5-2010

Transesterification and Recovery of Intracellular Lipids Using a Single Step Reactive Extraction

Daniel R. Nelson
Utah State University

TRANSESTERIFICATION AND RECOVERY OF INTRACELLULAR LIPIDS USING A SINGLE STEP REACTIVE EXTRACTION

by

Daniel R. Nelson

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

of

MASTER OF SCIENCE

in

Biological Engineering

Approved:

_____	_____
Sridhar Viamajala Committee Chair	Ronald Sims Committee Member
_____	_____
Kamal Rashid Committee Member	Byron Burnham Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2010

ABSTRACT

Transesterification and Recovery of Intracellular Lipids Using a

Single-Step Reactive Extraction

by

Daniel R. Nelson, Master of Science

Utah State University, 2010

Major Professor: Dr. Sridhar Viamajala
Department: Biological Engineering

A single-step, extractive reaction for extraction of lipids such as biodiesel components, omega-3 fatty acids, or other triglycerides from microbial cells was examined. Conventional methods for lipid extraction use toxic solvents, and require multiple steps and long processing times. When the goal is to produce fatty acid methyl esters or FAMEs, the extracted lipids are subjected to a separate transesterification reaction with simple alcohols in the presence of an acid or base catalyst. A simplified, single-step reactive extraction method can be applied that combines the sequential extraction followed by transesterification using acidified alcohols – a process known as *in situ* transesterification.

It was hypothesized that the *in situ* transesterification could be scaled-up for industrial processing by a systematic understanding of fundamental reaction parameters including temperature, catalyst concentration, and biomass/solvent ratios. The hypothesis

was tested using a marine fungus, *Schizochytrium limacinum* SR21. Growth of SR21 resulted in biomass yields of 0.3g-biomass/g-glycerol and accumulated high amounts of palmitic acid (C16:0, 0.255g-FAME/g-biomass), docosahexaenoic acid (DHA, C22:6, 0.185g-FAME/g-biomass), myristic acid (C14:0) (0.017g-FAME/g-biomass), and pentadecanoic acid (C15:0, 0.012g-FAME/g-biomass).

The bulk phase separation characteristics of the FAMEs were evaluated at high biomass concentrations. Recyclability of the acidified methanol in the system was also tested. A significant finding was that automatic phase separation of the FAMEs could be achieved. When FAME concentration reaches critical solubility, 22.7mg-FAME ml^{-1} methanol, all remaining FAMEs automatically phase separate. After FAME separation, the remaining methanol was recycled and used in subsequent *in situ* reactions. Upon recycling, greater than 85% of product extraction and recovery was achieved.

The kinetics of the transesterification reaction was evaluated under various acid and biomass/solvent conditions. Based on the fundamental reaction mechanism governing the *in situ* transesterification, a theoretic model was derived to predict the conversion of TAGs into FAMEs. Kinetic parameters were estimated by fitting the experimental data and the resulting model. The model derived closely resembled the observations in this study. Through understanding of the fundamental reaction kinetics and limitations during processing, a new, reliable, and cost-effective system for large scale lipid production can be developed for microbial biomass including oleaginous algae, fungi, and yeast.

(61 pages)

ACKNOWLEDGMENTS

I would like to thank the Department of Biological Engineering for supporting me throughout this research. I also would like to thank everyone involved in the project for their knowledge and support. I would especially like to thank Dr. Sridhar Viamajala, Dr. Ronald Sims, Dr. Kamal Rashid, and Dr. Brett Barney for their support and assistance.

Special thanks to my family and friends for their support, as well as the great outdoors for the adventures which allowed me to relax and focus on work.

Daniel R. Nelson

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF SYMBOLS, NOTATIONS, AND DEFINITIONS

Abbreviation Key

DCW	Dry cell weight
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detection
GC	Gas chromatography
HPLC	High performance liquid chromatography
PUFA	Poly unsaturated fatty acid
SFA	Saturated fatty acid
SFE	Supercritical fluid extraction
TAG	Triacylglyceride

CHAPTER 1

INTRODUCTION

In recent years, the significance of intracellular lipids has increased due to their implications in renewable energy, human nutrition, and health. Microorganisms, such as algae or fungi, have been shown capable of accumulating large amounts of lipids (saturated fatty acids (SFAs) and poly unsaturated fatty acids (PUFAs)). Therefore production of lipids using microorganisms for commercial use has gained the attention of the scientific community (Chisti, 2007; Vazhappilly and Chen, 1998).

The high energy demand in the industrialized world has driven an increasing need for alternative energy due to the limited supply of traditional petro chemically derived fuels as well as the environmental impact of these traditional sources. However, for alternative energy sources to be viable they must readily available, sustainable, economically competitive, technologically feasible, and environmentally acceptable (Meher et al., 2006). Areas of alternative energy research have included solar, geo-thermal, and biomass driven fuels cellulosic, corn ethanol, and algal biodiesel. Production of biodiesel from microorganisms such as algae, have reached a spotlight due to the potentially increased productivity of biodiesel they provide (Miao and Wu, 2006). The four most common ways of making biodiesel are direct use and blending, microemulsions, thermal cracking (pyrolysis), and transesterification (Ma and Hanna, 1999). Unfortunately, while the same processes which exist for extracting oils and converting them to fuel from plants do not necessarily apply to microorganism slurries. Therefore, new extraction technologies must be developed for microbial systems which are efficient and cost effective on large scales.

Along with the rising demands for renewable energies, is the increasing for nutritional supplements such as Omega-3 fatty acids. Omega-3 fatty acids belong to the group of polyunsaturated fatty acids (PUFAs) and several studies have shown that having a proper balance of PUFAs in the body helps to maintain proper organ function (Wu et al., 2005). For example, high DHA content in the brain and retina has been shown to be important for proper nervous system and visual function (Horrocks and Yeo, 1999). Also, reports have shown that these long chain omega-3 fatty acids can act as breast and colon cancer chemopreventive agents (Rose and Connolly, 1999; Senzaki et al., 1998). Omega-3 fatty acids are also believed to play key roles in alleviating the effects of arthritis and in preventing atherosclerosis and coronary heart disease (Barclay et al., 1994).

Mammals do not have the enzymes necessary for the synthesis of omega-3 fatty acids and therefore must supplement them in their diets (Cook, 1985). The most common source of omega-3 fatty acids is fish. However, most diets do not include sufficient amounts of fish. Therefore, diet supplements such as fish oil or omega-3-enriched food product intake is necessary to meet dietary needs (Linko and Hayakawa, 1996). In general, most fish contain less than 5% (w/w) lipid of which only 20-30% are omega-3 fatty acids (Bligh and Dyer, 1959). In addition, recovery of omega-3 fatty acids from fish oil is expensive (Belarbi et al., 2000). Due to the low concentrations of omega-3 fatty acids relative to fish mass, there is difficulty obtaining sufficient and inexpensive quantities of omega-3 fatty acids from fish. Therefore, in an increasingly nutrition-aware population, it is important to develop technologies capable of keeping up with the rising demand.

Analytical methods that strive to achieve rigorous and complete extraction of cellular lipids commonly use the well-known Bligh and Dyer method and other small variations. In this procedure, a mixture of chloroform, methanol, and water is used to perform the extraction and the solvent ratios are balanced such that a single phase is formed with the sample water (Bligh and Dyer, 1959). After completion of the extraction, phase separation is achieved by diluting the mixture with chloroform and water, allowing the extracted lipids contained in the chloroform phase to be removed. Most modifications or adaptations of this procedure involve changing the solvents and their ratios. For example, methylene chloride has been used as an alternative to chloroform and variations in extraction time, temperature, and order of solvent addition have been reported (Chin et al., 2006; Folch et al., 1957; Lewis et al., 2000; Perveen et al., 2006; Wu et al., 2005). Another emerging practice is the use of sonication during the reaction (Belarbi et al., 2000). Sonication helps to disrupt the cells and encourage better lipid solublization, but this method adds significant mechanical costs. Within all the derivatization of the Bligh and Dyer methods, the principle mechanisms of extraction remain the same.

Because fatty acid alkyl esters (typically fatty acid methyl ester, or FAME) are typically desired, such as for biodiesel, a second step following the Bligh and Dyer-based extraction of triglycerides is required to transesterify the lipids and is typically accomplished with the addition of an alcohol and a base or acid catalyst. Thus this overall two-step process requires significant amounts of solvents as well as processing time. Although these methods can potentially be scaled up to extract intracellular triglycerides, the cost and toxicity of the solvents are cause for concern.

Interest in lipid extraction and transesterification, and the lack of efficient processes, in some cases, has led to further studies on alternative extraction techniques. Of the new techniques under investigation, the microwave assisted, supercritical fluid, or *in situ* transesterification techniques have gained the most recognition (Carrapiso and Garcia, 2000). The use of microwaves provides a rapid extraction and does not require that the samples be previously dried (Pare et al., 1997). Supercritical fluid extraction (SFE) has gained interest because it significantly lowers the requirements for organic solvents, as well as the concerns for waste disposal after the reaction (Randolf, 1990). However, conditions affecting SFE, such as particle size and water content, must be improved to achieve efficient extractions (Eller and King, 1996). While many of these alternative extraction techniques have been developed for a lipid extraction for a wide variety of sources (meat, dairy, egg), they may be applied to microbial systems.

Finally, the alternate technique described in literature, the *in situ* transesterification method, combines the extraction and transesterification into a single step and thus lowers solvent use and processing time (Ashford et al., 2000; Chi et al., 2007; Lewis et al., 2000; Morita and Kumon, 2006; Yokochi and Honda, 1998). Although this method was first proposed in by Abel et al. in 1963, most investigations have only been performed in recent years. In this process, biomass is treated with a mixture of alcohol and acid or base resulting in the reactive extraction of lipids as FAMEs. Basic catalyzed reactions have been the focus in research because it is much faster than acid catalyzed reactions and the reaction conditions or more moderate (Vincente et al., 2004). However, base catalyzed reactions run the risk the formation of soaps from cellular free fatty acids (FFAs). The formation of soaps greatly increases the

cost of downstream processing as it causes the formation of gels, increases viscosity, and makes product separation more difficult (Carrapiso and Garcia, 2000; Ma and Hanna, 1999). Another factor affecting the use of base catalysis is the presence of water. When water is present in the system hydrolysis of the alkyl esters into FFAs is assumed, which leads to further soap formation. Soap formation can be avoided with the use of strong liquid acid-catalyzed processes where the FFAs are converted to the ester form through acid mediated esterification. Nevertheless, acid catalyzed reactions require higher temperatures and longer reaction times than base-catalysis (Freedman et al., 1984). Based on these considerations, the acid-catalyzed *in situ* transesterification process is likely to be more economically viable during large-scale processing. However, this method has been developed for laboratory scale reactions and is used for analytical quantification of lipids. For example, most laboratory methods use excess solvents and prolonged reaction times to ensure complete recovery (Lewis et al., 2000), factors that would need to be optimized for efficient large scale processing. Therefore, the method needs to be modified and optimized for large scale production.

In the research reported here, we have studied several key factors affecting the kinetics of the single step extractive reaction, as well as separation technologies for oil recovery and solvent recycling. The key factors examined were temperature, acid concentration, and biomass/solvent ratio. Our results suggest that temperature, acid concentration, and biomass/solvent ratio have a significant impact on the kinetics governing the reaction progress. From the results obtained, the single step extractive reaction offers a promising and cost efficient method for large scale extraction and recovery of fatty acid alkyl esters from microorganisms.

CHAPTER 2

IN SITU TRANSESTERIFICATION OF INTRACELLULAR LIPIDS

Introduction

In recent years, the significance of intracellular lipids has increased due to their implications in human nutrition, health, and renewable energy. Microorganisms, such as algae or fungi, have been shown capable of accumulating large amounts of lipids (saturated fatty acids (SFAs) and poly unsaturated (PUFAs) fatty acids). Therefore production of lipids using microorganisms for commercial use has gained the attention of the scientific community (Chisti, 2007; Vazhappilly and Chen, 1998).

Omega-3 fatty acids belong to the group of polyunsaturated fatty acids (PUFAs) and several studies have shown that having a proper balance of PUFAs in the body helps to maintain proper organ function (Wu et al., 2005). For example, high DHA content in the brain and retina has been shown to be important for proper nervous system and visual function (Horrocks and Yeo, 1999). Also, reports have shown that these long chain omega-3 fatty acids can act as breast and colon cancer chemopreventive agents (Rose and Connolly, 1999; Senzaki et al., 1998). Therefore, in an increasingly nutrition-aware population, it is important to develop technologies capable of keeping up with the demand.

Along with the rising demands for omega-3 fatty acids, is the increasing need for alternative energy. Production of biodiesel from microorganisms such as algae, have reached a spotlight due to the potentially increased productivity of biodiesel they provide. Unfortunately, the same technologies which exist for extracting oils and converting them to fuel from plants do not necessarily apply to microorganism slurries. Therefore, new

extraction technologies must be developed for microbial systems which are efficient and cost effective on large scales.

Analytical methods that strive to achieve rigorous and complete extraction of cellular lipids commonly use the well-known Bligh and Dyer method and other small variations. In this procedure, a mixture of chloroform, methanol, and water is used to perform the extraction and the solvent ratios are balanced such that a single phase is formed with the sample water (Bligh and Dyer, 1959). After completion of the extraction, phase separation is achieved by diluting the mixture with chloroform and water, allowing the extracted lipids contained in the chloroform phase to be removed. Most modifications or adaptations of this procedure involve changing the solvents and their ratios. For example, methylene chloride has been used as an alternative to chloroform and variations in extraction time, temperature, and order of solvent addition have been reported (Chin et al., 2006; Folch et al., 1957; Lewis et al., 2000; Perveen et al., 2006; Wu et al., 2005). Another emerging practice is the use of sonication during the reaction (Belarbi et al., 2000). Sonication helps to disrupt the cells and encourage better lipid solublization, but this method adds significant mechanical costs. Within all the derivatization of the Bligh and Dyer methods, the principle mechanisms of extraction remain the same.

Following the Bligh and Dyer-based extraction of triglycerides, a second step is required to transesterify the lipids and is typically accomplished with the addition of an alcohol and a base or acid catalyst. Thus this overall two-step process requires significant amounts of solvents as well as processing time. Although these methods can potentially be scaled up to extract intracellular triglycerides, the cost and toxicity of the solvents are cause for concern.

Interest in lipid extraction and the lack of efficient processes, in some cases, has led to further studies on alternative extraction techniques. Of the new techniques under investigation, the microwave assisted, supercritical fluid, or *in situ* transesterification techniques have gained the most recognition (Carrapiso and Garcia, 2000). The use of microwaves provides a rapid extraction and does not require that the samples be previously dried (Pare et al., 1997). Supercritical fluid extraction (SFE) has gained interest because it significantly lowers the requirements for organic solvents, as well as the concerns for waste disposal after the reaction (Randolf, 1990). However, conditions affecting SFE, such as particle size and water content, must be improved to achieve efficient extractions (Eller and King, 1996). While many of these alternative extraction techniques have been developed for a lipid extraction for a wide variety of sources (meat, dairy, egg), they may be applied to microbial systems.

Finally, the alternate technique described in literature, the *in situ* transesterification method, combines the extraction and transesterification into a single step and thus lowers solvent use and processing time (Ashford et al., 2000; Chi et al., 2007; Lewis et al., 2000; Morita and Kumon, 2006; Yokochi and Honda, 1998). Although this method was first proposed in by Abel et al. (1963), most investigations have only been performed in recent years. In this process, biomass is treated with a mixture of alcohol and acid or base resulting in the reactive extraction of lipids as fatty acid alkyl esters (typically fatty acid methyl ester, or FAME). Basic catalyzed reactions have been the focus in research because it is much faster than acid catalyzed reactions and the reaction conditions or more moderate (Vincente et al., 2004). However, base catalyzed reactions run the risk the formation of soaps from cellular free fatty acids (FFAs). The formation of soaps greatly

increases the cost of downstream processing as it causes the formation of gels, increases viscosity, and makes product separation more difficult (Carrapiso and Garcia, 2000; Ma and Hanna, 1999). Another factor affecting the use of base catalysis is the presence of water. When water is present in the system hydrolysis of the alkyl esters into FFAs is assumed, which leads to further soap formation. Soap formation can be avoided with the use of strong liquid acid-catalyzed processes where the FFAs are converted to the ester form through acid mediated esterification. Nevertheless, acid catalyzed reactions require higher temperatures and longer reaction times than base-catalysis (Freedman et al., 1984). Based on these considerations, the acid-catalyzed *in situ* transesterification process is likely to be more economically viable during large-scale processing. However, this method has been developed for laboratory scale reactions and is used for analytical quantification of lipids. For example, most laboratory methods use excess solvents and prolonged reaction times to ensure complete recovery (Lewis et al., 2000), factors that would need to be optimized for efficient large scale processing. Therefore, the method needs to be modified and optimized for large scale production.

The microorganism of choice, *Schizochytrium limacinum* SR21 (ATCC MYA 1381, referred to as SR21), is a unicellular marine fungus. This organism was chosen based on its ability to utilize glycerol as the primary carbon and energy source. Furthermore, previous studies have shown that during growth on glycerol, SR21 synthesizes FAMEs in high quantities relative to cell weight (Chi et al., 2007; Pyle et al., 2008). Another advantage to SR21 is that DHA and palmitic acid are the two major fatty acid products (Table 2-1), therefore kinetic modeling of two types of fatty acids (SFA's and PUFA's) were done simultaneously.

In the research reported here, several key factors affecting the kinetics of the single step extractive reaction have been studied. The key factors examined were temperature, acid concentration, and biomass/solvent ratio. The results suggest that temperature, acid concentration, and biomass/solvent ratio have a significant impact on the kinetics governing the reaction progress. From the results obtained, the single step extractive reaction offers a promising and cost efficient method for large scale extraction of fatty acid alkyl esters from microorganisms.

Materials and Methods

Chemicals

For all *in situ* transesterification and extraction experiments, sulfuric acid (98%), and HPLC grade chloroform, n-hexane, methanol were obtained from Sigma Aldrich (St. Louis, MO) and used as received. Individual fatty acid methyl ester standards (all greater than 95% purity) including methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C 16:0), and methyl docosahexaenoate (C 22:6) standards were obtained from Sigma Aldrich and used for gas chromatography calibrations. For culture media, yeast extract and Bacto-peptone were obtained from BD biosciences (San Jose, CA), glycerol (99.5%, Mallinckrodt Chemicals, Phillipsburg, NJ), and Instant Ocean brand sea salt (Aquarium Systems Inc, Mentor, OH) were used.

Microorganism, culture conditions and preparation of cells

A pure strain of *Schizochytrium limacinum* SR21 (ATCC MYA 1381, henceforth referred to as SR21) was used in this study. SR21 was grown in a complex medium containing 1g L^{-1} yeast extract, 1g L^{-1} peptone, 10g L^{-1} sea salt, and 6g L^{-1} glycerol. The

medium was adjusted to a pH of 7.0, after which 50 ml of medium was then transferred to 125 ml erlenmeyer flasks and autoclaved at 125°C for 60 minutes. The flasks were then inoculated with 100µl of freeze dried SR21 culture stock. The flasks were incubated at room temperature in an Innova 2300 platform shaker (New Brunswick Scientific, Edison, NJ) at 75 rpm for 4 days. The biomass was harvested using a Thermo IEC multi centrifuge (Thermo Scientific, Waltham, MA) at 3600rpm for 20 minutes. The samples were washed twice with a 0.85% sodium chloride solution, and re-centrifuged. The biomass was dehydrated using a FreeZone 4.5 freeze dryer (Labconco, Kansas City, MO) and stored in a Forma -80°C freezer (Thermo Scientific, Waltham, MA) at -80°C until used for experimentation. For all experiments, biomass is reported as a dry weight. To eliminate batch to batch variation, all biomass was grown under the same conditions, harvested as previously stated, and thoroughly mixed to ensure a homogenous biomass stock.

FAME recovery

To accurately quantify the FAMEs produced from the *in situ* transesterification reaction, the FAMEs must be recovered from the working solvent (acidified methanol). Common methods established in literature have been performed and preliminary results suggest they may be inefficient at extracting the total fraction of FAMEs present. Typically, a solvent such as chloroform or hexane is used to extract the FAMEs from the alcohol phase into the solvent phase (Indarti et al., 2005 Lewis et al., 2000; Zhou et al., 2007). The solvent/alcohol solution is mixed thoroughly for a period of time to allow the FAMEs to partition into the solvent phase, after which the solvent phase is either

analyzed or dehydrated (Yokochi and Honda, 1998). The reported recovery values can be as low as 80% when using these methods.

The subsequent recovery or partitioning step was done by extracting the FAMEs from the alcohol phase into a solvent phase (hexane). The recovery was measured by adding a known concentration, 0.05 mg ml^{-1}, of the FAMEs to methanol, extracted with hexane (1:10v/v methanol:hexane), and then analyzed using gas chromatography. The recovery was performed at 90°C with heating, and measurements were taken from 0 to 90 minutes. The objective was to obtain greater than 95% recovery.

Lipid and FAME extraction

The *in situ* reaction was performed by weighing biomass (33-125 mg) into 1.5 ml crimp top GC vials (Fisher Scientific). 0.5 ml of the acidified methanol solution (1-5% v/v H_2SO_4) was added and the vials were sealed. The reaction mixture in the vials was then heated at 60-100°C in a Hach DRB200 heating block for 90 minutes. The vials were removed every 10 minutes to be vortexed to ensure adequate mixing. The vials were then removed from the heat source and centrifuged to pellet the cell debris. The 0.5 ml working volume was then removed via gas syringe and placed in 5 ml serum bottle with 4 ml hexane for FAME recovery. 1ml hexane was then used to rinse the 1.5 ml GC vial to ensure all FAMEs were recovered; the 1 ml was then added to the 5 ml serum bottle bringing the final volume of hexane to 5 ml. The mixture was then heated for 20 minutes at 90°C, and then allowed to cool to room temperature. The hexane phase (upper) was then placed in GC vials for analysis.

A total lipid extraction was also performed on the biomass. This was done by weighing 20 mg biomass into a 15 ml glass tube and adding 5 ml mixture of

hexane:chloroform:tetrahydrofuran (1:1:1 v/v). The extraction mixture was then sonicated in 10 second intervals for 1 minute. The tube containing the reaction mixture was centrifuged and the supernatant was collected. The remaining biomass was then subjected to the addition of solvent followed with sonication 2 more times. The total volume, 15 ml, was then diluted 1:10 with the same solvent mixture to be analyzed.

Fatty acid methyl ester (FAME) and lipid analysis

The FAMEs were analyzed using gas chromatography equipped with flame ionization detection (GC-FID). A Shimadzu GC-2010 equipped with a Shimadzu AOC-20i auto injector and FID was used for all analyses. The GC was equipped with a Restek RTX-Biodiesel column with 0.53 mm guard. The FID detector was set at 370°C, with a column flow of 2.53 ml $minutes^{-1}$. The sample volume injected is 1µL and analysis begins at 60°C and increases 10°C $minutes^{-1}$ to 370°C, and holds constant at 370°C for 6 minutes. The FAME standards were prepared in a hexane solution at concentrations of 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 and 0.3 mg ml^{-1}. This linear range of concentration standards was used to develop a standard curve equation by which all unknown fatty acid concentrations were determined. Octocosane, C28:0, was used as an internal standard, 0.1 mg ml^{-1}.

Because the extracted biomass samples contained concentrated FAMEs, the samples were diluted with hexane to a ratio of 1:20 (sample:hexane). This was done to ensure the sample measurements remained within the calibrated range.

Reaction conditions

To study conditions affecting the recovery of lipids, the *in situ* transesterification reaction were examined as a function of temperature, acid concentration, and biomass/solvent ratio. Combinations of variables were tested in triplicate in a randomized manner to eliminate experimental bias.

Initially, rates of reaction were determined using dilute biomass solutions. This was done by keeping the acid concentration constant at 5% (v/v) H_2SO_4, and the biomass/solvent ratio constant at 66 mg ml^{-1}. The effect of temperature on the reaction rate was studied at 60, 80, 90, and 100°C.

The effect of acid or catalyst concentration was also a concern. Therefore, using a low biomass/solvent ratio of 66 mg ml^{-1} (3 times as much as some literature values; Lewis et al., 2000), at a constant temperature of 90°C, the acid concentration was tested at 1, 1.5, 2, and 5% (v/v) H_2SO_4.

Further tests were conducted using higher biomass to alcohol ratios for a comparison of reaction rates. During these studies the temperature was held constant at 90°C. Acid concentrations were held at 5% (v/v) H_2SO_4, and the biomass/solvent ratio levels were 66, 125, 200, and 250 mg ml^{-1}. These tests, in addition to establishing relevant key parameters for the transesterification reaction, have provided fundamental insight into the microscopic transport processes associated with the reactive extraction.

Results and Discussion

Fatty acid recovery for analysis

To study the reaction kinetics, a suitable and reliable method to quantify the amount of FAMEs present was needed. SR 21 biomass was grown and the FAMEs were analyzed as described in the materials and methods section. This organism was chosen based on its ability to produce high amounts of intracellular lipids relative to cell weight (Chi et al., 2007; Pyle et al., 2008). Table 2-1 represents the total FAMEs composition in SR21.

Table 2-1 Fatty Acid Methyl Ester Composition of *S. limacinum* SR21

	Fatty Acid Fraction: (% w/w) of Total Lipid			
	14:0	15:0	16:0	22:5, 22:6
Reference	Myristic	Pentadecanoic	Palmitic	DPA + DHA
Yokochi et al., 1998	2.7	7.6	34.2	51.7
Chi et al., 2007	4.0	nr	52.0	42.0
This Study	3.8±0.1	2.5±0.08	53.9±1.3	40.1±2.5

DPA = Docosapentaenoic acid, DHA = Docosahexaenoic acid, nr = not reported

Following the extraction-transesterification, or *in situ* transesterification reaction, the FAMEs were extracted from the acidified alcohol and analyzed using GC-FID. This was achieved by a liquid/liquid extraction using hexane. Known concentrations of FAME standards were dissolved in methanol, and subsequently extracted using hexane (1:10v/v methanol:hexane). The extraction was performed at 90°C, and the amount of FAME recovered into the hexane phase was measured from 0-90 minutes. Recovery of the FAMEs was analyzed and reported as the percentage recovery of the total FAME added to methanol. The experiments were done in triplicates and the standard deviation is reported as error bars.

As shown in Figure 2.1, 80% recovery of the FAMEs is seen immediately. FAMEs recovery continues to increase with time but eventually plateaus after 10 minutes. The extraction percentage reaches nearly 95% from 10-30 minutes. However after 30 minutes it appears that there is slight product degradation and loss possibly due to the effects of heating. Even with vigorous mixing and heating, fatty acid recovery can be as low as 80% if not given enough time for complete partitioning of the FAME into the hexane phase. Therefore, these results show that 10-30 minutes with adequate heat is required to obtain efficient extraction (>95%) of the FAMEs. For all further experiments, the extraction was conducted for 30 min.

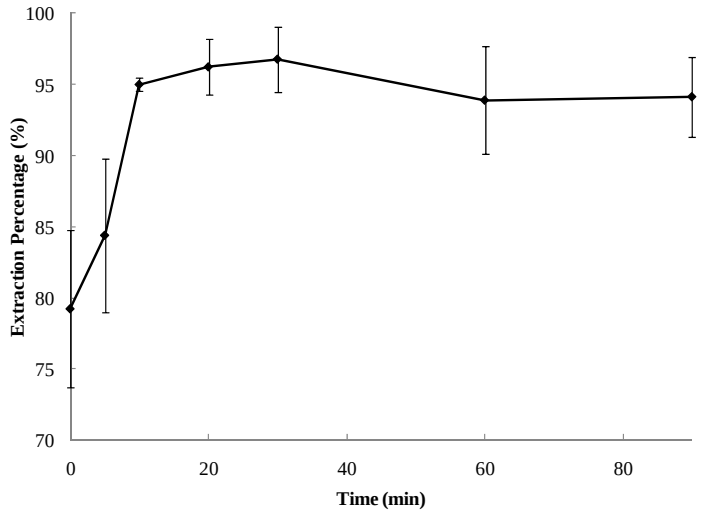

Figure 2.1: FAME recovery from methanol into hexane. FAME standards obtained from Sigma Aldrich were used to test the efficiency of extracting the FAME's from the working solvent (acidified methanol) into hexane. The standard deviation is shown.

Lipid profile analysis

The lipid profile in SR21 was analyzed separately in two ways. First, a total lipid extract was performed. Figure 2-2 shows the totally lipid extraction revealing the TAG region at approximately 30.0-35.0 minutes, with the presence of very little free fatty acids

and sterol compounds (15.0-30.0 minutes), with an internal standard of octacosane (C28:0, 0.1mg ml^{-1}) appearing at nearly 20.0 minutes.

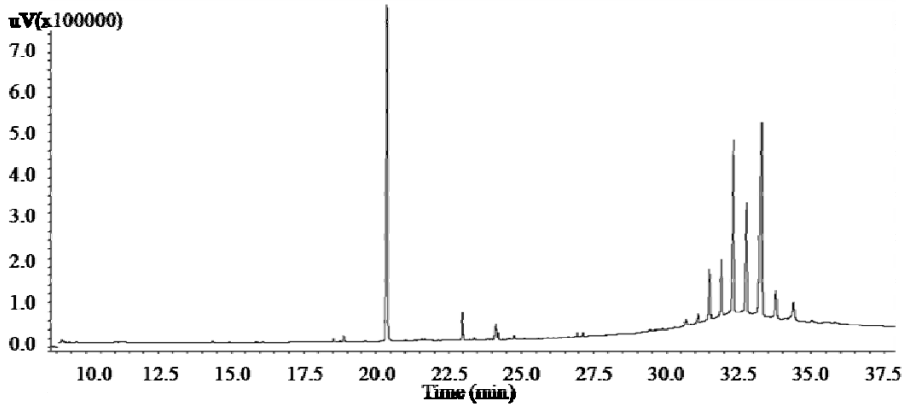

Figure 2: Total lipid extraction performed in SR21. This figure reveals the TAG region strongly at 30-35 minutes.

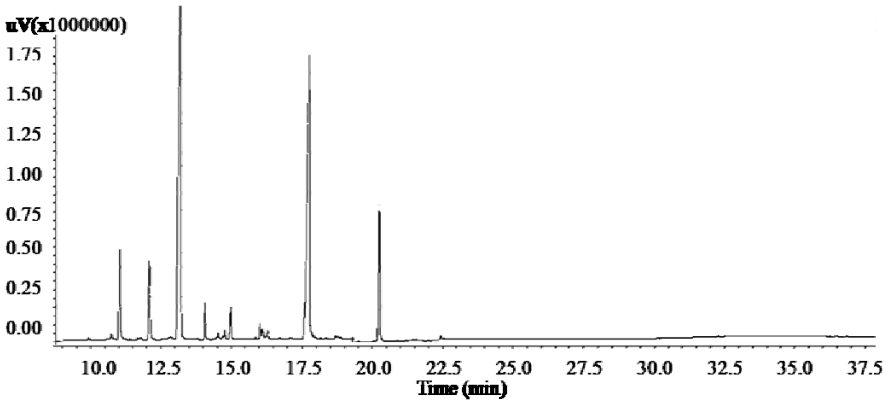

Figure 2-3: Individual fatty acid profile for SR21. An *in situ* transesterification reaction was performed on SR21 biomass and analyzed using GC-FID. The peaks corresponding to C14:0, C15:0, C16:0, DHA (C22:6), and the internal standard of octacosane (C28:0) can be seen at 11, 12, 13, 17.5, and 20 minutes approximately.

To determine the individual fatty acids which make up the TAG region of SR21, the *in situ* transesterification reaction was performed. Figure 2-3 is a representation of the individual fatty acid profile in SR21, including the TAG region. The peaks corresponding to C14:0, C15:0, C16:0, DHA (C22:6), and the internal standard of octacosane (C28:0) can be seen at 11, 12, 13, 17.5, and 20 minutes approximately. The TAG region was

included in the GC protocol to show that there are no remaining TAGs (30-35 minutes) that have not been transesterified and converted to FAMEs.

Effect of temperature

The effect of temperature was first studied to understand the reaction kinetics governing *in situ* transesterification. To conduct this study, biomass/solvent ratio was held constant at 66 mg ml^{-1}, and acid was held constant at 5% H_2SO_4. The temperature was increased from 60°C to 100°C, and the rate of FAME formation was measured as a function of time (Figure 2-4).

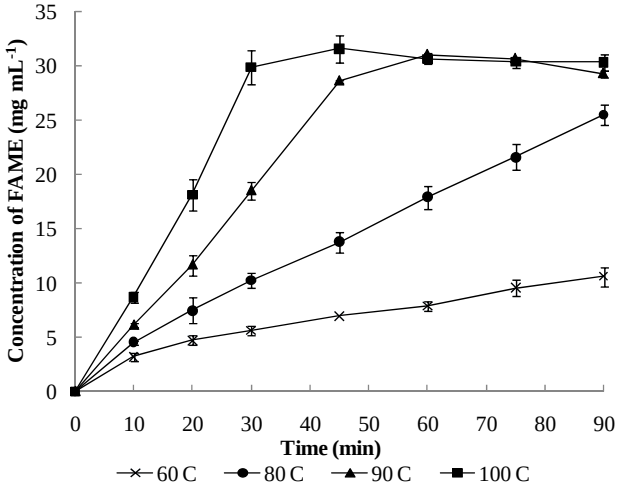

Figure 2-4: FAME concentration measured over time as a function of temperature. The total FAME concentration was analyzed using GC-FID. All experiments were conducted in triplicates and the standard deviation bars are shown.

The results from the effect of temperature on the rate of reaction demonstrate that as the temperature increases, the rate of reaction also increases. At higher temperatures (90, 100°C), the reaction reaches completion in 60 minutes or under. At lower temperatures (60, 80°C), the reaction is not completed within the 90 minutes allowed.

To further understand the effect of temperature, the rates of reaction at the different temperatures were modeled using the Arrhenius equation and are shown in Figure 2-5.

Using the linest function in excel, and also reiterated by performing the calculations by hand, the activation energy was determined to be 63.53 kJ mol^{-1}, with a standard deviation of 2.39 kJ mol^{-1}, and a 95% confidence interval of 5.85 kJ mol^{-1}.

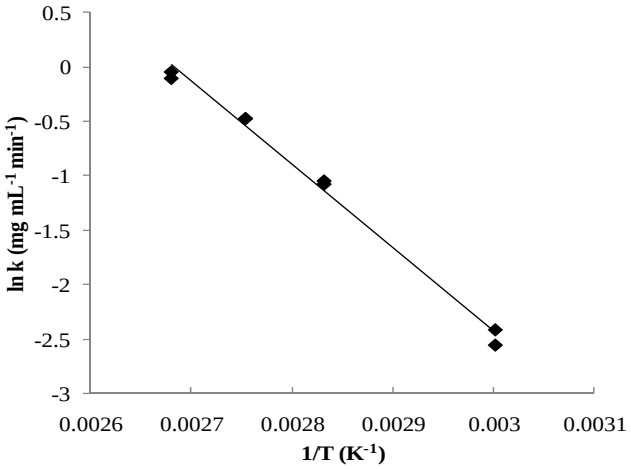

Figure 2-5: Reaction rates at the different temperatures (60, 80, 90, 100°C) were modeled using the Arrhenius equation. As temperature increases, the reaction rate also increases. Using the linest function in excel, and also by performing the calculations by hand, the activation energy was determined to be 63.53 kJ mol^{-1}, with a standard deviation of 2.39 kJ mol^{-1}, and a 95% confidence interval of 5.85 kJ mol^{-1}.

Effect of acid concentration

It was hypothesized that the concentration of the reaction catalyst would have a significant impact on the reaction time and efficiency. By maintaining the same biomass concentration, 66 mg ml^{-1}, the acid concentration was changed to 1, 1.5, 2, and 5% (v/v) H_2SO_4 in methanol. The FAME concentration was measured from 0-90 minutes, triplicates were performed and standard deviation bars are reported in Figure 2-6. At 5% acid, the appearance of TAGs was noticed at the early stages of the reaction (Figure 2-7). This was not apparent in the lower acid concentrations. One possible reason for the appearance of TAGs at this higher acid concentration is that the TAGs are extracted at faster rates than they are transesterified to FAMEs. Figure 2-7 is a chromatogram

showing the FAME region as well as the TAG region during the reaction at times of 10, 20, and 30 minutes. The magnified insert can be used to see the disappearance of TAGs as the reaction process (as time increases the TAG concentration or area decreases).

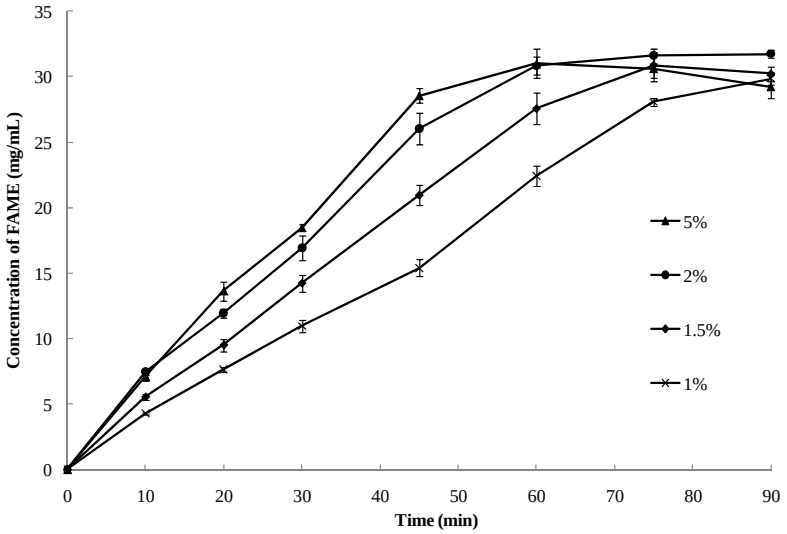

Figure 2-6: The effect of acid concentration was monitored at 90°C over 90 minutes using 66 mg ml^{-1} biomass/solvent ratio. The four acid concentrations tested were 1, 1.5, 2 and 5% H_2SO_4 in methanol (v/v).

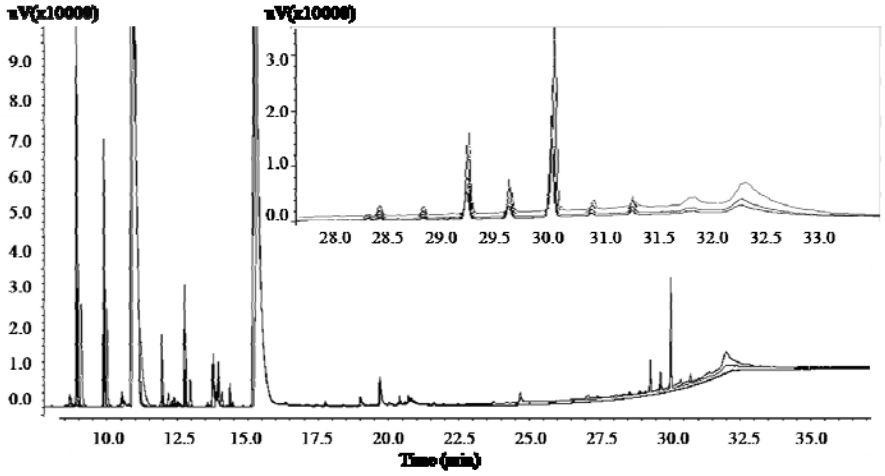

Figure 2-7: The reaction progress at 5% acid, for time intervals 10, 20, and 30 minutes. The magnified insert illustrates the disappearance of TAGs over time (the concentration or peak area decreases at time increases).

Figure 2-6 demonstrates the dependence of the reaction on the acid concentration. The acid concentration of 5% held the fastest rate of FAME extraction. For the acid concentration of 2% (v/v), the reaction was nearly equal to the rate at 5%, however the reaction takes 30 minutes longer to complete. However, as the acid concentration dropped below 2%, the reaction began to slow significantly.

Effect of biomass/solvent ratio

Current investigations of *in situ* transesterification have focused on low biomass/solvent ratios (1-20 mg ml^{-1}, Lewis et al., 2000). To develop a process suitable for large scale production, a major area of emphasis is to increase the amount of biomass the system can handle, without increasing the amount of solvent needed. Therefore, the reaction rates were studied at low, medium, and high biomass/solvent ratios. The biomass/solvent ratios tested in this study were 66, 125, 200, and 250 mg ml^{-1}.

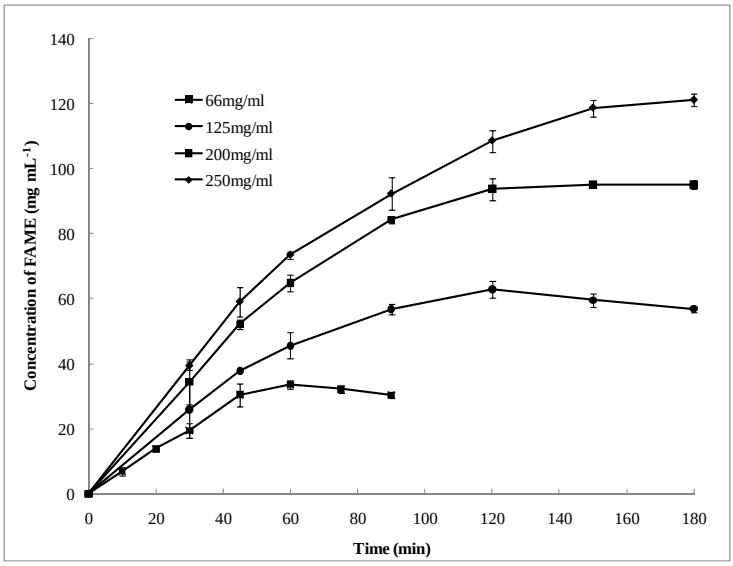

Figure 2-8: The effect of biomass/solvent ratio. As the biomass/solvent ratio increases, the rate of FAME formation also increases. All reactions were performed in triplicate and the standard deviations are shown.

Figure 2-8 illustrates the reaction rates at different biomass concentrations (66-250 mg ml^{-1}). It can be seen that as the biomass/solvent ratio increases, the reaction rate also increases. However, as the biomass/solvent ratio increases, the time to complete the reaction also increases. This is true as 66 mg ml^{-1} requires 60 minutes, 125 mg ml^{-1} requires 120 minutes, 200 mg ml^{-1} requires 150 minutes, and 250 mg ml^{-1} requires 180 minutes to achieve complete transesterification and recovery of the FAMEs. One reason for this increase in reaction time could be due to the increase in substrate, or initial TAG concentration. However, the relationship of the increase in biomass/solvent ratio is not directly proportional to the increase in reaction time. For instance, an increase of 66 to 250 mg ml^{-1} is nearly 4 fold, while the reaction only requires 3 times as long. Therefore, it is hypothesized that at higher biomass/solvent ratios there are other factors including bulk transfer of the reaction solvent, or catalyst activity may be involved.

Conclusion

It was hypothesized that the rates of reaction would be higher with increasing temperature and/or acid concentration. However, by using more severe reaction conditions product degradation was a concern. Therefore, these experiments were designed to establish appropriate temperature and acid ranges for achieving satisfactory (>95%) product yields without product loss.

By controlling and modifying the reaction variables, the solvent volumes can be decreased by a factor of 20 from literature values, with only minimal, if any, loss in efficiency of lipid conversion and recovery (greater than 95% recovery). This was seen by increasing the biomass/solvent ratios from reported literature values of 1-20 mg ml^{-1} to experimental values of 66-250 mg ml^{-1}. Therefore, the single step reactive extraction

may potentially be applied to larger scale applications. However, one consequence of increasing the biomass/solvent ratio however is the reaction requires longer processing times and this must be taken into consideration when scaling up.

The effect of temperature on the reaction rate was also determined. At higher temperatures, 100°C initially showed a faster rate of reaction, and reached completion around 45 minutes. However, after 30 minutes the reaction rate slowed. At 90°C, the reaction took 60 minutes to reach completion, and then remained stable for a longer period of time than at 100°C. While the reaction does take place more rapidly at 100°C, at this higher temperature the FAMEs begin to degrade. Thus, a reaction at 90°C may be more suitable as it is more stable and there is less risk of losing sample volume. In addition to temperature and biomass/solvent ratio, the acid concentration became a significant factor when the concentration was lowered. This is possibly due to the molar ratio of hydrogen ions to triglycerides. As the molecular availability of H^+ ions decreases, there is less interaction with the triglycerides, resulting in slower product formation. It appears that a critical level of acid concentration is reached at approximately 2%. Ideally, the acid concentration would be 5% or greater to keep the reaction rates higher. However, at 2% the reaction was not significantly slowed. Therefore, acid concentration is a significant factor that must be taken into consideration when scaling up.

Using these studies, an understanding of the fundamental reaction and limitations during processing, a new, reliable, and cost-effective system for large scale lipid production can be developed from microbial biomass including oleaginous algae, fungi, and yeast. Future work will include analysis of different organisms and strains. Also, future experimentation will also include modeling the effects of moisture content on the

reaction. Freeze drying biomass on large scales is not cost effective, therefore the study will be expanded to include air-dried biomass as well was wet biomass slurries.

CHAPTER 3

PHASE SEPARATION AND METHANOL RECYCLE

Introduction

Biodiesel and dietary omega-3 polyunsaturated fatty acids have experienced a worldwide increase in interest during recent years. This is reflected as the production of biodiesel in the United States has increased significantly; from 75 million gallons in 2005, to 450 million gallons in 2007 (National Biodiesel Board, 2007). The many factors driving this increase in interest can be ascribed to rising petroleum fuel costs, concern for environmental impact of combustible fossil fuels, and reliance on foreign oil sources (Nelson and Schrock, 2006). Also, the role of dietary omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been increasingly recognized for improved human health and for the prevention of diseases such as proper nervous system and visual function (Horrocks and Yeo, 1999), coronary heart disease (Barclay et al., 1994), and as breast and colon cancer chemopreventive agents (Rose et al., 1999; Senzaki et al., 1998). An analysis by Frost and Sullivan (2005) revealed that the US market revenue in 2004 for omega-3 and omega-6 fatty acids (e.g. docosahexaenoic acid) totaled $266 million, and is expected to reach a total of $671.9 million by the year 2011. Therefore production of lipids, such as biodiesel and omega-3 fatty acids, using microorganisms for commercial use has gained the attention of the scientific community (Chisti, 2007; Vazhappilly and Chen, 1998).

Biodiesel is typically produced via an acid- or base-catalyzed transesterification reaction of vegetable oils or animal fats the yields methyl esters, or fatty acid methyl

esters (FAME) (Knothe, 2005). However, using the traditional sources of the vegetable oils such as soybean and canola have raised concerns about providing enough feedstock for energy demands without damaging the food manufacturing industry. Therefore, among the most popular non-edible feedstocks investigated have included *Jatropha curcas* (Foidl et al., 1996), lard (Lee et al., 2002), and waste cooking oil (Kulkarni and Dalai, 2006). This study has focused on the use of microorganisms grown heterotrophically using glycerol, a by-product of biodiesel production. When 100kg of biodiesel is made, approximately 10kg of glycerol is produced (National Renewable Energy Laboratory, 2007). During conventional biodiesel production, this glycerol byproduct is contaminated with excess reagents (such as methanol and base) and soap-like compounds (salts of long-chain fatty acids) that are also byproducts of the biodiesel conversion process. Crude glycerol in its unrefined state has little economic value. Purification costs to produce food- or medical-grade glycerin are also now prohibitive due to its low market value as a result of this new and large supply source. The increase of available glycerol has caused the price of pure glycerin to decrease from $0.25/lb in 2004 to $0.025-0.05/lb in 2006 (Johnson and Taconi, 2007; Yazdani and Gonzalez, 2007). With the cost of refining crude glycerol being estimated at $0.20/lb (USDOE, 2007), it is not economical to recover pure glycerin. Due to this imbalance between demand and supply, costly disposal methods or alternative uses of crude glycerol are now needed.

The genus *Schizochytrium* was chosen for this study due to several reasons. *Schizochytrium* species, which have recently been reclassified as a member of *Thraustochytriacea,* among family of *Labyrinturomycota,* are known to have high

cellular contents of fatty acids (Morita and Kumon, 2006). The specific species of choice, *Schizochytrium limacinum* SR21, is a marine fungus and was isolated from the coral reef area in the Yap islands of the Federated States of Micronesia (Nakahara et al., 1996). This strain is capable of accumulating high amounts of biodiesel components and omega-3 fatty acids and is therefore a promising strain for large scale production. Another major advantage to this organism is that it produces relatively few lipid types. While many lipid and fatty acid producers accumulate several types of lipids including triacylglycerides (TAG), sterol esters, and phosphatidylcholines, *S. limacinum* SR21 has been shown to primarily produce TAGs (Morita and Kumon, 2006). Also, the overall fatty acids, including those contained in the TAGs, are relatively few (Table 3-1). This is advantageous for downstream processing because it will potentially reduce the number of steps and processes to separate and purify the desired FAMEs.

Basic growth conditions for SR21 have been established in previous studies. Wu et al. (2005) observed optimal cell growth and lipid production at pH 7.0 and also reported consumption of lipids and extracellular metabolic carbon during starvation. A study by Chi et al. (2007) showed that SR21 grew on glycerol from biodiesel waste but growth was inhibited when concentration of methanol (a contaminant in crude glycerol) was above 20g L^{-1}, likely due to methanol toxicity. The study also measured biomass and FAME yields in SR21 grown on glucose and glycerol and showed that the biomass and FAME productivities are similar on both substrates indicating that glycerol is as effective as other more traditional substrates for production of FAMEs.

While the studies done by Chi et al. (2007) show the potential for economical production of FAMEs through utilization of crude glycerol, there are few studies that

have adequately describe processes by which FAMEs could be extracted and purified on larger scales.

Therefore, processes have been developed to first extract the intracellular lipids from the original source, and then convert them to the more useful FAME form. Of the techniques developed, the well-known Bligh and Dyer method remains the most popular. In this procedure, a mixture of chloroform, methanol, and water is used to perform the extraction and the solvent ratios are balanced such that a single phase is formed with the sample water (Bligh and Dyer, 1959). After completion of the extraction, phase separation is achieved by diluting the mixture with chloroform and water, allowing the extracted lipids contained in the chloroform phase to be removed. Following this extraction, the lipids are subjected to a transesterification reaction in the presence of an acid or base catalyst to achieve the final FAME form. Of the newer techniques under investigation, the microwave assisted, supercritical fluid, or *in situ* transesterification techniques have gained the most recognition (Carrapiso and Garcia, 2000). The use of microwaves provides a rapid extraction and does not require that the samples be previously dried (Pare et al., 1997). Supercritical fluid extraction (SFE) has gained interest because it significantly lowers the requirements for organic solvents, as well as the concerns for waste disposal after the reaction (Randolf, 1990). However, conditions affecting SFE, such as particle size and water content, must be improved to achieve efficient extractions (Eller and King, 1996). While many of these alternative extraction techniques have been developed for a lipid extraction for a wide variety of sources (meat, dairy, egg), they may be applied to microbial systems.

Finally, the *in situ* transesterification method combines the extraction and transesterification into a single step and thus lowers solvent use and processing time (Ashford et al., 2000; Chi et al., 2007; Lewis et al, 2000; Morita and Kumon, 2006; Yokochi and Honda, 1998). Although this method was first proposed by Abel et al. in 1963, most investigations have only been performed in recent years.

However, the *in situ* method has been primarily developed for laboratory scale reactions and is used for analytical quantification of lipids. For example, most laboratory methods use excess solvents and prolonged reaction times to ensure complete recovery (Lewis et al., 2000); factors that would need to be optimized for efficient large scale processing. Therefore, the method needs to be modified and optimized for large scale production.

In the research reported here, several key factors have been studied that affect separation technologies for FAME recovery and solvent recycling. The key factors examined were biomass/solvent ratio, solubility of FAMEs in methanol, and recyclability of acidified methanol. Results show that automatic phase separation of FAMEs is achieved when the critical solubility concentration of FAMEs in methanol is reached. In addition, methanol may be recycled for use in further batch processing, thus reducing overall production costs. From the results obtained, under certain processing parameters the *in situ* transesterification process offers a promising and cost efficient method for large scale extraction and recovery of FAMEs from microorganisms.

Materials and Methods

For all *in situ* transesterification and extraction experiments, HPLC grade chloroform, n-hexane, methanol, and hydrochloric acid were obtained from Sigma Aldrich and used as received. Fatty acid methyl esters (FAMEs) including myristic acid (C14), pentadecanoic acid (C15), palmitic acid (C 16:0), and docosahexaenoic acid methyl ester (C 22:6) standards were obtained from Sigma Aldrich and used to determine GC calibration standards.

Microorganism and cell preparation

The microorganism of choice, *Schizochytrium limacinum* SR21 (ATCC MYA 1381, referred to as SR21), is a unicellular marine fungus. This organism was chosen based on its ability to produce high amounts of intracellular lipids relative to cell weight (Chi et al., 2007; Pyle et al., 2008).

SR21 biomass was grown in a complex medium containing 1g L^{-1} yeast extract, 1g L^{-1} peptone, 10g L^{-1} sea salt, and 6g L^{-1} glycerol. The medium is then adjusted to a pH of 7.0. The biomass was harvested using a Thermo IEC multi (Thermo Scientific, Waltham, MA, USA) at 3600rpm for 20 minutes. The samples were washed with a 0.85% saline solution, and re-centrifuged. The biomass was dehydrated using a FreeZone 4.5 freeze dryer (Labconco, Kansas City, MO, USA) and stored in a Forma -80°C freezer (Thermo Scientific, Waltham, MA, USA) at -80°C until used for experimentation. For all experiments, biomass is reported as a dry weight. To eliminate batch to batch variation, 16L of biomass culture was grown and harvested as previously stated, and thoroughly mixed to ensure a homogenous biomass stock.

In situ transesterification

The biomass was treated via an acid catalyzed *in situ* transesterification. This was done by weighing out biomass into vials individually to achieve biomass concentrations of 66, 125, 200, and 250 mg ml^{-1}. Each reaction was performed at 90°C and 10% H_2SO_4 (v/v). The samples were removed every 10 minutes and shaken vigorously. The optimal time for extraction has been previously determined (Nelson et al., 2010, in preparation). 3 replicates of each experiment were performed to reduce experimental error and variability.

After the reaction was stopped, the mixture was allowed to cool and the contents were transferred to a separate vial. The final contents were extracted using n-hexane in a ratio of 10:1 (v/v) of hexane to methanol. The mixture was shaken vigorously and heated at 90°C for 20 min. The total mixture was then allowed to cool to room temperature and 5ml of water was added and the aqueous phase was allowed to settle. The upper layer (hexane layer), containing the FAME's, was collected and stored for analysis.

FAME analysis

The FAMEs were analyzed using gas chromatography equipped with flame ionization detection (GC-FID). This common method is simple and rapid, and is thus advantageous for analysis. A Shimadzu GC-2010 equipped with a Shimadzu AOC-20i auto injector and FID was used for all experiments. The GC was equipped with a Restek RTX-Biodiesel column with 0.53 mm guard. The FID detector was set at 370°C, with a column flow of 2.53 ml $minutes^{-1}$. The sample volume injected is 1µL and analysis begins at 60°C and increases 10°C $minute^{-1}$ to 370°C, and holds constant at 370°C for 6 minutes. The FAME standards were prepared in a hexane solution at concentrations of

0.025, 0.05, 0.075, 0.1, 0.15, 0.2 and 0.3 mg ml^{-1}. This linear range of concentration standards was used to develop a standard curve equation by which all unknown fatty acid concentrations were determined.

Because the extracted biomass samples contained concentrated FAMEs, the samples were diluted with hexane to a ratio of 1:20 (sample:hexane). This was done to ensure the sample measurements remained within the linear range of the standard concentration tests to provide accurate results.

Phase separation

The phase separation of the FAMEs from the acidified methanol phase was studied. When the *in situ* transesterification reaction occurs, the intracellular lipids are extracted and transesterified simultaneously. As the lipids are removed they begin to accumulate and begin to separate automatically. It is believed that this automatic separation will continue to occur until the solution reaches equilibrium. Once this equilibrium has been reached, the remaining fatty acids within the methanol phase can be measured as well as the fatty acids which have separated. To do this, after performing the *in situ* transesterification, the mixture was allowed to cool to room temperature. While cooling the residual biomass settled to the bottom of the vial and the extracted FAME's floated to the top of the solution. The upper FAME layer was removed by syringe, measured volumetrically, and then analyzed as previously discussed. The remaining methanol phase was extracted with n-hexane as previously described to account for the remaining FAME's soluble in the methanol phase. This was done to determine the overall solubility of FAMEs in methanol and to study the bulk phase separation properties for

scale up of the overall process. The experiments were performed in triplicate for all biomass to solvent ratios.

Acidified methanol recycling

To reduce the overall cost of the process, it is desirable to recycle the methanol for subsequent reactions. After the phase separation of the FAMEs, the acidified methanol was then reused in a second transesterification reaction. This was done by directly transferring used acidified methanol into a second vial with previously weighed biomass of equivalent mass of the first reaction. The *in situ* transesterification process was repeated in the same fashion as the first step. The final mixture was then allowed to cool, and subsequently analyzed for all FAMEs in both the phase separated FAMEs as well as what remained in the methanol phase. Overall mass balances were kept to ensure all FAMEs in the biomass were accounted for.

The phase separation experiments showed there to be remaining FAMEs in the methanol phase. Therefore, the recycled methanol will contain soluble FAMEs, and potentially proteins and cell debris. Therefore, the reaction was monitored for both extraction efficiency and to measure the reaction rates to observe an increase or decrease in rates.

Table 3-1 Fatty Acid Methyl Ester Composition of *S. limacinum* SR21

	Fatty Acid Fraction: (% w/w) of Total Lipid			
	14:0	15:0	16:0	22:5, 22:6
Reference	Myristic	Pentadecanoic	Palmitic	DPA + DHA
Yokochi and Honda, 1998	2.7	7.6	34.2	51.7
Chi et al., 2007	4.0	nr	52.0	42.0
This Study	1.9±1.1	1.3±1.4	49.3±1.8	48.1±1.2

DPA = Docosapentaenoic acid, DHA = Docosahexaenoic acid, nr = not reported

Results and Discussion

Cell Preparation

SR21 biomass was grown and harvested as described earlier. The fatty acids contained within the biomass were then analyzed. Table 3-1 shows the fatty acid fraction in %w/w of the total intracellular lipids in SR21 as experimentally determined by previous studies.

Phase separation

Upon performing the *in situ* transesterification reaction, and allowing the mixture to cool, the FAMEs achieve automatic phase separation (Figure 3-1). The separated FAMEs are removed and the FAME concentration (mg mL^{-1}) in the methanol phase was measured and plotted against the total FAME concentration for each biomass to solvent ratio, 66, 125, 200, and 250 mg ml^{-1} (Figure 3-2). As shown in Figure 3-2, for all biomass/solvent ratios the concentration of FAMEs remaining in the methanol phase remained constant at nearly 22.7 mg ml^{-1}.

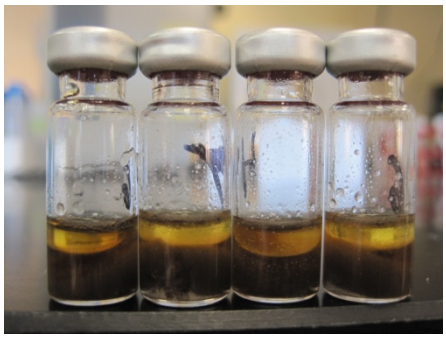

Figure 3-1: The *in situ* transesterification reaction at various biomass concentrations and recycling were allowed to cool and the FAMEs to achieve phase separation. In the figure the oil phase can be seen above the biomass and methanol layers.

These findings suggest that the overall solubility of FAMEs in methanol is near 22.7 mg ml^{-1}, and has no dependence on the total amount of FAMEs present. It can also

be seen that as the concentration of biomass increases from 66 to 250 mg ml^{-1}, the total concentration of FAMEs increases proportional to the biomass concentration.

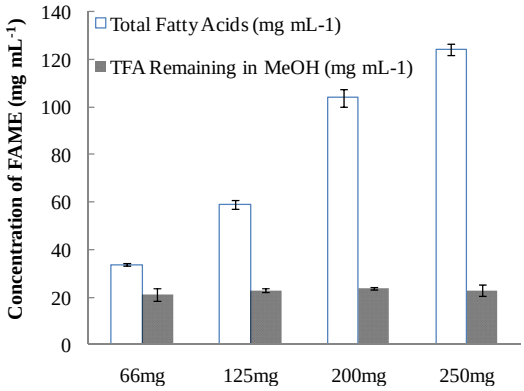

Figure 3-2: The concentration of FAMEs in the methanol phase was quantified along with total fatty acids extracted from the biomass during the *in situ* transesterification reaction. The soluble FAME concentrations remaining in 66, 125, 200, and 250 mg were 21.2, 22.9, 23.8, and 23.0 mg ml^{-1} respectively.

A representation of the individual composition of FAMEs in both the methanol phase and the overall FAME makeup is shown in Table 3-2. It can be seen that proportionately, the concentration of individual FAMEs in the methanol phase with respects to the overall composition does not change. Therefore, there is no bias in which type of fatty acid exists in each phase.

Table 3-2: The overall concentrations of individual fatty acids in both the soluble and in-soluble phases is shown. The total FAME concentration in each phase is also shown.

Biomass		DHA	C16	C14	C15	Total Fatty Acids
(mg mL-1)		(mg mL-1)	(mg mL-1)	(mg mL-1)	(mg mL-1)	(mg mL-1)
66	Soluble	9.61	11.11	0.30	0.16	21.17
	In-sol.	6.59	5.63	0.31	0.16	12.70
	TFA	16.20	16.74	0.61	0.32	33.87
125	Soluble	10.76	11.80	0.25	0.12	22.93
	In-sol.	17.31	16.56	1.48	0.82	36.17
	TFA	28.08	28.36	1.73	0.94	59.11
200	Soluble	10.91	12.27	0.42	0.21	23.81
	In-sol.	41.07	34.75	2.86	1.33	80.02
	TFA	51.99	47.02	3.28	1.55	103.83
250	Soluble	11.61	10.56	0.56	0.28	23.01
	In-sol.	50.72	45.27	3.08	1.88	100.96
	TFA	62.34	55.83	3.64	2.16	123.97

Acidified methanol recycling

Next, the FAME recovery when the methanol phase was recycled to a second *in situ* transesterification was plotted in Figure 3-3. In Figure 3-3, the graph on the left shows the overall FAME recovery from each biomass to solvent ratio. It also demonstrates the overall composition of the FAME concentration by distinguishing the individual fatty acids. It can be seen in this figure that there is no bias in the type of fatty acid recovered. Therefore, whether the fatty acid is saturated (C14:0, C15:0, C16:0) or unsaturated (DHA, 22:6), there is no discrepancy in the recovery. The right graph in Figure 2 represents the recovery percentage of the total available FAMEs present in the biomass. The highest recovery percentage corresponds to 96.9% at 66 mg, and a low of 84.4% at 250 mg. The decrease in recovery percentage may be attributed to several factors including residual biomass debris or proteins in the recycled methanol, or in the reduced amount of hydrogen ion concentration as a result of the hydrolysis of biomass and proteins and the transesterification process itself.

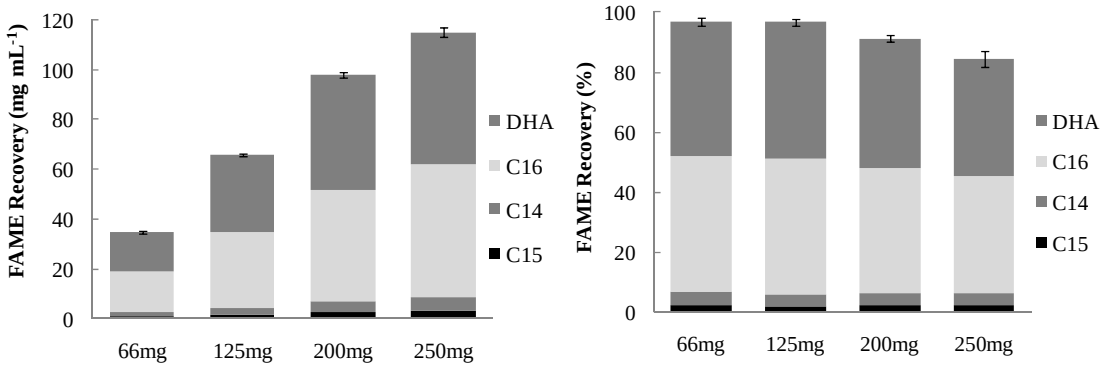

Figure 3-3: Left- The overall FAME recovery after 1 recycling of methanol. Right- The total recovery percentages of FAMEs for each level of biomass/solvent ratio. The recovery percentages for 66, 125, 200, 250 mg ml^{-1} are 96.9, 96.8, 91.3, and 84.4%, respectively.

Conclusion

It has been determined that through *in situ* transesterification of intracellular lipids, FAMEs can be automatically phase separated and the working solvent (acidified methanol), can be recycled for use in subsequent batch processing. The results indicate that the solubility of FAMEs in methanol is approximately 22.7 mg ml^{-1}. Once this constant is reached, the remaining FAMEs begin automatic phase separation and can be harvested without excessive input. Therefore, regardless of the FAME concentration within the system as a result of the amount of biomass present, any FAME concentration in excess of the critical solubility will phase separate. It was also shown that the phase separation is unbiased towards individual fatty acids. Regardless of whether the FAMEs are saturated or unsaturated, the same relative concentrations remain the same both the soluble and in-soluble phases.

It has also been shown that the methanol phase of the *in situ* reaction may be recycled and used in further extraction processes. Higher recovery percentages are achieved with lower biomass to solvent ratios, >95% for 66 and 125 mg ml^{-1}, and begins to decrease with increasing biomass. However, it is noted that by increasing the biomass to solvent ratio from 66 to 250 mg ml^{-1}, a nearly 4-fold increase, the recovery percentage only drops 10%. Therefore, when considering scaling up the process, it may be economically favorable to increase the biomass to solvent ratio and conserve the amount of solvent necessary. Future work can include conducting further recycling steps to determine long term effects of recycling the methanol.

CHAPTER 4

TRANSESTERIFICATION OF INTRACELLULAR LIPIDS: A KINETIC MODEL

Introduction

In recent years, the significance of lipids has increased due to their implications in renewable energy as well as for human nutrition and health. Lipids from sources such as vegetable oil can be converted to biodiesel through a transesterification reaction that produces fatty acid alkyl esters. Also, biodiesel has many technical advantages when used as a fuel source, such as high energy density, favorable combustion emission profile, and improved lubricating (Liu and Zhao, 2007). Most often, fatty acid methyl esters (FAMEs) are used as biodiesel due to the relatively lower cost of methanol – one of the reactants in the process. With increasing demand for biodiesel, conventional vegetable oil feedstocks derived primarily from oilseeds such as soy, canola, palm, rapeseed, and sunflower are unable to meet market needs resulting in an unsustainable pressure on agricultural practices and food sources. As a result, alternative feedstocks for lipids are needed.

Besides plants, microorganisms, such as algae or fungi, are also capable of accumulating lipids (such as mono-, di-, and tri- glycerides of fatty acids). In fact, laboratory research shows that algae are more efficient lipid producers than terrestrial plants and can yield at least two orders of magnitude higher lipid mass per acre per year than most conventional oil crops.

In addition, algae and other microorganisms are also capable of synthesizing large amounts of polyunsaturated fatty acids (PUFAs) that are important for human health and nutrition. PUFAs in the body help to maintain proper organ function (Wu et al., 2005). For example, high DHA content in the brain and retina has been shown to be important

for proper nervous system and visual function (Horrocks and Yeo, 1999). In fact, PUFAs are essential ingredients in infant diet and are added into most commercially available baby formula foods. In addition, PUFAs such as omega-3 fatty acids can also act as breast and colon cancer chemopreventive agents (Rose and Connolly, 1999; Senzaki et al., 1998). Therefore, in an increasingly nutrition-aware population, it is important to develop technologies capable of keeping up with the demand.

Unfortunately, the technologies that exist for extracting oils from plants and converting them to fuel do not necessarily apply to microbial biomass because of the differences in their physical properties. Therefore, new biodiesel production technologies must be developed for microbial systems which are efficient and cost effective on large scales (Meher et al., 2006).

Today, analytical methods that strive to achieve rigorous and complete extraction of cellular lipids commonly use the well-known Bligh and Dyer method (Bligh and Dyer 1959). In this procedure, a mixture of solvents, such as chloroform, methanol, and water is used to perform the extraction and the solvent ratios are balanced such that a single phase is formed with the sample water. After completion of the extraction, phase separation is achieved by diluting the mixture with chloroform and water, allowing the extracted lipids contained in the chloroform phase to be removed. Known modifications or adaptations of this procedure involve changing the solvents and their ratios. For example, methylene chloride has been used as an alternative to chloroform and variations in extraction time, temperature, and order of solvent addition may be beneficial (Chin et al., 2006; Folch et al., 1957; Lewis et al., 2000; Perveen et al., 2006; Wu et al., 2005). Another known emerging practice is the use of sonication during the reaction (Belarbi et

al., 2000). Sonication helps to disrupt the cells and facilitates faster lipid release from cells, but this method adds significant process costs. Microwaves can also be used to expedite the extraction process (Pare et al., 1997). Within all the adaptations of the Bligh and Dyer method, the principle mechanisms of extraction remain the same.

There are several disadvantages of the solvent extraction followed by transesterification approach described above. First, toxic solvents are used in this process (such as chloroform) that will need to be effectively recovered and recycled. Solvent recovery is an expensive process and will add to the cost of biodiesel production. Second, even with recovery processes in place, there is a high likelihood of generating hazardous waste that will incur additional treatment costs (Randolf, 1990). Third, the process requires two steps – an extraction step to obtain lipids from biomass followed by a reaction step to convert the lipids to biodiesel. Additional steps might be needed to recover the biodiesel from the solvent to provide a "fuel-ready" product. Fourth, due to the use of toxic solvents the process might be unsuitable for production of human nutrition products (e.g. PUFAs). Finally, the multiple steps involved lead to long processing times. Overall the known solvent extraction based methods require multiple energy intensive steps, incur high disposal costs and are unsuitable for generation of high-value nutraceuticals.

Supercritical fluid extraction (SFE) using carbon dioxide is an alternative approach that significantly lowers the requirements for organic solvents, as well as mitigates concerns for waste disposal after the reaction. However, conditions affecting SFE, such as particle size and water content, must be improved to achieve efficient extractions (Eller and King 1996). Also, the SFE option requires high initial capital

investment and continuously high energy inputs – two factors that render this method prohibitively expensive.

In the past, lab scale transesterification reactions have been reported for analytical description of cellular lipids (also known as lipid profile). In these tests reported in the literature, a low biomass to reactant (acidified methanol) ratio is used (typically ~20 mg biomass per ml of reaction). The FAMEs generated in such methods are very low in concentration and are completely soluble in the reaction mixture. If scale-up based on these existing protocols were to be done for commercial production of transesterified lipids, a separate solvent extraction (e.g. through use of hexane) would first be needed to retrieve FAMEs from the reaction mixture. The resulting FAME + hexane solution would then have to undergo additional processing, such as distillation, to recover the solvent. In this step, hexane would boil off (at ~70 °C, if carried out under atmospheric pressure) leaving behind an un-evaporated FAME product. Thus, this method would still require multiple steps (reaction, extraction and distillation) and use of solvents (hexane). Also, if this process were to yield a human nutrition product to generate a high value revenue stream, the distillation bottoms containing FAMEs would have to be very carefully stripped of all residual hexane that would require additional processing. Finally, processing biomass at the low concentrations prescribed in existing protocols would require prohibitively large-sized equipment. As an example, if algal biomass containing 50% lipid (by weight) were to be reacted at 20 mg ml^{-1}, a reaction vessel of 100 gallon volume would be needed to produce one gallon of product.

Finally, the alternate technique described in literature, the *in situ* transesterification method, combines the extraction and transesterification into a single

step and thus lowers solvent use and processing time (Ashford et al., 2000; Chi et al., 2007; Lewis et al., 2000; Morita and Kumon, 2006; Yokochi and Honda, 1998). Although this method was first proposed in by Abel et al. (1963), most investigations have only been performed in recent years. In this process, biomass is treated with a mixture of alcohol and acid or base resulting in the reactive extraction of lipids as fatty acid alkyl esters (typically fatty acid methyl ester, or FAME). Basic catalyzed reactions have been the focus in research because it is much faster than acid catalyzed reactions and the reaction conditions or more moderate (Lang et al., 2001; Vincente et al., 2004). However, base catalyzed reactions run the risk the formation of soaps from cellular free fatty acids (FFAs). The formation of soaps greatly increases the cost of downstream processing as it causes the formation of gels, increases viscosity, and makes product separation more difficult (Carrapiso and Garcia, 2000; Ma and Hanna 1999). Another factor affecting the use of base catalysis is the presence of water. When water is present in the system hydrolysis of the alkyl esters into FFAs is assumed, which leads to further soap formation. Soap formation can be avoided with the use of strong liquid acid-catalyzed processes where the FFAs are converted to the ester form through acid mediated esterification. Nevertheless, acid catalyzed reactions require higher temperatures and longer reaction times than base-catalysis (Freedman et al., 1984). Based on these considerations, the acid-catalyzed *in situ* transesterification process is likely to be more economically viable during large-scale processing. However, this method has been developed for laboratory scale reactions and is used for analytical quantification of lipids. For example, most laboratory methods use excess solvents and prolonged reaction times to ensure complete recovery (Lewis et al., 2000), factors that would need to be

optimized for efficient large scale processing. Therefore, the method needs to be modified and optimized for large scale production.

Very little research has been conducted to develop a model that could be used to understand and predict the conversion rates and factors influencing the transesterification reaction. The focus of this research was to develop a model capable of predicting the conversion of intracellular lipids into the useful FAME form using a model biomass organism *Schizochytrium limacinum* SR21 (referred to as SR21). Experiments were conducted at several biomass/solvent ratios, thus increasing the amount of substrate (TAGs) in the system. Therefore, kinetic modeling of the conversion rates of intracellular TAGs into FAMEs was possible.

Materials and Methods

Chemicals

For all *in situ* transesterification and extraction experiments, sulfuric acid (98%), and HPLC grade chloroform, n-hexane, methanol were obtained from Sigma Aldrich (St. Louis, MO) and used as received. Individual fatty acid methyl ester standards (all greater than 95% purity) including methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C 16:0), and methyl docosahexaenoate (C 22:6) standards were obtained from Sigma Aldrich and used for gas chromatography calibrations. For culture media, yeast extract and Bacto-peptone were obtained from BD biosciences (San Jose, CA), glycerol (99.5%, Mallinckrodt Chemicals, Phillipsburg, NJ), and Instant Ocean brand sea salt (Aquarium Systems Inc, Mentor, OH) were used.

Microorganism and cell preparation

A pure strain of *Schizochytrium limacinum* SR21 (ATCC MYA 1381, henceforth referred to as SR21) was used in this study. SR21 was grown in a complex medium containing 1g L^{-1} yeast extract, 1g L^{-1} peptone, 10g L^{-1} sea salt, and 6g L^{-1} glycerol. The medium was adjusted to a pH of 7.0, after which 50 ml of medium was then transferred to 125 ml erlenmeyer flasks and autoclaved at 125°C for 60 minutes. The flasks were then inoculated with 100µl of freeze dried SR21 culture stock. The flasks were incubated at room temperature in an Innova 2300 platform shaker (New Brunswick Scientific, Edison, NJ) at 75 rpm for 4 days. The biomass was harvested using a Thermo IEC multi centrifuge (Thermo Scientific, Waltham, MA) at 3600rpm for 20 minutes. The samples were washed twice with a 0.85% sodium chloride solution, and re-centrifuged. The biomass was dehydrated using a FreeZone 4.5 freeze dryer (Labconco, Kansas City, MO) and stored in a Forma -80°C freezer (Thermo Scientific, Waltham, MA) at -80°C until used for experimentation. For all experiments, biomass is reported as a dry weight. To eliminate batch to batch variation, all biomass was grown under the same conditions, harvested as previously stated, and thoroughly mixed to ensure a homogenous biomass stock.

Experimental design

The purpose of this study was to develop and test a kinetic model that could describe the transesterification of intracellular lipids into FAMEs at different biomass/solvent ratios as well as acid concentrations. The biomass was treated with an acid catalyzed *in situ* transesterification as described in a previous study (Nelson et al., 2010, in preparation). This was done by weighing out biomass into vials individually to achieve biomass concentrations of 66, 125, 200, and 250 mg ml^{-1}. For the

biomass/solvent ration tests, each reaction was performed at 90°C and 5% H_2SO_4 (v/v). The formation of FAMEs was measured over time as a function of biomass concentration. The formation of FAMEs was then used to back calculate the amount of TAGs, or substrate within the system during the reaction. The samples were removed periodically and shaken vigorously. Next, to determine the validity of the model created, the biomass/solvent ratio was held constant at 66mg ml^{-1}, and the acid concentration was tested at 1, 1.5, 2, and 5% H_2SO_4 (v/v). Again, the FAME formation was measured over time as a function of acid concentration and then used to back calculate the substrate, TAG concentration. Three replicates of each experiment were performed to reduce experimental variability. The samples were analyzed as described by Nelson et al. (2010, in preparation). The experimental data obtained was used to compare against the theoretic model derived from known reaction mechanisms.

Fatty acid methyl ester analysis

The FAMEs were analyzed using gas chromatography equipped with flame ionization detection (GC-FID). A Shimadzu GC-2010 equipped with a Shimadzu AOC-20i auto injector and FID was used for all analyses. The GC was equipped with a Restek RTX-Biodiesel column with 0.53 mm guard. The FID detector was set at 370°C, with a column flow of 2.53 ml min^{-1}. The sample volume injected is 1µL and analysis begins at 60°C and increases 10°C $minutes^{-1}$ to 370°C, and holds constant at 370°C for 6 minutes. The FAME standards were prepared in a hexane solution at concentrations of 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 and 0.3 mg ml^{-1}. This linear range of concentration standards was used to develop a standard curve equation by which all unknown fatty acid

concentrations were determined. Octocosane, C28:0, was used as an internal standard, 0.1 mg ml^{-1}.

The extracted biomass samples contained concentrated FAMEs, the samples were diluted with hexane to a ratio of 1:20 (sample:hexane). This was done to ensure the sample measurements remained within the calibrated range.

Results and Discussion

Model development

The reaction mechanism of an acid catalyzed transesterification involving fatty acids is outlined in Figure 4-1. A fundamental understanding of this reaction is necessary for an accurate model. The hydrogen ion, or catalyst, forms a complex with the existing fatty acid chain on the triacylglyceride (TAG). Once this complex has been formed, the alcohol may perform a nucleophilic attack on the carbon atom of the TAG (III). As this occurs, the existing bond between the fatty acid chain and the parent glycerol backbone are released and the hydrogen ion is regenerated. The model was developed using the acid catalyzed transesterification reaction mechanism as outlined in Figure 4-1.

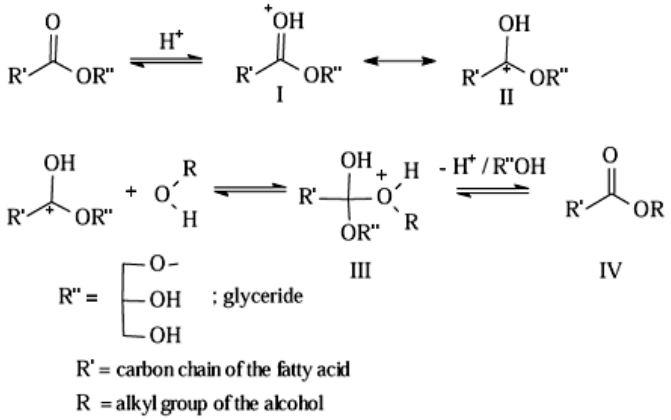

Figure 4-1. Acid catalyzed reaction mechanism for transesterification. (Meher et al., 2006)

Using the principal mechanism of the transesterification reaction, variables were assigned and the reaction was broken into steps to begin deriving equations that would describe the entire process. In the reactions below, S represents the TAG concentration, S' is methanol concentration, and H^+ is the hydrogen ion concentration. The overall reaction has been broken down into three steps and is represented as the following equations:

$$S + H^+ \quad \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} \quad SH^+ \tag{1}$$

$$SH^+ + S' \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} SS'H^+ \tag{2}$$

$$SS'H^+ \overset{k_3}{\rightarrow} MethylEster + Glycerol \tag{3}$$

For each of the three equations, the following relationships are established:

$$k_1[S][H^+] = k_1'[SH^+] \tag{4}$$

$$k_2[SH^+][S'] = k_2'[SS'H^+] \tag{5}$$

$$k_3[SS'H^+] = \frac{dP}{dt} = -\frac{d[S]}{dt} \tag{6}$$

where,

k_1, k_2, k_3 = zero-order rate constants for the formation of the complexes as illustrated.

[S] = specific stable TAG concentration at any time during the reaction (mg-TAG ml^{-1}-Methanol)

$[H^+]$ = specific stable H^+ concentration at any time during the reaction (mg-H^+ ml^{-1}-Methanol)

[S'] = is assumed to be a constant throughout the reaction

[SH^+] = specific stable TAG-H^+ complex at any time during the reaction (mg-TAG-H^+ ml^{-1}-Methanol)

[$SS'H^+$] = specific stable TAG-H^+-MeOH complex at any time during the reaction (mg-TAG-H^+-MeOH ml^{-1}-Methanol)

Since the first two reactions are assumed to be reversible, the equilibrium constants may be written as:

$$k_a = \frac{k_1'}{k_1} = \frac{[S][H^+]}{[SH^+]} \quad (7)$$

$$k_b = \frac{k_2'}{k_2} = \frac{[SH^+][S]}{[SS'H^+]} \quad (8)$$

$$k_3[SS'H^+] = \frac{dP}{dt} = -\frac{d[S]}{dt} = \frac{[SH^+][S]}{k_b} k_3 = \frac{k_3[S']}{k_b} * \frac{[S][H^+]}{k_a} \quad (9)$$

From these relationships:

$$\frac{dP}{dt} = -\frac{d[S]}{dt} = \frac{k_3}{k_a k_b}[S][H^+][S'] \quad (10)$$

Since [H^+] is unknown during the reaction, a mass balance of total proton concentration in the reactor can be written as:

$$[H^+]_0 = [H^+] + [SH^+] + [SS'H^+] \quad (11)$$

Applying the equations above, and using identities and substitution of variables, the overall equations are reduced to:

$$\frac{dP}{dt} = -\frac{d[S]}{dt} = \frac{k_3 S'[H^+]_0}{k_b} * \frac{1}{1+\frac{S'}{k_b}} * \frac{[S]}{\left(\frac{k_a}{1+\frac{S'}{k_b}}\right)+[S]} \quad (12)$$

where:

$$\frac{k_3 S'[H^+]_0}{k_b\left(1+\frac{S'}{k_b}\right)} = V_m \quad (13)$$

and,

$$\frac{k_a}{1+\frac{S'}{k_b}} = k_m \quad (14)$$

Under the experimental conditions, methanol concentrations (S') were much higher than the concentration of intracellular TAGs (S) and can be assumed to be constant. Also, if the experiments are performed using a fixed acid concentration ($[H^+]_0$, 5% in this case), k_M, and V_M, can be treated as constants. In terms of these new constants, the rate equation for the formation of FAMEs is:

$$\frac{dP}{dt} = -\frac{d[S]}{dt} = \frac{V_m S}{k_m+S} \quad (15)$$

From this expression, the rate of formation of FAMEs is dependent only on the concentration of intracellular TAGs. The model was fit to experimental data where FAME production was measured over time as a function of biomass concentrations using acidified methanol containing 5% (v/v) H_2SO_4. Single values of V_M and k_M that resulted

in the best fit were determined by minimizing the sum of the square of errors between experimental and predicted values. The results of the model fit are shown in Figure 4-2. It can be seen that a close correlation with experimental results was obtained using our model. Best fit values of V_M and k_M were determined to be 1.43 mg mL^{-1} $minute^{-1}$ and 23.15 mg mL^{-1}, respectively.

It was observed that there was rapid conversion of the TAGs into FAMEs for all biomass to solvent ratios. However, as the reaction neared completion it can be seen that the rates began to decrease. A decrease in reaction rate is observed at all biomass/solvent ratios. However the specific time is different for all biomass/solvent ratios. However, the reaction begins to slow for each biomass/solvent ratio when the initial substrate, or TAG concentration, reaches 15-20 mg ml^{-1}.

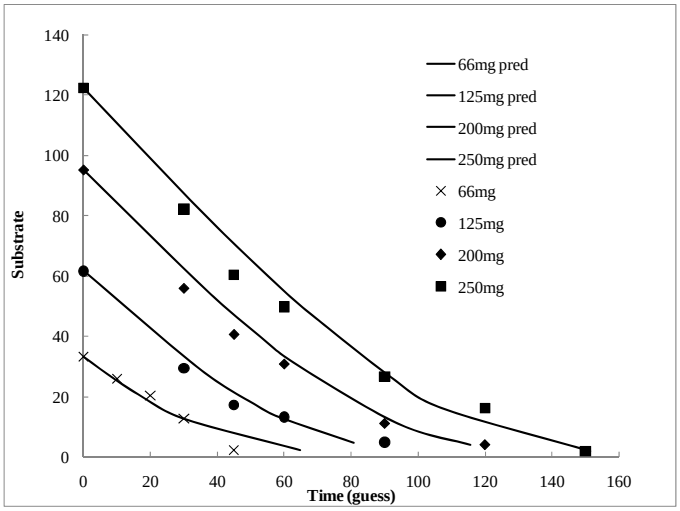

Figure 4-2: Transesterification of intracellular lipids (TAGs) into FAMEs measured over time, where experimental data was plotted against the predicted model.

To further verify its validity, the model was tested on experiments that were performed at constant biomass concentrations but had varying initial acid concentrations. Since k_M is independent of initial acid concentration, it was fixed at the previously

obtained value of 23.15 mg mL^{-1}. V_M was determined independently for each of the experiments carried out at 1, 1.5, 2 and 5% acid concentration and 66 mg mL^{-1} biomass. This was done by fitting the overall rate expression to experimental data by minimizing the sum of square of differences between measured and predicted values. From the expression of V_M, the value of this parameter would be directly proportional to initial acid concentrations, if the model was valid. Values of V_M obtained from best-fit model predictions were plotted against initial acid concentrations used in the experiment and these results are shown in Figure 4-3. The very good correlation obtained again validates the applicability of the model.

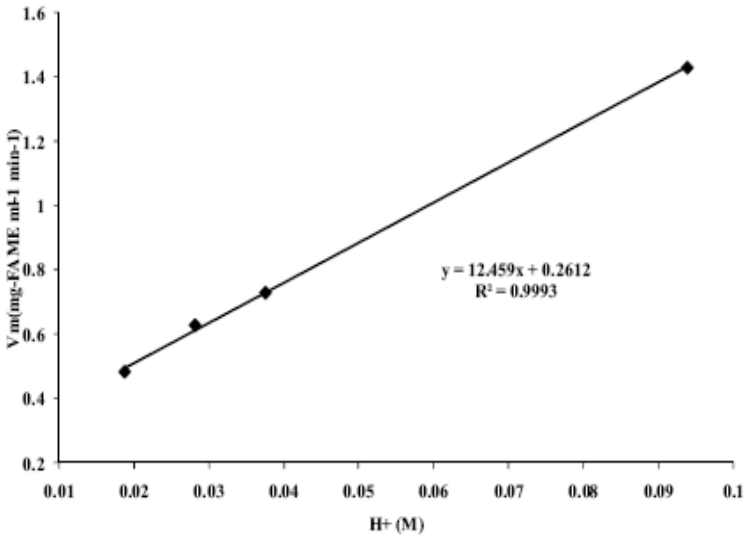

Figure 4-3: Variation of the reaction kinetic parameter, V_M with acid concentrations. The strong linear correlation validates the model assumptions.

Using the initial rates of reactions from Figure 4-2 (V_0), and knowing S_0, the values of V_M and k_M using both the Lineweaver-Burke and the Langmuir methods were

also calculated (Figure 4-4). However, using these values of V_M and k_M there was not a good fit to the experimental data.

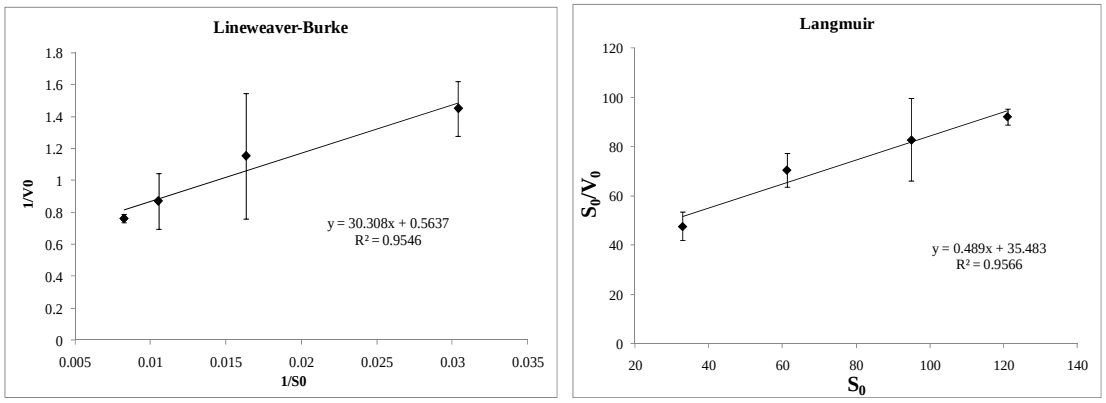

Figure 4-4: Left; Lineweaver-Burke plot where V_M = 1.77 mg ml^{-1} minute^{-1}, k_M = 17.08 mg ml^{-1}. Right; Langmuir plot where V_M = 2.05 mg ml^{-1} minute^{-1}, and k_M = 17.35 mg ml^{-1}.

Therefore, the models created in this study may potentially be applied to larger scale systems and used to determine the proper process conditions to obtain full transesterification of the lipids into FAMEs.

Conclusion

To make the large scale production system of FAMEs for use as biodiesel or as nutritional supplements economical, the organism must be able to accumulate large amounts of intracellular lipids in shorter growth times. Since the purpose of this study was to develop a model for large scale systems, SR21 was used as a model biomass source due to its relatively high accumulation of intracellular lipids (50% DCW). While there do exist multiple processes by which intracellular lipids may be converted to FAMEs, these processes require large amounts of mechanical input, as well as toxic solvents and reaction times. Therefore, the advantages of *in situ* transesterification have

warranted this study of the reaction kinetics. By using the fundamental reaction mechanism of the transesterification reaction, a model was derived that closely fit experimental data at increasing biomass/solvent ratios. Using this model, the coefficients V_M and k_M were successfully determined that could be used in Michaelis Menton type kinetics.

Finally, since the rate expression was derived from first principles, the model development and validation studies show that FAME production can be accurately described using fundamental reaction kinetics that are scale-independent. Thus, *a priori* design of large-scale reactors is possible by extrapolation of this laboratory data for *in situ* transesterification of microbial biomass.

CHAPTER 5

CONCLUSION

This work has shown that the *in situ* transesterification reaction can be modified to handle biomass/solvent ratios 20 times higher than previously defined protocols while still achieving greater than 95% extraction efficiency. This was seen by increasing the biomass/solvent ratios from 20 mg ml^{-1}, to greater than 200 mg ml^{-1}. Therefore, the single step reactive extraction may potentially be applied to larger scale applications. However, one consequence of increasing the biomass/solvent ratio however is the reaction requires longer processing times and this must be taken into consideration when designing larger systems.

The effect of temperature on the reaction rate has also been shown to have a significant impact on the reaction. At higher temperatures, 100°C initially showed a faster rate of reaction, and reached complete extraction and conversion of TAG to FAME around 45 minutes. However, after 30 minutes the reaction rate slowed. At 90°C, the reaction took 60 minutes to reach completion, and then remained stable for a longer period of time than at 100°C. While the reaction does take place more rapidly at 100°C, at this higher temperature there is evidence that the FAMEs begin to degrade. Thus, a reaction at 90°C may be more suitable as it is more stable and there is less risk of losing sample volume.

In addition to temperature and biomass/solvent ratio, the acid concentration became a significant factor when the concentration was lowered. This is possibly due to the molar ratio of hydrogen ions to triglycerides. As the molecular availability of H^+ ions

decreases, there is less interaction with the triglycerides, resulting in slower product formation. It appears that a critical level of acid concentration is reached at approximately 2%. Ideally, the acid concentration would be 5% or greater to keep the reaction rates higher. However, at 2% the reaction was not significantly slowed. Therefore, acid concentration is a significant factor that must be taken into consideration.

Because in many systems the types of fatty acids present can vary from short to long chain, and from saturated to unsaturated, it was important to determine the difference, if any, among their extractabilities. These experiments also demonstrated that the type of fatty acid present in the system did not affect the rate of reaction. The organism used in these experiments, SR21, had a higher SFA content (25% on dry basis) than PUFA (18.5% on dry basis). At these relative concentrations, both types of fatty acid present, palmitic acid (SFA), and DHA (PUFA), were extracted and transesterified and at the same rate. Thus, when designing a system based on the single step reactive extraction, the type and ratio of fatty acids present within the system will not affect the overall reaction kinetics.

Another significant advantage to using the *in situ* transesterification method described in this research is that FAMEs can be automatically phase separated and the working solvent (acidified methanol), can be recycled for use in subsequent batch processing. Therefore, the costs of overall production can be significantly reduced. Again, the results indicate that the solubility of FAMEs in methanol is approximately 22.7 mg ml^{-1}. Once this solubility constant is reached, the remaining FAMEs begin automatic phase separation and can be harvested without excessive input. Therefore, regardless of the FAME concentration within the system as a result of the amount of

biomass present, any FAME concentration in excess of the critical solubility will phase separate. The phase separation is unbiased towards individual fatty acids, whether long of short chained. Regardless of whether the FAMEs are saturated or unsaturated, the same relative concentrations remain the same both the soluble and in-soluble phases.

After reaction completion, the methanol phase of the *in situ* reaction may be recycled and used in further extraction processes. Higher recovery percentages are achieved with lower biomass to solvent ratios, >95% for 66 and 125 mg ml^{-1}, and begins to decrease with increasing biomass. However, it is noted that by increasing the biomass to solvent ratio from 66 to 250 mg ml^{-1}, a nearly 4-fold increase, the extraction and recovery percentage only drops 10%. Therefore, when considering scaling up the process, it may be economically favorable to increase the biomass to solvent ratio and conserve the amount of solvent necessary. Future work can include conducting further recycling steps to determine long term effects of recycling the methanol.

The last objective of this research was to develop a model to predict the extraction and transesterification of TAGs into FAMEs for large scale systems. While there do exist multiple processes by which intracellular lipids may be converted to FAMEs, these processes require large amounts of mechanical input, as well as toxic solvents and reaction times. Therefore, the advantages of *in situ* transesterification have warranted this study of the reaction kinetics via the *in situ* process. Using the fundamental reaction mechanism of the transesterification reaction, a model was derived that closely fit experimental data at increasing biomass/solvent ratios. Using this model, the coefficients V_M and k_M were successfully determined that could be used in Michaelis Menton type kinetics.

Since the rate expression was derived from first principles, the model development and validation studies show that FAME production can be accurately described using fundamental reaction kinetics that are scale-independent. Thus, *a priori* design of large-scale reactors is possible by extrapolation of this laboratory data for *in situ* transesterification of microbial biomass.

In conclusion, these studies provide an understanding of the fundamental reaction and limitations during the *in situ* transesterification process. From this understanding, a new, reliable, and cost-effective system for large scale lipid production can be developed from microbial biomass including oleaginous algae, fungi, and yeast. Future work may include analysis of different organisms and strains. Also, future experimentation could also include modeling the effects of moisture content on the reaction. Freeze drying biomass on large scales is not cost effective, therefore the study will be expanded to include air-dried biomass as well was wet biomass slurries.

REFERENCES

Able K, de Schmertertzing H, Peterson JI. 1963. Classification of microorganisms by analysis of chemical composition. J. Bacteriol. 85:1039-1044.

Ashford A, Barclay W, Weaver C, Giddings T, Zeller S. 2000. Electron microscopy may reveal structure of docosahexaenoic acid-rich oil within *Schizochytrium* sp. Lipids 35:1377-1385 No.12.

Barclay WR, Meager KM, Abril JR 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J. Appl. Phyc. 6:123-129.

Belarbi EH, Molina E, Chisti Y. 2000. A process for high yield and scalable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. Enzyme Microbial. Tech. 26:516-529.

Bligh E, Dyer W. 1959. A rapid method of total lipid extraction and purification. Canadian J. Biochem and Physiol 37:911-917 No. 8.

Carrapiso A, Garcia C. 2000. Development in Lipid Analysis: Some new extraction techniques and *in situ* transesterification. Lipids 35:1167-1177 No.11.

Chi Z, Pyle D, Wen Z, Frear C, Chen S. 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process Biochem. 42:1537-1545.

Chin C, Shen T, Su H, Ding S. 2006. *Schizochytrium limacinum* SR21 as a source of docosahexaenoic acid: optimal growth and use as a dietary supplement for laying hens. Australian J. Ag. Res. 57:13-20.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnol. Advances 25:294-306.

Cook H. 1985. Fatty acid desaturation and chain elongation in eukaryotes. In: D.E. Vance and J.E. Vance, editors. Biochemistry of Lipids and Membranes. Menlo Park: Benjamin/Cummings.

Eller FJ, King JW. 1996. Determination of fat content in foods by analytical SFE. Sem. Food Anal. 1:145-162.

Foidl N, Foidl G, Sanchez M, Mittlebach M, Hackel S. 1996. *Jatropha curcal* L. as a source for the production of biofuel in Nicaragua. Bioresource Technol. 58:77.

Folch J, Lees M, Stanley HS. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226,497

Freedman B, Pryde EH, Mounts TL. 1984. Variables affecting the yields of fatty esters from transesterified vegetable oils. J. Am. Oil Chem. Soc. 61:1638-1643.

Frost and Sullivan. www.food.frost.com (http://www.allbusiness.com/medicine-health/diet-nutrition-fitness-cholesterol/5168568-1.html). Accessed July 2005.

Horrocks L, Yeo Y. 1999. Health benefits of docosahexaenoic acid (DHA). Pharmacol. Res. 40:211-225.

Indarti E, Majid M, Hashim R, Chong A. 2005. Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. J. Food Comp. Anal. 18:161-170.

Johnson D, Taconi K. 2007. The glycerine gut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ. Prog. 26:338-348.

Knothe G. 2005. Introduction: What is biodiesel? In: Knothe G, Krahl J, Gerpen JV, editors. The Biodiesel Handbook. Illinois, AOCS Press. p 1-3.

Kulkarni MG, Dalai AK. 2006. Waste cooking oil- an economic source for biodiesel: A review. Ind. Eng. Chem. Res. 45:2901-2913.

Lang X, Dalai AK, Bakhshi NN, Reaney MJ, Hertz P. 2001. Preparation and characterization of bio-diesels from various bio-oils. Bioresour. Technol. 80:53-62.

Lee KT, Foglia TA, Chang KS. 2002. Production of alky esters as biodiesel fuel from fractionated lard and restaurant grease. J. Am. Oil Chem. Soc. 79:191-195.

Lewis T, Nichols P, McMeekin T. 2000. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. J. Microbiological Methods 43:107-116.

Linko Y, Hayakawa K. 1996. Docosahexaenoic acid: A valuable nutraceutical? Trends Food Science Technol. 7:59-63.

Liu B, Zhao Z. 2007. Biodiesel production by direct methanolysis of oleaginous microbial biomass. J. Chem. Technol. Biotechnol. 82:775-780.

Ma F, Hanna M. 1999. Biodiesel production: A review. Bioresour. Technol. 70:1-15.

Meher, LC, Sagar D. Vidya, Naik SN. 2006. Technical aspects of biodiesel production by transesterification—a review. Renewable and Sustainable Energy Reviews 10:248-268, Issue 3.

Miao X, Wu Q. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technol. 97:841-846.

Mortia E, Kumon Y. 2006. Docosahexaenoic acid production and lipid body formation in *Schizochytrium limacinum SR21.* Marine Biotechnol. 8:319-327.

National Biodiesel Board. 2007. Fuel Facts sheets. Accessed April 2007. http://www.biodiesel.org/pdf_files/fuelfactsheets/bacground.PDF

National Renewable Energy Laboratory. Accessed April 2007. http://www.eere.energy.gov/afdc/progs/vwbs 2.cgi?9626

Nakahara T, Yokochi T, Tanaka S, Yaguchi S, Honda D. 1996. Production of docosahexaenoic and docosapentaenoic acids by *Schizochytrium* sp. Isolated from the Yap Islands. J. Am. Oil Chemists' Soc. 73:1421-1426.

Nelson RG, Schrock MD. 2006. Energetic and economic feasibility associated with the production, processing, and conversion of beef tallow to a substitute diesel fuel. Biomass Energy 30:584-591.

Pare JRJ, Matni G, Belanger JMR, Li K, Rule C, Thibert B. 1997. Use of the microwave assisted process in extraction of fat from meat, dairy, and egg products under atmospheric pressure conditions. J. AOAC Intl. 80: 928-933.

Perveen Z, Ando H, Ueno A, Ito Y, Yamamoto Y, Yamada Y, Takagi T, Kaneko T, Kogame K, Okuyama H. 2006. Isolation and Characterization of a Novel Thraustochytrid-like microorganism that efficiently produces docosahexaenoic acid. Biotechnology Letters 28:197-202.

Pyle D, Garcia R, Wen Z. 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J. Agric. Food Chem. 56:3933-3939.

Randolf TW. 1990. Supercritical fluid extractions in biotechnology. Trends Biotechnol. 8:78-82.

Rose D, Connolly J. 1999. Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol. Ther. 83:217-244.

Senzaki H, Iwamoto S, Ogura E. 1998. Dietary effects of fatty acids on growth and metastasis of KPL-1 human breast cancer cells in vivo and in vitro. Anticancer Res. 18:1621-1627.

US Department of Energy. Accessed April 2007. http://www1.eere.energy.gov/biomass/pdfs/35523.pdf

Vazhappilly R, Chen F. 1998. Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgal and their heterotrophic growth. J Am Oil Chem Soc 75:393-397.

Vincente G, Martinez M, Aracil J. 2004. Integrated biodiesel production: A comparison of different homogenous catalysts systems. Bioresource Technol. 92:297-305.

Wu S, Yu S, Lin L. 2005. Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31. Proc. Biochemistry 40:3105-3108.

Yazdani S, Gonzalez R. 2007. Anaerobic fermentation of glycerol: a path to economic viability for the Biofuels industry. Curr. Opin. Biotechnol. 18:213-219.

Yokochi T, Honda D. 1998. Optimization of docosahexaenoic acid production by Schizochytrium limacinum SR21. Appl. Microbiol. Biotechnol. 49:72-76.

Zhou L, Lu Y, Zhou M, Zhao X. 2007. Enhanced production of docosahexaenoic acid using *Schizochytrium* sp. by optimization of medium components. J. Chem. Eng. of Japan 40:1093-1100 No. 12.