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Increased Production and Extraction Efficiency of Triacylglycerides from Microorganisms and an Enhanced Understanding of the Pathways Involved in the Production of Triacylglycerides and Fatty Alcohols

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INCREASED PRODUCTION AND EXTRACTION EFFICIENCY OF TRIACYLGLYCERIDES FROM MICROORGANISMS AND AN ENHANCED UNDERSTANDING OF THE PATHWAYS INVOLVED IN THE PRODUCTION OF TRIACYLGLYCERIDES AND FATTY ALCOHOLS

by

Robert M. Willis

A dissertation submitted in partial fulfillment
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ABSTRACT

Increased Production and Extraction Efficiency of Triacylglycerides from Microorganisms and an Enhanced Understanding of the Pathways Involved in the Production of Triacylglycerides and Fatty Alcohols

by

Robert M. Willis, Doctor of Philosophy

Utah State University, 2013

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The continued increase in the demand for fossil fuels combined with their ever dwindling supply has prompted the search for a suitable alternative fuel. The research contained within this dissertation seeks to increase the lipid content of cellular feedstocks, improve extraction efficiencies of lipids, and understand the pathways involved in the production of fatty alcohols and triacylglycerides from microbial feedstocks.

As part of this research the diatom, *Cheatoceros gracilis*, was grown at small and large scale to determine optimal growing conditions. No apparent nutrient stress trigger was required to initiate the accumulation of the biodiesel precursor triacylglyceride, unlike other documented algal strains. A follow-up to this project demonstrated that the microalga *C. gracilis* may utilize light intensity as a trigger for lipid production.

A major difficulty in the production of biofuels from microorganisms is the expensive process of dewatering, drying, and extracting the lipid compounds from the cells. As part of this research, a process has been developed that allows for lipid extraction to occur in the presence of water at a point as low as 2 percent solids or 98 percent water. This process utilizes a single organic solvent that mixes well with microbial lipids, but poorly with water allowing for efficient extraction of lipids and fast solvent to water separation. This process greatly decreases the cost of the microbial biofuels production associated with the removal of water from cell slurries.

Triacylglycerides and fatty alcohols are oleochemicals that are commonly used in industrial, pharmaceutical, and consumable processes. A predicted fatty acyl CoA reductase enzyme was cloned into an *E. coli* vector, expressed, characterized and shown to be active as a dual reductive enzyme reducing a fatty acyl CoA to its respective fatty alcohol, constituting the first enzyme of this type discovered in a bacterium.

The process of triacylglyceride production in microbes is fairly well understood; however, the process that regulates this production has not yet been fully explored. As part of this research, the model yeast organism, *Yarrowea lipolytica*, is utilized to identify essential genes for citrate transport that if removed could result in increasing triacylglyceride production *in vivo*.

(239 pages)

PUBLIC ABSTRACT

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The continued increase in the demand for fossil fuels combined with their ever dwindling supply has prompted the search for a suitable alternative fuel. The research contained within this dissertation seeks to increase the lipid (fat) content of cellular feedstocks, improve extraction efficiencies of lipids, and to understand the pathways involved in the production of fatty alcohols and triacylglycerides, compounds commonly used in many industrial processes, from microbial feedstocks. This work has been done in an attempt to increase the overall economic viability of microbial biofuels production.

The production of biofuels from microalgae used as a feedstock allows for the conversion of a waste gas, carbon dioxide, into a renewable biofuels source. As part of this research, the diatom *Cheatoceros gracilis*, was grown at small and large scale to determine optimal growing conditions. It is generally accepted that an essential nutrient must be withheld from the organism to cause them to produce the fat compound

triacylglyceride, however, this trigger does not appear to be present with this microalgae. A follow-up to this project demonstrated that high intensity light may be the trigger that this organism requires for fat production.

A major difficulty in the production of biofuels from microorganisms including algae, yeast, and bacteria is the expensive process of dewatering, drying, and extracting the lipid compounds from the cells. As part of this research, a process has been developed that allows for lipid extraction to occur in the presence of water at a point as low as 2 percent solids. This process utilizes a single organic solvent (exe. chloroform) that mixes well with microbial lipids, but poorly with water allowing for efficient extraction of lipids and fast solvent to water separation. This process greatly decreases the cost of microbial biofuels production associated with the removal of water from microbial feedstocks.

Triacylglycerides and fatty alcohols are oleochemicals (chemicals that are derived from a biological source) that are commonly used in industrial, pharmaceutical, and consumable processes. The understanding of how these chemicals are produced in different microorganisms was a focus as part of this work. An enzyme has been found in the bacterium *Marinobacter aquaeolei* VT8 that is responsible for producing fatty alcohols. This is the first enzyme of this type found in a bacterial organism.

The production of triacylglycerids in microorganisms is fairly well understood, however, the control of the cellular machinery that produces triacylglycerides has not been fully explored. As part of this research, the model yeast organism, *Yarrowea lipolytica*, is used to work toward determining genes that are necessary for citrate, a

triacylglyceride precursor in the cell, transport. The removal or chemical manipulation of these genes/enzymes should result in an increase in microbial lipid production. This understanding could thus be used to increase the overall lipid production of a microbial feedstock for biofuels production.

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I would especially like to thank my family for their never-ending support. My father for always encouraging me to be the best I could be. He has told me often through the years “If all you want to be in life is a ditch digger, then be the best ditch digger you

can be" quickly followed by "but of course I expect much more of you." I would like to thank my mother, for teaching me by her example to always be curious and question the world around me. I would like to thank my grandfather for teaching me to work hard and understand the inner workings of machinery. This initial curiosity about how machines function led me to my passion for studying how biological organisms function and ultimately my study of biochemistry. My Aunt Julie and Uncle Tom for their continued support both emotionally and financially. Without their support my academic carrier may very well have ended before it got started.

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Robert Malone Willis

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ABBREVIATIONS

FACoAR	Fatty Acyl-Co-Enzyme A Reductase
NADPH	Nicotinamide Adenine Nucleotide Phosphate Reduced
NADH	Nicotinamide Adenine Nucleotide Reduced
ACP	Acyl-Carrier Protein
CoA	Co-Enzyme A
FAR	Fatty Aldehyde Reductase
TAG	Triacylglyceride(s)
ATP	Adenosine Triphosphate
AMP	Adenosine Monophsophate
IMP	Inosine Monophosphate
TCA	Tri-carboxylic Acid Cycle
DW	Dry Weight
FAME	Fatty Acid Methyl Ester
DAF	Dissolved Air Flotation
FAACPR	Fatty Acid Acyl-Carrier Protein Reductase
IUPAC	Internation Union of Pure and Applied Chemistry
PBR	Photo-Bio-Reactor
FFA	Free Fatty Acid
GC	Gas Chromatograph
UTEX	University of Texas at Austin (Algal Culture Collection)
LB	Luria Burtani (LB Broth)

TLC	Thin Layer Chromotography
NIST	National Institues of Standards and Technology
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
THF	Tetrahydrafuran
UV	Ultraviolet
RPM	Revolutions Per Minute
SEM	Standard Error of the Mean
YPD	Yeast Peptone Dextrose (Yeast Media)
FID	Flame Ionization Detector
PTV	Programmed Temperature Vaporizer
MSDS	Material Safety Data Sheet
MJ	Mega Joules
mg	Milligrams
L	Liter
FEP	Fluorinated ethylene propylene
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
NADP+	Nicotinamide Adenine Nucleotide Phosphate Oxidized
NAD+	Nicotinamide Adenine Nucleotide Oxidized
NCBI	National Center for Bioinformatics
PCR	Polymerase Chain Reaction
DNA	Deoxyribose Nucleic Acid
BLAST	Basic Loacal Alignment Search Tool

MBP	Maltose Binding Protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
EDTA	Ethylenediaminetetraacetic acid
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylimide Gel Elecrophoresis
MS	Mass Specrophotometer
DMSO	Dimethyl Sulfoxide
NTB	2-nitro-5-thiobenzoate
FALDR	Fatty Aldehyde Reductase
HMG	3-hydroxy-3-methyl-glutamate
ITC	Isothermal Titration Calorimetry

CHAPTER 1

INTRODUCTION

The volatile petroleum market fluctuates on a daily basis and has an extreme effect on the prices of almost every consumable product purchased in the United States and other developed countries. In addition to this monetary effect, the physical amounts of petroleum available for use are rapidly decreasing resulting in economic uncertainty (*1–3*). At the current rate of consumption and acquirement, the fossil fuels that are utilized for liquid transportation which represent thirty-seven percent of the overall energy usage in the United States (Figure 1-1), will run out within the next 150 years (*3*). Both the increase in prices of consumables and the decrease in fossil fuel supply have created a demand for a substitute energy source that can overcome the negative effects of the current petroleum driven market.

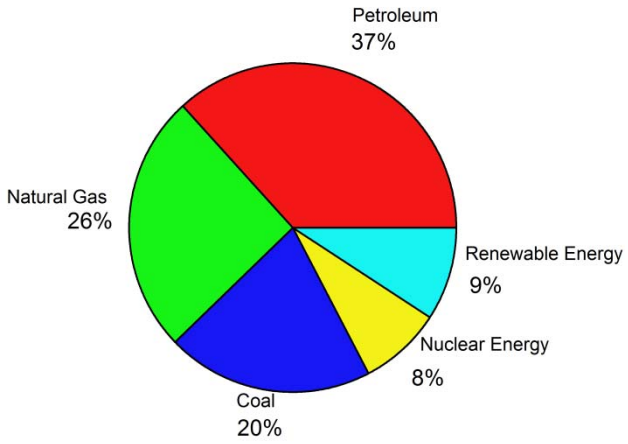

Figure 1-1. United States energy consumption by source for 2012**.** Adapted from information available at the US Energy Information Administration website (http://eia.gov).

. The search for this alternative energy source has inevitably led to the largest energy reserve in our solar system, the sun. Solar energy is abundant with over 4.3 x 10^{20} J of energy available at the earth's surface every hour, more than enough to supply the current yearly worldwide energy consumption of 4.25 x 10^{20} J (*3*). However, solar energy is a diffuse form of energy that unless harnessed appropriately cannot be effectively utilized. The utilization of solar energy has been the focus of scientific research for decades with two dominant forms of sunlight energy capture arising: photovoltaic and biological.

Photovoltaic capture of sunlight utilizes various compounds capable of harvesting light energy to produce electricity. Methods of light harvesting have been devised that utilize silicon based technologies in combination with rare earth metal materials to obtain photon capture efficiencies as high as 40% (*4*). Promising technologies that utilize abundantly available organic compounds for the capture and conversion of sunlight to electricity have also been developed and have efficiencies currently of about 10% with more efficient processes being developed (*5*). The combination of these technologies promises to greatly increase electrical energy production from sunlight in the near future. However, the low energy density of electrical storage devices limit the utility of photovoltaic cells in providing power to the transportation sector (*6*).

The biological capture of sunlight occurs throughout the world in organisms ranging from microscopic bacteria to towering redwood trees. The process of photosynthesis carried out by these organisms captures sunlight and utilizes the captured energy to reduce carbon dioxide to a myriad of useful compounds from sugars and fats to

petroleum like compounds (*7*). The major difficulty in utilizing biological organisms to capture sunlight is that much of the captured energy is stored in chemical forms, such as cellulose, that are difficult to use in the production of desirable energy rich compounds (*8*).

Regardless of the form of sunlight capture utilized, the petroleum substitutes produced must take into account the current infrastructures of energy usage in the United States and worldwide. The current transportation infrastructure has need of high density liquid fuels, similar to gasoline and diesel (*9–12*). The energy density of these fuels allows them to be used to travel longer distances on relatively small amounts of fuel as compared to equivalent amounts of natural gas or electricity. Any large scale deviation from this type of high density liquid fuel platform, such as the use of photovoltaics and battery powered electric transportation, would be costly and would likely take years to implement.

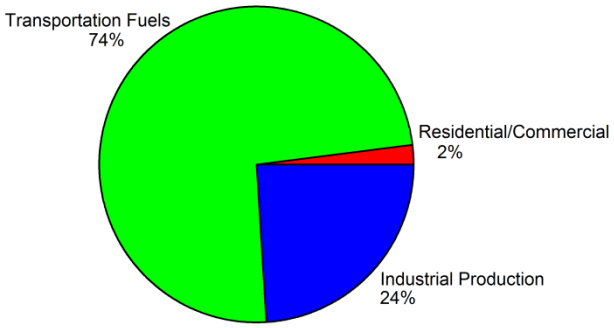

Figure 1-2. Petroleum usage based upon economic sector from 2012. Adapted from information available at the US Energy Information Administration website (http://eia.gov).

Although a large amount of concern is expressed over the issues surrounding the supply of fossil fuels for transportation, the large amount of petroleum utilized for industrial and consumable purposes is often less publicized. Many pharmaceuticals, health and beauty products, and other household chemicals utilize petroleum based chemicals as primary constituents (Figure 1-2) (*13*, *14*). As a result of this large industrial consumption, not only must a suitable substitute for petroleum fuels be determined to accommodate the transportation sector, but suitable substitutes for petrochemicals must be found to accommodate the industrial sector.

Initial steps have been taken by many researchers to develop appropriate substitutes for petroleum fuels and petrochemicals (Figure 1-3). First Generation biofuels, corn ethanol and soybean biodiesel, were developed as an answer to the problem of the increasing demand on fossil fuels. These biofuels utilize food crops and arable land to

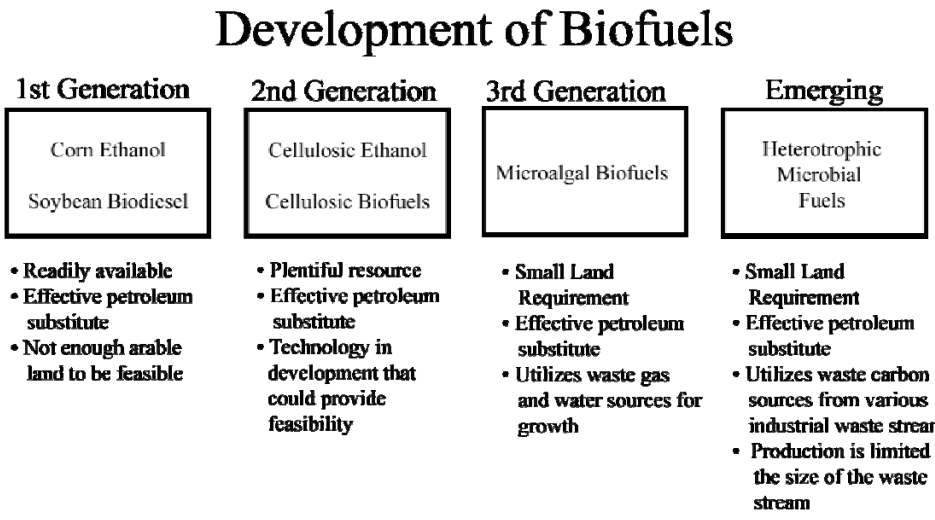

Figure 1-3. Historical development of various generations of biofuels. Adapted from information found in Brennan, *et al* (*17*).

produce a sustainable fuel (*15*, *16*). Though initially well received, many concerns have arisen as a result of this generation of biofuels. The main concerns are that the available production capability is too low to meet the current demand and the production of fuel compounds in this manner competes directly with food crops (*18*, *19*). To efficiently produce enough biofuels from the first generation sources to offset even the current usage of petroleum for transportation fuels in the United states would require more arable land than is available for growing crops, rendering this generation of biofuels obsolete as a sole source substitute for petroleum (*20*).

To overcome the shortcomings of the first generation of biofuels, a second generation of biofuels was developed utilizing non-food materials for their production. These biofuels often termed cellulosic biofuels, are produced from the sugars bound in cellulose and hemicellulose for the indirect production of fuels by microorganisms or direct conversion to fuels by catalytic means (*21*, *22*). Cellulosic biofuels may be generated from waste biomass produced as a result of farming, lumber production, and general waste production (*23*).

The production of cellulosic biofuels occurs through two major processes. The first method of utilizing the cellulosic biomass requires liberating the sugars from the complex compounds that are present in the biomass and making them available as reduced carbon sources for microbial biofuels production. These methods include hydrosylation, catalytic decomposition, and enzymatic breakdown of the biomass into its simpler monomeric parts (*24*, *25*). The major monomeric components present in the biomass such as glucose, xylose, and lignin, vary depending on the type of biomass (*26*).

Microorganisms capable of producing biofuels compounds utilizing these resulting sugars generally can be affected by higher concentrations of lignin compounds in the medium (*27*). This can create a problem when attempting to utilize these methods for biofuels production using biomass sources high in lignin such as woody biomass. However, cellulose sources low in lignin, such as straw and switchgrass, may provide promising sources of cellulosic derived sugars for the production of biofuels by microorganisms.

The second method of utilizing cellulosic biomass seeks to overcome the problem of lignin in the biomass. This method utilizes pyrolysis and gasification to convert biomass directly from its various carbohydrate and lignin components to oil compounds. These compounds consist of hydrocarbon and aromatic compounds with varying degrees of oxygenation (*28*). This method is the preferred method for conversion of woody biomass into biofuels; however, much work is yet to be done in determining how to efficiently separate the resulting mixture of compounds to produce a suitable diesel or gasoline substitute.

The overreaching problem with second generation biofuels is the same problem faced by first generation biofuels, which is the staggering amount of biomass required to produce a sufficiently large quantity of fuels capable of replacing petroleum diesel. Although biomass production does not compete directly with food crop production as first generation biofuels do, the amount of biomass required to supply the current demand for liquid transportation fuels would require biomass obtained from more than 75% of the available aerable land worldwide (*29*). Although theoretically possible, such an

undertaking is not necessarily feasible at this time. Further advancements in increasing the conversion efficiency of cellulosic biomass to the component sugars need to be made in order for cellulosic biofuels to become practical on a large scale.

Seeking to decrease competition with food crops and avoid the complications of second generation biofuels production, a third generation of biofuels, microalgal fuels, has been developed utilizing photosynthetic microorganisms. This method of biofuels production is promising in that the carbon the microorganisms require comes directly from carbon dioxide, which they fix to lipids and carbohydrates using energy derived from the sun. Growing microalgae requires very little land when compared to the amount of land needed for the growth of plant products required for first and second generation biofuels. The microalgal organisms can be grown in enclosed photobioreactor systems (PBR) or in large open ponds and raceways. It is estimated that the area of land required to produce enough biodiesel from microalgae to offset the current diesel demand would be about 3 million hectares or roughly the size of the state of Vermont (*30*).

Microalgae offer an attractive alternative for producing biofuels and as such have been extensively researched to determine the optimal growing conditions for lipid production and optimal large scale growth (*31*). However, microalgae have specific problems which hamper their widespread large scale growth; namely, low culture density, difficulty in maintaining pure cultures, and difficulty in obtaining large amounts of biofuels precursor compounds from produced biomass (*32*).

A great deal of research has been aimed at overcoming these difficulties and creating a viable process for biofuels production from microalgae. PBRs, as previously

mentioned, have been developed that allow for high light dispersion and penetration that can increase cell culture densities and prevent culture contamination by keeping the culture closed off from outside influences. This technology is promising and with more development may solve a majority of the difficulties associated with microalgal biofuels production (*33*). Difficulties in obtaining a high lipid production in produced biomass can be overcome by careful strain selection or genetic modification of algal organisms. Current systems for lipid production in microalgae rely on a dormant period of cell growth in which a major nutrient such as nitrogen becomes depleted in the cell culture. This triggers a large increase in lipid production while preventing or diminishing cell biomass formation (*34*). This is the case with some, but not all algal strains as is discussed in Chapters 2 and 3 of this dissertation. A careful consideration of the type of algal strain used can lead to the consistent non-nutrient stressed production of high lipid content in biomass.

Recently, attention for biofuels production has returned from the first through third generations of biofuels to the production of biofuels from microorganisms grown heterotrophically on niche waste carbon sources. This process, originally devised more than 70 years ago (*35*), utilizes sources of carbon for biofuels production from a large variety of waste streams of various production processes (*36*). The term niche oriented is used in this context to illustrate the fact that the supply of each waste stream is relatively small and the amount of biofuels that can be produced from each waste stream is small when compared to the overall amount of transportation fuels consumed worldwide.

This niche approach, despite being small and diverse, allows for the specialization of a single type of organism to each waste stream. In turn, this can allow for maximal production of a biofuel or specialty oleochemical from a particular waste stream. Recent research conducted in the Seefeldt lab at Utah State University (unpublished data) has shown that the yeast strain *Cryptococcus curvatus* grown on a waste carbon source from cheese production known as de-lac, can produce large amounts of biomass containing as much as 70% biodiesel precursors as measured from the dry weight of the cells. Given this level of production and the 550 million gallons of de-lac per day available nationwide, producing approximately 17 million gallons of biodiesel each day is a possibility (*37*). However, this production would only offset roughly 1% of the current diesel usage nationwide (*38*). Although this is a small amount overall, this niche method combined with other methods for biofuels production taken from the first three generations could come to fulfill the world's need for liquid transportation fuels. The overreaching goal of the research contained in this dissertation is aimed at developing novel ways to increase the viability of production of biofuels and oleochemicals from microorganisms that ultimately receive their energy from the sun.

BIOFUELS AND OLEOCHEMICALS

Oleochemicals are defined as "any chemical compounds that are derived from plant or animal fats or oils" (*39*). Given this definition, biofuel compounds such as biodiesel and ethanol can also be termed oleochemicals, but for the remainder of the discussions presented as part of this dissertation, the term oleochemicals will refer to specialty chemicals other than biofuels. From an applied standpoint, for biofuels and

oleochemicals to adequately function as substitutes for petroleum based products, the compounds will need to be able to satisfy the three major sectors of usage: transportation, industrial, and residential/commercial (*40*).

The need to fulfill the requirements of multiple sectors calls for a broad range of compounds to be produced in the form of oleochemicals. As seen in Table 1-1, each of the three major sectors utilizes specific classes of petroleum compounds to perform desired tasks. The classes of compounds used by various sectors have some points of overlap due to a further separation defined mainly by the density and boiling points of compounds used for various processes (*40*, *41*).

Table 1-1. Classes of compounds derived from petroleum classified by utilizing sector

Sector	**Major compound classes**[a]	**End products** [b]
Residential/Commercial	Paraffins and naphthalenes	heating oils
Transportation	Parafins, naphthalenes, and asphaltics	liquid fuels, lubricants, and road pavement
Industrial	olefins and aromatics	polymers, lubricants, pharmaceuticals, etc

[a] Major type of chemical compound produced broken down by IUPAC class identification.

[b] What the compounds are used to produce..

Adapted from Petroleum Products Handbook (*41*).

The challenge of finding a suitable substitute for petroleum lies not only in the production of a suitable liquid transportation fuel, but in the production of alternatives to

the aforementioned compounds required for the non-transportation uses of petroleum. (*42*). To accomplish this task a thorough review of the various compounds available from biological organisms must be undertaken to determine if adequate substitutes for all components of petroleum can be produced.

Many biological organisms produce oleochemicals that would be suitable as substitutes for petrochemicals. Plants produce a large range of compounds including paraffins, olefins, waxes, fatty alcohols, and fatty aldehydes (*43*, *44*). Animals and microorganisms alike have a surprising ability to produce many of these oleochemicals (*45*, *46*). As a result, each of the classes of petrochemical compounds has a similar compound or precursor available from a biological source (Figure 1-4).

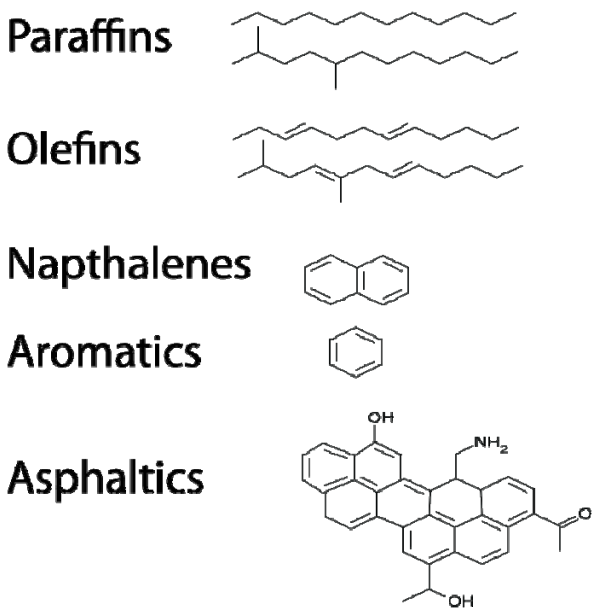

Figure 1-4. Example structures of compounds found in crude oil**.** Adapted from Petroleum Products Handbook (*41*)**.**

Parafins and Olefins. Paraffins are saturated hydrocarbon compounds that can be straight or branched chained. These compounds are commonly used for liquid transportation and heating fuels. Olefins are unsaturated hydrocarbon compounds that can be straight or branch chained. These compounds are commonly used in the industrial sector as precursors to the production of many different polymers, including plastics (*47*). Olefins and paraffins can be produced directly by many organisms or can be derived from paraffin and olefin like compounds such as free fatty acids. The production of paraffin and olefin compounds in biological organisms has been shown to occur through one of three possible mechanisms; dehydroxylation of a fatty alcohol (*48*), head to head fatty acid condensation (*49*), and decarbonylation (*50*).

The dehydroxylation of a fatty alcohol was reported by Park *et al.*, who showed that fatty alcohols present in the cells were further converted to alkane compounds by membrane fractions from *Vibrio furnissii* M1 (*48*). The authors reported that odd and even chained hydrocarbons were produced. This is contrary to what would be expected if the removal of a carbonyl group was occuring in each case given the fatty acid pool of the organism contains only even chained fatty acids. This led the researchers to theorize that some of the hydrocarbons must have been produced by a process removing the oxygen atom from the carboxyl end of the fatty acid such as dehydroxylation. It has been proposed that the reduction of an alcohol to an alkane or alkene could be possible since it would be the reverse of alkane oxidation that commonly occurs in oil degrading organisms (*51*). However, this work has not been able to be repeated and has fallen

under heavy scrutiny. The general consensus is that an alternative mechanism is responsible for the alkane production in biological organisms.

The combination of two fatty acid compounds to form an alkene in a head to head condensation type reaction has been reported by a group headed by Wackett (*49*). This work has shown that a series of enzymes coded by the genes known as *oleA, oleB,oleC* and *oleD* are required to produce a long chain alkene. These enzymes may function similarly to a polyketide synthase assembly (*52*) and are a new addition to the knowledge of olefin production in microorganisms. Further work has been done in characterizing the subunits of the complex as well as the compounds produced. As a result, enzymes of this class may soon find application in the industrial sector (*53*).

The decarbonylation of fatty aldehydes to form alkanes and alkenes is the most commonly studied and well understood method of paraffin and olefin production in biological organisms (*50*, *54–56*). The decarbonylation reaction generally utilizes a fatty aldehyde derived from the cellular fatty acid pool to produce an alkane or alkene compound. From the literature, it is apparent that two main types of decarbonylase enzymes are present in biological organisms. The first type utilizes a binuclear iron core active site to reduce a fatty aldehyde to an alkane generating formate (*56*). The second type utilizes a cobalt porphyrin ring to reduce a fatty aldehyde to an alkane generating carbon monoxide (*54*).

The binulcear iron core decarbonylase was first identified in cyanobacteria. A comparative genomics approach was taken to compare the genomes of several cyanobacteria known to produce alkanes and alkenes to a similar strain which did not.

The comparison found two genes that worked in series to reduce a fatty acid to an alkane or alkene: a fatty-acyl acyl carrier protein-reductase (FAACPR) and a fatty aldehyde decarbonylase . It was originally assumed due to prior research that the mechanism for this reaction involved carbon monoxide release; however, a recent manuscript demonstrated that the actual side-product released was formate and that the reaction was oxygen dependent (*57*).

The cobalt porphyrin type decarbonylase was first identified in the algal strain *Botryococcus braunii*, where it was noted that odd chain alkanes were produced. Investigations revealed that upon the addition of a fatty aldehyde substrate to cell lysate, carbon monoxide was evolved and alkanes were produced in stoichiometric quantities. Growth experiments done with ^{57}Co showed that radioactive cobalt co-localized with the purified enzyme and absorbance spectra suggested the presence of a porphyrin ring. However, no structural data has yet been obtained to verify the presence of the cobalt prophoryin ring (*54*).

Paraffins and olefins can also generated from oleochemical precursors by breaking down free fatty acids and triacylglyceride present in biologically derived oils to alkanes and alkenes through chemical means. Hydrotreating and cracking are common methods that can be used to saturate the carbon chains, remove oxygen groups, and produce alkane and alkene chains (*58*, *59*). These methods utilize high temperatures, 360 ºC and higher, combined with the presence of acid catalysts, such as zeolites, to accomplish the removal of oxygen and hydrogenation of the carbon chains (*60*). These

methods are commonly used in the petroleum industry and are in the process of being adapted to biofuels production.

Napthalenes and Aromatics. Naphthlenes and aromatics are compounds that consist of aromatic rings linked together and surrounded by various functional groups and carbon chains. Biologically similar compounds can be derived from fungal and woody biomass sources. Fungi have been shown to produce complex aromatic compounds through polyketide synthase enzyme complexes (*52*, *61*). These enzymes form long carbon chains with ketones placed regularly at every other carbon along the backbone. Various subunits of the enzyme complex can take the polyketide chain and form aromatic rings of various types from this initial chain.

Woody biomass contains a high proportion of lignin as discussed previously. These lignin compounds are oxygenated linked aromatic rings. The process of pyrolysis with appropriate catalysts can deoxygenate and break down the long lignin chains forming a mixture of aromatic compounds that could also be used as a substitute for petroleum derived napthalenes and aromatics (*62*).

Asphaltics. Fungi have cellular polyketide synthase enzyme systems capable of making many complex aromatic compounds, including compounds that could be as complex as asphaltic compounds. However, it is uncertain how the production of these compounds in microorganisms would affect the growth of the microorganisms. Also, this process would not be a cost effective method for producing low value asphaltic compounds (*52*, *63*).

Pyrolysis of woody biomass with a high lignin content as mentioned before can produce many complex aromatic compounds. Pyrolysis oil can be fractionated using a process similar to that used for crude oil in petroleum refining. The heavy fractions of pyrolysis oil have similar physical properties to asphaltic fractions of crude oil (*64*). It is possible that the pyrolysis of woody biomass could be utilized to produce asphaltic compounds and thereby offset or even replace petroleum based compounds as their supply decreases.

As discussed above, identical or similar chemicals from each of the main classes of oil compounds can be produced in or from biological organisms. The difficulty in producing these compounds on a large scale is finding a way to do so without interfering with the production of other commodities especially food production as previously stated. As a result, the use of plants and animals to produce these oleochemical petroleum substitutes is inadequate for large-scale production. Another method, such as the production of these compounds in microbes, is necessary in order to avoid competition for arable land.

OLEOCHEMICALS FROM MICROBIAL FEEDSTOCKS

Although many compounds can be produced from animal and plant feedstocks, the limited amount of land available for oleochemical production using first generation biodiesel production limits large scale oleochemical production from these sources. Oleaginous microorganisms, microorganisms capable of producing greater than 20% of their cell dry weight in lipid compounds, represent a promising source of lipid based oleochemicals (*65*). These organisms can produce various oleochemical compounds

endogenously and many are genetically tactile allowing for unlimited possibilities in the types of compounds produced through genetic modifications.

The production of oleochemicals from microorganisms generally follows the pathway shown below (Figure 1-5) with the process starting at the production of a feedstock rich in the desired oleochemical. This step then proceeds through to a dried feedstock from which the desired oleochemical can be obtained. The utilization of traditional approaches for the extraction of the oleochemical as demonstrated by multiple researchers requires the removal almost all of the water from the feedstock biomass. This is an energy intensive process, requiring amounts of energy comparable to the amount of energy that can be obtained from the oleochemical if used as a fuel.

Of the steps shown in the figure below (Figure 1-5), the initial production of a cellular feedstock of a high cellular density, low percent water, and high biofuels precursor or oleochemical content are undoubtedly the most important steps as this initial feedstock greatly influences the effectiveness of each subsequent step. This process requires a suitable native or genetically modified microorganism capable of producing the desired biofuels precursor or oleochemical (*67*). Essentially, the higher the biofuels

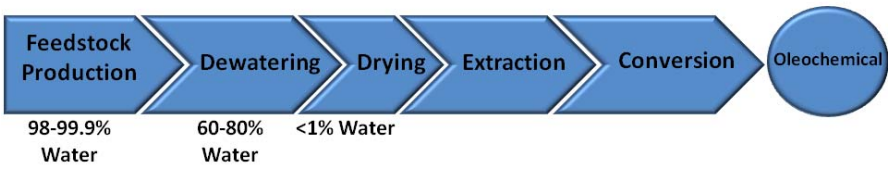

Figure 1-5. Traditional production scheme for oleochemicals obtained from microorganisms. Adapted from material found in (*66*).

precursor content or oleochemical content of the organism the more practical downstream processing becomes.

Work with oleochemical production from microorganisms began with the production of high-value pharmaceutical and nutraceutical compounds. Compounds such as citrate and beta-carotene were all produced first using microorganisms capable of producing the desired oleochemical compounds endogenously (*68–70*). After years of research into the production of these types of high value compounds endogenously, breakthroughs have been made in genetic and metabolic engineering greatly increasing the yield of these high value compounds (*71–73*). The high value of these compounds combined with the ability of many of these compounds to be excreted from the cells after production, has allowed for the high cost of the traditional process of growing, harvesting, and obtaining the oleochemicals to be economically feasible. This is not the case for lower value oleochemicals which are trapped within the cells and not excreted.

Increases in fossil fuel demand and cost over the past two decades have driven the field of microbial oleochemical production towards the production of compounds that behave similarly to fossil fuels. The production of oleochemicals from microorganisms in many cases requires some environmental trigger to cause production to begin. With microalgae it is generally accepted that a nutrient stress trigger, in the form of a nitrogen limitation, is required to initiate the production of the biodiesel precursor triacylglyceride (*74*). Similar endogenous triggers exist for other classes of microorganisms including yeast and bacteria (*75*). In the case of genetically modified organisms, the trigger for the

production of oleochemicals can be modified as well as the genes for oleochemical production.

Recently, researchers from Dr. Keasling's laboratory at UC Berkeley have demonstrated the utility of genetically modified organisms in oleochemical production. His team began by working with *E. coli*, genetically modifying the organism to produce biodiesel and wax ester compounds in vivo (*76*). This process required only the carbohydrates in the growth medium for oleochemical production, representing a breakthrough over earlier attempts which required the addition of exogenous fatty acid compounds to the medium for oleochemical production (*77*).

Keasling's group moved from work with *E. coli* to work with yeast cells. Yeast provided a platform capable of producing large amounts of oleochemical precursors i*n vivo*. Yeast also allowed for relatively fast genetic engineering and manipulation to produce large scale amounts of desired products. Other groups have worked with oleochemical production in yeast and have shown that the field is promising for future genetic research and manipulation for oleochemical production (*78*).

Regardless of the organism used and type of oleochemicals produced, another problem facing the field of microbial oleochemical production is the aquatic environment that the organisms grow in. Traditional methods for removing the oleochemicals depend on dewatering and drying the organisms. This is necessary because the majority of the oleochemical compounds are trapped within the cell membranes and surrounded by emulsifiers preventing efficient partitioning away from water (*79*). In order to efficiently

produce oleochemicals from microorganisms it is necessary to develop a method of extracting the oleochemicals from the microorganisms with minimal energy input.

EXTRACTION AND CONVERSION OF OLEOCHEMICALS FROM MICROBIAL FEEDSTOCKS

Once a high lipid content feedstock has been produced from microbial sources, the next step is to efficiently remove the lipids from the feedstock. This is traditionally done by first dewatering and then drying down the feedstock by a number of methods. These include centrifugation (*80*), dissolved air flotation (DAF) (*81*), belt drying (*82*), solar drying (*83*), lyophilization (*84*), and vacuum drying (*85*). Each of these methods have benefits and drawbacks, with some methods functioning more efficiently than others on a larger scale.

Dissolved air flotation (DAF) and belt drying are technologies originally developed for sewage treatment, but they have been adapted for use in harvesting algae that grow at low cellular densities in large volumes of water. DAF utilizes chemical coagulants such as alum or polymers that interact with the negatively charged cell membranes and cause them to clump together. A constant stream of fine air bubbles causes the clumped cells to float to the surface. This greatly concentrates the cells from greater than 99.9% water to <95 % water (*86*). The main difficulty with DAF is that this technology has not been demonstrated to work more efficiently on a large scale than other options currently available for harvesting. Another difficulty is that the coagulants used as a part of this process can interfere with extraction and conversion of oleochemicals as well as the use of extracted algal materials for animal feed purposes.

Belt drying utilizes filter membranes placed on a conveyer belt type system. Water is pumped onto the belt and heat and pressure are applied from a belt pressing down from above. The water is forced through the membrane leaving the resulting cell mass of up to 90% solids (10% water) behind. This method is relatively inexpensive to operate, however, a substantial capital cost is required making this process of a questionable value when tested at a larger scale (*87*).

Centrifugation is the most tried and tested of the traditional dewatering techniques. Centrifugation is currently used on large scale to remove solids from many industrial processes, including wine and beer production (*88*). Large, energy efficient centrifuges are available capable of processing thousands of gallons of liquid per hour, while concentrating the cells that are removed from the water stream to 90 to 80% water (10 to 20% solids). Of the water removal processes currently available for oleochemical producing cells, centrifugation is arguably the most reliable option for large scale harvest of microorganisms until further progress is made in increasing the large scale viability of DAF, belt drying, and other filtration techniques.

Once a cell mass is obtained by these dewatering methods, the remainder of the water must be removed to create a dry cell mass suitable for traditional extraction techniques. Lyophilization is used for lab scale water removal and allows for the perseveration of materials in their original state by keeping temperatures low. However, the energy required to remove the water in this manner is more than can be obtained from extracted microbial oils. Vacuum drying functions much like lyophilization, however, in this case heat is utilized to further speed the drying process. Unfortunately, the amount

of energy required to bring water from room temperature to a gas state away from the cellular material on a large scale deems these processes fairly energy expensive (*89*).

Solar drying still requires the same amount of energy to remove the water as other drying methods due to the state function nature of water evaporation, however in this case the energy may be obtained from sunlight. The main expenses with this method are the equipment costs to move the material around and the capital cost associated with green houses for drying the cellular mass. A major difficulty with this process is the product degradation that occurs with some oleochemicals over short periods of time, such as triacylglyceride, especially when working with algae (*90*, *91*).

Despite the costs of any drying method used to obtain cellular mass, an efficient traditional extraction process depends upon dried cell mass, that is cell mass that is >99% solids or less than 1 % water. Once cell mass has reached this level, three main types of extraction techniques can be used to remove the lipids; chemical extraction, mechanical extraction, and *in situ* transesterification.

Chemical extraction techniques are by far the most widely used for extracting oleochemicals from microbes. The most accepted method of total lipid extraction was published by Bligh and Dyer as a protocol for sampling total lipids in tissue samples (*92*). Other techniques similar to this, but varying in solvent ratios or types of solvents used have since been developed. All can be grouped under the classification of chemical extraction techniques, which can be further divided into three subclasses; namely, general, subcritical, and supercritical extractions.

In its general form, chemical solvent extractions involve the use of a solvent to extract lipids from a tissue sample with no special conditions applied. This may be done in combination with mixing of the solvent and sample by agitation (*93*), or in the case of the Soxhlet extraction, repeated exposure to a solvent wash (*94*). These types of methods are preferred for extracting total lipids from a particular sample. Though some small variation can occur in total lipids extracted depending on the solvent used (*95*), the generally accepted method when quantifying total lipids or comparing the efficiencies of extraction methods is the aforementioned Bligh and Dyer method (*92*).

Subcritical lipid extraction methods generally utilize a solvent system comprised of an organic solvent and an alcohol heated and pressurized to a level just before a critical point is reached. This allows for the organic solvent to assist in disrupting cell membranes and extracting cellular lipids into the solvent phase. This method requires the use of specialized equipment in order to reach elevated temperatures and pressures and so obtain the subcritical phase of the solvent system. Scaling up this equipment sufficiently to produce large quantities of oleochemicals would entail a high overhead cost. However, this method has been used to produce liter quantity amounts of algal oil from dry and even wet samples lending credence to its utility as an efficient extraction method (*96*).

Supercritical lipid extractions generally utilize inert compounds such as carbon dioxide that when combined with enough heat and pressure, reach a liquid phase that allows for miscibility with the lipid compounds contained in the feedstock materials (*97*). A large amount of research has been done into supercritical CO_2 extraction as its low

toxicity and utilization of an abundant waste gas are appealing (*98*). Though effective at extracting lipids from cells, this extraction method uses specialized equipment to attain increased temperatures and pressures and these have not adequately been demonstrated at large scale for lipid extraction.

Mechanical lipid extractions are most commonly used for oil seed extractions. These processes use heat and pressure to literally squeeze oil from the seeds. Mechanical extraction methods have been used for some types of microorganisms including algae, but the smaller cell size limits the effectiveness of the press at extracting oils. This resulting low extraction efficiency requires a post-processing solvent extraction to increase extraction efficiency to acceptable levels, making this process as a standalone somewhat inadequate for large scale oil extraction of microorganisms (*99*).

The process of combining mechanical extraction techniques with chemical extraction techniques has shown much promise for overcoming some of the weakness of both processes. A common method is to disrupt dried cells by bead milling, microwaving, sonication, grinding, or pressing, in combination with a solvent wash to remove lipids from lysed cells. This process has been used to great effect and is similar to the process used to extract oil from soybeans, as simple mechanical extraction is inefficient for this oil crop.

The most direct method of biofuels extraction is *in situ* transesterification. This method uses dried cells and combines the process of oil extraction with the final processing step of oil conversion. In this case the phospholipids, free fatty acids, and triacyglycerides within the cell are converted with high efficiency to fatty acid methyl

esters (FAME) using heat, sulfuric acid, and a large ratio of methanol to cell mass. The resulting FAME are readily separated from the mixture by a simple wash with solvent. This method has proven effective as a total lipid quantitation method at lab scale and for producing liter quantities of FAME for use in a biodiesel test engine (*100*).

One drawback that a majority of extraction methods have is their inability to extract lipids in the presence of water. As mentioned earlier, microbial cells grow in an aqueous environment of 98% to greater than 99.9% water. The removal of this water to a level compatible with traditional extraction methods requires the removal of 99% of water from the cells. As shown in the table below (Table 1-2), the energy cost for removing greater than 99% of the water from a cell culture with a density of 0.1% and cellular dry weight lipid content of 30% is approximately equal to the amount of energy available in the oil when standard harvesting and drying techniques are utilized.
To increase the ratio of energy output to energy input, ideally an extraction method would need to work in the presence of 80% or more of water. This process termed "wet cell lipid extraction", has been investigated by several researchers. The general conclusion has been that in order to efficiently extract lipids from wet cells, a solvent system which utilizes an alcohol with an organic solvent, must be used in conjunction with high temperatures and extraction time periods. This alcohol to solvent mixture is believed to allow for the penetration of the cellular membrane by both the solvent and alcohol in wet conditions resulting in the effective removal of lipids from the cells. The most effective extraction of this type was demonstrated with ethanol and hexane. To

Table 1-2. Energy balance for microbial oil production

Process	Energy Input[a]	Energy Input/Yield[b]
Evaporation/Dry Cell Extraction		
0.1% Solids (Evaporation, 30% DW Lipid)	7420 MJ	228.00
0.1% Solids (Evaporation, 60% DW Lipid)	3710 MJ	114.00
2% Solids (Evaporation, 60% DW Lipid)	184 MJ	5.60
20% Solids (Evaporation, 60% DW Lipid)	18.4 MJ	0.56
Pre-Concentration		
Continuous centrifugation [c] (0.1% - 20% Solids, 30% DW Lipid)	13.6 MJ	
Continuous centrifugation [c] (2% - 20% Solids, 60% DW Lipid)	340 kJ	

[a] Energy required to produce 1 L of oil using the described extraction method including water removal and solvent removal post extraction. A 100% extraction efficiency is assumed for the evaporation/Dry cell extraction scenarios. In the case of wet cell extractions, an efficiency of 85% is assumed. Energy consumed by mechanical mixing for extraction is assumed to be negligible.

[b] The ratio of the energy input to the energy output obtained from 1 L of oil (32500 kJ). A number larger than 1requires a larger energy input than energy yielded.

[c] Based on the energy consumption of a continuous centrifuge (Clara 80, Alfa Laval) and the amount of cell culture required to produce 1 L of oil. In the case of microalgae (30% lipid) 3,000 L of culture must be pre-concentrated (0.1% to 20% solids) prior to lipid extraction, for oleaginous yeast (2% solids, 60% lipid) just 76 L of culture (*101*).

accomplish this with cells that were 20% solids, heat and pressure was required, but greater than 88% of the extractable TAG was removed using this process (*96*).

Another process for wet extraction using the solvent dimethyl ether was developed with reported efficiencies of greater than 90%. This solvent is somewhat soluble in water and deomonstrates both cell penetration and lipid extraction abilities. The difficulty with this solvent is its separation from water after extraction (*102*). Little research has been done to date on wet cell lipid extraction using a single solvent that could easily be separated from water. A novel wet cell extraction technique capable of removing greater than 95% of the total TAG from cells in the presence of 80% water will be discussed in Chapter 4 of this dissertation.

The possibility of a highly efficient wet extraction technique would eliminate the need for the energy expensive process of drying. For those extracted oleochemical compounds that need further processing for use, including those used for biodiesel production, a few conversion methods are widely accepted. The accepted methods include an acid catalyzed and base catalyzed method for conversion of extracted oils to biodiesel.

The acid catalyzed method requires much longer processing times, 6- 8 hours, and has a low conversion efficiency, 60-80%. This method can be used on oils that have high free fatty acid content to prevent saponification of the fatty acids as would occur in the base catalyzed process (*103*). The base catalyzed method is most commonly used on a large scale and can reach efficiencies of greater than 90% in less than an hour of reaction time (*104*). The use of an effective wet extraction technique combined with a base

catalyzed process for biodiesel production would yield a net energy output that is not possible using more traditional extraction techniques.

TRIACYLGLYCERIDES

Triacylglycerides function as a common energy storage compound for many organisms throughout all the kingdoms of life. The triacyglyceride (TAG) molecule is composed of a glycerol backbone with three fatty acid chains esterified to the backbone as seen in Figure 1-6 below. These compounds can have heterogeneous or homogeneous fatty acid tails of various carbon chain lengths allowing for a large diversity in physical properties (*105*).

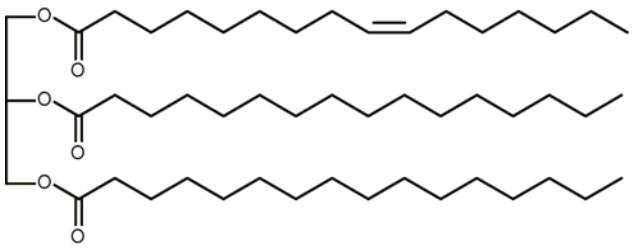

Figure 1-6. General structure of a triacylglyceride molecule**.**

In multi-cellular organisms, such as plants and animals, TAG are often stored in specific tissue and cell types known as adipose tissues and cells (*106*). These tissues function to regulate the amount and types of TAG that are stored within the organism. In single celled organisms TAG and other storage compounds are kept within the cells in lipid bodies (*107*, *108*). These lipid bodies are generally spherical droplets of lipid compounds that form within the cell. The lipid bodies often have specific proteins associated with them which are responsible for regulation of the TAG storage. These proteins form regulatory systems reminiscent of the more complex systems in multi-

cellular organisms (*109*). The production of TAG in higher animals and plants is well established (*110–113*). However, the understanding surrounding the regulation and production of TAG in microorganisms is lacking.

Microorganisms capable of producing greater than 20% of their cellular dry weight in TAG are known as oleaginous microorganisms. There are three main classes of oleaginous microbes: bacteria, microalgae, and unicellular fungi (yeast)(*65*). Some of the major details are known about the process of TAG production and its trigger in oleaginous microbes: however, reasons for differing levels of TAG production from one organism to another are poorly understood.

In yeast and bacteria the major pathways and trigger of TAG production are well understood as previously mentioned. The process begins with the limitation of nitrogen in the medium. As nitrogen in the medium is taken into the cells, extracellular concentrations of nitrogen become depleted. As cell proliferation continues, cytosolic nitrogen stores decrease leading to an increase in nitrogen scavenging activity within the cell. One of the most abundant sources of intracellular nitrogen is cyclic AMP. Cyclic AMP is broken down into IMP and ammonia by the action of AMP deaminase and results in decreased cyclic AMP levels in the cytosol and other organelles. Cyclic AMP is an allosteric activator of the TCA cycle enzyme isocitrate dehydrogenase. Without this activator in sufficient concentration, isocitrate dehydrogenase activity is inhibited, leading to increased levels of isocitrate and consequently citrate within the mitochondria (*114*).

Citrate is exported from the mitochondria by way of a membrane citrate transporter to the cytosol (*115*). Once in the cytosol citrate is cleaved by the enzyme ATP-citrate lyase, forming oxaloacetate and acetyl CoA. The resulting acetyl CoA is readily available for the production of fatty acids in the cytosol by fatty acid synthase enzymes (*116*). The fatty acids can then be esterified to the glycerol backbone through the Kennedy pathway, allowing for the formation of TAG (Figure 1-7) (*117*).

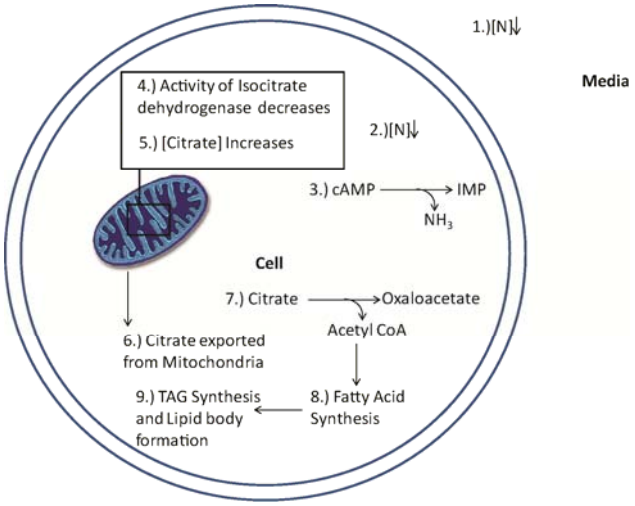

Figure 1-7. Diagram showing the trigger and pathway for TAG production from oleaginous yeast cells**.** Adapted from information found in (*114*)**.**

Although the major TAG production pathway is well established for bacteria and yeast, little is known about the same pathway in algae. Research into this pathway in algae is hindered by the difficulty of genetically manipulating algal organisms. Further research may help to determine more of the differences in algal lipid production and

could help to yield an organism capable of producing large amounts of cellular lipid comparable to those found in yeast.

Unlike other products produced endogenously by microorganisms, such as alcohols and citrate, TAG compounds are kept within lipid bodies or droplets in the cell as previously mentioned. These lipid bodies in some cases can function like organelles having proteins specifically found on the lipid body surface responsible for regulating the type and amount of TAG kept within the lipid body (*109*). It is the formation of these lipid bodies within the cells that necessitate the use of difficult extraction techniques for the removal of the oils form the cells.

FATTY ALCOHOLS

Fatty alcohols are compounds composed of a fatty acid chains ranging from 6 to 30+ carbons with a hydroxyl group attached to the terminal carbon as shown in the figure below (Figure 1-8). Fatty alcohols can have varying degrees of saturation, which stem from the fatty acid pool from which they are derived (*108*). Fatty alcohols are produced biologically in a wide variety of organisms including bacteria, plants, and animals (*118–122*). These compounds can also be produced from petroleum by the use of Ziegler and Oxo process (*123*). Fatty alcohols function in plants and animals as waterproofing and protective layers, such as is the case for plant cuticles (*124*) and sebaceous gland excretions in animals (*122*). In microbes, fatty alcohols function as precursors to wax compounds which are used for lipid storage (*125*).

Figure 1-8. General structure of a fatty alcohol molecule

Fatty alcohols are produced within cells by enzymes known as fatty acid reductases (FAR) or fatty acyl CoA reductases (FACoAR). FACoAR enzymes discussed in the literature are proposed to be closely related to one another by substrate usage and product formation. However, a wide variety of substrate specificities and activities have been reported for these enzymes (*118*, *119*, *126*, *127*). There are four major types of FACoAR enzymes discussed in the literature differing by products formed and substrates used. They include: NADPH-dependent medium chain FACoAR (*127*), NADPH-dependent long chain FACoAR(*118*, *126*), NADH-dependent FACoAR(*119*, *128*), and NADPH-dependent fatty acyl CoA /acyl ACP reductases (*56*).

The NADPH-dependent medium chain FACoAR enzymes are proposed to have activity similar to that of the *A. calcoaceticus* FACoAR (*127*). These types of enzymes have been shown to reduce the fatty acyl-CoA to an aldehyde *in vivo* and utilize a range of fatty acyl-CoA substrates with carbon lengths from 10-18 carbons. To date all bacterial FACoAR enzymes have fallen into this category (*125*, *127*, *129*). These enzymes are proposed to be used in bacterial systems ranging from luciferase activation (*129*) to wax ester production (*125*).

The NADPH-dependent long chain FACoAR are enzymes that function similarly to the FACoAR enzymes from the Jojoba and *Arabidopsis* plants (*118*, *126*). These enzymes require the use of NADPH as the medium chain reductases do, but have a

substrate specificity for fatty acyl-CoA compounds ranging from 20 to 32 carbons. Little to no activity is reported for carbon chains shorter than 20 carbons (*118*, *126*). These enzymes have been shown to reduce the fatty acyl-CoA to the corresponding alcohol *in vivo*. This is a 4 e^- reduction as opposed to the 2 e^- reduction performed by the known bacterial enzymes. These enzymes function in the production of the wax and alcohol components that make up the cuticle covering for the plant stems and leaves (*130*).

The NADH-dependent FACoAR are proposed to utilize NADH to reduce the fatty acyl-CoA. This type of enzyme has been isolated from *Euglena gracilis*, a protist, and the alga *Botryococcus braunii* (*119*, *128*, *131*). These enzymes reduce a fatty acyl - CoA to a fatty alcohol *in vivo* with an aldehyde intermediate formed that can be trapped by the addition of phenyl hydrazine as has been done by Kollatakudy and associates with the *E. gracilis* FaCoAR (*128*). These enzymes are believed to function in the wax ester production pathways for energy storage (*119*).

The most recently discovered type of FACoAR, the NADPH dependent fatty acyl-CoA / Acyl carrier protein (ACP) reductase, can reduce both acyl carrier protein bound fatty acids as well as the fatty acyl-CoA substrates to the respective aldehyde. These enzymes utilize both substrate types, but have a preference for the ACP bound substrates. This type of enzyme is believed to function in the alkane biosynthesis pathway of many cyanobacteria (*56*). Homologous FACoAR enzymes are found in many types of cyanobacteria and have been proposed by some researchers to exist in algae, plants, and mammals (*50*, *54*, *132*, *133*).

All four of these enzyme types have been studied to some extent, but have never been fully characterized for enzyme activity or substrate specificity. Furthermore, a bacterial FACoAR from *M. aquaeolei* VT8,which reduces a fatty acyl CoA to the fatty alcohol, does not fit into any of these four categories of enzyme activities and may constitute a new type of FaCoAR enzyme. The discovery of this novel FACoAR enzyme comprising a new enzyme type is discussed in Chapter 5 of this dissertation.

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CHAPTER 2

TRIACYLGLYCERIDE PRODUCTION IN THE MARINE DIATOM *CHAETOCEROS GRACILIS* GROWN IN BAG CULTURES AND RACEWAY PONDS WITHOUT NUTRIENT LIMITATION[1]

Abstract

Microalgae are widely promoted as a viable feedstock for the production of biofuels. Success in utilizing microalgae for biofuels will depend on developing strategies for the continuous and high production of the fuel precursor neutral lipids, specifically as TAG. Here, we report the continuous production of TAG (up to 30% (w/w) of dry weight) in the diatom *Chaetoceros gracilis* over extended periods (3 or more weeks) with semi-continuous harvesting in both 50 L bag reactors and as batch mode in 250 L raceways with no measured macronutrient limitations. The semi-continuous harvesting strategy allows extended production of TAG while minimizing costs associated with stationary phase growth required for classical nitrogen or phosphorous stress regiments currently utilized to induce lipid production in other algal strains.

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1. Introduction

Liquid transportation fuels derived from renewable feedstocks will play an ever increasing role in meeting future energy demands. Microalgae have been proposed as a potential feedstock for biofuels production, offering many advantages over traditional food crops, including significantly higher yields of biomass and neutral lipids per land area annually (Chisti, 2007; Hu et al., 2008).

Despite the potential promise, several substantial hurdles remain to be overcome before photosynthetic microbes such as cyanobacteria or microalgae (including diatoms) can be utilized on a commercial scale for the production of biofuels. These obstacles include *i*) the maintenance of a specific algae as the dominant species in outdoor cultures, *ii*) cost-effective solutions to address issues of harvest and separation of biomass from growth media, and *iii*) manipulation of culture conditions to maximize accumulation of neutral lipids (specifically TAG) that serve as the primary feedstock for biofuel production.

For most microalgae, a limiting macronutrient stress is requisite to trigger the increase in cellular accumulation of TAG. A typical growth strategy involves the culture of algae to high cell density, at which point a specific macronutrient (usually nitrogen or phosphorous) is removed or limited in the media, followed by a further incubation period to induce lipid production (Hu, 2004; Hu et al., 2008). During these periods of stress, the cell density of the culture does not typically increase (and in some cases may actually decrease), while cells divert their metabolic energy from growth related functions to lipid accumulation. A more ideal strategy for algal lipid production would be to identify an

organism and conditions that would allow continuous high lipid production in actively growing cells without requiring extended periods of nutrient limitations. This would allow for the harvest of cells continuously without the large cost of emptying, cleaning, and refilling the growth system or maintaining cultures for extended periods of time in a stationary growth phase to induce lipid formation.

Some microalgae species are reported to have very rapid doubling times, and are known to accumulate neutral lipids under an array of growth and stress conditions (Krichnavaruk et al., 2007; Parrish and Wangersky, 1990). Of these, *Chaetoceros gracilis,* a marine diatom that is used extensively by the aquaculture industry as an essential food for rearing all stages of bivalve mollusks, such as clams, mussels, and scallops, in addition to shrimp and a variety of fish (Krichnavaruk et al., 2007; Parrish and Wangersky, 1990) is a prime candidate for biodiesel production due to it's ability to accumulate large amounts of neutral lipids. Several studies of neutral lipid accumulation related to nutrient stress were reported for *C. gracilis* during growth in both small and large scale cage culture turbidostats under low light with high media exchange rates (Lombardi and Wangersky, 1991; Parrish and Wangersky, 1990). Additional studies of *Chaetoceros muelleri* have demonstrated large-scale production in both airlift photobioreactors (Krichnavaruk et al., 2007) and in vertical plate reactors (Zhang and Richmond, 2003), the latter being performed under full sunlight.

Herein, we describe a detailed study of the diatom *C. gracilis* (UTEX strain LB 2658) grown in 50 Liter bag bioreactors and small scale raceway ponds. As part of these studies, all cultures were maintained in sequential batch mode in a bag reactor or as a

single batch culture in a small raceway pond to determine the potential for long term and large scale growth, maintenance, and neutral lipid accumulation. The results of these experiments and a discussion of the implications of these studies and findings are presented in this chapter.

2. Materials and Methods

2.1 Growth media and conditions

A seed stock of the marine diatom *C. gracilis* (UTEX LB 2658) was maintained as 250 mL cultures in 500 mL baffled flasks under constant shaking at 140 rpm. Light was provided by a bank of fluorescent bulbs (T12) located overhead with approximately 180 μmol photons m^{-2} s^{-1} on a 14 hour light and 10 hour dark cycle. Seed stocks were transferred every 4 weeks to fresh media to maintain a unialgal culture. The diatom was grown on a simulated seawater media containing 18 g/L NaCl, 600 mg/L KCl, 1.3 g/L $MgSO_4 \bullet 7H_2O$, 100 mg/L $CaCl_2 \bullet 2H_2O$, 250 mg/L K_2HPO_4, 25 mg/L $CaSiO_3$, 150 mg/L $NaNO_3$, and 5 mg/L ferric ammonium citrate. Media was supplemented with 1 mL/L of a trace metals solution containing 600 mg/L $B(OH)_3$, 250 mg/L $MnCl_2 \bullet 4H_2O$, 20 mg/L $ZnCl_2$, 15 mg/L $CuCl_2 \bullet 2H_2O$, 15 mg/L $Na_2MoO_4 \bullet 2H_2O$, 15 mg/L $CoCl_2 \bullet 6H_2O$, 10 mg/L $NiCl_2 \bullet 6H_2O$, 2 mg/L V_2O_5, and 10 mg/L KBr. Innoculants for larger cultures were prepared in Celstir® flasks (Wheaton Science Products, Millville, NJ) containing 5 L of total media, and mixed at very slow speeds (~50 rpm). Air supplemented with carbon dioxide (1 % v/v) was provided through a stainless steel bubbler tube containing small pinholes. The cell culture flask was placed between two banks of fluorescent lights and

grown to a standard density (approximately 500 mg/L dry weight). This was then utilized to inoculate the cultures in the bags as described below.

2.2 Bag construction and conditions

Bags were constructed to contain 50 L of culture media. Bag material consisted of polyethylene (6 mil, 16 inch polybag rolls (U-Line S2942)) cut to 56 inch lengths. The bottoms of the bags were sealed with a heat press (impulse sealer) and the top was wrapped around a piece of polycarbonate with grooves provided to run silicone tubing into the bags. The polycarbonate spacer ran the width of the bag, so that no access to the internal volume of the bag was available other than through the silicone tubing. The bag was pressed against the polycarbonate spacer and held in place with gum rubber and a series of bolts attached to pieces of aluminum angle iron. Aeration was provided through a long stainless steel tube (1/4" diameter) containing a series of pinholes connected by silicone tubing to the air supply line. In temperature controlled experiments, an additional piece of stainless steel tube was provided as a long coil to act as a heat exchanger, and was connected to an external heating/cooling water bath. Air was provided to the bags at a flow of 2700 mL/min from an external oil-less air pump (GAST Model #DOA-P707-FB-4Z02Y) and was supplemented with carbon dioxide (1 % v/v). Cultures were started by combining 5 L of *C. gracilis* culture from Celstir® flasks with an equal volume of fresh media. The culture was then increased daily with 10 L aliquots of fresh media until a volume of 50 L was obtained. The culture was maintained at approximately this same 50 L volume for the duration of the experiment, by removing set volumes of media and replacing with fresh media.

2.3 Raceway growth conditions

Raceway growths were conducted using a 250 L capacity fiberglass raceway built by Separation Engineering (Escondido, CA). The raceway was pH controlled and mixed by a paddle wheel. Media was prepared as described in concentrated stocks, mixed with 20 L of UV treated reverse osmosis water and poured directly into the raceway. The volume was then brought to 200 L with UV treated reverse osmosis water. The raceway was inoculated with a 50 L culture from a bag reactor, as described above, bringing the final volume to 250 L. Final harvest was performed using a Sharples MT-41-23 continuous flow centrifuge (Sharples, New York City, NY).

2.4 Cell density and nutrient analysis

Culture density was routinely monitored by performing an absorbance scan of the culture in the range of 300 to 900 nm using a 1 cm pathlength quartz cuvette. A 1 to 5 L aliquot of the culture media was removed and the cells concentrated by centrifugation at 7000 g. The supernatant was analyzed for pH and used to track nitrate and phosphate levels using test strips (Hach Chemical, Loveland, CO) to confirm that the culture was not limited for either of these media components. Further analysis was conducted on the supernatant using a Quickchem 8500 analyzer (Lachat Instruments, Loveland, CO). To determine nutrient concentrations within the supernatant, samples were filtered through a 0.2 μm filter, and analyzed for total nitrogen, total phosphorous, and total silicon.

2.5 Lipid analysis

The wet cell paste was collected and frozen in 50 mL plastic Falcon tubes, then dried on a lyophilizer (Labconco Freezone 4.5L, Kansas City, MO) to determine residual

dry weight and prepare samples for lipid analysis. Neutral lipids from the samples were extracted using a solvent mixture containing equal volumes of chloroform, tetrahydrofuran (THF), and hexane, by removing a small sample of dry cells (200 mg) and extracting 3 times with 5 mL of the solvent mixture using a Branson 450 microtip sonicator (Danbury, CT), with a series of 5 second bursts to disrupt the cells. The cell debris was separated from the supernatant using a tabletop centrifuge (Fisher Scientific Model 228, Pittsburgh, PA), and the extract removed using a gas tight syringe with a long (6 inch) needle so as not to disturb the cell pellet. The extracts were combined, and brought to a final volume of 15 mL for quantitative analysis.

Gas chromatographic (GC) analysis was conducted using a Shimadzu GC-2010 (Shimadzu, Columbia, MD). A 1 mL aliquot of the extract was derivatized by adding 50 µL of *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) and allowing the solution to sit at room temperature for 30 minutes. Then a 1 µL sample was injected from each sample onto an Rtx-Biodiesel TG column (15 m x 0.32 mm ID with 0.1 µm film (Restek, Belfonte, PA)) and run using a temperature program of 60 ºC for 1 min, followed by a gradient of 10 ºC/min to 370 ºC and held at the final temperature for 6 min with a constant velocity of 50 cm/min with helium as the carrier gas. A set of standards composed of triolein, tripalmitin, and palmitic acid (Nu-chek Prep Inc., Elysian, MN) were prepared to quantify components of the lipids in *C. gracilis*. These standards covered a range between 0.1 and 1.0 mg/mL for the TAG with a minimum of a 5 point standard curve. Data analysis was performed using the GC solutions program (Shimadzu) and total areas were calculated and fit to a standard curve with R^2 values greater than

0.99. To analyze the fatty acid composition of the TAG, the sample was dried to remove any residual solvent, 1 mL of methanol and 18 µL of concentrated sulfuric acid was added, 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. The sample was then cooled rapidly, and approximately 1 mL of chloroform was added. The sample was washed twice with 3 mL of distilled water to remove the methanol and sulfuric acid. The chloroform layer (containing the fatty acid methyl esters) was then analyzed. Mass spectral analysis was conducted using a Shimadzu GCMS-QP2010S. A 1 µL sample was injected onto a Stabilwax-DA column (15 m x 0.25 mm ID with 0.1 µm film (Restek, Belfonte, PA)) and run using a temperature program of 100 ºC for 1 min, followed by a gradient of 10 ºC/min to 235 ºC and held at the final temperature for 10 min with a constant velocity of 50 cm/min with helium as the carrier gas. Spectra obtained were compared to the National Institute of Standards and Technology (NIST) database 05 for compound identification.

3. Results

3.1 Properties of neutral lipids obtained from C. gracilis

Neutral lipids were extracted from dried cells using the ternary solvent system described, resulting in an extract consisting primarily of TAG, sterols, and free fatty acids (FFA). This solvent system was selected in part because it allowed for the further

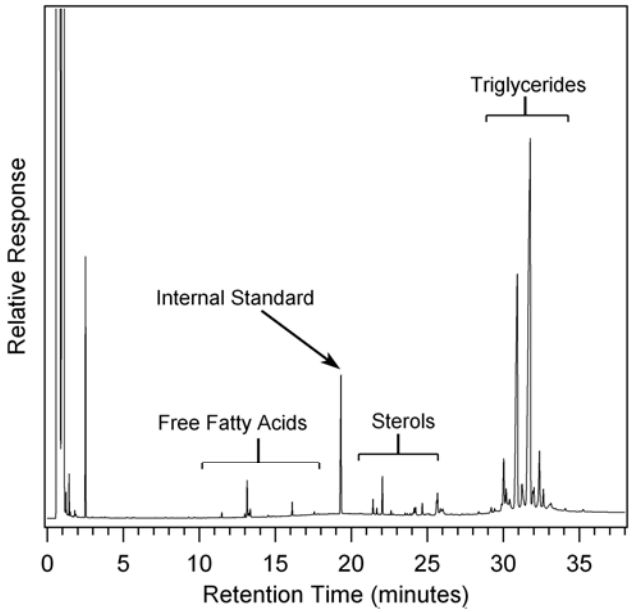

Figure 2-1. Chromatogram of neutral lipids extracted from *C. gracilis***.** Shown is a gas chromatogram of the hydrocarbons extracted using the protocol for isolation of neutral lipids described in the materials and methods section. TAG elute from the column between 29 and 35 minutes. An internal standard is included (at approximately 19 minutes) for sample quality control and assurance. Derivatized fatty acids elute between 12 and 18 minutes, while sterols elute between 20 and 26 minutes. Peaks were confirmed by comparison to external standards and by separate analysis using a mass spectrometer.

derivatization of FFA, which improved peak resolution and quantification of the FFA. Using a high temperature column and oven profile, TAG could be resolved and quantified directly (Figure 2-1), similar to an approach taken by Lombardi with an Iatroscan (Lombardi and Wangersky, 1991). This approach was preferred to other lipid quantification techniques that monitor lipid indirectly. In addition to the GC method, the various components could be separated by thin layer chromatography (TLC), or the

solvent could be removed from the extract, and the extract further treated to produce fatty acid methyl esters (FAME) for analysis by mass spectrometry as shown in Figure 2-2.

The fatty acid composition of the TAG fraction of *C. gracilis* did not vary appreciably throughout these studies. The predominant fatty acids found were C16:0, C16:1, C14:0 and C20:5. The analysis uncovered only minor amounts of C18 fatty acids (saturated or unsaturated). These findings are similar to those reported for other *Chaetoceros* species previously (Renaud et al., 2002), and are similar to TAG

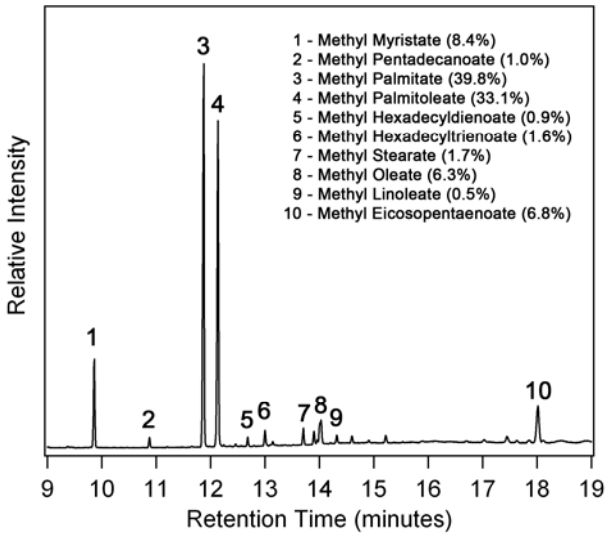

Figure 2-2. Fatty acid methyl ester profile of TAG produced by *C. gracilis***.** Shown is a gas chromatogram of the TAG derived FAME converted using a standard acid methyl transesterification protocol as described in the materials and methods section. The peaks identified by this method are labeled, showing a predominance of C16:0 (methyl palmitate), C16:1 (methyl palmitoleate) and C14:0 (methyl myristate) as the major components of the fatty acids accumulated in the neutral lipids from this species.

compositions reported for several other diatom species (Alonso et al., 2000; Anderson et al., 1978). The results obtained for the TAG determination were used as the primary indication of neutral lipid accumulation for the remainder of the comparisons provided, and polar lipids are not accounted for or considered in this approach.

3.2 Growth of C. gracilis in 50 L bag cultures

The primary objective of these studies was to determine the potential to grow the marine diatom *C. gracilis* in bag cultures and raceway ponds to produce high yields of TAG. This strain was selected for these trials based on a consistent TAG content found during preliminary studies of a variety of strains of green algae and diatoms. While *C. gracilis* did not produce the highest content of TAG of the algae screened in the initial studies, it did produce a reasonable and consistent amount of TAG in virtually every growth and analysis performed. Additionally, *C. gracilis* did not appear to require the long periods of nutrient deprivation to initiate accumulation of TAG, as compared to a number of green algae strains tested.

During the initial growths performed with *C. gracilis*, bags containing 50 L of culture were maintained for periods exceeding 60 days, following a regimen where a portion of the culture was removed and replaced with fresh media every 2 or 3 days to determine an adequate sampling schedule for the more stringent experiments presented here, confirming the potential to maintain this diatom as a unialgal culture in a simple bag system for extended periods of time with limited environmental controls. During the initial growths in bags, the cultures were monitored for pH, biomass dry weight and percent neutral lipids (as TAG). Adjustments of pH or temperature were not regularly

made as part of the initial studies, so that the robustness of the strain could be tested under these initial settings with the media selected. While efforts were made to further induce lipid accumulation during later weeks of the growth by limiting specific nutrients, the most significant amounts of TAG were found during the first few weeks of growth of the culture, when neither nitrogen nor phosphate appeared to be limiting growth. Studies were then undertaken to track neutral lipids and culture nutrients with more rigorous sampling and environmental management, as described below.

In the primary bag studies presented here (Figure 2-3), cultures were scaled up from 5 L culture flasks to 50 L bags as described in the material and methods over the course of 5 successive days. While the culture was not axenic, the culture was monitored by microscopy to look for contamination by green algae or cyanobacteria over the course of the growth. Contamination by bacteria could not be avoided, as the strain obtained from the culture collection is not a pure culture, even in the seed stock, and attempts to clean the strain have been unsuccessful, possibly due to a requisite association between this diatom and a specific bacterium.

In general, the density of the cultures reached a final concentration of approximately 0.6 g dry mass/L of culture in this particular bag design, though higher densities (as much as 1.5 g/L) have been obtained by ourselves using plate reactor systems or reactors with more surface area for light penetration, similar to previous reports for other *Chaetoceros* strains (Zhang and Richmond, 2003). During the months of April and May, temperature control was achieved by maintaining a constant air temperature in a greenhouse with cell densities being maintained in the range of 0.6 g/L

dry weight (data not shown). During the summer months, internal cooling, as described in Materials and Methods, was applied to keep the culture from getting too hot, with densities between 0.3 to 0.6 g/L dry weight being maintained.

3.3 Biomass production and nutrient utilization in C. gracilis

There have been many reports related to lipid production in various green algae that describe a need to stress cultures by depriving them of critical nutrients (typically nitrogen or phosphorus) to induce the production of neutral lipids (Gouveia et al., 2009; Hu, 2004; Hu et al., 2008; Li et al., 2008; Spoehr and Milner, 1949). We have observed similar results for a number of green algae grown in our laboratory with other strains (including *Chlorella*, *Scenedesmus*, and *Nannochloris* species), where the percentages of TAG during the early log phases of growth is very low (below detection in some species). However, for some species of diatoms, such as *C. gracilis*, a different phenomenon was observed.

During the continuous bag growth studies presented here, each bag was monitored daily for several parameters, and cultures were prepared and operated in a sequential batch growth mode, where cells were removed and the bag was replenished daily with fresh media, replacing 10% of the culture volume, similar to the culture management approach reported for vertical plate reactors (Zhang and Richmond, 2003). The results for one specific bag are presented in Figure 2-3.

In the studies with *C. gracilis* presented here, even during the first several days when the culture was growing in log phase with significant daily dilutions (of as much as 50%), a considerable percentage of TAG was obtained from the cells. While it is not possible to

say that this organism was not limited for specific micronutrients, it is clear from this analysis that *C. gracilis* was never stressed for nitrogen or phosphorus (Figure 2-3, bottom panel). Silicon was provided as silicate to these cultures at 200 μM concentrations (5.6 mg/L total silicon) similar to those reported by others (Zhang and Richmond, 2003). The silicon concentration selected was based on silicon nutrient dependence experiments performed in our laboratory as preliminary work which revealed that concentrations of silicate between 100 and 400 μM resulted in optimal cell densities for *C. gracilis*, and concentrations greater than 500 μM resulted in a decrease in cell densities. Similar amounts of silicate were added in the bag and raceway studies detailed here. Furthermore, the analysis of total silicon (Figures 2-3 and 2-4 bottom panels) revealed that available silicon measured from the collected supernatant had total silicon levels between 0.5 and 3 mg/L total silicon (~18 to 107 μM) throughout the experiments, and never showed signs of depletion below detection in any of the experiments tested.

3.4 Neutral lipid production in C. gracilis

A primary interest in these studies was to track the percentage of neutral lipid (as TAG) during the growth of *C. gracilis* in 50 L bags to evaluate the potential of this species as a feedstock for biofuels. Bag cultures were found to contain significant amounts of TAG (10-15% of dry weight), even during the log phase of the growth cycle. In addition to TAG, FFAs were also found, but at much lower percentages ranging from 0.5-2% of the dry weight. The percentage of TAG remained relatively constant between 10-20% of the dry weight mass for the first few weeks of culture with semi-continuous

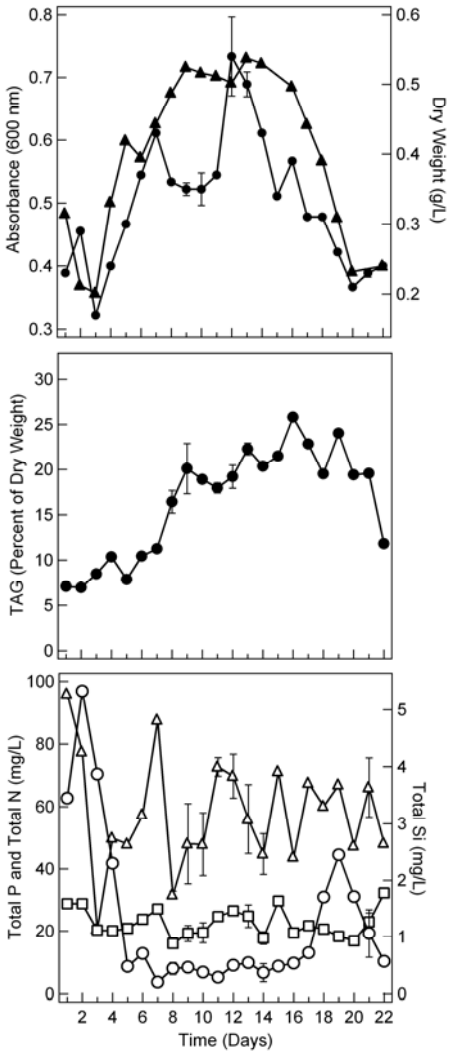

Figure 2-3. Growth parameters from a specific *C. gracilis* bag study**.** The top panel shows the measured absorbance taken at 600 nm (▲) and the dry weight (●) plotted against the time after inoculation for one specific bag. The middle panel shows the measured TAG as a percent of dry weight (●). The bottom panel shows the amounts of total nitrogen (△), total phosphorus (□) and total silicon (○). This bag reactor was operated in a sequential batch manner, as described in the materials and methods. This result is typical of what is seen for multiple bag reactors.

harvesting, but gradually dropped down to less than 3% after nearly 2 months time in longer studies (data not shown), indicating that some factor related to culture age or environmental conditions had led to a decrease in lipid accumulation, while cell density remained relatively constant. This finding for *C. gracilis* is in contrast to the general observation that neutral lipid accumulation in algae primarily occurs during the later phases of the growth cycle due to nutrient stress.

The percent TAG reached a higher value in cultures grown outside in direct sunlight compared to cultures grown inside a greenhouse, which reached up to 16% TAG. The outdoor bag culture reached more than 25% TAG (Figure 2-3, middle panel) during the course of this experiment, which does not account for polar lipids present in the cell membranes. Several other factors were explored during these experiments, including whether the TAG percentages deviated significantly throughout the course of the day. Since cells were harvested at different times each day, this was of particular interest. Samples drawn in the early morning, early afternoon and late afternoon were harvested and immediately frozen on the same day, to see if shifts were seen throughout the day. These results showed little (less than 10%) difference between the samples, indicating that levels of TAG were not fluctuating significantly on shorter (hourly) time scales for *C. gracilis* under these conditions.

3.5 Growth of C. gracilis in a raceway pond

Following a series of growths of *C. gracilis* in bags, an experiment was prepared to utilize a 50 L bag culture to inoculate a small raceway (250 L) to compare and contrast the growth in a larger system grown in batch mode, and to determine the potential for the

culture to remain unialgal without engineering controls to minimize contamination. The raceway growth was conducted for 11 days with daily sampling as described for large cultures in the Materials and Methods section. The growth steadily increased for the first six days following inoculation, before entering a log phase of growth the last 5 days with a high correlation between absorbance and dry weight as shown in the top panel of Figure 2-4. The TAG content also steadily increased with no apparent nutrient deficiencies for any of the nutrients monitored (Figure 4-2, lower panel). The growth was halted after 11 days due to signs of contamination by a possible cyanobacterium that was detected in daily analysis of cell culture. The final harvest of the raceway culture yielded 0.7 g dry biomass/L, and contained 29% (w/w) TAG, similar to the results obtained growing *C. gracilis* in bags. Importantly, and similar to the findings in the bag studies, the highest rate of growth was seen between days 7 and 11, which paralleled the primary accumulation of TAG, indicating that the cells did not require an extended stationary growth period after nutrient depletion to initiate the accumulation of TAG in *C. gracilis* under these conditions, and were accumulating significant quantities of TAG during log phase of growth.

4. Discussion

In this chapter, we describe specific studies aimed to demonstrate the continuous unialgal culture of the marine diatom *C. gracilis* (UTEX LB 2658) under conditions that produced significant percentages of TAG without the generally required macronutrient limitation regimen required to induce stress conditions and lipid accumulation in other species of microalgae. Preliminary experiments as part of a broad screening process

revealed that *C. gracilis* yielded significant quantities of TAG among a variety of green algae and diatoms tested, similar to the findings from a recent screening protocol reported by Araujo et al (Araujo et al., 2011), and our initial studies in 50 L bags revealed that this strain could be maintained as long as 60 days in bag reactors without significant contamination, an aspect that is important for commercial production and a necessary requirement for the detailed analysis of TAG accumulation over extended periods of time.

The results for TAG accumulation in *C. gracilis* (Figures 2-3 and 2-4) demonstrate that *C. gracilis* produces high quantities of TAG even when the primary macronutrients phosphorus, nitrogen or silicon were available at sufficient levels to sustain log phase growth. Our results also demonstrated that *C. gracilis* continued to accumulate lipids even during the exponential growth phase of the culture in raceway experiments, and were maintained for extended periods of time in bags. The TAG levels only began to drop after 20 days in bag studies as the culture eventually declined as a result of parameters independent of any changes in the three key macronutrients tracked in the culture. This affect is attributed to a likely aging issue within the culture, as it occurred in each of the extended bag growths that were performed as part of this work.

The primary drawback found in culturing this specific strain of *C. gracilis* as part of these studies is related to overall cell densities that were obtained in larger cultures. Higher cell yields have been reported for other strains of *Chaetoceros* grown in alternative bioreactor systems. However, the goal of these studies was to test the growth and lipid production of this specific strain of *C. gracilis* which was found to produce

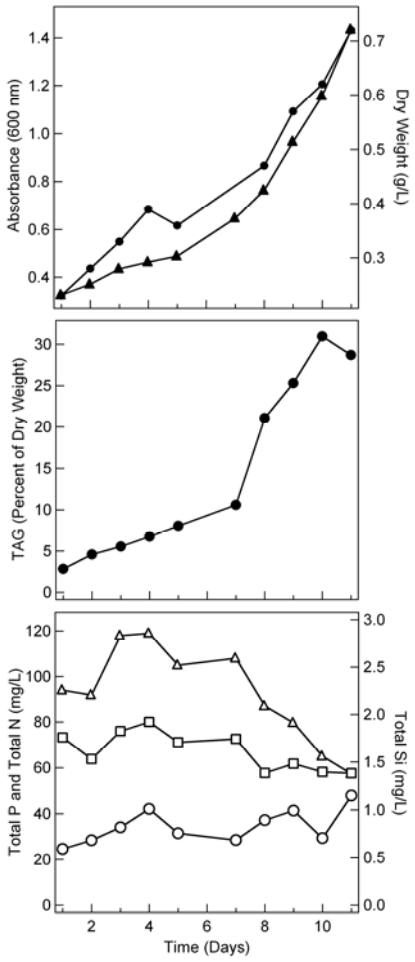

Figure 2-4. Growth parameters of *C. gracilis* raceway growth. The top panel shows the measured absorbance taken at 600 nm (▲) and the dry weight (●) plotted against the time after inoculation for an open raceway experiment. The middle panel shows the measured TAG as a percent of dry weight (●). The bottom panel shows the amounts of total nitrogen (△), total total phosphorus (□) and total silicon (○). This culture was run in a full batch mode. A 50 L bag of inoculum was added to 200 L of media to initiate culture growth. A 1 L sample was taken daily and the water level was maintained by the addition of UV treated reverse osmosis water to compensate for evaporation with no additional nutrients added.

higher levels of TAG than other *Chaetoceros* or green algal strains in our own algal strain collection. While others have reported higher cell mass yields for *Chaetoceros* (Krichnavaruk et al., 2007; Zhang and Richmond, 2003), they did not directly track TAG levels over the course of their growth or the cultures were grown under conditions with extensive exchange of culture media (Lombardi and Wangersky, 1991) using approaches that might prove difficult to achieve in simple mass culture systems such as raceways. It is possible that further studies could improve cell densities in the future with this specific strain, or that other factors such as photoinhibition limited cell growth or that light limitation could be responsible for the induction of lipids. However, cultures grown under lower light intensities with greater culture surface area resulted in only slight improvements in cell density as part of supporting experiments with more complex bioreactor systems. TAG accumulation in this strain was found under every growth condition tested for this species, regardless of overall illumination levels.

Successful commercialization of any algal strain for biofuels will require significant production of lipids, and specifically the production of neutral lipids such as TAG, which are easier to convert to transportation fuels than polar lipids obtained directly from cell membranes. While others have reported lipid yields of 50% or higher for some algal strains, such reports should be carefully considered in the context of the methods used to quantify the lipids. The determination of lipid content in algae can be accomplished through a range of techniques. Many laboratories quantify lipids by extracting dried cells with specific solvents, and then removing the solvent to gravimetrically determine the remaining material mass, similar to the techniques used by

Bligh and Dyer (Araujo et al., 2011; Bligh and Dyer, 1959; Li et al., 2008). While a broadly accepted technique, simplified gravimetric methods do not provide the identity of the individual lipid component classes, and could be subject to errors from other solvent extractable components, including sterols, FFAs, chlorophyll and other non-polar cellular components that may not contribute to optimal fuel blends and do not necessarily constitute convertible polar lipids in the same manner as TAG. These gravimetric methods might also measure portions of the polar lipid fraction derived from cellular membranes, which may prove difficult to convert to biofuels using conventional processes.

To fully characterize neutral lipid accumulation in *C. gracilis*, an accurate and reproducible method of quantifying specific lipids was established as part of this work, with a focus on the direct quantification of TAG using a high-temperature GC method. This provided results similar to more precise approaches taken by others (Lombardi and Wangersky, 1991). The method used here can discriminate between FFA, TAG and sterols, but did not measure the polar lipid fraction, which could further increase the overall levels of total lipid obtained, yielding higher percent lipid results more similar to those reported by laboratories using an indiscriminating method based on gravimetric analysis of total extractable lipid. Indeed, when a method was utilized that is capable of converting polar lipids in addition to neutral lipids, yields using this strain of *Chaetoceros* were higher than could be accounted from just the TAG component quantified here (Wahlen et al., 2011).

Alternative lipid quantitation techniques have been reported that utilize special lipophilic dyes such as Nile Red, which can provide relative numbers related to neutral lipid accumulation through fluorescence assays (Chen et al., 2009; Cooksey et al., 1987). While Nile Red protocols provide a rapid result and are potentially more specific for neutral lipids than the simple gravimetric methods, precise quantification of TAG requires direct methods that separate and quantify the TAG component. These methods are similar to the approaches used by (Gardner et al., In Press) to validate the results obtained by Nile Red using a direct GC method.

The lipid fraction obtained from *C. gracilis* consisted primarily of C14:0, C16:0 and C16:1 fatty acids, with minor contributions from C18:1 and C20:5 fatty acids. This lipid profile is similar to those found for other diatoms tested in our laboratory, and is similar to other reports in the literature (Alonso et al., 2000; Anderson et al., 1978; Renaud et al., 2002). It should be noted that the lipid profile reported here was obtained for the TAG and FFA, and does not account for other lipids that may be present in the polar lipid fraction derived from the membranes of these diatoms. While high levels of FFA present in the lipid fraction could be of concern for traditional base catalyzed conversions of TAG to biodiesel, acid catalyzed methods are well suited for generating biodiesel from mixed feedstocks high in FFA (Wahlen et al., 2008) resulting in near complete conversion of both the TAG and FFA components, as has been reported using biomass obtained from these experiments (Wahlen et al., 2011). The suitability of this mixture of fatty acid methyl esters (FAME) as a biodiesel alternative will be the focus of future experiments.

The raceway experiments pursued during this work aimed to evaluate whether diatoms such as *C. gracilis* might serve as a potential candidate for large-scale production of algal biofuels in open ponds. The primary benefit of utilizing this strain over green algae relates to the lack of a required lipid accumulation phase during growth, since TAG accumulated in these studies even during the log phase growth, and consistently yielded a significant quantity of TAG in each culture grown. This is important from an economic standpoint, as cultures would not require extended dormant periods where the total biomass accumulation is essentially stationary during TAG accumulation required by other strains to achieve significant lipid accumulation, encumbering land area that could not be utilized for the production of additional biomass during the stress induced lipid accumulation. The culture density in the open raceway system did achieve a dry weight comparable to that achieved in bags, and the percentage of TAG in the raceway culture was actually higher than that obtained in the bags, prior to harvest of *C. gracilis* at the culmination of the raceway growth. These results indicate a strong potential for the growth of *C. gracilis* as a strain in outdoor mass cultures, albeit under stringent growth regimen to minimize contamination.

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CHAPTER 3

EFFECT OF LIGHT INTENSITY AND WAVELENGTH ON THE GROWTH AND LIPID PRODUCTION OF FOUR SPECIES OF OLEAGINOUS MICROALGAE

Abstract

Microalgae comprise a diverse group of organisms which have the potential to become valuable sources of pharmaceuticals, fuels, feed additives, and food additives. The use of microalgae for biofuels production has become a high point of interest in the last decade. However, the cost effective production of biofuels from microalgae is met with many difficulties. Of these difficulties, the low conversion efficiency of sunlight to biofuels precursor compounds is a major hurdle to be overcome. To work toward accomplishing this, a set of experiments was devised and conducted to determine the effect of both the intensity and wavelength of light on microalgal growth and transesterifiable lipid production. The results of these experiments have further demonstrated the diversity of microalgae with all four strains utilized reacting differently to the experimental parameters. These experiments demonstrate that in the case of *C. gracilis,* high light intensity leads to the increased production of transesterifiable lipids. As well, wavelengths in the 500-600 nm range are not required for efficient growth of all of the microalgae tested, allowing for wavelengths of light in this range to be utilized for other purposes, such as photovoltaics.

1. Introduction

Microalgae represent a highly diverse group of microorganisms capable of producing high value products solely from carbon dioxide and sunlight (Mata et al., 2010). It is this characteristic in particular that has brought these organisms to the forefront of biofuels research, as their ability to photosynthetically produce biofuels precursors from carbon dioxide may represent a truly carbon neutral fuel source (Brennan and Owende, 2010). However, much like the first and second generations of biofuels that preceded it (Naik et al., 2010), many hurdles remain to be overcome before the economically viable production of algal biofuels can become a reality (Gallagher, 2011).

Despite difficulties evident in the harvesting (Shelef et al., 1984), extracting (Demirbas, 2008), and converting of biofuels precursors into biodiesel and similar compounds (Demirbas and Fatih Demirbas, 2011), arguably the most challenging step of algal biofuels production is the obtaining of an initial feedstock of algae with sufficient cell density and lipid content to facilitate an affordable downstream process (Hu et al., 2008). This difficulty is due in part to the inefficiency of the algal organism in capturing and converting sunlight and carbon dioxide into the desirable lipid compounds.

Some studies have looked into the wavelength utilization of various strains of algae (Brody and Emerson, 1959; Kaushik and Kumar, 1970; Wallen and Geen, 1971), and some reports have hinted at a positive effect of light intensity on the growth of specific algal organisms contrary to the bleaching effect normally seen (Orcutt and Patterson, 1974; Renaud et al., 1991), but an in depth study of the effects of light intensity and wavelength on algal growth and lipid production over a wide variety of organisms has not been attempted to date.

This chapter seeks to overcome this knowledge gap by determining the effect of light intensity and wavelength utilization of four varied strains of algae. The ultimate goal of this research is to determine how to optimize the photosynthetic efficiency of these algal organisms and to find which wavelengths of light are necessary for algal growth and lipid production. This in turn would allow for the use of un-necessary wavelengths of light for the production of electricity by photovoltaic cells which could offset some of the costs associated with the production of biofuels from microalgae.

2. Materials and methods

2.1. Light reactor construction

A spectral light reactor was constructed with 14 columns of solux 47K/36º BEAM 12V 50 W lamps (Solux Eiko, Japan) with 12 lamps per column (Figure 3-1). Each column of lamps was spaced evenly so as to be centered with a 1.2 L glass reactor tube with the final panel dimension being 24 inches by 44 inches. A total of 14 tube holders

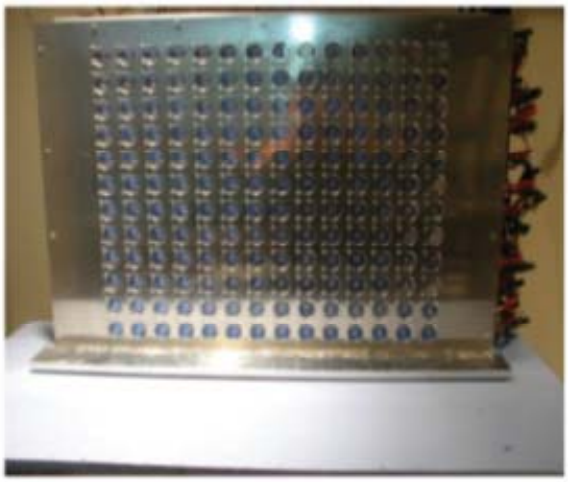

Figure 3-1. Constructed light plate for photosynthetic growth reactor.

were placed in a water bath in front of the rows and columns of lamps, however, the two end positions, 1 and 14, were not used as the photosynthetic photon flux at these positions was found to be much lower than that of tube positions 2-13. High heat generated by the lamps was dissipated by fans installed around the reactors to constantly stream away hot air. To keep cultures at ideal growth temperatures, the 1.2 L glass reactor tubes were placed in a water bath composed of 3/8 inch plate glass with dimensions of 44 inches x 24 inches x 4 inches with plates sealed to each other by the use of silicon caulking. The water bath was temperature controlled by the addition of stainless steel cooling coils inserted at the bottom of the water bath and an in-line coolant refrigerator model 13271-212 (VWR, Radnor, PA, USA) constantly circulating a 50/50 mix of water and propylene glycol (Prestone, Danbury, CT, USA) through the coils. Further temperature control was achieved by utilizing an Isotemp 2150 in tank coil heater (Fischer, Hampton, NH, USA) to balance the cooling effect when lights were not in operation.

Reactor tubes were handmade in house from scientific grade tubular glass and were capped with silicon stoppers with four separate holes for the insertion of a gas line needle, sampling needle, a pH probe, and an exhaust port. Sampling needles (Popper & Sons, New Hyde Park, NY, USA) extended 12 inches into the tube and were topped with a Luer Lock attachment for ease of removing samples with a sterile syringe. Gas line needles (Popper & Sons, New Hyde Park, NY, USA) extended the entire length of the tube (approximately 36 inches) and were fitted at the top with 3/8 inch silicon tubing (Cole-Parmer, Vernon Hills, IL, USA) which connected to an air regulator that supplied

air flow at 1 L/min from house air filtered through a 0.25 μm filter (GE Health Sciences, Piscataway, NJ, USA). At intervals triggered by increasing pH carbon dioxide was mixed with house air from a separate regulator at 1% concentration at an in-line joint.

A narrow tipped pH probe (Cole-Parmer, Vernon Hills, IL, USA) was inserted into the pH probe port on the cap and extended 8 inches into the culture. pH probes were sterilized by washing for 10 min in 95% ethanol and air drying before insertion into culture. pH probes were connected to a controller box (Hanna Instruments, Smithfield, RI, USA) which was set to a specific pH depending on the organisms optimal pH described in the growth section. If the pH increased above the set pH, a solenoid was activated allowing for the carbon dioxide to flow into the tube at the preset 1% concentration.

2.2. Growth of inoculum cultures

All inoculum cultures were grown with 250 mL volumes in 500 mL baffled flasks (Chemglass, Vineland, NJ, USA) with shaking at 250 rpm on an Innovo 2100 shaker table (New Brunswick Scientific, Edison, NJ, USA) under exposure to 300 μmol photons m^{-2} s^{-1} of light provided by two fluorescent lamps above the table. Unless otherwise stated, media was autoclaved at 121 ºC for 45 minutes, mixed as described below by aliquoting stocks into sterile flasks, and this was followed by re-capping the flask with a loose fitting polypropylene cap (VWR, Randor, PA, USA). All media components were purchased through Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Media preparation was performed by first autoclaving stock solutions of micronutrients, major components, a 100x potassium phosphate dibasic solution, 100x

potassium phosphate monobasic, and 100x sodium metasilicate nonahydrate solution in separate containers. The components were then added to a sterile flask as described above in the appropriate proportions, exe. 2.5 mL of a 100x solution for a total of 250 mL volume. Final pH was adjusted by the addition of sterile 1M sodium hydroxide or 1 M hydrochloric acid to 7.5 for *Chaetoceros gracilis*, *Neochloris oleoabundans*, and *Scenedesmus dimorphus*. The media for *Synechococcus elongatus* was adjusted to a pH of 8.0 after media mixing.

Chaetoceros gracilis growth media was a modified broad seawater media composed of 18 g/L of sodium chloride, 0.25 g/L potassium phosphate dibasic, 0.07 g/L sodium metasilicate nonahydrate, 0.85 g/L sodium nitrate, 1.3 g/L magnesium sulfate heptahydrate, 0.1 g/L calcium chloride dehydrate, 0.6 g/L potassium chloride, 0.015 g/L ferric ammonium citrate and supplemented with 1 mL of micronutrient solution per 1 L of final media composed of 0.6 mg/L boric acid, 0.25 mg/L Manganese chloride tetrahydrate, 0.041 mg/L of zinc sulfate heptahydrate, 0.079 mg/L copper sulfate pentahydrate, 0.015 mg/L cobalt chloride hexahydrate, 0.01 mg/L nickel chloride hexahydrate, 0.002 mg/L vanadium pentoxide, 0.01 mg/L potassium bromide, and 0.039 mg/L of ammonium molybdate pentahydrate.

Neochloris oleoabundans and *Scenedesmus dimorphus* growth media was composed of 0.05 g/L of sodium chloride, 0.15 g/L potassium phosphate dibasic, 0.35 g/L potassium phosphate monobasic, 0.85 g/L sodium nitrate, 0.15 g/L magnesium sulfate heptahydrate, 0.05 g/L calcium chloride dehydrate, 0.015 g/L ferric ammonium citrate and supplemented with 1 mL of micronutrient solution per 1 L of final media

composed of 2.86 mg/L boric acid, 1.81 mg/L manganese chloride tetrahydrate, 0.22 mg/L of zinc sulfate heptahydrate, 0.079 mg/L copper sulfate pentahydrate, and 0.039 mg/L of ammonium molybdate pentahydrate.

Synechococcus elongatus growth media was composed of BG-11 media which includes 1.5 g/L sodium nitrate, 0.075 g/L magnesium sulfate heptadhydrate, 0.06 g/L ferric ammonium citrate, 0.036 g/L calcium chloride dehydrate, 0.006 g/L citric acid, 0.001g/L sodium EDTA, 0.03 g/L potassium phosphate dibasic, 0.02 g/L sodium carbonate and supplemented with 1mL of a trace metals solution composed of 2.86 mg/L Boric acid, 1.81 mg/L manganese chloride tetrahydrate, 0.222 mg/L zinc sulfate tetrahydrate, 0.079 mg/L copper sulfate pentahydrate, 0.05 mg/L cobalt nitrate.

2.3 Growth and monitoring of cultures in reactor

Reactor cultures were put together in the same manner as the innoculum cultures. The reactor tubes were assembled with the exception of the pH probe with all ports capped with aluminum foil. The stopper was fastened to the tube by the use of autoclave tape and the tubes were autoclaved for 45 min on a dry cycle. Prior to autoclaving all tubes were filled with water to 1.2 L and a permanent mark was made at the bottom of the 1.2 L meniscus before being drained to facilitate later media filling.

After autoclaving the reactor tubes were placed one at a time in the water bath of the light reactor. The tubes were filled with sterile media with all necessary ingredients added as for the inoculum growths. Care was taken to keep the lid on the tube as much as possible during the filling process to prevent contamination. All air and pH equipment was then connected and the pH of each reactor was brought to its optimum point by the

addition of sterile 1 M hydrochloric acid or 1 M sodium hydroxide added through the sampling port. The reaction tubes were then allowed to equilibrate for 1 hr while inoculums were prepared.

Each inoculum culture was checked for cell density by performing cell counts. Cell counts were performed using a Brightline hemocytometer (Hausser, Horsham, PA, USA). For consistency all inoculums were adjusted with fresh sterile media to a fixed cell density for each type of organism: 3.7×10^7 cells/mL for *C. gracilis*, 4.1×10^8 for *S. dimorphus*, 3.1×10^8 for *N. oleoabundan*, and 3.1×10^9 for *S. elongatus*. The inoculum was then added to each culture to give a 5 % final inoculum to media concentration using the same inoculum culture for each of the 3 replicates in a given experiment.

Cultures were monitored daily for dry weight and absorbance between 200 and 900 nm. Dry weight was determined by filtering a 10 mL sample through a 0.6µm filter (GE Life Sciences, Piscataway, NJ, USA) and washing with a 0.5 M sodium formate solution. The weight of the filter was determined before filtering by a 24 hr equilibration in a desiccating chamber followed by weighing to the filter to $1/10^{th}$ of a mg. This process was repeated after filtering the samples and the weight of the filter before was subtracted from the weight of the filter after to determine the final dry weight of the sample.

2.4 Use of filters to lower light intensity and omit wavelengths from the light spectrum

The high intensity light produced by the lamps exceeds 2300 µmol photons m^2 s^{-1} as measured by a spatial light meter (Apogee, Logan, UT, USA) placed inside a water filled culture reactor tube. To adjust this intensity and to vary the wavelengths of light

available to the cultures, various light filters, known as gels, were used. All gel filters were purchased from Rosco laboratories unless otherwise stated (Rosco, Stamford, CT, USA). These gels each have varying pigments designed to uniformly lower light intensity or specifically omit certain wavelengths of light. To uniformly lower the light intensity, a series of neutral density filters were utilized. These filters, designated #3, #6, #9, and # 15, uniformly lowered the light intensity from the approximately 2300 µmol photons m^2 s^{-1} to 1000, 500, 250, and 1250 µmol photons m^2 s^{-1}, respectively.

Filters that modify the various wavelengths were also obtained with omissions of wavelengths in the photosynthetic absorbance ranges being determined by a spectral radiometer (Apogee, Logan, UT, USA). Three wavelength omitting filters were utilized in these experiments designated #19, #346, and #2007. Filter #19 blocks wavelengths in the photsynthetically active region from 400 to about 600 nm effectively. Filter #346 blocks wavelengths in the photosynthetically active region from about 500 to 600 nm effectively. Filter #2007 blocks wavelengths in the photosynthetically active region from about 500 to 700 nm effectively. Light intensities were kept at a constant 250 µmol photons m^2 s^{-1} for wavelength omission experiments. To keep light intensities at this appropriate 250 µmol photons m^2 s^{-1} wavelength omitting gels were put paired with neutral density gels. Filter #2007 was paired with Filter #15, filter #19 was paired with filter #3, and filter #346 was paired with filter #6. Resulting light intensities were confirmed by the use of a spectral radiometer (Apogee, Logan, UT, USA) to be 250 µmol photons m^2 s^{-1}.

2.5 Final harvest and analysis of cultures

Cultures were harvested after various lengths of time ranging from 9-14 days depending on the apparent health of the culture. Harvesting was performed by pouring tube cultures into 500 mL polypropylene centrifuge bottles and harvesting in a high speed centrifuge at 10 000 rpm for 10 min. The resulting cell pellet was frozen at -80 ºC and subsequently lyophilized on a Freezone lyophilzer (Labconco, Kansas City, MO, USA).

Lyophilized cell samples were tested for energy content in a combustion calorimeter (C 2000 basic version 1, IKA, Wilmington, NC). The calorimeter was calibrated in isoperibolic mode with Benzoic acid with a starting temperature of 25 ºC. All samples were run in triplicate with 500 mg of dried cells. All samples were combusted with oxygen at 435 pounds per square inch (psi) in a quartz crucible inside the decomposition vessel.

Cell samples were also analyzed for transesterifiable lipid content by an *in situ* analysis for total fatty acid methyl esters (FAME). A 100 mg sample of dried biomass was placed in a reaction vial. A 2 mL of a methanol solution containing 1.7% sulfuric acid was to the vial and the vial was placed in the chamber of a scientific microwave (CEM Discover, Matthews, NC). The sample was heated at 125 ºC for 40 min to give a complete conversion of all tranesterifiable lipids to FAME. The resulting mix was cooled to room temperature and then the FAME was extracted by adding 2 mL of chloroform and 2 mL of water to the reaction tube and shaking vigorously. The mixture was then centrifuged in a small table top centrifuge (Fisher Scientific Model 228, Pittsburgh, PA) and the chloroform layer was removed to a 10 mL volumetric vial. This entire extraction

process was repeated two more times and the final volume was brought to 10 mL in the volumetric vial.

The FAME was quantified by adding a 1: 10 dilution of the extracted 10 mL FAME solution, 100 µL of solution to 900 µL of chloroform, to a GC vial. The sample was then analyzed using a gas chromatograph (Shimadzu GC-2010, Columbia, MD) equipped with a programmable temperature vaporizer (PTV) injector and a flame ionization dectector (FID). An Agilent 123-BD11 column (15m x 0.32mm ID with 0.1µm film thickness) (Agilent, Santa Clara, California) was installed on the GC. Analysis was conducted by injecting 1 µl of the sample into the PTV, which followed a temperature program of 60 °C for 1 min, followed by a gradient of 10 °C min^{-1} up to 370 °C, then held at this temperature for 6 min. The oven followed an identical temperature program. The detector was maintained at a temperature of 380 °C. Helium was used as the carrier gas and the flow was controlled in constant velocity mode at 30 cm s^{-1}. The FID detector was calibrated using FAME standards of methyl myristate, methyl palmitate, and methyl oleate (Nu-chek Prep Inc., Elysian , MN) at six concentrations ranging from 0.1 to 1 mg/mL. Data collected for standards utilized the same GC program. FAME compounds were found to elute and retention times from 9 to 16 min. These peaks were integrated using GC Solution Postrun v. 2.3 (Shimdzu Scientific, Columbia, MD) and linear regression analysis of peak area was used to determine the final FAME concentration.

3. Results and Discussion

3.1 Effect of light intensity on culture growth and transesterifiable lipid production

Cultures of four strains of algae were grown under 3 different light intensities measuring approximately 250, 500, and 1250 μmol photons m^2 s^{-1}, respectively. The intensity of the light reaching the inside of each reactor tube was determined to be approximately the levels shown in the figure below (Figure 3-2). Filter #3 was not used to grow cultures to test for the effects of light intensity on growth as it has a similar light intensity to filter #15. Growths of cultures were attempted without filters, but culture growth did not occur at this light intensity.

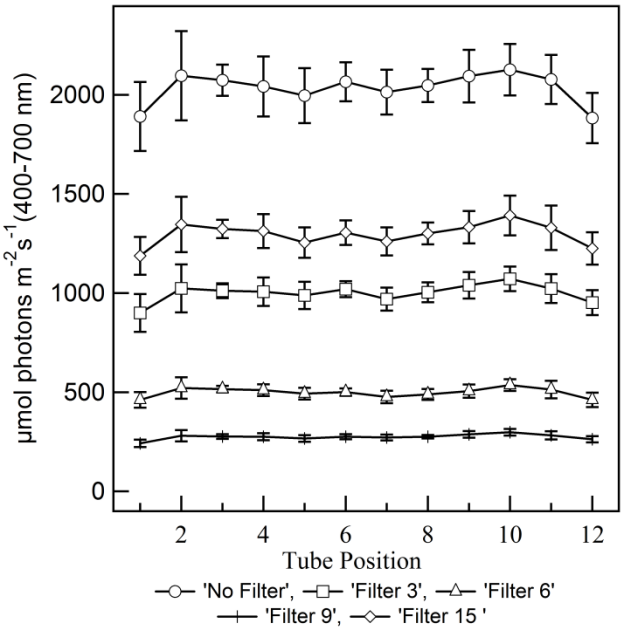

Figure 3-2. Light intensities seen at each reactor tube utilizing various neutral density filter gels. Density in filter gels ranged from approximately 250 μmol photons $m^{-2}s^{-1}$ with the highest density filter up to approximately 2250 photons $m^{-1}s^{-1}$ with no filter.

Cultures grown at various light intensities were monitored over time for both absorbance at 750 nm and dry weight as described in the materials and method section. As can be seen in Figure 3-3, the dry weight of *Scenedesmus dimorphus* and *Neochloris oleoabundans* decreases significantly with increasing light intensity and the dry weights of *Synechococcus elongatus* growths change little with increasing light intensity. Surprisingly, the dry weights of the *Chaetoceros gracilis* growths significantly increase with increasing light intensity. This could be due to differences in natural growing depths of the respective organisms as *S. dimorphus* and *N. oleoabundans* are surface growing algae adapted to regular optimal sunlight. Whereas diatoms such as *C. gracilis,* grow at lower depths and are most likely adapted to be able to utilize any available light with increased pigment contents and light catching antennae (Vuorio et al., 2003).

After the final harvest of the cultures, the cell mass was lyophilized and subjected to total tranesterifiable lipid analysis and bomb calorimetry as described in the methods section. Table 3-1 shows the total energy content from each growth along with the total transesterifiable lipids. As shown in the table, with the exception of *C. gracilis*, all organisms demonstrate a decrease in total energy content as well as transesterifiale lipids as light intensity increases. Contrary to the other organisms studied, *C. gracilis* shows a marked improvement in energy content and transesterifiable lipid production. The total energy and total transesterifiable lipid content for all organisms tended to follow the dry weight of each of the growths with energy content and lipid content increasing or decreasing with the dry weight measurements.

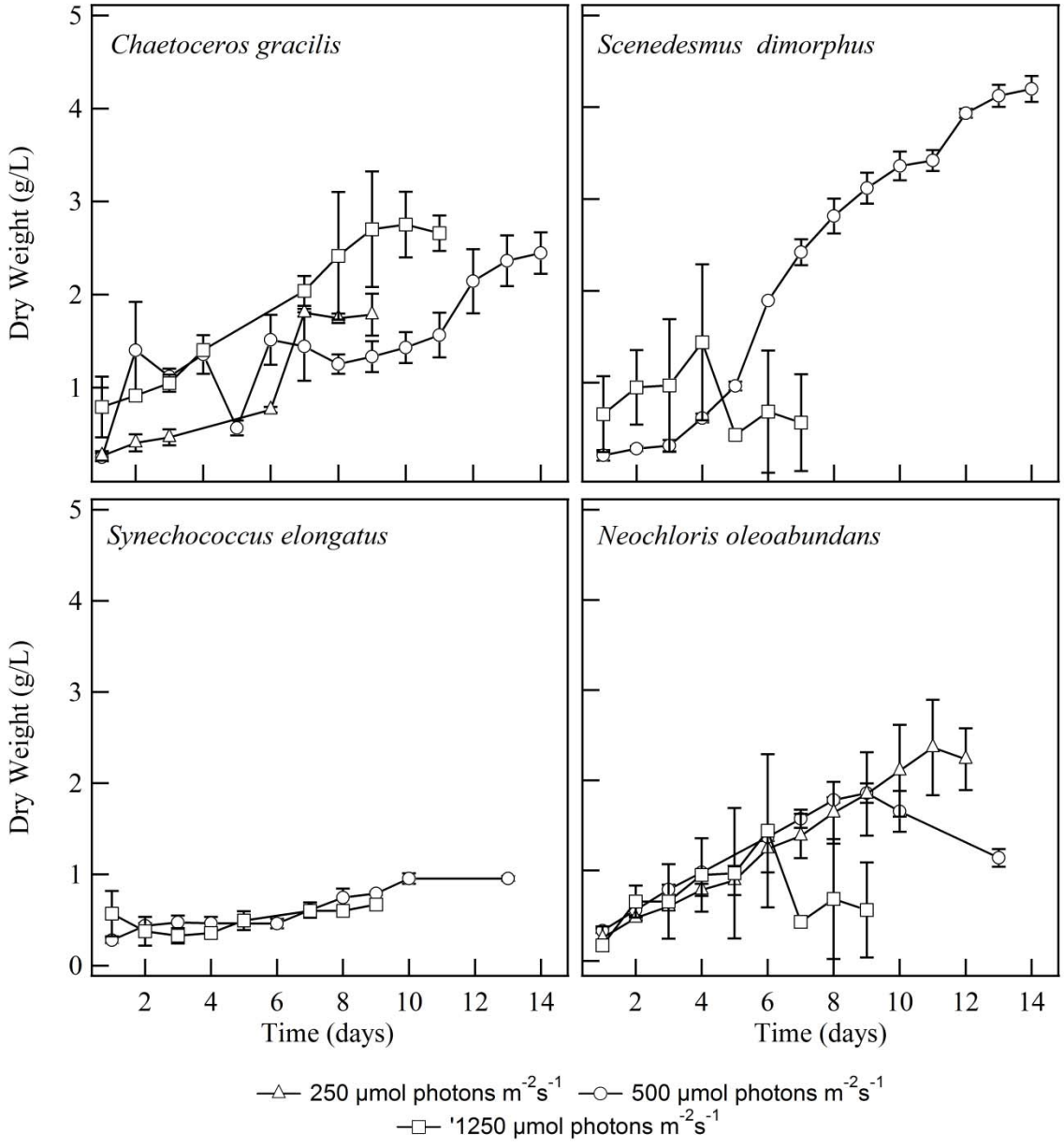

Figure 3-3. Effect of light intensity on growth of algal cells. Cell growth as determined by dry weight of the culture growths is depicted for the four studied algal organisms at light intensities of 250 µmol photons m^{-2} s^{-1} (△), 500 µmol photons m^{-2} s^{-1} (○), 1250 µmol photons m^{-2} s^{-1} (□)

Table 3-1. Energy and transesterifiable lipid content of organisms grown at varying photosynthetic photon flux

Organism	Energy content (Cal/g)			Biodiesel Content (% mass)		
	Filter 3[a]	Filter 6[b]	Filter 15[c.]	Filter 3[a]	Filter 6[b]	Filter 15[c]
Chaetoceros gracilis	4378 (±193.5)	5390 (±257.8)	5344 (±177.2)	16.0 (±1.94)	25.0 (±1.84)	30.2 (±1.17)
Neochloris oleoabundans	4998 (±276.7)	5135 (±279.9)	4644 (±119.9)	17.8 (±0.97)	19.4 (±0.29)	18.2 (±1.02)
Scenedesmus dimorphus	ND[d]	5256 (±96.2)	5010 (±331.4)	ND[d]	23.0 (±0.78)	11.9 (±0.47)
Synechococus elongatus	5411 (±44.1)	ND[e]	5132 (±27.3)	10.8 (±0.96)	ND[e]	6.04 (±0.35)

[a] Cultures were grown under an average light intensity of 250 μmol photons m^{-2} s^{-1}

[b] Cultures were grown under an average light intensity of 500 μmol photons m^{-2} s^{-1}

[c] Cultures were grown under an average light intensity of 1250 μmol photons m^{-2} s^{-1}

[d] *Scenedesmus dimorphus* was not included in Experiment #1.

[e] Not determined, insufficient material.

This marked difference between *C. gracilis* and the other organisms may in part be due to a bleaching effect noted for many strains of algae as they are exposed to high light intensities. In plants bleaching occurs as a result of an overabundance of electrons being produced without an electron sink large enough to absorb them. As a result the conjugated bonds of many of the pigment compounds become reduced or broken resulting in a loss of color to the plants (Kasahara et al., 2002). Although not specifically identified in algae, a similar process is most likely at work.

In the case of *C. gracilis*, an effective electron sink is most likely in place that allows for the absorption of these excess electrons generated by the organisms' photosystems. As determined in Chapter 2 of this dissertation, *C. gracilis* appears to

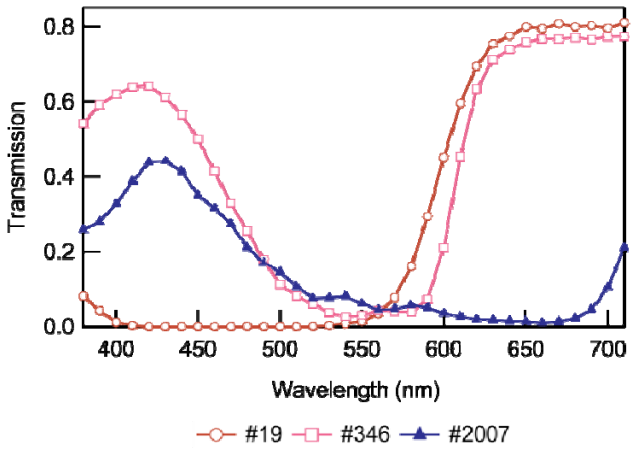

Figure 3-4. **Transmission of various photosynthetically active wavelengths of light through colored filter gels**. Filters tested were #19 (○), #346 (□), and #2007(▲). Filters tested selectively blocked wavelengths in the range of visible light where chlorophyll a, chlorophyll b, and/or phycobillins absorb light.

produce lipid without a nutrient stress from nitrogen, phosphorous, or silicate, as is common in other algal strains. Given the data collected as a part of this work, it is plausible that *C. gracilis* produces transesterifiable lipid compounds in response to light intensity as a means of removing excess electrons.

3.2. Effect of the wavelength of light on algal growth and lipid production

The effect of the wavelengths of light on the growth of the organisms was also determined as a part of this work. As shown in Figure 3-4, three filters were used which eliminated various wavelengths of photosynthetically active light. This was done to determine if the algae could grow well and produce lipids without exposure to certain wavelengths of light. The organisms used in these experiments produce a variety of pigments, but the two major pigments, chlorophyll a and chlorophyll b, have very specific absorption maxima within the 400-500 nm and 600-700 nm wavelengths. The overall goal of this set of experiments was to utilize wavelengths from 500 to 600 nm, if not necessary for algal growth, to produce electricity from photovoltaic cells (Li et al., 2005).

All four stains of algae were grown in the presence of filtered light with a constant 250 μmol photons m^{-2} s^{-1} for each filter condition. As shown in Figure 3-5, the wavelength of light used had a significant effect on the growths of *N. oleoabundans* and *S. dimorphus* while little significant effect was seen on the growths of *C. gracilis* and *S. elongatus*. The dry weights for the filters #346 and/or #19 depending on the organism are similar to those obtained under 250μmol photons m^{-2} s^{-1} with a neutral density filter, indicating that the 500-600 nm range of light is not necessary for the growth of the algal

organisms and could be utilized for other purposes. For the case of *N. oleoabundans* the removal of wavelengths in the range of 400-500 nm resulted in a dramatic decrease in growth. This can be attributed to the high ratio of chlorophyll a to chlorophyll b found in this organism (Pruvost et al., 2009). The small amount of chlorophyll b available is most likely unable to sustain the organisms' growth. *S. dimorphus* suffers a similar fate when wavelengths of light from 400-500 nm are omitted, but some detectable growth still occurs. The same outcome to a slightly lesser extent occurs in this organism when light from 600-700 nm is omitted. These results can again be correlated back to the ratio of chlorophyll a to chlorophyll b in this organism with approximately equal proportions of the two compounds occurring in species of this genus and both ranges from 400-500 and 600-700 nm are required for optimal growth (Humbeck et al., 1988).

Growths of *S. elongatus* and *C. gracilis* showed no significant difference regardless of the wavelength of light to which they were exposed to. This could be due in part to the various additional pigments contained within these organisms in sufficient quantities to make up for the lack of function of either chlorophyll a or chlorophyll b. Another possibility is that these organisms have the ability to adjust their pigment content as a consequence of the light to which they are exposed such as is the case with blue light adjusted organisms (Sagert and Schubert, 1995).

As with the light intensity growths, total energy content and total transesterifiable lipid measurements were obtained for the filtered light growths. Table 3-2 shows the data obtained from these experiments. As shown below, no significant difference

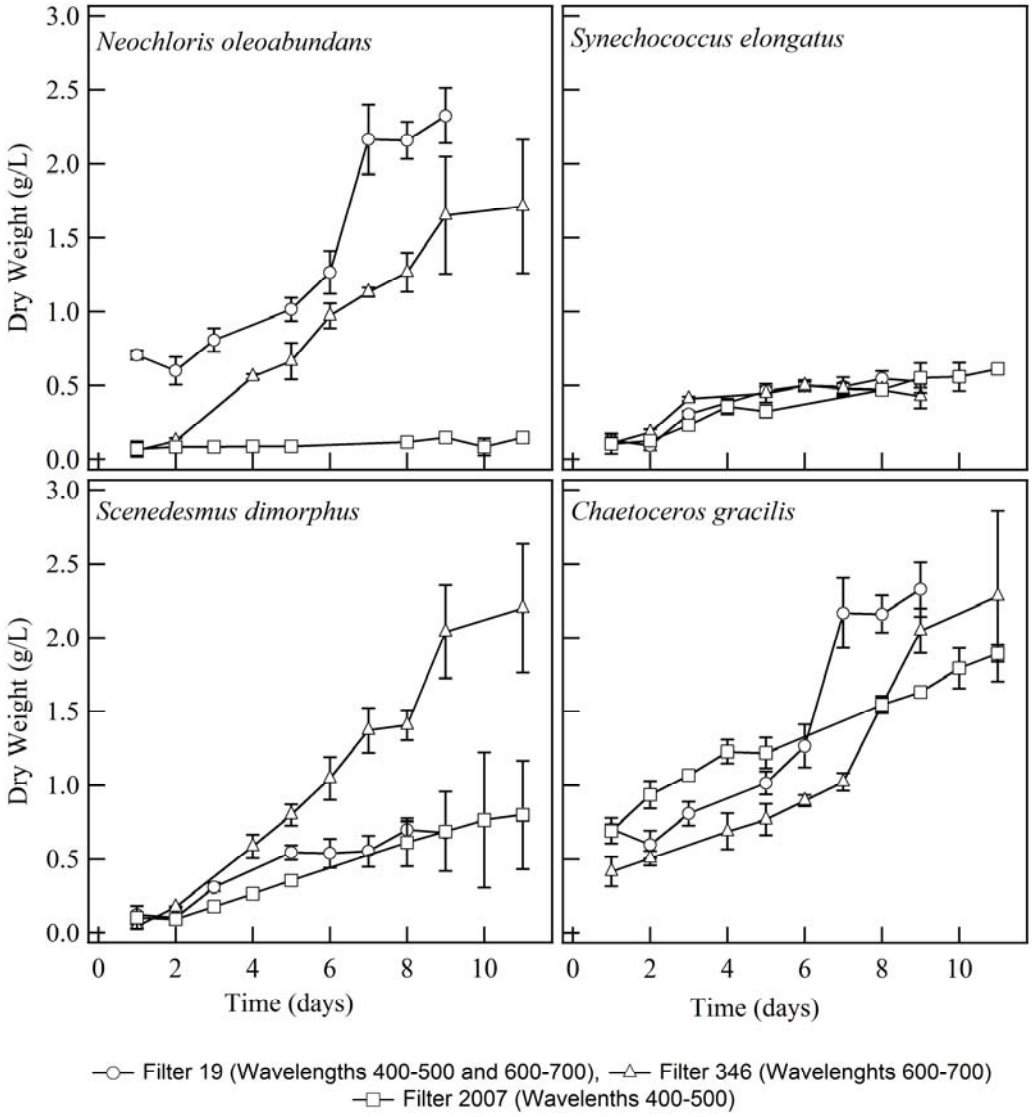

Figure 3-5. Effect of the wavelength of light on the growth of algal cells. Cell growth as determined by dry weight of the culture growths is depicted for the four studied algal organisms using light filters, Filter # 346 (△), Filter # 19 (○), Filter # 2007 (□) to block specific wavelengths of light.

Table 3-2. The effect of wavelength filtered light on energy and transesterifiable lipid content of photosynthetic microorganisms

Organism	Energy content (Cal/g)			Biodiesel Content (% mass)		
	Filter 19[a,b]	Filter 346[a,c]	Filter 2007[a,d]	Filter 19[a,b]	Filter 346[a,c]	Filter 2007[a,d]
Chaetoceros gracilis	4285 (±133)	4260 (±192)	4510 (±126)	13.2 (±0.44)	16.3 (±2.45)	17.4 (±0.98)
Neochloris oleoabundans	5063 (±276.7)	5110 (±279.9)	5360 (±119.9)	19.6 (±0.97)	19.2 (±0.29)	16.5 (±1.02)
Scenedesmus dimorphus	4808 (±690)	5230 (±107)	5320 (±93)	14.5 (±2.28)	15.3 (±0.44)	13.6 (±0.44)
Synechococcus elongatus	5265 (±78.6)	5180 (±89.6)	5120 (±99)	8.44 (±0.39)	7.6 (±0.24)	7.37 (±0.66)

[a]Cultures were grown under an average light intensity of 250 μmol photons m^{-2} s^{-1}

[b] Cultures were grown with filtered light allowing wavelengths of 600-700 nm through the filter.

[c] Cultures were grown with filtered light allowing wavelengths of 400-500 and 600-700 nm through the filter.

[d] Cultures were grown with filtered light allowing wavelengths of 400-500 nm through.

between energy content and transesterifiable lipid content is seen in the growths. This result is contrary to what was noted for the effect of increased light intensity on energy

and transesterifiable lipid content indicating that the wavelength of light the organisms are exposed to has little to no effect on the lipid production of the organism.

4. Conclusions

Microalgae are a diverse group of organisms capable of growing at high densities and producing large amounts of useful compounds under a myriad of growth conditions. However, the efficiency at which the algal organisms capture the available light energy and convert it into useful compounds is low. As well, little is known about the effect of light intensities and wavelengths on algal growth and lipid production. A system utilizing algae and photovoltaics to capture more of the photosynthetically active wavelengths of light could greatly improve the overall efficiency of energy production.

Given that each of the four algal strains tested behaved differently under different light intensities and wavelengths, various systems could be devised for growing algae for the production of high value compounds and collecting wavelengths unnecessary for growth to use in the production of electricity by photovoltaic cells. In the case of *C. gracilis*, light intensity appears to be a major trigger for the production of lipids allowing for high light intensities to be used to produce high value lipid compounds and electricity from wavelengths not used for algal growth. These insights taken together with prior research findings could allow for the production of high levels of valuable products from algae paired with electricity production which could offset the high energy costs associated with biofuels and value added products production from microalgae.

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CHAPTER 4

HIGHLY EFFICIENT LIQUID TO LIQUID EXTRACTION OF TRIACYLGLYCERIDES FROM THE WET CELLS OF OLEAGINOUS MICROBES THROUGH THE USE OF SOLVENT SELECTION AND MECHANICAL MIXING[1]

ABSTRACT

Microbially derived biofuels comprise an important replacement to petroleum based fuels. Traditional biofuels produced from land crops compete with food production and drive increases in food prices, while microbial biofuels can be produced without utilizing arable land or competing with food crops. A major difficulty in the process of producing microbial biofuels is the high cost of dewatering and drying the microorganisms to efficiently extract cellular lipids. To eliminate some of the dewatering and drying steps while maintaining extraction efficiency, a wet cell extraction method was developed by screening hundreds of solvent candidates for their ability to extract the class of microbial lipids TAG from water based on the thermodynamic relative selective parameters found in ASPEN's database, boiling point, health hazard, and cost. Optimal solvents identified using this approach were utilized in a mechanical mixing process with wet cells to determine lipid extraction efficiency. Herein is reported a simple and energetically favorable liquid-liquid extraction process using three of the most promising

[1] Coauthored by Willis, Robert M., McCurdy, Alex T., Ogborn, Mariah K., Wahlen, Bradley D., Pease, Leonard, Seefeldt, Lance C.

solvents determined from the solvent selection search and mechanical mixing analysis, chloroform, methylene chloride, and tert-butyl chloride, to extract lipids from oleaginous yeast, bacteria, and microalgae with greater than 95% efficiency compared to the Bligh and Dyer extract on dry cell material. The optimal extraction solvent of the three tested was determined to be chloroform with optimal extraction conditions being a 4:1 solvent to cell slurry ratio, 20% solids cell slurry concentration, mix time of 2 min, and Reynolds mixing number of 5×10^6. This process allows for the highly efficient and energy favorable extraction of wet cells ranging from 2 to 20 percent solids while utilizing a single solvent which prevents the complication of downstream processing and solvent recovery resulting from the use of multiple solvents.

INTRODUCTION

Continually rising worldwide demand for petroleum combined with an ever decreasing petroleum supply, has increased the need for the development of adequate fossil fuel alternatives. First and second generation biofuels have shown promise as replacements to petroleum sources, however, the production of the alcohols and biodiesel, from grains and oil seeds competes directly with the same land utilized to grow food crops.[1] The microbial production of biofuels has been shown to be an advantage over traditional biofuels production in that the organisms can be grown in large reactors or open tanks and produce large amounts of lipid compounds with limited land usage.[2–4] The use of microorganisms has further advantages in that many can be grown on waste carbon sources including carbon dioxide, for phototrophic growth, and waste carbon streams from various industrial processes such as milk lactose and hydrolysates, for

heterotrophic growth. [5] This combination of growth on non-arable land and the utilization of waste carbon sources can greatly decrease the overhead costs of fuel production when compared to traditional biofuels.[6]

Several different types of microorganisms can produce large amounts of oils, including algae, yeast, fungi, and bacteria, with some producing greater than 70% of their cell dry weight as lipids.[3] This high percentage of lipid production combined with the ease of growth and utilization of waste carbon sources as aforementioned has created a great deal of interest in utilizing microorganisms for fuel production. However, a major drawback to use of these organisms for fuel production lies in the aquatic environment in which they thrive, which depending on the organism, can contain as much as 99.9% water.[6,7] This water can impede the lipid extraction process significantly by preventing the interaction of non-polar solvents generally used for lipid extraction with the lipid containing cells found in the aqueous phase.

Traditional methods of lipid extraction involve separating the water from the cells by centrifugation or tangential filtration. The cell pellet obtained from these methods is then further dewatered using either heat or lyophilization to remove greater than 99 percent of the water. [8,9] The lipids can then be extracted from the cells by utilizing a variety of methods including mechanical agitation, chemical solvents, *in situ* transesterification, and super and subcritical solvents.[3,7,10–17] The energy required to remove greater than 99% of the water is substantial and represents a sizable portion of the production costs.[18] This fact has spawned a great deal of research into the extraction of lipids from oleaginous microorganisms still in an aqueous solution with limited

concentration and drying. However, of all the wet and dry based lipid extractions available, solvent based extractions, similar to hexane based extractions used to extract oil from soybeans, have the potential to be implemented readily on an industrial scale without the need for dramatic changes in industrial infrastructure.[19]

A recent report has shown that with enough heat, pressure, and time greater than eighty eight percent of the extractable lipids could be removed from wet microalgal cells (40% solids) utilizing a solvent system comprised of hexane and ethanol.[9] This is a valuable step toward the extraction of lipids from water mixed with microorganisms, however, this process utilizes a two solvent system that is commonly used for extraction processes in high value carotenoid and lipid compounds,[20] heat, pressure, and a specialized vessel. The added cost of recovery for two solvents, the heat and pressure requirement, as well as the reaction vessel itself would have relatively high expenses when scaled to sufficiently extract lipids from large amounts of microorganisms.

A second process utilizing dimethyl ether to extract lipids from algal cells in a liquid slurry suggested that a liquid to liquid extraction may be feasible on a large scale; however this report lacked optimization of extraction parameters and only demonstrated extraction of lipid from one type of oleaginous microorganism. As well, this process utilized a solvent that is somewhat soluble in water increasing the difficulty in downstream processing and solvent recovery.[21]

An ideal method for lipid extraction would utilize two liquids, water and single a solvent, that are sparingly soluble in each other to allow for easy separation, and with the solvent having a high affinity for microbial lipids. The solvent used would need to have

a low boiling point as to be easy to remove from microbial lipids which have a high boiling point. The solvent would also need to be a minimal risk to the environment and to workers handling the solvents during the extraction process or be able to be easily contained.[8] Using the program Aspen, we sought to determine solvents that fit these criteria for a liquid to liquid mechanical extraction process. The solvents obtained from this process as well as the parameters of mixing, extraction time, and solvent ratios for optimal extraction of lipids from the microorganisms are reported here in.

EXPERIMENTAL SECTION

Materials. All solvents were purchased from Alfa Aesar for use in the extraction experiments unless otherwise stated.

Strains and Culture Conditions. Representative oleaginous strains of microalgae, yeast, and bacteria were used to develop the process of extracting lipids directly from cell culture. *Cryptococcus curvatus* (ATCC# 20509) obtained from the American Type Culture Collection (ATCC, Manassas, VA) was used as a representative oleaginous yeast strain. *C. curvatus* was stored at -80 °C in YPD and 20% glycerol. Two single colonies from a YPD plate were used to start two 8 mL YPD cultures incubated at 30 °C overnight with moderate shaking. Two 8 mL of overnight cultures were used to inoculate each of five 750 mL cultures in 2.8 L Fernbach flasks using media described previously modified to utilize sucrose in place of glucose.[22] Cultures were incubated at 30 °C with moderate shaking (300 rpm) until an OD_{600} of approximately 5. All of the 750 mL cultures were then used to inoculate a 50 L fermenter containing 46 L of identical

media. The culture was incubated for 5 days at 30 °C, with an aeration rate of 42 L min^{-1} of compressed air and agitated at a rate of 220 rpm.

The microalgae *Chaetoceros gracilis* (UTEX# LB 2658), was obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX). *C gracilis* was grown as described previously,[12] with the exception of the culture being grown in 1.2 L tubular air lift reactors, aerated with compressed air supplemented with 1% CO_2 at rate of approximately 1 L min^{-1}. Tube reactors were illuminated by a bank of fluorescent lights (300 μmol s^{-1} m^{-2}) on a 14:10 light:dark cycle. *C. gracilis* growth was monitored by measuring the optical density of the culture at 600 nm. Cultures were harvested when optical density did not increase for two consecutive days.

Rhodococcus opacus PD630 (DSM# 44193), an oleaginous bacterium, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained as instructed. *R. opacus* cultures were grown on defined phosphate media adapted from Kurosawa *et al* and Chartrain *et al* using sodium nitrate and sucrose instead of ammonium sulfate and glucose respectively.[23] Batch cultures for lipid production were initiated with a 10% (% v/v) starting inoculum at an optical density (660 nm) of 0.4 and were grown in the presence of 6.43 g L^{-1} sodium nitrate and 80 g L^{-1} sucrose. *R. opacus* cultures reached maximum lipid and biomass production after 120 hours.

Cell Dewatering and Sample Preparation. Yeast cells were harvested using a continuous centrifuge (LE model, CEPA, Lahr, Germany) to obtain a cell mass of 41 percent solids. Bacterial and microalgal cells were harvested at 8000 RPM using an SLC-

4000 rotor for 10 minutes in a superspeed centrifuge (Thermo Fisher Scientific, Waltham, MA). Harvested biomass was frozen at -80 °C and a small sample of each was retained and dried by lyophilization (Freezone 4.5, labconco, Kansas City, MO) to determine the percent solids of the biomass. Samples of differing biomass content were achieved by thawing the frozen biomass and resuspending it in an appropriate volume of a 10mM potassium phosphate buffer to achieve samples containing 20, 40, 80, 120, 200, and 400 g biomass per liter of solution. Once each solution was thoroughly mixed, aliquots of 30 mL each were placed into 50 mL conical tubes and snap frozen in liquid nitrogen then stored at -80 °C until needed. Consistency in each batch was measured by lyophilizing a 30 mL sample from the beginning, middle, and end of the aliquot process and measuring its dry mass content. These samples were also used to establish the lipid content of the batch. The effect of repeated freezing and thawing cycles on extraction efficiencies was determined using control experiments with fresh and frozen samples from all stages of sample preparation.

Total Lipid Analysis. Total TAG content was determined for each sample by extracting the oil and measuring the concentration of TAG molecules by GC. This differs from the common practice of determining the general lipid content gravimetrically, which can overestimate the amount of saponifiable lipid in the biomass. Total lipids were extracted from dried microbial cells using a protocol similar to one developed by Bligh and Dyer. [24] A total of 200 mg of dry microbial biomass was mixed with 4 mL of a 2:1 chloroform:methanol mixture. Samples were sonicated (Branson Sonifier 250, Dabury, CT) continuously for 30 seconds with the sonifier set at an output

of 6. Approximately 6 mL of water was added to each sample to force a separation of methanol from chloroform into two separate phases. The samples were then centrifuged to rapidly establish the phase separation using a tabletop centrifuge (Fisher Scientific Model 228, Pittsburgh, PA). The aqueous layer was removed using a transfer pipette and the resulting chloroform layer was removed using a gas tight syringe with a long (6 inch) needle so as not to disturb the cell debris and placed in a clean volumetric flask. Four mL of the chloroform methanol mixture was again added to the remaining cell debris and the sonication and separation was repeated. This process was performed a total of three times to effectively remove all extractable lipids. The final volume of chloroform was brought to 10 mL in the volumetric flask and a sample was prepared by diluting the chloroform extract 1:10 with fresh chloroform. Samples were analyzed using a gas chromatograph (Shimadzu GC-2010, Columbia, MD) equipped with a programmable temperature vaporizer (PTV) injector and a flame ionization dectector (FID). An Agilent 123-BD11 column (15m x 0.32mm ID with 0.1μm film thickness) (Agilent, Santa Clara, California) was installed on the GC. Analysis was conducted by injecting 1 μl of the sample into the PTV, which followed a temperature program of 60 °C for 1 minute, followed by a gradient of 10 °C min^{-1} up to 370 °C, then held at this temperature for 6 minutes. The oven followed an identical temperature program. The detector was maintained at a temperature of 380 °C. Helium was used as the carrier gas and the flow was controlled in constant velocity mode at 30 cm s^{-1}. The FID detector was calibrated using tripalmitin (Nu-chek Prep Inc., Elysian , MN) at six concentrations ranging from 0.065 and 0.65 mg/mL. Data collected for standards utilized the same GC program. Triglyceride

compounds were found to elute from 27.5 minutes to 32.5 minutes. These peaks were integrated using GC Solution Postrun v. 2.3 (Shimdzu Scientific, Columbia, MD) and linear regression analysis of peak area was used to determine triglyceride concentration.

Solvent Selection through Aspen Analysis. The modeling program Aspen (Aspen Technology, Burlington, MA) was used to perform a search through a large range of solvents for suitability for use in a mechanical mixing liquid-liquid extraction process. Using the standard databases of chemical properties and suite of solvent interaction equations available in the Aspen program, the degree of solubility a solvent has with water or with the lipid compound triolean was determined in the form of activity coefficients. The ratio of the activity coefficient of the solvent with triolean divided by the activity coefficient of the solvent with water gave a number which was deemed the β factor. For these calculations the larger the β factor the more efficiently the solvent would be predicted to extract triolean from the cell slurry.

The analysis performed with the Aspen software allowed each solvent to be ranked according to its β factor, but did not take into consideration the cost, toxicity, or explosion hazard of the solvent. As each of these factors would be important to consider for a commercial process, a subset of solvents was selected that had a low risk of explosion, scored a 2 or lower on the material safety data sheet (MSDS) toxicity and were found to be lower cost solvents. Ultimately the number of solvents was narrowed to 14. The selected solvents were then screened for their ability to extract TAG from microbial biomass. This initial analysis consisted of mixing the solvent and a cell slurry (20% solids) of oleaginous biomass at a 2:1 solvent:cell slurry ratio at high speeds (20 000

rpm) for 2 minutes. Samples were then processed as described below and total TAG extracted was determined by GC as described above.

Calculation of Reynolds and Peclet Mixing Numbers. The Reynolds mixing number was calculated using the Reynolds equation for mixing by an impeller[25] shown below in equation 1.

$$Re_i = N_i D_i^2 \rho/\mu \quad (1)$$

Where Re_i is the dimensionless Reynolds number for mixing, N_i is the stirrer speed ($m\text{-}s^{-1}$), D_i is the impeller diameter (m), ρ is the fluid density in (kg/m^3), and μ is the fluid dynamic viscosity (kg/ms).

The Peclet mixing number was calculated using the Peclet equation for mass diffusion[26] shown below in equation 2.

$$P_e = LU/D \quad (2)$$

Where P_e is the dimensionless Peclet mixing number, L is the radius of the mixing blade in meters, U is the velocity in $m \bullet s^{-1}$, and D is the mass diffusion coefficient calculated by using equation 3.

$$D = kT/(3\pi\mu d) \quad (3)$$

Where *D* is the mass diffusion coefficient, *k* is planks constant 1.38×10^{-23} m^2 kg s^{-2} K^{-1}, *T* is the temperature of the system in Kelvin, π is the standard value for *pi* of 3.14159, μ is viscocity in kg $m^{-1}s^{-1}$, and *d* is the diameter of the particle in solution (in this case a range is used from a full yeast cell at 10 μm down to a TAG molecule at 9.94×10^{-10}m). The diameter of the TAG molecule, Triolean, was calculated using an online chemical geometry program (Chemicalize.org by axon). Viscosity and density for the

yeast cell samples were determined using a viscometer (SVM 3000, Anton Paar Stabinger, Richmond, VA). The viscosity and density of the solvents were determined from the MSDS of each. To determine the approximate viscosity of the mixture of yeast cells and solvent, the Refutas set of equations were employed.[27] The approximate density of the mixture of solvent and yeast cells was determined by averaging the density and ratio of the solvent to yeast cells as described by Lapayerouse.[28] The stirrer speed was controlled by a potentiometer (Staco Energy Products, Dayton, Ohio), and the RPM value for each analysis was confirmed by a Red Lion blender tachometer (York, PA) designed to work with the blender used. The impeller diameter was measured to be 5.24 cm. As viscosities varied between solvents, the value of the Reynolds number was held constant between samples by adjusting the RPM of the blender.

Wet Cell Lipid Extraction. Wet cell extractions were conducted by placing both solvent and cell slurry in a stainless steel blender cup and mixing the contents using a blender (Waring 7011Hg, Conair, Stamford, CT). Several variables, including solvent:cell slurry ratio, solids content of cell slurry, mixing time, mixing speed, and type of solvent, were tested to determine the optimal conditions for efficient lipid extraction. Throughout all of the experiments the volume of the cell slurry was held constant at 30 mL. A potentiometer (Staco Energy Products, Dayton, Ohio) was used to control the rate of speed, which was displayed by the previously described tachometer. In order to determine the importance of mixing the cell slurry in the presence of a solvent, two sets of control samples were performed. In the first set, the cell slurry was mixed at 20000 RPM with no solvent present and mixed vigorously by inversion in the centrifuge bottles

afterwards with solvent for 2 minutes. In the second set, cells were mixed vigorously with the solvent by inversion in the centrifuge bottle for 2 minutes, without high speed mixing.

After mixing, samples were emptied into a 250 mL Teflon FEP centrifuge bottle (Thermo Scientific, Waltham, MA) and sealed with a sealing cap assembly (Thermo Scientific, Waltham, MA) designed for the bottle. The bottles were centrifuged at 4000 g for 10 minutes to rapidly establish the separation of the organic and aqueous layers. The organic layer was then removed to a clean 11 dram vial and brought to 40 mL by the addition of clean solvent. The vials were prepared by marking the meniscus of 40 mL of deionized water. The vials were then dried and sealed with PTFE lined caps. If the organic layer contained more than 40 mL of solvent, the solvent was dried down under house air until a total of 40 mL could be collected. The samples were then stored at -20 ºC until the TAG could be quantified by GC. All samples were conducted in triplicate to allow for standard error of the mean (SEM) calculations.

Measurement of Extracted Oil Quality Parameters. Several milliliters of oil were obtained from each type of solvent extraction by distilling off the solvent, leaving only the extracted oil. Oil extracted by the solvents chloroform, methylene chloride, and tert-butyl chloride were analyzed to determine the relative percentages of FFA, phospholipids, sterols, and TAG extracted by each solvent. Prior to GC analysis 50 µL of *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) (Sigma Aldrich, St. Louis, Mo) was added to a 1 mL sample of oil diluted 1:100 with chloroform and allowing the solution to sit at room temperature for 30 minutes. MSTFA derivitizes free fatty acids improving their resolution on the GC. Standards for TAG (Tripalmitin, Nu-chek Prep

Inc., Elysian, MN), FFA (palmitic acid, Sigma Aldrich, St. Louis, Mo), and phospholipid (Phosphatidyl choline, Nu-chek Prep Inc., Elysian, MN) were all prepared for analysis with 1mg/mL final sample size and likewise treated with MSTFA. The samples and standards were then run on the GC/FID as described earlier. Peaks were integrated and total areas were used to determine the relative amounts of each major type of compound in the form of a percentage of the total area under the peaks.

Total protein content of the various oil extractions and control samples of soybean oil and extra virgin olive oil were calculated using a modified method from Ramazzotti et al [29] with a 50 mM PBS solution and a 3:1 oil to buffer ratio (3 mL of oil to 1 mL of buffer). The buffer and oil mixture was placed in a laboratory microwave (Discover S, CEM, Mathews, NC) and heated at 55 ºC with vigorous stirring for 24 hours. The sample was then centrifuged to rapidly establish a phase separation and the resulting aqueous phase was removed and analyzed for protein content using the Pierce BCA protein concentration assay kit (Thermo Fisher Scientific, Rockford, IL)

RESULTS

Solvent Selection. The objective of this study was to develop an efficient solvent extraction method to recover oil directly from a culture of oleaginous microorganisms without completely removing the water (2 to 40% solids). The process, outlined in Figure 4-1, would ideally utilize a single solvent that is sparingly soluble in water, but has a high capacity for TAG. In addition to its ability to extract TAG, selecting a solvent with a low boiling point would facilitate its recovery with minimal energy input. As each cell is surrounded by a cell wall and membrane, the ability of the solvent to cross this barrier or

facilitate its disruption will be important to the success of the process. Choosing the ideal solvent based on these characteristics has the potential to lower the cost associated with single cell oil production by minimizing the costs associated with dewatering the culture and drying the microbial biomass.

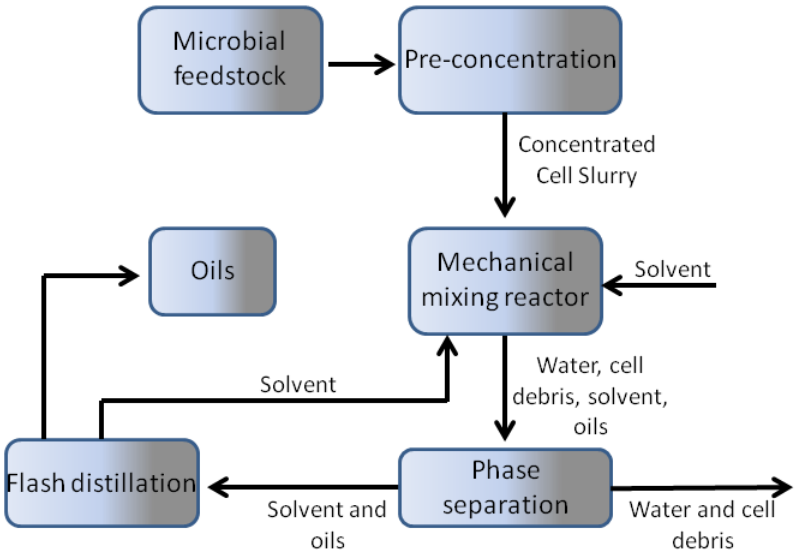

Figure 4-1. Proposed liquid to liquid extraction process. The proposed extraction process shown with minimal dewatering and no drying required before extraction with mechanical mixing. The final oil product can be converted to fuels using standard processes.

The determination of which solvents would be suitable for this task was performed with the use of the program Aspen. This program allows for the calculation of activity coefficients which are directly correlated to how well two liquid compounds mix together. A ratio of the activity coefficient for a solvent and TAG to the activity coefficient for a solvent and water gives a β factor that can be used to predict the solvents ability to remove TAG and be separated from water. The larger the β factor the better the

solvent would be predicted to behave in this system. Aspen was used to calculate the β factor of several hundred solvents. The system was defined as triolein dispersed in an aqueous solution. Triolein was used instead of a microbial cell in order to simplify the calculation of β factors. Of the solvents screened, about 300 had β factors deemed large enough to function as effective solvents in the proposed liquid-liquid extraction process. Additional selection criteria included examining the health and explosion hazard of each solvent found in each solvents MSDS. Finally the cost of each solvent was used to further down select the number of potential solvents. Although cost does not affect the technical ability to extract TAG from a liquid culture, it will certainly be an important consideration for the industrial application of the process at scale. Based upon these criteria, the list of candidate solvents was narrowed to fourteen. These were then tested under a fixed set of conditions for lipid extraction capability using a high speed mechanical mixer. The conditions were as follows for initial screening of selected solvents: 60 mL of the solvent being tested, 30 mL of a 20% solids cell slurry, and a mixing speed of 20 000 rpm for 2 min.

The results of the initial solvent screening (Table 4-1) indicate that the Aspen ranking by β factor does not correlate well with the ability of the solvent to extract TAG from cells still in solution. A good example is pentane, which is ranked 33rd among the solvents screened. Despite its favorable ranking, it was only capable of extracting 46% of the available TAG, while 2-chlorobutane was capable of extracting 87% of the available TAG and had a similar Aspen ranking (29th). The most effective solvent was n-

Table 4-1. Aspen solvent search results

Solvent	Aspen Rank[a]	Health Hazard[b]	Cost per L[c]	Boiling Point (°C)	Viscocity mPa-s (@20 °C)	Percent TAG Extracted [d]
n-Propylmercaptan	8	2	$74.60	67	0.385	92 ± 1
1-Chlorobutane	17	1	$49.00	77	0.422	90 ± 1
***Tert*-butyl-chloride**	**27**	**0**	**$49.00**	**51**	**0.510**	**87 ± 1**
2-Chlorobutane	29	0	$102.00	68	0.360	87 ± 1
Chloroform	**22**	**2**	**$37.40**	**61**	**0.537**	**86 ± 1**
Methylene chloride	**168**	**2**	**$33.00**	**40**	**0.413**	**82 ± 1**
Dimethyl sulfide	3	2	$42.60	36	0.284	79 ± 1
Diethyl ether	35	2	$48.00	35	0.224	79 ± 1
2-Methyl-1,3-butadiene	12	1	$78.80	34	0.225	78 ± 2
1,1-Dichloroethylene	42	2	$81.00	30	0.840	71 ± 2
Isopropyl chloride	34	1	$91.40	34	0.303	71 ± 3
Allyl Chloride	7	2	$76.80	45	0.354	50 ± 1
Pentane	33	0	$55.50	35	0.224	46 ± 1
n-Hexane	138	2	$36.20	69	0.300	22 ± 1

[a]Solvents were ranked by the βfactor described in the Methods section.

[b]Health hazard number for each solvent was obtained from the corresponding material safety data sheet.

[c]The cost per liter of each solvent was obtained from Alfa Aeser catalog prices for 1 L or 500 mL volumes.

[d]The percentage of the total TAG available recovered by the wet extraction technique for a given solvent.

propylmercaptan (92 % of TAG) and the least effective was n-hexane, extracting only 22% of available TAG. Several of the top solvents contained electron withdrawing atoms such as Cl, S, or O giving them a slight polarity when compared to hexane though most are classified as non-polar. The solvents with the highest extraction efficiency had one or more of these atoms in their composition indicating that the presence of these atoms or the polarity that they imbue could be a reason for increased extraction efficiency.

Based on the criteria described, three solvents, *tert*-butyl chloride, chloroform, and methylene chloride, were selected for optimization studies to improve their efficiency of lipid extraction over the initial test conditions. These solvents were chosen for their high extraction efficiency (87%, 86%, 82% of available TAG, respectively) in initial tests, low boiling points, relative low cost, and in the case of *tert*-butyl chloride for its low toxicity according to the MSDS. Optimization of this process involved determining the effect of the following variables on extraction efficiency: percent solids of the cell slurry, the ratio of solvent:cell slurry volume, and mixing speed and time.

Influence of Solids Content on Extraction Efficiency. In order to determine the influence that cell slurry solids content has on extraction efficiency, 30 mL samples were prepared with percent solids that ranged from 2 to 40% and subjected to an identical extraction procedure. Each 30 mL sample was mixed with 60 mL of solvent for 2 minutes. Mixing speeds for each sample were varied to maintain a constant Reynolds number of 6×10^6 for chloroform, 4×10^6 for *tert*-butyl chloride, and 7×10^6 for methylene chloride. As can be seen in Figure 4-2A, the percent lipid extracted for all of the solvents appears to have a parabolic shape showing that as the amount of water in the

cell slurry decreases (from 2 to 20% solids) the amount of lipid extracted increases. However, as the percent solids increases to 40%, the percentage of available TAG extracted is reduced from 79% (20% solids) to 49% for *tert*-butyl chloride. This drop–off in extraction efficiency is consistent with the solvent reaching a carrying capacity for TAG. Both chloroform and methylene chloride experience a similar decrease in extraction efficiency, although to a lesser extent (86% to 79% and 82% to 75%, respectively). In the case of these three solvents, each had nearly identical extraction efficiencies at 20% solids; however substantial differences can be noted for *tert*-butyl chloride which resulted in significantly lower extraction efficiencies at 2% solids and 40% solids than either chloroform or methylene chloride.

The total amount of TAG extracted for each percent solids sample is plotted in Figure 4-2B. The total amount of TAG extracted by chloroform plotted against the percent solids of the samples forms a near linear line demonstrating that the solvent has ample capacity to extract TAG under these conditions. The solvent *tert*-butyl chloride and to a lesser extent methylene chloride do not have the same linear correlation between TAG extracted and increasing solids content. This could be interpreted as the solvent reaching a carrying capacity for TAG (Tables 4-2 and 4-3).

Determination of Optimal Solvent to Cell Slurry Ratio. Due to the apparent limitation to the amount of TAG that a solvent could extract, experiments were conducted to determine the optimal ratio (v:v) of solvent to cell slurry. The ratio of solvent:cell slurry was varied from the point of having half as much solvent as cell slurry 0.5:1) to having 8 times as much solvent as cell slurry (8:1) (Figure 4-3). To conduct this

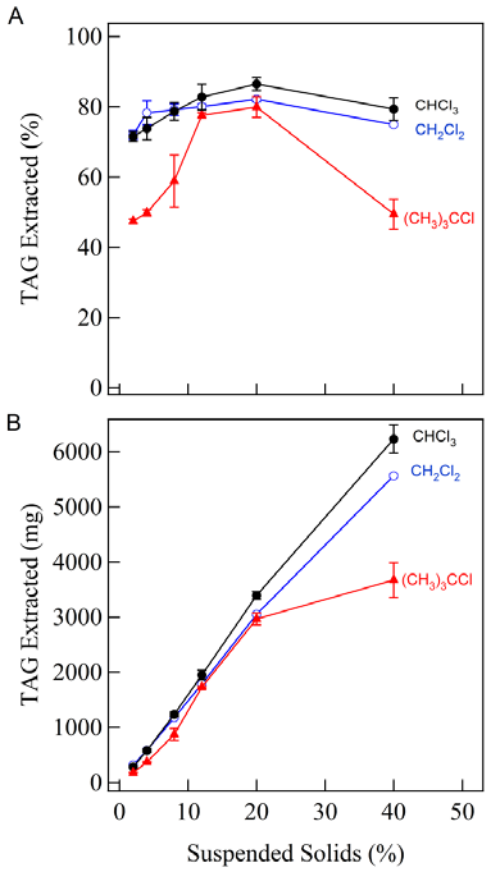

Figure 4-2. Effect of suspended solids concentration on extraction efficiency. **A**. The amount of extracted TAG relative to the amount available is shown plotted against the concentration (% w/v) of suspended solids. **B**. The total TAG extracted from the cells plotted against the concentration of suspended solids (% w/v). Error bars are shown with standard error of the mean (SEM). In each case 30 mL of cell slurry (with varying % solids) was extracted with 60 mL (2:1 solvent:cell slurry) of either chloroform (● $CHCl_3$), methylene chloride (○ CH_2Cl_2), or *tert*-butyl chloride (▲ $(CH_3)_3CCl$) by mixing for 2 min. Reynolds number of 6 x 10^6 for chloroform and methylene chloride and 4 x 10^6 for *tert*-butyl chloride. The total TAG extractable was determined from the modified Bligh and Dyer extraction performed on dry cell samples.

Table 4-2. Optimum percent solids for highest extraction efficiency

Percent Solids[a]	Chloroform (%TAG Recovered)	Methylene chloride %TAG Recovered	*Tert*-butyl-chloride %TAG Recovered
40	79 ± 3	75 ± 1	49 ± 4
20	86 ± 2	82 ± 1	79 ± 3
12	83 ± 4	80 ± 1	77 ± 1
8	79 ± 3	79 ± 2	59 ± 7
4	74 ± 3	78 ± 3	50 ± 1
2	72 ± 1	72 ± 1	47 ± 1

[a] Percent dry solid cell material in the aqueous sample.

[b] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, Reynolds number of 6 x 10^6 for chloroform and methylene chloride and 4 x 10^6 for *tert*-butyl chloride (a lower number had to be used because of equipment limitation, max RPM 22000), and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

Table 4-3. Total TAG extracted from 2-40% solids

Percent Solids[a]	Chloroform (TAG Recovered (mg))	Methylene chloride (TAG Recovered (mg))	*Tert*-butyl-chloride (TAG Recovered (mg))
40	6232 ± 252	5564 ± 28	3674 ± 315
20	3393 ± 71	3050 ± 26	2968 ± 108
12	1949 ± 86	1783 ± 14	1729 ± 17
8	1235 ± 40	1176 ± 24	874 ± 110
4	579 ± 25	582 ± 25	370 ± 7
2	281 ± 5	315 ± 5	176 ± 2

[a] Percent dry solid cell material in the aqueous sample.

[b] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, Reynolds number of 6 x 10^6 for Chloroform and methylene chloride and 4 x 10^6 for *tert*-butyl chloride (a lower number had to be used because of equipment limitations, max RPM 22000), and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

experiment, 30 mL samples of 20% solids were mixed for 2 min at a speed that resulted in Reynolds numbers of 9 x 10^6 for chloroform, 7 x 10^6 for *tert*-butyl chloride, and 1x 10^7 for methylene chloride. Identical Reynolds numbers could not be achieved due to variations in solvent viscosity and density, and speed limitations of the blender, therefore the contstant rpm of 20 000 resulted in different Reynolds numbers for each solvent.

Under these conditions, extraction with chloroform at a 4:1 or greater ratio resulted in removal of approximately 100% of the available TAG. To achieve the same result with methylene chloride, a 6:1 ratio is required. However, in the case of *tert*-butyl chloride further increasing the solvent to cell slurry ratio past 2:1 does not result in an increase in the percent of the TAG extracted (Table 4-4).

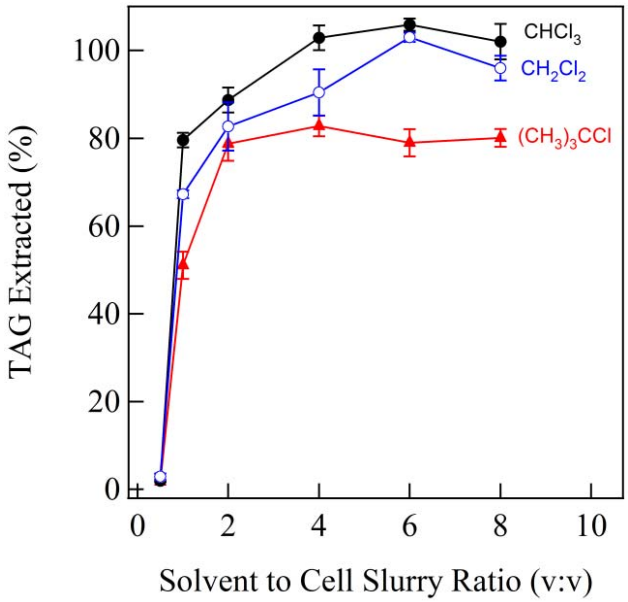

Figure 4-3. Determination of the optimal solvent to cell slurry ratio. The amount of TAG extracted relative to the amount available is plotted against a varying solvent:cell slurry ratio. A fixed volume of cell slurry (30 mL, 20% solids) was mixed with varying volumes of chloroform (● $CHCl_3$), methylene chloride (○ CH_2Cl_2), or *tert*-butyl chloride (▲ $(CH_3)_3CCl$) for 2 min and Reynolds numbers for each solvent were 9 x 10^6, 1 x 10^7, and 6 x 10^6 respectively. Error bars are shown with SEM. Reynolds numbers vary between solvents due to differences in density, viscocity, and mechanical limitations of the equipment (max RPM 20 000).

Determination of Optimal Mixing. The optimal mixing number was determined as shown in Figure 4-4 by varying the Reynolds mixing number from 9 x 10^5 (2000 rpm) to 9 x 10^6 (20 000 rpm) for chloroform, 1 x 10^6 (2000 rpm) to 1 x 10^7 (20 000 rpm) for methylene chloride, and 6 x 10^5 (2000 rpm) to 6 x 10^6 (20 000 rpm) for *tert*-butyl chloride. The results show a hyperbolic curve for all three solvents that starts at about 10 percent extraction efficiency performed using a control of low mixing approximating a

Table 4-4. Optimal Solvent to Cell slurry volume ratio

Solvent to Cell Slurry Ratio[a.]	Chloroform (% TAG Recovered[b])	Methylene chloride (% TAG Recovered[b])	*Tert*-butyl-chloride (%TAG Recovered[b])
0.5	2 ± 0.3	3 ± 1	2 ± 0.2
1	80 ± 2	67 ± 1	51 ± 3
2	89 ± 3	83 ± 5	79 ± 3
4	103 ± 3	90 ± 5	83 ± 2
6	106 ± 1	103 ± 1	79 ± 3
8	102 ± 4	96 ± 3	80 ± 2

[a] Ratio of the amount of solvent used to the cell slurry liquid volume fixed at 30 mL

[b] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, RPM value of 20000, and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

Reynolds number of zero, which increases to a plateau of approximately 80, 85 and 90 percent of extraction efficiency at maximum Reynolds mixing numbers for chloroform, methylene chloride, and *tert*-butyl chloride respectively. The optimal Reynolds numbers for chloroform, methylene chloride, and *tert*-butyl chloride were determined to be 4 x 10^6 (8000 rpm), 4 x 10^6 (8000 rpm), and 3 x 10^6 (10 000 rpm) respectively (Tables 4-5, 4-6, and 4-7).

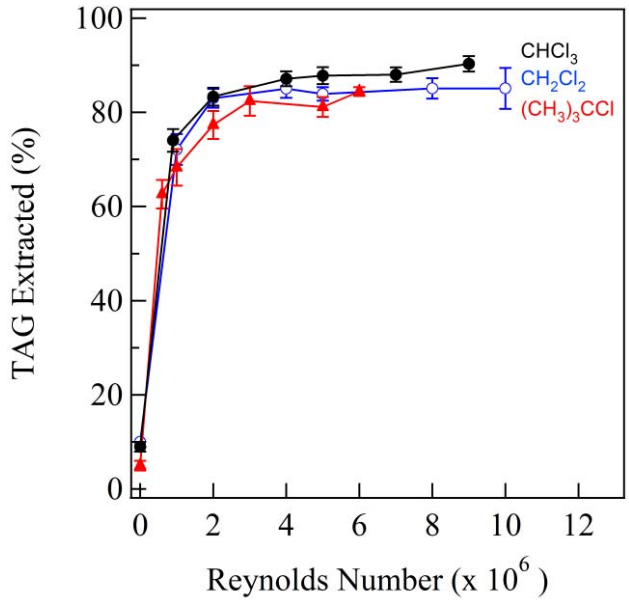

Figure 4-4. Determination of the optimal Reynolds number for mixing. Chloroform (● $CHCl_3$), methylene chloride (○ CH_2Cl_2), or *tert*-butyl chloride (▲ $(CH_3)_3CCl$) were mixed for 2 min with 30 mL of cell slurry (20% solids) at a 2:1 solvent:cell slurry ratio. The percentage of the total TAG extracted is plotted against the Reynolds number. Error bars are shown with SEM.

Table 4-5. Optimal Mixing for Chloroform

Reynolds Number [a]	Peclet Number Range [b]	Chloroform (%TAG Recovered [c])
Approx. 0	Approx. 0	9 ± 1
9×10^5	$3.4 \times 10^8 - 3.4 \times 10^{12}$	74 ± 2
2×10^6	$6.8 \times 10^8 - 6.8 \times 10^{12}$	83 ± 2
4×10^6	$1.3 \times 10^9 - 1.3 \times 10^{13}$	87 ± 2
5×10^6	$1.7 \times 10^9 - 1.7 \times 10^{13}$	87 ± 2
7×10^6	$2.5 \times 10^9 - 2.5 \times 10^{13}$	88 ± 2
9×10^6	$3.4 \times 10^9 - 3.4 \times 10^{13}$	90 ± 2

[a] Reynolds number calculated as described in the Methods section

[b] Peclet number calculated as described in the Methods section, range calculated using the diameter of a TAG molecule and the diameter of a yeast cell in the calculations to give the minimum and maximum values, respectively

[c] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids , RPM values varying from 2000 to 20000, and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

Table 4-6. Optimal Mixing for Methylene Chloride

Reynolds Number [a]	Peclet Number Range [b]	Methylene chloride (% TAG Recovered [c])
Approx. 0	Approx. 0	10 ± 1
1×10^6	$2.6 \times 10^8 – 2.6 \times 10^{12}$	72 ± 3
2×10^6	$5.2 \times 10^8 – 5.2 \times 10^{12}$	83 ± 2
4×10^6	$1.0 \times 10^9 – 1.0 \times 10^{13}$	85 ± 2
5×10^6	$1.3 \times 10^9 – 1.3 \times 10^{13}$	83 ± 1
8×10^6	$2.0 \times 10^9 – 2.0 \times 10^{13}$	85 ± 2
1×10^7	$2.6 \times 10^9 – 2.6 \times 10^{13}$	85 ± 4

[a] Reynolds number calculated as described in the Methods section

[b] Peclet number calculated as described in the Methods section, range calculated using the diameter of a TAG molecule and the diameter of a yeast cell in the calculations to give the minimum and maximum values, respectively

[c] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, RPM values varying from 2000 to 20000, and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

Table 4-7. Optimal Mixing for *Tert*-Butyl Chloride

Reynolds Number [a]	Peclet Number [b]	*Tert*-butyl chloride %TAG Recovered [c]
Approx. 0	Approx. 0	5 ± 1
6×10^5	$3.2 \times 10^8 – 3.2 \times 10^{12}$	63 ± 3
1×10^6	$6.4 \times 10^8 – 6.4 \times 10^{12}$	68 ± 4
2×10^6	$1.3 \times 10^9 – 1.3 \times 10^{13}$	77 ± 3
3×10^6	$1.6 \times 10^9 – 1.6 \times 10^{13}$	82 ± 3
5×10^6	$2.4 \times 10^9 – 2.4 \times 10^{13}$	81 ± 2
6×10^6	$3.2 \times 10^9 – 3.2 \times 10^{13}$	84 ± 1

[a] Reynolds number calculated as described in the Methods section

[b] Peclet number calculated as described in the Methods section, range calculated using the diameter of a TAG molecule and the diameter of a yeast cell in the calculations to give the minimum and maximum values, respectively

[c] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, RPM values varying from 2000 to 20000, and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section

The amount of time required to extract the maximum amount of TAG from the cell slurry is an important parameter to consider to reduce operating costs at larger scales. Cell slurry samples (30 mL) containing 20% solids were extracted with 60 mL of solvent for 0.5, 1, 2, 3.5, and 5 minutes at Reynolds numbers of 9 x 10^6, 1 x 10^7, and 6 x 10^6 for chloroform, methylene chloride, and *tert*-butyl chloride respectively (Figure 4- 5).

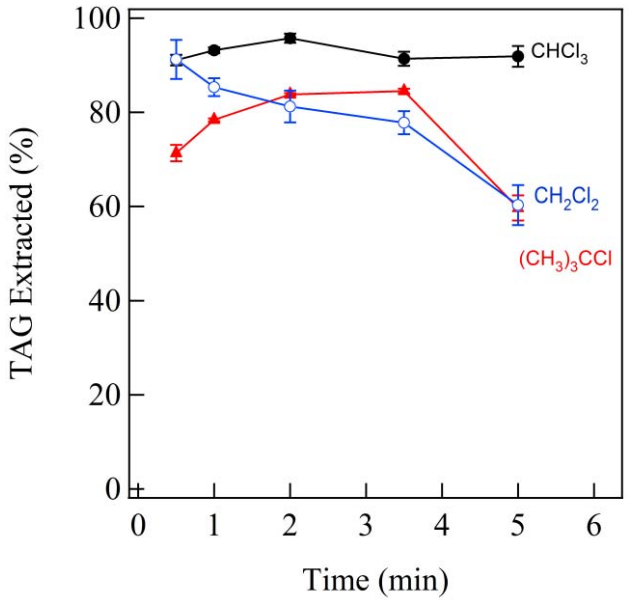

Figure 4-5. Determination of the optimal mixing time. The amount of TAG extracted relative to the total TAG available is plotted against time of mixing. 30 mL of cell slurry (20% solids) is mixed with either chloroform (● $CHCl_3$), methylene chloride (○ CH_2Cl_2), or *tert*-butyl chloride (▲ $(CH_3)_3CCl$) at a 2:1 solvent:cell slurry ratio for varying lengths of time with Reynolds numbers of 9 x 10^6, 1 x 10^7, and 6 x 10^6 respectively. Error bars are shown with SEM. The Reynolds numbers vary between different solvents due to differences in viscosities, densities and mechanical limitations of the equipment used.

The effect of extending mixing times on extraction efficiency was unique for each solvent. In the case of chloroform the mixing times ranging from 30 seconds to 5 minutes resulted in high extraction efficiencies with little variation (91-96%). In contrast, *tert*-butyl chloride extractions show significant improvement from 30 sec to 2 minutes with a sudden decrease in extraction efficiency occurring at 5 minutes. Methylene chloride extraction reaches a maximum at 30 seconds followed by a steady and significant decrease in extraction efficiency with increased mixing (Table 4-8).

Table 4-8. Optimum mixing times for solvents

Time (min)	Chloroform (%TAG Recovered[a])	Methylene chloride (%TAG Recovered[a])	*Tert*-butyl-chloride (%TAG Recovered[a])
0.5	91 ± 1	91 ± 4	71 ± 2
1	93 ± 1	85 ± 2	78 ± 1
2	96 ± 1	82 ± 3	84 ± 1
3.5	91 ± 2	78 ± 2	84 ± 1
5	92 ± 2	60 ± 4	60 ± 3

[a] Amount of the TAG recovered utilizing a 2:1 ratio of solvent to cell slurry volume, 20% solids in the sample, RPM value of 20000, and extraction time of 2 min as described in the Methods section. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

Table 4-9. Properties of extracted oils compared to food grade oils

	Percent Free Fatty Acids [b]	Percent Sterols [b]	Percent Phospholipids [b]	Percent TAG [b]	Protein Content (µg/mL)[c]
Solvent used to extract					
Chloroform[a]	0.29%	0.57%	1.44%	97.7%	140 ± 4
Methylene chloride[a]	0.35%	0.70%	1.34%	97.6%	133 ± 2
Tert-butyl chloride[a]	0.67%	0.58%	1.30%	97.4%	267 ± 30
Reference standard					
Soybean Oil	ND	0.42%	0.90%	98.6%	146 ± 17
Extra Virgin Olive Oil	0.98%	0.20%	2.10%	96.7%	495 ± 29

[a]Yeast oil obtained by solvent extraction of wet cells.

[b] Percent of the integration area relative to the total integration area of the peaks from the GC/FID analysis, percentages are approximately equal to the weight percent.

[c] The protein content of each oil was determined by the BCA protein assay.

Qualitative Analysis of Extracted Oil. It is unknown how each solvent might affect the quality of the oil. The method described in this study is unlike any commercial method for extracting oils. As a result, it is possible that this method of extracting the oil from cells suspended in solution could also extract other biomolecules reducing the value of the oil. We analyzed oil extracted by each of the three solvents (*tert*-butyl chloride, chloroform, and methylene chloride) for the presence of free fatty acids, sterols, phospholipids, triglycerides, and protein. Commercially obtained soybean oil and olive oil were used as reference oils and were analyzed by the same manner. A total of 100 mL

oil was obtained by extraction with each solvent. As shown in Table 4-9, the percentage of fatty acids in the solvent extracted yeast oil was lower than that observed in olive oil (0.98%) in each case, with an average of 0.43%. Sterols observed in the solvent extracted oils did appear to be slightly higher than observed in the two reference oils but were still low with the methylene chloride extracted oil containing the highest level of all oils tested (0.70%). Each oil was found, as expected, to be primarily composed of TAG. The solvent extracted yeast oil did contain more phosholipids (average of 1.36%) than soybean oil (0.9%) but less than extra virgin olive oil (2.1%). Protein content of the extracted yeast oil varied among the solvents used for extraction with methylene chloride extracted oil containing the least (133 μg/mL) and oil extracted by *tert*-butyl chloride the most (267 μg/mL). Protein content of soybean oil (146 μg/mL) was similar to our solvent extracted yeast oil and extra virgin olive oil had a higher protein content (495 μg/mL).

Processing of Microalgal and Bacterial Feedstocks. Having developed a successful process for the extraction of oils from yeast cells in solution, the optimal parameters determined were extended to oleaginous bacterial and microalgal organisms to determine the general applicability of the method. A cell solution, containing 20% solids of either *R. opacus* or *C. gracilis* cultures were mixed with chloroform at a 4:1 solvent:cell slurry ratio for 2 minutes at 20 000 rpm. Under these conditions greater than 90% of available TAG was extracted from each organism (Table 4-10). Differences in extraction efficiency between the three organisms did exist, with the efficiency of extraction of bacterial oil reaching 91%, lower than both microalgae (95%) and yeast

Table 4-10. Extraction efficiencies of oils from oleaginous microbes

Species	Extraction efficiency (%) [a]	TAG content (% dry cell weight)[b]
C. curvatus (yeast)	103 ± 3	62 ± 2
R. opacus (bacteria)	91 ± 6	19 ± 1
C. gracilis (microalgae)	95 ± 1	38 ± 1

[a]Ratio of extracted TAG to total available triacylglycerids expressed as a percentage. Extraction performed with a 4:1 solvent to cell slurry (20% solids) ratio mixing at 20 000 RPM for 2 min.

[b]The total lipid content of cells expressed as a percentage of dry cellular weight. Lipid quantity was measured by a modified Bligh and Dyer method.

(103%). The result of this experiment demonstrated the effectiveness of the method on different classes of oleaginous microorganisms.

Energetic Cost of Oil Extraction. To determine how this liquid-liquid extraction process fairs in terms of net energy usage, thermodynamic energy calculations were performed. The culture volume processed was normalized to the amount required to produce 1 L of oil from the microorganism in question and depended heavily upon the cell density of the culture and cellular lipid content. For the case of microalgae, 3,000 L of culture at 0.1% solids and 30% (dry weight) TAG would be required to produce 1 L of biodiesel, whereas only 77 L of yeast culture at 2% solids and 60% TAG content would be required for the production of 1 L of biodiesel. The amount of energy used to concentrate each culture to 20% solids was calculated based on the energy requirements of a continuous centrifuge (Clara 80, Alfa Laval, Lund Sweden) with an electrical usage

Table 4-11. Energy balance for oil production process from microbes

	Energy Input[a]	Energy Input/Yield[b]
Evaporation/Dry Cell Extraction		
0.1% Solids (Evaporation, 30% DW Lipid)	7420 MJ	228.00
0.1% Solids (Evaporation, 60% DW Lipid)	3710 MJ	114.00
Wet Cell Extraction		
2% Solids (chloroform extraction, 60% DW Lipid)	69 MJ	2.10
20% Solids (chloroform extraction, 60% DW Lipid)	6.900 MJ	0.21
2% Solids (dichloromethane extraction, 60% DW Lipid)	115 MJ	3.40
20% Solids (dichloromethane extraction, 60% DW Lipid)	11.5 MJ	0.34
2% Solids (*tert*-butyl chloride extraction, 60% DW Lipid)	49.6 MJ	1.50
20% Solids (*tert*-butyl chloride extraction, 60% DW Lipid)	5.0 MJ	0.15
Pretreatment		
Centrifugation [c] (0.1% to 20% Solids, 30% DW Lipid)	13.7 MJ	
Centrifugation [c] (2% to 20% Solids, 60% DW Lipid)	0.34 MJ	

[a] Energy required to produce 1 L of oil using the described extraction method including water removal and solvent removal post extraction. A 100% extraction efficiency is assumed for the evaporation/Dry cell extraction scenarios. In the case of wet cell extractions, an efficiency of 85% is assumed. Energy consumed by mechanical mixing for extraction is assumed to be negligible.

[b] The ratio of the energy input to the energy output obtained from 1 L of oil (32500 kJ). A number larger than 1requires a larger energy input than energy yielded.

[c] Based on the energy consumption of a continuous centrifuge (Clara 80, Alfa Laval) and the amount of cell culture required to produce 1 L of oil.

of 10.1 kWh per 8000 L processed. For wet cell extractions, a conservative 85% extraction efficiency was assumed keeping with the 2:1 solvent to cell slurry ratio results determined as a part of this work. The extraction efficiency is expressed within the energy input to remove each type of solvent from the oil.

The results of this analysis (Table 4-11) show that the use of a wet solvent extraction method could reduce the energy expenses by more than half. In the best case scenario utilizing *tert*-butyl chloride for wet extraction, the removal of water by centrifugation to 20% solids followed by a wet solvent extraction with solvent recovery included in the energy cost would require approximately 18500 KJ of energy to produce 1 L of oil. This is essentially the energy cost of drying the water off of the cells and extracting with solvent.

DISCUSSION

Single cell oil production was introduced as a promising solution to overcome the difficulties of arable land usage and competition for food products associated with the traditional biofuels production of corn ethanol and soybean biodiesel.[30] Initial research into the growth of oleaginous bacteria, microalgae, and yeast organisms for biofuels production has shown an ability to produce biofuels from organisms grown on waste carbon sources with as little as 3.8% of the land used for traditional biofuels from soybeans, 0.3% of the worldwide land availability,[31] and has the potential to offset 100 % of the diesel used worldwide.[32]

Unfortunately the great promise microorganisms possess for fuel production is hampered by the limitations of the processes responsible for obtaining biomass and

extracting the desired compounds from the biomass used to produce biofuels.[5] In the case of microalgal biofuels production, biomass harvesting and lipid extraction can account for a large percentage of the cost of fuel production with 50% of the total fuel production costs coming from the overall process of oil extraction.[33] The high cost of oil extraction is due largely to the aquatic environment in which the organisms grow and produce lipids, and the high energy and monetary cost of removing the cells from this environment. As a result, several research groups have undertaken methods to efficiently remove the lipids from cells while still in their aqueous environment. These experiments have included extractions performed with subcritical mixtures of solvents, [10] supercritical carbon dioxide,[15] mechanical agitation followed by solvent extraction,[11] water miscible solvents,[21] and wet cell fractionation.[34] All of these extraction methods resulted in various amounts of success in the extraction of cellular lipids with all but two [10,21] reaching extraction efficiencies well below that possible with dry cells. Further, many of these processes are complicated by the use of multiple solvents and the requirement of large reactor chambers, heat, and pressure for high throughput processing.

The use of either multiple solvents in the form of a solvent system or a solvent that is fairly soluble in water from wet microbial cells is commonly performed.[10,21] The addition of an alcohol or use of a partially water soluble solvent allows for the increased mixing of the oil containing cells with the solvent, thus allowing for a greater extraction efficiency.[16,35] However, this use of a solvent system or partially water soluble solvent greatly increases the difficulty in downstream processing as the separation of the alcohol

or solvent from the water adds extra energy expense to the solvent recovery and oil purification.

Further, it is important to note that there are several different types of compounds that can be extracted from microbes and not all are convertible directly to fuel compounds.[36] These compounds include, but are not limited to TAG, phospholipids, free fatty acids, sterol compounds, and pigment based compounds *(eg.* Chlorophyll). Of these compounds the general objective is to increase the amount of TAG (and free fatty acids if producing biodiesel) extractable while minimizing the amounts of the other compounds extracted into the oil. Phospholipid compounds can be transesterified to produce biodiesel as has been previously demonstrated,[12] however, the extraction of these compounds from an organism containing these as a majority of the lipids present, as can be done with wet cell fractionation, can be very time and energy expensive when compared to the extraction of cells containing a high percentage of TAG.[34] A focus of this work was to determine how much of the TAG could be removed by a particular solvent as well as how to minimize the energy input required for the lipid extraction so that the extraction would be thermodynamically favorable.

The processes described as part of this work sought to approach the problem of increasing solvent to cell contact from a different angle. Rather than attempting to increase solvent to cell slurry interaction by using solvent systems to change solvent solubilities, a method of increasing cell to solvent contact through physical mixing was devised. If the mixing speed is sufficiently high even two non-compatible liquids, such as an organic solvent and water, can form an essentially homogeneous mixture that would

separate once the mixing ceased. This would allow for the use of a solvent to extract cellular oils without adding the complications of additional solvents and a clean separation would be easily obtainable.

Following this initial concept yeast cells of *C. curvatus*, chosen for their high TAG content and high culture density, [37] were subjected to initial tests with the common solvents chloroform and hexane. When these showed great promise as candidates for wet cell extractions, a process was developed to determine other solvent candidates that could be utilized. Using the modeling program Aspen and a GC method for quantifying TAG in a cell sample both described in the Methods section, Table I was generated showing several solvents that could be utilized for oil extraction from a cell slurry.

From this table, some trends surrounding the solvent's physical properties became evident. Non-polar solvents such as hexane and pentane had very low extraction efficiencies when compared to even slightly more polar solvents tested under the same conditions. This could be due to the increased ability of the compounds substituted with Cl, S, or O atoms to aid in penetrating the phospholipid membrane of the cells and access the available oils inside. To test this possibility, control tests were performed by mixing the cells at 20 000 RPM with no solvent present and then mixing vigorously by shaking with chloroform, methlyene chloride, or *tert*-butyl chloride. The results, seen in Table 4-12, show that when the experiment was performed in this manner approximately half of the TAG extracting efficiency was observed for each solvent type indicating the presence of a solvent while mixing was necessary to obtain higher extraction efficiencies. Not only does this result indicate that the atomic composition of the solvent may be necessary for

Table 4-12. Control Blending Experiments

Solvent	% TAG Extracted[a]
Chloroform	55 ± 2
Methylene chloride	33 ± 3
Tert-butyl chloride	45 ± 2

[a] Amount of the TAG recovered by blending 30 mL of a 20 percent solids cell slurry at 20000 RPM with no solvent present followed by shaking to mix vigorously by hand for 2 min. Number shown is the percent of the total available TAG extracted with the total available TAG being calculated by the modified Bligh and Dyer extraction of dry cells as described in the Methods section.

high extraction efficiency to occur, but that the process of disrupting the cells followed by mixing with a solvent in a process known as cell disruption[38] is not enough to obtain high extraction efficiencies at least in the case of wet yeast cells.

Of the solvents listed in Table 4-1, three were chosen to be optimized for percent solids, solvent to cell slurry ratio, mixing numbers, and mixing time. The solvents chosen were chloroform, methylene chloride, and *tert*-butyl chloride. Chloroform and methylene chloride were chosen because of their low cost, ready availability, high extraction efficiency, and stepwise difference in Cl atom substitution. The solvent *tert*-butyl chloride was chosen because of its low toxicity, low cost, and relatively high extraction efficiency in initial tests. Each of these solvents was subjected to the same conditions for testing with the exception of differences in the Reynolds number for mixing. The Reynolds number depends on the viscosity and density of the solvent. Given the large viscosity and density differences between solvents, to fix the Reynolds number at an

equally high value for all three solvents would be difficult given the constraints of the equipment used. To simplify this, the maximum RPM of the mechanical mixer was used for the condition that would give the highest Reynolds number possible for each solvent and condition. The Reynolds number for each subsequent condition and solvent in the set was fixed from that point and the RPM of the mechanical mixer was adjusted accordingly.

To ensure that multiple freezing and thawing of samples, as was required for sample preparation, did not affect extraction efficiency, samples of fresh cells, frozen once cells, and frozen twice cells were subjected to extraction conditions identical to those described for analysis in Table 4-1. The results showed no significant effect of multiple cycles of freezing and thawing on lipid extraction efficiencies from the cells.

Using each of the three solvents described previously, cell slurries of percent solids ranging from 2 to 40% were tested to determine at what point the optimal lipid extraction would take place. As seen in Figure 4-2A, the extraction efficiency for all three solvents increases with increasing percent solids of the cell slurry until 20% solids at which point the extraction efficiency begins to drop off for each solvent. This is the most exaggerated for the solvent *tert*-butyl chloride which drops off significantly more than either chloroform or methylene chloride. It is hypothesized that this is due to a carrying capacity of each solvent for the TAG compounds present in the cells. This is especially probable due to the high dry weight percentage of TAG in the yeast cells of 61 percent.

If the carrying capacity for the solvent was the reason for the drop off in efficiency as the cell slurry percent solids climbed to 40 percent, then it would stand to reason that increasing the volume of solvent while keeping all other parameters constant would increase the extraction efficiency. As seen in Figure 4-3, this is the case for both chloroform and methylene chloride. Both of these compounds reach a point at which all of the extractable TAG is removed as the ratio of solvent: cell slurry increases to 4:1 and 6:1 respectively. *Tert*-butyl chloride, however, reaches a plateau point at 2:1 and increases little past that point leading to a conclusion that this solvent may have another unknown complication with removing more TAG from the cells besides carrying capacity.

Having established an optimal percent solids and solvent to cell slurry volume for each of the solvents it became necessary to minimize the mixing input. This set of experiments was performed by controlling the RPM of the mechanical mixer with a potentiometer. A fixed set of RPM values was used for all three solvents ranging from 2000 to 20000 RPM. This gave a varying set of Reynolds and Peclet mixing numbers as can be seen in Figure 4-4 and Tables 4-5, 4-6, and 4-7 depending on the solvent used. The resulting extraction efficiencies increased significantly for each solvent from an RPM of approximately 0 to an optimal RPM of 8000 to 10000 depending on the solvent demonstrating that minimal mixing could be obtained that was below the maximum output of the equipment used.

The final parameter investigated was the mixing time required to remove the largest percent TAG possible. Ideally the mixing time would be as short as possible as to

minimize energy inputs. As seen in Figure 4-5, the mixing time varies greatly with each solvent. This could be due at least in part to the differences in boiling points of the solvents. As seen in Table 4-1, the boiling points of chloroform, methylene chloride, and *tert*-butyl chloride are 61°C, 40°C, and 51°C, respectively. The equipment used was unsealed and all experiments were conducted in a fume hood. With a boiling point as low as 40°C, it is possible that enough of the methylene chloride leaks out as vapors during the course of the experiment to give a decrease in extraction efficiency over time. With higher boiling points, it is possible that much less of the chloroform and *tert*-butyl chloride solvents are lost. This is supported by the small decrease seen with chloroform over a 5 minutes extraction versus the much larger decrease seen with *tert*-butyl chloride which has a 10°C lower boiling point. Further experiments with chloroform (data not shown) have demonstrated that after 8 minutes of blending time the extraction efficiency of chloroform decreases to about 60%. Given this information it is possible that if a sealed unit were utilized methylene chloride could give much higher extraction efficiencies. Also, shorter mixing times approaching 30 seconds could be used to minimize the loss of solvent due to evaporation. Other solvents with lower boiling points could be tested in a similar manner and be found to function well as solvents. Overall these experiments indicate that in the case of chloroform and methylene chloride 30 seconds of blending time is more than sufficient to obtain the highest extraction efficiency. *Tert*-butyl chloride requires 2 minutes of mixing time to reach the optimum extraction efficiency.

After testing samples extracted with all three chosen solvents, an analysis of the extracted oil was conducted. This was to determine how the extracted oils compared to commercially available sources of oil in terms of the composition of the lipids and protein content. It is apparent from the GC/FID analysis that the lipid composition is slightly different than commercially available soybean oil in the percentages of sterols, phospholipids, and free fatty acids. The common base catalyzed method for biodiesel production would most likely not be suitable for the oil obtained from these reactions directly given the increased levels of free fatty acids over soybean oil. However, a simple acid pre-treatment could be optimized to remove the free fatty acids and allow for a base catalyzed reaction to occur efficiently.[39]

The protein content of the extracted oils varied little from that of soybean oil with the exception of *tert*-butyl chloride which was more than double the protein content of soybean oil. This demonstrates that very little protein is present that would contaminate and make the biodiesel produced from these extracted oils unsuitable for engine use.

After optimizing the extraction conditions for the yeast organisms, we desired to see how these conditions applied to other organisms. The bacterium *R. opaceus* and the diatom *C. gracilis* were both subjected to lipid extraction using the solvent chloroform and the maximum RPM of the mechanical mixer (20000 RPM), a 2:1, 4:1, and 6:1 solvent to cell slurry ratio, a percent solids of 20 percent, and mixing time of 2 min. The resulting extraction efficiencies as shown in Table 4-10, demonstrate that in the case of the yeast cells and the microalgae cells extraction efficiencies greater than 95% efficiency can be achieved. However, in the case of the bacterial cells only 91% can be achieved.

To further investigate the differences seen in extraction efficiency at a 4:1 ratio between the yeast and bacteria, microwave assisted *in situ* transesterification reactions were conducted as described by Wahlen *et al*.[12] These reactions indicated that the yeast cells contained approximately 71 percent transesterifiable lipids compared to the 62 percent TAG available for extraction as determined by modified Bligh and Dyer extraction. The microalgal cells were similar with approximately 44 percent transestreifiable lipids compared to the 38 percent TAG available for extraction as determined by the modified Bligh and Dyer extraction. These numbers are within an acceptable range as the in situ method removes as much as 10 percent more methyl esters from the cells as available TAG presumably drawing from free fatty acids and phospholipids available in the cells. When the bacterial cells were subjected to the same analysis approximately 38% transesterifiable lipids of the dry weight were obtained compared to the 18% from the modified Bligh and Dyer reaction, indicating that the bacterial cells being gram positive are more difficult to extract lipids from. To attempt to overcome this a higher ratio of chloroform at 6:1 solvent to cell slurry volume was run under the same conditions as mentioned previously for bacterial extraction and approximately 120% of the total extractable lipids as determined form the Bligh and Dyer extraction were removed from the cells (data not shown). This demonstrates that although more difficult to extract with this method, conditions could be found that would allow for a highly efficient extraction of oils from gram positive bacterial cells.

As previously mentioned, the drying and extraction of post harvested microbial cells represents a large percentage of the production costs for microbial oils. To

demonstrate the viability of this process to generate a net energy output in the production of 1 L of microbial oil. Table 4-11 shows the energy calculations for various processes involved in the extraction of lipids from microbes. These calculations take into account two different types of feedstock, the first is a microalgal feedstock that has a concentration of 0.1% solids (1 g/L dry weight) coming from the growth phase with 30 percent TAG in the cellular dry weight. The second shows a yeast culture that contains 2% solids (20 g/L dry weight) coming from the growth phase with 60% TAG in the cellular dry weight. Centrifugation was chosen as the preferred method for cell concentration as it is a readily commercially available process for the harvest of cells. As shown for microalgae at 30% lipid of the dry weight, to complete the standard cell concentration from 0.1% solids to 20% solids followed by evaporation of the remaining water and solvent extraction of the lipids would require 33,680 kJ of energy at a minimum to produce 1 L of oil. This is more energy than could be obtained from the oil, which is approximately 32,500 kJ of energy (unpublished data). Comparing this to the method described as a part of this manuscript, the use of chloroform to extract from 20% solids wet cells requires 6900 kJ of energy giving a total processing energy of approximately 20 600 kJ. The result of utilizing the wet cell extraction gives a net energy production allowing room for biodiesel conversion without the entire process becoming net energy negative.

The process developed as part of this work represents a method capable of quickly removing greater than 95% of the extractable TAG from cells in a water slurry containing 20 percent cell solids by dry weight. The ability to obtain this extraction

efficiency by utilizing only one solvent in a 30 second time period represents a milestone in wet cell extraction technology. Further, the use of this extraction is thermodynamically favorable unlike traditional extraction techniques. As shown in Table 4-11, using microalgae at 30% lipid of the dry weight and 0.1% solids, performing this wet extraction with *tert*-butyl chloride can produce 1 L of biodiesel using approximately 24 000 kJ of energy allowing for a net energy gain from the oil of 8500 kJ. There are improvements that can be made and are being undertaken at this time to remove greater than 95% of the lipids using a continuous extraction process with multiple stages. This would allow for the use of less toxic solvents to obtain the same extraction efficiencies. Overall this work provides understanding of a new wet extraction process and will allow for an increase in the viability of microbial biodiesel production by decreasing the overall energy and economic cost of lipid extraction.

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CHAPTER 5

CHARACTERIAZATION OF A FATTY ACYL-COA REDUCTASE FROM *MARINOBACTER AQUAEOLEI* VT8: A BACTERIAL ENZYME CATALYZING THE REDUCTION OF FATTY ACYL-COA TO FATTY ALCOHOL[1]

ABSTRACT

Fatty alcohols are of interest as a renewable feedstock to replace petroleum compounds used as fuels, in cosmetics, and in pharmaceuticals. One biological approach to the production of fatty alcohols involves the sequential action of two bacterial enzymes: (i) reduction of a fatty acyl-CoA to the corresponding fatty aldehyde catalyzed by a fatty acyl-CoA reductase, followed by (ii) reduction of the fatty aldehyde to the corresponding fatty alcohol catalyzed by a fatty aldehyde reductase. Here, we identify, purify, and characterize a novel bacterial enzyme from *Marinobacter aquaeolei* VT8 that catalyzes the reduction of fatty acyl-CoA by four electrons to the corresponding fatty alcohol, eliminating the need for a separate fatty aldehyde reductase. The enzyme is shown to reduce fatty acyl-CoAs ranging from C8:0 to C20:4 to the corresponding fatty alcohols, with the highest rate found for palmitoyl-CoA (C16:0). The dependence of the rate of reduction of palmitoyl-CoA on substrate concentration was cooperative, with an apparent $K_m \approx 4$ μM, $V_{max} \approx 200$ nmol $NADP^+$ min^{-1} (mg protein)$^{-1}$, and $n \approx 3$. The enzyme also reduced a range of fatty aldehydes with decanal having the highest activity.

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[1] Coauthored by Robert M. Willis, Bradley D. Wahlen, Lance C. Seefeldt, and Brett M. Barney (2011) *Biochemistry* **50** (48) 10550-10558. Copyright American Chemical Society. Reprinted with Permission.

The substrate *cis*-11-hexadecenal was reduced in a cooperative manner with an apparent K_m of ~50 μM, V_{max} of ~8 μmol $NADP^+$ min^{-1} $(mg\ protein)^{-1}$, and n ~2.

INTRODUCTION

Long chain fatty alcohols are widely used in cosmetics and soaps and are of interest as biofuels to substitute for petroleum-derived compounds (*1-4*). Fatty alcohols are produced biologically in plants where they are used as components of the plant cuticle and in bacteria where they are condensed with fatty acids to make wax esters that function as energy storage compounds. In many plants, fatty acyl-CoAs are reduced to the corresponding alcohol either by two consecutive reduction steps (two electrons each) with the formation of the fatty aldehyde intermediate or by a single four-electron reduction (Figure 5-1). In pea leaves (*Pisum sativum* L.), two distinct fatty acyl-CoA reductases (FACoAR) have been identified, with one catalyzing the four-electron reduction to the fatty alcohol and the other the two-electron reduction to the fatty aldehyde (*5*). In the Jojoba plant and *Arabidopsis thaliana*, FACoAR enzymes have been reported that are capable of the four-electron reduction of a long chain fatty acyl-CoA directly to the corresponding alcohol (*6, 7*).

In contrast to the four-electron reductions catalyzed by several plant FACoAR enzymes, the FACoAR enzyme reported for the green algae *Botryococcus braunii* only reduces fatty acyl-CoA substrates to the corresponding fatty aldehydes (*8*). Likewise, the

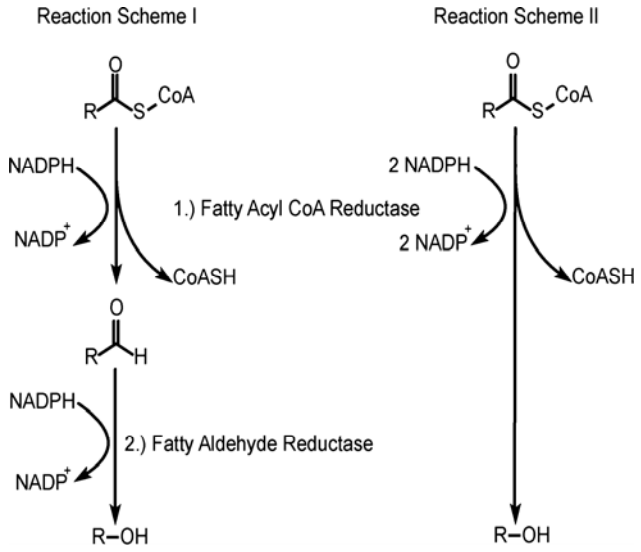

Figure 5-1. Proposed reaction schemes for fatty acyl-CoA reductase. Scheme I shows the two-step reduction mechanism proposed for known bacterial fatty acyl-CoA reductases. Scheme II shows the single-step reduction proposed for the fatty acyl-CoA reductase enzymes of many higher eukaryotes.

bacterial FACoAR from *Acinetobacter calcoaceticus* only reduces fatty acyl-CoA substrates to the corresponding fatty aldehyde (*9*). A recent report identified a FACoAR from the cyanobacterium *Synechococcus elongatus* PCC7942 that catalyzes the reduction of a fatty acyl carrier protein (ACP) to a fatty aldehyde (*10*). The enzyme was named a fatty acyl–acyl carrier protein reductase, as the enzyme had a lower K_m for fatty acyl-ACP versus fatty acyl-CoA,(*10*) which differentiates this from the FACoAR from *A. calcoaceticus* that does not utilize fatty acyl-ACP (*9*).

We recently characterized an enzyme from the marine bacterium *Marinobacter aquaeolei* VT8 that was found to reduce fatty aldehydes to fatty alcohols and had a higher specificity for long chain aldehydes than for shorter aldehydes (*11*). We sought to

also characterize the upstream enzyme in this pathway, a putative FACoAR, from the same organism. The FACoAR from *M. aquaeolei* VT8 is of particular interest because in addition to the C-terminus that shares similarity (74% similar and 53% identical between residues 370 and 660 of *M. aquaeolei* VT8 FACoAR) to the majority of the FACoAR from *A. calcoaceticus* (Figure 5-2) that was shown to reduce fatty acyl-CoA to the fatty aldehyde (*9*), it also contains a separate N-terminal domain that is distinctive from the FACoAR of *A. calcoaceticus*. Here, we report the purification and characterization of the FACoAR from *M. aquaeolei* VT8 and show that this enzyme has broad substrate specificity and catalyzes the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohols, making it unique among the characterized bacterial FACoAR enzymes.

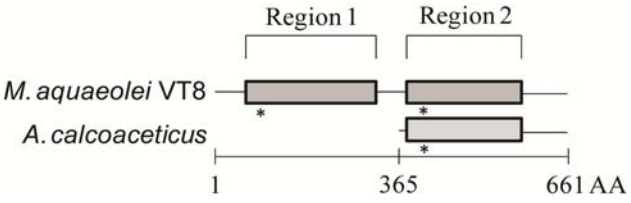

Figure 5-2. Domain arrangement of *M. aquaeolei* VT8 FACoAR compared to *A. calcoaceticus* FACoAR. Schematic domains are shown with the 661 amino acid *M. aquaeolei* VT8 and the 295 amino acid *A. calcoaceticus* sequence. Conserved regions are outlined with the C-terminal domain of the *M. aquaeolei* VT8 enzyme aligning with high similarity (53% identical and 74% similar) to the majority (residues 9 to 295) of the *A. calcoaceticus* enzyme. Denoted by asterisks are the conserved pyridine nucleotide binding regions found in each enzyme, which have the conserved sequence GXGX(1-2X)G.

MATERIALS AND METHODS

Reagents. 5′-Dithiobis(2-nitrobenzoic acid), also referred to as Ellman's reagent or DTNB, was purchased from TCI America (Portland, OR). Coenzymes (NADPH, NADH, $NADP^+$, and NAD^+), various fatty acyl-CoAs, fatty aldehydes and fatty alcohols, and all other reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Cloning and Gene Expression. The protein sequence of the fatty acyl-CoA reductase (FACoAR) from *Acinetobacter calcoaceticus* (ZP_06058153.1)(*9*) was used to perform a BLAST search of the NCBI database for a corresponding gene in *Marinobacter aquaeolei* VT8. The search identified a gene (YP_959769.1) whose protein product contained 661 amino acids with ~50% identity (73% similarity) over a region that corresponded to about 280 residues of the C-terminus of the protein. The gene was cloned by PCR from purified genomic DNA isolated from *M. aquaeolei* VT8 using primers (GACGA***GAATTC***AATTATTTCCTGACAGGCGGCACCGG) and (TCGAC***TCTAGA***CTCCAGTATATCCCCCGCATAATC) and the failsafe PCR kit (Epicenter, Madison, WI). The PCR product was ligated into the *Eco*RI and *Xba*I sites of a pUC derivative plasmid. The entire cloned insert was sequenced to confirm that no mistakes were introduced. The gene was then moved to a pMAL-c4x plasmid derivative (New England Biolabs, Ipswich, MA) containing an insert for incorporation of an 8X His-tag following the in-frame insertion after the *Xba*I site. This resulted in the final plasmid pPCRMALD8 that contains the FACoAR from *M. aquaeolei* VT8 with an N-terminal maltose binding protein (MBP) fusion and a C-terminal His-tag. This construct contains a Factor Xa cleavage site immediately following the MBP protein to facilitate

removal of the maltose binding protein following purification. The plasmid was transformed into the *E. coli* TB1 strain (New England Biolabs, Ipswich, MA) for protein expression.

Protein was expressed by growing 1 L cultures in Luria–Bertani broth (LB) supplemented with 100 mg/L ampicillin from an 8 mL starter culture. The culture was grown with shaking at 37 °C until the culture reached an optical density of ~0.6 at 600 nm. Protein expression was induced by the addition of 50 mg/L of isopropyl-β-thiogalactopyranoside (IPTG), and the culture was grown for 3–4 h before harvesting by centrifugation. Collected cell pellets were frozen and stored at −80 °C.

Protein Purification. Cell pellets of ~5 g were resuspended in 30 mL of lysis buffer composed of 20 mM Tris-HCl pH 7.0, 50 mM NaCl, and 1 mM EDTA. The resuspended cells were placed in a 50 mL conical tube and placed in a water–ice mixture to keep the cells cold during lysis. The cells were passed through a French pressure cell three times at 1000 lb/in^2. Whole cell lysate was centrifuged at 10000*g* for 20 min to separate the cell debris from the soluble extract.

Soluble cell lysate was passed over an amylose column (P/N E8201L, New England Biolabs, Ipswich, MA) to bind the fusion protein and washed with 3 column volumes of lysis buffer, followed by a wash step with 2 column volumes of lysis buffer supplemented with 1.0 M NaCl to interrupt nonspecific binding. The column was then washed with 3 column volumes of equilibration buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl), and the bound protein was eluted with 2 column volumes of equilibration buffer supplemented with 10 mM maltose. The relative protein concentrations of the collected

fractions were determined by Nanodrop (Thermo Scientific, Wilmington, DE). Fractions containing significant amounts of protein were then pooled and added to a metal affinity column (P/N 17-0575-01, GE Healthcare, Upsalla, Sweden) charged with nickel. The metal affinity column was washed with 3 column volumes of equilibration buffer followed by a wash with 2 column volumes of equilibration buffer supplemented with 85 mM imidazole to disrupt nonspecific binding. The column was eluted with 2 column volumes of a 500 mM imidazole solution in equilibration buffer. Resulting fractions were analyzed on a 12% SDS-PAGE gel. Fractions containing a purified protein whose migration in the SDS-PAGE was consistent with a 116 kDa protein, according to the protein marker, were pooled and applied to a G25 Sephedex column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with equilibration buffer. Desalted fractions were flash frozen and stored in liquid nitrogen. Protein concentration was determined using the Pierce BCA protein concentration assay kit (Thermo Fisher Scientific, Rockford, IL).

Initial Activity Assays. Initial activity assays were conducted using thin layer chromatography (TLC) and a gas chromatography (GC) assay similar to that described previously (11). To test activity, 0.3 mg of protein was added to a reaction vessel along with 200 μM palmitoleyl-CoA and 800 μM NADPH, NADH, $NADP^+$, or NAD^+ in reaction buffer containing 20 mM Tris-HCl, pH 7.0, and 50 mM NaCl. Reactions were allowed to proceed for 1 h before extraction with 2 mL of hexane. The hexane water mixture was vortexed vigorously for 30 s before phase separation by centrifugation. The hexane phase was removed to a clean container, and the solvent was removed under a

stream of argon gas. The resulting residue was resuspended in 100 µL of hexane and spotted on a TLC silica plate along with 5 µL each of palmitoleyl alcohol (10 mg/mL) and *cis*-11-hexadecenal (10 mg/mL) standards. Any unreacted palmitoleyl-CoA substrate would partition in the water phase and would not appear on the TLC plate. The TLC plate was developed in a 2:15:90 volumetric ratio of glacial acetic acid:ethyl ether:hexane. After development, visualization was performed in a sealed jar with iodine crystals for 10 min. The TLC results were verified by GC/MS analysis of the sample prepared in the same way. GC/MS results were compared to retention times and mass spectrum of known standards.

The GC/MS analysis was performed using a Shimadzu GCMS-QP2010S. A 1 µL sample was injected onto a Stabilwax-DA column (30 m × 0.25 mm i.d. with 0.25 µm film thickness (Restek, Belfonte, PA)) and run using a temperature program of 100 °C for 1 min, followed by a temperature gradient of 10 °C/min to 235 °C and held at the final temperature for 10 min. Helium gas was used as the carrier and flow was controlled by maintaining a constant velocity of 50 cm/min. Mass spectra obtained were compared to the National Institute of Standards and Technology (NIST) database 05 for peak identification.

Continuous Spectrophotometric NADPH Assay. All assays were conducted in a total volume of 1 mL. A buffer containing 50 mM NaCl, 20 mM Tris-HCl pH 7.0, and 0.5 mg/mL bovine serum albumin was prepared along with a 1 mM stock of aldehyde dissolved in dimethyl sulfoxide (DMSO) or a 0.1 mM stock of the acyl-CoA and a 2.0 mg/mL stock of NADPH. All components including protein were degassed in sealed

vials and placed under an argon atmosphere. NADPH was degassed as the solid prior to the addition of degassed buffer. Each assay was conducted by adding 75 μL of the NADPH stock, 58 μg of protein for acyl-CoA assays or 15 μg of protein for aldehyde assays, varying concentrations of aldehyde or acyl-CoA, and buffer to bring the final volume to 1 mL. Each sample was continuously monitored for the decrease of NADPH at 340 nm on a Varian 50 Bio UV–vis spectrophotometer (Walnut Creek, CA). Initial rates were calculated in Excel (Microsoft, Redmond, WA) using the linear initial rates of reaction obtained from the spectrophotometric assays by obtaining the slope from the best fit line and calculating nmol of NADPH oxidized per second. These initial rates were used to calculate the apparent K_m and V_{max} values using the Igor Pro software package (Wavemetrics, Lake Oswego, OR) fitting the initial rates to the Hill equation (*12*). NADPH specific activity assays were conducted identically as described above using a fixed 60 μM concentration of the various aldehyde substrates or 5 μM of the various acyl-CoA substrates.

Continuous Spectrophotometric DTNB Assay. Buffers and solutions were prepared as described above for NADPH assays. Each assay was conducted by adding 75 μL of the 2 mg/mL NADPH stock solution in buffer, 58 μg of protein, and 10 μL of a 10 mg/mL solution of DTNB in DMSO, varying concentrations of acyl-CoA and buffer to bring the volume to 1 mL. Reduction of acyl-CoA substrate was monitored by following the increase of the 2-nitro-5-thiobenzoate (NTB^{2-}) dianion concentration at 412 nm. Initial rates were calculated in Excel (Microsoft, Redmond, WA) using the linear initial rates of reaction obtained from the spectrophotometric assays by obtaining the slope from

the best fit line and calculating nmol of NTB^{2-} dianion formed per second using the extinction coefficient of the NTB^{2-} dianion of 14 150 M^{-1} cm^{-1} (*13, 14*). These initial rates were used to calculate the apparent K_m and V_{max} values using the Igor Pro (Wavemetrics, Lake Oswego, OR) software package fitting the initial rates to the Hill equation (eq 1) where v is the initial velocity, V_{max} is the maximum calculated velocity, $[S]^n$ is the concentration of substrate, n is the Hill coefficient, and $K_{0.5}{}^n$ is the approximation of K_m or the approximate substrate concentration at which half of V_{max} is obtained at a specific value of the Hill coefficient n. In the case of enzyme inhibition, a modified version of the Hill equation allowing for cooperative inhibition (eq 2) where all of the coefficients are defined as for the Hill equation and the additional $[i]^n$ is the concentration of inhibitor and n is the Hill coefficient. The K_i^n is the approximate concentration of inhibitor it takes to double the $K_{0.5}$ at a specific value of the Hill coefficient n (*12, 15*).

$$v = \frac{Vmax[S]^n}{K_{0.5}^n + [S]^n} \qquad (1)$$

$$v = \frac{V_{max}[S]^n}{K_{0.5}^n\left(1+\frac{[i]^n}{K_i^n}\right)+ [S]^n} \qquad (2)$$

pH Studies. Optimal pH was determined by assaying over a range of pH values from 5.5 to 9.0. A buffer composed of 50 mM MES, 50 mM MOPS, 50 mM TAPS, 150 mM NaCl, 0.5 mg/mL BSA was made, and the pH was adjusted by adding either NaOH or HCl. Assays were conducted using the NADPH continuous spectrophotometric assay described above.

Verification of Activity without MBP Tag. To determine activity of the enzyme with the maltose binding protein (MBP) removed, 500 μg of protein was digested with 10 units of Factor Xa (New England Biolabs, Ipswich, MA), according to the protocol provided by New England Biolabs. The resulting protein was used in a set of assays conducted according to the DTNB and NADPH protocols described above. The kinetic curves produced were compared to the established curves.

Determination of Quaternary Structure. Three milligrams of purified protein was exchanged into a buffer containing 150 mM NaCl and 20 mM Tris-HCl pH 7.0. This protein was loaded onto a size exclusion column (High Load 2660 Superdex 200 GE Healthcare) equilibrated with a buffer containing 20 mM Tris-HCl pH 7.0 and 150 mM NaCl along with standards of known native molecular weight using the GE high molecular weight standard kit (General Electric Healthcare, Uppsala, Sweden) to determine the size of the resulting protein and run at 0.7 mL/min flow rate, and protein elution was monitored continuously at 280 nm.

RESULTS

The putative FACoAR from *M. aquaeolei* VT8 was cloned as described in the Materials and Methods section following an approach similar to that used to clone and isolate active fatty aldehyde reductase (FALDR) from *M. aquaeolei* VT8 (*11*). The only variation in the approach taken here was the addition of a C-terminal 8X His-tag to the N-terminal maltose binding protein-FACoAR fusion protein. This approach allowed a rapid two-step purification of the enzyme using amylose affinity and metal affinity chromatography (Figure 5-3). The migration of the protein on an SDS gel agreed well

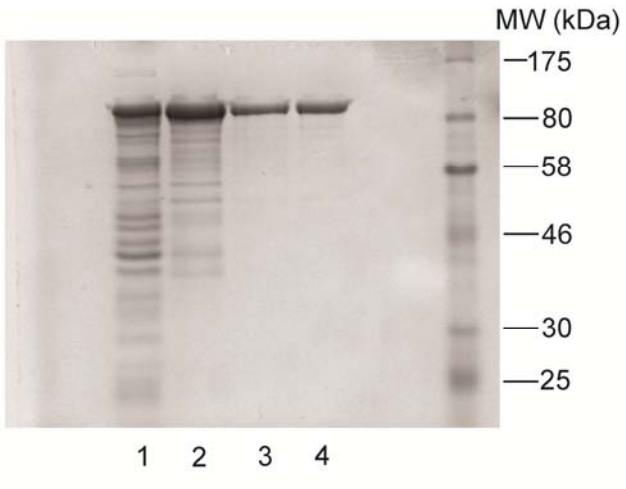

Figure 5-3. Purification of the FACoAR. Shown is an SDS-PAGE of the purification scheme of the FACoAR from *M. aquaeolei* VT8 expressed from *E. coli*. A protein of approximately 116 kDa is obtained after each affinity purification step. Lane 1 contains the soluble cell free lysate. Lane 2 contains the elution from the amylose affinity resin. Lane 3 contains the elution from metal affinity resin charged with nickel. Lane 4 contains the elution from the G25 sephadex column. The far right lane contains the protein standards. Protein is >95% pure following the G25 sephadex column purification.

with the molecular mass of 116 kDa predicted from the amino acid sequence. Previous attempts to purify FACoAR proteins encountered solubility problems and resulted in inactive enzymes, similar to what was found for the FALDR (*9, 11*). By expressing the FACoAR from *M. aquaeolei* VT8 as a fusion with the maltose binding protein, the FACoAR was soluble and active.

An initial assessment of fatty acyl-CoA reduction activity was achieved by utilizing thin layer chromatography (TLC) to determine substrate and product identities

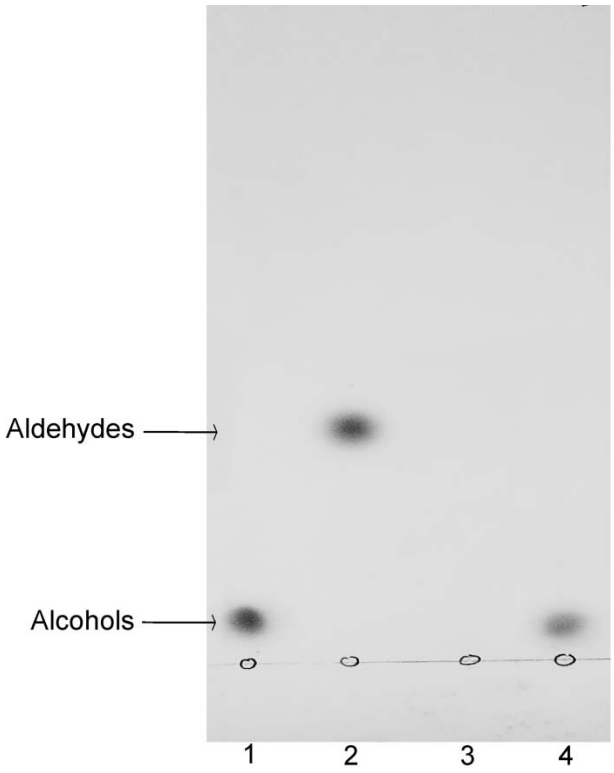

Figure 5-4. TLC plate of fatty acyl-CoA reductase products of reaction. Lane 1 contains 5 μL of a palmitoleyl alcohol (10 mg/mL in hexane) standard. Lane 2 contains 5 μL of a *cis*-11-hexadecenal (10 mg/mL in hexane) standard. Lane 3 contains heat inactivated FACoAR from *M. aquaeolei* VT8 incubated with palmitoleyl-CoA and NADPH and extracted as described in the Materials and Methods. Lane 4 is identical to lane 3, except that the FACoAR was not heat inactivated. Samples were allowed to react for 1 h with gentle shaking at room temperature before extracting with hexane and spotting on TLC. The solvent front is the top of the image. The drawn circles indicate the point at which the samples were blotted before developing the TLC plate.

and requirements for reductant. These initial assays established that reduction of a fatty acyl-CoA substrate by FACoAR required NADPH. When NADH was substituted for NADPH, no reaction product was detected. Further, while fatty acyl-CoAs were a substrate, no detectable reduction was found with free fatty acids. The TLC analysis revealed exclusively the fatty alcohol product, with no aldehyde detected (Figure 5-4).

The products of the FACoAR reduction of acyl-CoA were next analyzed by gas chromatography with flame ionization detection (GC/FID). Product identities were also confirmed by gas chromatography with detection by mass spectrometry (GC/MS). These assays verified that the only detectable product from palmitoleyl-CoA reduction by FACoAR was palmitoleyl alcohol. The requirement for NADPH agrees well with the findings by Reiser and Somerville for the FACoAR from *A. calcoaceticus*, which was also found to utilize NADPH as the reductant (*9*), while eukaryotic FACoAR enzymes have been reported to use either NADPH for Jojoba (*Simmondsia chinensis*) and honey bee (*Apis mellifera*) or NADH for the unicellular protist *Euglena gracilis* (*6, 16, 17*).

To further examine the activity of the FACoAR, two real-time, spectrophotometric assays were developed. In one, the release of free CoA from reduction of an acyl-CoA was monitored by reaction of the CoA with Ellman's reagent (DTNB), which results in the production of the NTB^{2-} dianion that absorbs at 412 nm. In the second assay, the oxidation of NADPH was monitored at 340 nm. By using either the NADPH oxidation or the CoA release assay, the activity of the enzyme could be followed with a range of substrates. Optimal activity was found for the enzyme at pH 7.0, with activity falling dramatically below pH 6.5 and above 9.0 (data not shown). Treatment of

the FACoAR with the factor Xa protease to remove the maltose binding fusion protein did not result in a significant change in enzyme activity versus an uncleaved control (Figure 5-5). All further assays were done with the intact maltose binding protein–FACoAR fusion.

The fatty acyl-CoA substrates used in these experiments have relatively low critical micelle concentration (CMC) values (*18*). The CMC values depend on the buffer ionic strength, temperature, and protein concentration present. For fatty acyl-CoA substrates, the generally accepted ranges for CMC values are between 10 and 40 μM. All specific activities were determined using fatty acyl-CoA concentrations below the published CMC values (*18-20*). Experiments were conducted with higher concentrations of several fatty acyl-CoA substrates, resulting in the expected lower activity with higher substrate concentration (data not shown).

Figure 5-6 shows the specific activity for palmitoyl-CoA reduction versus the concentration of substrate, tracking either the formation of NTB^{2-} (the chromophore produced following reaction of DTNB with the free thiol of CoA) or the oxidation of NADPH. The data in both assays were best fit to a sigmoidal curve, indicating possible allosterism or cooperativity. Fitting the data to the Hill equation (*12*), an apparent K_m of ~4 μM was obtained for the reduction of palmitoyl-CoA with a value of n ~2.8. The V_{max} was calculated to be ~115 nmol CoA released min^{-1} mg $protein^{-1}$ or ~197 nmol $NADP^+$ min^{-1} mg $protein^{-1}$, suggesting that the enzyme was catalyzing the four-electron reduction of the acyl-CoA to the corresponding alcohol. In contrast to this FACoAR from *M. aquaeolei* VT8, Reiser et al. reported levels of activity for the *A. calcoaceticus*

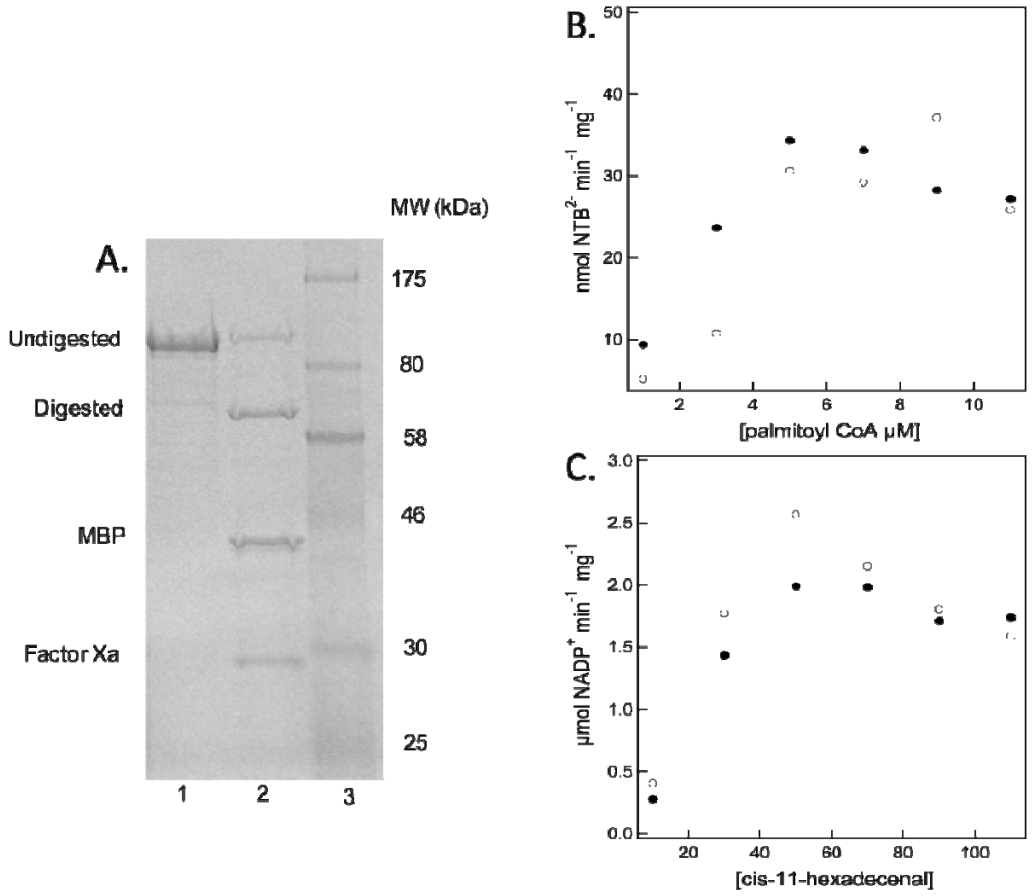

Figure 5-5: Factor Xa digest of fatty acyl CoA reductase from *M. aquaeolei* VT8. A. Shows the SDS-PAGE gel of the FACoAR digest with lane 1 containing the undigested intact Maltose Binding Protein (MBP) fusion with FACoAR, lane 2 containing the fusion protein digested with Factor Xa protease in a reaction for 8 hr, and lane 3 containing the protein marker with MW in kDa as indicated. **B**. Shows the DTNB assay of the enzyme using palmitoyl-CoA. The control samples are denoted by (○) and the digested samples are denoted by (●). **C**. Shows the NADPH assay of the enzymes using cis-11-hexadecenal with control and digested samples denoted by the same symbols as in B. All assays were done with a single sample.

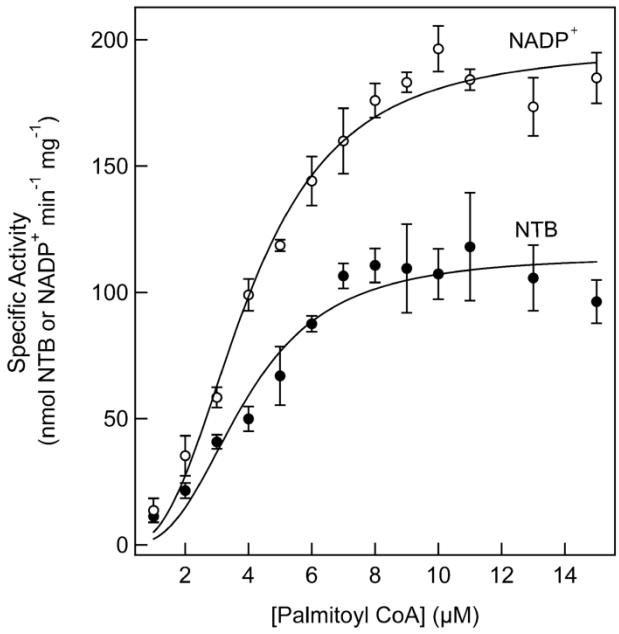

Figure 5-6. Kinetic parameters of fatty acyl-CoA reductase from *M. aquaeolei* VT8 indicating direct production of palmitoyl alcohol from palmitoyl CoA. The (●) indicate points for the DTNB assay measuring the release of free CoA. V_{max} of 115 ± 7 nmol NTB $^{-2}$min $^{-1}$ mg of protein $^{-1}$, apparent K_m of 4 ± 0.3 μM, and a n of 2.8 ± 0.7. The (○) indicate points for the NADPH assay measuring the enzymatic utilization of NADPH. V_{max} of 197 ± 8 nmol $NADP^+$ min^{-1} mg of protein $^{-1}$, apparent K_m of 4.0 ± 0.2 μM, and n of 2.6 ± 0.3. All kinetic parameters were calculated using a triplicate data set with Igor Pro software shown with error bars representing the standard error of the mean (SEM) fit to eq 1 in the Materials and Methods.

FACoAR of ~0.1 pmol min^{-1} μg $protein^{-1}$ (0.1 nmol min^{-1} mg $protein^{-1}$), although this was the activity reported for the unpurified cell extract, as purification was not achieved with their enzyme (*9*).

The above data indicate that the *M. aquaeolei* VT8 FACoAR catalyzes the four-electron reduction of an acyl-CoA to the corresponding alcohol, and thus the reaction is expected to pass through the two-electron reduced aldehyde intermediate. In an attempt to trap a possible aldehyde intermediate, assays were performed in the presence of phenylhydrazine or hydrazine using an approach similar to that taken to isolate aldehyde intermediates from crude preparations of *E. gracillis* FACoAR (*17*). Assays were conducted in the same manner as described in the Materials and Methods section for both NADPH and DTNB spectrophotometric assays, except that 5 mM phenylhydrazine or hydrazine was included in the buffer to potentially trap any free aldehyde. The results showed little difference between either the initial rate of NADPH oxidation or the total quantity of NADPH consumed between the sample with hydrazine and the control run without hydrazine (data not shown). To verify the reaction of hydrazine, samples were prepared with fatty aldehyde as substrate in the presence of 5 mM hydrazine or phenylhydrazine, and the reaction rate was measured by the oxidation of NADPH. Approximately 90% inhibition was measured in the samples containing hydrazine compared to the control without hydrazine. The lack of inhibition by hydrazine of the fatty acyl-CoA reduction to fatty alcohol indicates that a free fatty aldehyde intermediate is not accessible to hydrazine and likely is remaining bound to the enzyme and is therefore inaccessible to hydrazine reaction during the time frame of the assay.

The carbon length preference of fatty acyl-CoA substrates for the FACoAR from *M. aquaeolei* VT8 was also determined by examining the rates of substrate reduction for a series of fatty acyl-CoA molecules (Table 5-1). These results show palmitoyl-CoA as

Table 5-1. Specific Activity toward Acyl-CoA Substrates

Substrate	**Carbon Chain Length**	**Specific Activity (nmol NTB min^{-1} mg^{-1})[a]**	**% Specific Activity of Palmitoyl-CoA Reduction[b]**
octanoyl-CoA	C8	15 ± 1	9
lauroyl-CoA	C12	34 ± 9	26
myristoyl-CoA	C14	34 ± 3	60
palmitoyl-CoA	C16	57 ± 6	100
palmitoleyl-CoA	C16:1	57 ± 3	99
stearoyl-CoA	C18	40 ± 2	69
oleyl-CoA	C18:1	44 ± 6	78
arachidonoyl-CoA	C20:4	49 ± 9	86

[a] Reactions were performed as described in the Materials and Methods section using 5 μM of the respective CoA and are reported as nmol of the NTB^{2-} anion produced per min per mg of fatty acyl-CoA reductase protein.

[b] All values are reported as a percent of the specific activity for the reduction of palmitoyl-CoA by the fatty acyl-CoA reductase enzyme.

the best substrate. Good rates of reduction for longer (C20:4) and shorter (C8) fatty acyl-CoA groups were also found. These activities contrast with the results reported for the FACoAR from *A. calcoaceticus*, which showed significantly lower activity with substrates greater than C18 and no detectable activity with substrates smaller than C14 (*9*).

In addition to determining whether this FACoAR from *M. aquaeolei* VT8 could reduce fatty acyl-CoA substrates, the ability of the enzyme to reduce fatty aldehydes to the corresponding alcohol was determined. Such activity would be similar to the activity of the FALDR from this same species (*11*). This activity was measured using the previously described spectrophotometric assay to track the disappearance of NADPH but utilized a range of fatty aldehydes as substrates (Table 5-2). The best substrate was decanal (C10), with lower rates observed for shorter (to C6) and longer (to C16) aldehydes. Figure 5-7 shows the specific activity as a function of the concentration of *cis*-11-hexadecenal. Again, the products were analyzed by both TLC and GC/MS. A fit of these data to the Hill equation reveals an apparent K_m ~50 μM and V_{max} of ~8 μmol min^{-1} mg $protein^{-1}$, with cooperativity indicated by n ~1.9. The specific activity is significantly higher than the activity found for the recently characterized FALDR enzyme from *M. aquaeolei* VT8 (*11*) and more than 100-fold higher than the specific activity for reduction of palmitoleyl-CoA substrate (Table 5-1). Thus, the FACoAR described here can reduce either fatty acyl-CoA or fatty aldehyde substrates to the corresponding fatty alcohols.

For each of the fatty acyl-CoA substrates analyzed as part of this work (see Table 5-1), the quantity of NADPH oxidized during the reaction was found to be approximately twice the amount of NTB^{2-} (product of the reaction with DTNB and free CoA) produced. This result indicates that any fatty aldehyde formed from the reduction of fatty acyl-CoA is immediately further reduced to the fatty alcohol.

As is seen in Figures 5-6 and 5-7, the activities of the FACoAR from *M. aquaeolei* VT8 do not follow standard Michaelis–Menten kinetics. Instead, sigmoidal kinetics are observed that are best fit to the Hill equation. Cooperativity is observed with other enzymes that utilize acyl-CoA substrates and are active in the synthesis of lipid compounds. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductases from human, mammalian, and bacterial sources alike have been shown to demonstrate similar kinetic behavior (*21-23*).

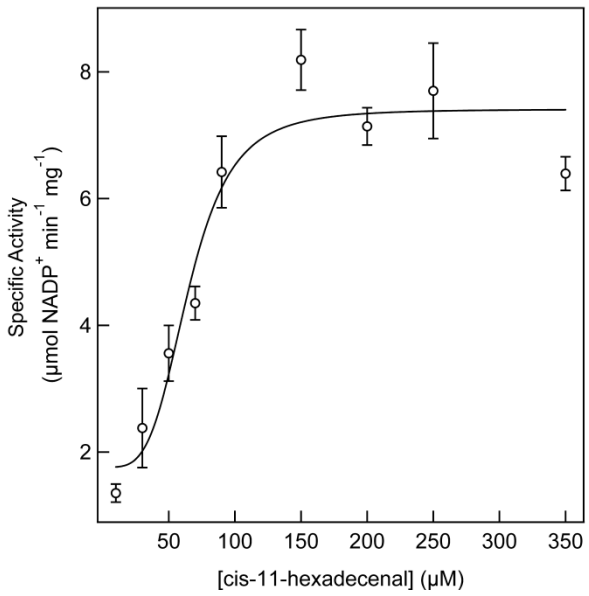

Figure 5-7. Kinetic parameters of fatty acyl-CoA reductase from M. Aquaeolei VT8 showing reactivity toward cis-11-hexadecenal. NADPH assay measuring the decrease in absorbance at 340 nm. V_{max} of 7.7 ± 0.6 μmol min^{-1} mg of protein $^{-1}$, apparent K_m of 48 ± 7 μM, and a n of 2 ± 0.8. Data shown with SEM. Calculated using Visual Enzymics and Igor pro Software fit to eq 1 in the Materials and Methods.

Table 5-2. Specific Activity toward Aldehyde Substrates

substrate	**carbon chain length**	**specific activity (nmol $NADP^+$ min^{-1} mg^{-1})[a]**	**% specific activity of decanal reduction[b]**
acetaldehyde	C2	9 ± 1	<1
propanal	C3	2 ± 1	<1
hexanal	C6	1200 ± 60	15
octanal	C8	3600 ± 670	45
decanal	C10	8000 ± 630	100
dodecanal	C12	7500 ± 570	93
cis-11-hexadecenal	C16:1	6300 ± 200	79
2-naphthaldehyde		460 ± 90	6

[a] Reactions were performed as described in the Materials and Methods section using 60 μM of the respective aldehyde and are reported as nmol of $NADP^+$ produced per min per mg of fatty acyl-CoA reductase protein.

[b]All values are reported as a percent of the specific activity of decanal reduction by the fatty acyl-CoA reductase enzyme.

As a part of the characterization of the *M. aquaeolei* VT8 FACoAR, we probed the nature of the sigmoidal kinetics shown in Figures 5-6 and 5-7 by attempting to alleviate the sigmoidal response of activity as a function of substrate concentration by the addition of reducing agents and detergents, which have been shown to affect the cooperativity in the enzyme HMG-CoA reductase (*24*). The use of detergents (isopropanol, Triton X-100) and reducing agents (β-mercaptoethanol) at varying

concentrations failed to alleviate the sigmoidal character of the kinetics of the *M. aquaeolei* VT8 FACoAR and in each case inhibited activity (data not shown). This seems to indicate the potential of some inter- or intra-protein interaction that is necessary for activity to occur.

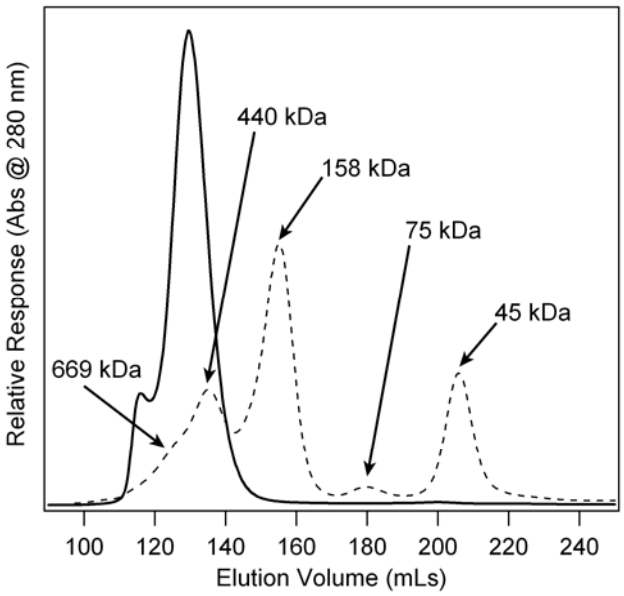

Figure 5-8. Native Protein Size Determination**.** Shown is the chromatogram of the FACoAR from *M. aquaeolei* VT8 run on a size exclusion column. A set of standards (dashed line) are overlaid and were used to determine potential oligomerization of the purified protein. Fractions were taken to determine activity of the enzyme for the early fractions versus the primary band at about 130 mL. SDS-PAGE analysis of the fractions showed a single subunit of high purity in all fractions, indicating that the majority of the protein is likely a homotetramer, with a small fraction of the protein existing as inactive higher oligomers.

To further investigate this cooperativity, the protein was analyzed by size exclusion chromatography to determine the native size of the protein using a Superdex 200 size exclusion column (Figure 5-8). The protein traveled through the column as a single peak with a small amount of contaminating higher oligomers or aggregates traveling just ahead as a shoulder on the main peak. When compared to specific molecular weight standards, the primary peak ran with an apparent size ~4 times that of the predicted monomeric state, indicating a possible tetrameric form of the enzyme. When collected fractions were run on an SDS gel, all fractions were shown to contain only the single pure monomer of the FACoAR protein, yet only the fraction corresponding to the possible tetrameric state showed the highest levels of activity, while the higher contaminating oligomers were significantly lower in activity. This indicates that the enzyme likely requires a higher oligomeric state (tetramer) for activity but loses activity upon aggregation. These findings support the hypothesis that both the fatty acyl-CoA reduction and further fatty aldehyde reduction are performed by the same enzyme and are not the result of two different proteins, as the activity for both reactions remained constant through each step of the protein preparation.

To further probe the hypothesis that both fatty acyl-CoA reduction and fatty aldehyde reduction are performed by the same enzyme, and not the result of a contaminating protein, inhibition experiments were devised. Since the specific activity for fatty acyl-CoA is lower (100-fold lower) than the specific activity for aldehyde reduction, the addition of fatty acyl-CoA would be expected to inhibit fatty aldehyde reduction in a concentration-dependent manner. Further, if both substrates are utilizing

the same enzyme (or potentially even the same active site), then the substrates would be expected to act as reversible inhibitors of one another and should shift the apparent K_m, but not change the V_{max}. In fact, it is observed that the addition of increasing concentrations of palmitoyl-CoA (1, 2, and 4 μM) significantly lowered the enzymatic activity for aldehyde reduction with an increase in the apparent K_m (Figure 5-9) and was estimated based on a fit using eq 2 to have a K_i of ~2 μM. This inhibition could be overcome with the addition of higher concentrations of aldehyde substrate, suggesting competition within the same enzyme by the palmitoly-CoA.

The inverse inhibition experiment tracking the effect of added fatty aldehyde on fatty acyl-CoA reduction using the DTNB assay (which is specific for tracking CoA release) was also of interest to determine if fatty aldehyde addition would alter the apparent K_m for the fatty acyl-CoA. However, concentrations of fatty aldehyde required (greater than 150 μM to saturate the enzyme) for this experiment resulted in elevated background interference of the aldehyde with the DTNB reagent, making it difficult to have sufficient confidence in the results obtained.

DISCUSSION

The fatty acyl-CoA reductase (FACoAR) from *A. calcoaceticus* (ZP_06058153.1) was the first FACoAR described in a bacterium (*9*) and was utilized as an initial template to search for a similar enzyme in *Marinobacter aquaeolei* VT8. An enzyme from *M. aquaeolei* VT8 was found (YP_959769) with amino acid sequence similarity to the FACoAR from *A. calcoaceticus*, although the *M. aquaeolei* VT8 enzyme appears to have an additional N-terminal domain. The first domain on the N-terminal end of this enzyme

shares a very slight sequence similarity (41% similar and 22% identical over the region between residues 4 and 145) with the fatty aldehyde reductase (FALDR) previously characterized from *M. aquaeolei* VT8 (*11*), while the second domain on the C-terminal

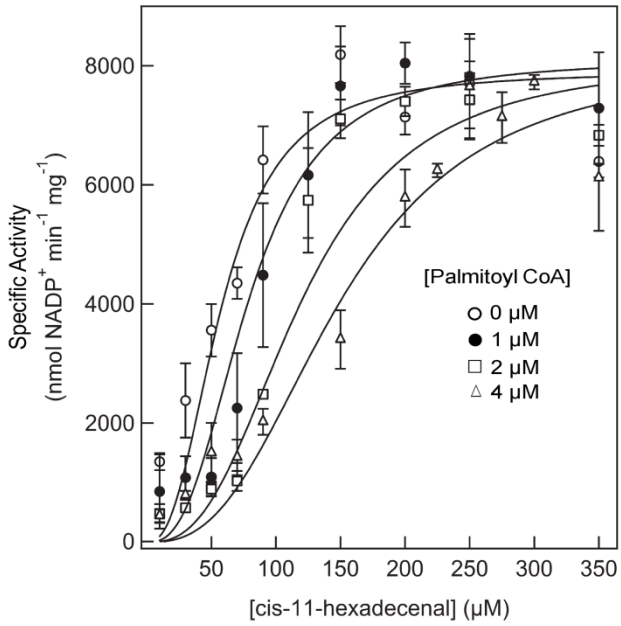

Figure 5-9. Palmitoyl CoA inhibition of fatty aldehyde reduction. The reduction of the fatty aldehyde *cis*-11-hexadecenal is inhibited in a competitive manner with an apparent K_i of 1.9 ± 0.26 μM palmitoyl-CoA. The (○) denote assays performed with 0 μM palmitoyl-CoA, (●) denote assays performed with 1 μM palmitoyl-CoA, (□) denote assays performed with 2 μM palmitoyl-CoA, and (△) denote assays performed with 4 μM palmitoyl-CoA. Specific activities are plotted in units of nmol $NADP^+$ min^{-1} mg $protein^{-1}$. All assays were conducted in triplicate and are shown with error bars indicating SEM fit to eq 1 in the Materials and Methods section. The apparent K_i was determined by fitting all four data sets to eq 2 in the Materials and Methods.

end (beginning at ~375 residues and proceeding to the end of the protein) aligns well (74% similar and 53% identical) with the FACoAR from *A. calcoaceticus* (Figure 5-2). These similarities are consistent with the *M. aquaeolei* VT8 FACoAR enzyme catalyzing the four-electron reduction of a fatty acyl-CoA substrate to the corresponding fatty alcohol as reported here, making this bacterial enzyme more similar in reactivity to the FACoAR described from the plant Jojoba (*6*). Although the *M. aquaeolei* VT8 FACoAR does not align well with known acyl-CoA reductases from plants, a high sequence similarity for the entire sequence to a range of proteins from many other bacteria was noted, including enzymes found in lipid accumulating bacteria such as *Rhodococcus* (*25, 26*), *Alcanivorax* (*27*), and several strains of *Mycobacterium* (*28*).

The FACoAR from *M. aquaeolei* VT8 was found to catalyze the NADPH-dependent reduction of fatty acyl-CoA substrates ranging from 8 to 20 carbons in length (both saturated and unsaturated) as well as fatty aldehyde substrates to the corresponding fatty alcohol. The *M. aquaeolei* VT8 FACoAR activity is significantly higher than what was reported for FACoAR from *A. calcoaceticus* (*9*). The *M. aquaeolei* VT8 FACoAR also exhibited an independent fatty aldehyde reductase activity, which was not reported for the *A. calcoaceticus* FACoAR enzyme. The *M. aquaeolei* VT8 FACoAR only acted on fatty acyl groups bound to CoA and did not react with free fatty acids directly.

The FALDR from *M. aquaeolei* VT8 characterized previously in our laboratory (*11*) shares a minimal sequence similarity with the CER4 protein from *Arabidopsis* (*7*) and also shares a very minimal similarity with the N-terminal domain for the FACoAR described here. The FALDR enzyme was previously tested against a range of fatty

aldehydes and for the same substrate as shown in Figure 5-7 (*cis*-11-hexadecenal) was found to have a K_m of 177 μM and a V_{max} of ~60 nmol min^{-1} mg $protein^{-1}$(*11*). From these results, the FACoAR described here appears to have a lower apparent K_m and a much higher V_{max} overall than was reported for the *M. aquaeolei* VT8 FALDR for fatty aldehyde reduction. To verify that the FALDR previously isolated from *M. aquaeolei* VT8 (11) did not have activity toward fatty acyl-CoA substrates, it was subjected to the same DTNB and NADPH assays described here for the FACoAR. Activity was confirmed for fatty aldehyde reduction as previously reported, but no activity was detected (<1.0 nmol min^{-1} mg^{-1}) for fatty acyl-CoA reduction, indicating a clear difference for the substrate profiles of these two enzymes.

M. aquaeolei VT8 is a member of the hydrocarbonoclastic bacteria, a group of cosmopolitan oil-degrading marine bacteria that have a narrow substrate range that includes alkanes, polycyclic aromatic hydrocarbons, and small organic acids (*29*). Hydrocarbon degradation would require a range of enzymes capable of oxidizing alkanes to serve as a source of energy, and thus the FACoAR could presumably be involved in degradation pathways in addition to production of fatty alcohol. To test whether FACoAR might play a role in oxidative reactions, the reversibility of this enzyme was tested using $NADP^+$ and various substrates. No activity was detectable following the reduction of $NADP^+$ at 340 nm (<1.0 nmol min^{-1} mg $protein^{-1}$) in the presence of fatty alcohols, fatty aldehydes, or combinations of each with free CoA, though this may simply be the result of an equilibrium that strongly favors the alcohol and $NADP^+$ products.

A possible mechanism of action for the *M. aquaeolei* VT8 FACoAR can be considered by comparison to mechanisms proposed for related enzymes. Although there are no reports of enzymes fully kinetically characterized with high sequence identity to this enzyme, the HMG-CoA reductase provides a model for comparison. HMG-CoA reductase is an important enzyme in the cholesterol synthase pathway that catalyzes the four-electron reduction of HMG-CoA to mevalonate via mevaldehyde as an intermediate. In HMG-CoA reductase, the two reduction steps are believed to occur at one active site where the CoA substrate is reduced to the alcohol completely, with the mevaldehyde intermediate not leaving the active site. Figure 5-10 shows a possible reaction scheme for the FACoAR catalyzed reduction of a fatty acyl-CoA substrate that parallels the mechanism for the HMG-CoA reductase and is supported by the experimental data presented herein. The lack of an effect of either hydrazine or phenylhydrazine on FACoAR rates of fatty acyl-CoA reduction supports a model where the intermediate aldehyde does not diffuse away from the enzyme before being further reduced to the alcohol. As each of the domains of the *M. aquaeolei* VT8 FACoAR contain a conserved pyridine nucleotide binding region, there is a potential for two separate active sites in the enzyme, though further experiments involving truncations will be required to adequately test this possibility.

The V_{max} for fatty aldehyde reduction by the FACoAR is significantly higher than the V_{max} obtained for fatty acyl-CoA substrate reduction in this FACoAR, although the apparent K_m for fatty acyl-CoA was somewhat lower (~4 μM for palmitoyl-CoA versus ~50 μM for *cis*-11-hexadecenal, Figures 5-6 and 5-7). This higher rate of reduction for

the aldehyde could be a key factor in minimizing the loss of a potential intermediate aldehyde that could be toxic to the cell. Inhibition experiments using combinations of both fatty aldehyde and fatty acyl-CoA revealed that small concentrations of fatty acyl-CoA inhibited fatty aldehyde reduction in a concentration-dependent manner that could be overcome with higher concentrations of fatty aldehyde (Figure 5-9). These data, along with a consistent ratio of activities for both fatty aldehyde reduction and fatty acyl-CoA reduction in all steps of the purification including the size exclusion chromatography step, support the hypothesis that both activities are the result of the same enzyme and support a likelihood that both reactions occur either at the same active site or at active sites that are strongly cooperative with one another. The second hypothesis is supported by the presence of two conserved pyridine nucleotide binding regions within the protein and may indicate that even with two active sites, only one of the active sites is able to catalyze the related reaction at a time.

The sigmoidal character of the rate versus substrate concentration (Figures 5-6 and 5-7) can be explained by protein–protein interactions that might occur in a multimeric form of the protein, such as the homotetramer that is indicated from our results. The interacting proteins would be cooperating to allow enzymatic function much like what is seen with HMG-CoA reductase (*24*). For HMG-CoA reductases, cleavage of sections of protein with freeze/sheer solubilization led to elimination of the sigmoidal kinetics, indicating a loss of cooperativity (*24*). To test whether portions of the N- or C-terminus of the protein are responsible for the exhibited cooperativity and necessary for

catalysis, truncated versions of *M. aquaeolei* VT8 FACoAR could be constructed as part of future work.

In this chapter, we have described a novel bacterial enzyme from *M. aquaeolei* VT8 that catalyzes the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohol, in contrast to other reports for bacterial FACoAR enzymes that only reduce fatty acyl-CoA by two electrons to the fatty aldehyde (9). The C-terminal domain of the FACoAR from *M. aquaeolei* VT8 shares sequence similarity with

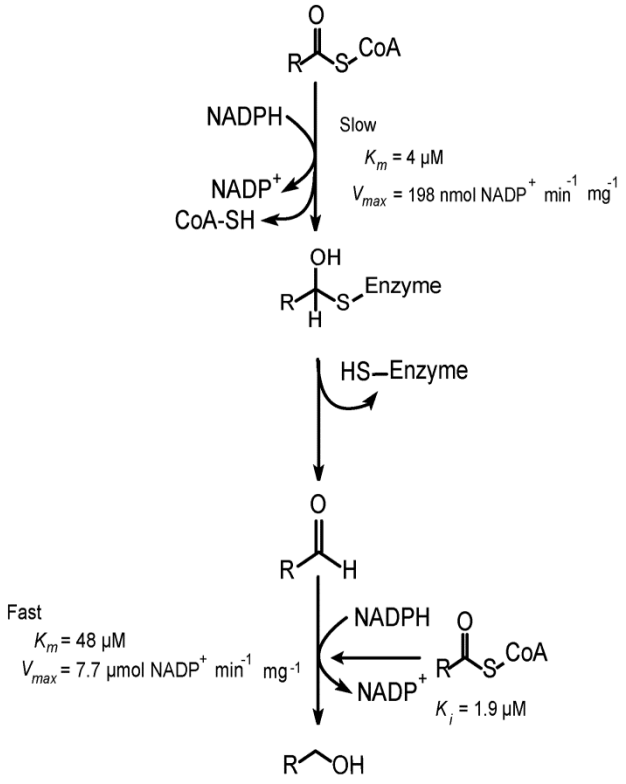

Figure 5-10. Proposed reaction mechanism for fatty acyl-CoA reductase. This figure shows the reduction of the fatty acyl-CoA occurring within the same active site in a two-step reduction. The inhibition of aldehyde reduction by fatty acyl-CoA is shown. The reaction could proceed through an aldehyde intermediate.

the FACoAR from *A. calcoaceticus*, while the unique N-terminus domain appears to have little homology to other known FACoAR enzymes. Further, the substrate specificity for the *M. aquaeolei* VT8 FACoAR is broader than the relatively narrow specificity reported for the vast majority of other FACoAR enzymes previously characterized (*6, 9, 16, 30*). Homologues to the *M. aquaeolei* VT8 FACoAR are found in a variety of other bacteria, including those that are known to accumulate wax esters, indicating that this enzyme may constitute an additional class of bacterial FACoAR enzymes in contrast to those only sharing similarity with the single domain FACoAR from *A. calcoaceticus*.

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CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The worldwide demand for energy is increasing at an alarming rate with energy demand expected to outstrip production with current methods by the year 2050 (*1*). Given this looming difficulty it has become necessary to determine new methods of energy production to meet the coming demand. The current energy infrastructure relies heavily on liquid fuels with these supplying 33% of the total energy consumption worldwide (*2*). As well, approximately 23% of the liquid petroleum utilized worldwide is allocated to the production of petrochemicals used in plastics, cosmetics, pharmaceuticals, and many other materials (*2*). With less than a 200 year supply of the petroleum remaining at current rates of consumption, the discovery of new and renewable sources of liquid fuels and petrochemicals has become of key interest to governments and global corporations alike.

As part of this search, biofuels and oleochemicals have become of great interest for supplying the increasing need for a renewable fuel and preparing for the eventual loss of petroleum (*3–5*). Given current technology, no one method of biofuels and oleochemical production can hope to supply all of the current and future needs for fuels and chemicals. The most probable solution for biofuels replacing current and future energy needs requires the use of all possible methods for biofuels and oleochemicals production combined to give a large biofuels and oleochemicals industrial sector.

This sector would include all three generations of biofuels with the current focus remaining on continuing innovation for increased production and efficiencies. As the

work contained in this dissertation has focused on the microbial production of biofuels, the remainder of this summary will discuss future directions of research that the author believes will most benefit the future of microbial biofuels production and further the viability of renewable liquid fuels.

The production of biofuels and oleochemicals from microbes comprise the third generation of biofuels as has been discussed in the Chapter 1 of this dissertation. Regardless of the desired compound produced or organism utilized for production, the process of producing microbial biofuels follows five main steps including: Feedstock production, dewatering, drying, extraction, and conversion. For microbial biofuels and oleochemicals production to be effective each of these steps must be optimized to minimize waste and maximize product yield (*6*). The research contained in this dissertation sought to alleviate difficulties associated with feedstock production and extraction methods. The remainder of this discussion will focus on future directions for improving these areas of microbial biofuels production.

PRODUCTION OF INCREASED BIOFUELS PRECURSORS IN MICROBES

The feedstock produced in the case of microbial biofuels is generally a cell slurry. The density of the dry cellular material in the cell slurry ranges from 0.1 to greater than 2 percent solids depending on the organism grown and carbon source utilized. For phototrophically grown algae, percent solids of the cell slurries generally range from 0.1 to 1 percent solids (*7*). On the other end of the spectrum, heterotrophically grown microbes range from 1.5 to greater than 10 percent solids (*8*).

The ultimate goal when producing a microbial feedstock is to maximize the percent solids of the cell slurry as well as the amount of the desired product present in the cells. Current projects which produce biofuels and oleochemicals in large quantities use microbes selected for their ability to naturally produce the desired compound of choice (exe. example TAG or fatty acids) (*9*). This method generally results in a tradeoff between cell mass production and production of the desired compound under certain growth conditions. Though careful control of nutrients can give a midpoint result for many organisms which results in acceptable levels of both the cell mass and the desired compound, new approaches are necessary to fully maximize the potential of the feedstock for production (*10*).

The production of high value compounds, including fuels, from microalgae has been a focus of research since the mid 1970's as part of the aquatic species program. What was identified as part of this program was the lack of knowledge about the trigger for lipid production which precedes large lipid accumulation. As well, it was noted that consistent high level production of precursor lipids was a limiting factor in the production of biofuels from algae (*11*).

Research groups have focused mostly on the production of biofuels precursors from green algae strains which require a nitrogen depletion period before lipid accumulation can occur (*12–14*). This method creates further difficulties in that an increase in lipid production in this manner is often directly correlated to a decrease in biomass production usually resulting in an overall decrease in biodiesel productivity when high lipid growths are compared to low lipid growths (*12*). As noted in Chapter 2

of this dissertation, the research presented herein has taken a different approach utilizing a brown algal strain, the diatom *C. gracilis*.

C. gracilis, and other diatoms, differ distinctly from green algae in their position within the water column in natural habitats and light usage patterns. These organisms are capable of growing with less light lower in the water column and have the ability to use a broader range of wavelengths of light due to the unique set of pigments they produce (*15*). Experiments performed in the Seefeldt lab have demonstrated that *C. gracilis* consistently produced a high level of lipid biodiesel precursors without the need for a nutrient stress period.

In Chapter 2 of this dissertation it was demonstrated that growths of *C. gracilis* in large scale reactors (50 to 200 L) were capable of producing biodiesel precursors up to 30% by dry weight of the algal biomass with no apparent nutrient stress from nitrogen, phosphorous, or silicate. This is counter to what has been shown for green algal species which appear to require complete nitrogen depletion in the media for a period of days to reach maximum lipid production in the cells (*16*). This finding sparked interest into the cause or trigger of lipid production for this organism.

A group working with *C. mueleri*, a diatom similar to *C. gracilis*, noted a 50 % increase in lipid production between a flat plate culture grown outside under full sun and a culture grown inside under simulated light conditions (*17*). A similar result was seen by research conducted in the Seefeldt lab with *C. gracilis*, showing that a culture grown with indoor light, 300 μmol m^{-2} sec^{-1}, produced approximately 50% less biodiesel precursors than a culture grown at full outdoor light (unpublished data). Given this

evidence, it was believed that the trigger for the production of lipid in *C. gracilis* could be found by studying the wavelengths and intensity of light to which the organism was exposed under varying conditions.

The search for this light trigger is discussed in Chapter 3 of this dissertation. Experiments were conducted using a complex growth reactor system designed to manipulate the available light wavelengths and light intensities. The results of these experiments indicated that increased light intensity on *C. gracilis* resulted in both increased biomass and increased levels of biodiesel precursors. A negative effect on both biomass and biodiesel precursor levels were noted for all green algal strains tested. This is the first evidence obtained for a direct effect of light intensity on the increase in lipid production in a microalgal organism.

This effect of light intensity is not unheard of in algal organisms. For example the alga *Dunelliella salina* is known to produce large amounts of β-carotene in response to a combination of nitrogen limitation, high salinity, and high light intensity exposure (*18*). It is hypothesized that the organism produces this conjugated compound as a means of absorbing excess electrons produced as a result of exposure to high intensity light including UV rays. A similar process may be at work in the diatom *C. gracilis*. This organism naturally grows below the surface of the water where light intensity is greatly decreased. The increased production of lipids from this organism, especially the highly unsaturated 20 carbon fatty acids produced as discussed in Chapter 3, could be the result of a similar response to high intensity light.

Experiments should be conducted using this organism and the same equipment utilized in Chapter 3 of this dissertation to determine more definitively which wavelengths of light at higher intensities may be causing the increase in lipid production. With the use of appropriate filters the effect of UV wavelengths in the range from 200 to 400 nm could be investigated and may be found to trigger the increase in lipid production as part of an anti-photo-bleaching defense. As well, the intensity of light at wavelengths ranging from the 400 to 700 nm range should be increased to higher levels to see if an effect of intensity at these wavelengths on the production of lipid could be identified.

Despite the successes of the manipulation of growth conditions commonly used to increase lipid production in microorganisms, processes of this type have tradeoffs in terms of biomass and lipid production as previously mentioned. Genetic modification of microorganisms for the production of oleochemicals and biofuels products has begun to allow for the production of larger amounts of compounds in organisms that can grow to very high densities, upwards of 10% solids in culture (*8*). With the further development of genetic tools for manipulating microorganisms, specifically algae, great advances could be made by circumventing biological controls and natural triggers to increase biomass and lipid production in these organisms.

INCREASE IN EFFICIENCY OF DOWNSTREAM PROCESSING OF MICROBIAL FEEDSTOCKS

The production of the desired microbial feedstock results in a culture that is greater than 95% water in most cases. For other downstream processes such as extraction and conversion to occur efficiently, the amount of water in the feedstock must be limited

to less than 1% (*19*). This is generally accomplished in two steps, the first being a general dewatering and the second a drying step, both of which are energy intensive.

As a result of these energy expensive steps, which in some cases can account for as much as 50% of the cost of the production of the product (*20*), several techniques have been implemented to extract oleochemicals from wet microbial biomass including: solvent extraction (*21*), subcritical extraction (*22*), supercritical extraction (*23*), mechanical disruption (*24*), and cellular excretion (*25*). All of these techniques have positive and negative aspects, however, most likely to be implemented widely on a large scale are the solvent based extractions which are already utilized in agricultural based oil extraction processes (*26*). Due to this need for solvent based extraction, the focus of further research regarding extraction techniques as part of this dissertation centers mainly on wet cell solvent based extractions.

The overall approach to wet cell extraction techniques utilizes a solvent system which under particular temperatures (*27*) and pressures (*28*) results in the increased mixing of the solvent(s) with the cells in the water layer. The majority of these approaches can achieve efficiencies at or above 50% extraction efficiency when compared to dry cell extraction techniques with some achieving as high as 88% (*22*). These approaches have a few drawbacks which are difficult to overcome including the high cost of recovering multiple solvents in downstream processes and high energy and time requirements for many systems to achieve high extraction efficiency.

As part of the research in this dissertation discussed in Chapter 4**,** a system was developed to overcome many of these difficulties with wet solvent extraction. Contrary

to the high temperatures, pressures, and solvent mixtures which current systems utilize to create mixing between the water layer and the solvent layer for wet lipid extraction, the approach utilized in Chapter 4 of this dissertation uses solvent selection and direct mechanical mixing. The advantages of this approach include the use of a single solvent and the elimination of steps including high temperatures and pressures for lipid extraction.

A solvent selection process was developed utilizing the chemical systems modeling program Aspen. This program utilizes multiple equations to allow for the calculation of liquid to liquid interactions of solvents and various solutes in the form of activity coefficients. These activity coefficients were calculated for the interaction of a particular solvent and water as well as the interaction of that same solvent with the common microbial fat storage compound triolean. The ratio of the mixing of the solvent with triolean to the mixing of the solvent with water gives a value that was deemed a β factor. The larger the β factor the better the solvent is predicted to work in a single solvent extraction system.

In determining an optimal solvent for a wet lipid extraction process, it is important to not only consider how well the solvent will extract the lipid, but the ease of solvent recovery, toxicity to the environment, and cost of the solvent (*29*). Noting these constraints, over one thousand solvents were screened through the Aspen computer program to test for predicted lipid recovery. These solvents were then narrowed down by selecting for a boiling point less than 60 C, but higher than 30 C. Finally, any toxic solvents with an MSDS health hazard number of 3 or higher, or solvents with prohibitive

purchasing costs were eliminated. The resulting list of 14 solvents compiled showed a broad range of extraction efficiencies when tested under experimental conditions. It was apparent that the experimental extraction efficiencies did not correlate well with the predicted extraction efficiencies calculated by the program. This lack of correlation was attributed to the inability of the Aspen program to take into account the presence of the cell walls, membranes, and other cellular components mixed with the water and lipids.

Despite this lack of correlation, the program did succeed in identifying several solvents that were capable of extracting a high percentage of lipid from the cells when compared to the Bligh and Dyer extraction (between 80 and 100%). As well, this approach utilizes a single inexpensive solvent to achieve high extraction efficiency and allows for a much simpler solvent recovery regimen.

As with any solvent based extraction method, this method of wet extraction has issues that cause difficulties in secondary processes. For example, oil used for food production must meet specific quality standards, and the use of certain solvents, such as chloroform, for extraction may lead to contamination of the oil products and make them unfit for human consumption (*30*). In a similar way, the extracted wet biomass is generally dried down and used for the isolation of protein for human consumption or as animal feed. Levels of solvents in these materials must also not exceed federal regulations and guidelines (*31*).

To overcome these difficulties, more environmentally friendly solvents need to be utilized, such as hexane. Currently, a countercurrent extraction method similar to what is used to extract soybeans, but adapted to the high speed mixing approach described as a

part of this work is being developed by the Seefeldt lab in collaboration with the Pease lab at the University of Utah (*32*). This method will allow for the use of widely accepted extraction solvents, such as hexane, to extract lipids from wet microorganisms with high efficiency. The general theory is that if the feedstock flows in the opposite direction of the solvent then the solvent saturated with the least amount of lipid comes in contact with the feedstock with the most amount of lipid at all times. Given enough mixing chambers this process would allow for a high efficiency extraction to be obtained with a low efficiency, but widely accepted solvent.

An alternative to counter current extraction using a low efficiency solvent, would be using a two solvent system. If an alcohol is used in conjunction with hexane the extraction efficiencies with this process have been shown to increase from approximately 20% to almost 90%. It will be necessary to use as little alcohol as possible as the recovery of a second solvent from the water phase will increase production costs. Both methods used have the potential to overcome the high energy and monetary expense associated with traditional microbial lipid extraction from dry material, however, further experiments regarding both methods should be conducted.

UNDERSTANDING THE PRODUCTION OF HIGH VALUE COMPOUNDS IN FEEDSTROCKS FROM MICROBES

As has been previously mentioned, despite improvements that may be made to increase the efficiency of intermediary processes in oleochemical production from microbes, the simplest and most efficient way to improve all aspects of the process is to improve the amount of lipid or desired oleochemical compound in the feedstock biomass

which is produced. To effectively accomplish this, an understanding of the biochemistry behind the production of the oleochemical of interest must be obtained. While this is currently difficult to accomplish for many algal species, a great deal of understanding can be had by studying bacteria and yeast that produce oleochemicals.

Fatty Alcohol Production in Bacteria. Chapter 5 of this dissertation discusses the specific enzymes involved in the production of the fatty alcohol class of oleochemicals by bacterial organisms. Fatty alcohols are of great interest for pharmaceutical (*33*), cosmetic (*34*), and industrial (*35*) purposes. Until the publication of the manuscript encompassing Chapter V of this dissertation it was widely accepted that the pathway for fatty alcohol production in bacteria passed through an intermediate aldehyde which was further reduced by an enzyme separate from the one which reduced the fatty acyl CoA substrate, an FALDR (*36*). As shown as part of this work, some bacterial species possess an enzyme capable of reducing a fatty acyl CoA substrate directly to a fatty alcohol (*37*).

This work was able to conclude that this enzyme is able to act on both aldehyde and fatty acyl CoA substrates either through a single active site or through two closely interacting active sites. The most active form of this enzyme was determined to be a heterotetramer by size exclusion chromatography and as a result of this, the enzyme exhibits non-Michaelis-Menten activity as shown by activity graphs displaying a best fit line to the Hill equation (*38*).

To further elucidate the existence of one or two active sites within this enzyme as was undetermined form the information discussed in Chapter 5 of this dissertation, further investigation into the amino acid sequence and predicted structure were

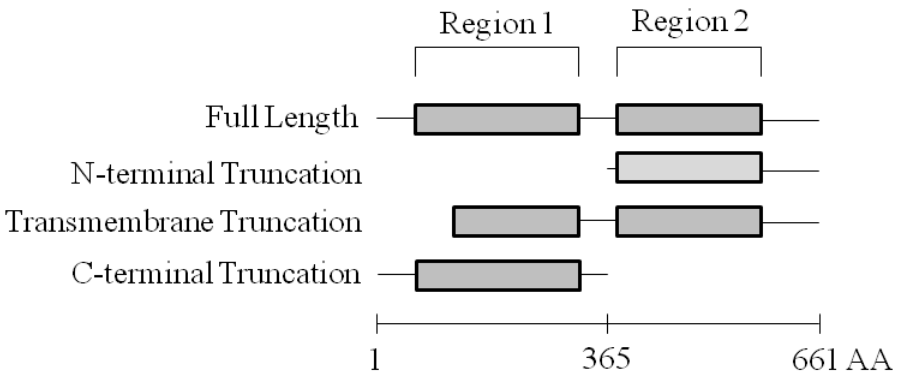

Figure 6-1. Construction of truncations of the terminal ends of the M. aquaeolei VT8 FACoAR enzyme. Truncations are shown in cartoon fashion with the N-terminal truncation removing amino acid residues 1-365 comprising region 1, Transmembrane truncation removing residues 1-85 comprising a predicted transmembrane region, and the C-terminal truncation removing residues 366-661 comprising region 2.

conducted. When the amino acid sequence was blasted against multiple homologous enzymes two apparent domains became visible. The appearance of these two apparent domains gave some credence to the two active site theory and further tests were devised.

As shown in Figure 6-1, three truncation constructs were produced which removed various terminal amino acids from the wild type full length enzyme. Given that region 2 showed high similarity to the *A. calcoaceticus* enzyme which is known to reduce a fatty acyl-CoA to a fatty aldehyde (*36*) and both region 1 and region 2 contain conserved Rossman folds for pyridine nucleotide binding sites, it was originally theorized

that the N-terminal domain contained an active site capable of reducing a fatty aldehyde to a fatty alcohol. A second possible hypothesis was that the C-terminal domain contained an active site capable of reducing both the fatty acyl CoA and the fatty aldehyde and the N-terminal domain played a role essential/or non-essential in the overall enzyme activity, such as regulating or modulating activity.

Upon purification of all three constructs shown above in Figure 6-1, assays were conducted utilizing both cis-11-hexadecenal and palmitoleyl-CoA for substrates in the presence of NADPH as described in Chapter 5 of this dissertation. The resulting assays showed no significant activity of any truncation construct, suggesting that the N-terminal region may function as a facilitator for the activity of the C-terminal active site. Alternatively, two active sites may still exist, but may require the presence of the other to function correctly either through facilitated protein folding or active site residue interactions.

To further determine whether this enzyme contains 1 or 2 active sites, experiments studying the binding of the substrates to the two Rossman fold sites should be conducted. Through the use of Isothermal Titration Calorimetry (ITC), it is possible to determine binding and dissociation constants. Each of the truncation mutants and the wild type enzyme should be tested for binding affinity with the substrates/products NADPH, $NADP^+$, free CoA, fatty acyl CoA, fatty aldehyde, and fatty alcohol.

These experiments performed with NADPH would give evidence for the existence of more than one active site. If NADPH bound to the N-terminal truncation mutant, wild-type, and C-terminal truncation mutant with similar binding affinities, then

the possibility of separate, but interacting active sites would be very high. In contrast if NADPH only bound to the wild type and either the C-terminal truncation mutant or the N-terminal truncation mutant, then that would indicate the presence of only one active site. However, this evidence would help to elucidate which region the active site was contained in.

The use of $NADP^+$ in these experiments would indicate if the active site had difficulty releasing the $NADP^+$ after the reduction reaction had taken place. If it were found that two separate active sites were present, and both bound $NADP^+$, an increased binding affinity for $NADP^+$ in one truncation mutant could help to show why aldehyde reduction occurs at a rate almost 100 times faster than fatty acyl-CoA reduction. If the $NADP^+$ were slow to come off of the active site because of higher binding affinity it would greatly decrease the overall reaction rate of that part of the reaction.

ITC testing for the binding affinity of fatty aldehyde and fatty acyl CoA with the different truncation mutants along with the wild-type enzyme would also work to demonstrate if one or two active sites exist in this enzyme. If the aldehyde or fatty acyl CoA binds to one truncation mutant, but not the other and vice-versa, this would indicate the presence of two active sites. However, if both the aldehyde and fatty acyl-CoA bind to same truncation mutant, this indicates the presence of only one active site. Further, if the binding of one substrate, the fatty aldehyde, is determined to be much more favorable than the binding of the other substrate, fatty acyl-CoA, to the same active site, this result would explain in part the differences in activity between the two substrates.

Finally, testing using ITC for the binding affinity of the two co-products of the reaction, free CoA and fatty alcohol, with the various truncation mutants and wild-type enzyme would give evidence to support the theory for one or two active sites. If the free CoA or fatty alcohol binds to one active site, but not the other and vice versa, this would indicate the presence of two active sites. As well, if the binding affinity of free CoA for the wild type enzyme and/or truncation mutants was relatively high, this would help to explain the slow rate of fatty acyl-CoA reduction compared to fatty aldehyde reduction. As a result, it would help to identify the slow step in the reaction mechanism if this were found to be the case.

In addition to determining the presence of one or two active sites, the order of substrate addition and product release should be determined for this enzyme to find the exact enzymatic mechanism. To accomplish this kinetics curves for NADPH, fatty acyl-CoA, and fatty aldehyde need to be determined at sub-saturating concentrations of the other substrates to establish the true kinetics constants of this reaction.

In the case of the FACoAR enzyme described in Chapter 5 of this dissertation, there are essentially 4 substrates and 4 products of reaction. Two molecules of NADPH are converted to $NADP^+$, a fatty acyl-CoA is converted to a fatty aldehyde and free CoA, and the fatty aldehyde is further converted to a fatty alcohol. To simplify the determination of these reaction kinetics, the experiments should be run in two sets. The first set would test the binding and interaction of the fatty acyl-CoA and NADPH, the second set would test the binding and interaction of the fatty aldehyde and NADPH.

For the two substrate determination of NADPH and fatty acyl-CoA kinetics within this enzyme, a range of fatty acyl-CoA substrate concentrations from 1 to 10 μM should be tested against a range of fixed NADPH concentrations ranging from 10 μM to 1 mM. This would give you a graph containing multiple Hill equation curves with increasing apparent V_{max} values and/or apparent K_m values. This data could then be fit using Sigmaplot software to a set of equations that would describe the mode of binding for this bimolecular part of the reaction. This portion of the reaction may occur in ping-pong, ordered-sequential, or random-sequential binding modes for either substrate. The Hill version of the equations as contained in Sigmaplot would help to describe the exact reaction mechanism for how the substrates bind and products come off of the enzyme.

The second half of the reaction could be studied in a similar fashion by adding a range of fatty aldehyde concentrations from 10 to 150 μM with fixed NADPH concentrations ranging from 10μM to 1 mM. The resulting graph would give Hill curves with increasing apparent K_m and/or apparent V_{max} values. This data could in turn be fit to a set of equations using Sigmaplot software to again determine if this part of the reaction occurs with ping-pong, ordered-sequential, or random-sequential kinetics for substrate binding and product release. These two half reactions could then be put together to form a complete reaction mechanism for this enzyme.

The determination of binding affinities by ITC and kinetic parameters using the real-time activity assays described in Chapter 5 of this dissertation would give evidence to support either one or two active sites within this enzyme. As well, a definitive and complete reaction mechanism could be determined explaining and describing all of the

data presented as a part of Chapter 5 in this dissertation. The work which has been described herein could be used to develop a manuscript for publication that would reach the level of biochemistry as Chapter 5 has done.

As well as determining binding constants and kinetics, further research into this enzyme should include the determination of a crystal structure with and without various substrates trapped. The determination of a crystal structure with NADPH, fatty acyl CoA, or fatty aldehyde within the crystal structure would allow for direct determination of an active site location for each substrate. Such crystals have been obtained with the wild-type enzyme with no substrates added, however these crystals were unable to defract x-rays, as the crystal quality was poor. Further screens for crystallization conditions suitable to produce diffracting crystals have been undertaken and are currently monitored monthly. Further efforts should be placed on obtaining protein crystals of diffraction quality.

TAG Production in Yeast. The production of TAG commonly used in the production of biofuels and food additives by microorganisms is poorly understood in algae, but is well understood in yeast organisms. A small group of yeast which are capable of producing greater than 20% or their cell dry weight in lipid compounds are referred to as oleaginous yeast (*39*). These oleaginous yeast have a well defined pathway for TAG production (*40*), however, some nuances of the pathways which cause dramatic differences between the lipid production of different species are not well understood. For example the yeast *Yarrowia lipolytica* produces less than 12% of its dry weight under nitrogen limiting conditions as transesterifiable lipid with glucose as the carbon source

(unpublished data Seefeldt lab). A second oleaginous yeast, *Cryptococcus curvatus*, produces greater than 70% of its dry weight as transesterifiable lipid under the same conditions (unpublished data Seefeldt lab). The large difference between these yeast strains with similar lipid producing machinery in regards to lipid production represents a lack of understanding in how the quantity of lipid production occurring in cells is controlled.

To study this difference in lipid production, the literature was screened to determine what metabolites each organism produces. It was determined that *Y. lipolytica* produces a large amount of citrate which is excreted into the media (*41*). Citrate is a precursor carbon compound to the production of TAG in yeast cells. It is possible that this large exportation of citrate greatly limits the ability of the cells to produce TAG due to the lack of substrate. Two possibilities exist to explain this phenomenom. Either a transporter removes citrate before it can be converted, or the export of citrate is a result of the cells lipid production machinery being unable to handle high concentrations of citrate within the cell.

To test these hypotheses, the levels of citrate exported into the media were tested for both *Y. lipolytica* and *C. curvatus*. These results showed that *Y. lipolytica* exported, in some cases, greater than 10g/L of citrate, while *C. curvatus* exported no detectable levels

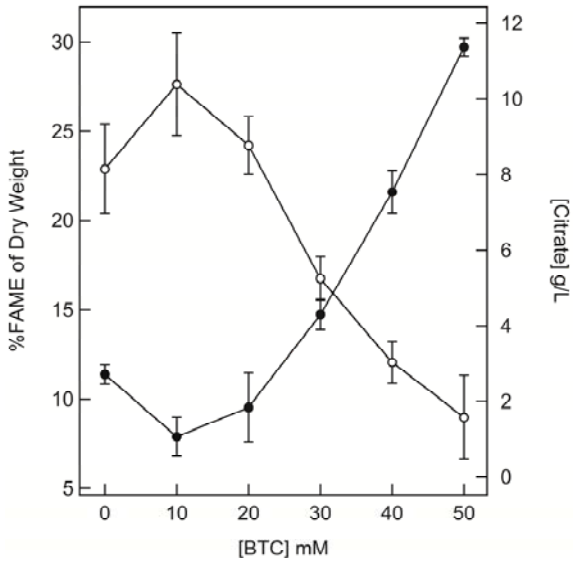

Figure 6-2. Effect of Benzene 1,2,3-tricarboxylic acid (BTC) on citrate transport in *Y. lipolytica*. The concentration of citrate in g/L is shown by the (○) symbol and is measured along the left y-axis. The percent FAME of the dry weight is shown by the (●) and is measured on the left y-axis.

of citrate (unpublished data from Seefeldt lab). The differences in citrate export indicate a clear difference in carbon handling within the cells.

The hypotheses were further tested by adding a compound benzene 1,2,3 tricarboxylic acid (BTC) which is a known inhibitor of citrate export in the fungi *Aspergilus nigerus* (*42*). The resulting growths, seen in Figure 6-2, demonstrate that as the level of citrate exported decreases in *Y. lipolytica*, the amount of lipid produced increases. However, even at near lethal levels of BTC, citrate export still occurs with only a moderate level of lipid production as compared to *C. curvatus*.

To completely block citrate export in *Y. lipolytica* and determine if citrate transport is the sole or only part of the cause of decreased lipid production in this yeast

when compared to *C. curvatus*, a knockout of the citrate transporter gene is currently being generated. A gene in *Y. lipolytica* (YALI0E31064g) was found to be homologous to a rat plasma membrane citrate transporter gene (*43*). The resulting knockout will be tested to determine if lipid production is increased further by preventing any efflux of citrate from the cell.

Alternatively, the cellular enzymes responsible for converting citrate to TAG may have different kinetic parameters, such as a higher K_m and lower V_{max} when compared to *C. curvatus* enzymes, which prevent them from functioning under the cellular conditions and necessitate the export of the citrate from the cell. Experiments including the expression and characterization of the enzyme(s) responsible for citrate conversion into TAG should be conducted starting with the first enzyme in the pathway ATP-citrate lyase. The characterization of these enzymes to determine their kinetic parameters would definitively demonstrate why lipid production varies so widely between species of oleaginous yeast and give direction to further research into the metabolic engineering of yeast and other microbial cells for increased lipid production.

Production of Microbial Protein. Of all the high value compounds that can be produced by microbes, the most overlooked product is the very material that is often considered a side product, the cellular proteins left in the extracted carcasses. Proteins from these microbes often termed single cell proteins represent a commodity of untapped potential and profit (*44*). The cellular carcasses of extracted microbes are generally composed of at least 50% protein. If the protein were to be purified, as is the case with

dairy proteins, a commodity worth up to 7 times that of biodiesel can be produced given the current market value of whey protein and biodiesel (*45*).

Further experiments into the production of protein as a side product of lipid production should be considered. A combination of the manipulation of growth conditions and genetic modification could yield an organism capable of producing a large amount of lipid and still leave the remaining material with a high amount of protein for use in other processes. Methods for simultaneously extracting lipid to an organic phase and protein to an aqueous phase from microbial organisms would easily facilitate protein concentration by membrane filtration, as is commonly done with whey protein. While pursuing the future of oleochemicals and biofuels produced from microorganisms, it will become ever more important to explore all possibilities of products that may be produced including protein from the extracted materials.

CONCLUSION

Worldwide the demand for energy continues to rise while the supplies dwindle ever lower. The almost insatiable energy market demands the production of newer and more efficient forms of energy with the transportation industry requiring high density liquid energy sources. To meet these increasing demands, more breakthroughs will be required in the production of high quality and high density liquid fuels. Oleaginous microbes may hold the key to producing the required high energy density compounds as biofuels research continues into the future. The work conducted as part of this dissertation helps to continue innovation by garnering further understanding into the production of oleochemicals in oleaginous microalgae, bacteria, and yeast. As well,

extraction techniques have been developed which allow for increased extraction efficiency while alleviating many of the problems associated with standard microbial oleochemical processing. The future of microbial oleochemicals will depend on the ability of researchers to continue to push the boundaries of what is currently possible and develop new ways of economically producing high value oleochemicals.

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APPENDIX

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Willis, R.M., Wahlen, B.D., Barney, B.M., Seefeldt, L.C. Traicylglyceride production in the marine diatom *Chaetoceros gracilis* grown in bag cultures and raceway ponds without nutrient limitation.

Willis, R.M., McCurdy, Alex T., Ogborn, Mariah K., Wahlen, Bradley D., Pease, Leonard, Seefeldt, Lance C. Highly efficient liquid to liquid extraction of Triacylglycerides from the wet cells of oleaginous microbes through the use of solvent selection and mechanical mixing.

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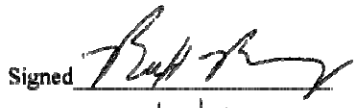
Signed 

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Author: Robert M. Willis, Bradley D. Wahlen, Lance C. Seefeldt, and Brett M. Barney

Publication: Biochemistry

Publisher: American Chemical Society

Date: Dec 1, 2011

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Robert M. Willis

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Professional Overview

Accomplished graduate research scientist and mentor to undergraduate students. Experience with a wide variety of biochemical and chemical techniques including but not limited to: protein purification, molecular biology, lipid biochemistry, microbiology, GC/MS, GC/FID analysis, experience with Aspen, and experience with analysis of solvent properties for modeling for various lipid extraction methods.

Core Qualifications

- Highly experienced with protein purification and activity assays utilizing various forms of product detection
- Highly experienced with lipid and solvent based quantitation and identification utilizing GC/FID and GC/MS
- Understand the principles and general use of LC/MS systems
- Highly experienced with molecular biology, including PCR, DNA analysis, expression vector construction, and bacterial and yeast transformation.
- Highly experienced with algal cultivation, harvest, and protein and lipid analysis
- Able to design efficient and concise experiments and interpret the outcome of experiments to facilitate further implementation of experimental results
- Proficient in understanding and writing scientific literature
- Experienced with the basic use of Aspen and with solvent modeling for use in lipid extraction method development
- Proficient with the Microsoft Office Suite and IGOR Pro w/visual enzymics
- Hard working, dedicated, and loyal

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Kaskaskia College 2005

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- Title: Increased production and extraction efficiency of triacylglycerides from microorganisms and an enhanced understanding of the pathways involved in the production of triacylglycerides and fatty alcohols.
- Advisor: Dr. Lance Seefeldt

Experience

Graduate Research Assistant

January 2009 to Current

Utah State University – Logan, UT

- Determine research focuses
- Report progress periodically to supervisor
- Guide and direct undergraduate research assistants
- Write quarterly reports of research
- Keep up to date on relevant scientific literature
- Primary writer of scientific literature describing research
- Submit written manuscripts to appropriate journal for publication

Graduate Teaching Assistant

August 2008 to December 2008

Utah State University- Logan, UT

- Guide undergraduate students in completing lab assignments
- Determine proficiency of students at completing tasks
- Report determined grades to general lab professor

Phlebotomist/Medical Laboratory Assistant

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St. Mary's Hospital– Centralia, IL

- Worked directly with patients to obtain samples for analysis
- Worked with laboratory technicians to analyze samples and prepare reports

Undergraduate Research Assistant

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VA Research Hospital – Boise, Idaho

- Worked directly with principle investigator researching the effects of chemotherapeutic agents on the heart
- Responsible for analyzing protein content and activity of samples
- Responsible for writing scientific reports of findings weekly

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Publications

Wahlen, B. D., **Willis, R. M**., and Seefeldt, L. C. (2011) Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures, Bioresource Technology 102, 2724–2730.

Willis, R. M., Wahlen, B. D., Seefeldt, L. C., and Barney, B. M. (2011) Characterization of a fatty acyl-CoA reductase from Marinobacter aquaeolei VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol, Biochemistry 50, 10550–10558.

Wahlen, B. D., Morgan, M. R., McCurdy, A. T., **Willis, R. M**., Morgan, M. D., Dye, D. J., Bugbee, B., Wood, B. D., and Seefeldt, L. C. (2012) Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions, *Energy Fuels*.

Presentations

Presentation of a poster at USU Chemistry/Biochemistry Hansen Retreat September 2011. Characterization of a two-step bacterial fatty acyl-CoA reductase from Marinobacter aquaeolei VT8.

Poster presented at USU Chemistry/Biochemistry Hansen Retreat September 2012. Highly efficient liquid to liquid extraction of triacylglycerides from microbial cells using mechanical mixing.

Poster presented at National Biodiesel Board Conference February 2013. Highly efficient liquid to liquid extraction of Triacylglycerides from microbial cells using mechanical mixing.

Patent Applications

Patent pending, application number US-2012-0184006-A1filed July 19, 2012. Reductase Enzymes. Utility patent filed. **Robert M. Willis**, Bradley D. Wahlen, Lance C. Seefeldt, Brett M. Barney.

Patent pending, application number 13/233,676 filed September 15, 2011. Methods for production of biodiesel. Utility patent filed. Bradley D. Wahlen, **Robert M. Willis**, Lance C. Seefeldt.

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