≥ 6	4
Rice bran oil	≥ 40	25
Madhuca indica	20	22

[a]Free fatty acid as % (w/w) of oil.

the yield of biodiesel production from feedstocks with varying free fatty acid content. Reaction times and product analysis were accelerated by the use of a microwave heated reaction that resulted in high conversion efficiencies in short times coupled with product quantification by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Parameters examined included the molar ratio of alcohol to triacylglyceride, temperature and time of reaction, the type and concentration of catalyst, and the type and molar ratio of alcohol. The optimal conditions were used in a traditional heating method to produce biodiesel in high yield from lipids containing high free fatty acids.

MATERIALS AND METHODS

Reagents. Soybean oil was food grade. Oleic acid (NF grade), sulfuric acid, methanol, ethanol, n-propanol, and n-butanol were purchased from Mallinckrodt (Hazelwood, MO) and were used without any further purification. Absolute ethanol was dried over 4 Å molecular sieve (Mallinckrodt, Hazelwood, MO) overnight prior to use.

Microwave reaction procedures. Analytical scale reactions were performed in a monomode microwave reactor (CEM Discover, Matthews, NC), with programmable power output over the range of 0 to 300 watts, temperature monitoring via an internal infrared sensor, and fully automated pressure control.[8] The reaction tubes used are sealed and can handle pressures up to 300 psi, allowing temperatures to be used above the boiling points of the alcohols. Reactions were stirred by means of an integrated magnetic stirrer and a Teflon-coated stir bar in the vessel. The reaction parameters of temperature, pressure, and power output were monitored in real-time via the instrument display. Esters of oleic acid were synthesized by placing 1 gram of oleic acid in a 10 mL sealed vial with the noted volume of alcohol. The oleic acid and alcohol were premixed, and concentrated sulfuric acid (18 M) was added to equal 5% (w/w) of the oil. The sealed vial was then inserted into the microwave cavity, the temperature was increased to the reaction temperature by the application of 25 watts of power, and the reaction was held at the reaction temperature for the programmed period of time. At the end of the reaction, the sample was rapidly cooled with an internal stream of air. Sodium bicarbonate was added to the reaction from a saturated solution to neutralize the remaining acid. The sample was then washed with water and centrifuged to separate the biodiesel from the water/alcohol layer. Biodiesel was prepared from soybean oil by the same method

described above, except that the soybean oil was substituted for the oleic acid.

Benchtop acid catalysis. A 6:1 molar ratio of n-butanol (30.6 grams) and soybean oil (60 grams) was heated in a three-necked 250 mL round bottom flask with constant mixing via a Teflon coated stir bar. When the reaction was at 80°C, 1.8 grams of concentrated sulfuric acid was added resulting in a final concentration of 3% (w/w) relative to oil. The reaction was allowed to proceed at 120°C with the temperature monitored by a thermometer inserted through a septum in one neck of the flask. Samples were removed through a second septum inserted in another neck of the flask, using a gas tight syringe. A condenser was attached to the third neck to retain solvent during heating. Samples (2 mL) were withdrawn every 10 min during the course of the reaction to monitor the reaction progress. Each sample was neutralized with a saturated sodium bicarbonate solution, washed with water, and centrifuged at 3300 rpm (Centrific Model 228, Fisher Scientific, Pittsburgh, PA) as described above to separate the biodiesel from the aqueous phase. After 70 minutes of refluxing at 120°C, the reaction was allowed to cool. The resulting biodiesel was then neutralized with sodium bicarbonate, and rinsed twice with 300 mL of water. The contents were allowed to separate prior to final analysis.

^{1}H-NMR analysis. ^{1}H-NMR experiments were performed at 7.05 T on a JEOL ECX spectrometer using $CDCl_3$ as solvent. ^{1}H (300 MHz) spectra were recorded at 23°C with a pulse angle of 45 degrees, and a relaxation delay of 2 s. The final spectrum was the average of 16 scans. The percentage yield (% Y) of methyl ester for the reaction between methanol and either oleic acid or soybean oil was calculated using the following equation:[9]

$$\% Y = 100\,(2I_4/3I_3) \quad (1)$$

where I_4 refers to the integrated peak area of the three methoxy protons (4, **Figure 2-1**) present only in the methyl esters and I_3 refers to the integrated peak area of the two α-methylene protons (3, **Figure 2-1**) present in free fatty acids, triacylglycerides, and methyl esters. The percentage yield (% Y) of alkyl ester for the mixed feedstock reaction with n-butanol and for the reaction between oleic acid and longer chain alcohols (other than methanol) was calculated from equation 2,

$$\% \, Y = 100 \, (I_2/I_3) \qquad (2)$$

where I_2 refers to the integrated peak area of the two methylene protons immediately adjacent to the oxygen atom (2, **Figure 2-1**) present in alkyl esters longer than methyl ester and I_3 is defined as above.[7]

The extent of reaction between soybean oil and longer chain alcohols cannot be calculated as described above. The signals resulting from the three glyceryl methylenic protons (1, **Figure 2-1**) overlaps with the signals from the methylene protons immediately adjacent to the oxygen atom (2, **Figure 2-1**) in the alkyl ester. Ghesti *et al.* developed an equation (3) to calculate the percentage yield (% Y) of alkyl ester for the reaction of triacylglycerides and longer chain alcohols that utilizes the methylenic protons from glycerol.[10]

$$\% \, Y = 100 \, [(I_R - I_L)/((I_R - I_L) + 2I_L)] \qquad (3)$$

In this equation, I_R is the sum of the integrated peak areas of the methylene protons immediately adjacent to the oxygen atom (2, **Figure 2-1**) and the glyceryl methylenic protons (1, **Figure 2-1**) from 3.95 to 4.20 ppm and I_L is the integrated peak area of the

glyceryl methylenic protons (1, **Figure 2-1**) from 4.20 to 4.35 ppm.

Biodiesel analysis. A sample of biodiesel produced from soybean oil and n-butanol using the method described above was subjected to testing by Bentley Tribology Services (Minden, NV) to determine key American Society for Testing and Materials (ASTM) parameters for biodiesel including: viscosity at 40°C (ASTM D445) and pour point (ASTM D97).

RESULTS

Acid catalysis of free fatty acids to biodiesel. In order to establish parameters that influence the conversion of triacylglycerides containing large amounts of free fatty acids to biodiesel using an acid catalyst, we first examined the efficiency of conversion of a free fatty acid alone or a pure triacylglyceride alone to the respective alkyl ester under various conditions. In these studies, conditions were selected that resulted in maximization of the differences between the variables as a way to probe the different conditions. Maximizing biodiesel yields was optimized in later experiments.

Oleic acid (*cis*-9-octadecenoic acid) was chosen as a representative fatty acid and its reaction with methanol was first examined. In these experiments, the extent of reaction was determined by comparing the integrated signal intensity for protons recorded in the ^{1}H-NMR spectrum monitoring the methoxy ($-OC\mathbf{H}_3$) protons (4, **Figure 2-1**), and the α-methylene (-$C\alpha\mathbf{H}_2$-) protons (3, **Figure 2-1**).[9] Reaction parameters that were examined included the molar ratio of alcohol to fatty acid, the type of alcohol, the reaction temperature, the amount of catalyst, and the time of reaction.

To approach the parameter of the ratio of alcohol to fatty acid, the amount of sulfuric

acid (H_2SO_4) was held constant at 5% (w/w) relative to fatty acid and a reaction temperature of 60°C was selected, which is just below the boiling point of methanol. The concentration of sulfuric acid used was comparable to the concentration used in earlier studies.[7] The effect of methanol to oleic acid molar ratios on reaction efficiency was evaluated by performing reactions at molar ratios of 2:1, 4:1, 7:1, and 10:1 (methanol:oleic acid). Four separate reactions at 1, 4, 8, and 16 minutes were performed for each selected molar ratio (**Figure 2-2**). The results of this experiment showed an increase in the conversion efficiency over time with the greatest efficiency observed at the highest ratio of methanol to fatty acid.

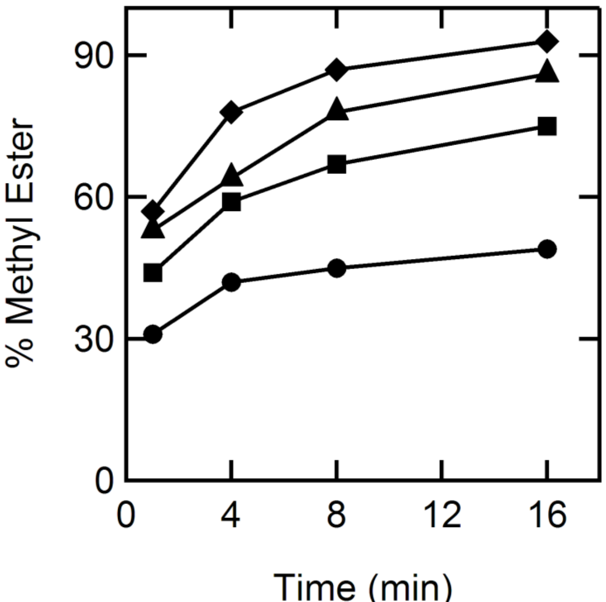

Figure 2-2. Effect of the methanol to oleic acid ratios on yield of methyl ester formed. All reactions were performed at 60°C with 5% (w/w) H_2SO_4 relative to fatty acid added. The percentage of methyl ester formed based on the maximum possible yield is plotted against the time of reaction. The methanol to oleic acid molar ratios were: ● 2:1, ■ 4:1, ▲7:1, ♦ 10:1.

Next, the effects of varying the type of alcohol on the reaction efficiency were examined. Methanol, ethanol, n-propanol, and n-butanol were reacted with oleic acid at an alcohol:oleic acid molar ratio of 4:1, with 5% (w/w) sulfuric acid, and a reaction temperature of 80°C (**Figure 2-3**). This reaction temperature was chosen to be between the boiling points for the selected alcohols. The percentage alkyl ester formation was determined as described in the Materials and Methods section. As can be seen in **Figure 2-3**, each alcohol resulted in effective conversion (85-90%) of oleic acid to the alkyl-ester within 16 min. Further, the results reveal that longer chain alcohols result in greater conversion efficiency under these

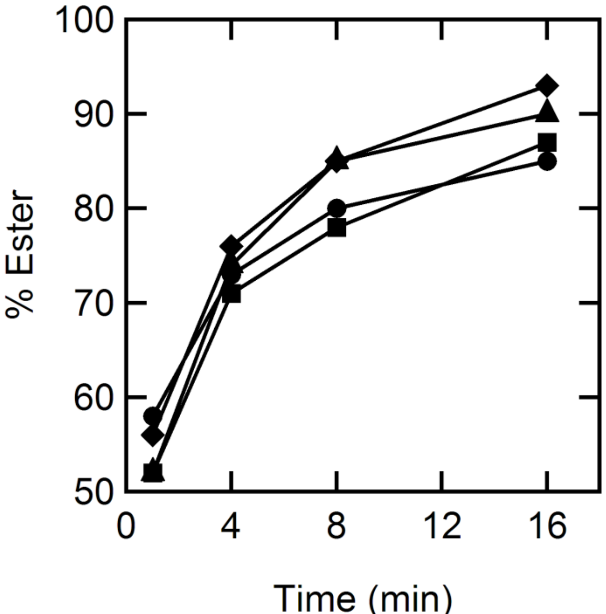

Figure 2-3. Effect of the alcohol type on the yield of alkyl ester formed with oleic acid. All reactions were performed at 80°C with 5% (w/w) H_2SO_4 relative to fatty acid added and a 4:1 alcohol:oleic acid molar ratio. The percentage of alkyl ester formed based on the maximum possible yield is plotted against the time of reaction. Alcohols used were: ● methanol, ■ ethanol, ▲ *n*-propanol, and ♦ *n*-butanol.

conditions than do shorter chain alcohols. The most effective alcohol was n-butanol.

Acid catalysis of triacylglycerides to biodiesel. Next, the conditions that influence the conversion of triacylglycerides (represented by soybean oil) to biodiesel were examined. The reaction of methanol with soybean oil as a function of time was first examined at a ratio of 30:1 (10:1 ratio of methanol to fatty acid) and a reaction temperature of 60°C with 5% (w/w) sulfuric acid. It was discovered that these conditions resulted in less than 2% conversion to the methyl ester even after 32 min. The inability of methanol to react with soybean oil under the same conditions utilized with oleic acid led us to evaluate the performance of longer chain alcohols in the transesterification of soybean oil.

Methanol, ethanol, n-propanol, and n-butanol were mixed with soybean oil at a 12:1 alcohol:soybean oil molar ratio (4:1 ratio of alcohol to fatty acid) and reacted at 80°C for 1, 4, 8, 12, and 16 minutes (**Figure 2-4**). The efficiency of reaction was found to increase with time for all of the alcohols, however the methanol and ethanol reactions were found to result in very low conversion efficiencies even after 16 min (less than 18%). In contrast, the n-propanol and n-butanol reaction efficiencies were much greater, achieving approximately 50% conversion over 16 min. For the reactions with the longer chain alcohols, the extent of reaction was determined by ^{1}H-NMR spectroscopy (**Figure 2-5**) as described in the Materials and Methods section.

Optimization of acid catalysis with n-butanol. Since n-butanol showed the best conversion efficiency for both oleic acid and triacylglycerides, reaction conditions with this alcohol were further optimized. One goal in this optimization was to minimize the amount of n-butanol used, as a way to lower material costs and to improve final purification of the biodiesel. In order to determine the minimum amount of n-butanol required to effectively

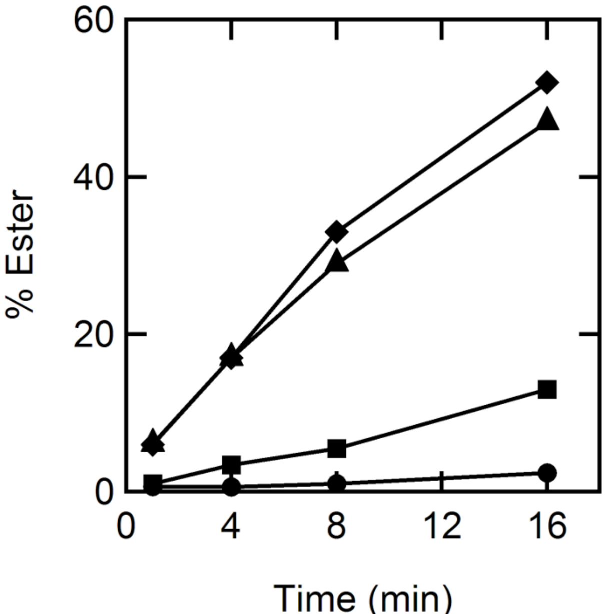

Figure 2-4. Effect of the alcohol type on the yield of alkyl ester formed with soybean oil. All reactions were performed at 80°C with 5% (w/w) H_2SO_4 relative to oil added and a 12:1 alcohol:soybean oil molar ratio. The percentage of alkyl ester formed based on the maximum possible yield is plotted against the time of reaction. Alcohols used were: ● methanol, ■ ethanol, ▲ *n*-propanol, and ♦ *n*-butanol.

esterify oleic acid, reactions were conducted with n-butanol:oleic acid molar ratios of 2:1 and 4:1 at times of 1, 4, 8, and 16 minutes at a temperature of 110°C using 5% (w/w) H_2SO_4 as catalyst (**Figure 2-6**). Under these conditions, even the lowest molar ratio of n-butanol to oleic acid (2:1) showed high conversion efficiency (> 95%) within 4 min of reaction. These results contrast with the slow reaction of methanol with oleic acid shown in **Figure 2-2**.

The transesterification of soybean oil with n-butanol was evaluated to determine what molar ratio of butanol to soybean oil is necessary to efficiently form biodiesel with acid catalysis. **Figure 2-7** shows the efficiency of conversion with molar ratios of n-

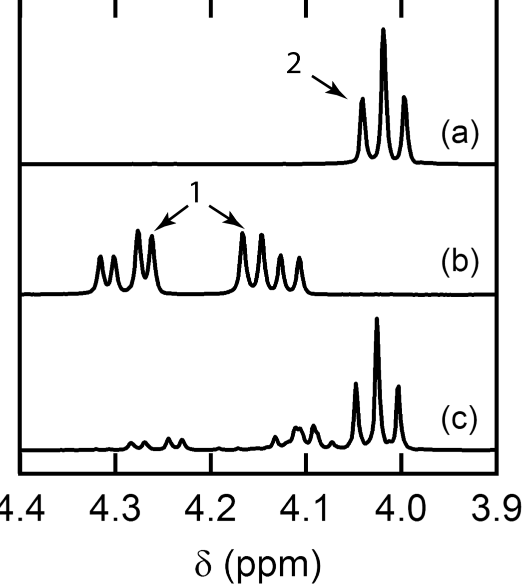

Figure 2-5. **^{1}H-NMR spectra**. Shown are ^{1}H-NMR spectra between 3.90-4.40 ppm chemical shift for: (a) pure butyl ester, (b) pure soybean oil, and (c) product of the transesterification of soybean oil with n-butanol. Numbers correspond to protons labeled in **Figure 2-1**.

butanol:soybean oil of 6:1, 12:1, and 30:1 (2:1, 4:1, and 10:1 ratio of n-butanol to fatty acid) at 110°C with 5% (w/w) sulfuric acid catalyst. After 8 minutes of reaction, each molar ratio had achieved greater than 90% butyl ester formation. It is noteworthy that under these conditions, all molar ratios of n-butanol to soybean oil showed nearly identical conversion efficiencies, indicating that the lowest ratio is sufficient.

Acid catalysis of combined triacylglycerides and fatty acids using n-butanol. Considering the results presented in the previous sections with free fatty acids or pure triacylglycerides alone, it was of interest to determine the efficiency of biodiesel conversion

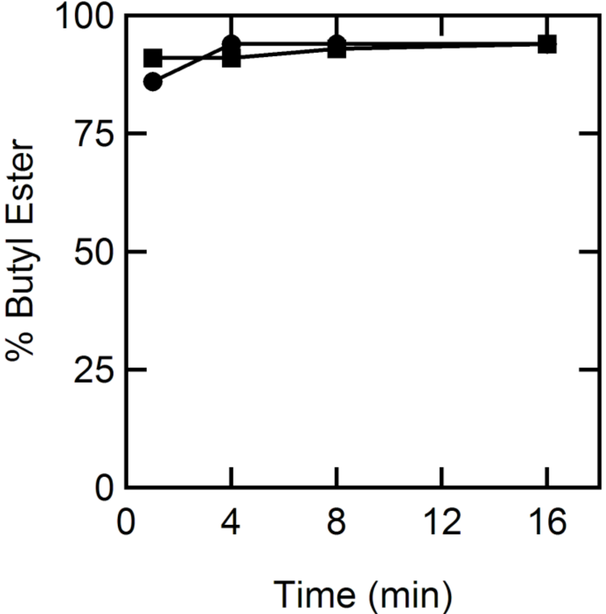

Figure 2-6. Effect of the ratio of *n*-butanol to oleic acid on butyl ester formation. All reactions were performed at 110°C with 5% (w/w) H_2SO_4 relative to fatty acid. The percentage of butyl ester formed based on the maximum possible yield is plotted against the time of reaction. The n-butanol to oleic acid molar ratios were: ● 2:1, ■ 4:1.

from mixtures of triacylglycerides and free fatty acids. Such a mixed feedstock was created by adding various amounts of oleic acid to soybean oil with molar ratios ranging from 5:1 to 1:5 (oleic acid to soybean oil). A minimal ratio of 2:1 n- butanol to fatty acid was used assuming a soybean oil molar mass of 870 g/mol. The reaction temperature was 110°C, and a 5% (w/w) concentration of H_2SO_4 relative to total lipid was used. The formation of butyl esters from the mixed feedstock was monitored by ^{1}H NMR analysis as described previously.[7] The efficiency of butyl-ester formation was found to be nearly 90% for all tested ratios of fatty acid to soybean oil ranging from 15% to 80% after 16 min of reaction.

Benchtop acid catalysis of soybean oil with n-butanol. The optimal conditions that

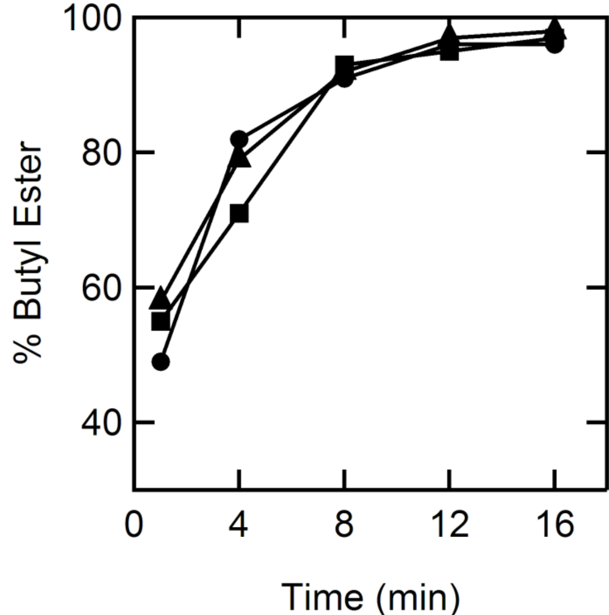

Figure 2-7. Effect of the ratio of *n*-butanol to soybean oil on butyl ester formation. All reactions performed at 110°C with 5% (w/w) H_2SO_4 relative to oil. The percentage of butyl ester formed based on the maximum possible yield is plotted against the time of reaction. The n-butanol to soybean oil molar ratios were: ● 6:1, ■ 12:1, ▲ 30:1.

were deduced from the experiments above in the microwave reactor were next applied to a "benchtop" reaction of soybean oil with n-butanol with conventional heating, a method that is scalable. A molar ratio of 6:1 n-butanol to soybean oil was used along with 3% (w/w) H_2SO_4 relative to oil. Samples were withdrawn at different times during the heating process and were analyzed for the percentage butyl-ester formed (**Figure 2-8**). The first sample was taken at 10 min after the addition of the acid when the reaction temperature was at 110°C. Subsequent samples were withdrawn after the reaction temperature had stabilized at 120°C. Under these conditions, the reaction was found to be > 96% complete after 30 min and 98% complete after 50 min. This experiment demonstrated that the acid catalyzed method to

convert triacylglycerides with n-butanol to biodiesel is scalable utilizing existing technology.

Properties of biodiesel produced by acid catalysis with n-butanol. The butyl-biodiesel produced from soybean oil using the acid catalyzed method described above was analyzed for key ASTM properties. The viscosity at 40°C (ASTM D445) was found to be 4.50 $mm^2\ s^{-1}$ well within the range of 1.9 – 6.0 $mm^2\ s^{-1}$ typical of methyl-ester biodiesel. The pour point (ASTM D97) was determined to be -13°C.

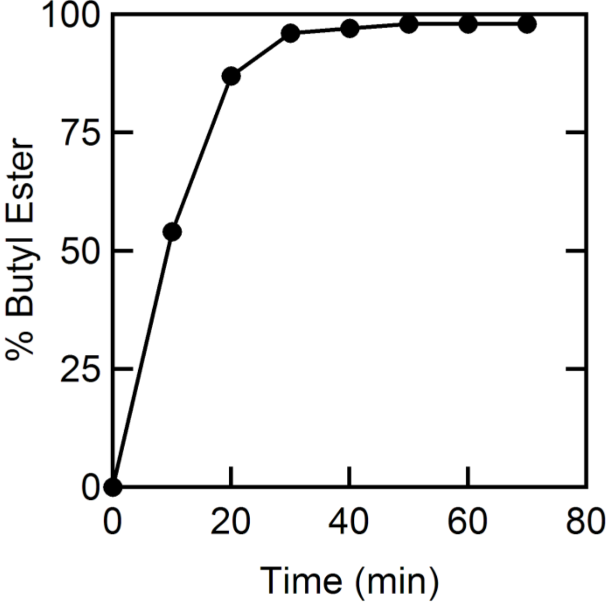

Figure 2-8. Biodiesel production with conventional heating. A larger scale (110 mL) reaction of n-butanol and soybean oil (6:1 molar ratio) with 3% (w/w) H_2SO_4 relative to oil was conducted with conventional heating. The percentage butyl ester formed based on the maximum possible yield is plotted against the time of reaction. The sample taken at 10 minutes was at 110°C, while all subsequent samples were taken after the reaction temperature had stabilized at 120°C.

DISCUSSION

Biodiesel is an attractive alternative liquid transportation fuel for a variety of reasons that have been articulated elsewhere.[11] There are challenges to its widespread penetration into the liquid fuel market, however, including cost and dependence on plant derived oils as feedstock.[1,12,13] In the US, soybean oil is the dominant feedstock used for biodiesel production. Soybeans are currently yielding approximately 48 gallons of oil per acre.[14] At this rate of production, the entire US soybean crop,[15] if turned into biodiesel, could only displace ~ 10% of the petroleum diesel currently consumed in the US.[1,16] Further, a recent study has indicated that the use of soybeans as the feedstock for biodiesel production results in a net increase in the CO_2 output when compared to the use of an equal amount of petroleum.[17,18] In order for biodiesel to significantly displace petroleum diesel usage, a wider range of feedstocks must be utilized in the future. Many of these alternative feedstocks contain high concentrations of free fatty acids in addition to triacylglcerides (**Table 2-1**), which complicates biodiesel production using a base catalyzed method.

One approach for dealing with such mixed feedstocks is to first remove the free fatty acids, either by washing or by acid reaction, followed by base catalysis of the remaining triacylglycerides to biodiesel.[3,19-23] This approach is costly and potentially wastes a significant fraction of the available lipids.[13] Another approach that has been explored is the use of an acid catalyzed reaction, which is capable of simultaneously converting both the free fatty acids and the fatty acids attached to glycerol to biodiesel.[4,6,24,25] A number of short chain alcohols have been shown to effectively form biodiesel from vegetable oil by acid catalysis.[7,24] This approach, using methanol as the alcohol, however, has several noted shortcomings. In particular, it has been reported that these reactions take very long times (>

69 h) with low conversion efficiencies.[5,6,26,27] The results presented in the present work are consistent with these findings using methanol as the alcohol.

In order to optimize the conversion of mixed feedstock lipids into biodiesel using an acid catalyzed procedure, a wide range of reactions conditions were examined. This was facilitated by the utilization of a microwave reactor, which has the capability of rapidly heating and cooling reaction solutions.[7,8,28] Conventional heating of these test reactions would have been much slower. Further, ^{1}H-NMR spectroscopy provided a rapid method for accessing the extent of conversion to the product alkyl-esters.[7,9,10,29-31] This method is capable of quantification of products with an accuracy of +/- 2 %, with typical data acquisition taking only 10 min.[9]

Using these approaches, it was possible to explore conditions for the rapid and high efficiency conversion of mixed feedstock lipids into biodiesel using an acid catalyzed reaction. Key parameters that were identified that control the reaction speed and efficiency were the alcohol type, ratio of alcohol to fatty acid, the temperature of the reaction, and the acid catalyst concentration. For the conversion of oleic acid to its methyl ester using methanol, the reaction was most efficient at the highest ratios of methanol to fatty acid (highest ratio tested was 10:1). There was a marginal improvement in going from 7:1 to 10:1 after 16 min, suggesting that saturation for the reaction occurs near the 10:1 ratio of methanol to oleic acid.

The type of alcohol used in the conversion of oleic acid to the alkyl ester also had an effect on the efficiency of the reaction. Longer chain alcohols were found to result in greater efficiency when the reaction was conducted at 80°C. This effect of alcohol type on the reaction efficiency was more pronounced for soybean oil conversion. As shown in **Figure 2-**

4, only the longer chain alcohols (n-propanol and n-butanol) resulted in high conversion to the respective alkyl-ester when compared to ethanol and methanol after 16 min reaction time at 80°C. The branched alcohol iso-butanol gave similar results to those found for n-butanol. A likely explanation for this result is the increased miscibility of the longer chain alcohols in soybean oil. Both methanol and ethanol are marginally miscible with soybean oil, whereas the more hydrophobic n-propanol and n-butanol are much more soluble. This was observed as two separate phases when methanol and ethanol were first mixed with the soybean oil following initial stirring. In contrast, n-propanol, n-butanol, and iso-butanol rapidly mixed with the soybean oil, forming a homogeneous solution. The speed of the chemical reaction is expected to be much faster for the homogeneous solutions with the longer chain alcohols than for the heterogeneous solutions with the shorter chain alcohols.

Another benefit to using the longer chain alcohols is that this allows a higher reaction temperature to be used, since the boiling temperatures for the alcohols increases with chain length (methanol (65°C) < ethanol (78°C) < n-propanol (97°C) < n-butanol (117°C)).[32] The ability to run the transesterification reactions at higher temperatures (just below the boiling temperature of the alcohols) has a significant impact on the speed and efficiency of conversion of soybean oil. A comparison of the results shown in **Figure 2-4** and **Figure 2-7** illustrate for soybean oil transesterification with n-butanol that increasing the reaction temperature from 80°C to 110°C significantly increases the kinetics of the reaction, with greater than 95% conversion at the higher temperature within 8 min compared to 50% conversion after 16 min at 80°C.

The higher reaction temperature achievable with the longer chain alcohols, coupled with the improved miscibility of the alcohols with the hydrophobic oil triacylglycerides, also

allows lower ratios of alcohol to fatty acid to be utilized, thus minimizing costs and the quantity of unreacted materials in the final product. With n-butanol as the alcohol, a ratio of 6:1 n-butanol to triacylglyceride gave excellent conversion efficiencies and times (**Figure 2-7**). These conditions were found to be in good agreement with the optimal conditions reported in a recent study of microwave assisted formation of biodiesel from soybean oil and n-butanol via an acid catalyzed method.[7] The stoichiometric minimum ratio of alcohol to triacylglyceride is 3:1 to allow all three fatty acids on the triacylglyceride to be converted to the alkyl-ester. A 2:1 molar ratio of n-butanol to fatty acid was found to provide sufficient excess of alcohol to allow high efficiency conversion to biodiesel, resulting in minimal excess alcohol in the final product.

The reaction conditions of alcohol type, concentration, and temperature next allowed an analysis of the conversion efficiency for a mixed feedstock containing a range of free fatty acid concentration. Using the higher reaction temperature and n-butanol as the alcohol at a ratio of 2:1 to total fatty acid, it was demonstrated that the same level of conversion to the butyl-ester could be achieved in feedstocks containing from 15% to 80% free fatty acid in triacylglycerides. This wide range of free fatty acid content over which the reaction remains effective should allow high efficiency conversion of many different feedstocks that contain free fatty acids.

Lastly, the reaction conditions developed in the microwave reactor for conversion of soybean oil to the butyl ester were translated into a more traditionally stirred and externally heated “benchtop” reactor. As can be seen in **Figure 2-8**, a high efficiency conversion (98%) could be achieved within thirty minutes. This is slower than in the microwave heated reaction as expected given the slower heating using conventional methods.[7] However, the

near completion of the reaction at modest alcohol to fatty acid ratios contrasts with the earlier observations of acid catalyzed reactions with methanol taking > 69 hours.[5]

The reaction conditions examined here should allow rapid and high efficiency conversion of mixed feedstock oils containing high concentrations of free fatty acids into biodiesel. A central conclusion from this work is that the optimal conditions for acid catalyzed transesterification of soybean oil described previously can also be applied successfully to feedstocks containing large amounts of free fatty acids.[7] In addition, we demonstrate that the increased rate of reaction observed in the acid-catalyzed transesterification of soybean oil using n-butanol as the alcohol is not limited to just reactions utilizing microwave heating but is also observed when conventional heating technologies are employed. Obvious questions about using longer chain alcohols in biodiesel production include the costs and availability of the alcohols, and the properties of the butyl-esters as biodiesel. At present, butanol costs more than methanol. However, the cost of butanol including iso-butanol are likely to drop sharply in the near term as a joint project DuPont and British Petroleum is expected to begin producing large quantities of butanol within the next two years.[33] Additionally, the increased cost of butanol can, in part, be offset by using low cost, high free fatty acid feedstocks in the production of biodiesel.

While there are limited data currently available regarding the properties of butyl-esters as biodiesel, it has been reported that butyl-esters of select fatty acids have comparable cetane numbers to the methyl-esters of the same fatty acids.[34,35] The viscosity of the butyl-ester biodiesel was determined to be 4.50 $mm^2 s^{-1}$, well within the range of 1.9 – 6.0 $mm^2 s^{-1}$ allowed by the ASTM D445 method. Finally, the pour point (ASTM D97) of the n-butyl-ester biodiesel was found to be -13°C, a value considerably lower than the typical value of

0°C measured for methyl-ester biodiesel. This depressed gel point for butyl-ester is consistent with earlier reports that longer chain alcohol esters have lower gel points than do methyl-esters,[35,36] thus making butyl-esters better choices for cold weather locations. It has been shown that biodiesel produced from branched alcohols exhibits improved cold flow properties over biodiesel produced from straight-chain alcohols without significantly affecting cetane numbers.[35,37]

The conditions reported in the present work could be applied to the efficient and high yield conversion of mixed feedstock lipids into biodiesel using longer chain and branched chain alcohols resulting in biodiesel with favorable properties. The information presented here should facilitate the further development of non-traditional oil feedstocks (e.g, from algae) as viable sources for biodiesel production.

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CHAPTER 3

BIODIESEL PRODUCTION BY SIMULTANEOUS EXTRACTION AND CONVERSION OF TOTAL LIPIDS FROM MICROALGAE, CYANOBACTERIA, AND WILD MIXED-CULTURES[1]

Abstract

Microalgae have been identified as a potential biodiesel feedstock due to their high lipid productivity and potential for cultivation on marginal land. One of the challenges in utilizing microalgae to make biodiesel is the complexities of extracting the lipids using organic solvents followed by transesterification of the extracts to biodiesel. In the present work, reaction conditions were optimized that allow a single step extraction and conversion to biodiesel in high yield from microalgae. From the optimized conditions, it is demonstrated that quantitative conversion of triglycerides from several different microalgae and cyanobacteria could be achieved, including from mixed microbial biomass collected from a municipal wastewater lagoon. Evidence is presented that for some samples, significantly more biodiesel can be produced than would be expected from available triglycerides, indicating conversion of fatty acids contained in other molecules (e.g., phospholipids) using this approach. The effectiveness of the approach on wet algae is also reported.

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1. Introduction

Biodiesel is a renewable fuel that can be produced from biological oils derived from plants, animals, or microbes (Williams and Laurens, 2010; Graboski and McCormick, 1998). The oils (triglycerides) are converted by transesterification using alcohols (e.g., methanol) and catalyst (base or acid) to yield glycerol and the fatty acid alkyl ester or fatty acid methyl ester (FAME) if methanol is the alcohol. For a number of reasons, there is interest in developing different feedstocks to provide triglycerides as a source for biodiesel production other than traditional oilseed crops (*i.e,* soybean and canola) (Dismukes et al., 2008). Microalgae offer many potential advantages as a non-food feedstock for biodiesel production (Chisti, 2007, 2008; Hu et al., 2008), although this potential has yet to be realized because of several remaining technical barriers. For example, life cycle analysis conducted on the process of biodiesel production from microalgae indicates that 90% of the process energy is consumed by oil extraction, indicating that any improvement in lipid extraction will have a significant impact on the economics of the process (Lardon et al., 2009). Many microalgae are known that accumulate significant quantities of triglycerides (20-50% of total dry weight). One approach to converting algal triglycerides to biodiesel requires that the lipids are first extracted using organic solvents (e.g., hexanes, chloroform, methanol). The solvents are removed by distillation and the triglycerides are then reacted with acid or base and an alcohol (typically methanol) to make the FAME (Xu et al., 2006; Cheng et al., 2009).

It would be valuable to be able to extract and convert triglycerides in microalgae into biodiesel in a single step, bypassing the use of large quantities of organic solvents. Such *in situ* or direct transesterification approaches have been used as an analytical technique to prepare FAMEs for the determination of the fatty acid composition of lipid containing tissues (Park and Goins, 1994; Lepage and Roy, 1984; Rodríguez-Ruiz et al., 1998). Likewise, there have been reports of direct transesterification of oil palm pulp (Obibuzor et al., 2003) and other materials where traditional extraction techniques were

inadequate (Qian et al., 2008). It has been reported that such direct methods can result in greater FAME yields than are achieved in the two step extraction followed by transesterification approach (Lepage and Roy, 1984, 1986; Siler-Marinkovic and Tomasevic, 1998; Lewis et al., 2000; Vicente et al., 2009). A direct approach has been shown to be effective in making biodiesel from both pure (Lewis et al., 2000; Johnson and Wen, 2009; Vicente et al., 2009) and mixed cultures of microorganisms (Dufreche et al., 2007; Mondala et al., 2009). In addition to producing FAMEs from TAG and free fatty acids, one study suggests that *in situ* transesterification reduced the amount of phospholipids in a sample to a level below detection (Vicente et al., 2009). Here, we have developed an approach for the direct production of FAMEs by *in situ* transesterification from high TAG accumulating microalgae such as *Chaetoceros gracilis*. The approach has been applied to a number of different microalgae, including a mixed culture from a wastewater lagoon.

2. Materials and Methods

2.1. Strains and culture conditions

Strains used in this study are listed in **Table 3-1**. Stocks of each culture were maintained in 250 mL of media in 500 mL baffled flasks, rotating at 140 rpm, and illuminated from overhead by Cool White fluorescent lighting (180 μmol photons m^{-2} s^{-1}) on a 14:10 (light:dark) photoperiod. Unless otherwise stated, larger cultures were grown in 5 L Cell-Stir flasks (Wheaton Industries, Inc., Millville, NJ) illuminated by Cool White fluorescent lights (280 μmol photons m^{-2} s^{-1}), with slow stirring (~ 50 rpm) and aeration at the bottom of the vessel with air supplemented with 1% (v/v) CO_2. Artificial seawater media used to culture marine strains contained the following components per liter: NaCl (18 g), KCl (0.6 g), $MgSO_4 \cdot 7H_2O$ (1.3 g), $CaCl_2 \cdot 2H_2O$ (100 mg), K_2HPO_4 (250 mg), $CaSiO_3$ (25 mg), $NaNO_3$ (150 mg), and ferric ammonium citrate (5 mg). In addition, 1 mL of the following trace element solution was added per liter of media: H_3BO_3 (600 mg

L^{-1}), $MnCl_2 \cdot 4H_2O$ (250 mg L^{-1}), $ZnCl_2$ (20 mg L^{-1}), $CuCl_2 \cdot 2H_2O$ (15 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (15 mg L^{-1}), $CoCl_2 \cdot 6H_2O$ (15 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (10 mg L^{-1}), V_2O_5 (2 mg L^{-1}), and KBr (10 mg L^{-1}). Freshwater microalgal strains were grown using Bristol's medium modified to include ferric ammonium citrate (15 mg L^{-1}) (Bold, 1949). Cyanobacteria (Hu et al., 2000) were grown in BG-11 media containing the following composition per liter: $NaNO_3$ (1.5g), $MgSO_4 \cdot 7H_2O$ (75 mg), $CaCl_2 \cdot 2H_2O$ (36 mg), citric acid (6 mg), H_3BO_3 (2.86 mg), $MnCl_2 \cdot 4H_2O$ (1.81 mg), $ZnSO_4 \cdot 7H_2O$ (222 μg), $Na_2MoO_4 \cdot 2H_2O$ (390 μg), $CuSO_4 \cdot 5H_2O$ (79 μg), $Co(NO_3)_2 \cdot 6H_2O$ (49.4 μg), Ferric ammonium citrate (6 mg), Na_2CO_3 (20 mg), and KH_2PO_4 (30.5 mg) (Rippka et al., 1979).

In addition to growth in 5 L culture flasks, *C. gracilis* was cultured in a 220 L raceway (Seperation Engineering, Escondido, CA). The inoculum for the raceway was prepared by increasing the volume of a 5 L *C. gracilis* culture to 50 L in a polyethylene bag (#S2942, U-Line, Waukegan, IL). The bag culture was mixed vertically by air supplemented with 1% CO_2. The raceway was maintained at a pH of 7.5 by the introduction of CO_2 and mixed by a paddle wheel. The raceway and bag culture were positioned in a greenhouse where ambient solar light was supplemented with sodium vapor lamps. Once the *C. gracilis* raceway culture reached stationary growth phase, cells were harvested by centrifugation, frozen immediately, and lyophilized (Labconco, Kansas City, MO) prior to lipid analysis or experimental reactions. Wild cultured cells were obtained by centrifugation of water from the Logan city (Utah) wastewater lagoon.

2.2. In situ transesterification

Experiments to determine the optimal conditions for biodiesel production were conducted with 100 mg of lyophilized algal biomass. Methanol containing sulfuric acid as a catalyst was added to the reaction vessel containing the algae and a PTFE coated stir bar (50 mm). Both the volume of methanol and the amount of sulfuric acid was varied to

Table 3-1. Strains used in this study.

Strains	Media	Source
Chaetoceros gracilis	Artificial seawater	UTEX[a] (LB 2658)
Phaeodactylum tricornutum	Artificial seawater	UTEX[a] (640)
Tetraselmis suecica	Artificial seawater	UTEX[a] (LB 2286)
Neochloris oleoabundans	Bristol media	UTEX[a] (1185)
Chlorella sorokiniana	Bristol media	UTEX[a] (1602)
Synechocystis sp. PCC 6803	BG-11	Hu et al. 2000
Synechococcus elongatus	BG-11	Hu et al. 2000

[a]The Culture Collection of Algae at the University of Texas at Austin.

determine the amount necessary for optimal biodiesel production. Reactions were conducted in a commercial scientific microwave (CEM, Matthews, NC), where conditions of time and temperature could be controlled with precision (Leadbeater and Stencel, 2006; Wahlen et al., 2008). Once completed, reactions were stopped by the addition of chloroform to the reaction vessel forming a single-phase solution with the methanol. Phase separation was then accomplished by washing the methanol-chloroform solution with water (~5 mL) followed by centrifugation. The methanol and sulfuric acid partitioned with the water in the upper phase, while FAME, TAG, and other lipids partitioned with chloroform in the lower, organic phase. The residual biomass formed a layer at the boundary between these two phases. The chloroform phase was removed with a gas tight syringe to a 10 mL volumetric flask. The remaining biomass was washed twice with 2 mL of chloroform to recover residual FAMEs and lipids. The total volume of chloroform was brought to 10 mL and mixed by inversion. Reaction conditions of time, temperature, catalyst concentration, and methanol volume were varied to determine optimal parameters for maximal biodiesel production from algal biomass by *in situ* transesterification.

2.3. Total lipid analysis

Total lipids were extracted from dry algal biomass by using a

chloroform:methanol (2:1) solvent mixture similar to that used by Folch (Folch et al., 1957). Dried algae samples (200 mg) were sonicated (Sonifier 250, Branson, Danbury, CT) in 5 mL of chloroform:methanol (2:1) for approximately 30 s. The biomass was then collected at the bottom of the test tube by centrifugation and the solvent was removed to a weighed vial (EP scientific P/N 340-40C, Miami, OK). Extraction of biomass was repeated twice as described above. The organic extractions, pooled into a weighed vial, were dried by blowing a stream of argon gas for 12 h. The dried vials were weighed to establish the total lipid.

2.4. Triglyceride content determination

The TAG content of each algal sample was determined by gas chromatography (GC) analysis of the total lipid extraction. Total lipid extraction was conducted on a 100 mg sample size. Each sample was placed in a 10 mL microwave reaction tube along with a PTFE coated stir bar and 3 mL of chloroform:methanol (2:1) solution. Samples were maintained at 60°C by microwave irradiation for 5 min. Once cooled, samples were centrifuged to collect the biomass at the bottom of the test tube to facilitate removal of the solvent, which was then removed to a 10 mL volumetric flask. Extraction was repeated twice and the final volume was adjusted to 10 mL with chloroform. The resulting solution was mixed by inversion and 1 mL was added to a GC vial for analysis.

2.5. Lipid quantification

Triglyceride (TAG) and fatty acid methyl ester (FAME) content of each algal sample was determined with a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with a programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID), mass spectrometer (MS) (GCMS-QP2010S, Shimadzu Scientific, Columbia, MD), and autosampler. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32 mm ID, 0.10 μm film thickness, Restek, Bellefonte, PA)

using a temperature program of 60°C for 1 min followed by a temperature ramp of 10°C per minute to 360°C for 6 min. Constant velocity of helium as a carrier gas was set at 50 cm/sec in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380°C. Each sample contained octacosane (10 μg/mL) as an internal standard. FID detector response to FAME and TAG was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) at concentrations ranging from 0.1 mg/mL to 1 mg/mL and tripalmitin at concentrations ranging from 0.05 mg/mL to 0.5 mg/mL. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., Elysian MN) and were diluted with chloroform to obtain the needed concentrations. A standard (GLC-68A, Nu-Chek Prep Inc.) containing methyl esters ranging from methyl tetradecanoate (C14:0) to methyl nervonate (C24:1) was used to identify the retention time window for FAME peak integration. Peaks within this region were integrated using GCsolution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis. TAG concentration of samples was determined in a similar manner.

2.6. GC/MS analysis

The fatty acid composition of the biodiesel obtained from each strain was determined by GC/MS analysis, using the Shimadzu 2010 gas chromatograph described above. Samples were prepared by *in situ* transesterification as described above, using 2 mL methanol (2.0% H_2SO_4) and 100 mg biomass. Samples were heated to 80°C for 20 min in the microwave. Samples were processed as described above for the *in situ* transesterification reactions. 1 μL of each sample was injected in the split-injection mode with a split ratio of 1:2. The split/splitless injector was connected to a stabilwax column (30 m, 0.25 mm ID, and 0.10 μm film thickness, Restek, Belafonte, PA) that interfaced with a mass spectrometer (GCMS-QP2010S, Shimadzu Scientific). The temperatures of the injector,

interface and ion source were 235, 240, and 200°C, respectively. Helium was used as the carrier gas set at a constant velocity of 50 cm/s in velocity mode. Initially, the oven temperature was maintained at 100°C for 1 min, and increased at a rate of 10 C° min^{-1} to 235°C and held for 10 min. The mass range scanned was 35 to 900 m/z at a rate of 2000 scan s^{-1}. Peak identification was accomplished by comparing mass spectra to the National Institute of Standards and Technology (NIST) 2005 mass spectral library (NIST, Gaithersburg, MD).

3. Results and Discussion

3.1. Optimization of algal biodiesel production

A common method for establishing the biodiesel potential of an algal strain is to determine the total lipid content. This is accomplished by extracting the biomass with a solvent mixture of both non-polar and polar solvents such as the mixture of chloroform and methanol in a 2:1 ratio described by Folch *et al*. (1957). Because the resultant extract includes all lipid-soluble compounds from within the cell, it does not accurately represent how much biodiesel could be produced from a given sample. The Folch method has also been shown to under report the amount of lipid present in some samples (Lepage and Roy, 1984). Lepage *et al*. developed a single-step method to prepare FAME from the total lipids of a sample (Lepage and Roy, 1984). The method, termed direct transesterification or *in situ* transesterification, allowed for the quantification and characterization of the total fatty acids of a sample without requiring a separate extraction method. Direct transesterification has been successfully used to produce biodiesel from materials for which traditional solvent extractions are not efficient (Obibuzor et al., 2003; Lewis et al., 2000; Vicente et al., 2009; Dufreche et al., 2007). In addition to simplifying the production process, direct transesterification results in improved yields of FAME when compared to a conventional extraction followed by conversion approach (Siler-Marinkovic and Tomasevic, 1998; Lewis et al., 2000).

We sought to optimize the production of FAME specifically from microalgae using a direct transesterification approach by exploring a range of reaction parameters. Cells of the diatom *C. gracilis* were used as a representative feedstock for the development of this method due to their high TAG content (27% CDW). Reactions were performed utilizing a precision microwave instrument to allow for rapid screening of reaction conditions with a great degree of reproducibility. Each reaction was analyzed by gas chromatography using a method that allowed for the determination of FAME yield as well as quantification of residual TAG. Key parameters essential to the successful conversion of algal lipids to biodiesel by direct transesterification were identified as the alcohol type used, the amount of alcohol per unit of biomass, temperature of reaction, and catalyst concentration.

3.1.1. Alcohol Selection

In the *in situ* transesterification process, alcohols perform a vital role, acting as both the solvent, extracting the lipids from the biomass and as the reactant, converting the lipids to fatty acid alkyl esters. Prior studies of *in situ* transesterification have investigated the efficiency of alcohols such as methanol, ethanol, 1-propanol, and n-butanol at extracting and converting biodiesel from soybean, rice bran, and sunflower seed (Kildiran et al., 1996; Georgogianni et al., 2008). To determine which alcohol would perform better in the production of biodiesel from the diatom *C. gracilis*, methanol, ethanol, 1-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol were used to determine the ability of each alcohol to both extract the oil and convert it to the corresponding fatty acid alkyl ester. Extracting *C. gracilis* cells with methanol removed significantly less TAG from the algal biomass than did the other four alcohols (**Table 3-2**). Interestingly, when the same procedure was performed in the presence of 1.8% (v/v) H_2SO_4 as catalyst, the type of alcohol used did not have a significant effect on the amount of fatty acid alkyl esters obtained. Approximately equal amounts of fatty acid alkyl esters resulted from the *in situ*

transesterification of algal biomass with each alcohol (**Table 3-2**). As a result, the lowest cost alcohol (methanol) was selected for optimization of the *in situ* transesterification reaction.

3.1.2. The effect of methanol volume

Previous studies of acid-catalyzed conversion of vegetable oil to FAME have shown that the extent of TAG conversion is influenced by the volume of methanol used per unit of oil (Canakci and Van Gerpen, 1999; Freedman et al., 1984, 1986; Wahlen et al., 2008). It was expected that the volume of methanol used per unit of algal biomass would have a similar effect on the FAME yield from total algal lipids by *in situ* transesterification. To identify the optimal methanol to biomass ratio, 100 mg samples of *C. gracilis* cells were incubated with methanol (1 mL, 2.5 mL, and 5 mL) containing 1.2% (v/v) H_2SO_4, at 60°C for 25 to 150 min at 25 min intervals (**Figure 3-1**). Samples reacted with 1 mL methanol increased from 4.1 mg of FAME at 25 min to a maximum of 14.8 mg at 125 min. Increasing the volume of methanol used for each 100 mg sample to 2.5 mL increased the yield of FAME to 7.4 mg at 25 min and a maximum yield of 22.6 mg at 150 min. Increasing the volume of methanol further to 5 mL did not result in an increase in the yield of FAME.

Freedman *et al*. determined that an excess of methanol to TAG (30:1 methanol:TAG molar ratio) is required for the efficient conversion of soybean oil to FAME (Freedman et al., 1984, 1986). Here, it was determined that the volume of methanol necessary for maximal direct conversion of algal lipids to FAME (**Figure 3-1**) is even higher than reported by Freedman *et al*. (Freedman et al., 1984, 1986). While high ratios of methanol are essential for optimal direct conversion of lipids in algae to biodiesel, the direct method completely eliminates the need for n-hexane extraction prior to transesterification. Additionally, the unreacted methanol can be reused to continue processing algal biomass. The elimination of the n-hexane extraction combined with the

Table 3-2: Effectiveness of different alcohols at extracting and converting algal oil

Alcohol	mg TAG extracted[a]	mg FAME per sample[b]
methanol	3.1	35.6
ethanol	20.2	30.8
1-butanol	18.9	36.9
2-methyl-1-propanol	19.5	28.7
3-methyl-1-butanol	19.1	36.4

[a]100 mg biomass extracted with 1 mL of alcohol heated to 60°C by microwave irradiation for 10 min with constant stirring. [b]100 mg of biomass was heated to 60°C for 100 min with 2 mL of alcohol and 1.8% (v/v) sulfuric acid.

potential to reuse excess methanol should significantly reduce the financial impact of the direct transesterification method compared to the traditional two step process.

3.1.3 The effect of temperature

Prior studies of conventional and *in situ* methods for converting TAG to FAME demonstrated that increasing the reaction temperature decreases the amount of time necessary to reach a maximal yield of FAME (Özgül-Yücel and Türkay, 2002). To increase the efficiency of the reaction without requiring temperatures significantly higher than the boiling point of methanol, experiments were performed to identify the lowest temperature required to reach a maximal yield of FAME in short reaction times of 10 and 20 min. Reactions to study the influence of temperature on FAME yield were carried out with 100 mg samples of *C. gracilis* and 2 mL of methanol containing 2% (v/v) H_2SO_4 at temperatures ranging from 60 to 110°C in increments of 10 C° for either 10 or 20 min (**Figure 3-2**). Reaction times of 10 min yielded 23.7 mg of FAME at a temperature of 60°C and reached a maximum yield of 33.7 mg FAME at a reaction temperature of 90°C. By increasing the reaction time to 20 min a maximal FAME yield of 34.1 mg was reached with a temperature of 80°C. Significant improvements in FAME yield were

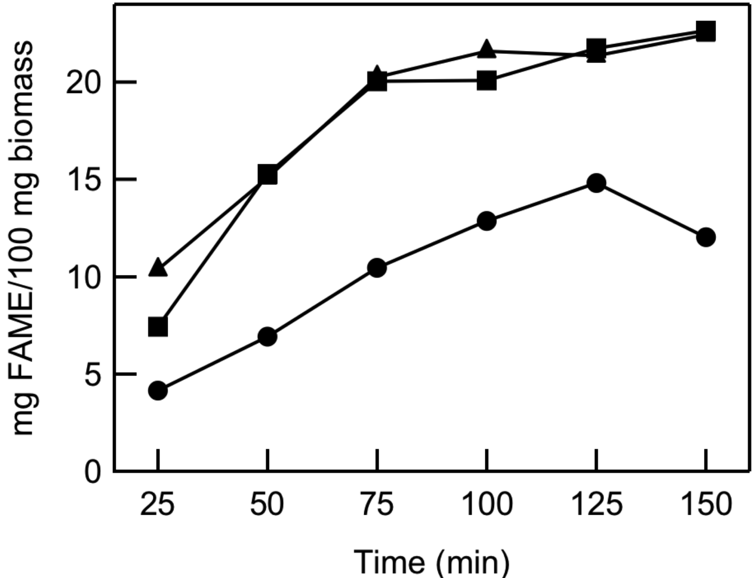

Figure 3-1. The effect of methanol concentration on the extraction and conversion of algal lipids to biodiesel. All reactions were performed by heating 100 mg of lyophilized *C. gracilis* cells to 60°C with varying volumes of methanol containing 1.2% (v/v) H_2SO_4. The total yield of fatty acid methyl esters obtained per reaction with 100 mg biomass is plotted against the time of reaction. Volumes of methanol tested were ●, 1 mL; ■, 2.5 mL; and ▲, 5 mL.

observed when the temperature was increased from 60°C to 80°C for 20 min reactions. No additional increase in product yield was observed for temperatures higher than 80°C. The increased rate of reaction with increasing temperature observed here is consistent with other reports of acid catalyzed transesterification reactions conducted either directly on biomass or performed on extracted oil (Canakci and Van Gerpen, 1999; Wahlen et al., 2008; Özgül-Yücel and Türkay, 2002).

3.1.4 Concentration of the catalyst

In addition to time, temperature of reaction, and concentration of reactants, an important variable in the efficient conversion of lipids to FAMEs is the concentration of the catalyst. *In situ* transesterification reactions, like conventional reactions, utilize either acidic or basic catalysts. Sulfuric acid was chosen as the catalyst for these studies because acid-catalyzed reactions have been shown to be effective at converting both TAG and free fatty acids (FFA) into FAME (Wahlen et al., 2008). This is an important consideration as the presence of FFA has been observed as minority constituents of algal lipids (data not shown). Among *in situ* transesterification studies that utilized sulfuric acid as the catalyst, the amount used varied (Mondala et al., 2009; Özgül-Yücel and Türkay, 2002). Because of this variation, it was unclear what effect varying the concentration of sulfuric acid in methanol would have on FAME yield using the *in situ* transesterification method. Samples of *C. gracilis* cells (100 mg) were incubated with 2 mL of methanol containing varying percentages (1.2% - 2.4% v/v) of H_2SO_4 (conc.) for 10 min at 80°C (**Table 3-3**). Samples reacted with methanol containing 1.2% H_2SO_4 yielded 28.2 mg of FAME while the highest concentration of H_2SO_4 in methanol tested (2.4%) yielded 31.7 mg (**Table 3-3**). Varying the concentration of the catalyst had a modest effect on the production of biodiesel, similar to the report of the direct transesterification of rice bran oil (Özgül-Yücel and Türkay, 2002).

3.3 Maximal FAME yield

Traditionally the efficiency of the biodiesel production is determined by monitoring the disappearance of TAG, the substrate. As is demonstrated in **Table 3-4**, all strains of algae yielded more FAME than expected based on the TAG content. Because of this, knowing whether the reaction is complete presents a challenge. It is possible to have converted all TAG to FAME without approaching the maximal biodiesel yield. To examine whether the conditions reported here result in the maximal production of

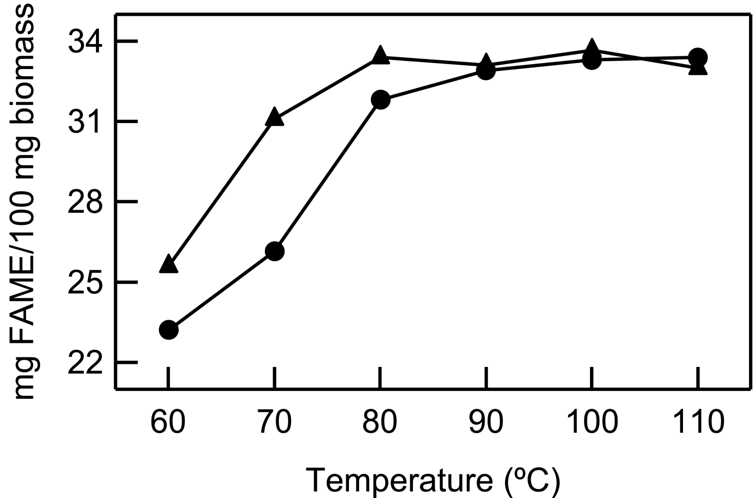

Figure 3-2. Temperature dependence of the extraction and conversion of algal lipids to methyl esters. All reactions were performed with 100 mg of lyophilized *C. gracilis* cells and 2 mL of methanol containing 2.0% (v/v) H_2SO_4. The total yield of fatty acid methyl esters obtained per reaction is plotted against the temperature of the reaction. Reactions were performed for ●, 10 min; and ▲, 20 min.

biodiesel, a sample of the green alga *N. oleoabundans* (100 mg) containing 3.4% (CDW) TAG, was incubated with 2 mL methanol containing 1.8% (v/v) H_2SO_4 at 80°C for times ranging from 5 to 35 min in 5 min increments (**Figure 3-3**). The FAME yield increased from a minimum of 19.4% CDW at 5 min to a maximum of 28.2% CDW at 20 min. Increasing the time of incubation beyond 20 min did not affect the total yield of FAME obtained.

3.3 Application of the method to other phototrophic microorganisms

By varying reaction parameters, such as the volume of methanol relative to algal

biomass in each reaction, the temperature of reaction, and the catalyst concentration, optimal conditions were determined for the effective conversion of total algal lipids from the marine diatom *C. gracilis*. Although the reaction parameters determined here proved effective at converting lipids from *C. gracilis*, which accumulates significant quantities of TAG (27% of cellular dry weight, (CDW)), it remained unclear whether the same reaction conditions would be effective at converting lipids from other phototrophic microorganisms with differing TAG content and diverse cell wall compositions. A group of organisms (**Table 3-1)** were selected to determine how effective the optimal reaction conditions were at converting total lipids to FAME regardless of TAG content. The samples used in this study were not necessarily optimized for lipid production but are rather representative samples of the given organism or organisms from a given environment. In addition to the organisms of **Table 3-1**, a sample of diverse phototrophic organisms was obtained from a wastewater treatment lagoon to demonstrate the wide applicability of the method to produce biodiesel. The TAG and total extractable lipid content of each organism was determined. TAG content was determined by GC analysis of chloroform:methanol (2:1) extraction of each organism or mixture of organisms. The total extractable lipid content of each sample was established by gravimetric analysis of chloroform:methanol (2:1) extracted lipids. To determine the total FAME content of each

Table 3-3. Effect of catalyst concentration on fatty acid methyl ester yield

Catalyst Concentration[a] (% v/v)	FAME (mg per 100 mg biomass)[b]
1.2	28.2
1.4	30.4
1.6	31.1
1.8	31.8
2.0	32.9
2.2	32.9
2.4	31.7

[a]The amount of H_2SO_4 (conc.) added to methanol, reported as % (v/v).
[b]Result of a 100 mg sample heated to 80°C for 10 min with 2 mL of methanol containing varying concentrations of H_2SO_4.

sample, the lyophilized biomass (100 mg) was reacted with 2 mL of methanol containing 1.8% (v/v) H_2SO_4 for 20 minutes at 80°C. FAMEs obtained from this reaction were then quantified by GC. The results are reported in **Table 3-4**.

The TAG content of each sample varied greatly. The diatom *C. gracilis* had the highest TAG content (27.3% CDW), while the sample obtained from the municipal wastewater lagoon had the lowest (<1% CDW) (**Table 3-4**). The green algae, *Chlorella sorokiniana* and *Tetraselmis suecica*, had TAG contents (7.6% CDW and 6.7% CDW, respectively) in between the values obtained for *C. gracilis* and the wastewater sample. No TAG was detected in the cyanobacteria used in this study. Total extractable lipid content of the algae samples followed a similar trend; *C. gracilis* had the highest lipid content of 44% CDW, while the municipal wastewater sample contained the least amount (14.4% CDW). Surprisingly, the two cyanobacteria, *Synechocystis* sp PCC 6803 and *S. elongatus,* had total extractable lipid contents of 18.4% CDW and 17.7% CDW respectively, despite their inability to produce TAG. Often the total extractable lipid

Table 3-4. Application of method to other phototrophic organisms.

Organism	Description	TAG (mg per 100 mg biomass)[a]	Extractable lipid content (mg per 100 mg biomass)[b]	FAME (mg per 100 mg biomass)[e]	Percent FAME of extractable lipid
Chaetoceros gracilis	Diatom	27.3 (±0.67)	44 (±0.87)	36 (±0.32)	82
Tetraselmis suecica	Green alga	6.7 (±0.23)	23 (±1.1)	18[f]	78
Chlorella sorokiniana	Green alga	7.6 (±0.12)	23.5 (±1.36)	18[f]	77
Synechocystis sp. PCC 6803	Cyanobacterium	ND[d]	18.4 (±0.58)	7.1 (±0.19)	39
Synechococcus elongatus	Cyanobacterium	ND[d]	17.7[c]	7.1 (±0.12)	40
Municipal wastewater lagoon	Mixed culture	< 1	14.4 (±0.42)	10.7 (±0.32)	74

[a]Total TAG content determined by triplicate solvent extractions of 100 mg samples with chloroform:methanol (2:1). Analyses performed in triplicate.
[b]Total lipid content determined by gravimetric analysis of solvent (chloroform:methanol, 2:1) extractable material from a 200 mg sample. Analysis performed in triplicate.
[c]Result of the gravimetric analysis of solvent (chloroform:methanol, 2:1) extractable material from a single 200 mg sample.
[d]Not detected.
[e]FAME content of cells determined by GC analysis of the chloroform extraction of 100 mg lyophilized algal samples heated to 80°C for 20 min in the presence of 2 mL of acidified (1.8% conc. H_2SO_4 (v/v)) methanol. Unless otherwise stated samples were performed in triplicate.
[f]Result of the direct transesterification of a single sample.

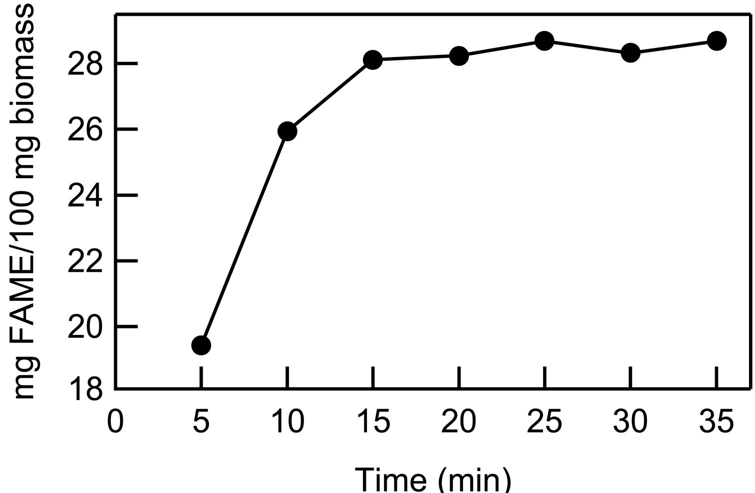

Figure 3-3. The extent of methyl ester formation from *N. oleoabundans* cells. The TAG content of the cells was 3.4% of the total dry weight. Reactions were performed with 100 mg of *N. oleoabundans* cells at 80°C with 2 mL of methanol containing 2.0% (v/v) H_2SO_4. The total yield of methyl esters is plotted against the time of reaction.

content is reported as an indicator of the suitability of a given strain for biodiesel production even though some strains, such as the cyanobacteria used in this study, do not contain TAG, the principal biodiesel feedstock. To accurately identify whether a strain could be used as a biodiesel feedstock, the FAME potential must be determined. To do this, the optimal *in situ* transesterification reaction parameters determined in this study were applied to each strain, and the results are listed in **Table 3-4**. As expected, *C. gracilis*, the strain with both the highest total lipid and TAG content, also had the highest FAME yield (36% CDW). Unexpectedly the FAME yield exceeded the total TAG present. The FAME yield for each of the other algal strains tested was also higher than the amount of TAG available. The strain *T. suecica* exhibited the largest difference between the FAME yield (18% CDW) and the amount of extractable TAG (6.7% CDW)

available for transesterification. The method of *in situ* transesterification was also applied to two species of cyanobacteria, *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus*. No TAG was expected to be found in these species and none was detected by GC analysis of their total lipid extraction. Despite the lack of TAG, the FAME yield obtained from the *in situ* transesterification of the two cyanobacteria was 7.1% CDW. Finally the mixed culture obtained from the wastewater lagoon was subjected to *in situ* transesterification. Though the wild mixed culture contained less than 1% (CDW) TAG, a FAME yield of 10.7% (CDW) was obtained through *in situ* transesterification.

The data described in **Table 3-4** indicates the effectiveness of the direct transesterification at producing biodiesel from a diverse range of organisms. In each case, the direct transesterification method resulted in conversion of all of the triglycerides to biodiesel. Somewhat surprising, however, was the observation that the FAME that could be captured actually exceeded the available triglycerides in each case. For the two green algae, three-fold more biodiesel was obtained compared to that expected from the triglyceride content. This discrepancy could be explained by an ineffective extraction technique used to establish the TAG content, leaving a substantial amount of the compound in the cell. The inclusion of acid in direct transesterification reactions could also facilitate a more efficient extraction by breaking down the cell wall of the organism. A second explanation could be that other sources of fatty acids such as membrane lipids can be converted to biodiesel by this method and contributed to the total FAME yield. To explore the possibility of membrane lipids being converted to biodiesel by direct transesterification, two strains of cyanobacteria, *Synechocystis* PCC 6803 and *Synechococcus elongatus*, were selected for direct transesterification. Cyanobacteria are photoautotrophic bacteria and are not known to accumulate TAG (Alvarez and Steinbüchel, 2002). Although TAG was not detected in the total lipid extract for these samples, each cyanobacterium had total extractable lipid contents comparable to the two strains of green algae (~18% CDW to 23% CDW respectively). Despite the fact that

these two cyanobacteria do not contain triglycerides, significant biodiesel could be made, with 7% of the total dry cellular weight and 40% of the total extractable lipid being converted to biodiesel. To confirm the potential for phospholipids to act as substrates for the transesterification reaction, the membrane lipids 1,2-dipalmitoyl-sn-glycero-phosphate and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (Sigma Aldrich, St. Louis, MO) were reacted using the same conditions as used for direct transesterification, resulting in the production of FAME. The production of FAME from both cyanobacteria and purified membrane lipids demonstrates the feasibility of biodiesel production from phospholipids using this approach. This is consistent with the observation of others that higher yields of FAME were obtained for direct transesterification reactions compared to a two-step extraction followed by conversion approach (Siler-Marinkovic and Tomasevic, 1998; Lewis et al., 2000). A reduction of phospholipids in a sample has also been observed for *in situ* transesterification reactions (Vicente et al., 2009; Haas and Scott, 2007).

Although the strain with the highest biodiesel yield in our studies was the strain with highest TAG, it is significant to note that even samples with low or no triglycerides can be used to make large quantities of biodiesel using the direct approach described here. This observation indicates that any source of microbes might be a viable feedstock for biodiesel production. To test this possibility, samples were collected from the wastewater lagoon (Logan, UT). This is a large, open pond system, with a very high proportion of phototrophic microbes at the effluent to the system. Even though the total TAG content of this mixed sample was less than 1%, greater than 10% of the cellular dry weight could be converted to FAME. Although this is three-times less than the amount of biodiesel produced from *C. gracilis*, the production of the wastewater algae has the benefit of not requiring any input for growth or maintenance. The studies presented here raise the question of whether maintaining a unialgal culture for biodiesel production will be cost effective compared to an unmanaged, mixed culture as is found in many

wastewater lagoon systems. The current work should motivate a thorough life cycle analysis of a variety of growth conditions.

3.4 Direct conversion of wet algae biomass

An energy intensive step in the use of microalgae for biodiesel is drying of the water from the algal biomass prior to transesterification. Previous studies of the transesterification of vegetable oil have determined that water present at very low concentrations has a detrimental effect on the yield of FAME (Canakci and Van Gerpen, 1999). Here, we examined how water might affect the yield of FAME from microalgae using the direct transesterification approach. Distilled water was added back to dry *C. gracilis* cells, ranging from 10 to 100% (w/w) of the biomass. The water was allowed to rehydrate the cells for a minimum of 30 min.

The optimal reaction conditions discovered for dry biomass (2 mL methanol, 1.8% (v/v) H_2SO_4, 80°C for 20 min) were then applied to the wet biomass and the FAME yield was determined as a function of the water content (**Figure 3-4**). Increasing water content progressively decreased the yield of FAME, with equal water and biomass (100% w/w) yielding only 50% of the expected FAME. Increasing the volume of methanol to 3 mL per 100 mg of biomass did not significantly improve FAME yields. However, when the volume of methanol was increased to 4 mL a significant improvement in the yield of FAME was observed. FAME yields of 84% of the expected yield were observed in the sample rehydrated with an equal amount of water when 4 mL of methanol were added. This corresponds to 30 mg of FAME from a 100 mg sample, exceeding the biodiesel that could be produced from the TAG (27 mg, **Table 3-4**) alone using a conventional approach. Higher water content could be partially compensated by adding more methanol. When methanol volumes of 5, 6, and 7 mL were used with 400% water rehydrated samples, the FAME yields were 54%, 61%, and 69%, respectively, of the expected. It is expected that increasing the methanol volume beyond 7 mL would allow

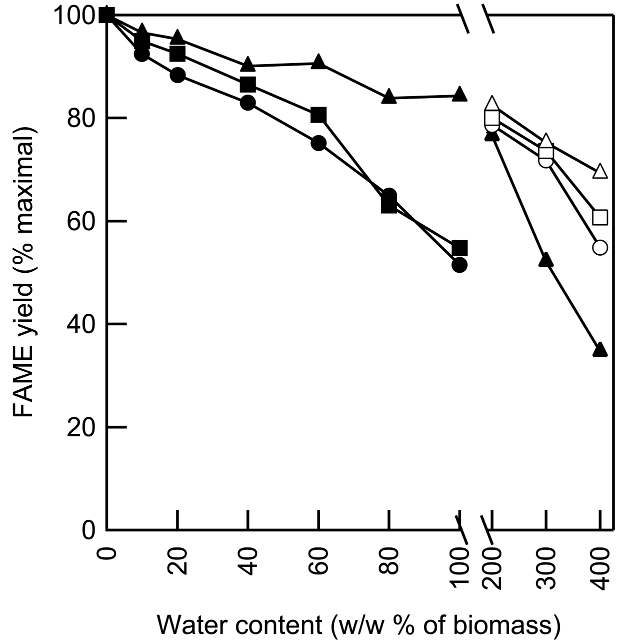

Figure 3-4. The effect of water content on FAME yield from algae. Water content of biomass was varied by adding distilled water to freeze dried *C. gracilis* cells. Water content is reported as a percentage of the dry algal biomass (w/w %). Wet biomass samples were reacted with varying volumes of methanol containing 1.8% (v/v) H_2SO_4 by heating the mixture to 80°C for 20 min. The FAME yield observed for samples containing water, reported as a percentage of the dry sample FAME content, is plotted against the water content of the algal biomass in each sample. Volumes of methanol analyzed were ●, 2 mL; ■, 3 mL; ▲, 4 mL; ○, 5 mL; □, 6 mL; and △, 7 mL.

ever more water in the biomass sample, however, at some point the volume of methanol added would become impractical. Although increasing the volume of methanol in the direct transesterification reaction has the potential to reduce costs associated with drying the biomass, it is uncertain whether these savings will offset the cost of the increased volume of methanol. Additional studies will be required to understand the economic

viability of the process to produce biodiesel from algae. Such an analysis is beyond the scope of this study.

4. Conclusion

The parameters for the direct conversion of microalgal lipids presented here are applicable to diatoms, green algae, cyanobacteria, and a wild mixed culture, despite their diverse lipid compositions. The direct transesterification approach surprisingly yielded more biodiesel than was expected from the triglyceride content, indicating capture of fatty acids from membrane phospholipids. The realization that a direct transesterification approach can be used to generate biodiesel from mixed cultures that do not make significant TAGs should motivate a more careful analysis of the cost and benefit approaches being used in the pursuit of using algae as a renewable biofuel feedstock.

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CHAPTER 4

FACTORS AFFECTING THE ACCUMULATION AND COMPOSITION OF WAX ESTERS IN *MARINOBACTER AQUAEOLEI* VT8[1]

Abstract

Marinobacter aquaeolei VT8 is the type strain for a genus of moderately halotolerant bacteria capable of degrading a range of hydrocarbons. In addition to the degradation of hydrocarbons, *M. aquaeolei* VT8 also contains a collection of genes that are utilized in certain microorganisms to accumulate wax esters, a high value lipid component. We report here that when supplied with simple organic acids as the only carbon source under physiologically stressed conditions of nitrogen limitation, *M. aquaeolei* VT8 accumulates a range of wax esters similar to those previously derived from commercial whaling sources. The degree of saturation and the composition of the accumulated waxes obtained during growth were found to be influenced by the salt content of the growth media, and could be altered by changing the salinity of the media. Additionally, significant differences were found between the fatty alcohol and fatty acid components of the wax esters, indicating a differing degree of selectivity or separate pools of substrates for these two components. Further, the wax ester types could be altered by growing the bacteria in the presence of a variety of exogenously provided alcohols, ranging from medium chain alcohols such as isoamyl alcohol to fatty alcohols such as tetradecanol, demonstrating the potential of this bacterium as a model system for *in vivo* studies of wax ester production.

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Introduction

The accumulation of reduced or partially reduced carbon compounds is a physiological feature of many biological systems when grown under conditions of nutrient limitation. In bacteria, polyhydroxyalkanoates (PHAs) are the most common compound for energy storage (Anderson and Dawes, 1990). While PHAs are predominant, a small selection of bacteria can accumulate alternative storage compounds, including various lipids that are common in many eukaryotes. For example, some bacteria are known to accumulate neutral lipids such as triacylglycerides (Alvarez and Steinbüchel, 2002) while others produce wax esters (Ishige *et al*., 2003). Triacylglycerides are esters formed from fatty acids and the polyol glycerol, while wax esters are the esters of fatty acids and fatty alcohols. Triacylglycerides can be utilized for a broad array of purposes including cooking oil, and can be converted to biodiesel, while wax esters have utility in cosmetics, pharmaceuticals, and as a high value lubricant (Jetter and Kunst, 2008).

Recent reports have identified a new class of enzyme found in select bacteria that is capable of catalyzing the formation of both triacylglycerides and wax esters from diacylglycerides and fatty acyl-CoA or from fatty alcohols and fatty acyl-CoA, respectively (Figure 4-1) (Kalscheuer and Steinbüchel, 2003; Stöveken *et al*., 2005). The terminal enzyme in the proposed pathway leading to these two types of compounds in bacteria is known as the wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase (WS/DGAT). Some initial characterization of the enzyme has recently been completed from the bacterium *Acinetobacter calcoaceticus* ADP1 (Kalscheuer and Steinbüchel, 2003; Stöveken *et al*., 2005). Homologs of the gene responsible for this

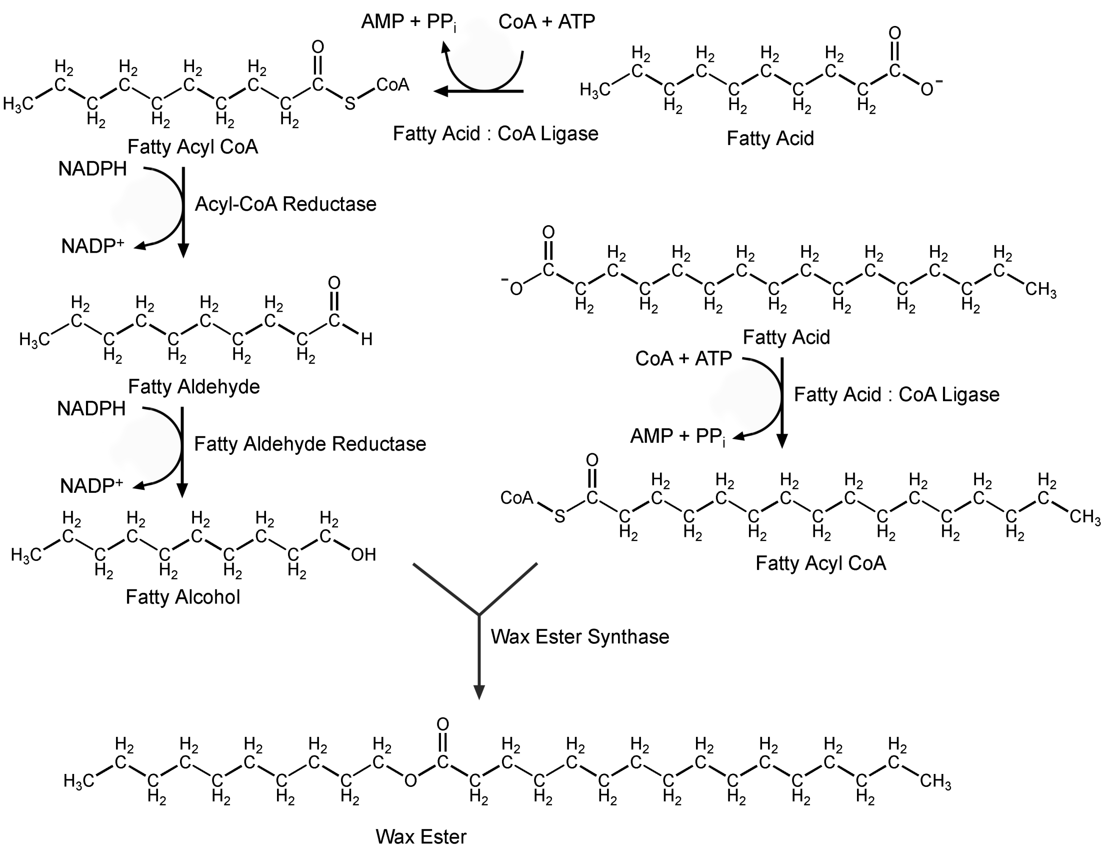


Figure 4-1 Putative wax ester synthesis pathway. Proposed bacterial pathway for wax ester synthesis from fatty acids.

enzyme have since been found in a selection of other bacteria, including *Rhodococcus* (Wältermann *et al*., 2007; Alvarez *et al*., 2008) and many species of *Mycobacterium* (Daniel *et al*., 2004; Wältermann *et al*., 2007). Several other bacteria, whose genomes have been recently sequenced, including *M. aquaeolei* VT8 (Huu *et al*., 1999; Wältermann *et al*., 2007), *Hahella chejuensis* KCTC 2396 (Jeong *et al*., 2005; Wältermann *et al*., 2007), and several species of *Psychrobacter* (Bakermans *et al*., 2006; Wältermann *et al*., 2007) have also been found to contain homologs of these genes.

Marinobacter species have been shown to accumulate isoprenoid wax esters when grown on the isoprenoid compounds phytol, squalene, and 6, 10, 14-trimethylpentadecan-2-one (Rontani et al., 1999; Rontani et al., 2003). Genes from *Marinobacter hydrocarbonoclasticus* DSM 8798 homologous to the *Acinetobacter* WS/DGAT gene were found to be active with the isoprenoid substrates phytol and phytenoyl-CoA (Holtzapple and Schmidt-Dannert, 2007). In addition to producing wax esters when grown on isoprenoid compounds, *Marinobacter hydrocarbonoclasticus* SP17 is capable of producing wax esters when grown on alkanes (Klein *et al*., 2008). Each of these reports described the production of wax esters from energy rich hydrocarbons, but did not investigate whether *Marinobacter* species accumulate wax esters when grown on minimal media.

Here, we report a detailed study of the growth conditions and stresses that result in the accumulation of wax esters in the bacterium *Marinobacter aquaeolei* VT8, similar to those found in other reported species (Gallagher, 1971; Bryn and Jantzen, 1977; Russell and Volkman, 1980; Fixter *et al*., 1986; Ishige *et al*., 2003). To characterize the possible enzymes involved in the formation of these wax esters, we compared the types and percentages of the fatty acid component and the fatty alcohol component over a range of salt concentrations. We further probed if the wax ester composition (type of alcohols and fatty acids in the wax ester) could be modified by the inclusion of different exogenously added alcohols. These experiments indicate that the fatty acid and fatty alcohol components of the wax esters are either derived from two separate pools of initial substrate or the initial substrate is possibly modified for the synthesis of wax esters. They

further demonstrate the potential to use this organism for the production of a broad range of wax esters having a wide array of properties.

Results

Neutral Lipid Extraction – A primary focus of these studies was to determine whether *M. aquaeolei* VT8 would accumulate neutral lipids such as wax esters or triacylglycerides (TAGs) when grown on simple organic acids. To test if *M. aquaeolei* VT8 might accumulate significant amounts of neutral lipid under minimal nutrient growth conditions, this bacterium was cultured on a variation of ATCC Media 2084 (described below), also known as *Halomonas* media (Vreeland *et al*., 1980; Huu *et al*., 1999). In this minimal media, amounts of nitrogen were varied by lowering the concentrations of nitrogen containing components. The cells were grown to stationary phase and held for a day prior to harvest, drying and extraction. An initial analysis by gas chromatography using a flame ionization detector and high temperature column confirmed the presence of small quantities of several peaks in the region of the chromatogram where wax esters would be expected (Figure 4-2 and Figure 4-3), but little evidence of anything in the region corresponding to TAGs. The protocol used here to extract wax esters is routinely used to extract TAGs from a variety of different bacteria, algae and diatoms, and would be expected to extract these neutral lipids from *Marinobacter* as well if they were present. This protocol has been tested against the Bligh and Dyer method (Bligh and Dyer, 1959), with comparable extraction profiles and amounts of wax ester. In none of the various growths and extractions performed as part of this work were significant signs of TAGs (above trace levels) found in any of the samples

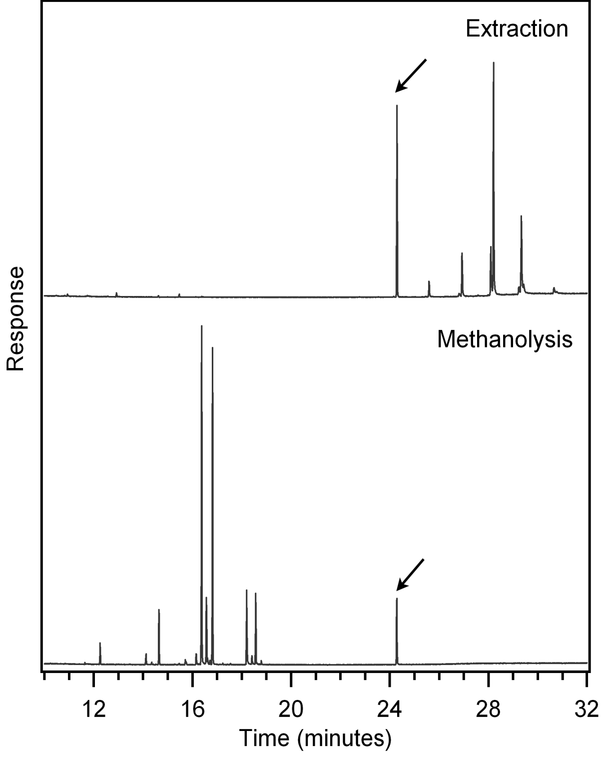


Figure 4-2 Wax ester composition analysis. Shown are the GC chromatograms of the extracts from *M. aquaeolei* VT8 (top trace). The wax ester containing portion was obtained and dried down and used to perform a methanol:acid treatment to yield the methyl esters of the fatty acids and the fatty alcohols (bottom trace). Chromatograms shown were analyzed using GC/MS as described in the methods section. All peaks in the traces were identified and used to quantify the percent composition of the fatty alcohols and fatty acids in the wax esters obtained. An internal standard (marked with an arrow) is included in each sample for analytical reproducibility purpose.

analyzed. One specific peak in the wax ester region matched the retention time of an external standard of hexadecyl hexadecanoate, and gave a positive match when analyzed

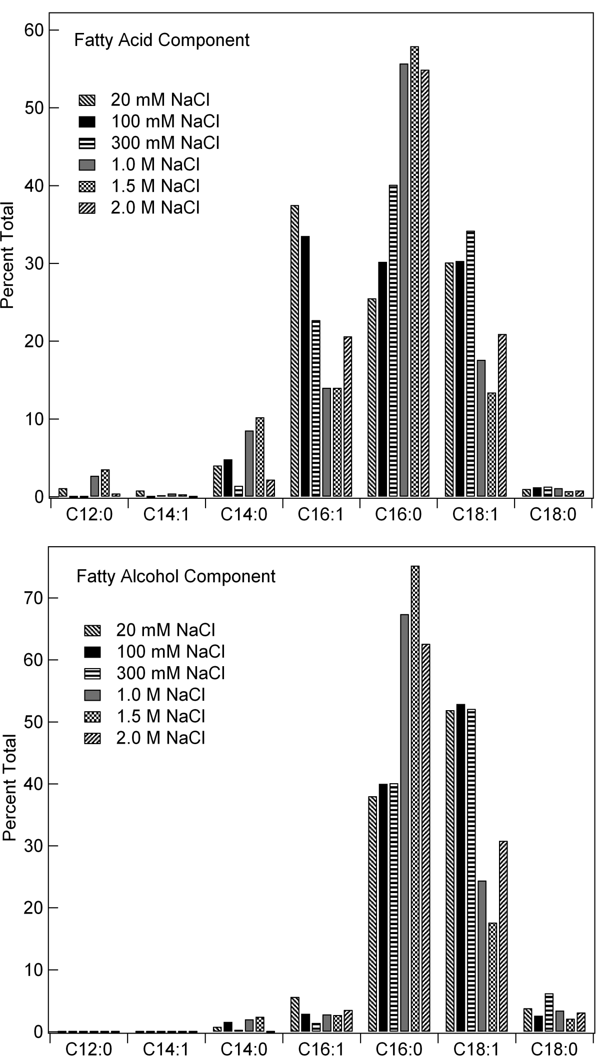


Figure 4-3 Wax ester compositions under different salt concentrations. Shown are the percent composition of the fatty acids (top) and fatty alcohols (bottom) obtained from cells grown under different salt concentrations. Percentages were calculated following identification of all components in the chromatograms of the methanol:acid treated wax esters under the various growth conditions. Samples were compared to external standards obtained from Nu-Chek Prep or Sigma-Aldrich. Results are presented as bar graphs for simple comparisons between the fatty acid and fatty alcohols of the same size (x-axis) under the different salt concentrations.

by mass spectrometry, indicating that the inclusion of long chain alkanes or other larger hydrocarbons are not necessarily required for the production and accumulation of wax esters in this species, and that simple organic acids could support the growth of the organism and the production of these high energy compounds.

Media Minimization Studies for Lipid Accumulation in *Marinobacter* – As an initial study of the stress conditions required for wax ester accumulation in *M. aquaeolei* VT8, a minimal media was developed using the *Halomonas* media (ATCC 2084) as a template. A first effort was to eliminate complex components such as yeast extract and protease peptone, and to lower components such as casamino acids. Several carbon sources were tested, including glucose, acetate, succinate, citrate, malate, pyruvate, fumarate and lactate. For each of these carbon sources, only 4.0 g/L of material was added, and only 100 mg/L of casamino acids with vitamins was included. All other media components were free of fixed carbon. Comparable rates of growth and final cell densities were found for each carbon source listed above, except glucose, which gave a much lower rate of growth.

In addition to the tests of carbon sources, the amount of nitrogen in the form of urea added was varied to limit the total nitrogen available to the cells in attempts to induce the storage of wax esters or TAGs. In these experiments, it was found that lowering the urea concentrations from 1.0 g/L to 100 mg/L was sufficient to result in high yields of wax esters (see below). Further experiments substituted the source of nitrogen with nitrate, nitrite, and ammonia at concentrations equivalent to the molar nitrogen content of 100 mg/L of urea, and each nitrogen source was found to support growth with sufficient induction of wax ester accumulation.

Quantification of the wax esters was done by extracting lipids followed by gas chromatography using a range of commercially obtained wax ester, fatty acid, and fatty alcohol standards (Sigma Aldrich and Nu-Chek Prep). Additionally, several wax esters were synthesized using the protocol described in the materials and methods to identify specific wax esters found in *M. aquaeolei* VT8 by retention time and mass spectrometry comparisons. The amounts of wax ester obtained were compared to a gravimetric analysis of the wax esters extracted and dried down with reasonable agreement (based on the absence of any additional peaks in the chromatogram, and the final weight of waxy material obtained after overnight drying at room temperature). Based on both GC and gravimetric analysis, wax compositions of ~25 mg total wax esters from 200 mg of dry cells were obtained from most of the nitrogen limited growths.

Wax Ester Compositional Analysis – The composition of wax esters found naturally in biological samples is difficult to define using only extraction and gas chromatography, even when combined with mass spectrometry. The reason for the lack of specificity is related to the fact that most of the high temperature columns required to separate these wax components resolve the compounds primarily based on the total number of carbons in the wax ester, such that a C16 alcohol and C18 acid based wax ester would have a similar retention time to a C18 alcohol and C16 acid based wax ester. A clear evaluation of the wax ester composition requires first hydrolyzing the ester bond, to break the wax ester down into a fatty acid and fatty alcohol. This is especially important when the wax ester contains one or more points of unsaturation, as it is difficult to assign whether this unsaturated bond is present in the alcohol derived portion, or the fatty acid derived portion. The identification of the principle components that wax esters

are derived from could give some clue as to the upstream processing or specificity of the enzymes responsible for supplying the fatty alcohols and fatty acids utilized to produce wax esters in a specific species.

To characterize the wax esters derived from *M. aquaeolei* VT8, we first developed and tested a protocol to convert commercially available wax ester into the component fatty acid methyl ester and fatty alcohol using an acid catalyzed transesterification in the presence of an excess of methanol, utilizing a protocol similar to that published previously (Wahlen et al., 2008) and detailed in the materials and methods section here. The technique was applied to a sample of approximately 5 mg of wax ester extracted from *M. aquaeolei* VT8 grown under various salt concentrations (from 20 mM to 2.0 M NaCl). The resulting products (containing both the fatty acid methyl esters and the free fatty alcohols) were analyzed by gas chromatography coupled to mass spectrometer to identify and quantify the component peaks (Figure 4-2). Each peak was identified by comparing its fragmentation pattern and parent ion mass against the NIST 05 mass spectral library and its elution profile.

The resulting composition of the fatty acid and fatty alcohol components obtained when *M. aquaeolei* was grown over a range of salt concentrations was identified and quantified using GC/MS, and the results are depicted in Figure 4-3. For the fatty acid component of the wax esters (Figure 4-3 A), it was observed that the fatty acids shift from higher levels of unsaturation for lower salt concentrations (20 to 300 mM NaCl) to higher levels of saturation for high salt concentrations (1.0 to 2.0 M NaCl). The fatty acids also cover a larger range of sizes, from C12:0 to C18:1.

In contrast to the results for the fatty acid component (Figure 4-3 A), the fatty alcohol component shows a much lower degree of diversity (Figure 4-3 B). For the fatty alcohol component, the alcohols consisted primarily of two separate alcohols, C16:0 and C18:1. While small amounts of C16:1 alcohol were found (1 to 5 % of the total), the trend does not follow the results seen for the fatty acid component, which is a much higher percentage (approximately 15 to 40 %) of the total fatty acids. It is not expected that this result is due to chemical modification during the methanolysis treatment, as the C18:1 alcohol would have likely been affected as well. Thus, the results reveal stark differences in the composition of the two different components, which are discussed below.

While it is not possible to identify the precise identity of different wax esters based only on the wax ester peaks by mass spectrometry, it is possible to determine the overall size and level of saturation from the analysis. In general, for most of the wax ester profiles, we found 5 groups of peaks with each group consisting of two peaks that do not clearly resolve. These wax ester groups ranged in size from C28 wax esters to C36, with the unsaturated wax esters eluting from the column first, and the saturated wax esters following. The most common wax esters found were either C32 (labeled as peak 2 in Figure 4-4) or C34, which supports the hypothesis that the wax esters result from random selection of substrates and the low fidelity of the WS/DGAT enzyme that is the terminal enzyme in the production of these compounds.

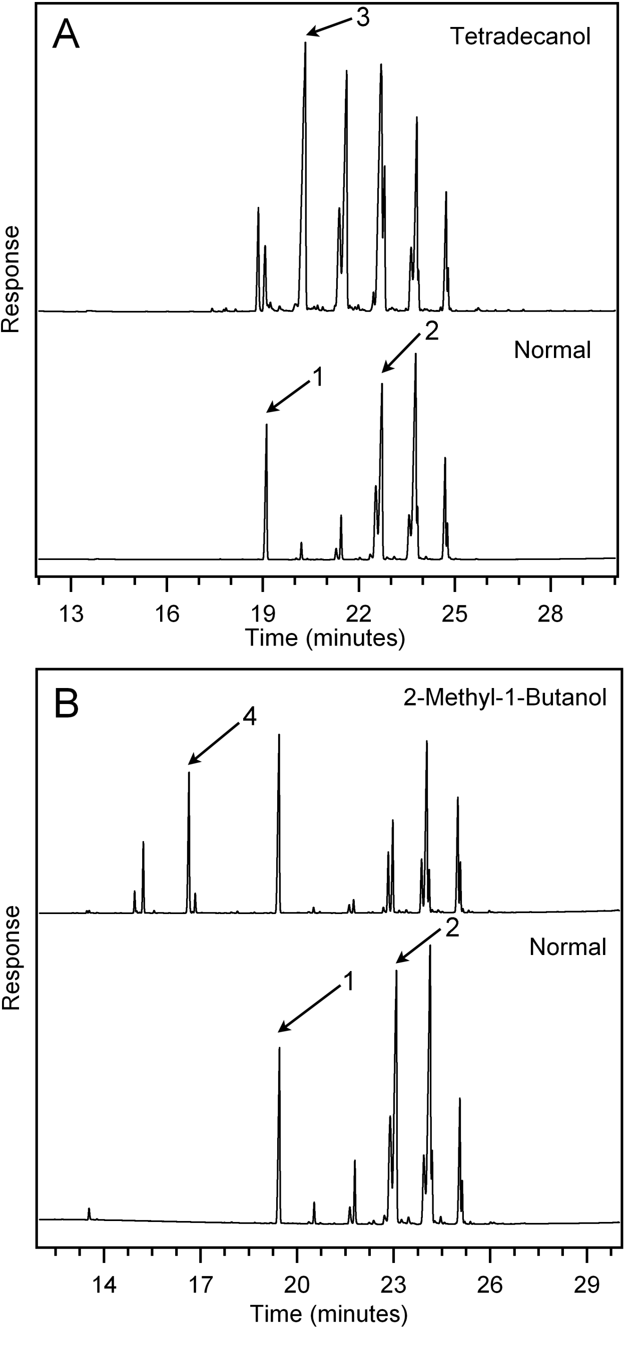
Alterations to Wax Ester Composition by Exogenously Added Alcohols – An additional interest in this work was to determine whether the wax ester profile in *M.*

aquaeolei VT8 could be altered through the addition of exogenous alcohols, ranging from alcohols found as low percentages in the natural wax esters (tetradecanol) to much smaller alcohols such as isobutanol or n-butanol. These studies are of interest for both biosynthetic applications, and also for cellular assays of enzyme activity.

For initial experiments, *M. aquaeolei* VT8 was grown under normal nitrogen limiting conditions, to induce the accumulation of wax esters in the cell. Shortly after inoculation, 200 mg of tetradecanol (solid crystals) was added to the culture, while it was left out of the control. In the media, the tetradecanol floated on the surface of the solution during incubation, and more was added only if it was no longer visible on the surface. Inclusion of tetradecanol resulted in a shift in the profile of the wax esters that were accumulated by *M. aquaeolei* VT8 (Figure 4-4 A), leading to a strong increase in the peaks correlating to C28 wax esters and C30 wax esters, from 1.4% to 24% and from 5.3% to 25% of the total wax esters found, respectively. Several other smaller alcohols (octanol, decanol and dodecanol) were also included, and each showed a similar type of shift in the wax ester profile of the waxes that were accumulated (results not shown). These results indicate that *M. aquaeolei* VT8 is capable of utilizing a broad range of long chain alcohols and follows a general activity profile *in vivo* favoring longer chain alcohols, similar to the results that have been reported for *in vitro* experiments (Kalscheuer and Steinbüchel, 2003; Stöveken *et al*., 2005).

Further efforts sought to determine if *M. aquaeolei* VT8 could utilize much smaller alcohols such as n-butanol and the branched alcohol isobutanol when included in the growth medium. Initial attempts showed slight alterations with several new peaks found near the region of the chromatogram where biodiesel (methyl and ethyl esters)

Figure 4- 4 Wax esters obtained during growth with exogenously provided alcohols. Shown are the GC chromatograms of the solvent extracts of dried *M. aquaeolei* VT8 cells grown in the presence of tetradecanol, versus a control without the addition of the tetradecanol (A). Several peaks are highlighted from the chromatogram, which were identified by retention time and mass spectrometry to confirm identities. Peak 1 corresponds to an internal standard (octacosane) added to monitor analytical performance. Peak 2 corresponds to the peak for hexadecyl hexadecanoate. Peak 3 was identified as a C28 wax ester, composed of tetradecanol and tetradecanoate. A similar experiment is shown for 2-methyl-1-butanol (B), where addition of the exogenous alcohol resulted in the production of smaller wax esters, with properties similar to biodiesel. Peak 4 corresponds to the 2-methyl-1-butyl ester of oleic acid (C18:1)


A
Tetradecanol
3
Normal
1
2
Response
13
16
19
22
25
28
Time (minutes)
B
2-Methyl-1-Butanol
4
Normal
1
2
Response
14
17
20
23
26
29
Time (minutes)

would be expected, which correlated well with the expected product for a butyl or isobutyl ester. Building on this initial success, several other alcohols that can be derived from alternative pathways such as the Ehrlich pathway (Hazelwood *et al*., 2008) were also tested as media components. As this strain is reported to grow in the presence of various hydrocarbons (Huu *et al*., 1999; Soltani *et al*., 2005) it was of interest to see if different alcohols would be inherently toxic to growth of this bacterium. Alcohols tested included ethanol, isopropanol, n-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenyl ethanol. For each alcohol, ~100 μL of the alcohol was added starting the second day of the growth following inoculation, and additions continued each day after to maintain a significant amount of the alcohol in solution over the course of the growth. *Marinobacter* grew well on all of the alcohols, with only isopropanol inducing any morphological differences in the cells obtained, observed as poor packing into dense pellets during the centrifugation. While n-butanol resulted in only trace quantities of these compounds in the chromatogram, the branched isobutanol resulted in significantly higher amounts. Of the various alcohols tested, several resulted in large alterations in the quantities and compositions of the wax ester fractions, with results similar to those shown in Figure 4-4 B.

Similar to *in vitro* reports for the WS/DGAT enzyme from *Acinetobacter* (Kalscheuer and Steinbüchel, 2003; Stöveken *et al*., 2005), the *Marinobacter* cells seemed to follow a profile where increasing amounts of the corresponding esters were produced as larger and more branched alcohols were added in the media. The inclusion of the alcohols 2-methyl-1-butanol and 3-methyl-1-butanol, which can be produced through the Ehrlich pathway (Sentheshanmuganathan and Elsden, 1958; Sentheshanmuganathan,

1960) from the amino acids isoleucine and leucine, were able to alter the percentages of the wax esters produced in the cells, so that when the alcohol 2-methyl-1-butanol (Figure 4-4 B) was included in the growth medium, as much as 26% of the total wax ester fraction was represented by esters derived from this alcohol. Mass spectrometry analysis of the four new primary peaks indicated that these were primarily (approximately 60%) the alcohol ester of oleic acid, and secondarily (approximately 25%) the alcohol ester of palmitic acid. Since all these growths were done in the presence of 1.0 M NaCl, these results are unexpected, as oleic acid and palmitic acid comprise 20% and 55% of the total fatty acid composition of wax esters obtained at this salt concentration when grown in the absence of the added alcohols. This indicates that the inclusion of these smaller branched alcohols altered the composition and degree of saturation of the cellular components of these esters, a result supported by the wax ester profile for the components not derived from these alcohols (less hexadecyl hexadecanoate). Whether this is the result of adaptation due to toxicity issues from the presence of these alcohols in the growth media, or a change in lipid production due to differential fractionation of the waxes obtained, could not be ascertained from the data.

Discussion

The moderately halophilic bacterium *M. aquaeolei* VT8 is classified as a hydrocarbon degrading species and was isolated from an oil rich environment (Huu *et al*., 1999). Many *Marinobacter* species have been reported to utilize hydrocarbons for their growth (Gauthier *et al*., 1992; Huu *et al*., 1999; Lattuati *et al*., 2002). Based on this phenotype, *M. aquaeolei* joins a range of organisms that can degrade and utilize

hydrocarbons as a source of carbon for growth. It has also been reported previously that *Marinobacter* species accumulate wax esters when grown on phytol (Rontani et al., 1999; Rontani et al., 2003) or on alkanes (Klein *et al*., 2008), but wax ester production starting from simple organic acids or simple carbohydrates (*de novo*) in *Marinobacter* has not been described. This is of interest because one of the proposed functions of the WS/DGAT enzyme in species such as *M. aquaeolei* VT8 is to accumulate wax esters and TAGs as an energy storage compound in the cell under periods of cellular stress. The *de novo* biosynthesis of wax esters has been reported for *Acinetobacter calcoaceticus* when grown on succinate, acetate, or glucose (Fixter *et al*., 1986; Wältermann *et al*., 2005). Wax ester synthesis in *Alcanivorax borkumensis* was reported to be dependent on the presence of alkanes, while *Alcanivorax jadensis* is capable of wax ester production when grown on sodium pyruvate as well as alkanes (Kalscheuer *et al*., 2007). Species of *Marinobacter* contain several variant genes homologous to the genes encoding the wax ester synthase enzymes studied previously in *Acinetobacter* (Kalscheuer and Steinbüchel, 2003; Stöveken *et al*., 2005; Holtzapple and Schmidt-Dannert, 2007), and thus it seemed probable that *M. aquaeolei* VT8 would be capable of *de novo* wax ester synthesis.

In this work, we demonstrate that the type strain *M. aquaeolei* VT8 does indeed accumulate a series of straight chain wax esters (not isoprenoid derived) when grown on simple organic acids such as citrate and succinate. Similar to reports for other bacteria and algae systems, where lipid accumulation is induced by stress conditions requiring ample carbon but a limited nutrient such as nitrogen (Hu *et al*., 2008), *M. aquaeolei* VT8 accumulated significant amounts of these wax esters under nitrogen limited stress conditions, and showed little evidence of these compounds when grown using an

enriched media such as LB broth. Basic media growths yielded between 0.6 to 0.8 g dry weight per liter after 4 days of growth, while similar growths with media compositions containing a greater concentration of a nitrogen source yielded between 1.1 and 1.4 g cellular dry weight per liter.

Wax esters differ from fatty acid and TAGs (the latter serving as a storage compound in some species), in that they are composed of a second component derived from a fatty alcohol that is presumed to be derived from a reduced carbon stream (possibly from fatty acids as well, as depicted in Figure 4-1). While this is a well known feature of wax esters, little information has been presented in the literature about the composition of the two separate components in natural wax esters accumulated by most bacteria, and the profiles of these species is of interest in our efforts to understand the pathways and specificity of the enzymes that are involved in wax ester biosynthesis. Thus, comparisons of the fatty acids from these bacteria alone does not give a clear picture of how these compounds are formed. This work provides a detailed analysis of the profile of these unique neutral lipids from *M. aquaeolei* VT8, and reveal the changes that can be induced in the properties of the lipids by simply changing the salt content in the media, leading to a higher degree of saturated wax esters in brine conditions (1.0 M NaCl) versus a higher degree of unsaturated wax esters when grown in conditions more similar to freshwater or seawater (300 mM NaCl).

One of the most striking findings from this research is related to the stark differences uncovered between the fatty acid and fatty alcohol components of the wax esters obtained from *M. aquaeolei* VT8. While the fatty acids cover a broader range, the fatty alcohol is comprised primarily of two different alcohols (C16:0 and C18:1), and

lacks the same level of diversity. This result indicates a clear difference between the two components. There are several explanations that could account for these differences. The first explanation is related to selectivity of the two unique enzymes that are proposed to convert fatty acids to fatty alcohols. The first enzyme, the fatty acyl-CoA reductase, has not been studied in any great detail. While reports have identified this enzyme from another species (Reiser and Somerville, 1997), the purification of active enzyme has not been described, and the range of substrates has not been studied in detail. A version of this enzyme in *Marinobacter* has not yet been confirmed, though investigations are underway in our laboratory. The second enzyme, the fatty aldehyde reductase, has been studied previously in our laboratory (Wahlen *et al*., 2009), and could have a specific activity toward individual substrates. Additionally, we have not ruled out the interaction of a broad range alcohol dehydrogenase, which is also present in *M. aquaeolei* VT8. However, it would be quite interesting if one of these enzymes were selective for the C16:0 derived aldehyde over the C16:1, but was not as selective for the C18:0 derived species over C18:1. Other possible explanations for the change include possible downstream processing to reduce any unsaturated bonds in the C16:1 derived alcohol. However, based on the presence of smaller fatty acids (C12:0 and C14:0), but the absence of the corresponding C12:0 alcohol, it is difficult to propose that this is the result of a downstream processing to the final substrate or final wax ester product. It is also possible that the fatty alcohols present in this species are derived from a completely different pool of reduced carbon species, though the presence of homologs and enzymes with activity toward acid derived products (Reiser and Somerville, 1997; Wahlen *et al*., 2009), lead us to favor a single pool of reduced carbon from fatty acid synthesis that are modified to

yield the fatty alcohol (Figure 4-1). Future experiments are planned to investigate the profiles of enzymes present during wax ester accumulation to develop a clearer picture of this pathway.

Reports of the enzyme responsible for the terminal step in wax ester synthesis in the cell, the WS/DGAT enzyme, indicate that this class of the enzymes is amenable to the utilization of a broad range of substrates (Kalscheuer and Steinbüchel, 2003; Kalscheuer *et al*., 2003; Stöveken *et al*., 2005; Uthoff *et al*., 2005). Indeed, the WS/DGAT enzyme can catalyze the formation of esters with alcohols as small as ethanol, and as large as the C30 alcohol triacontanol (Stöveken *et al*., 2005). We have purified two of the WS/DGAT enzymes present in *M. aquaeolei* VT8, and determined that these enzymes have activity toward the range of substrates found here (unpublished data), and others have reported similar activities for related enzymes (high homology) in other species of Marinobacter (Holtzapple and Schmidt-Dannert, 2007). While it is of interest which specific WS/DGAT enzyme or enzymes might be functioning during the induction described in this work, this question was not the focus of the current work, and will be investigated by other means in the future.

Since *Marinobacter* species are known to grow in very complex media that include an array of hydrocarbons (Gauthier *et al*., 1992; Huu *et al*., 1999; Lattuati *et al*., 2002; Klein *et al*., 2008), we tested the feasibility of altering the profile of the wax esters accumulated in the cell by providing exogenous alcohols in the media. Inclusion of alcohols present in *Marinobacter* wax esters such as tetradecanol (found to be about 1% of the alcohol composition) led to a dramatic shift in the profile of the wax esters that were accumulated, indicating that this compound persisted within the cell (even when

provided as a hydrophobic solid) and was utilized by the enzyme as a substrate *in vivo*. It also appears that tetradecanol can be converted to serve as the fatty acid component in the reaction, presumably through enzymatic steps oxidizing the alcohol to the acid, where it can then be converted to the fatty acyl-CoA required to serve as the fatty acid component substrate in the general biosynthetic scheme (Figure 4-1).

Further, we sought to determine whether the WS/DGAT enzymes present in *M. aquaeolei* VT8 are also capable of using much smaller alcohols that would result in an ester with properties similar to traditional biodiesel. Inclusion of medium chain alcohols such as isobutanol and isoamyl alcohol resulted in a significant percentage of the wax ester profile consisting of these derived alcohol esters (Figure 4-4 B). These results indicate that the alcohols were not significantly toxic to the cells at the concentrations utilized, and that there is a significant potential for the production of a broad range of esters in bacteria such as *Marinobacter*. It further demonstrates that *M. aquaeolei* VT8 could be a candidate for the *in vivo* accumulation of a fuel such as biodiesel.

In summary, we demonstrate here that under nutrient limitation conditions, *M. aquaeolei* VT8 can be induced to accumulate a significant amount of wax ester (as dry weight), similar to reports in other bacteria (Bryn and Jantzen, 1977; Fixter *et al*., 1986; Ishige *et al*., 2003). Further, we show that the degree of unsaturation and overall composition of these wax esters is altered based on the amount of salt present in the media. The bacterium is also capable of utilizing a range of alcohols provided in the media to alter the internal composition of the wax esters that are accumulated. This feature could lead to various biosynthetic applications with this microbe.

Experimental Procedures

Materials – N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and all other reagents used were obtained from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Fatty acid methyl ester and fatty alcohol standards were obtained from Nu-Chek Prep (Elysian, MN).

Bacterial Growth – *M. aquaeolei* VT8 (ATCC 700491) was obtained from the American Type Cultures Collection and grown on either ATCC Media: 2084 *Halomonas* media (Vreeland *et al*., 1980; Huu *et al*., 1999) or Luria-Bertani (LB) agar plates supplemented with NaCl (40 g/L added). All growths in liquid culture were done using a basic media with modifications derived from the 2084 *Halomonas* media. This media contained the following per liter unless otherwise stated; NaCl (50 g), casamino acids with vitamins (100 mg), sodium succinate (7 g), urea (100 mg), $MgSO_4 \cdot 7H_2O$ (5 g), K_2HPO_4 (500 mg), $CaCl_2$ (200 mg), and $FeSO_4 \cdot 7H_2O$ (35 mg) adjusted to a final pH of 7.3. Cells were grown as 1 L volumes in a 2 L Erlenmeyer flask on a shaker table at 28°C and 200 rpm. All growth experiments were run for 3 days, unless stated otherwise. Cells were harvested by centrifugation at 7000 g. The effect of different carbon sources on the growth of *M. aquaeolei* and on the production of wax esters was evaluated by replacing succinate in the media above with an equimolar amount of an alternative carbon source. The following compounds were used as the sole carbon source: sodium acetate, sodium citrate, malic acid, sodium pyruvate, sodium lactate, sodium fumarate, and hexadecyl hexadecanoate. The ability of *M. aquaeolei* VT8 to grow utilizing a variety of nitrogen sources was evaluated by substituting for urea at twice the molar concentration of the urea described in the basic media. The nitrogen sources examined were as follows:

sodium nitrite, sodium nitrate, glutamic acid, and ammonium chloride. As previous reports have indicated that wax ester production is nitrogen concentration dependent, the consequence of varying the concentration of urea from 1.67 mM (basic media) to 17 mM was determined. The effect of varying NaCl concentrations (20 mM to 2.0 M) in the media as well as the starting pH (6.3 to 8.3) was also evaluated. A variety of small, medium, and long chain alcohols were added to *M. aquaeolei* VT8 cultures to evaluate their effect on growth and lipid profile. The alcohols selected included each of the following: ethanol, 2-propanol, 1-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, phenyl ethanol, octanol, decanol, dodecanol, and tetradecanol.

Wax Ester Extraction – The extraction of neutral lipids from *Marinobacter* was performed by drying the wet cell paste using a freeze drier apparatus (Labconco Lyph-Lock 4.5 L) to remove residual water. During the extraction, all steps were performed in glass test tubes or I-Chem bottles (VWR Scientific, West Chester, PA) to avoid contact with any other plastics, which could lead to residual paraffin contamination. For each sample, 200 mg of dried cell material was broken into a fine powder with a metal spatula, and placed in a glass test tube. To this, 5 mL of solvent mixture (1:1:1 (v/v) chloroform:hexane:tetrahydrofuran) was added. This solvent combination was selected for a broad solvent polarity range that does not significantly interfere with the ability to use MSTFA for free acid and alcohol derivitizations. The cells and solvent were subjected to sonication in three separate 10 sec bursts using a sonifier (Branson sonifier 250, Danbury, CT) equipped with a microtip probe, set at constant duty cycle. Sonication was performed in a fume hood. The cell debris was centrifuged using a simple clinical tabletop centrifuge (Centrific 228, Fisher Scientific, Hampton, NH) for 1 min, and the

organic phase was removed with a 5 mL gas tight syringe to avoid disturbing the pelleted cell debris. The process was repeated two more times with 5 mL of the solvent mixture each time, and the extracts were combined and brought to 15 mL final volume. Extractions using this method were compared to the amounts of neutral lipid extracted when using the methanol and chloroform extraction protocol of Bligh and Dyer (Bligh and Dyer, 1959) with a good agreement in the quantities of wax ester obtained with both protocols. The solvent mixture selected here seems to be preferential for the extraction of the neutral lipid wax component of the cell, and did not appear to harvest a significant amount of the membrane fraction, as control samples with high concentrations of nitrogen in the media mix did not show any peaks related to combustible hydrocarbons throughout the chromatogram using an FID detector.

Wax Ester Synthesis – Due to the limited availability of specific wax esters from chemical suppliers, we developed a simple procedure for the rapid synthesis and purification of wax esters. The wax esters, tetradecyl hexadecanoate, hexadecyl hexadecanoate, hexadecyl oleate, oleyl hexadecanoate and oleyl oleate, were each synthesized by combining 1 gram of the appropriate fatty acid with 1 gram of the fatty alcohol in a test tube along with 18 μL of concentrated sulfuric acid (~18 M). Samples were then heated to 125˚C by microwave irradiation in a scientific microwave (CEM Discover, Matthews, NC) and held at that temperature for 20 minutes. After heating, samples were neutralized by the addition of a saturated sodium bicarbonate solution. Synthesized wax esters were purified by preparative TLC on silica gel 60 F_{254} plates (0.2 mm, EMD, Darmstadt Germany) using the solvent mixture hexane:THF:acetic acid 85:15:1. Plates were visualized by exposure to iodine vapors, using oleyl oleate as a

control. Regions of the plate corresponding to wax esters were removed and eluted with chloroform for gas chromatography and mass spectrometry (GC/MS) analysis.

Hydrocarbon Analysis – All analysis was performed on a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with both a flame ionization detector for quantification and a separate mass spectrometer (GCMS-QP2010S, Shimadzu Scientific) for peak identification. Analytes were separated on an RTX-Biodiesel column (10 m, 0.32 mm ID with a guard column, Restek, Bellefonte, PA) using a temperature program of 60°C for 1 min followed by a temperature ramp of 10°C per minute to 360°C for 6 min, and a constant velocity of 50 cm/sec with helium gas as the carrier. Samples were injected into a programmable temperature vaporizer (PTV) injector to simulate on-column injection that followed an identical temperature program to that listed above for the column. Injections of 1 µL were made from a GC vial with Teflon coated septa, which also contained octacosane (10 µL of a 10 mg/mL stock solution) as an internal standard. For derivitazations of free acids and alcohols, 50 µL of MSTFA was added and allowed to react at room temperature for a minimum of 60 min prior to injection. Integrations of peaks were done using GCsolution postrun version 2.3 (Shimadzu).

Wax Ester Composition Analysis – Once the wax ester was extracted from a known quantity of dried cells (~200 mg), the remaining solvent was removed under a stream of argon. To the dried wax ester sample was added 1mL of methanol and 18 µL of concentrated sulfuric acid, and the sample was heated at 125°C for 20 minutes by microwave irradiation in a scientific microwave (CEM Discover, Matthews, NC) to prepare the fatty acid methyl esters and free fatty alcohols from the wax ester. The sample

was then cooled rapidly, and approximately 1 mL of chloroform was added. The sample was then washed twice with 3 mL of distilled water to remove the methanol and sulfuric acid. The chloroform layer (containing the fatty acid methyl esters and the fatty alcohols) was then analyzed by GC/FID to quantify the fatty acid methyl esters and fatty alcohols, and by GC/MS to identify each component using the National Institute of Standards and Technology (NIST) '05 mass spectral library. Using this method, the sample can be further derivatized using the reagent MSTFA, which reacts with the remaining free fatty alcohols to yield the trimethylsilyl ether of the corresponding alcohol. This resulted in a shift in retention time of the corresponding alcohol peaks (see Figure 4-2), while not changing the retention time or amounts of the fatty acid methyl esters, and adds an additional level of certainty to the results. The amounts of fatty acids and fatty alcohols were determined as an area percent following identification of all major peaks in the chromatogram. Using this technique analysis, it was possible to identify and determine relative amounts of each fatty alcohol and fatty acid derived from the natural wax esters.

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CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF AN NADPH-DEPENDENT FATTY ALDEHYDE REDUCTASE FROM *MARINOBACTER AQUAEOLEI* VT8: ROLE IN BACTERIAL WAX ESTER PRODUCTION[1]

Abstract

Wax esters, ester linked fatty acids and long chain alcohols, are important energy storage compounds in select bacteria. The synthesis of wax esters from fatty acids is proposed to require the action of a four enzyme pathway. An essential step in the pathway is the reduction of a fatty aldehyde to the corresponding fatty alcohol, although the enzyme responsible for catalyzing this reaction has yet to be identified in bacteria. We report here the purification and characterization of an enzyme from the wax ester accumulating bacterium *Marinobacter aquaeolei* VT8, which is a proposed fatty aldehyde reductase in this pathway. The enzyme, a 57 kDa monomer, was expressed in *E. coli* as a fusion protein with the maltose binding protein on the N-terminus and was purified to near homogeneity using amylose affinity chromatography. The purified enzyme was found to reduce a number of long chain aldehydes to the corresponding alcohols coupled to the oxidation of NADPH. The highest specific activity was observed for the reduction of decanal (85 nmol decanal reduced/min/mg). Short chain and aromatic aldehydes were not substrates. The enzyme showed no detectable catalysis of the reverse reaction, the oxidation of decanol by $NADP^+$. The mechanism of the enzyme

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was probed with several site-specific chemical probes. The possible uses of this enzyme in the production of wax esters are discussed.

Introduction

Wax esters, long chain fatty acids linked to long chain alcohols, serve many different functions in biological systems (4, 14). In plants, wax esters are a primary constituent of the outer waxy layer of leaves, where they function as a hydrophobic barrier to prevent water loss. Wax esters are also found in high concentration in the spermaceti organ of the sperm whale, where they are thought to aid in regulating buoyancy. The unique properties of this family of compounds have made them valuable as additives in cosmetic and medical formulations, as well as high grade lubricants and food additives. A small group of microbes have been shown to accumulate wax esters, probably as energy storage compounds (3, 5). There is interest in understanding the pathways and enzymes utilized in bacterial wax ester synthesis as a possible way to produce wax esters of interest to biotechnology (4, 6, 7).

Early work on bacterial wax synthesis indicates a four enzyme pathway starting from long chain fatty acids (**Figure 5-1**) (7, 21). The first step in the pathway involves the formation of fatty acyl CoA from a fatty acid by the action of a fatty acid: CoA ligase that also utilizes CoA and MgATP. The fatty acyl CoA is reduced to the corresponding fatty aldehyde by the NADPH-dependent acyl-CoA reductase (11). It is proposed that the fatty aldehyde is then reduced to a fatty alcohol by a fatty aldehyde reductase (FALDR), although this enzyme has yet to be indentified in any bacteria. The final step in wax ester

formation is catalyzed by a wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase (WS/DGAT) (5). This enzyme has been purified from bacteria and partially characterized (5, 16, 19). The enzyme shows a broad substrate range for alcohols ranging from ethanol to triacontanol and for acyl-CoAs of various lengths. Branched and aromatic alcohols are also substrates (8, 16, 19). The wide substrate range of this enzyme offers the possibility of producing a number of wax esters biologically, including diesel substitutes (6).

A significant obstacle in utilizing the WS/DGAT enzymes has been the inability to identify a fatty aldehyde reductase in a bacterial system that would supply the fatty alcohol required for wax ester synthesis (7). With such an enzyme in hand, a multienzyme expression system could be developed that would allow biological production of wax esters as high valued oils and waxes, all derived from renewable resources.

In this work, we have cloned and expressed a gene from the marine bacterium *Marinobacter aquaeolei* that is predicted to be a fatty aldehyde reductase (FALDR) in the pathway for wax ester synthesis. The gene for this putative FALDR was identified by searching known bacterial genomes with the FALDR gene (CER4) sequence from the plant *Arabidopsis* (13). *Marinobacter* species are known to accumulate branched wax esters when grown in a media supplemented with phytol, but detailed reports regarding production of wax esters from simple sugars have not been reported (1). The product of the putative FALDR gene in *M. aquaeolei* shares primary sequence identity with the CER4 gene from Arabidopsis and is thus predicted to serve a similar function in wax ester synthesis (13). The FALDR gene from *M. aquaeolei* has been cloned and purified as

Figure 5-1: Proposed bacterial pathway for wax ester synthesis from fatty acids.

a fusion protein with the maltose binding protein. A description of this enzyme and its substrate specificity is reported.

Materials and Methods

Materials – All reagents were purchased from Sigma-Aldrich Company (St. Louis, MO) unless otherwise specified. Restriction enzymes, T4 DNA ligase and *Escherichia coli* strain TB1 were obtained from New England Biolabs (Ipswich, MA).

Bovine serum albumin (BSA) was fraction V (Sigma P/N A2153) and was prepared fresh daily in the same buffer as the assay. $NADP^+$-dependant alcohol dehydrogenase from *Thermoanaerobium brokii* (Sigma P/N A8435) was prepared fresh the day of use for control experiments.

Strains and Plasmid Constructions – *M. aquaeolei* VT8 was obtained from the American Type Cultures Collection (ATCC), and was grown initially on ATCC medium 2084 (*Halomonas* medium (2)) at 30ºC. Plates of the 2084 medium were prepared by adding 1.5% Bacto Agar (BD, Franklin Lakes, NJ). Genomic DNA was isolated by first growing 1 L of cells to an optical density of 0.6 at 600 nm followed by collection of the cells by centrifugation at 7000 g. The cell pellet was washed once with 50 mM phosphate buffer, pH 7.2. The cells were then suspended in 5 mL of 50 mM Tris-HCl buffer at pH 7.8 with 2% Triton X-100 and 5 mg of lysozyme. The suspension was allowed to sit for 10 minutes at room temperature followed by incubation in boiling water for 5 minutes. The solution was centrifuged at 10,000 g for 10 minutes. The supernatant was retained, and an equal volume of isopropanol was added to precipitate the DNA. The DNA was washed once with 10 mL of isopropanol, and then twice with 10 mL of ice-cold ethanol. The DNA was allowed to air dry and was then subjected to restriction digest and purification following the desalting protocol from the Qiaex II kit (Qiagen, Valencia, CA).

A gene (accession NC_008740.1) proposed to code for a fatty aldehyde reductase from *Marinobacter aquaeolei* VT8 was amplified from genomic DNA using the primers BBP244 5’ GATGAGGAT<u>CCATGG</u>AGCAATACAGCAGGTACATCACGCTGAC and BBP245 5’ GACTG<u>GAATTC</u>AGGCAGCTTTTTGCGCTGGCGCGC following the

Failsafe protocol (Epicenter, Madison, WI) using buffer G and an annealing temperature of 60˚C. The PCR product was purified using the Qiaex II desalting protocol (Qiagen, Valencia, CA) and was digested with EcoRI and NcoI along with the plasmid pBB052 (a pUC19 derivative with kanamycin in place of ampicillin for selection, and an N-terminal His-Tag followed by an NcoI site). The reaction was terminated by heat inactivation at 65˚C, followed by ligation. Plasmids were maintained in *E. coli* strain JM109 unless specified otherwise. The gene encoding for the N-terminal His tagged protein was then transferred to the pET-30A vector to express the fatty aldehyde reductase protein in *E. coli* strain BL21. Additionally, the EcoRI site following the fatty aldehyde reductase gene in the original vector was removed by digestion with EcoRI, filling in with T4 DNA polymerase, and ligation of the blunt-end product. A new EcoRI site was then introduced just upstream of the second codon of the gene by PCR amplification, removing the methionine start codon, and preparing the gene to be placed in-frame with an EcoRI site following a maltose binding protein from the pMAL-c2x plasmid (New England Biolabs, Ipswich, MA). The modified gene was then transferred to the pMAL-c2x plasmid, and sequenced to confirm that it contained no mistakes, prior to transferring this plasmid to the *E. coli* strain TB1 for expression of the maltose binding protein fused to the fatty aldehyde reductase.

Enzyme Purification – One liter of LB medium supplemented with ampicillin (100 µg /mL) in a 4 L flask was inoculated with 16 mL of an overnight culture of *E. coli* TB1 transformed with the pMAL-c2x vector expressing the maltose binding protein-fatty aldehyde reductase fusion (MBP-FALDR) and was grown for approximately five hours at 37˚C prior to induction by the addition of 50 mg of IPTG (isopropyl β-D-1-

thiogalactopyranoside), after which the culture was grown for an additional two hours at room temperature. Cells were harvested by centrifugation and were immediately frozen for later use. The cells were suspended in 30 mL of column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) supplemented with 0.1 mM phenylmethylsulfonyl fluoride. Cells were lysed by passing the suspended cells through a French pressure cell (SLM Aminco) three times in the presence of DNAse (P/N DN-25, Sigma-Aldrich). Soluble protein was collected by centrifuging the cell lysate at 17,500 g for 10 min. The supernatant was diluted three-fold with column buffer and was then applied to a column containing a 10 mL column bed of amylose resin (New England Biolabs, Ipswich, MA) and the column was washed with 30 mL of column buffer containing 1 M NaCl, followed by a second wash with 30 mL of column buffer. The fusion protein was eluted with 15 mL of column buffer containing 10 mM maltose.

Preliminary Assays of Fatty Aldehyde Reductase Activity – For initial assessment of the activity of the purified FALDR enzyme, 50 μg of purified FALDR protein was added to a reaction mixture containing 100 mM of Tris buffer at pH 7.9, 100 mM NaCl, 2.4 mM of either NADPH or NADH as a reductant, and decanal, oleic acid, and hexadecanol as possible substrates. The assays were run under an argon atmosphere in septa sealed vials overnight at room temperature with constant gentle mixing. The products of the reactions were then extracted from the buffer by adding an equal volume of hexane and organic layer components were analyzed by gas chromatography equipped with a flame ionization detector (Forte HT5 column, SGE Analytical Science, Austin, TX), 30 meter by 0.32 mm ID with 0.5 μm film thickness, with argon as a carrier and a temperature ramp from 60˚C to 360˚C increasing at 10˚C per minute). Samples

containing new peaks not present in a control sample were also run through a gas chromatograph equipped with a mass spectrometer (Shimadzu GC-2010 and GCMS-QP2010S) to assign the identity of the product.

Fatty Aldehyde Continuous Spectrophotometric Assay Development – All assays were performed in a sealed quartz cuvette with a 1 cm pathlength. The cuvette and solutions were first degassed with argon on a manifold to remove oxygen from the headspace and solutions. Substrates were initially disbursed into a buffer solution containing BSA at a concentration of 0.5 mg/mL by sonication for three ten second intervals using a microtip sonicator (Branson, Danbury, CT). The initial assay for pH optimization included a buffer of 100 mM MOPS, 100 mM MES, and 100 mM TAPS. For reduction assays, 75 μL of a 2 mg/mL NADPH stock was added to bring the initial concentration of NADPH in the cuvette to approximately 200 μM, and an approximate absorbance at 340 nm of 1.2 based on the reported extinction coefficient of 6220 M^{-1} cm^{-1} for NADPH (22). In all assays, the reaction was run for at least two minutes with the NADPH and substrate present, prior to the addition of any enzyme, to obtain a background oxidation rate of the NADPH. The absorbance reading at 340 nm was read every 0.5 seconds, and the reaction was run until a steady-rate was achieved for at least 60 seconds. A linear fit of the data was then used to establish the rate, and the initial background rate was subtracted, to determine the rate associated with substrate reduction by the enzyme.

When the assay was used to check for activity with NADH in place of NADPH, this compound was simply substituted at the same concentration, and the assay run as described above. Reactions using either $NADP^+$ or NAD^+ used the same concentration,

but instead followed the increase in absorbance monitored at 340 nm. Once the pH optimum was established for the enzyme, TAPS and MOPS were removed from the buffer, and only MES was used.

Results and Discussion

Expression of a Proposed Fatty Aldehyde Reductase – A previous report by Rowland *et al.* proposed that the gene CER4 from *Arabidopsis thaliana* coded for a wax ester biosynthetic enzyme (13). When this gene was disrupted in *Arabidopsis*, a phenotype resulted with significant decreases in concentration of measured primary alcohols and wax esters and slightly elevated levels of aldehydes found in the waxy cuticle that coats the aerial surfaces of the plant (13). Based on this evidence, the CER4 gene was tentatively assigned as a fatty aldehyde reductase. In an effort to find a similar enzyme in bacteria, this gene was used here in a BLAST search of several completed bacterial genomes that are known to contain the wax ester synthase (WS/DGAT) gene. Two species of marine bacteria, including *Marinobacter aquaeolei* VT8, were found to contain an open reading frame (ORF) with moderate similarity (48% positive and 27% identical for one species) to the CER4 gene.

The gene for the putative FALDR enzyme from *M. aquaeolei* VT8 was first cloned into a pUC19 derivative vector with an N-terminal 8 His-Tag. This gene was further modified to remove various restriction sites through silent mutations while maintaining the integrity of the protein sequence, and the modified gene was inserted into a Novagen pET vector (EMD Chemicals, Inc., San Diego, CA) to include the N-terminal histidine tag. While initial expression experiments using the pET vector showed a high

level of expression of the protein, initial attempts to purify the protein were hampered by low solubility. In all cases, the majority of the expressed protein associated with the cell debris following cell disruption and centrifugation (see **Figure 5-2**). Efforts to improve solubility by inclusion of a variety of common detergents (Tween 20, Triton X-100, Dodecyl Maltoside, and CHAPS) in varied concentrations were met with limited success, and was eventually abandoned for other approaches, as the majority of the FALDR seemed to remain in the insoluble pellet.

Several approaches were undertaken to improve the solubility of the FALDR enzyme. Eventually, the solubility problem was overcome by creating a fusion protein utilizing the highly soluble maltose binding protein (MBP). This was accomplished by inserting the modified gene into the multiple cloning site of the pMAL-c2x vector (New England Biolabs), which when expressed in the proper *E. coli* host strain, produced a protein that remained predominantly in the soluble fraction, even without the inclusion of any detergents (see **Figure 5-2**). This approach allowed a quick purification by using an amylose resin (New England Biolabs, Ipswich, MA) to bind the maltose binding protein portion of the fusion protein, and maltose for elution, resulting in a relatively pure protein (approximately 90% pure by SDS-PAGE analysis). The other minor protein components seen in the preparation are presumed to be related to the FALDR, either resulting from proteolytic degradation or premature termination of expression by the host system, as the associated bands were specific to the expression of the FALDR protein.

Initial Assessment of the Activity of the Fatty Aldehyde Reductase – Preliminary experiments were run with the FALDR enzyme to probe possible substrates by mixing either NADH or NADPH with an aldehyde (decanal), alcohol (hexadecanol)

or fatty acid (oleic acid) and then looking for changes in concentration of one or more of the potential substrates by GC analysis. The assays were allowed to run overnight in a sealed vial under an atmosphere of argon to maintain the reduced forms of the nicotinamide coenzymes. Of the possible substrates run in this experiment, only the vial containing NADPH showed a decrease in the decanal peak, and the generation of a new peak, which correlated to a retention time for decanol, and was confirmed by mass spectrometry. The reverse reaction using $NADP^+$ and hexadecanol or decanol showed no detectable levels of hexadecanal or decanal production. This initial result pointed to the likelihood that the enzyme is an NADPH-dependent fatty aldehyde reductase, and further experiments were run to characterize this activity. While none of the experiments

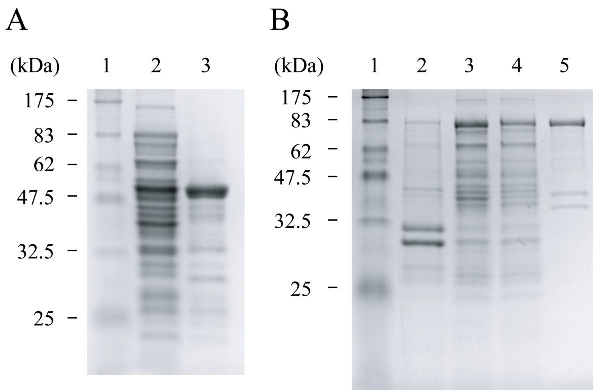

Figure 5-2: FALDR SDS-PAGE. (A) Solubility of N-terminal histidine tagged FALDR. Lane 1, molecular weight markers; sizes as indicated. Lane 2, soluble fraction of total cell lysate. Lane 3, insoluble fraction of total cell lysate. (B) Purification of MBP-FALDR fusion protein. Lane 1, molecular weight markers; sizes as indicated. Lane 2, insoluble fraction of total cell lysate. Lane 3, soluble fraction of total cell lysate. Lane 4, amylose column flow through. Lane 5, protein eluted with 10 mM maltose.

performed as part of this work indicated that the enzyme is capable of oxidizing the product decanol using $NADP^+$, it is possible that this reaction is extremely slow versus the reduction of the aldehyde, and thus is below the level of detection. The activity of medium chain alcohol dehydrogenases from a broad range of species show rates of oxidation of alcohols that are 10% of the rates of the reduction of the corresponding aldehyde, so it is possible that this rate is too low to detect (10).

Continuous Assay of Fatty Aldehyde Reductase Activity – Having established that the FALDR would utilize NADPH as a substrate, it was possible to employ a continuous spectrophotometric assay to monitor substrate reduction rates based on the loss of absorbance at 340 nm when NADPH is oxidized to $NADP^+$. Using this assay, it was possible to establish the pH dependence of the reduction reaction. The rate of reaction was highest at pH 6.3, with a steep increase in activity going from 8.0 to 6.3. The rate at 6.3 was only slightly greater than at 6.5 (30 percent higher). Several factors had to be considered in establishing the optimal pH of the assay. The first was the background oxidation of the NADPH without any enzyme present. NADPH naturally degrades to $NADP^+$ at neutral and acidic pH values, and is most stable under alkaline conditions (12, 23). The assays were run under an argon atmosphere to minimize oxidation of the NADPH by O_2. A further consideration was the rapid degradation that occurs at the lowest pH values. While the highest enzyme rates were observed at pH 6.3, the rates were highly sensitive to slight pH variations at this value. So, for standard assays used here, a pH of 6.5 was selected, where the activity was minimally affected by slight variations in pH.

Substrate Specificity and Kinetic Parameters – Six commercially available substrates were examined for reduction by the FALDR enzyme. The long-chain aldehydes decanal and dodecanal were examined, as well as the smaller aldehydes butanal, hexanal and octanal. The larger, unsaturated aldehyde, *cis*-11-hexadecenal was also tested. Testing of other long chain aldehydes will require those to be synthesized and purified. In addition to these straight chain aldehydes, activity was also tested with the aromatic aldehyde benzaldehyde.

Utilizing the continuous assay for fatty aldehyde reductase activity, the dependence of rate on substrate concentration was determined for all of the commercially available substrates described above. Each assay was repeated multiple times to confirm the results from each single determination. The results obtained for *cis*-11-hexadecenal are shown in **Figure 5-3** and are representative of the results obtained for repeated determinations and for the other substrates. The data were fit to the Michaelis-Menten equation, revealing a K_m of approximately 177 μM and a maximum velocity of 63 nmol/min/mg. The activity obtained for this substrate was slightly lower than that obtained for decanal. It is difficult to benchmark these rates as no comparable enzyme has been purified. In addition, several factors could limit the observed activity. All of the substrates tested have limited solubility in aqueous solutions, and must first be suspended in solution via sonication. Even under these conditions, it is likely that the substrates exist inside micelles. The sonication process utilized here could result in the partial degradation of the substrate over time. Additionally, the suspension of the substrate in solution is only temporary, and the substrate slowly separates from aqueous solution. Due to these limitations, the measured activity is likely to be an underestimate of the actual

activity. In the cell, the enzyme and substrate are likely associated with the hydrophobic membranes. The K_m values obtained for each of the substrates that showed activity were in the micromolar range, indicating that the enzyme should be active in the cell even at low substrate concentrations. It was also observed that the enzyme was unstable over time, even when stored at 4ºC, losing as much as 50% of the activity over a period of a week. Though the purification is quite rapid, it is uncertain how much activity is lost during preparation.

As can be seen in **Table 5-1**, the FALDR enzyme required a minimal chain length C8 aldehyde to show significant activity. The shorter substrates butanal (C4) and hexanal (C6) showed no apparent activity under these conditions, while the activity for octanal (C8) was approximately half the activity obtained for decanal (C10), which had the highest activity of the substrates tested (85 nmol/min/mg). A further investigation of the octanal activity revealed that the apparent K_m for this substrate may be very close to the concentration tested here (~750 μM), so that the activity would be greater with higher concentrations of substrate. The activity was lower with dodecanal (C12), though the apparent K_M was lower (~200 μM) than that for octanal and similar to decanal (~100 μM). In this instance, the solubility may play a more important role, as dodecanal was the longest saturated aldehyde commercially available that was used in this work. The substrate *cis*-11-hexadecenal (C16) showed activity comparable to decanal, though this substrate does contain a site of unsaturation, which could change accessibility to the active site versus dodecanal. It is also possible that the results here are related more to substrate solubility and availability to the enzyme. As a primary wax ester found in *M. aquaeolei* VT8 grown under nitrogen deficient growth conditions is hexadecyl

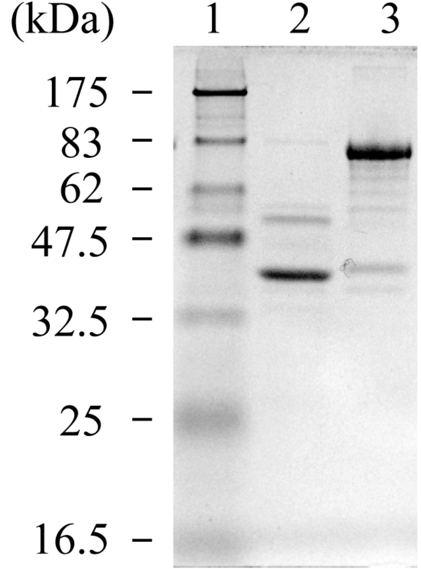

Figure 5-3: Factor Xa cleavage of the MBP-FALDR fusion protein. Lane 1, molecular weight markers; sizes as indicated. Lane 2, MBP-FALDR incubated overnight with Factor Xa. Lane 3, MBP-FALDR control incubated overnight without Factor Xa. Both protein samples were tested for fatty aldehyde reductase using decanal following incubation, and found to have comparable activities over a range of decanal concentrations.

hexadecanoate (unpublished data), it would be expected that hexadecanal would be a likely natural substrate of this enzyme. Future work will include efforts to produce adequate quantities of purified hexadecanal for kinetic studies. Finally, the aromatic ring containing aldehyde benzaldehyde showed no apparent activity when assayed at similar concentrations, indicating that the active site may be specific for straight chain aldehydes (saturated or unsaturated).

Table 5-1: Fatty aldehyde reductase substrate comparisons

Substrate*	Substrate Molecular Structure	Relative Activity (Percent of Decanal)
Butanal		< 1
Hexanal		< 1
Octanal		52
Decanal		100
Dodecanal		55
cis-11-hexadecenal		87
Benzaldehyde		< 1

Since all the assays were conducted with the MBP-FALDR fusion protein, a principle concern was whether the fusion protein accurately represented the activity of the wild-type enzyme. To examine whether the maltose binding protein affected the activity of the wild-type enzyme, the maltose binding protein was removed from the N-terminus of the FALDR protein by Factor Xa cleavage. The near complete cleavage of maltose binding protein was verified by SDS-PAGE (**Figure 5-4**). This cleavage is facilitated by the incorporation of a factor Xa cleavage site (Ile-Glu-Gly-Arg) just upstream of the EcoRI site of the pMAL-c2x vector. Assays conducted with the cleaved

FALDR protein and decanal did not reveal any loss or improvement in the rate of substrate reduction from the rate exhibited by the MBP-FALDR fusion protein.

Reversibility of the Enzyme with Fatty Alcohols – To test the possible reversibility of the FALDR, the oxidation of a number of alcohols to the corresponding aldehyde using $NADP^+$ as the oxidant was tested. In these assays, the increase in absorbance at 340 nm was followed. Under no conditions did any reduction of $NADP^+$ occur in the presence of the fatty alcohols tested. As a control, the alcohol dehydrogenase from *Thermoanaerobium brokii* was followed with 2-propanol and the same stock of $NADP^+$ to confirm the integrity of the assay. These results showed no evidence that FALDR is capable of catalyzing the reverse reaction, the oxidation of a fatty alcohol to a fatty aldehyde. Similarly, reactions were followed in the same manner by substituting NADH or NAD^+ for NADPH or $NADP^+$ using decanal and decanol, respectively, with no indication of activity over background with any combination except with decanal and NADPH. Based on this, it is proposed that the enzyme exhibits activity only for the reduction of fatty aldehydes in an NADPH-dependent reaction.

Inhibition Studies – To probe the possible mechanism of the FALDR, some potential chemical inhibitors were tested for effects on the reduction of decanal by the enzyme in the presence of NADPH. In all cases, the compound was tested initially at concentrations of 1.0 mM, and if a significant inhibition was found, lower concentrations were also tested (**Table 5-2**). The metal chelator EDTA, which is used during purification of the enzyme to limit the activity of metalloproteases, had little effect on the activity. Reductants such as ascorbic acid, dithiothreitol and β-mercaptoethanol, also showed little effect (less than 25% decrease in activity). Only dithionite showed a significant decrease

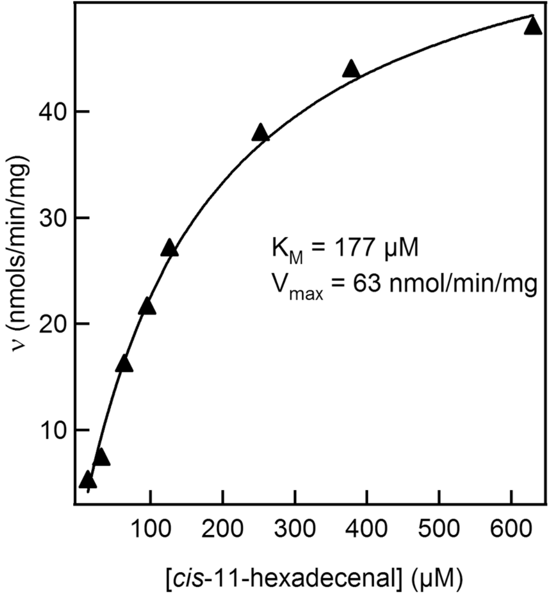

Figure 5-4: Substrate saturation for the MBP-FALDR fusion protein. Aldehyde reductase activity of MBP-FALDR was measured spectrophotometrically by monitoring the oxidation of NADPH continuously at 340 nm. Absorbance versus time measurements were used to determine initial rates of aldehyde reduction at each concentration of cis-11-hexadecenal. The initial rates were fit to the Michaelis-Menten equation to determine V_{max} and K_m. Experiments were repeated three times to confirm reproducibility. The results shown here represents the data obtained from a single set of data.

in activity. At higher concentrations, this was difficult to assess fully, as the dithionite interferes with the absorbance at 340 nm where activity is measured. At the lower concentration of 250 µM, the interference is lower, and the inhibition is more pronounced. This could be an indication of an active site residue or cofactor that is

susceptible to reduction. The two metal chelators dipyridyl and diethyldithiocarbamate showed only a moderate inhibition of activity at elevated concentrations of 1.0 mM. This would indicate that if a transition metal is involved in the catalysis, it is not readily accessible to such chelators. Finally, the ability of decanol to inhibit reduction of decanal was also tested. Here, inhibition of almost 45% at the two concentrations tested was observed, indicating a possibility that product inhibition can regulate activity, even though the enzyme is apparently not reversible. Future studies will investigate the nature of this inhibition further, and will also utilize group specific reagents to determine the nature of the active site chemistry utilized by this enzyme.

Table 5-2 : Inhibition of decanal reduction

	Inhibitor Concentration	
	1.0 mM	0.25 mM
Potential Inhibitor	(percent activity)	(percent activity)
EDTA (disodium salt)	81	ND*
ascorbic acid	76	ND*
dithiothreitol	76	ND*
β-mercaptoethanol	75	ND*
dithionite	25	8
diethyldithiocarbamate	50	61
dipyridyl	46	59
dodecanol	54	55

Assays were run in 100 mM MES buffer pH 6.5 with 200 µM NADPH, 250 µM decanal and 125 µg of fatty aldehyde reductase fusion protein. All compounds were dissolved in buffer except dipyridyl, which was dissolved in dimethyl sulfoxide (DMSO). Inclusion of DMSO did not result in decreased activity at the same concentration. Activity is reported as the activity remaining in the presence of each compound as a percentage of the activity without any inhibitor present. *Not determined. Each compound was tested multiple times to confirm reproducibility. The remaining activity presented here is the result from a single set of data.

Contrasting the Fatty Aldehyde Reductase and Alcohol Dehydrogenases – As a means of comparison, medium chain alcohol dehydrogenases (MCADH) can reduce a broad range of aldehydes, including propanal, butanal, hexanal, octanal and benzaldehyde (9, 17, 20). Studies conducted with the MCADH enzyme from *Saccharomyces cerevisiae* did not test the activity of the enzyme with longer chain substrates such as decanal and dodecanal. For the substrate octanal, the largest saturated aldehyde tested with this MCADH, the activity was reported to be approximately 150 μmol/min/mg (9), which is higher than the activities found here for FALDR. An MCADH from *Acinetobacter,* however, was shown to be active with the long chain aldehyde tetradecanal (17). However, this enzyme exhibited, with the substrate tetradecanal, only 7.2 percent of its maximal activity obtained with the substrate heptanal (17). Larroy *et. al.* demonstrated, with a multiple sequence alignment, that the MCADH enzyme from both *S. cerevisiae* and *Acinetobacter* exhibited strict conservation of residues typical of MCADH (9). Using the FALDR amino acid sequence from *M. aquqeolei* (YP_959486), a BLAST 2 Sequences (18) search was conducted (using the BLOSUM62 matrix with a default expect value of 10.0) against the amino acid sequences for both the MCADH from *S. cerevisiae* (NP_014051) and *Acinetobacter* (BAB12270). No significant similarity was found.

Even though the MCADH from *Acinetobacter* exhibited activity toward the fatty aldehyde tetradecanal, its effective participation in the formation of wax esters would likely be limited as MCADH enzymes are soluble and would be partitioned from the lipophilic substrates necessary for wax synthesis. Additionally, these enzymes are highly active with other smaller aldehydes, such as butanal and hexanal, which were poor

substrates for the FALDR. *M. aquaeolei* does contain a gene with high similarity to the reported MCADH enzyme class, and efforts to clone and characterize this gene are also underway. Those efforts will include characterization with longer aldehydes (decanal, etc) that were not tested for the MCADH enzyme from *Saccharomyces cerevisia* (9), but were for the *Acinetobacter* MCADH (17).

Summary – This work has demonstrated that *M. aquaeolei* contains a gene that encodes a fatty aldehyde reductase. The enzyme is specific for NADPH, and does not appear to be reversible. In the native form, the enzyme is relatively insoluble, and partitions with the membrane fraction, where substrates would also likely accumulate. It is possible that this enzyme may be involved in the synthesis and accumulation of wax esters under conditions of nutrient deficiency. Previous reports have identified genes involved in the synthesis of fatty aldehydes from species of *Acinetobacter* (11, 15), but to our knowledge, this is the first report of a bacterial enzyme that is capable of reducing fatty aldehydes to the corresponding alcohol. The enzyme is able to reduce a range of aldehydes, although a minimum size is required (> hexanal) and no activity was found for the reduction of the aromatic compound benzaldehyde. The highest activity found here was for the substrate decanal. The activity, while lower than that found for medium chain alcohol dehydrogenases, is significantly higher than that reported for the acyl-CoA reductase from *Acinetobacter* (11), which is believed to catalyze the preceding step in the pathway, the reduction of a fatty acid coenzyme A substrate to the fatty aldehyde. The FALDR enzyme activity was not impacted significantly by reductants such as ascorbic acid, but was dramatically lowered when dithionite was included in the buffer. The discovery of this fatty aldehyde reductase in *M. aquaeolei* VT8 now paves the way for

inclusion of this enzyme along with others in the pathway as a way to synthesize wax esters of specified lengths in bacteria.

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CHAPTER 6

TOWARD THE CHARACTERIZATION OF WS/DGAT PROTEINS FROM *M. AQUAEOLEI* VT8 AND THEIR EXPRESSION IN *SYNECHOCYSTIS* TO PRODUCE BIODIESEL *IN VIVO*

Abstract

Biodiesel is a renewable fuel that can be used in place of petroluem derived diesel. It is produced by the transesterification of vegetable oils with a short-chain alcohol, producing fatty acid alkyl esters. The potential of biodiesel to meet the demand for transportation fuels is very low due to the large amount of land that would be required to cultivate a sufficient amount of oilseeds. This highlights the need to develop sources of biodiesel that utilize marginal land and the energy from the sun. The cyanobacterium *Synechocystis* sp. PCC 6803 is a photoautotrophic microorganism amenable to genetic modification. The introduction of a single gene (*ws/dgat*) into the genome of *Synechocystis* will confer the ability to produce biodiesel from a short-chain alcohol added to the growth media. The marine bacterium, *Marinobacter aquaeolei,* produces biodiesel when grown in the presence of short-chain alcohols. Four homologous WS/DGAT proteins are encoded by the genome of *M. aquaeolei* VT8. Each of these four *ws/dgat* genes were cloned and will be characterized to determine the optimal *ws/dgat* gene for introduction into *Synechocystis.* A description of the develoment of a *Synechocystis* strain is discussed. I describe here an approach to develop a photoautotrophic organism that is capable of producing biodiesel *in vivo* on marginal

lands using only sunlight, water, and CO_2.

Introduction

The energy that society uses for transportation comes exclusively from petroleum in the form of gasoline or diesel. The use of petroleum for fuel is undesirable, as much of the world's supply exists in politically unstable regions of the world. The combustion of the petroleum fuels, gasoline and diesel, produces hazardous particulate emissions and produces carbon dioxide, a powerful greenhouse gas. The increasing concentrations of CO_2 in the atmosphere is contributing to rising temperatures globally. A changing climate will broadly affect the environment, ecosystems, and the economy. In order to slow the rate of climate change and obtain energy independence, governments worldwide are investing in renewable energy research.

Initial efforts to replace fossil fuels has utilized traditional agricultural crops, such as corn and soybean, to produce the biofuels ethanol and biodiesel, respectively. These first generation biofuels, while being technically capable of replacing fossil fuels, are incapable of meeting the worldwide demand for fuel. Dedicating the entire US corn and soybean crops for ethanol and biodiesel production could only meet 12% of gasoline demand and 6% of diesel demand, leaving nothing for food and livestock feed (*1*).

Recently, renewable feedstock sources for biodiesel production have received a lot of attention (*2*). Obtaining oil from microalgae, for instance, can be improve the sustainably of biodiesel, because microalgae has a higher rate of production than oilseed crops and can be cultivated on marginal lands (*2*). Microalgae are more efficient at converting the energy from the sun to oil than are terrestrial plants (*3*). The production of

biodiesel from microalgae is not currently economically viable because of the high cost of harvesting the microscopic organisms from water (*4*). Once harvested, many oil extraction techniques would require the algae to be first dried, an energy intensive process. With the oil extracted, conversion is then required to produce a useable fuel.

An approach that has the potential to eliminate the need to harvest, extract and convert was recently demonstrated by Kalscheuer *et al.* This group engineered an *E. coli* strain to produce biodiesel *in vivo*, calling the product microdiesel (*5*). Essential to microdiesel is the action of a dual function enzyme with wax ester synthase (ws) activity as well as diacylglycerol acyltransferase (dgat) activity (*6*). The WS/DGAT enzyme accepts a broad range of alcohols substrates, but is most active toward long-chain alcohols (*7*, *8*). When short-chain (C2-C5) alcohols are used as substrates, molecules with similar characteristics and properties to biodiesel are produced. Kalscheuer *et al.* took advantage of this feature of the WS/DGAT enzyme and engineered an *E. coli* strain to express the pyruvate decarboxylase and alcohol dehydrogenase genes, from the ethanol producing bacterium *Zymomonas mobilis*, in conjunction with the *ws/dgat* gene from *Acinetobacter* sp. ADP1 (*5*). The resulting strain was capable of producing biodiesel *in vivo*. The overall yield of fatty acid ethyl esters was low, which the authors attributed to the low activity of the enzyme with ethanol. The Keasling group also demonstrated the utility of the WS/DGAT protein to produce fatty acid ethyl esters *in vivo* (*9*). Their yields were also reduced by the low activity of the WS/DGAT protein with ethanol. By producing biodiesel through fermentation these two examples still rely on agricultural products and do not directly convert energy from the sun. This approach has the same basic limitation of first generation biofuels. Not enough sugar is available to produce

enough biofuel by fermentation.

Marinobacter aquaeolei VT8 is a marine bacterium whose genome codes for four proteins homologous to the WS/DGAT protein from *Acinetobacter*. The activity of the *M. aquaeolei* proteins toward short-chain (C2-C5) alcohols has not been characterized. Identification of a WS/DGAT enzyme with improved activity toward short-chain alcohols would improve the feasibility of producing biodiesel *in vivo*. In this paper I seek to determine the potential of WS/DGAT proteins from *M. aquaeolei* to produce biodiesel *in vivo* by characterizing each purified protein using a real-time spectrophotometric assay. The limitation of the microdiesel approach to produce biodisel molecules directly from the sun is addressed by discussing the potential of cyanobacteria as a microdiesel platform.

Materials and Methods

<u>Strains</u>

Marinobacter aquaeolei VT8 obtained from the Amercan Type Culture Collection (ATCC) (*10*). Cells were grown on modified ATCC 2084 media (*Halomonas* media) (*11*). Cultures contained per liter the following: 50 g NaCl, 100 mg casamino acids (vitamin assay), 7 g sodium succinate, 100 mg urea, 200 mg $CaCl_2$, 20.0 mg $MgSO_4{\cdot}7H_2O$, 500 mg K_2HPO_4, 35 mg $FeSO_4{\cdot}7H_2O$. Solid media, when needed was obtained by the inclusion of 1.5% agar. For alcohol incorporation studies 1 mL of an alcohol was added to the media prior to innoculation, an additional 1 mL was added 48 hrs later. Alcohols used in this study were: 2-phenylethanol, n-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol. *E. coli* Jm109 was utilized for

molecular biology and *E. coli* TB1 was used for expression of WS/DGAT proteins. *E. coli* cells were grown in Luria-Bertani media with the appropriate antibiotic unless otherwise stated. *Synechocystis* sp. PCC 6803 was cultured in 200 mL of BG-11 media (*12*) in 500 mL baffled flasks with constant shaking at 140 rpm. The culture was illuminated by cool-white fluorescent lights on a 14:10 light:dark cycle with an intensity of 200 μmol photons m^{-2} s^{-1}. BG-11 solid media was prepared by the addition of 1.5% agar, TES-OH to 0.1 mM final concentration, and 3g L^{-1} sodium thiosulfate.

Plasmids

Four genes from *M. aquaeolei* VT8, whose protein products are homologous to the acinetobacter WS/DGAT protein (gene accession #AF529086), were cloned from genomic DNA. DNA was isolated from *M. aquaeolei* VT8 using standard molecular biology techniques. Genes of interest were amplified by PCR following the Failsafe protocol (Epicenter, Madison, WI) using primers listed in **Table 6-1**. Each PCR product was purified using a commercial column purification technology (Zymo Research, Orange, CA). The first of the *ws/dgat* genes (YP_957462.1) was amplified using primers BBP242 and BBP243 and cloned into pUC19 by restricting the plasmid and PCR product with KpnI and HindIII. The resultant plasmid was further modified by site-specific mutagenesis using primers BBP247 and BBP248 to remove an internal NcoI site, while maintaining the wild type amino acid sequence. The *ws/dgat*1 sequence was then moved to pBB052, a pUC derivative with Kan^R, by cutting with the restriction endonucleases NcoI and HindIII. The sequence was further modified to add an XbaI site in place of the HindIII site to accommodate cloning the gene into pMal-CHis3 (Amp^R). The gene was

then incorporated into pMal-CHis3 by restriction with NcoI and XbaI. This modification created an N-terminal fusion protein with the maltose binding protein (MBP) to aid in solubility and purification. **Figure 6-1** diagrams the features of the MBP-WS/DGAT1 construct. The second *ws/dgat* gene (YP_960328.1) was cloned using the primers BBP539 and BBP516 and incorporated into pMal-CHis3 directly by restricting both plasmid and PCR product with EcoRI and XbaI. The third *ws/dgat* gene (YP_958134.1) was cloned with primers BBP756 and BBP757 and the cloning of the fourth *ws/dgat* gene (YP_960629.1) used the primer pair of BBP758 and BBP759. Both *ws*3 and *ws*4 were incorporated into pMal-CHis3 by restriction with NcoI and XbaI. Sequences of each of these plasmids were verified by sequence analysis.

The preparation of the integration platform plasmid pPSBA2 (*13*) to incorporate the *ws/dgat*1 gene into the genome of *Synechocystis* was begun by the PCR amplification of this gene from genomic DNA using the primers BBP764 and BBP765. The PCR amplicon was first incorporated into pUC19 to give pPCRWSyn1. An NdeI site within the gene was subsequently removed by site-specific mutagenesis using primers BBP249 and BBP250 to yield pPCRWSyn2. This plasmid then served as the template for the removal of an internal BamHI site using primers BBP766 and BBP767 to produce pPCRWSyn3. Each site-directed mutagenesis reaction preserved the primary amino acid sequence of the gene product. The sequences of plasmids pPCRWSyn1-3 have been verified correct by sequence analysis. The *ws/dgat*1 will soon be moved from pPCRWSyn3 to the pPSBA2 plasmid using BamHI and NdeI restriction endonucleases. The pPSBA2 plasmid contains a 500 bp region upstream of the *psbAII* gene which includes the start codon of the gene integrated into an NdeI site and a 500 bp region

Table 6-1: Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or oligonucleotide primer	Relevant feature or sequence	Source or Reference
Strains		
E. coli Jm109	F′ *traD36* $proA^{+}B^{+}$ $lacI^{q}$ *Δ(lacZ)M15/ Δ(lac-proAB) glnV44* $e14^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	New England Biolab
E. coli TB1	F^{-} *ara Δ(lac-proAB) [Φ80dlac Δ(lacZ)M15] rpsL*(Str^{R}) *thi hsdR*	New England Biolab
M. aquaeolei VT8	Sequence strain	ATCC
Plasmids		
pUC19	Amp^{R} , *lacZ*, cloning vector	New England Biolabs
PBB052	Kan^{R}, *lacZ*, cloning vector	Brett Barney
pMal-CHis3	Amp^{R}, variation of pMal-c4x which incorporates 8x-His tag onto gene	Brett Barney
pMal-c4x	Plasmid conaining maltose binding protein to facilitate fusion protein creation	New England Biolabs
pPCRWS1	Contains *ws/dgat*1 gene cloned into pMal-Chis3.	This study
pPCRWS2B	Contains *ws/dgat*2 gene cloned into pMal-Chis3.	This study
pPCRWS3B	Contains *ws/dgat*3 gene cloned into pMal-Chis3.	This study
pPCRWS4B	Contains *ws/dgat*4 gene cloned into pMal-Chis3.	This study
pPCRWSyn1	*ws/dgat*1 gene for incorporation into pPSBA2.	This study
pPCRWSyn2	*ws/dgat*1 gene for incorporation into pPSBA2 with internal ndeI site removed.	This study
pPCRWSyn3	*ws/dgat*1 gene for incorporation into pPSBA2 with both the internal ndeI site and BamHI site removed.	This study
pPCRWSyn4	*ws/dgat*1 incorporated into pPSBA2 by restriction with NdeI and BamHI	This study
pPSBA2	Contains the upstream and downstream regions of the *psbAII* gene.	Reference 10
pPSBA2KS	Contains an *aphX-sacB* construct inserted into pPSBA2	Reference 10
pPCRWSyn4	Incorporation of pPCRWSyn3 into pPSBA2	This study
Primers		
BBP242	`5' gacatGGTACCATGGAACGCCCCTGAATCCCACTGACCAG 3'`	This study
BBP243	`5' gactgAAGCTTACAGACCGGCGTTGAGCTCCAGC 3'`	This study
BBP247	`5' CGAACGACTGTTAAAAATCCACCAcGGCATGGAAGAGG 3'`	This study
BBP248	`5' CCTCTTCCATGCCGTGGTGGATTTTTAACAGTCGTTCG 3'`	This study
BBP481	`5' CTGGAGCTCAACGCCGGTCTagAAGCTTGGCGTAATCATGGTTCC 3'`	This study
BBP482	`5' GGAACCATGATTACGCCAAGCTTctAGACCGGCGTTGAGCTCCAG 3'`	This study
BBP539	`5' gagaGAATTCAAACGTCTCGGAACCCTGGATGCCTCCTGG 3'`	This study
BBP516	`5' gatggTCTAGACTCTTGCGGGTTCGGGCGCGCTTCTTCG 3'`	This study
BBP756	`5' gatggTCTAGACTCCTTCTGAATTTGCCCAGCCCACC 3'`	This study
BBP757	`5' gagaCCATGGGCGTCAGCTGTCGGAACTGGATGCC 3'`	This study
BBP758	`5' gagaCCATGGGTCAGCAAAACGGACGGCCATGACC 3'`	This study
BBP759	`5' gatggTCTAGACTGGAGGCCTGGCGGAAACCG 3'`	This study
BBP764	`5' ctgtGAATTCCATATGCATGTGGGCGGCCTC 3'`	This study
BBP765	`5' ctgtGAATTCGGATCCTTACAGACCGGCGTTGAGCTCC 3'`	This study
BBP766	`5' TCAGGCCCGGAAGGAcCCGGCCTACGACTCC 3'`	This study
BBP767	`5' GGAGTCGTAGGCCGGgTCCTTCCGGGCCTGA 3'`	This study
BBP249	`5' CAGCGCTACCGGCAcATGAGCCCGGAG 3'`	This study
BBP250	`5' CTCCGGGCTCATgTGCCGGTAGCGCTG 3'`	This study

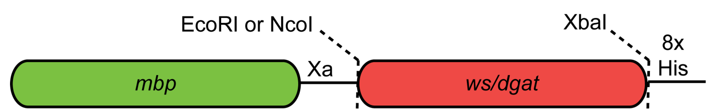

Figure 6-1: Diagram of maltose binding protein-*ws/dgat* fusion protein. Xa designates a factor Xa cleavage site to facilitate the removal of maltose binding protein. Insertion of *ws/dgat* into XbaI site, in frame, incorporates an 8xHis tag to facilitate purification.

downstream of the *psbAII* gene that includes the stop codon of the gene (*13*). Once *ws/dgat*1 has been incorporated into pPSBA2, the gene will be integrated into the chromosome of *Synechocystis* sp. PCC 6803 by homologous recombination.

Transformation of *Synechocystis*

Transformation of *Synechocystis* sp. PCC 6803 was performed as previously described (*13*). *Synechocystis* was grown and maintained as described above. The growth of *Synechocystis* was monitored spectrophotometrically by measuring the optical density at 730 nm. Cultures were used for transformation before the OD_{730} reached a value of 0.5. Aliquots of the culture for transformation experiments were collected and concentrated to an OD_{730} by centrifugation. 10 μg of pPSB2KS (*13*) plasmid DNA was added to 0.5 mL aliquots of culture and were incubated in the light at ~30ºC for 6 hrs. Cells from the transformation experiment were collected by centrifugation and plated onto 50 mL BG-11 plates without antibiotic. Using a sterilized gas-tight syringe, 2.5 mg dissolved in sterile water was added to the bottom of the plate. This allowed for the slow development of an antibiotic concentration gradient. Colonies that developed over the course of two to three weeks, were restreaked twice prior to PCR analysis. Primers

5'psbAII$_{up}$ and 3'psbAII$_{down}$ from Legarde *et al.* were used demonstrate chromosome integration (*13*).

Wax ester extraction

M. aquaeolei cultures grown in the presence of different alcohols were harvested and immediately frozen. The frozen cells were dried by lyophilization prior to lipid analysis. Neutral lipids were extracted from dried cells using a 1:1:1 hexane:chloroform:tetrahydrofuran (THF) solvent mixture. The extraction of each sample was performed by adding 5 mL of the solvent mixture to a glass test tube containing 200 mg of dried cells. A microtip probe from a sonifier set at a constant duty cycle (Branson sonifier 250, Danbury, CT) was used to apply three separate 10 sec bursts to the sample and solvent to aid the extraction process. Cellular material was collected by centrifugation and the organic phase was then removed with a gas tight syringe. The extraction process, as described, was repeated two more times, combining the extracts and bringing the final volume to 15 mL.

Neutral Lipid Analysis

All analysis was performed on a gas chromatograph (Model 2010, Shimadzu Scientific, Columbia, MD) equipped with both a flame ionization detector for quantification and a separate mass spectrometer (GCMS-QP2010S, Shimadzu Scientific) for peak identification. Analytes were separated on an RTX-Biodiesel column (10 m, 0.32 mm ID with a guard column, Restek, Bellefonte, PA) using a temperature program of 60°C for 1 min followed by a temperature ramp of 10°C per minute to 360°C for 6 min, and a constant velocity of 50 cm/sec with helium gas as the carrier. Samples were

injected into a programmable temperature vaporizer (PTV) injector that followed an identical temperature program to that listed above for the column. Injections of 1 μL were made from a GC vial with Teflon coated septa, which also contained octacosane (10 μL of a 10 mg/mL stock solution) as an internal standard.

Peak identification was accomplished by mass spectrometry (Shimadzu GCMS-QP2010S). Analytes were separated using a 15 m Stabilwax-DA fused silica capillary column with an internal diameter of 0.25 mm a film thickness of 0.1 μm (Restek, Belfonte, PA). One μL of sample was injected into a split/splitless injector set to a temperature of 240ºC in direct mode. Oven temperature program was set to an intial temperature of 100ºC held for 1 min, followed by a gradient of 10ºC/min to 235ºC held for 10 min. Helium was used as the carrier gas and flow was controlled by constant velocity set at 50 cm/min. Individual compounds were identified by comparing the mass spectra obtained for individual peaks with spectra in the National Institute of Standards and Technology (NIST) database 05.

Enzyme Purification

Of the four *ws/dgat* genes that have been cloned as MBP-WS/DGAT fusion constructs, two have been purified (WS/DGAT1 and WS/DGAT2). Each of the four MBP- *ws/dgat* fusion plasmids have been transformed into *E. coli* TB1 for expression. Expression experiments of each plasmid have been performed by SDS-PAGE analysis of two identical cultures, one grown in the presence of the inducer IPTG (isopropyl β-D-1-thiogalactopyranoside) and the other grown in the absence of IPTG. In each case a band, at the approximate position expected, was present in the total cellular protein of the

sample containing IPTG that was not found in the other. One liter of LB medium supplemented with ampicillin (100 μg /mL) in a 4 L flask was inoculated with 16 mL of an overnight culture of *E. coli* TB1 transformed with the plasmid containing a MBP-*ws/dgat* fusion gene and was grown for approximately five hours at 37˚C prior to induction by the addition of 50 mg of IPTG , after which the culture was grown for an additional two hours at room temperature. Cells were harvested by centrifugation and were immediately frozen for later use.

The cells were suspended in 30 mL of phosphate buffer (125 mM PO_4^{2-}, 150 mM NaCl pH 7.4). The re-suspended cells were lysed using a French pressure cell (SLM Aminco) in the presence of DNAse (P/N DN-25, Sigma-Aldrich). Soluble protein was collected by centrifugation of the cell lysate at 17,500 g for 10 min. The supernatant was diluted three-fold with column buffer and was then applied to a column containing 10 mL of amylose resin (New England Biolabs, Ipswich, MA). The column was washed with 30 mL of phosphate buffer containing 1 M NaCl, followed by a second wash with 30 mL of phosphate buffer. The fusion protein was eluted with 15 mL of phosphate buffer supplemented with 10 mM maltose. Fractions containing significant amounts protein as determined by UV absorbance at 280 nm, were pooled together and applied to a nickel affinity column equilibrated with phosphate buffer. The column was washed with a 70 mM imidazole phoshate buffer (pH 7.4). This was followed by the elution of protein by the addition of 1.5 column volumes of phosphate buffer (pH 7.4) containing 500 mM imidazole. Fractions containing WS/DGAT enzyme, as determined by SDS-PAGE analysis were pooled together and flash frozen in liquid nitrogen.

WS/DGAT Activity Assays

Wax ester synthase activity assays were developed to quantify the amount of free coenzyme A in solution using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) or Ellman's reagent (*14*, *15*). The TNB^{2-} anion that develops when DTNB reacts with free sulfhydryl groups, has a maximal absorbance at 412 nm. DTNB is included in the reaction assay at a concentration of 0.1 mM. Other reaction components include assay buffer (125 mM PO_4^{2-}, 150 mM NaCl, and 4.5 mg mL^{-1} bovine serum albumin (BSA)), acyl-CoA at varying concentrations and an alcohol dissolved in DMSO. Care must be taken to avoid adding acyl-CoA molecules in sufficient concentration to reach their critical micelle concentration.

Results and Discussion

Fatty Acid Alkyl Esters Production *in vivo*

It has been noted by several groups that the WS/DGAT family of enzymes accepts a wide range of alcohol substrates for the acyltransferase reaction they catalyze (*6–8*, *16*). In particular, it has been observed that WS/DGAT enzymes will utilize a short chain alcohol, such as ethanol, as an acyl acceptor, forming molecules with characteristics and properties similar to biodiesel fuel (*5*). As discussed in the introduction this attribute of the WS/DGAT enzyme has been exploited to engineer microbes such as *E. coli* and *S. cerevisiae* to produce biodiesel and specialty chemicals *in vivo* (*5*, *9*). In these applications, the alcohol substrate, ethanol, was produced *in vivo*. Kalscheuer *et al.* observed that low levels of fatty acid ethyl esters were produced despite the addition of exogenous fatty acids to the media and high levels of *in vivo* ethanol production (*5*).

The reason for the low production of fatty acid ethyl esters was identified by activity assays conducted with the *Acinetobacter* WS/DGAT enzyme (*7*). The enzyme exhibited high activity with fatty alcohols 12-18 carbons in length with maximal activity occurring with Oleyl alcohol (C18:1). Decreasing activity was observed for alcohols with carbon chain lengths greater than 18 carbons. Enzyme assays with shorter chain alcohols (C4-C10) exhibited ~30% of the average activity observed with fatty alcohol substrates (C12-C18), while the wax ester synthase activity of WS/DGAT with ethanol was less than 10%.

M. aquaeolei contains four *ws/dgat* genes, designated *ws/dgat*1, *ws/dgat*2, *ws/dgat*3, *ws/dgat*4 (**Table 6-1**). The primary amino acid sequence encoded by these genes is similar to the primary amino acid sequence of the WS/DGAT protein from *Acinetobacter* sp. ADP1. The similarity between the WS/DGAT proteins from *M. aquaeolei* and the WS/DGAT protein from *Acinetobacter* varies from 20-44%, while similarity among *M. aquaeolei* WS/DGAT proteins ranges from 20-37%. The WS/DGAT protein from *Acinetobacter* is the only representative of this class of enzymes to have its activity towards short chain alcohols determined (*7*). This raises the possibility that an enzyme homologue could have improved activity toward shorter alcohols. A WS/DGAT enzyme with improved activity toward short alcohols would have a significant biotechnological benefit.

M. aquaeolei was chosen as our model organism to identify WS/DGAT homologues with higher activity toward short-chain alcohols as it was observed to incorporate exogenous short-chain alcohols into fatty acid alkyl esters (**Figure 6-2**). The following alcohols were added separately to the growth medium of *M. aquaeolei:* 2-

phenylethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, or 2-methyl-1-propanol. The neutral lipids were extracted from the freeze-dried *M. aquaeolei* cells and analyzed by GC. New peaks with earlier retention times than those from normal growth *M. aquaeolei* cells were visible in the chromatogram of each lipid extract. The new peaks appeared in the region where fatty acid methyl esters appear. The lipid extracts were further subjected to GC/MS analysis, which identified the compounds as fatty acid in ester linkage with the added alcohols. The concentration of these new alkyl esters relative to the wild-type wax esters was higher for the 2-methyl-1-butanol growth extracts. The longer-chain, branched alcohols seemed to be preferred as additional growth experiments with ethanol or n-butanol added did not result in the significant accumulation of fatty acid esters of these alcohols.

It is tempting to declare that the WS/DGAT enzymes from *M. aquaeolei* are most active with 2-methyl-1-butanol. However, sufficient control over variables are not possible in whole cell assays, such as the one conducted here, to make that conclusion. Nothing is known about enzyme concentration within the cell or even which WS/DGAT enzyme is most catalytically active with the short-chain alcohols. The fate of the alcohol in solution is unknown, other cellular processes may utilize one alcohol preferrentially, thus reducing its availability to WS/DGAT enzymes. The different alcohols may also be transported across the cell membrane at a different rate than another, limiting its intracellular concentration. Protein purification and kinetic characterization is the only approach that will determine which alcohol is the ideal substrate and which of the four WS/DGAT proteins encoded by the *M. aquaeolei* genome is most active toward short-chain alcohols.

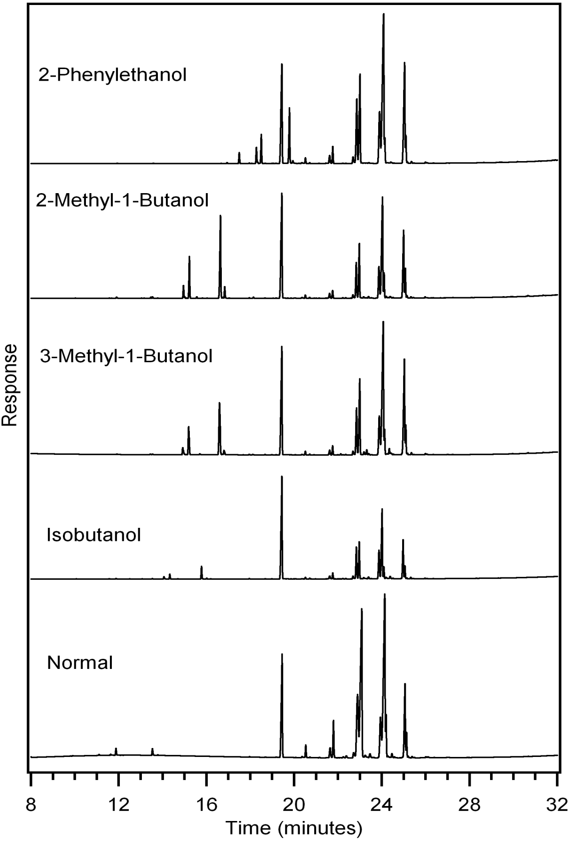

Figure 6-2: Wax esters obtained during growth with alternative alcohols. Shown are the GC chromatograms of the neutral lipid extractions obtained from dried *M. aquaeolei* cells grown in media alone (bottom trace) and when grown in the presence of various alcohols (as labeled). The peaks present in the region between 14 and 20 minutes correspond to fatty acid alkyl esters of the added alcohol. The peak at approximately 19 minutes is an internal standard. The peaks with retention times between 20 and 26 minutes are the natural, wild type wax esters (each sample).

Purification of WS/DGAT proteins

Initially the *ws/dgat*1 was cloned by PCR with an added six histidine tag to accommodate a rapid metal affinity purification technique. The gene was placed in a pET vector and was found to express well in the presence of the inducer IPTG, but did not express in its absence. Purification of this protein, however, was not possible. When the cells expressing WS/DGAT1 were disrupted and the membranes collected by centrifugation, it was found that the vast majority of the protein partitioned with the membrane. Inclusion of a variety of detergents common to biochemistry (e.g. tween 20, triton x-100, n-dodecyl-β-D-maltoside) did not improve the solubility of the protein. This result was not too surprising as the enzyme utilizes fatty acid substrates and others have reported that the protein is a membrane associated protein (*17*) and contains a putative transmembrane domain (*6*). Purification of this protein resulted in very low yields and was inadequate for further analysis. The size and location of the histidine tag was altered without affecting solubility.

The solubility problem was finally overcome by creating a fusion protein with the maltose binding protein (MBP) and WS/DGAT. The MBP fusion protein was constructed (**Figure 6-1**) as an N-terminal fusion to WS/DGAT and featured a C-terminal 8x histidine tag to facilitate purification of the protein to homogeneity. Expression of this protein resulted in a soluble protein. In addition to enhancing the solubility of the WS/DGAT protein, the MBP fusion will aid in the purification of the protein. MBP binds, as its name suggests, maltose. Amylose resin is commercially sold for this application. MBP-fusion proteins applied to an amylose column can simply be eluted by adding maltose. Purification the MBP-WS/DGAT fusion proteins to homogeneity can be

accomplished in two stages, using metal affinity chromatography and amylose affinity chromatography. The fusion protein also contains a factor Xa cleavage site in the linker region between MBP and WS/DGAT to facilitate the removal of MBP. The protease cleavage site was not chosen for the project but rather came included on the plasmid supplied by New England Biolabs. The inclusion of a factor Xa site in the fusion construct is unfortunate as the cleavage of MBP from a very large growth is not possible due to the cost of factor Xa. In the future the construction of a MBP vector containing a tobacco etch virus protease recognition site will be considered.

The construction of each vector containing a *ws/dgat* gene is described in detail in the material and methods section. The sequence of each construct was verified by sequence analysis and tested for expression of the encoded MBP-WS/DGAT fusion protein by SDS-PAGE analysis. WS/DGAT1 and WS/DGAT2 have been purified and activity verified (development of an activity assay is described below). WS/DGAT3 and WS/DGAT4 have not yet been purified. The plasmids containing the genes corresponding to these proteins have only recently been obtained. The activity will be verified in the near future.

Development of Real-time Assay

Initial publications discussing the activity of WS/DGAT proteins utilized a heroic assay to determine the enzymes activity toward a range of substrates (*6*, *7*). The alcohol substrate and an acyl-CoA molecule with a ^{14}C carbonyl atom were added with the WS/DGAT protein and were allowed to react for a fixed amount of time before the reaction was stopped by the addition of solvent to extract the lipid components of the

assay. The extracted product and substrates were then separated by TLC. The spot corresponding to wax esters was then scraped off of the plate and the radiation was quantified by scintillation counting to determine how much was product was formed. This approach did not allow for real-time analysis, and the radiolabeled acyl-CoA substrate would be difficult and expensive to obtain.

Holtzapple *et al.* recently published an investigation of the activity of WS/DGAT proteins from *Marinobacter hydrocarbonoclasticus* toward isoprenoid compounds such as phytol and phytenoyl-CoA using a real-time assay (*14*). In the process of the acyltransferase reaction catalyzed by WS/DGAT, an alcohol substrate attacks the carbonyl of a fatty acyl-CoA molecule forming, as products, a wax ester and free coenzyme A. Holtzapple *et al.* used a compound known as Ellman's reagent to quantify free coenzyme A. Ellman's reagent (DTNB) is a disulfide molecule which reacts rapidly with free thiols such as the thiol found on free coenzyme A to produce a mixed disulfide with conenzyme A and 5-thio-2-nitrobenzoic acid (TNB^{2-}) (**Figure 6-3**). TNB^{2-} imparts a yellow color to an aqueous solution and absorbs light maximally at 412 nm. Product formation can be followed in real-time by monitoring the change in absorbance at this wavelength. This assay has performed well in initial experiments and the kinetic characterization of the four WS/DGAT proteins from *M. aquaeolei* can now begin. The focus of the characterization will be to determine the WS/DGAT protein with the lowest K_m for each substrate. The WS/DGAT protein best suited for the production of biodiesel *in vivo* will need to have good activity with dilute substrates.

Figure 6-3: Wax ester synthase activity assay. Coenzyme A released in the production of wax esters reacts with DTNB producing a mixed disulfide and the TNB anion, which absorbs maximally at 412 nm.

<u>Preparation of *Synechocystis psbAII* Knockout</u>

Synechocystis has been used for biotechnological applications such as the overproduction of the carotenoid pigment zeaxanthin sp. PCC 6803 (*13*). *Synechocystis* is a naturally competent cyanobacterium. DNA added to a solution of cells will be readily taken up by the organism. Tools for genetic manipulation in this organism are well established. Many strong promoter from *Synechocystis* have been used to overexpress foreign proteins. The promoter for the *psbAII* gene is particularly valuable. The *psbAII* gene encodes the D1 protein that forms, in part, the reaction center complex of photosystem II. This gene is absolutely essential to photoautotrophic growth and is highly expressed when the cyanobacterium is exposed to light. In *Synechocystis* this gene is encoded by three genes (*psbAI, psbAII,* and *psbAIII*). Deletion of one of the three *psbA* genes does not adversely affect the ability of *Synechocystis* to grow

photoautotrophically.

The plasmid pPSBA2, generously shared by Dr. Wim Vermaas of ASU, contains a 500 bp segment of DNA found upstream of the *psbAII* gene including the start codon and a 500 bp segment of DNA found downstream which flank a multiple cloning site to facilitate the insertion of genetic material (*13*). The plasmid pPSBA2KS, also provided by Dr. Vermaas, contains the *aphX* and *sacB* genes flanked by the upstream and downstream regions. This plasmid is thus designed to integrate into the chromosome and replace *psbAII* with *aphX* and *sacB*, conferring resistance to the antibiotic kanamycin and lethal sensitivity to sucrose respectively. The *psbAII* knockout plasmid (pPSBA2KS) was added to prepared *Synechocystis* culture. Transformants were selected on BG-11 plates with kanamycin added to the bottom of the plate. This allowed cells to begin to grow with a kanamycin concentration that was initially low but gradually increased as the antibiotic diffused through the agar. This resulted in a uniform "lawn" of *Synechocystis* cells on the surface of the plate after a week of growth. Individual colonies began to appear in time as the antibiotic diffused through the agar. Ultimately, ~30 transformants appeared as single colonies on the plate. Four individual transformants were picked and segregated by restreaking twice onto BG-11 plates containing kanamycin. Restreaking of transformants is required to segregate mutant genomes from the wild type genome as *Synechocystis* contains multiple copies of its genome. PCR, using primers from Lagarde *et al.* and DNA from each transformant, was used to verify complete segregation (**Figure 6-4**); wild type *Synechocystis* DNA was used as a template for a control PCR. Complete segregation was verified for each of the transformants analyzed.

With the *psbAII* gene successfully knocked out in *Synechocystis*, replacement of

the *aphX/sacB* genes with a *ws/dgat* gene will accomplish our goal of developing a photoautotrophic organism capable of producing wax esters or biodiesel *in vivo*. The insertion of the *sacB* gene in addition to kanamycin resistance will be essential to successfully completing this task. The expression of the *sacB* gene in *Synechocystis* (and many other bacteria) is lethal when grown in the presence of sucrose (*18*). The *sacB* gene encodes a levansucrase enzyme that catalyzes the hydrolysis of sucrose and the synthesis of levans, high molecular weight polymers of fructose (*19*). The toxicity is thought to arise from either the interference of normal cellular process by the high molecular weight levans or the inappropriate transfer of a fructose monomer to another molecule. Our *Synechocystis* knock-out strain will serve as the DNA acceptor in a

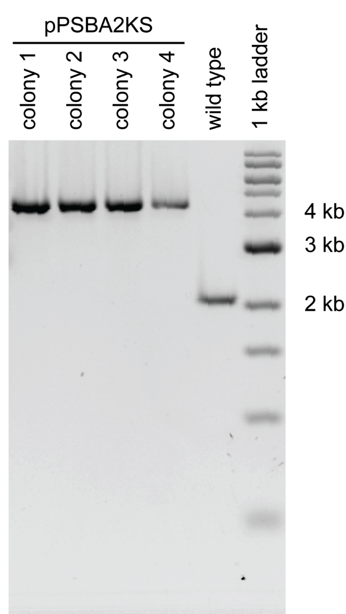

Figure 6-4: PCR analysis of pPSB2KS *Syncechocystis* transformants. The genomic DNA from each transformant was used as a template for PCR using primer 5'psbAII$_{up}$ and 3'psbAII$_{down}$ from reference 10.

second round of transformation. The *ws/dgat*1 gene will be inserted into the pPSBA2 plasmid to create pPCRWSyn4. The resulting plasmid will be added to a culture of the *psbAII* knock-out strain and plated on BG-11 media containing 5% sucrose. Only the *Synechocystis* cells that successfully integrate pPCRWSyn4 and completely segregate will survive on the sucrose pates. This approach allows for the successful gene knockout and subsequent insertion of a gene of choice without leaving a selection marker in the organism's chromosome.

Summary

The goal of this project is to develop a cyanobacterial strain that is capable of producing biodiesel *in vivo*. As a first step to accomplishing this, I have determined that WS/DGAT proteins within *M. aquaeolei* have activity toward shorter chain alcohols (C2-C5). This organism contains four putative *ws/dgat* genes. It is unknown which of these genes encodes the protein responsible for the production of fatty acid alkyl esters *in vivo* from exogenous short-chain alcohols. Each of these genes have been cloned and expressed as MBP-WS/DGAT fusion proteins. Two (WS/DGAT1 and WS/DGAT2) have been over-expressed in *E. coli* and purified to homogeneity. The activity of these two proteins has been verified by a real-time activity assay that does not require radiolabeled substrates. The capability to alter the genome of *Synechocystis* sp. PCC 6803 has been demonstrated by replacing the *psbAII* gene with a kanamycin resistance marker and the *sacB* gene. The *psbAII* knockout strain will serve as the recipient of a *ws/dgat* gene. The transformants containing the *ws/dgat* gene will be counterselected on sucrose plates, having lost sensitivity to sucrose by the replacement of *sacB* with *ws/dgat*. The work

described in this chapter does not constitute a complete project. Although, a substantial amount of preliminary work has been accomplished. The tasks that remain to complete the story are not significant, although they will be time consuming.

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CHAPTER 7

SUMMARY

We currently obtain the bulk of our energy from fossil fuels, presenting society with several challenges that are not easily solved. Consumption of fossil fuels releases the greenhouse gas CO_2 into the atmosphere contributing to global climate change, which threatens to affect the world's economies (*1*). Significant reductions in our emissions must take place just to maintain current atmospheric levels of carbon dioxide and avoid further climate change. Unfortunately, the energy produced from fossil fuels has become a foundation of the world economy and a reduction in the output of energy would adversely impact the world economy and ultimately its people. Petroleum is a finite resource and cannot meet the demand for energy indefinitely. As reserves of this resource either dwindle or become increasingly difficult to obtain, renewable alternatives must be developed to meet future demand.

Worldwide energy demand is large and a renewable source of energy equally large is required to meet society's needs. As discussed previously (Chapter 1), the amount of solar energy that reaches the surface of the earth far exceeds our current energy demands. A key challenge to the utilization of this energy is the ability to capture the sun's radiant energy and convert it to a form that can be readily used (*2*). Photovoltaic cells are the best known technology for capturing solar energy, producing electricity from the sun's rays. This form of energy is ideal for commercial and industrial applications but has limited utility for transportation. Here, liquid hydrocarbon fuels are needed as they are portable and contain a significant amount of energy in a small volume (*3*, *4*). Nature,

through the process of photosynthesis, has developed the process of capturing and storing solar energy in the form of chemical bonds. Plants through photosynthesis can synthesize a diverse array of chemical compounds essential to life using only the energy from the sun and CO_2 as the starting material.

As previously mentioned (Chapter I), biodiesel can be used in place of petroleum diesel offering greatly improved emissions with minimal reductions in performance (*5*, *6*). Biodiesel from agricultural crops, such as soybeans, are limited in their potential to meet demand for diesel fuel due to the amount of land their cultivation requires. Critics of biofuels have raised concerns that diverting agricultural land away from food production could cause food prices to increase rapidly. Increasing the use of food crops for energy production may actually result in greater CO_2 emissions through land use change caused by increased demand for energy crops (*7*, *8*). The food vs. fuel crisis highlights the need to produce biodiesel from non-food sources (*9*).

Two approaches can be taken to increase the biodiesel supply, sufficiently, to meet demand for petroleum diesel without impacting the food supply. First, the range of feedstocks available for biodiesel production can be expanded to include non-edible oils that typically contain a high content of free fatty acids (FFA) through the development of conversion methods that accommodate the FFA contained in these oils (*10*). Oils high in FFA include used vegetable oil, rice bran oil, and Jatropha oil (*11–13*). The second approach, involves developing new feedstocks that do not utilize agricultural land and have higher productivity than traditional oilseed crops. These new feedstocks could include oleaginous microorganisms such as microalgae, fungi, and bacteria (*14–16*). These organisms can accumulate greater than 20% of their cellular dry weight (CDW) as

triglyceride (*17*). The content of this dissertation is focused on producing biofuels from non-food oils and oleaginous microorganisms.

Biodiesel from Oils High in Free Fatty Acid

Vegetable oil with a high free fatty acid (FFA) content can be a challenge to biodiesel production (*18*). The typical transesterification reaction utilizes methanol and a base catalyst to convert the triglyceride found in the oil (*19*). However, this method of biodiesel production is not efficient with oils high in FFA, resulting in poor removal of glycerol due to the formation of soap (*18*). Exchanging the base catalyst used in the process with an acid catalyst allows for the esterification of FFA but does so at the expense of efficiency, complete conversion requires much longer reaction times (*20*, *21*). An alternative approach requires a two-stage reaction approach that involves, first, the esterification of the oil with an acid catalyst followed by neutralization, then transesterification with a base catalyst (*10*). Although this method takes advantage of the FFA material it requires additional processing steps to complete the conversion to FAME. A simple single step method for utilizing low cost oil high in FFA is described in Chapter II simultaneously conducts the esterification and transesterification of these oils without extended reaction times (*22*). It was found that the acid catalyzed reaction is greatly accelerated when longer-chain alcohols (e.g. *n*-butanol) are used. This improvement in the rate of conversion occurs because longer-chain alcohols (longer than ethanol or methanol) are miscible with vegetable oil. It appears that the acid catalyzed transesterification reaction is more sensitive to the two phase reaction that exists with vegetable oil and methanol, than is the base catalyzed reaction. Simply creating a single

phase reaction with a longer chain alcohol greatly enhanced the reaction rate. Using such an approach, many low value feedstocks, high in FFA, could be efficiently converted to biodiesel.

Biodiesel from Oleaginous Microorganisms

Species of oleaginous microorganisms can be found among microalgae, bacteria, and fungi. An organism is considered oleaginous if it accumulates greater than 20% CDW as triglyceride (TAG) (*17*). The ability to accumulate oil in this quantity is not a widespread attribute among microalgal, fungal, or bacterial species. Due to their oil accumulating ability, these organisms have been used commercially to produce polyunsaturated fatty acids for the neutraceutical market and are considered potential sources of oil for biodiesel production (*16*, *23*, *24*). Oleaginous microbes grow rapidly and have been shown to accumulate up to 80% CDW as TAG (*25–27*). In the sections that follow a brief description of each group of oleaginous microorganism (microalgae, fungi, and bacteria), their potential benefit, a summary of completed work, and remaining questions within the field will be discussed.

Microalgae

In the 80's, the US Department of Energy (DOE) began a program to understand how aquatic species, such as microalgae, could be used as a feedstock for biodiesel production (*28*). The work was initiated in response to the high oil prices of the late 70's. The aquatic species program isolated several thousand strains of microalgae and characterized their oil producing potential. Many strains were identified that showed tremendous potential as a feedstock for biodiesel production. Estimates of microalgae's

capacity to produce lipids vary from the unrealistically large 14,600 gal of biodiesel per acre (*29*) to a more reasonable 2,640 gal of biodiesel per acre (*30*). As a comparison, the average yield of oil from an acre of soybeans is significantly less (~48 gal) (*29*). The dramatic enhancement in productivity that microalgae offers over oilseed crops would reduce the area of land required to displace the amount of petroleum diesel consumed yearly. Microalgae with their higher lipid productivity rates, ability to be cultivated on land unsuitable for agriculture, and tolerance for seawater and wastewater, have great potential to meet our current and future transportation energy needs without impacting our ability to produce food. However, the ultimate adoption of microalgae as a biodiesel feedstock will depend on its economic viability.

The process of producing biodiesel from microalgae is a six step process. This process includes; *i*) organism selection, *ii*) cultivation at a commercially relevant scale, *iii*) nutrient management to achieve maximal lipid productivity, *iv*) harvesting and dewatering, *v*) extraction of lipids, and *vi*) conversion of lipids to biodiesel. Each step of the process will require innovation to achieve commercial success. Chapter 3 describes a process for producing biodiesel from microalgae in a single step. This process combines the steps of extraction and conversion through direct or *in situ* transesterification. The algal biomass is heated in the presence of an alcohol (e.g. methanol) and an acid (e.g. sulfuric acid) to simultaneously extract and convert algal lipids. It was found that this approach led to higher yields than would be possible using the traditional extract then convert method (*31*). The higher yields were attributed to the conversion of phospholipids that are not extracted by organic solvents. This was demonstrated by using biomass from cyanobacteria, which do not make triglyceride, in a direct

transesterification reaction. The result of this reaction was the formation of biodiesel that amounted to ~7% CDW, all from membrane lipids. Furthermore, when pure phospholipids (1,2-dipalmitoyl-sn-glycerophosphate and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine) were used in place of algal biomass in a direct transesterification reaction, large amounts of methyl palmitate were produced.

The ability of this method to produce biodiesel from phospholipids will not only increase the overall yields of biodiesel but will also simplify the cultivation process. A challenge to producing biodiesel from microalgae at large scales is managing the nutrients to achieve a dense culture with high lipid content. High lipid content algae is frequently achieved by depriving the microalgae for an essential nutrient, often reduced nitrogen. Implementing this stress as part of the cultivation strategy can result in lower biomass yields and longer cultivation times (*32*). Because the direct transesterification method reported in Chapter 3 can produce biodiesel from phospholipids, high lipid contents may not result in maximal lipid productivity. This may occur when biomass production is at its highest without stringent management practices. This concept was demonstrated using an extreme example. Wild microalgae, which required zero input (nutrients or culture management), were collected from a municipal wastewater treatment facility and were subsequently used as a feedstock for direct transesterification. A low but significant biodiesel content of 10% CDW was obtained from these cells that required zero management (*31*). This method has the potential to improve how microalgal cultures are managed to achieve maximal lipid productivity.

A discussion of the viability of microalgal biodiesel could not be complete without addressing the issue of harvesting and dewatering. A dense microalgal culture

typically contains 1 g/L (dry weight, DW) of algal cells, which is 0.1% (w/w) of the solution. An outdoor raceway culture in stationary growth phase is often less dense (unpublished data). For every gallon of biodiesel produced, 3,000 gallons of water must be processed (assuming a culture density of 1 g L^{-1} DW and a lipid content of 30% CDW). Because of this tremendous amount of water, efficient harvesting and dewatering will determine economic viability of microalgal biodiesel. Separation technologies such as centrifugation and tangential flow filtration are both mature effective technologies that are capable of recovering the algal biomass but are both costly in terms of equipment and operation. The economic viability of algal biofuels will likely depend on innovations in this area.

On the surface, the issue of harvesting algae appears to be an engineering problem that must be solved by the development of a new mechanical approach to removing water and collecting microalgal biomass. The problem can, however, be addressed biologically by developing or isolating strains of algae that either grow to greater densities in culture or actually harvest themselves. A "dense" culture of microalgae appears black. This is due to the very efficient nature of their light harvesting pigments (e.g. chlorophyll) making the culture appear more dense than it really is. Microalgae use specialized light harvesting antenna complex (LHC), which consist of pigments and associated proteins, to capture solar energy and transfer it to the photosynthetic reaction centers (*33*). Photosynthetic organisms have evolved large LHC to efficiently capture energy in low light conditions. In large scale microalgal culture, which would be necessary for commercialization of microalgal fuels, large LHC reduces the overall biomass productivity as light is absorbed by the surface algae shading the rest

of the culture (*34*). The excess energy absorbed by the surface algae is not used productively and is released as heat or fluorescence (*35*). Microalgae with smaller light harvesting complexes absorb less light allowing the light to penetrate deeper into the culture and is used more efficiently for microalgal growth, leading to higher photosynthetic efficiencies (*36*). Efforts are underway to develop strains with reduced antenna size that will allow the culture to attain greater photosynthetic efficiency and possibly grow to higher densities, reducing the energy expended to harvest the microalgae (*37–39*).

Some strains of microalgae and cyanobacteria are easier to remove from culture than others. Differences in strains could be exploited to make harvesting easier. Mixing is often required to keep the algae in solution. Allowing the cells to settle prior to harvest would concentrate the cells, minimizing the volume of water to be processed. Algae could also be actively concentrated through a process called flocculation, essentially the clumping of cells into a much larger mass (*40*, *41*). The wastewater treatment industry often induces flocculation by the addition of a material often polymeric or a cationic in nature (*42*). Addition of a flocculant adds expense and may impact the quality of the fuel produced from the algae. The development of a strain that auto-flocculates in response to an external stimuli would be tremendous innovation. Particular strains of microalgae begin to produce extracellular polymers in response to stress conditions that cause them to begin to flocculate (*40*). Flocculating strains of algae could be cultured along with oleaginous strains of algae to induce flocculation, once flocculated the algae can be simply collected from the bottom of the culture vessel (*41*).

Heterotrophic Microorganisms for Biodiesel production

The primary advantage of using microalgae as a feedstock for biodiesel production is its ability to utilize only the energy from the sun and CO_2 from the atmosphere. However, due to the technical challenges related to its growth and harvest, algae derived biodiesel remains a distant albeit promising reality. Oleaginous bacteria and fungi are a more viable alternative to microalgae for producing a biodiesel feedstock in the near term. These organisms can be grown on simple sugars obtained either from food processing waste, such as molasses from sugar refining (*43*) and whey permeate from cheese production (*44*), or from the sugars contained in cellulosic biomass (*45*). Recent advances in biomass pretreatment are reducing the cost of obtaining simple sugars directly from cellulosic materials (e.g. agriculture and forestry residues) (*46*, *47*). The process for producing biodiesel using heterotrophic organisms is similar to the approach taken to produce biodiesel from microalgae, with two important differences. Heterotrophic organisms can grow much more dense because their growth is not dependent upon light penetration. Therefore, heterotrophically grown organisms can achieve densities greater than 50 g L^{-1} under optimal conditions (*25–27*). As a result, less water msut to be removed from solution to process the microbial biomass for biodiesel production, significanlty reducing the cost of this energy intensive step. The harvested bacterial/fungal biomass can then be processed in the same manner as microalgal cells to produce biodiesel (*48*, *49*).

The following sections will provide a summary of accomplished work in this dissertation related to oleaginous bacteria as well as provide an overview of the potential that both oleaginous bacteria and fungi present for the production of fuels, fuel

precursors, and oleochemicals. A detailed discussion of the knowledge gaps that exist will follow, which if filled will improve the production of lipids from low-cost sugars.

Bacteria

In regard to biofuel production, bacteria are more commonly associated with ethanol production than they are with the production of biodiesel. Most bacteria store energy in the form of poly hydroxyalkanoates (*50*). However a few species of bacteria from the actinomycetes group, such as species of *Rhodococcus*, *Mycobacterium*, and *Acinetobacter,* accumulate instead wax esters and triglyceride as a form of energy storage (*51*). Essential to the production of both of these lipids is the dual function enzyme, wax ester synthase/acyl coenzyme A (CoA):diacylglycerol acyltransferase (WS/DGAT) (*52*). This enzyme, first identified in the bacterium *Acinetobacter calcoaceticus* ADP1, transfers an acyl group from fatty acyl-CoA to either a fatty alcohol (wax ester synthesis) or a diacylglycerol (triglyceride synthesis). Bacterial strains containing this enzyme can produce either wax esters, triglycerides, or a combination of both (*53*). While the exact reason why some organisms produce exclusively wax esters while others produce triglycerides is not known, it is thought that it could be due to the availability of the fatty alcohol substrate (*54*, *55*).

The fatty alcohol substrate is produced from fatty acyl-CoA molecules in two steps of two electron reductions. Whether this reduction occurs at the active site of a single enzyme or whether it is the action of two separate enzymes remains unclear (*54*, *55*). Reiser and Somerville identified an enzyme essential to wax ester production in *Acinetobacter baylyi* that, when deleted, abolished wax ester production in this strain

(*56*). The purified enzyme was found to have acyl-CoA reductase activity, reducing fatty acyl-CoA to the corresponding aldehyde. However, no fatty alcohol was formed in *E. coli* cells heterologously expressing this enzyme. This finding was surprising given that plants were found to catalyze the reduction of fatty alcohols from fatty acyl-CoA with a single enzyme (*57*). Reiser and Somerville were unable to identify the second enzyme in the bacterial system that catalyzes the reduction of fatty aldehyde to fatty alcohol (*56*).

In Chapter 5 the characterization of an enzyme with fatty aldehyde reductase (FALDR) activity is described. This enzyme was cloned from *Marinobacter aquaeolei* VT8 and heterologously expressed in *E. coli* as a fusion protein with the maltose binding protein (*55*). The FALDR enzyme was found to be NADPH dependent and was most active with *cis*-11-hexadecenal producing *cis*-11-hexadecenol. The activity of the enzyme decreased with decreasing chain length of the aldehyde substrate. Activity with longer-chain aldehydes was not easily accomplished due to the lack of commercially available long-chain aldehydes. No activity was found with aromatic aldehydes nor was any activity observed with short chain aldehydes, such as butyraldehyde. The ability of this enzyme to utilize fatty acyl-CoA as a substrate was tested repeatedly with multiple preparations of the enzyme without any evidence of substrate reduction. A very recent report indicates that this enzyme is capable of reducing fatty acyl-CoA substrates (*58*). We have been unsuccessful in our efforts to observe this activity. The reverse reaction or oxidation of fatty alcohols such as 1-hexadecanol in the presence of $NADP^+$ was not observed, as a result the enzyme was termed a fatty aldehyde reductase (FALDR).

The FALDR enzyme is suspected to be responsible for the formation of fatty alcohol substrates for the WS/DGAT enzyme, although there is no direct evidence for its

involvement in wax ester production. The development of a FALDR deletion strain would be an important step to understanding its role in wax ester production. Efforts to produce such a strain through deletion by homologous recombination were not successful. This effort is complicated by the lack of a genetic system in *M. aquaeolei*. Although evidence presented in this dissertation suggests that two separate enzymes are responsible for fatty alcohol substrates in wax ester accumulating bacteria, this does not exclude the possibility of a separate enzyme that performs the two step reduction of fatty acyl-CoA to fatty alcohol. Recently the Seefeldt lab presented evidence that a separate enzyme from *M. aquaeolei* catalyzes the reduction of fatty acyl-CoA substrates to the corresponding alcohol (*54*). As with the FALDR enzyme, no direct evidence for the involvement of this enzyme in the wax ester biosynthetic pathway is available.

The enzymes involved in the wax ester biosynthetic pathway have important applications in biotechnology (*59*). The WS/DGAT enzyme is particularly useful. This enzyme has a broad substrate range capable of accepting the native long chain alcohol substrates as well as non-native short chain alcohols such as ethanol (*60*). The ability of this enzyme to catalyze the transfer of an acyl group to short chain alcohols, such as ethanol, enables the production of biodiesel, *in vivo* (*61*). Kalscheuer et al expressed a *ws/dgat* gene in a strain of *E. coli* modified to produce ethanol. The result was a recombinant strain that produced fatty acid ethyl esters *in vivo*. The authors of this study termed the fuel “microdiesel.” This same enzyme has been used since in studies aimed at designing microbial strains to produce drop-in fuels directly (*62*).

Homologues of this enzyme exist among a few bacteria (*63*). In some cases, as many as fifteen homologues are present in a single strain. In each case the enzymes share

approximately 25% sequence identity. The putative active site motif HHXXXDG (*64*) is, however, strictly conserved. With significant variation present within this class of enzymes it is possible that one particular WS/DGAT homologue might be better suited for the production of microdiesel than another. An alcohol other than ethanol might be better suited for microdiesel production as ethanol is a poor substrate for the enzyme (*60*, *61*). Studies of the composition of wax esters (Chapter 4) obtained from *M. aquaeolei* grown in the presence of either ethanol, 1-butanol, 2-methyl propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, or phenyl ethanol reveal that the WS/DGAT enzymes from *M. aquaeolei* appear to be more active toward four and five carbon alcohols than ethanol. These wax ester composition studies merely indicate which alcohols might be better substrates. Assays with the purified enzyme will be required to determine which alcohols are the better substrates and which enzyme has the higher activity and lower K_M values. Results of these studies will better inform researchers, developing microorganisms to produce biodiesel *in vivo*, which *ws/dgat* gene should be included in their designer strain and which alcohol should be produced to serve as substrate for the WS/DGAT protein. Each of the alcohols used in the wax ester compositional studies (Chapter 4) can be made within the cell by re-engineering the amino acid biosynthetic pathway (*65–69*). A better understanding of the differences in substrate preference and activity among WS/DGAT homologues will lead to the development of synthetic organisms with increased capacities to produce microdiesel.

In addition to their utility in producing microdiesel, WS/DGAT enzymes allow oleaginous bacteria to accumulate large amounts of triglyceride (*51*). *Rhodococcus opacus* and *Rhodococcus jostii* are two examples of oleaginous bacteria that accumulate

impressive amounts of intracellular triglyceride amounting to 68% CDW and 57% CDW respectively (*25*, *70*). Given the high lipid content, these bacterial strains could be a potential source of biodiesel feedstock. Although the heterologous expression of WS/DGAT in *E. coli* for the production of microdiesel is clever, yields were not impressive (*61*). *E. coli* cells expressing *ws/dgat* only yielded 1.26 g/L of fatty acid ethyl esters. The simultaneous production of an ethanol and a fatty acid substrate is challenging for the organism. Ethanol and other small alcohols are toxic to organisms, perhaps contributing to low yields and limiting the effectiveness of microdiesel production. In contrast to ethanol, triglyceride, a metabolite native to the bacterium, is a non-toxic compound. *Rhodococcus* cells accumulate up to 68% of their dry weight as TAG with no apparent affect on growth (*25*). Improved understanding of the biology of lipid accumulation in oleaginous bacteria could serve to improve the conversion rate of sugar to product. While both *R. opacus* and *R. jostii* have sequenced genomes, the metabolic engineering of these organisms to improve lipid accumulation will be limited by a lack of genetic tools.

Fungi

Oleaginous fungi represent a third microbial platform for lipid production (*24*, *71*). Although no work related to the production of fuels from oleaginous fungi is described in this dissertation, their potential as a biodiesel platform deserves mention here and constitutes an important avenue for future research. Like oleaginous bacteria, fungi can utilize a low value carbon source found in food production waste and cellulosic biomass to grow and accumulate large amounts of intracellular triglyceride which can

account for 70% of the cell's dry weight (*14*). Oleaginous species of fungi are uncommon and include yeast such as *Cryptococcus curvatus*, *Rhodosporidium toruloides, Lypomyces starkii*, and *Yarrowia lipolytica*, and the filamentous fungus *Mortierella alpina*. Common industrial species of yeast such as *Saccharomyces cerevisiae*, and *Pichia pastoris* do not have the ability to accumulate substantial amounts of triglyceride.

Oleaginous yeast and fungi could serve as ideal model organisms for eukaryotic triglyceride accumulation (*72*, *73*). A thorough understanding of the biochemistry of lipid accumulation in microalgae is prevented at present because of the lack of a good model organism. Genome sequences of microalgae are available and a robust genetic system exists for *Chlamydomonas reinhardtii* (*74*, *75*). However, this organism does not naturally accumulate triglyceride. The lack of genetic tools, in relevant strains, severely limits the ability of researchers to understand the factors that contribute to lipid accumulation in microalgae and how this process could be manipulated to increase productivity or to produce a desired product. The oleaginous yeast, *Yarrowia lipolytica*, on the other hand could be an ideal model organism to understand the process of lipid accumulation in a single cell eukaryote (*72*, *73*). The genome sequence of this oleaginous yeast and a robust genetic system is readily available (*73*, *76*, *77*). The genetic tools available for *Y. lipolytica* could be applied to four areas of research with the aim to increase lipid production in oleaginous microorganisms: *i*) Carbon utilization, *ii*) nitrogen metabolism, *iii*) triglyceride formation, and *iv*) elimination of side reactions.

The ideal organism for biofuel production would be one capable of producing biofuels at a large enough scale to meet demand for liquid transporation fuels. An

important characteristic would be the ability to simultaneously metabolize the mixture of hexose and pentose sugars found in biomass and directing the flow of its carbon efficiently to the formation of fuels or fuel precursors. The fermentation of biomass derived sugars to ethanol has long been a challenge as industrial organisms lack the appropriate biochemistry (*78*, *79*). Woody biomass is composed of varying amounts of cellulose (40-50%), hemicellulose (30-40%), and lignin (20-30%) (*78*). Cellulose is made up entirely of $\beta_{1\rightarrow4}$ linked D-glucose monomers, while hemicellulose is primarily composed of the five carbon sugar xylose. Fermentation of cellulosic materials requires a pretreatment step to enhance the availability of these sugars and is the focus of intense research (*80–82*). Native *Saccharomyces cerevisae*, the choice organism for commercial ethanol production, is unable to utilize xylose to an appreciable extent. *S. cerevisae* modified to express xylose reductase (Xyl1) and xylitol dehydrogenase (Xyl2) from *Pichia stipitis* enables this strain to utilize xylose through the pentose phosphate pathway (PPP). Two major hurdles must be overcome in using lignocellulosic sugars for fuel production. First, organisms preferentially use glucose and xylose utilization is repressed in the presence of significant glucose concentrations. Glucose concentrations must first be reduced below a threshold level before significant xylose utilization occurs (*83*). As a result, fermentations using biomass derived sugars are not as efficient as a fermentation with glucose or xylose alone. The second, and greater, challenge results from the cofactor requirements of Xyl1 and Xyl2. Xyl1 requires NADPH or NADH with a strong preference for phosphorylated nicotinamide cofactor (*84*), while Xyl2 has an absolute requirement for NAD^+ (*85*). Under the anaerobic conditions of xylose fermentation for ethanol production, redox imbalance ensues due to the host organism's inability to

regenerate NAD^+ (*86*). Many researchers are reengineering the metabolism of the xylose utilizing strains to develop a variety of new approaches to regenerate the necessary cofactor. Xylose utilization under aerobic conditions, however, does not cause a redox imbalance as NAD^+ can be readily regenerated through respiration (*86*, *87*). The development of xylose utilizing strains capable of producing fuels under aerobic conditions could eliminate concerns of cofactor imbalance. The production of fuels based on fatty acid biosynthesis is one example of a fuel generating pathway that occurs under aerobic conditions. Further research could explore the production of a variety of fuels that either exploit fatty acid biosynthesis or utilize another pathway amenable to aerobic conditions.

In addition to a complex mixture of hexoses and pentoses, biomass hydrolysates, obtained by treating the biomass with enzymes, heat, and acid, also contain many compounds that inhibit microbial growth (*81*, *82*). Acetic acid is produced by the hydrolysis of hemicellulose as the carbohydrates in this compound are heavily acetylated (*88*). Lignin is an amorphous polymer whose basic building blocks are hydroxylated aromatic compounds (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol). Pretreatment of biomass to make the carbohydrates available releases lignin derived compounds which inhibit microbial growth (*89*). It is of extreme importance to develop strains of yeast that can tolerate the inhibitors present in biomass hydrolysates.

Oleaginous microorganisms begin to produce lipids once the reduced nitrogen has been depleted (*90*, *91*). The mechanism of lipid induction and how it is coupled to nitrogen depletion is poorly understood. In oleaginous yeast, nitrogen depletion in the media results in the scavenging of nitrogen from cellular molecules such as adenosine

monopohosphate (AMP) by AMP deaminase, forming inosine monophosphate (IMP), lowering cellular levels of AMP in the process (*91*, *92*). AMP deaminase activity was found to be significantly higher in cells grown under nitrogen limiting conditions (*91*). It is uncertain how low nitrogen levels affect the activity of AMP deaminase and whether the cells respond to extracellular or intracellular nitrogen levels. Understanding the nature of the cell's response to low nitrogen levels could enable researchers to take advantage of this response to improve lipid production. The activity of AMP deaminase has a profound effect on the accumulation of lipid. In some strains of yeast isocitrate dehydrogenase (ICDH), responsible for the oxidative decarboxylation of isocitrate to form α-ketoglutarate, has an absolute requirement of AMP for activity (*92*). As a consequence of low AMP concentrations and ICDH activity, isocitrate and consequently citrate concentrations begin to increase. The increased mitochondrial citrate concentration is then shuttled to the cytoplasm where ATP citrate lyase catalyzes the production of acetyl-CoA and oxaloacetate from citrate for fatty acid biosynthesis and lipid begins to accumulate (*17*, *71*, *93*).

The goal of cultivating oleaginous yeast is to produce as much triglyceride as possible. A well used strategy to increase the production of a metabolite is to both “push” and “pull” substrates. This approach serves to create more product by increasing the rate at which the initial substrate is formed (push) and increasing the rate at which substrate is depleted (pull). As an example, the first committed step of fatty acid biosynthesis is the formation of malonyl-CoA from acetyl-CoA and carbonate catalyzed by acetyl-CoA carboxylase (ACCase). Increasing the level of expression of this enzyme or its rate of reaction could result in more malonyl-CoA available for fatty acid biosynthesis and

ultimately more fatty acid molecules being produced (*94*). This effectively "pushes" more carbon into the fatty acid biosynthetic pathway. A build up of product could potentially inhibit the rate of product formation. In order to avoid this inhibition, the product needs to be removed or "pulled." To increase TAG production, the fatty acid substrate could be removed at a higher rate by increasing the activity of any or all of the enzymes that function to transfer the fatty acyl group to glycerol. Overexpression of such acyl transferases has led to increased lipid content in the oleaginous yeast strain *Yarrowia lipolytica* (*95*). The example discussed here is simplified and there is much that is unknown about the regulation of triglyceride production in yeast. One genome sequence from an oleaginous strain is currently available. As more strains are sequenced and genetic tools are developed, a greater understanding of this process will be attained. This knowledge will undoubtedly contribute to the development of industrial strains to produce triglyceride economically.

The maximum yield of triglyceride will be obtained when all of the excess carbon is being channeled into the appropriate biosynthetic pathway and undesireable non-essential diversions of carbon are either eliminated or minimized. The oleaginous yeast *Y. lipolytica* is well known to excrete large amounts of citrate into the medium (up to 200 to 240 g L^{-1}) (*96*, *97*). With regard to triglyceride production, secreted citrate represents lost carbon and negatively impacts the yield of the desired product. To maximize lipid production, citrate excretion needs to be minimized. In oleaginous yeast citrate is thought to be a necessary intermediate in lipid production. Alternatively, the export of citrate could be eliminated and the rate of acetyl-CoA generation could be increased by altering the expression level of ATP citrate lyase. First steps in this effort will be to

identify and eliminate the gene encoding the membrane protein responsible for citrate export. With citrate export reduced or eliminated, triglyceride productivity and efficiency will increase.

The availability of a sequenced genome coupled with a robust genetic system makes *Yarrowia lipolytica* an important model organism for lipid accumulating eukaryotes. Advancements in our understanding of the biochemistry associated with lipid production in this organism will aid the development of industrial strains capable of producing biofuels economically. Key to this possibility will be an understanding of how to effectively utilize low-cost carbon, while tolerating inhibitory compounds present in biomass hydrolysate. The management of nutrients, such as reduced nitrogen, in commercial reactors will be informed by an understanding of how the organism responds to nutrient levels and how this response impacts the final TAG yield. An industrial organism that efficiently directs the flow of carbon to the production of TAG will be a key feature to the success of microbial biodiesel production.

Conclusion

The finite nature of petroleum and its role in contributing to global climate change has increased the interest in developing biobased alternatives. Biodiesel is a non-toxic, renewable fuel that can be effectively used in place of petroleum diesel, with little to no modification to existing engines. The availability of feedstock for the production of biodiesel is a significant barrier to its wider adoption. Limited agricultural resources, such as arable land, prevent oilseed crops from meeting feedstock demand alone. The work described in this dissertation has sought to address the supply of biodiesel feedstock

through the development of new methods to utilize existing resources as well as new biodiesel feedstocks. Oleaginous microorganisms have the potential to deliver a sufficient quantity of high quality biodiesel feedstock, although many challenges exist to their implementation. A greater understanding of the biochemistry of oleaginous microbes will be important to their adoption as a platform for biodiesel and oleochemical production.

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Education

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Mentor, 2008 InTech Summer Internship. July 2008.

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Presentation on Biofuels, Cache Valley Homeschool Science Day. 2007.

Mentor, 2007 InTech Summer Internship. July 2007.

Presentations

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Patent Applications

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