ABSTRACT

KILLENS, RUSHYANNAH RYCHELLE. Biochemical Characterization of Extremophile Fatty Acid Metabolism Enzymes for Use in Algal-Based Biofuel Production. (Under the direction of Dr. Amy M. Grunden.)

There is a renewed interest in renewable energy due to concerns over long-term fossil fuel supply, global warming, and global human population growth. Although promising alternative fuel sources have been derived from food crops (first-generation) and lignocellulose biomass (second generation), these feedstocks are not feasible for commercial use due to competition with food supplies and a requirement for technology development (pretreatment of biomass, enzymatic saccharification of the pretreated biomass, etc.) for the affordable conversion of lignocellulose biomass to fuel. For these reasons, there is a push to produce clean and renewable energy derived from algal biomass. The research reported here is focused on the biochemical characterization of fatty acid synthesis enzymes to augment microalgae-biofuel. Among microalgae, the highly productive, halophilic chlorophytes Dunaliella spp. are a rich source of lipids and have strong potential to be an economically viable source for renewable oil production.

To modify carbon flux through the fatty acid biosynthesis pathway and capture fatty acids incorporated into triacylglycerides, lipid biosynthesis genes from extremophiles were selected and biochemically characterized to establish their compatibility for functioning in Dunaliella to increase microalgal oil production. To this end, the acetyl-coenzyme A carboxylase (ACCase) from the bacterial halophile Chromohalobacter salexigens BAA-138 was recombinantly expressed in Escherichia coli to provide sufficient enzyme for biochemical characterization. The ACCase enzyme carries out the rate-limiting step during
fatty acid synthesis (FAS), and it has been shown that increased ACCase expression in bacteria leads to elevated rates of FA production. Based on its rate-limiting role in the formation of fatty acids, it is proposed that by expressing a bacterial ACCase in microalgae the FAS limiting effects of transcriptional repression and feedback inhibition of the native microalgal ACCase could be mitigated.

In addition to increasing lipid production in microalgal strains, thermoactive thioesterases were also identified so that during high temperature conversion of lipids to fuel they could release the free fatty acids (FFAs) previously sequestered during algal cell growth as part of triacylglycerides. Therefore, heat stable thioesterases from *Metallosphaera sedula* DSM5348 and *Sulfolobus solfataricus* P2 were recombinantly expressed in *E. coli* to enable their biochemical characterization and evaluation for suitability for use in microalgae for improved biofuel production.
Biochemical Characterization of Extremophile Fatty Acid Metabolism Enzymes for Use in Algal-Based Biofuel Production

by
Rushyannah R. Killens

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APPROVED BY:

_________________________________________    ________________________________
Dr. Amy M. Grunden                               Dr. Heike Winter-Sederoff
Committee Chair

_________________________________________    ________________________________
Dr. James Brown                                  Dr. Jonathan Olson
DEDICATION

This work is dedicated to my son, Cory Letrai Cade, Junior. My dearest son, it was through love, faith, and persistence that I produced this work. And it is my hope that you will use all three to fulfill your life’s purpose and dreams. As a scientist, I know the world is yours to behold. Aim beyond the stars!
BIOGRAPHY

Conceived out of love to the union of Robert Killens and the late Karen Killens, both active military, Rushyannah Killens-Cade was born on the post of Fort Bragg, North Carolina. She is the oldest of five children and was happily raised in Lumberton, NC where she learned the importance of family. She attended the University of North Carolina at Chapel Hill where she obtained a Bachelor of Arts degree in Psychology. Upon obtaining her undergraduate degree, Rushyannah enrolled at North Carolina Agricultural and Technical State University (NCAT) to complete a Master of Science (MS) degree in Chemistry. Rushyannah joined Dr. Kanipes’ lab where she isolated and characterized the lipooligosaccharide biosynthetic locus of Campylobacter coli MK100. While completing her MS degree, Rushyannah received a National Science Foundation fellowship from North Carolina State University (NCSU). By participating in the Bridge to the Doctorate program, Rushyannah received the support she needed to matriculate into a doctoral program at NCSU. After graduating from NCAT, she began working on her Ph.D. under the direction of Dr. Amy M. Grunden in the department of Microbiology at NCSU. Through the guidance of Dr. Grunden and the support of her wonderful colleagues, Rushyannah has streamlined her focus in research. During the journey to completing her doctoral degree, Rushyannah married her heart’s heart, Cory Cade, and had a precious son, Cory Cade, Junior. Rushyannah Killens-Cade will work in service to humanity through her contribution in the field of science.
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TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................... ix
LIST OF FIGURES .................................................................................................................. x

CHAPTER 1: Literature Review ............................................................................................... 1
1.1 FIRST-GENERATION BIOFUELS .................................................................................. 1
1.1.1 BIOETHANOL ........................................................................................................... 1
1.1.2 BIODIESEL .............................................................................................................. 2
1.1.3 METHANE/BIOGAS ............................................................................................... 4
1.1.4 CHALLENGES OF 1ST GENERATION BIOFUELS ................................................ 5
1.2 SECOND-GENERATION BIOFUELS ............................................................................ 6
1.2.1 LIGNOCELLULOSE ............................................................................................... 7
1.2.2 CONVERSION PROCESS FOR 2ND GENERATION BIOFUELS .................................. 8
1.2.3 MAIN CHALLENGES OF 2ND GENERATION BIOFUELS ........................................ 10
1.3 THIRD-GENERATION BIOFUELS ................................................................................ 11
1.3.1 METABOLIC ENGINEERING OF MICROALGAE FOR ENHANCED LIPID BIOSYNTHESIS ........................................................................................................ 18
1.3.2 EXTREMOPHILIC ENZYMES ................................................................................ 28
1.3.2.1 PROPERTIES OF HALOPHILIC ENZYMES .................................................... 29
1.3.2.2 PROPERTIES OF THERMOPHILIC ENZYMES ........................................... 30
1.4 CONCLUSION ............................................................................................................... 32
1.5 REFERENCES ............................................................................................................... 32

CHAPTER 2: Recombinant expression and biochemical characterization of an acetyl-CoA carboxylase from the halophilic bacterium Chromohalobacter salexigens BAA-138 ......................................................................................................................... 66

   ABSTRACT ..................................................................................................................... 67
2.1 INTRODUCTION .......................................................................................................... 69
2.2 MATERIALS AND METHODS ...................................................................................... 71
LIST OF TABLES

CHAPTER 1: Literature Review ........................................................................................................1
  Table 1-1: Comparison of microalgae with other oilseed crops ..................................................53
  Table 1-2: Fuel properties comparison ......................................................................................54
  Table 1-3: Composition of microalgal oil ..................................................................................55
  Table 1-4: Lipid content and productivity found in different microalgae species .................56
  Table 1-5: Genetic modification of FAS pathway in vascular plants .........................................58
  Table 1-6: Proposed application for Dunaliella species ..............................................................60

CHAPTER 2: Recombinant expression and biochemical characterization of an acetyl-CoA carboxylase from the halophilic bacterium Chromohalobacter salexigens BAA-138........................................................................................................................................66
  Table 2-1: Primers and expected products generated in this study ...........................................86

CHAPTER 3: Characterization of a thermostable, recombinant carboxylesterase from the hyperthermophilic archaeon Metallosphaera sedula DSM5348 .....................................................................91
  Table 3-1: Effect of detergents on the activity of M. sedula carboxylesterase variants .....116
  Table 3-2: Effect of organic solvents on the activity of M. sedula carboxylesterase variants ..........................................................................................................................117
# LIST OF FIGURES

## CHAPTER 1: Literature Review

- Figure 1-1: Conversion processes for biofuel production from microalgal biomass ........................................... 61
- Figure 1-2: Pathway of TAG biosynthesis microalgae .......................................................... 62
- Figure 1-3: Reaction mechanism for *E. coli* ACCase ................................................................. 63
- Figure 1-4: Pathway of fatty acid biosynthesis in bacteria .......................................................... 64
- Figure 1-5: The active cleft of *E. coli* TAP solved by crystallography ........................................... 65

## CHAPTER 2: Recombinant expression and biochemical characterization of an acetyl-CoA carboxylase from the halophilic bacterium *Chromohalobacter sallexigens* BAA-138

- Figure 2-1: SDS-PAGE of partially purified *C. sallexigens* ACCase ........................................... 87
- Figure 2-2: *Cs_accD* complementation study ........................................................................... 88
- Figure 2-3: Effect of pH on the activity of partially purified *Cs_ACCase* ................................. 89
- Figure 2-4: Effect of salt concentration on the activity of partially purified *Cs_ACCase* ... 90

## CHAPTER 3: Characterization of a thermostable, recombinant carboxylesterase from the hyperthermophilic archaeon *Metallosphaera sedula* DSM5348

- Figure 3-1: Alignment of *M. sedula* carboxylesterase with other esterases ......................... 118
- Figure 3-2: SDS-PAGE of purified *M. sedula* carboxylesterase variants ............................ 119
- Figure 3-3: Specific activity of *M. sedula* carboxylesterase variants toward various pNP-esters ..................................................................................................................... 120
- Figure 3-4: Effect of temperature on the activity of *M. sedula* carboxylesterase variants .... 121
- Figure 3-5: Effect of pH on the activity of *M. sedula* carboxylesterase variants ............... 122
- Figure 3-6: Effect of metal ion and chelating agent on the activity of *M. sedula* carboxylesterase variants ..................................................................................................................... 123
CHAPTER 4. Recombinant expression and biochemical characterization of a thermostable esterase from Sulfolobus solfataricus P2 to augment production of microalgal-derived biofuel

Figure 4-1: Alignment of S. solfataricus esterase with other esterases ..........................................................141
Figure 4-2: SDS-PAGE of purified S. solfataricus esterase ..........................................................142
Figure 4-3: Specific activity of S. solfataricus esterase toward various pNP-esters............143
Figure 4-4: Effect of temperature on the activity of S. solfataricus esterase .....................144
Figure 4-5: Effect of pH on the activity of S. solfataricus esterase .................................145
CHAPTER 1

Literature Review

1.1 FIRST-GENERATION BIOFUELS

First generation biofuels are derived from sugar crops (sugarcane, sugarbeet), starch crops (corn, sorghum), oilseed crops (soybean, canola), and animal fats to produce bioethanol, biodiesel, and biogas. A fermentation process is used to convert sugar and starch crops into bioethanol. Biodiesel is created via the transesterification of oils and animal fats. Wet organic wastes are converted through an anaerobic digestion process to form biogas. First generation biofuels are commercially produced and are projected to continue to have growth in production and consumption (European Commission 2007).

1.1.1 BIOETHANOL

Bioethanol is produced from sugar crops (sugarcane, wheat, beet root, fruits, and palm juice) and grain (corn, wheat, barley, rice, sweet sorghum, potato, and cassava) through fermentation and distillation of sugar compounds using classical or genetically modified organisms including yeast (Saccharomyces species), bacteria (Zymomonas species), and mold (mycelium). In the case of carbohydrate sources (such as starch from corn), a preliminary hydrolysis step is necessary to liberate the sugars that can then be fermented to ethanol. The enzyme mostly used in this pretreatment step is α-amylase.
Bioethanol can serve as a full substitute for the petroleum-derived oil in flexible-fuel vehicles (widely used in Brazil) as well as blended easily with gasoline. Typically, no engine modification is needed to use the blend. Ethanol can also be used as an octane-boosting, pollution-reducing additive in unleaded gasoline.

The cost of producing bioethanol varies depending upon crop types, agricultural practices, land and labor costs, plant sizes, processing technologies and government polices (Demirbas and Demirbas 2011). For instance, the cost of producing the biofuel in a United States’ dry mill plant totals $1.65/gallon (Demirbas 2009). Yet, in developing countries with warm climate, producing ethanol from sugar canes is considerably cheaper, so much so that it is increasingly becoming a cost-effective alternative to petroleum fuels. This difference could be due to the further processing of ethanol derived from cellulosic feedstock using enzymatic hydrolysis when compared to starch or sugar-based feedstock.

1.1.2 BIODIESEL

Biodiesel is a substitute of diesel and is derived from oil plants (e.g., rapeseed, palm oil, soybean, sunflower, jatropha, coconut, peanut) and animal fats through transesterification. The process of transesterification involves converting extracted oils into fuel by breaking ester bonds linking the long carbon chain fatty acids (FAs) to glycerol. This conversion is performed with a short-chain alcohol (methanol or ethanol) in the presence of a base, an acid, or an enzyme catalyst. The purpose of transesterification is to lower the viscosity and oxygen content of the vegetable oil and is impacted by several factors such as
the type of catalyst employed (alkaline or acid), molar ratio of alcohol/vegetable oil, temperature, water content (purity), and free fatty acid (FFA) content. Due to serious drawbacks from these factors jeopardizing the economical advantages of biodiesel, there are different alternative techniques to biodiesel production that are being studied such as employing a heterogeneous catalyst, lipase catalyst, or supercritical alcohol (Balat and Balat 2010; Gomez-Castro et al. 2010; Kiss et al. 2010; Lee and Saka 2010; Ye et al. 2010).

The cost of this renewable fuel source varies depending on the feedstock, geographic area, alcohol prices, and seasonal variability in crop production (Leung et al. 2010; Balat and Balat 2010; Ganapathy et al. 2009; Shu et al. 2010; Wen et al. 2010). With minor engine modifications, biodiesels can serve as a full substitute for diesel (B100) or blended with common diesel fuel at any concentration (Knothe 2010). In fact, in the United States, diesel fuel is blended with biodiesel at 20% whereas in Germany, diesel fuel is supplemented with 7% biodiesel (Bunger et al. 2012). This alternative diesel fuel is non-toxic, sulfur-free, and biodegradable in nature. It also displays a desirable flash point and aromatic content properties. With this in mind, biodiesel is known to extend engine life while reducing the cost of maintenance as a result of its desirable lubricity (Luque et al. 2008). When compared to regular petrol-based diesel, this alternative fuel results in less combustion emission, carbon monoxide, and unburned hydrocarbons (Gerpen 2005).
1.1.3 METHANE/BIOGAS

Biogas, or biomethane, is a fuel produced through anaerobic digestion of wet organic wastes such as animal manure, sewage effluent, and food crop processing waste. The use of biogas as an alternative fuel source is attractive because of its environmentally friendly status, potentially providing an opportunity for complete recycling of minerals and nutrients from the soil. Sugars, starches, lipids, and proteins present in organic wastes can easily be degraded by a series of bacteria and archaea such as hydrolytic fermentative, hydrogen producing acetogenic, hydrogen consuming acetogenic, carbon dioxide reducing, and aceticlastic methanogenic species (Tauseef et al. 2013). These microorganisms rely on the following three metabolic reactions for the generation of biomethane: hydrolysis, acidogenesis, and methanogenesis (Park et al. 2005; Charles et al. 2009). Although organic waste can be digested via aerobic digestion, the anaerobic process is more advantageous due to a low energy requirement for operation and a low biomass production, and it is considered a viable technology in the competent treatment of organic waste (Wang et al. 1999; Steyer et al. 2002; Angenent et al. 2004; Kim et al. 2006; DeBaere 2006; Jingura and Matengaifa 2009). Yet, since biogas is carried out by an array of microorganisms and depends on various factors like pH, temperature, hydraulic retention time, carbon/nitrogen ratio, it is a relatively slow process. A few other limitations associated with biogas include lack of process stability, low loading rates, slow recovery after failure and specific requirements for waste composition.
1.1.4 CHALLENGES OF 1ST-GENERATION BIOFUELS

Although mature commercial markets for use of 1st-generation biofuels are in place, the sustainable and economic production of these alternative fuel sources are of great concern. The use of agricultural products (sugar crops, grains, and oil plants) as feedstock for first generation biofuel production is a major source of contention because these agriculture commodities are also used for food and feed production. It is believed that the use of biofuel raw materials reduces the availability of food supply; thus, increases food prices. According to the International Food Policy and Research Institute, about 30% of U.S. maize production went into ethanol in 2008 rather than into world food and feed markets. In addition to crops used for biofuel feedstock being more prone to erosion and a burden to the water supply, they often alter cropping patterns. With this in mind, as the demand for biofuel feedstock increases (in particular corn), there are more frequent plantings of corn in crop rotation, resulting in an increase in corn acreage at the expense of wheat and the plowing up of grasslands (Wiesenthal et al. 2006; Steenblik 2007).

The rise in demand for and production of biofuels has resulted in a number of effects on grain supply-and-demand systems (Rosegrant 2008). The detrimental effects on grain supply-and-demand systems have resulted in an underinvestment in agricultural research and technology and rural infrastructure, especially irrigation in developing countries, as well as increasing pressure on the natural-resource base (land and water) (Rosegrant 2008). Ongoing deforestation is a constant concern for many developing countries as more land is converted from permanent forest cover to agriculture. An example of this conversion can be
seen in Indonesia. Between 2000 and 2005, the country cleared 1.8 million hectares of forest for the creation of oil palm plantations (Colbran and Eide 2008). At such a large scale of deforestation, there is a loss of biodiversity (species), ecosystem goods and services, common pool resources, and the indigenous people’s traditional food sources. The destruction of forests can contribute to greenhouse gas emissions through upfront costs incurred from the loss of carbon stored in above-and below-ground biomass when land is cleared; and/or opportunity costs from the loss of carbon sequestration service of converted land-uses (Koh and Ghazoul 2008). In addition to deforestation, an increased use of scarce fresh water for irrigation can lead to competition for water. With this in mind, ethanol plants require approximately 3 to 6 liters of water per liter of ethanol produced (Stanich 2007). Press reports of local concerns over the use of their water supplies are appearing with increased regularity (Kirchhoff 2007; Wilson 2007).

1.2 SECOND-GENERATION BIOFUELS

The concerns for sustainability and environmental impacts of 1st-generation biofuels have stimulated interest in developing 2nd-generation biofuels produced from non-food, cheap, and readily available plant biomass such as lignocellulosic feedstock materials (cereal straw, sugar cane bagasse, forest residues), wastes (organic components of municipal solid wastes), and dedicated feedstocks (purpose-grown vegetative grasses, short rotation forest, and other energy crops). Lignocellulose is the building block of all plants as it accounts for 75% of the cell wall; thus, the main component of plant biomass. Although the structure and
composition of plant cell walls differs considerably among plant species and cell types within a species, the main constituents of lignocellulosic materials are cellulose, hemicelluloses, and lignin.

1.2.1 LIGNOCELLULOSE

Cellulose is the most abundant biopolymer as it is estimated that approximately $1.5 \times 10^5$ kilograms of the biomaterial is produced annually (Crawford 1981; Deguchi et al. 2006). This polysaccharide is a simple and linear polymer that is composed of $\beta$-1,4-linked D-glucose units. For bioethanol production, cellulose can be converted into glucose via enzymatic action of cellulases.

Hemicellulose is the second most abundant polysaccharide in nature as it constitutes 20%-30% of dicotyledonous plant biomasses, up to 50% for some tissues of monocotyledonous plants, and approximately 25% of the available biomass of the bioenergy-specific crops Miscanthus, switchgrass, fescue, and fiber sorghum (Schadel et al. 2010; Timell 1967; Ebringerova et al. 2005). Hemicelluloses are closely associated with cellulose microfibrils via hydrogen bonds (keeping the fibers from aggregating and adding flexion to the cell wall) and cross-linked to lignin (Gomez et al. 2008). Generally, this polysaccharide is heterogeneously branched (with degrees of polymerization of 200 or less) and consists of a variety of sugar monomers (mainly xylans and mannans). The degree and type of branching and monomer proportions vary among plant species and cell wall types (Schadel et al. 2010; Li L et al. 2006).
Lignocellulose is also comprised of 15-30% lignin as it forms a matrix that is closely associated with cellulose filaments, and is covalently attached to hemicelluloses. Lignin is a complex aromatic heteropolymer consisting of \( p \)-hydroxyphenyl (H)-, guaiacyl (G)-, and syringyl (S)-phenylpropanoid units, which are derived from three monolignols, \( p \)-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, respectively (Higuchi 1985; Boerjan et al. 2003). The ratio of G:S:H units varies from species to species and are linked together via several ether and C-C bonds. Due to this linkage (prohibiting hydrolytic attacks), most treatments do not extensively degrade lignin resulting in lignocellulose being resistant to chemical and biological degradation.

1.2.2 CONVERSION PROCESS FOR 2\textsuperscript{ND}-GENERATION BIOFUELS

The generation of biofuels from lignocellulosic feedstocks can be obtained through two different processing pathways, biochemical and thermo-chemical. The biochemical route can be divided into three main steps: pretreatment, saccharification of cellulose, and the fermentation of sugars into ethanol. The pretreatment step, which disrupts the encasement of cellulose filaments in lignin, is critical for improving the configuration and efficiency of the subsequent steps. The options for pretreatment can be classified into biological, physical, chemical, or a combination, each with variations having different temperatures and reaction times (Yang and Wyman 2008). Generally, after pretreatment, two approaches are used for saccharification of cellulose: enzymatic (cellulases and hemicellulases) or chemical hydrolysis (Sun and Cheng 2002; Chornet et al. 2010). Finally, the products from hydrolysis
are separated and fermented in the presence of microorganisms (bacteria and yeast) to produce ethanol and various by-products.

The thermo-chemical route includes direct combustion, gasification, liquefaction, and pyrolysis technologies. Essentially, this pathway produces synthesis gas (syngas) by subjecting lignocellulosic biomass feedstocks to an extremely high temperature in the presence of a controlled amount of air. This pathway leads to the conversion of biomass into the following fractions (depending on the temperature): biochar, pyrolytic oil/bio oil (containing chemical products of lignocelluloses, biomass like aliphatic alcohols/aldehydes, furanoids, pyranoids, benzenoids, FAs and high molecular mass hydrocarbons), and syngas (comprised of carbon monoxide, hydrogen, short chain alkanes, and carbon dioxide). At low temperatures (250 °C – 350 °C) and in the absence of oxygen, the biomass undergoes a torrefaction process (involving water elimination, bond breakage, appearance of free radicals, and formation of chemically organic functional groups), generating biochar (Shafizadeh 1982). Pyrolysis (either fast or slow depending on the heat exchange rate with biomass) occurs at temperatures of 550 °C to 750 °C and in an anaerobic environment to produce bio-oil. When biomass is processed at even greater temperatures (750 °C – 1200 °C) and with limited inputs of oxygen, gasification takes place to produce mostly syngas (by either the catalytic or non-catalytic routes) and biochar and bio oils as by-products. Syngas can then be transformed into liquid hydrocarbons (biodiesel and bio-kerosene), bio-dimethyl ether (DME), and/or bio-methane.
1.2.3 MAIN CHALLENGES OF 2ND-GENERATION BIOFUELS

Although 2nd generation biofuels were expected to be superior to 1st generation biofuels in terms of land requirement and competition for land, food, fiber, and water, 2nd generation biofuel technologies present technical barriers. When compared to other sugar- and starch-based feedstocks, the conversion rate of lignocellulosic ethanol to ethanol is significantly lower, ranging from 30% to 60% (depending on the technologies). The major challenges of lignocellulosic ethanol production (via the biochemical route) are pretreatment, cellulose enzyme production, and fermentation technologies. Among the three major components of lignocellulose, cellulose has the highest conversion rate to ethanol, 85% - 90%; hemicelluloses 30% - 85%; lignin 0% (Cheng and Timilsina 2011). A pretreatment step must be used to liberate cellulose and hemicellulose from lignin for their conversion to glucose monomers. Thus, the concentration of lignin content determines the extent of pretreatment needed. Current pretreatment technologies depend on high temperature or high pressure to maximize yields of cellulose and hemicellulose while reducing inhibitors (lignin), resulting in a high cost for the process. The effective hydrolysis of cellulose, hemicellulose, and lignin requires several cellulases (endoglucanase, exoglucanase, and β-glucosidase) to convert the carbohydrates into sugars. Trichoderma, Penicillium, and Aspergillus, all wood-rotting fungi, are the most common producers of cellulases; however, their production cost remains relatively high in comparison with amylases (the enzymes used in the starch to ethanol process). The main products of hemicellulose hydrolysis include hexoses, pentoses, and xylose. Although the fermentation of hexoses (C6) is already employed in large-scale corn-
to-ethanol industries, the fermentation of pentose (C5) sugars such as xylose is much more complicated due to the absence of natural organisms able to efficiently convert pentose sugars to ethanol at high yields. It should also be noted that acids used during the hydrolysis process may inhibit the fermentation process, requiring an additional operation for detoxification; hence, an increase in production cost.

1.3 THIRD-GENERATION BIOFUELS

At present, the production systems of 1st generation biofuels have considerable economic and environmental limitation and the generation of second generation biofuels is not cost-effective because there are a number of technical barriers that need to be overcome before their potential can be realized. Therefore, based on current knowledge and technology projections, 3rd generation biofuels derived from microalgae are viewed as a viable alternative energy resource that is devoid of the significant drawbacks associated with 1st and 2nd generation biofuels. These photosynthetic microorganisms (prokaryotic or eukaryotic) have simple growth requirements (light, sugars, CO₂, N, P, and K), resulting in the production of lipids, proteins, and carbohydrates that can be further processed into biofuels and valuable co-products.

Microalgae are thallophytes (plants lacking roots, stems, and leaves) that have chlorophyll a as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells (Lee 2008). There are two basic types of microalgae, prokaryotic (i.e. cyanobacteria) and eukaryotic (i.e. green algae, red algae, and diatoms).
Membrane-bound organelles (plastids, mitochondria, nucleus, Golgi bodies, and endoplasmic reticulum) are absent from prokaryotic algal cells (cyanobacteria). Eukaryotic algal cells are surrounded by a cell wall composed of polysaccharides and do contain the aforementioned organelles which control the functions of the cell.

Microalgae are more efficient than land plants at accessing water and other nutrients because their cells grow in aqueous suspension (Sheehan et al. 1998). Generally, these microorganisms can also convert solar energy into chemical energy to create biomass more efficiently than land plants owing to their simple cellular structure (unicellular), although the mechanism of photosynthesis is similar in both groups. Through photosynthesis, microalgae are able to fix the dissolved inorganic carbon and CO₂ in the gaseous effluents (e.g. atmosphere, discharge gases from heavy industry, and soluble carbonates) to form chemical energy. Microalgae-based CO₂ fixation is at rates an order of magnitude higher than those of land plants. Microalgae can have many types of metabolisms (e.g. mixotrophic, autotrophic, heterotrophic, photoheterotrophic). Photosynthesis is the main energy source for mixotrophs; however, they can also use organic substances to meet their requirements for energy and carbon. Autotrophic microalgae can only utilize inorganic compounds for growth. Organic compounds generate the carbon and energy sources needed for heterotrophs to grow. Photoheterotrophic microalgae are capable of using dissolved organic substrates and harvesting light energy.

Often referred to as ‘miniature sunlight-driven,’ biochemical factories, microalgae can provide feedstock for several renewable liquid fuels such as biomethane, biohydrogen, bioethanol, biobutanol, biodiesel, bio-oil, syngas, and biojet derived from microalgal oils.
(Meier 1955; Golueke and Oswalk 1959; Golueke et al. 1957; Uziel et al. 1975; Benemann et al. 1977; Borowitzka 1990; Roessler et al. 1994b; Sheehan et al. 1998; Banerjee et al. 2002; Gavrilescu and Chisti 2005; Ghirardi et al. 2000; Melis 2002; Akkerman et al. 2002; Fedorov et al. 2005; Kapdan and Kargi 2006) (Figure 1-1). The concept of using microalgae as a source of fuel is not new; however, a new interest in the production of biofuel from microalgae has developed due to rising prices of petroleum and the emerging concern about global warming that is associated with pollutant emissions and greenhouse gases (Gavrilescu and Chisti 2005).

Recent studies have shown that microalgae biomass appears to be one of the promising sources of renewable biofuel that is capable of meeting the global demand for transportation fuel. The advantages of using microalgae-derived biofuel include the following: (1) microalgae are capable of year-round production, therefore, oil productivity of the microorganism exceeds the yield of the best oilseed crops (Table 1-1); (2) they require less water than terrestrial crops for growth, therefore, reducing the load on freshwater sources; (3) they can be cultivated in saline/brackish water/coastal seawater on non-arable land, and therefore, may not incur land-use changes, minimizing associated environmental impacts, while not competing for resources with conventional agriculture; (4) depending on the algal strain, microalgae can synthesize and accumulate large quantities of neutral lipids/oils [20 – 50% dry cell weight (DCW)] and grow at high rates, e.g. 1 – 3 doublings/day; (5) microalgae can sequester CO$_2$ from flue-gases emitted from fossil-fuel combustion, thereby reducing emissions of a major greenhouse gas (1 kg of dry algal biomass uses about 1.83 kg of CO$_2$); (6) the microorganism can utilize nitrogen and
phosphorus from a variety of wastewater sources, therefore, aiding in wastewater bioremediation; (7) neither herbicides nor pesticides are needed for the cultivation of microalgae; (8) they can produce value-added co-products or by-products, e.g. biopolymers, proteins, polysaccharides, pigments, animal feed, and fertilizers, and; (9) by varying growth conditions, the biochemical composition of the algal biomass can be altered; therefore, the oil yield may be significantly enhanced (Schenk et al. 2008; Dismukes et al. 2008; Searchinger et al. 2008; Chisti 2007; Metting 1996; Spolaore et al. 2006; Cantrell et al. 2008; Rodolfi et al. 2009; Hirano et al. 1997; Qin 2005; Ghirardi et al. 2000).

Generally, microalgae produce lipids that include neutral lipids, polar lipids, wax esters, sterols, and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines, and pyrrole derivatives such as chlorophylls. These lipids can be grouped into two categories, storage lipids (non-polar lipids) and structural lipids (polar lipids). In microalgae, the physiological roles of lipids used for biofuels include energy storage, structural support as membranes, and intercellular signaling (Weselake 2005). With this in mind, storage lipids differ from structural lipids in that they are mainly composed of triacylglycerol (TAG). TAGs consist of three FA chains esterified via the hydroxyl groups of a glycerol backbone which can be transesterified to produce biofuels. The fatty acyl chains are chemically similar to the aliphatic hydrocarbons that make up the bulk of the molecules found in gasoline, diesel, and jet fuel (Table 1-2). The composition of FAs is important when selecting an algal strain for biofuel purposes because they have a significant effect on the characteristics of biofuel produced including viscosity, cloud point, flash point, oxidative stability, ignition delay, and combustion quality.
The composition of FAs from microalgae varies among species. Generally, microalgae synthesize FAs that are either unsaturated or saturated and mainly have chain lengths that range from C_{14} to C_{18} (Roessler et al. 1994b) (Table 1-3). Typically, saturated and mono-unsaturated FAs are predominant in most algae examined (Borowitzka 1988).

Naturally, the accumulation of lipids in microalgae can reach 75% by weight of dry biomass but such high levels of oil production are often associated with low biomass productivities (Mata et al. 2010). This is because biomass productivity and lipid accumulation are not necessarily correlated; hence, an increase in lipid accumulation will not lead to an increase in lipid productivity (Rodolfi et al. 2009; Sheehan et al. 1998). Most common algae, including *Dunaliella*, have lipid accumulation between 20 – 50% (Table 1-4), but higher lipid production can be reached by optimizing the growth determining factors such as the control of nitrogen level, light intensity, pH, temperature, salinity, CO_{2} concentration, mineral salts, harvesting procedures as well as subjecting the microorganism to stressful or unfavorable conditions (Hu et al. 2008; Widjaja et al. 2009; Weldy and Huesemann 2007; Roessler 1990; Wu and Hsieh 2008; Qin 2005; Chiu et al. 2009; de Morais and Costa 2007; ). Wu and Hsieh found that there was a 76% increase in production of lipids for specific growth conditions (studying salinity, nitrogen concentration and light intensity on lipid productivity) when compared to typical growth processes (2008). Likewise, Hu reported that when the cells of oleaginous green algae were subjected to unfavorable culturing conditions such as photo-oxidative stress or nutrient starvation, the average total lipid content increased from 25.5% DCW to 45.7% DCW (Hu et al. 2008).
During optimal growth conditions, algae will generate a large amount of biomass but with relatively low lipid content, which constitutes about 5 - 20% of their DCW, including the esterification of FAs into glycerol-based membrane lipids containing medium-chain (C_{10}-C_{14}), long-chain (C_{16}-C_{18}), and very long-chain (≥C_{20}) species and FA derivatives (Hu et al. 2008). However, under unfavorable environmental or stress conditions, many algae will alter their lipid biosynthetic pathways, switching from the synthesis of membrane lipids to the formation and accumulation of neutral lipids (20 - 50% DCW), resulting in the total lipid content in the cell consisting of 80 % TAGs and allowing the microorganism to withstand these adverse conditions (Klyachko-Gurvich 1974; Suen et al. 1987; Tonon et al. 2002; Tornabene et al. 1983). It is believed that this accumulation of TAGs likely occurs as a means of providing an energy deposit that can be readily catabolized in response to a more favorable environment (Harwood et al. 1988).

Our current knowledge of lipid metabolism, specifically the biosynthetic pathway of FAs and triacylglycerols (TAGs), in algae is still in its infancy when compared to vascular plants and bacteria. It is believed that these biosynthetic pathways in algae are highly similar to those of vascular plants based on sequence homology and some common biochemical characteristics of a number of genes and/or enzymes involved in lipid metabolism (Hu et al. 2008). There are three major steps involved in the synthesis of TAGs: (1) the generation of malonyl-CoA from acetyl-CoA by acetyl coenzyme A carboxylase (ACCase); (2) the elongation of the acyl chain by FA synthase; and (3) the formation of TAGs. In algae, the de novo synthesis of FAs occurs mainly in the chloroplast by a type II FAS system, involving a group of dissociated enzymes that catalytically elongate a growing FA by two carbon units in
an iterative pathway (Ryall et al. 2003). Beginning with the carboxylation of acetyl-CoA to form malonyl by ACCase, the principal reactions leading to the production of FAs in algal FAS are very much like those discussed for bacteria (Section 1.3.5.2). Generally, the pathway produces a carbon chain length of 14 to 18, saturated or mono-unsaturated FA (Harwood 1998; Roessler 1990). However, there are some exceptions to this generalization in which very long chain (>20) PUFAs (polyunsaturated FAs) are synthesized and portioned into TAGs which occurs in the green alga *Parietochloris incise* and freshwater red microalgae *Porphyridium cruentum* (Bigogno et al. 2002; Cohen et al. 2000). To produce an unsaturated FA, a double bond is introduced by the soluble enzyme stearoyl ACP desaturase.

The completion of *de novo* FAS is accomplished by either the removal of the acyl group from ACP by an acyl-ACP thioesterase that hydrolyzes the acyl ACP (releasing FFA) or the transfer of FA directly from ACP to glycerol-3-phosphate or monoacylglycerol-3-phosphate by acyltransferases in the chloroplast (Ohlrogge and Browse 1995). Thus, the enzymes involved in the termination of the FAS determine the final FA composition of individual algae.

Triacylglycerol biosynthesis in algae has been proposed to occur via the direct glycerol pathway (*Kennedy pathway*) (Figure 1-2) (Ratledge 1988). Fatty acids generated in the chloroplast are sequentially transferred from CoA to the sn-1 and sn-2 position of glycerol 3-phosphate (G3P) by glycerol 3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT), respectively, leading to the formation of the central metabolite phosphatidic acid (PA) (Chen and Smith 2012). The dephosphorylation of PA at the sn-3 position is then catalyzed by phosphatidic acid phosphatase (PAP) leading to
the production of \( sn \)-1,2-diacylglycerol (DAG). In the last and only committed step in TAG biosynthesis, a third FA is transferred to the vacant position of DAG, catalyzed by diacylglycerol acyltransferase (DGAT). DGAT is unique to the TAG biosynthetic pathway (Ohlrogg and Jaworski 1997). The acyltransferases involved in the biosynthesis of TAG appear to exhibit preference for specific acyl-CoA, as in plants. Thus, it is believed to play a vital role in determining the final acyl composition of TAG molecules (Hu et al. 2008).

Although the Kennedy pathway is believed to be the main pathway for TAG biosynthesis, Dahlqvist et al. (2000) reported a non-acyl-CoA-dependent mechanism that utilizes phospholipids as acyl donors and DAG as the acceptor by the catalytic action of phospholipid:diacylglycerol acyltransferase (PDAT), similar to that observed in some plants and yeast. This pathway is thought to be critical in the regulation of membrane lipid composition in response to different environmental and growth conditions since during various stress conditions, algae generally experience rapid degradation of the photosynthetic membrane with concomitant occurrence and accumulation of cytosolic TAG-enriched lipid bodies (Hu et al. 2008).

### 1.3.1 METABOLIC ENGINEERING OF MICROALGAE FOR ENHANCED LIPID BIOSYNTHESIS

Although environmental stresses have been shown to increase lipid accumulation in microalgae during cell growth, the growth rate of the stressed cells is generally greatly reduced (Roessler et al. 1994a). For microalgae-derived biofuel to be commercially viable,
the microorganism must achieve enhanced lipid production under high growth rate conditions (Borowitzka 1992). Hence, there is a great need to use metabolic engineering to genetically modify the lipid biosynthetic pathway in microalgae for an increase in lipid concentration during normal cell growth. This idea is not new as it has been performed in vascular plants (Table 1-5). Hence, strategies to engineer the lipid biosynthetic pathway toward more compatible lipid profiles have involved the following: (1) the overexpression of FA biosynthetic enzymes; (2) increasing the availability of precursor molecules, such as acetyl-CoA; (3) the downregulation of FA catabolism by inhibiting β-oxidation, or lipase hydrolysis; (4) altering saturation profiles through the introduction or regulation of desaturases; and (5) optimizing FA chain length with thioesterase(s) (Beer et al. 2009).

To date, the mechanisms involved in the fatty acid biosynthesis (FAB) pathways in microalgae have not been extensively studied and most information has been gathered from studies on plant metabolism (Adarme-Vega et al. 2012). However, genes encoding key enzymes involved in the FAB have been identified such as in Ostreococcus tauri, Thalassiosira pseudonana, Phaeodactylum tricornutum, Cyclotella cryptica, Isochrysis galbana, and in particular the model organism Chlamydomonas reinhardtii (Wagner et al. 2010; Xu et al. 2009; Tonon et al. 2005a; Tonon et al. 2005b; Domergue et al. 2002; Domergue et al. 2003; Roessler et al. 1994a; Livne and Sukenik 1990; Chi et al. 2008). For our study, we have identified extremophilic archaeal and bacterial lipid biosynthetic genes for the expression in Dunaliella species to maximize its FA synthesis as a means to overcome transcriptional repression and alleviate feedback inhibition.
The unicellular, photosynthetic and motile biflagellate microalgae *Dunaliella* is classified in the order Chlorophyceae, Volvocales, which includes a variety of ill-defined marine and fresh water unicellular species (Ben-Amotz 2004). The best-known species of *Dunaliella* are *D. salina*, *D. tertiolecta*, *D. primolecta*, *D. viridis*, *D. bioculata*, *D. acidophyla*, *D. parva*, and *D. media* (Tafreshi and Shariati 2009). Generally, members of the genus *Dunaliella* are characterized by an ovoid cell volume usually in the shape of a pear, wider at the basal side and narrow at the anterior flagella top. Cells may change shape with changing conditions, often becoming spherical under unfavorable conditions (Preisig 1992). Like other members of Volvocales, the microorganism has one cup-shaped chloroplast which mostly has a central pyrenoid surrounded by starch granules, a few vacuoles, mitochondria, Golgi apparatus, a membrane-bound nucleus, a nucleolus, and an eyespot (Ben-Amotz 2004). The cell is enclosed only by an elastic plasma membrane covered by a mucus surface coat (Tafreshi and Shariati 2009). The absence of a rigid cell wall permits rapid cell volume changes in response to extracellular changes in osmotic pressure.

Species in the genus are isolated from a wide range of marine habitats such as oceans, brine lakes, salt marshes, salt lagoons, and salt water ditches near the sea, predominantly in water bodies containing more than 2M salt and high-levels of magnesium. The ability of *Dunaliella* to grow and thrive in a variety of salt concentrations, from as low as 0.1 M to salt saturations > 4 M, makes this alga the most halotolerant eukaryote known (Ben-Amotz 2004). This microorganism can thrive in these seemingly harsh conditions because of its ability to change its intracellular concentration of glycerol. Although there are many proposed applications for *Dunaliella* (Table 1-6), it has many characteristics making it an
ideal candidate for producing biofuels. These favorable characteristics include that it has been mass-cultured successfully in bioreactors and open ponds for commercial β-carotene production; that it does not require aseptic growth conditions; that it has no outer cell wall which facilitates lipid extraction; and that it requires saltwater rather than freshwater, limiting further demands for freshwater resources for energy production (Graham and Wilcox 2000; Minowa et al. 1995; Pak et al. 1991; Pak et al. 1993; Park et al. 1998; Pick 1998; Tafreshi and Shariati 2006; Tornabene et al. 1980; Webber 2008; Weldy and Huesemann 2007). One method to potentially improve algal-based biofuel derived from *Dunaliella*, genetic engineering efforts is to target the fatty acid biosynthesis pathway with the expression of bacterial and archaeal FAS genes so that there is an increase in lipid accumulation during active cell growth.

Fatty acid synthase (FAS) systems are grouped into two classes, type I and type II. Different from the mammalian type I FAS system, which utilizes a large multifunctional enzyme, the type II FAS system uses a series of monofunctional proteins, and each catalyzes one step in the biosynthesis pathway. The type II FAS system has been extensively investigated in an *Escherichia coli* model system and is found mostly in bacteria, mitochondria, and chloroplasts (Rock and Cronan 1996; Rock and Jackowski 2002).

The first committed and rate limiting step in the *de novo* FAS pathway is catalyzed by Acetyl Coenzyme A Carboxylase (ACCase). ACCase is highly conserved in its function in most living organisms including Archaea (which have isoprenoid lipids in place of lipids based on FAs), bacteria, and the chloroplasts of most plants. It is important to note that small amounts of FFA or their derivatives and homologs of bacterial FAS enzymes are found in
archaea; however, the importance of FA in archaea remains unknown (Kates et al. 1968; Langworthy et al. 1974; Boucher 2007; Dibrova et al. 2011). The bacterial form has three separate components: biotin carboxylase (BC; a homodimer of 49.4 kDa monomers encoded by \textit{accC}), biotin carboxyl carrier protein (BCCP; a homodimer of 16.7 kDa monomers encoded by \textit{accB}), and carboxyltransferase (CT; a heterotetramer of 35.1 kDa and 33.2 kDa monomers encoded by \textit{accA} and \textit{accD}, respectively). Roessler et al. (1994a) showed that the native ACCase isolated from \textit{C. cryptic} (microalgae) has a molecular mass of approximately 740 kDa and appeared to be composed of four identical biotin-containing subunits. Similarly, the molecular mass of the native ACCase from \textit{I. galbana} was estimated at 700 kDa, consisting of four identical subunits (Livne and Sukenik 1990). This biotin-dependent enzyme catalyzes the carboxylation of acetyl-CoA to malonyl-CoA in two distinct half reactions (Figure 1-3). In the first reaction, BC catalyzes the MgATP-dependent carboxylation of a biotin cofactor (covalently coupled through an amide bond to a lysine residue on BCCP), at the N1 atom in the ureido ring, forming carboxybiotin (Tong 2005). The reaction uses bicarbonate as the CO\textsubscript{2} donor. The \textit{accB} and \textit{accC} genes form an operon in the genome of most prokaryotes. The second half reaction involves the CT transferring the carboxyl group from the N1 atom of biotin to the methyl group of acetyl-CoA, producing malonyl-CoA (Li and Cronan 1992; Blanchard and Waldrop 1998; Polakis et al. 1974).

The most crucial role of ACCase, in bacteria/microalgae, is the biosynthesis and metabolism of long-chain FAs. Long-chain FAs are generated via a continual cycle of reactions involving the condensation, reduction, dehydration, and reduction of carbon-carbon bonds (Rock and Cronan 1996; Campbell and Cronan 2001). The central player in this
continuous cycle is acyl carrier protein (ACP), a small acidic protein that shuttles all of the intermediates as thioesters attached to the terminus of its 4’-phosphopantetheine prosthetic group (Rock and Jackowski 2002). The malonyl-CoA product of ACCase is used as a building block to extend the chain length of FAs. In order to deliver malonyl-ACP for initiation of FASII and to supply each round of FA elongation, malonyl-CoA:ACP transacylase, encoded by the fabDimat gene, catalyzes a transthioesterification of malonate from CoA to ACP (Harder et al. 1974). FabH/KASIII (β-ketoacyl-ACP synthase III/3-ketoacyl ACP synthase III) then catalyzes the initial condensation reaction of acetyl-CoA with malonyl-ACP, utilizing acyl-CoA primers, to initiate cycles of FA elongation. Differing from other elongation condensing enzymes, this condensing enzyme couples CoA thioesters with acyl-CoA units in the initial elongation step as opposed to only the ACP thioesters consumed in the subsequent condensation steps (FabB and FabF) (Handke et al. 2011; Jackowski and Rock 1987).

The central machinery of bacterial/microalgal FASII is represented by the elongation module, consisting of four enzymes, that works in a cyclic process to extend the saturated fatty acyl chain by two carbon units during each cycle until a final length of 16 or 18 carbons is reached (Figure 1-4). The FabH product enters the elongation cycle to form β-hydroxyacyl-ACP due to the catalysis of NADPH dependent β-ketoacyl-ACP reductase (FabG/KAR [3-ketoacyl-ACP reductase]). The next step in the elongation cycle is dehydration of the β-hydroxyacyl-ACP to the trans-2-enoyl-ACO by FabA and FabZ/HD (3-hydroxyacyl-ACP dehydratase). These enzymes are distinguished by the fact that FabZ only catalyzes the dehydration reactions, whereas FabA not only dehydrates, but also is capable of
isomerizing the double bond in the 10-carbon intermediates as the first step in unsaturated FAB (Rock and Jackowski 2002). The final step in the elongation cycle is catalyzed by the enoyl-ACP reductase gene fabI/enr, yielding acyl-ACP bound by a saturated acyl chain extended by two carbon units compared with the start of the cycle.

Subsequent rounds of FA elongation, in bacteria, are catalyzed by either β-oxoacyl synthase I or II (KAS), having similar substrate specificities to FabH. During each of the condensation reactions, the acyl chain is removed from ACP and attached in a thioester bond by a cysteine residue in the active sites of FabH, FabB, and FabF (White et al. 2005). The acyl chain is added to the tip of the malonyl after an extender malonyl-ACP enters the active site, resulting in the loss of a CO₂ group in the process. Hence, as the additional carbon groups are placed at the base of the acyl chain rather than the tip, the acyl chain is built from the “inside out” (Chan and Vogel 2010). Upon reaching a saturated fatty acid (SFA) of 16-18 carbons, the majority of acyl-ACPs are incorporated into the membrane. FabB (KAS I) and FabF (KAS II) are also important determinants of the product distribution of the pathway. All FASII pathways have this basic set of enzymes to initiate and elongate acyl chains, and the diversity of products is accomplished by variation of this theme.

In addition to the production of SFAs, each organism can also synthesize unsaturated fatty acids (UFAs). In bacterial FASII, UFAs are generated by the introduction of cis double bond(s) into the growing acyl chain. The amount of UFAs in the membrane phospholipids influences its membrane fluidity and function, and high levels of UFAs can disruptively disorder the membrane (Cronan and Vagelos 1972). Two genes, fabA (β-hydroxyacyl dehydratase) and fabB (3-Ketoacyl-ACP synthase I), are vital players in the UFA pathway,
and they are typically co-localized in bacterial genomes. The first step in the production of these acyl chains involves the *fabA*-encoded dehydrase/isomerase which catalyzes a reaction at the branch point of the two pathways: the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP (Heath and Rock 1996b; Leesong et al. 1996). The isomerase function is exclusively performed on C10 substrates in which the double bond is added. FabB is required to elongate the FabA product and determines the cellular UFA content (Clark et al. 1983; Cronan et al. 1969; Zhang et al. 2002). These key players are co-transcribed in a *fabA-fabB* operon in some organisms, such as *Pseudomonas aeruginosa* (Hoang and Schweizer 1997).

In *E. coli*, owing to the functionality of FabB and FabI, the relative abundance of these two enzymes determines the ratio of SFA to UFAs in biological membranes (Chan and Vogel 2010). Accordingly, due to the nature of FA synthesis, only a small number of specific UFAs are produced in *E. coli*. This leads to most of the UFAs being palmitoleic and vaccenic acids (Cronan 1967; Morein et al. 1996). FabA and FabB are not widely found in bacteria, with recent studies indicating that they may be restricted to alpha and gamma proteobacteria (Campbell and Cronan 2001; Lu et al. 2004). In fact, variations to the described UFA synthesis process exist and are notably different in Gram-positive organisms (Marrakchi H et al. 2002).

Extensive research has been conducted to gain an understanding of how type II FAS is regulated and coordinated with carbon-chain synthesis. A noteworthy experiment was the demonstration that the overexpression of a cytosolic form of *E. coli* thioesterase I (TesA’, a version of TesA with the N-terminal signal peptide deleted), which removes the fatty acyl moiety from the fatty acyl-ACPs, releasing them as FFA, relieved inhibition of FA synthesis.
brought on by cessation of phospholipid biosynthesis. Overexpression of *E. coli* TesA' also eliminated accumulated acyl-ACP pools and reduced the synthesis of abnormal length FAs (Barnes and Wakil 1968; Jiang and Cronan 1994). Generally, in stationary phase, FA synthesis ceases; however, in the presence of ectopically expressed thioesterases, the cells continue to generate FAs that are excreted from the bacteria (Jiang and Cronan 1994). These findings confirmed the extremely tight regulation by long chain acyl-ACPs in the FAS pathway, which act as feedback inhibitors of key enzymes. These key regulated enzymes are ACCase and to lesser degrees FabH and FabI, all confirmed by *in vitro* studies (Davis and Cronan 2001; Heath and Rock 1996b). Inhibition of ACCase prevents the generation of malonyl-CoA, which is the extender unit needed for the elongation of the growing acyl chains (Davis and Cronan 2001). FabH catalyses the initial step in FA elongation; thus, regulation at this step would prevent the initiation of new acyl chains and limit the total number of FAs produced (Heath and Rock 1996a; Heath and Rock 1996b). Lastly, FabI reduces enoyl-ACP, which is important for the completion of the acyl chain elongation cycle due to the reversible nature of the preceding dehydration reaction (Heath and Rock 1996b). Hence, inhibition of FabI would slow the rate of FA formation.

TesA from *E. coli* belongs to the α/β hydrolase family of serine carboxylesterases and lipases (Nardini and Dijkstra 1999; Ollis et al. 1992; Schrag and Cygler 1997). As an α/β hydrolase, the fold structure of the enzyme consists of an alpha/beta sheet where seven or eight beta-sheets wrap around five or six alpha-helices (Ollis et al. 1992). Lipases (EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1) are enzymes that catalyze the hydrolysis or synthesis of ester bonds in lipids (Jaeger and Eggert 2002). As hydrolases, these lipolytic
enzymes act on the ester bonds of the triglycerides to liberate FAs and glycerol. Although both enzyme types share structural characteristics, the fundamental difference between carboxylesterases and (true) lipases is that the former acts on shorter chain triglycerides (shorter than 10 carbon atoms) which are water-soluble, whereas the latter hydrolyzes longer chain triglycerides (Gilham and Lehner 2005; Jaeger et al. 1999).

These enzymes are also often classified as belonging to the family of SGNH-hydrolases due to the presence of four strictly conserved residues, Ser-Gly-Asn-His, in four conserved blocks I, II, III, and V, respectively (Mølgaard et al. 2000; Li et al. 2000; Dalrymple et al. 1997; Lee et al. 2006). Each of the four residues plays a key role in the catalytic function of the enzyme (Figure 1-5). In block I, the catalytic Ser\(^{10}\), a tetrahedral-carbon-covalent acyl-enzyme intermediate, acts as the nucleophile and proton donor to the oxyanion hole. The Gly\(^{44}\) residue in block II and the Asn\(^{73}\) in block III act as two other proton donors to the oxyanion hole. The His\(^{157}\) residue in block V acts as a base to make active Ser more nucleophilic by the deprotonation of the hydroxyl group, thus, stabilizing the oxyanion-intermediate conformation (Lee et al. 2006). Another unique feature in block V is the presence of Asp\(^{154}\) located at the third amino acid preceding His\(^{157}\) (i.e., DxxH) (Akoh et al. 2004). Both Asp\(^{154}\) and His\(^{157}\) combine with Ser\(^{10}\) to form the catalytic triad.
1.3.2 EXTREMOPHILIC ENZYMES

Since ACCase is the primary regulated and rate-limiting step for FA synthesis in microalgae, it is thought that increasing the activity of this multi-subunit enzyme should result in a significant increase of lipid accumulation as has been seen in bacterial systems (Rock and Jackowski 2002; Davis et al. 2000). However, little alteration in lipid production has been seen in microalgae with elevated levels of ACCase activity (Dunahay et al. 1995; Sheehan et al. 1998; Dunahay et al. 1996; Roesler et al. 1997; Klaus et al. 2004). It may be that the overexpression of a native ACCase suffered from feedback inhibition by acyl-ACP (Davis and Cronan 2001). One way to reduce this inhibition could be to select and express a non-native ACCase gene that is not recognized by the acyl-ACP of the targeted organism. This selection should be based on growth parameters of the targeted organism so that the non-native protein can have optimal activity in those growth conditions such as the expression of a protein isolated from a halophilic bacterium (e.g. Chromohalobacter salexigens) in a halophilic microalgae (e.g. Dunaliella spp). With this in mind, because Dunaliella is halophilic and growth at increased salinity has been shown to improve its total FA synthesis, the acetyl coenzyme A carboxylase (ACCase) from a moderate halophilic bacterium was identified.
1.3.2.1 PROPERTIES OF HALOPHILIC ENZYMES

Halophiles are microorganisms that can thrive in high-salt environments and are found in all three domains of life: Archaea, Bacteria, and Eukarya. There are two different adaptive strategies that allow halophilic microorganisms to cope with the osmotic pressure induced by the high salt concentrations found in their habitat, the “high-salt in” strategy and the “low-salt, organic-solutes-in” strategy (Madigan and Oren 1999; Oren 2002). The “low-salt, organic-solutes-in” strategy is used by most moderate halophilic bacteria and eukaryotes. These microorganisms limit the entry of inorganic salts (such as NaCl) into the cell via active ion transporters on their surface and synthesize small organic molecules, known as osmolytes, to balance the osmotic pressure (Nieto and Vargas 2002). Extremely halophilic archaea and bacteria survive using the “high-salt in” strategy by requiring their proteins to carry adaptations that allow them to remain stable and functional in high concentrations of inorganic salts. These microorganisms accumulate inorganic ions in the cytoplasm (K\(^+\), Na\(^+\), Cl\(^-\)) to balance the osmotic pressure of the medium. Halophilic proteins can function in high salt concentrations because the hydrophobic and electrostatic interactions of amino acids for proper folding and maintaining stability are strengthened by the presence of the salt (Reed et al. 2013). When compared to non-halophilic proteins, halophilic proteins have more acidic residues, such as glutamic and aspartic acid, on the surface of the protein and different hydrophobic residues. Reed et al. (2013) have postulated that the role of the acidic residues in a halophilic protein may be to increase the proteins flexibility by having a large number of nearby charges that repel each other; thus, making it
easier for a halophilic protein to change its conformation despite having a more rigid hydrophobic core. Interestingly, salt concentrations have a substantial effect on the folding, structure, and catalytic property of a halophilic enzyme; however, enzyme activity is dependent on the nature of the salt (Hecht et al. 1989; Mevarech et al. 1977; Madern et al. 2000).

1.3.2.2 PROPERTIES OF THERMOPHILIC ENZYMES

In addition to expressing the ACCase enzyme isolated from a halophilic bacterium, type I thioesterase from an acidophilic hyperthermophile archaeon was identified to produce FFAs captured from TAGs with shorter FA composition. For this study, the objective was to express the thermostable thioesterase during active algae growth, having optimal activity during the conversion of lipids to jet fuel. This conversion is done at high temperature and pressure (Wang et al. 2013).

Thermophiles thrive in extreme environments and thermodynamic conditions. These extremophiles can be grouped on the basis of cardinal growth temperatures: moderate thermophiles (T_{opt} \sim 60^\circ C), extreme thermophiles (T_{opt} \sim 75^\circ C), and hyperthermophiles (T_{opt} \sim 85^\circ C) (Segerer et al. 1993). Moderate thermophiles are commonly found in warm environments such as hot desert soils, natural hydrothermal waters, industrial wastewaters, and domestic water sources (Segerer et al. 1993). Extreme- and hyper-thermophiles proliferate in terrestrial (such as solfataric fields) and marine hydrothermal (such as hot fumaroles, springs, and deep sea vents) systems.
Typically, non-thermophilic proteins undergo irreversible unfolding at the aforementioned temperatures, resulting in aggregation. Thermophilic proteins have many adaptations that allow them to retain their structure and function at elevated temperatures. One such adaptation is the aberrancies in quaternary structure. Generally, structural flexibility in protein structure is unfavorable in thermostable enzymes. To achieve rigidity, some proteins will increase their oligomeric state to form octomers, while others will achieve conformational rigidity by organizing all the necessary components into a single subunit, creating structural rigidity, and promoting tighter packing of the hydrophobic core (Mayer et al. 2012; Brasen et al. 2005; Park et al. 2013). Another structural adaptation observed in thermostable proteins is disulfide bridging. Studies have shown that disulfide bonds increase stability within thermophilic proteins and play a role in preventing alteration of quaternary structure (Cacciapuoti et al. 1994; Cacciapuoti et al. 2012; Boutz et al. 2007). These structural elements have also been shown to be important in oligomerization as it confers stability within the dimer by disallowing separation of the individual subunits. Salt bridges (ion pairs), a source of thermodynamic and kinetic stability, are important to the structural stability of thermostable proteins as they increase the thermal capacity of proteins using favorable charge-charge interactions (Chan et al. 2011; Lee et al. 2005). On the average, thermotolerant proteins have 8 to 9 salt-bridges per 100 amino acids, a percentage about twice larger then in mesophilic proteins (Karshikoff and Ladenstein 2001). Thermostable proteins have also adapted to higher content of surface charged amino acid residues to stabilize the proteins by preventing aggregation at higher temperatures (Fukuchi and Nishikawa 2001).
1.4 CONCLUSION

Microalgae have emerged as one of the most promising feedstocks for biofuels due to their fast growth rates (compared to vascular plants), high lipid content, and ability to grow in a broad range of environments. However, many microalgae only achieve maximal lipid production in the presence of an environmental stressor, resulting in slow growth rates and the production of lipid compositions not ideal for biofuel production. Thus, metabolic engineering of the lipid biosynthetic pathway in microalgae is necessary to help increase algal oil production. This study is focused on the biochemical characterization of extremophile fatty acid synthesis and processing enzymes that could be introduced into microalgae such as *Dunaliella*. Specifically, an acetyl-CoA carboxylase from a halophile and thioesterases from thermophiles were recombinantly expressed in *Escherichia coli* to enable their purification, biochemical characterization, and evaluation for suitability for use in marine microalgae.

1.5 REFERENCES


<table>
<thead>
<tr>
<th>Plant Source</th>
<th>Seed oil content (% oil by wt in biomass)</th>
<th>Oil yield (L oil/ha year)</th>
<th>Land use (m² year/kg biodiesel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn/Maize (Zea mays L.)</td>
<td>44</td>
<td>172</td>
<td>66</td>
</tr>
<tr>
<td>Hemp (Cannabis sativa L.)</td>
<td>33</td>
<td>363</td>
<td>31</td>
</tr>
<tr>
<td>Soybean (Glycine max L.)</td>
<td>18</td>
<td>636</td>
<td>18</td>
</tr>
<tr>
<td>Jatropha (Jatropha curcas L.)</td>
<td>28</td>
<td>741</td>
<td>15</td>
</tr>
<tr>
<td>Camelina (Camelina sativa L.)</td>
<td>42</td>
<td>915</td>
<td>12</td>
</tr>
<tr>
<td>Canola/Rapeseed (Brassica napus L.)</td>
<td>41</td>
<td>974</td>
<td>12</td>
</tr>
<tr>
<td>Sunflower (Helianthus annuus L.)</td>
<td>40</td>
<td>1070</td>
<td>11</td>
</tr>
<tr>
<td>Castor (Ricinus communis)</td>
<td>48</td>
<td>1307</td>
<td>9</td>
</tr>
<tr>
<td>Palm oil (Elaeis guineensis)</td>
<td>36</td>
<td>5366</td>
<td>2</td>
</tr>
<tr>
<td>Microalgae (low oil content)</td>
<td>30</td>
<td>58,700</td>
<td>0.2</td>
</tr>
<tr>
<td>Microalgae (medium oil content)</td>
<td>50</td>
<td>97,800</td>
<td>0.4</td>
</tr>
<tr>
<td>Microalgae (high oil content)</td>
<td>70</td>
<td>136,900</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 1-2 Fuel properties comparison (adapted from Lee et al. 2008).

<table>
<thead>
<tr>
<th>Fuel Type</th>
<th>Major Components</th>
<th>Important Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gasoline</td>
<td>$C_4$-$C_{12}$ hydrocarbons</td>
<td>Octane number</td>
</tr>
<tr>
<td></td>
<td>Linear, branched, cyclic, aromatics</td>
<td>Energy content</td>
</tr>
<tr>
<td></td>
<td>Anti-knock additives</td>
<td>Transportability</td>
</tr>
<tr>
<td>Diesel</td>
<td>$C_9$-$C_{23}$ (average $C_{16}$)</td>
<td>Cetane number</td>
</tr>
<tr>
<td></td>
<td>Linear, branched, cyclic, aromatics</td>
<td>Low freezing temperature</td>
</tr>
<tr>
<td></td>
<td>Anti-freeze additives</td>
<td>Low vapor pressure</td>
</tr>
<tr>
<td>Jet Fuel</td>
<td>$C_8$-$C_{12}$ hydrocarbons</td>
<td>Very low freezing temperature</td>
</tr>
<tr>
<td></td>
<td>Linear, branched, cyclic, aromatics</td>
<td>Net heat of combustion</td>
</tr>
<tr>
<td></td>
<td>Anti-freeze additives</td>
<td>Density</td>
</tr>
</tbody>
</table>
Table 1-3 Composition of microalgal oil (adapted from Meng et al. 2009).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Chain length: Number of Double Bonds</th>
<th>Oil Composition (w/total lipids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic Acid</td>
<td>C16:0</td>
<td>12–21</td>
</tr>
<tr>
<td>Palmitoleic Acid</td>
<td>C16:1</td>
<td>55–57</td>
</tr>
<tr>
<td>Steric Acid</td>
<td>C18:0</td>
<td>1–2</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>C18:1</td>
<td>58–60</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>C18:2</td>
<td>4–20</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>C18:3</td>
<td>14 – 30</td>
</tr>
</tbody>
</table>
Table 1-4 Lipid content and productivity found in different microalgae species (adapted from Mata et al. 2010).

<table>
<thead>
<tr>
<th>Marine and freshwater microalgae species</th>
<th>Lipid content (% dry weight biomass)</th>
<th>Lipid productivity (mg/L/day)</th>
<th>Volumetric productivity of biomass (g/L/day)</th>
<th>Areal productivity of biomass (g/m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankistrodesmus sp.</td>
<td>24.0-31.0</td>
<td>-</td>
<td>-</td>
<td>11.3-17.4</td>
</tr>
<tr>
<td>Botryococcus braunii</td>
<td>25.0-75.0</td>
<td>-</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>Chlorella minutissima</td>
<td>33.6</td>
<td>21.8</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>Chlorella calicivora</td>
<td>14.6-16.4/39.8</td>
<td>17.6</td>
<td>0.04</td>
<td>-</td>
</tr>
<tr>
<td>Chlorella emersonii</td>
<td>25.0-63.0</td>
<td>10.3-50.0</td>
<td>0.036-0.041</td>
<td>0.91-0.97</td>
</tr>
<tr>
<td>Chlorella protothecoides</td>
<td>14.6-57.8</td>
<td>12.14</td>
<td>2.00-7.70</td>
<td>-</td>
</tr>
<tr>
<td>Chlorella sorokiniana</td>
<td>19.0-32.0</td>
<td>44.7</td>
<td>0.23-1.47</td>
<td>-</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>5.0-58.0</td>
<td>11.2-40.0</td>
<td>0.02-0.20</td>
<td>0.87-0.95</td>
</tr>
<tr>
<td>Chlorella sp.</td>
<td>10.0-48.0</td>
<td>42.1</td>
<td>0.02-2.5</td>
<td>1.61-16.47/25</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>2</td>
<td>-</td>
<td>2.90-3.64</td>
<td>72.5/130</td>
</tr>
<tr>
<td>Chlorella</td>
<td>18.0-57.0</td>
<td>18.7</td>
<td>-</td>
<td>3.50-13.90</td>
</tr>
<tr>
<td>Chlorocyclus sp.</td>
<td>19.3</td>
<td>53.7</td>
<td>0.28</td>
<td>-</td>
</tr>
<tr>
<td>Chromatococciun cohnii</td>
<td>20.0-51.1</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Dunaliella salina</td>
<td>6.0-25.0</td>
<td>116</td>
<td>0.22-0.34</td>
<td>1.6-3/20-38</td>
</tr>
<tr>
<td>Dunaliella primolaeta</td>
<td>23.1</td>
<td>-</td>
<td>0.09</td>
<td>14</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
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<td>-</td>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td>Dunaliella sp.</td>
<td>17.5-67.0</td>
<td>33.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ellipsocephalus sp.</td>
<td>27.4</td>
<td>47.3</td>
<td>0.17</td>
<td>-</td>
</tr>
<tr>
<td>Euglena gracilis</td>
<td>14.0-20.0</td>
<td>-</td>
<td>7.7</td>
<td>-</td>
</tr>
<tr>
<td>Haematococcus plumaris</td>
<td>25</td>
<td>-</td>
<td>0.05-0.06</td>
<td>10.2-36.4</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>7.0-40.0</td>
<td>-</td>
<td>0.32-1.60</td>
<td>-</td>
</tr>
<tr>
<td>Isochrysis sp.</td>
<td>7.1-33</td>
<td>37.8</td>
<td>0.08-0.17</td>
<td>-</td>
</tr>
<tr>
<td>Melosira subtilissima</td>
<td>16</td>
<td>30.4</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>Mesodinium salmoni</td>
<td>20.0-22.0</td>
<td>-</td>
<td>0.08</td>
<td>12</td>
</tr>
<tr>
<td>Nanochloris sp.</td>
<td>20.0-56.0</td>
<td>60.9-76.5</td>
<td>0.17-0.51</td>
<td>-</td>
</tr>
<tr>
<td>Nanochloropsis oculata</td>
<td>22.7-29.7</td>
<td>84.0-142.0</td>
<td>0.37-0.48</td>
<td>-</td>
</tr>
<tr>
<td>Nanochloropsis sp.</td>
<td>12.0-33.0</td>
<td>37.0-90.0</td>
<td>0.17-1.43</td>
<td>1.9-5.3</td>
</tr>
<tr>
<td>Marine and freshwater microalgae species</td>
<td>Lipid content (% dry weight biomass)</td>
<td>Lipid productivity (mg/L/day)</td>
<td>Volumetric productivity of biomass (g/L/day)</td>
<td>Areal productivity of biomass (g/m²/day)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------</td>
<td>------------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Neochloris oleobundans</td>
<td>29.0-65.0</td>
<td>90.0-134.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitzschia sp.</td>
<td>16.0-47.0</td>
<td>-</td>
<td>-</td>
<td>8.8-21.6</td>
</tr>
<tr>
<td>Oocystis pusilla</td>
<td>10.5</td>
<td>-</td>
<td>-</td>
<td>40.6-45.8</td>
</tr>
<tr>
<td>Pavlova salina</td>
<td>30.9</td>
<td>49.4</td>
<td>0.16</td>
<td>-</td>
</tr>
<tr>
<td>Pavlova lutheri</td>
<td>35.5</td>
<td>40.2</td>
<td>0.14</td>
<td>-</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>18.0-57.0</td>
<td>44.8</td>
<td>0.003-1.9</td>
<td>2.4-21</td>
</tr>
<tr>
<td>Porphyridium cruentum</td>
<td>9.0-18.8/60.7</td>
<td>34.8</td>
<td>0.36-1.50</td>
<td>25</td>
</tr>
<tr>
<td>Scenedesmus obtusus</td>
<td>11.0-55.0</td>
<td>-</td>
<td>0.004-0.74</td>
<td>-</td>
</tr>
<tr>
<td>Scenedesmus quadricauda</td>
<td>1.9-18.4</td>
<td>35.1</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>Scenedesmus sp.</td>
<td>19.6-21.1</td>
<td>40.8-53.9</td>
<td>0.03-0.26</td>
<td>2.43-13.52</td>
</tr>
<tr>
<td>Skeletonema sp.</td>
<td>13.3-31.8</td>
<td>27.3</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>13.5-51.3</td>
<td>17.4</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td>Spirulina platensis</td>
<td>4.0-16.6</td>
<td>-</td>
<td>0.06-4.3</td>
<td>1.5-14.5/24-51</td>
</tr>
<tr>
<td>Spirulina maxima</td>
<td>4.0-9.0</td>
<td>-</td>
<td>0.21-0.25</td>
<td>25</td>
</tr>
<tr>
<td>Thalassosira pseudonana</td>
<td>20.6</td>
<td>17.4</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>8.5-23.0</td>
<td>27.0-36.4</td>
<td>0.12-0.32</td>
<td>19</td>
</tr>
<tr>
<td>Tetraselmis sp.</td>
<td>12.6-14.7</td>
<td>43.4</td>
<td>0.3</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1-5 Genetic modifications of vascular plants and their resulting changes in FA content (adapted from Yu et al. 2011).

<table>
<thead>
<tr>
<th>Modification</th>
<th>Organism</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression of a cytosolic variant of endogenous ACCase</td>
<td><em>Brassica napus</em></td>
<td>5% increase in seed oil content</td>
<td>Töpfer et al. 1995</td>
</tr>
<tr>
<td>Expression of KASIII from <em>Spinacia oleracea</em></td>
<td><em>Brassica napus</em></td>
<td>Increased palmitic acid proportion, decreased total fatty acids 5-10%</td>
<td>Thelen et al. 2002</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae G3p dehydrogenase (gpd1) expression</td>
<td><em>Brassica napus</em></td>
<td>40% increase in seed oil content</td>
<td>Bao and Ohlrogge 1999</td>
</tr>
<tr>
<td>Carthamus tinctorius G3p acyltransferase (GPAT) expression</td>
<td><em>Arabidopsis thaliana</em></td>
<td>10-21% increase in seed oil content</td>
<td>Nikolau et al. 2003</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae su-2 acyltransferase (SLC1-1) expression</td>
<td><em>Brassica napus</em></td>
<td>53-121% increase in erucic acid content</td>
<td>Dehesa et al. 2001</td>
</tr>
<tr>
<td>Arabidopsis thaliana diacylglycerol acyltransferase (DGAT1) expression</td>
<td><em>Brassica napus</em></td>
<td>Increases in oil content and seed weight</td>
<td>Ohlrogge and Jaworski 1997</td>
</tr>
<tr>
<td>Down regulation of FAD2 desaturase and FatB hydrolase</td>
<td><em>Glycine max</em></td>
<td>85% increase in oleic acid levels</td>
<td>Taylor et al. 2002</td>
</tr>
</tbody>
</table>


Table 1-5 (con’t).

<table>
<thead>
<tr>
<th>Modification</th>
<th>Organism</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression of <em>Coriandrum sativum</em> Δ4palmitoyl ACP desaturase</td>
<td><em>Nicotiana tabacum</em></td>
<td>&lt; 10% of total fatty acid became palmitoleic acid</td>
<td>Stoumas et al 1995</td>
</tr>
<tr>
<td>Expression of <em>Thunbergia alata</em> Δ6 ACP desaturase</td>
<td><em>Arabidopsis thaliana</em></td>
<td>&lt; 10% of total fatty acid became palmitoleic acid</td>
<td>Knothe and Dunn 2003</td>
</tr>
<tr>
<td>Expression of <em>Umbellularia californica</em> lauryl-ACP thioesterase</td>
<td><em>Arabidopsis thaliana</em></td>
<td>24% of total fatty acid converted to laurate</td>
<td>Schultz and Ohlrogge 2001</td>
</tr>
<tr>
<td>Expression of <em>Umbellularia californica</em> lauryl-ACP thioesterase</td>
<td><em>Brassica napus</em></td>
<td>58% of total fatty acid converted to laurate</td>
<td>Cahoon et al. 1997</td>
</tr>
<tr>
<td>Expression of <em>Cuphea hookeriana</em> FatB1 thioesterase</td>
<td><em>Brassica napus</em></td>
<td>Fatty acid content changed to 11% caprylate and 27% caprate</td>
<td>Cahoon et al. 1998</td>
</tr>
<tr>
<td>Co-expression of <em>Cuphea hookeriana</em> FatB1 thioesterase and KAS (ketoacyl ACP synthase)</td>
<td><em>Brassica napus</em></td>
<td>30-40% increase in short chain fatty acid content over FatB1 expression only</td>
<td>Cahoon and Shanklin 2000</td>
</tr>
<tr>
<td>Co-expression of <em>Cuphea hookeriana</em> FatB1 thioesterase and LPAAT from <em>Cocos nucifera</em></td>
<td><em>Brassica napus</em></td>
<td>67% of total fatty acid content converted to laurate</td>
<td>Davies 1993</td>
</tr>
</tbody>
</table>
Table 1-6 Proposed applications for *Dunaliella* species (adapted from Tafreshi and Shariati 2009).

<table>
<thead>
<tr>
<th>Species</th>
<th>Proposed Application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Removing of heavy metal, treating wastewater</td>
<td>Tekinbas et al. (1996), Talea and Kumar (1999), Hunts et al. (2001), Taeji et al. 2002</td>
</tr>
<tr>
<td></td>
<td>Food supplement</td>
<td>Kay (1992)</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>Source of carotenoids, vitamins, and antioxidants</td>
<td>Chidambaram Murthy et al. (2005), Milko (1963)</td>
</tr>
<tr>
<td></td>
<td>Source of PUFA (oil, polyunsaturated fatty acids)</td>
<td>Abd El-Baky et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Biocatalytic reactors</td>
<td>Geng et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Single cell protein (SCP), minerals</td>
<td>Spermarthy et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Enzymes</td>
<td>Ben Amoutz and Avron (1980)</td>
</tr>
<tr>
<td><em>D. tertiolecta</em></td>
<td>SCP, minerals</td>
<td>Fobregas and Herrero (1985)</td>
</tr>
<tr>
<td></td>
<td>Ecological indicators</td>
<td>Hall and Golding (1993)</td>
</tr>
<tr>
<td></td>
<td>Source of bioactive compounds</td>
<td>Borzechska (1995)</td>
</tr>
<tr>
<td><em>D. parvalecta</em></td>
<td>Source of antibiotic substances</td>
<td>Chang et al. (1993)</td>
</tr>
<tr>
<td><em>D. viridis</em></td>
<td>Source of oxygenated carotenoids</td>
<td>Moulton and Dunford (1990)</td>
</tr>
</tbody>
</table>
Figure 1-1 Conversion processes for biofuel production from microalgal biomass (adapted from Brennan and Owende 2010).
Figure 1-2 Simplified schematic demonstrating the TAG biosynthetic pathway in microalgae (1) Cytosolic glycerol-3-phosphate acyl transferase, (2) lyso-phosphatidic acid acyl transferase, (3) phosphatidic acid phosphatase, and (4) diacylglycerol acyl transferase. (adapted from Hu et al. 2008).
Figure 1-3 The ACCase reaction consists of two distinct half reactions, BC and CT activity, respectively (A). Biotin is located at the end of a “swinging arm,” which allows for its translocation between the active sites of BC and CT (B) (adapted from Tong 2005).
Figure 1-4 Pathway of fatty acid biosynthesis in bacteria (adapted from Heath and Rock 2002). Fatty acid biosynthesis starts with the carboxylation of acetyl-CoA with malonyl Co-A by acetyl-CoA carboxylase. Once malonyl-ACP is produced, an iterative cycle of chain elongation commences by the actions of FabG, FabA/FabZ, and FabI, each turn resulting in a net addition of two carbons to the growing fatty acid.
Figure 1-5 The active cleft of *E. coli* TAP (*tesAI/apeAI/pldC*) solved by crystallography (adapted from Lee et al. 2006).
CHAPTER 2

Recombinant expression and biochemical characterization of an acetyl-CoA carboxylase from the halophilic bacterium Chromohalobacter salexigens BAA-138

Rushyannah Killens-Cade\textsuperscript{1}, Caroline Smith\textsuperscript{2}, Amy Grunden\textsuperscript{1}

\textsuperscript{1}Department of Plant and Microbial Biology, North Carolina State University, Raleigh, USA 27695
\textsuperscript{2}Department of Genomic Science, North Carolina State University, Raleigh, USA 27695
ABSTRACT

Fatty acid synthesis (FAS) is a highly conserved and tightly regulated process in all three domains of life: Archaea, Bacteria, and Eukarya. The first committed step in FAS is the carboxylation of acetyl-coenzyme A (acetyl-CoA) to produce malonyl-CoA by a biotin-dependent enzyme, acetyl-CoA carboxylase (ACCase). This step consists of two distinct half reactions and is the rate-limiting and primary regulated step for FAS. Its rate-limiting role in the formation of fatty acids makes this enzyme an attractive target for increasing fatty acids that can act as feedstock for biofuel production such as microalgal triacylglycerides. Davis et al. (2000) have shown that increasing ACCase expression, in bacterial systems, results in a concomitant increase in the intracellular level of malonyl-CoA. Although this increase in malonyl-CoA production in ACCase overexpressing bacterial strains has been shown, this increase in malonyl-CoA production has yet to be demonstrated for microalgae with increased native ACCase expression. The unaltered lipid production observed when native algal ACCase expression is increased is most likely the result of feedback inhibition by acyl-ACP and transcriptional FAS regulation. The expression of bacterial ACCase genes may aid in overcoming transcriptional repression and alleviate feedback inhibition in microalgae. Since bacterial ACCase enzymes are sufficiently homologous to microalgal ACCase, we propose that bacterial ACCase activity will reinforce the microalgal ACCase activity, thus increasing the pool of malonyl-CoA for flux through the FAS pathway. In this study, ACCase from the moderately halophilic Chromohalobacter salexigens was recombinantly
expressed in *Escherichia coli*, partially purified, and evaluated for activity in response to pH and salinity to demonstrate its compatibility for use in marine microalgae *Dunaliella*. 
2.1 INTRODUCTION

Acetyl-CoA carboxylase (ACCase) is a biotin-dependent enzyme that catalyzes the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA to malonyl-CoA during de novo fatty acid biosynthesis. In prokaryotes, the enzyme employs three distinct and separate functional subunits: the biotin carboxyl carrier protein (BCCP), a biotin carboxylase (BC) domain, and carboxyltransferase (CT) complex subunits α and β (αCT and βCT) (Jitrapakdee and Wallace 2003). The ACCase reaction consists of two distant half reactions. In the first reaction, the BC domain catalyzes the ATP-dependent fixation of CO₂ to the biotinylated lysine residue of the BCCP subunit, forming carboxybiotin (Polakis et al. 1974). During the second half-reaction, CT binds the carboxyl group, from the carboxybiotin, and transfers it to acetyl-CoA to form malonyl-CoA (Polakis et al. 1974). The net reaction is the formation of malonyl-CoA from acetyl-CoA with ATP and bicarbonate as cofactors.

Fatty acids are ubiquitously found in living organisms as cell membrane components in the form of ester- or ether-linked lipids. The fatty acid synthesis (FAS) pathway involves the repeated cycle of condensation, reduction, dehydration reactions, and the subsequent reduction of carbon-carbon bonds. This pathway is highly conserved within the kingdoms of life. In its rate-limiting role in the FAS pathway, the overexpression of ACCase has been shown to increase the metabolic flux toward fatty acid production (Davis et al. 2000). Thus, there is an increased effort to modify the metabolic pathway in microbes, by targeting the ACCase, to improve fatty acid synthesis.
Algae-based lipid-derived hydrocarbons such as olefins, methyl esters, and alkanes hold promise as feedstock for biofuel production because they have the following advantages when compared to other plant oils: renewability, short production cycle, less labor required, less affection by venue, and easier to scale-up (Li et al. 2008). Although studies have demonstrated that enhanced production of fatty acids can result from the overexpression of ACCase in *Escherichia coli*, little change in lipid production has been seen in lipid-producing microalgae with increased levels of this multi-subunit enzyme activity (Davis et al. 2000; Dunahay et al. 1995; Dunahay et al. 1996; Lu et al. 2008; Roesler K et al. 1997; Klaus et al. 2004; Sheehan et al. 1998). This unaltered lipid production is likely due to the overexpression of native versions of the ACCases which are subjected to feedback inhibition by acyl-ACP as well as microalgal FAS regulation (Davis and Cronan 2001). One way to reduce this inhibition and/or regulation could be to select and express a non-native ACCase protein that is not recognized by the acyl-ACP of the targeted organism. In this work, our goal was to recombinantly express and characterize the acetyl-CoA carboxylase (ACCase) from the moderately halophilic bacterium *Chromohalobacter salexigens* BAA-138 which was isolated from a saltern in the Netherlands (Arahal et al. 2001; Coperland et al. 2011). Our research findings provide information that can be used for the metabolic engineering of halophilic algae such as *Dunaliella* for enhanced biofuel production.
2.2 MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Reagents

The *Chromohalobacter salexigens* BAA-138 strain (ATCC) was cultured in nutrient broth with 10 % NaCl and used for the isolation of genomic DNA. The *Escherichia coli* XL1-Blue strain (Novagen, EMD Biosciences) was used for cloning and maintaining the recombinant plasmid. *E. coli* BL-21(λDE3)LysS (Novagen, EMD Biosciences) cells were used for recombinant ACCase expression. The *E. coli* LA1-6 strain, which contains a temperature sensitive lesion in the β subunit of the carboxyltransferase, was purchased from the *E. coli* Genetic Stock Center. The expression plasmid pET-21b was obtained from Novagen, EMD Biosciences. For the over-expression of the biotin ligase, pBirAcm was kindly provided by Dr. Paul Hamilton. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. iProof™, a high-fidelity DNA polymerase, PCR kit was obtained from Bio-Rad. For overlap extension PCR, a LongRange PCR kit was purchased from Qiagen. Synthetic oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). The PCR products were purified using a QIAquick PCR purification kit (Qiagen). The plasmids were purified using a QIAprep spin miniprep kit (Qiagen). The radioactively labeled metabolite, NaH$^{14}$CO$_3$ (56 mCi/mmol), was purchased from MP Biomedicals. Acetyl-CoA was obtained from Sigma-Aldrich. Q Sepharose™ (GE Healthcare Life Sciences) was utilized for the partial purification of ACCase.
Cloning of an Acetyl-CoA Carboxylase from *Chromohalobacter salexigens*

The *Csal_2284* (*accB*), *Csal_2285* (*accC*), *Csal_1263* (*accD*), and *Csal_0579* (*accA*) genes were amplified from genomic DNA by the polymerase chain reaction (PCR). For the amplification of PCR products, iProof™ high fidelity DNA polymerase was used in a 50 µl PCR reaction solution containing 10 µl 5x iProof™ HF buffer, 1 µl dNTPs (10 mM), 1 µl forward primer (40 µM), 1 µl reverse primer (40 µM), and 1 µl *C. salexigens* DNA (60 ng/µl). The PCR reactions were carried out using a thermal cycler (Bio-Rad) under the following conditions: initial denaturing at 98 °C for 30 sec; 30 cycles at 98 °C for 10 sec (denaturing), annealing at various temperature (see Table 2-1) for 45 sec, and 72 °C for 30 sec (extension); a final extension cycle of 72 °C for 7 min, and preservation at 4°C. The four PCR products were purified and visualized using a 1 % (w/v) agarose gel stained with ethidium bromide.

A second round of PCR was done to prepare the ends of the purified amplicons for overlap extension PCR. The PCR reaction mixture and conditions were carried out as described above with the exception of the annealing temperatures (see Table 2-1). The four PCR amplicons (*Cs_accB*, *Cs_accC*, *Cs_accD*, and *Cs_accA*) were purified (separately) and visualized using a 1 % (w/v) agarose gel stained with ethidium bromide.

The purified products were pooled for a two-step assembly process. These two rounds of PCR resulted in the formation and amplification of *Cs_accBC*, *Cs_accCD*, and *Cs_accDA* gene fragments. The first step annealed the DNA fragments, without the presence of primers. The PCR parameters for this step involved an initial denaturing step at 98 °C for 30 sec; 10
cycles at 98 °C for 10 sec (denaturing), annealing at 67.5 °C for 30 sec, and extension at 72 °C for 45 sec; and a final extension cycle of 72 °C for 7 min. The initial assembly PCR mixture was the same as described above with the exception that no primers were included in the reaction mix and 0.6981 pmol of purified product was used. The second step permitted the amplification of the fused gene fragments. For this step, 2µl of the first reaction and primers were added to 46 µl of fresh PCR reaction mixture (as described above). The PCR amplification parameters for the second step were as follows: pre-denaturation at 98 °C for 30 sec, followed by 40 cycles of denaturation at 98 °C for 10 sec, annealing at various temperatures (see Table 2-1) for 30 sec, and polymerization at 72 °C for 1 min, and a final extension cycle of 72 °C for 7 min. The three PCR products were purified (separately) and visualized using a 1 % (w/v) agarose gel stained with ethidium bromide.

To generate a full-length DNA sequence (Cs_ACCase), the assembled gene fragments were used as template in an overlap extension PCR method. Overlap extension PCR was performed in a 50 ul reaction containing 2U LongRange PCR enzyme mix, 1X LongRange PCR buffer with Mg2+, 500 µM dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, and 125ng DNA (Cs_accBC, Cs_accCD, and Cs_accDA). Touchdown PCR was conducted under the following conditions: a 3 min initial denaturation at 93 °C; 10 cycles of 93 °C for 15 sec, 60 °C for 30 sec, and 68 °C for 5 min; 20 cycles of 93 °C for 15 sec, 50 °C for 30 sec, and 68 °C for 5 min; and a final extension at 68 °C for 7 min. The * denotes that the temperature decreased by 1 °C at each cycle. The full-length PCR amplicon (Cs_accBCDA) was purified and visualized using a 1 % (w/v) agarose gel stained with ethidium bromide. The purified DNA product was subjected to a double digestion, over-
night, with *Nde*I and *BamHI* to render sticky ends needed for cloning. For construction of pCs_accBDCA, the plasmid (pET-21b) was digested with *Nde*I and *BamHI*, gel purified, and ligated to the compatible sticky end DNA molecule of *accBCDA*. CaCl$_2$-competent cells of *E. coli* XL1-Blue were transformed with the ligated reaction and plated on solid LB agar media supplemented with ampicillin (100µg/ml) for selection of pCs_AccBDCA. A positive clone was confirmed by restriction mapping (*Nde*I, *EcoRI*, *NotI EcoRV*, and *BamHI*) and then sequenced by Eurofins MWG Operon (Huntsville, AL) to ensure that no mutations were generated during the amplification process. The sequence information was analyzed by using MacVector (Accelrys) computer software.

**Cs_accD Complementation Test**

*E. coli* LA1-6 [*accD6(Ts)*] cells were transformed with either an empty vector (pET-21b) or plasmid pCs_accBCDA (Harder et al. 1972). After growing overnight at 30 °C on LB agar plates, the transformants were streaked onto minimal growth (M63) agar plates and incubated at either 30 °C (permissive temperature), 37 °C (permissive temperature), 39 °C (non-permissive temperature), or 42 °C (non-permissive temperature). The transformants were grown overnight on M63 as described by Harder et al (1972), supplemented with isopropyl β-D-1-thiogalactopyranoside (0.2 mM) and ampicillin (100 µg/ml).
Over-expression of Recombinant ACCase

*C. saleigens* ACCase was expressed by transforming *E. coli* BL21(λDE3)pLysS cells with pCs_AccBCDA and pBirAcm. For the control expression sample, an empty pET-21b and pBirAcm were also used to transform *E. coli* BL21(DE3)pLysS. A freshly transformed colony was then used to inoculate 1 L of Luria Bertani (LB) media in a 2 L flask. Each flask contained 10 μg/ml chloramphenicol and 100 μg/ml ampicillin. The cultures were incubated at 37 °C with shaking until reaching mid-log phase at which point protein expression was induced by the addition of 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Biotin (50 uM) was also added at the time of induction for the biotinylation of AccB. After a 4 h induction with shaking at 37 °C, the cells were harvested by centrifugation (9,500 rpm, 50 min, 4 °C) and stored at -20 °C prior to preparing cell lysates. The expression of targeted recombinant proteins was evaluated by SDS-PAGE using a 12.5 % polyacrylamide gel with the Bio-Rad Mini-PROTEAN® apparatus and visualized using Coomassie brilliant blue R250. The molecular mass of the recombinant Cs_ACCase subunits was determined to be approximately 18.6 kDa (AccB), 57.1 kDa (AccC), 33.3 kDa (AccD), and 35.2 kDa (AccA) by SDS-PAGE. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).
Partial Purification of Recombinant Acetyl-CoA Carboxylase

The *E. coli* BL21(λDE3)pLysS cell pellets containing the targeted proteins (Cs_ACCase)/BirA-overexpressing and empty pET-21b plasmid/BirA-overexpressing were resuspended in 20mM Tris-HCl, pH 8.0 (binding buffer) containing 1mM benzamidine-HCl. The cell suspensions were lysed by three passages of the lysate through a chilled French pressure cell (20,000 lb/in²). Cellular debris was removed by centrifugation (15,000 rpm, 45 min, 4 °C). The supernatants were collected and filtered with a 0.45μM filter from Millipore. The filtered supernatants were loaded onto a 5 ml HiTrap™ DEAE anion exchange column equilibrated with 20 mM Tris-HCl buffer (pH 8.0) and eluted with a linear 0 -to- 1 M NaCl gradient. The control sample cell extract was applied to the DEAE anion exchange column and fractionated prior to column fractionation of the recombinant ACCase containing cell extract. All fractions were visualized by SDS-PAGE using a 12.5 % polyacrylamide gel. The fractions containing *C. saleigens* acetyl-CoA carboxylase subunits and corresponding control sample fractions were pooled (separately) and dialyzed against the storage buffer, 50 mM Tris-HCl buffer (pH 8.0), for 16 h at 4 °C. Both samples were concentrated with Amicon® Ultra centrifugal filter device (molecular mass cutoff of 30000) (Millipore) and stored at 4 °C until use.
The activity of Cs_ACCase was determined by measuring the acetyl-CoA-dependent incorporation of $^{14}\text{C}\text{O}_2$ from $[^{14}\text{C}]$ bicarbonate into the acid-stable product malonyl-CoA as previously described (Davis et al. 2000; Focke et al. 2003). The reaction mixture contained 100mM Tris-HCl buffer (pH 7.5), 1mM ATP, 100mM KCl, 2.5mM MgCl$_2$, 39mM NaH$^{14}\text{CO}_3$ (1.17 μCi), 1mM dithiotheritol, and 0.3mM acetyl-CoA in a total reaction volume of 100 μl. The reaction was initiated by the addition of NaH$^{14}\text{CO}_3$, permitted to proceed at 30 °C for 15 min, and stopped with 100 μl of 6N HCl. The contents of the tubes were then evaporated to dryness at 95 °C. The residue was suspended in 100 μl of 6N HCl and transferred to a scintillation vial containing 1 ml of Economical Biodegradable Counting Cocktail (Research Products International Corporation). The acid-stable radioactivity was counted using a Beckman multi-purpose scintillation counter. Negative controls without acetyl-CoA were included to determine the nonspecific carboxylation and radiochemical background. Nonspecific CO$_2$ fixation by crude extract was assayed in the absence of acetyl-CoA. $^{14}\text{C}$ radioactivity was determined for 5 min. Cs_ACCase samples were assayed in 3 technical replicates. One unit of enzyme activity catalyzed the incorporation of 1 μmol $^{14}\text{C}$ into acid-stable products/min. This assay was used to determine the optimum pH (5 -to- 11) and salt concentration (0 -to- 900 mM KCl) for Cs_ACCase activity.
2.3 RESULTS AND DISCUSSION

Amino Acid Sequence Comparison

*C. saleigens* acetyl-CoA carboxylase, comprised of four subunits, was assembled by overlap extension PCR. For the expression study, each protein retained its ATG initiation codon and TAG stop codon. Due to their distance from the ribosomal binding site found on the expression plasmid (pET-21b), a RBS was engineered in the nucleotide sequence to permit the expression of *Cs_accC*, *Cs_accD*, and *Cs_AaccA*.

The *Cs_ACCase* protein has a calculated molecular weight of 306.3 kDa and an isoelectric point (pI) of 4.72. The BLASTP tool was used to find identities of *Cs_ACCase* among the non-redundant protein sequence data deposited at the National Center for Biotechnology Information database. The deduced amino acid sequence for the *C. saleigens* ACCase genes showed 82 %, 77 %, and 77 % identity with those from a *Halomonas elongate* DSM 2581, *Pseudomonas aeruginosa* PAO1, and *Azotobacter vinelandii* DJ, respectively. Some conserved interface sites involving the interaction between *Cs_AccB* and *Cs_AccC* were identified. Sequence alignment revealed the presence of the highly conserved Cys\(^{50}\) residue from AccC which has two interactions with AccB, (1) forming a hydrogen bond with the peptidic NH of Phe\(^{146}\) and (2) the peptidic NH of Cys\(^{50}\) forming a hydrogen bond with the carboxylate group of Glu\(^{145}\) (Broussard et al. 2013). Sequence alignment revealed that AccC contains the residue Glu\(^{297}\) that extracts the proton from bicarbonate and Arg\(^{338}\) which stabilizes the biotin enolate oxyanion. Sequence alignment showed that the amino acid
residues Ala\textsuperscript{118}-Met\textsuperscript{119}-Lys\textsuperscript{120}-Met\textsuperscript{121} (a highly conserved biotinylation motif) are present in the C. \textit{salexigens} AccB sequence (Chou et al. 2009; Cronan and Waldrop 2002).

Expression and Enrichment of Recombinant Acetyl-CoA Carboxylase from C. \textit{salexigens}

The recombinant Cs\_ACCase subunits were heterogeneously expressed in \textit{E. coli} BL-21 (\lambda DE3)pLysS cells under the control of the T7 promoter to allow for a high level of expression. The expression of the targeted proteins was induced by IPTG while culturing in LB media. After induction, cells from a two liter culture were incubated for 4 h at 37 °C, harvested, and lysed by passage through a French pressure cell. Anion exchange chromatography was used to partially purify \textit{C. salexigens} ACCase (Kroeger et al. 2011). The Cs\_ACCase fractions were pooled according to the elution profile and SDS-PAGE gel (Figure 2-1). The control sample (containing an empty pET-21b vector) was processed using the same purification steps as used for the Cs\_ACCase and pooled according to the fractions collected for Cs\_ACCase.

It has been shown that the AccB subunit requires biotinylation for the holoenzyme to be catalytically active (Soriano et al. 2006). Prior to the partial characterization of Cs\_ACCase, mass spectrometry was used to analyze the biotinylation of Cs\_AccB (fused with a 6X-histidine tag). Intact protein mass spectrometry analyses indicated that approximately 70% biotinylation was achieved after co-expression of Cs\_AccB with BirA (data not shown). Mass spectrometry analyses also determined that biotinylated Cs\_AccB is
18.79 kDa (data not shown). In contrast, Cs_AccB samples that were not subjected to the biotinylation protocol had only approximately 0 – 5% biotinylation. This low level of biotinylation in Cs_AccB samples was presumably due to endogenous E. coli BirA activity. Thus, during the expression of Cs_AccBCDA, the biotin ligase was co-expressed.

Functional Analysis of Cs_accD Using a Growth Complementation Method

The ability of C. salexigens accD to complement E. coli LA1-6 [accD6(Ts)] (Li and Cronan 1993) was investigated. Generally, the mutant is viable at permissive temperatures (≤ 39 °C), but not at higher temperatures. E. coli LA1-6 was transformed with the pCs-accBCDA plasmid and an empty pET-21b plasmid (control). The transformants were allowed to incubate, overnight, at 30 °C, 37 °C, 40 °C, and 42 °C. Only the transformants of strain LA1-6 carrying pCs_accBCDA could grow at 40 °C and 42 °C (Figure 2-2). These results indicated that the C. salexigens accD gene was able to complement the β subunit of carboxyltransferase in E. coli LA1-6. This finding suggested that there should be full translation of all four subunits because accD is the third gene on the synthetic operon (pCs_accBCDA).

Catalytic Properties of C. salexigens ACCase in Response to pH and Salinity

The catalytic properties of the partially purified Cs_ACCase were studied at 30 °C because Dunaliella can grow within a broad optimal temperature range of 25 and 35 °C (30
"C being in the middle) (Ben-Amotz 1995). The activity of the control sample (fraction that contained empty vector) was comparable to the negative control.

Although a broad pH range for activity was observed for Cs_ACCCase, the optimum pH of enzyme activity was 11 (Figure 2-3). Halophilic environments can vary in pH, with subsets of these environments being highly alkaline (Reed et al. 2013). C. salexigens can grow from pH 5 to 10 on liquid media containing 10 % total salt, with optimal growth at pH 7.5 (Arahal et al. 2001). Dunaliella species have a wider range of pH tolerance, from pH 0 to 11. The optimum pH for D. salina is between 9 and 11 (Tafreshi AH and Shariati M 2009). The ability of the microalgae to thrive in a highly alkaline environment is due to the photosynthetic fixation of CO$_2$ with NO$_3^-$ uptake which contributes to further release of OH$^-$ (Ben-Amotz and Avron 1989). It is important to note that culturing the microalgae at such high pH can result in precipitation by several calcium salts and flocculation of the algal biomass; thus, most research studies avoid culturing the cells at a pH above 8 (Ben-Amotz and Avron 1989). Although halophiles can thrive in alkaline environments, they have cellular mechanisms to maintain a more neutral pH in their cytoplasm, usually with a range from 7 to 8.5 (Horikoshi 1999).

As a moderately halophilic bacterium, C. salexigens can grow in media containing NaCl concentrations between 0.5 M and 4M, with an optimum at 2 – 2.5 M (Ventosa et al. 1998; Arahal 2001). The salt concentration requirement for growth of Dunaliella is comparable to C. salexigens. Thus, the enzymatic activity response of Cs_ACCCase was measured in various salt concentrations. Cs_ACCCase exhibited the maximum activity when there was no KCl present in the reaction; although, activity was observed over a broad range
of salinity (0 mM -to- 900 mM). Organisms that thrive in extremely salty environments must maintain a balance between the solute concentration inside their cells and their environment to prevent dehydration of their cytoplasm. C. salexigens, like most halophiles, accumulates compatible solutes and has efficient sodium antiporters as a means to maintain normal intracellular salt concentration, therefore it is not necessarily expected that intracellular enzymes demonstrate high activity at elevated salt concentrations (Oren 2002).

2.4 CONCLUSION

In summary, we have expressed, partially purified, and characterized C. salexigens acetyl-CoA carboxylase (Cs_ACCase) activity in response to pH and salinity. Based on the broad pH and salt concentration range over which the recombinant Cs_ACCase was shown to be active, it will likely be able to functional if expressed in marine microalgae such as Dunaliella, and its expression in Dunaliella could increase malonyl-CoA production in the algae, and therefore, improve fatty acid synthesis and algal oil production.

2.5 ACKNOWLEDGEMENTS

The authors thank Dr. Michael Sikes for the use of his lab and help with the radioactive assay. This work was supported by the National Science Foundation EFRI program under Grants EFRI 093772 and 1332341.
2.6 REFERENCES


Table 2-1 Primers and expected products generated in this study.

<table>
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<th>Primer Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size (bp)</th>
<th>Annealing Temperature (°C)</th>
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<tr>
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The bolded nucleotides of the primer sequences correspond to **NdeI** (accB), **NdeI** (Cs_accB), **NdeI** and **BamHI** (accC), **BamHI** (Cs_accC), **BamHI** and **SacI** (accD), and **SacI** and **HindIII** (accA) restriction sites.

The italicized nucleotides of the primer sequence denote the ribosomal binding site (RBS) engineered into the sequence.
Figure 2-1 SDS-PAGE of partially purified *C. salexigens* ACCase. L: Molecular Weight Marker (in kDa), Lane 1: Whole cell extract (*C*_ACCase), Lane 2: Cell-free extract (*C*_ACCase), Lane 3: pooled DEAE fractions containing *C*_ACCase (3 μg), Lane 4: Control sample (empty vector) (5 μg).
Figure 2-2 The temperature-sensitive *Escherichia coli* mutant strain LA1-6, transformed with either an empty vector or the pCs_accBCDA construct, was grown on minimal growth media as described by Harder et al. (1972). All plates are shown with cells containing empty vector grown on the left side of the plate and cells with the pCs_accBCDA construct are grown to the right. The complementation study shows that when the temperature shifts from permissive to non-permissive temperatures, only the cells containing the Cs_accBCDA construct are able to grow.
Figure 2-3 Effect of pH on the activity of enriched Cs_ACCase was determined over a pH range from 4 to 11, at 30 °C. Assays contained 100mM Tris buffer, 1mM ATP, 2.5mM MgCl₂, 100mM KCl, 39mM NaH¹⁴CO₂ (1.17 μCi), 1mM dithiothreitol, and 0.3mM acetyl-CoA in a total reaction volume of 100 μl. Error bars represent standard error.
Figure 2-4 Salt concentration (KCl) profile of partially purified Cs ACCase. Enzyme activity for *C. salexigens* ACCase was determined over a salt concentration range from 0 mM to 900 mM, using acetyl-CoA as substrate. The reaction mixture consisted of 100 mM Tris-HCl buffer, 1 mM ATP, 2.5 mM MgCl₂, 39 mM NaH¹⁴CO₃ (1.17 μCi), 1 mM dithiothreitol, and 0.3 mM acetyl-CoA in a total reaction volume of 100 μl. Error bars represent standard error.
CHAPTER 3

Characterization of a thermostable, recombinant carboxylesterase from the hyperthermophilic archaeon Metallosphaera sedula DSM5348

Rushyannah Killens-Cade¹, Rachel Turner², Christine MacInnes², Amy Grunden¹

¹Department of Plant and Microbial Biology, North Carolina State University, Raleigh, USA 27695
²Department of Biology, North Carolina State University, Raleigh, USA 27695

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ABSTRACT

Lipid-producing microalgae are emerging as the leading platform for producing alternative biofuels in response to diminishing petroleum reserves. Optimization of fatty acid production is required for efficient conversion of microalgal fatty acids into usable transportation fuels. Microbial lipases/esterases can be used to enhance fatty acid production because of their efficacy in catalyzing hydrolysis of esters into alcohols and fatty acids while minimizing the potential poisoning of catalysts needed in the biofuel production process. Although studies have extensively focused on lipases/esterases produced by mesophilic organisms, an understanding of lipases/esterases produced by thermophilic, acidic tolerant microbes, such as Metallosphaera sedula, is limited. In this work, the esterase from Metallosphaera sedula DSM5348 encoded by Msed_1072 was recombinantly expressed in Escherichia coli strain BL21 (λDE3). The purified enzyme either with a hexahistidine (His₆)-tag (Msed_1072Nt and Msed_1072Ct) or without the hexahistidine (His₆)-tag (Msed_1072) was biochemically characterized using a variety of substrates over a range of temperatures and pH and in the presence of metal ions, organic solvents, and detergents. In this study, the fusion of the protein with a hexahistidine (His₆)-tag did not result in a change in substrate specificity, but the findings provide information on which enzyme variant can hydrolyze fatty acid esters in the presence of various chemicals, and this has important implication for their use in industrial processes. It also demonstrates that Metallosphaera sedula Msed_1072 can have application in microalgae-based biofuel production systems.
3.1 INTRODUCTION

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are enzymes that catalyze the synthesis of ester compounds and hydrolyze triglycerides (Jaeger and Eggert 2002). As hydrolases, these lipolytic enzymes act on the ester bonds of the triglycerides to liberate fatty acids and glycerols. Although both enzyme types share structural characteristics, the fundamental difference between esterases and (true) lipases is that the former acts on shorter chain triglycerides (shorter than 10 carbon atoms) which are water-soluble, whereas the latter hydrolyzes longer chain triglycerides (Gilham and Lehner 2005; Jaeger et al. 1999). Based on their regio- and stereo-specific properties, cofactor-independent activity, broad substrate specificity, and stability in the presence of organic solvents, ester-hydrolyzing enzymes are attractive biocatalysts (Jaeger et al. 1999). As such, they are used widely in a number of industrial applications, including medical biotechnology, resin removal from pulp, detergent production, organic synthesis, flavor and aroma synthesis, food related processes, and biofuel production (Panda and Gowrishankar 2005; Salameh and Wiegel 2007).

The increasing consumption of energy, population growth, and environmental concerns have resulted in an urgent need to explore more sustainable energy sources. Lipid-producing microalgae are emerging as a leading platform for producing alternative sustainable energy, mainly because of desirable characteristics such as rapid growth rate, production of valuable co-products, and cultivation in various marine and freshwater environments that do not compete with arable crop lands (Menetrez 2012; Pienkos and Darzins 2009; Guschina and Harwood 2006). Their adaptability to a range of growth environments may result in the ability to produce lipids of diverse compositions (Guschina
and Harwood 2006). These include membrane lipids as well as lipids involved in carbon and energy storage such as triacylglycerides (TAG), which are neutral lipids that consist of three fatty acids esterified to glycerol. In addition to having a diversified lipid composition that may aid survival in harsh environments some algae may also alter lipid biosynthetic pathways leading to the accumulation of TAG during unfavorable environmental or stress conditions (Hu et al. 2008).

Recent genetic and metabolic engineering research has focused on increasing lipid yields in microalgae by targeting the carbon flux through the fatty acid synthesis pathway. However, for microalgae to be a cost effective and reliable alternative energy source, the process for the direct conversion of lipidic biomass feedstock to combustible fuel must become more efficient. Specifically, there is a need to save energy during the conversion process and avoid extensive degumming. A synthetic biology approach which targets increasing the yield and quality of algal feedstock is one way of overcoming these difficulties. By transforming algae to express thermostable lipases/esterases, fatty acid release from TAGs that are stored in lipid bodies can be achieved during the high temperature conversion of lipids to fuel. Having enzymes that are active during this process can reduce the time required for lipid to fuel conversion, thereby saving energy and improving cost effectiveness.

For our microalgae based biofuel system, we selected a homolog of an Escherichia coli acyl-ACP thioesterase, TesA. TesA, expressed in an E. coli expression system, has been shown to hydrolyze long chain fatty acyl-ACPs (products of the fatty acid synthesis pathway), resulting in relief of feedback inhibition and increased production of total free fatty
acids (FFAs) (Liu et al 2010; Voelker and Davies 1994). It also has been shown that TesA has a wide substrate specificity and is able to hydrolyze long-chain fatty acyl-ACPs to produce FFAs containing 12 to 18 carbons (Barnes and Wakil 1968). This hydrolysis profile works to the advantage of biofuel systems because aromatic hydrocarbons with 12 to 14 carbon atoms are ideal for production of petroleum-derived jet fuels.

To evaluate its application for algae transformation and subsequent biofuel production, we have recombinantly expressed and characterized variants of a putative alpha/beta hydrolase (Msed_1072) from the hyperthermoacidophilic archaeon Metallosphaera sedula DSM5348 (optimal growth at 75 °C and a pH range of 1.0 and 4.5) which was isolated from an acidic drain of a hot water pond at Pisciarelli Solfatara, near Naples, Italy (Huber et al. 1989). M. sedula Msed_1072, originally targeted for analysis based on its similarity to E. coli TesA (19% similarity; conserved catalytic triad amino acids Ser-Asp-His) was fused with a hexahistidine (His$_6$)-tag and produced recombinantly in E. coli. It has been shown that fusing a thioesterase with a C-terminal hexahistidine (His$_6$)-tag can result in a shift in substrate specificity (Lee et al. 1999); therefore, N-terminal histidine-tagged, C-terminal histidine-tagged, and untagged variants of Msed_1072 were evaluated in order to satisfy the overall goal of this study which was to identify a thermophilic carboxylesterase with an affinity towards long carbon chain substrates.
3.2 MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Reagents

The genomic DNA of *Metallosphaera sedula* DSM5348 was kindly provided by Dr. Robert Kelly of NCSU. The *Escherichia coli* XL1-Blue strain (Novagen, EMD Biosciences) was used for cloning and maintaining the recombinant plasmid. BL21(DE3)LysS (Novagen, EMD Biosciences) cells were used for over-expression of the proteins. The expression plasmids pET21b and pET28a were obtained from Novagen, EMD Biosciences. Restriction endonucleases, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs. Synthetic oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). The PCR products were purified using a QIAquick PCR purification kit (Qiagen). The plasmids were purified using a QIAprep spin miniprep kit (Qiagen). All assay substrates were purchased from Sigma-Aldrich. The following chromatography columns were utilized for the enzyme purification step: 5 ml HisTrap Nickel Sepharose™, HiTrap Q Sepharose™, and HiTrap SP Sepharose™ from GE Healthcare Life Sciences.

Cloning of a Thermoactive Carboxylesterase from *Metallosphaera sedula*

The *Msed_1072Nt* and *Msed_1072* genes were amplified using the forward primer: 5’ TTAATACGACCATATGCCCCCTACATCCAGAGGTAGAAATTAC-3’, with an NdeI site (bold), and the reverse primer: 5’-GATACTTCCGGATCCGTGGATAGGTTCATCTCGG-
3’, with a *BamHI* site (bold). The *Msed_1072Ct* gene was amplified using the forward primer:

5’

ATACGACCATATGGCCCCTACATCCAGAGGTAAGAAATTACTTTCCCAGCAGAG
GTAAGAAATTAC- 3’, with an *NdeI* site (bold), and the reverse primer: 5’-

TACGGGATCCAGGACAGATCTCAGAACCCCA-GCAATGTG 3’, with a *BamHI* site (bold) and Phusion® high fidelity DNA polymerase in a 50 µl PCR reaction solution containing 10 µl 5x Phusion® HF buffer, 1 µl dNTPs (10 mM), 0.5 µl forward primer (40 uM), 0.63 µl reverse primer (40 uM), 0.5 µl Phusion® DNA polymerase, and 2 µl *M. sedula* genomic DNA (50 ng/ µl). The PCR reactions were carried out using a thermal cycler (Bio-Rad) under the following conditions: an initial denaturing step at 98 °C for 30 sec; 30 cycles at 98 °C for 10 sec (denaturing), annealing at 64.4 °C [Msed_1072 and Msed_1072 Nt]/67.9 °C [Msed_1072Ct] for 30 sec, and extension at 72 °C for 30 sec; a final extension cycle of 72 °C for 7 min, and preservation at 4°C. The amplicons were purified and visualized using a 1 % (w/v) agarose gel. The purified DNA products were subjected to a double digestion, overnight, with *NdeI* and *BamHI* to render sticky ends needed for cloning. For construction of pET21b-Msed_1072, pET28a-Msed_1072Nt, and pET21b-Msed_1072Ct the plasmids were digested with *NdeI* and *BamHI*, gel purified, and ligated to the compatible sticky end DNA molecule of Msed_1072, Msed_1072Nt and Msed_1072Ct, respectively. CaCl₂-competent cells of *E. coli* XL1-Blue were transformed with the ligated reaction and plated on solid LB agar media supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) for selection of pET21b-Msed_1072, pET21b-Msed_1072Ct and pET28a-Msed_1072Nt, respectively. A positive clone of each construct was sequenced by Eurofins MWG Operon (Huntsville, AL)
to ensure that no mutations were generated during the amplification process. The sequence information was analyzed by using MacVector (Accelrys) computer software.

Over-expression of Recombinant Thermoactive Carboxylesterase Msed_1072

For the over-expression of *M. sedula* carboxylesterase, each construct and the rare arginine, leucine, and isoleucine tRNA encoding plasmid pRIL were used to transform *E. coli* BL21(DE3)pLysS cells. For the control expression samples, the plasmids without the *Msed_1072* gene were also used to transform *E. coli* BL21(DE3)pLysS. The transformants were plated and selected on LB agar plates containing the appropriate antibiotics after incubation overnight at 37 °C. The *E. coli* BL21(DE3)pLysS cells harboring the recombinant plasmids were used to inoculate a 2 L culture of auto-induction media supplemented with the appropriate antibiotics for large scale expression of the protein (Studier 2005). The cells were incubated with shaking at 200 rpm for 16 h at 37 °C. Cells were harvested by centrifugation (9500 rpm, 50 min, 4 °C) and stored at -20 °C prior to preparing cell lysates. The expression of the recombinant protein was evaluated by SDS-PAGE using a 12.5 % polyacrylamide gel with the Bio-Rad Mini-PROTEAN® apparatus and visualized using Coomassie brilliant blue R250. The molecular mass of the recombinant carboxylesterase was determined to be approximately 33.2 kDa by SDS-PAGE. The molecular weight of the poly-histidine tagged proteins was determined to be approximately 36.10 kDa by SDS-PAGE. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).
Recombinant Thermoactive Carboxylesterase Purification

The *E. coli* BL21(DE3)pLysS cell pellets containing the Msed_1072, Msed_1072Nt, and Msed_1072Ct, proteins were resuspended and lysed for 10 min with 10 mL B-PER® bacterial protein extraction reagent (Pierce, Rockford, IL) containing 1 mM benzamidine-HCl. The use of the nonionic detergent in the initial lysing step was critical for the solubility of the protein. The cells were further lysed by three passages of the lysate through a French pressure cell (20,000 lb/in$^2$). Cellular debris was removed by centrifugation (15,000 rpm, 45 min, 4 °C). The supernatant was collected and heated at 70 °C for 15 min. The heat-treated supernatant was centrifuged (15,000 rpm, 15 min, 4°C) to remove denatured proteins.

The supernatant containing the solubilized his-tagged recombinant proteins (Msed_1072Nt, Msed_1072Ct) was added to a 5 ml HisTrap Ni Sepharose™ column for purification. Prior to the addition of the protein, the Ni$^{2+}$-charged column was equilibrated with 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, and 10 mM imidazole (buffer A). After binding the protein to the metal charged column, the column was washed with the equilibration buffer to remove all unbound protein. The thermostable esterase bound to the affinity column was then eluted with a linear 10 mM to 250 mM imidazole gradient. The peak fractions were visualized using 12.5 % SDS-PAGE gels. To remove the imidazole, fractions containing the recombinant proteins were pooled and dialyzed against the storage buffer, 50 mM Tris-HCl buffer (pH 7.0), for 16 h at 4 °C.

The supernatant containing solubilized untagged Msed_1072 was loaded onto a 5 mL HiTrap™ Q anion exchange column equilibrated with 20 mM Tris-HCl buffer (pH 8.0),
0.025 % Tween-20, and 0.1 % CHAPS (buffer B) and eluted with a linear 0-to-1 M NaCl gradient. The fractions containing carboxylesterase activity were collected and dialyzed against 50 mM MES (pH 6.0), 0.025 % Tween-20, and 0.1 % CHAPS (buffer C) overnight for purification by a 1 mL HiTrap™ SP cation exchange column. The column was equilibrated with the same buffer (buffer C). Bound protein was eluted by linearly increasing NaCl concentration from 0 to 1.0 M. The fractions were collected and analyzed for carboxylesterase activity. Active fractions containing carboxylesterase activity were pooled and dialyzed against the storage buffer, 50 mM Tris-HCl buffer (pH 8.0) and 0.025 % Tween-20, for 16 h at 4 °C.

Measurement of Carboxylesterase Activity

Enzyme activity was determined by a spectrophotometric assay using 4-nitrophenyl-octanoate as substrate, which was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 M. The reaction mixture (1 ml) consisted of 50 mM MOPS buffer, pH 7.0, 0.025 % Tween-20, and 1 mM substrate. The reaction mixture was pre-heated with enzyme at 65 °C for 5 min to thermo-activate the enzyme. The reaction was initiated by the addition of substrate and allowed to proceed for 5 min at determined optimal temperatures. The enzyme reaction was stopped by adding 97.5 µl of chilled 0.25 M sodium carbonate. Controls were carried out as above, minus the addition of enzyme, to monitor the background hydrolysis of the substrate. The amount of liberated p-nitrophenol (pNP) was determined by reading absorbance of the sample at 410 nm. Esterase samples were assayed in 12 technical
replicates. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per min per mg of protein; thus, activities were expressed in units per milligram of protein.

For assays conducted at different pH values, the following buffers were used at a final concentration of 50 mM: pH 4.0 and 5.0, sodium acetate; pH 6.0, MES; pH 7.0 MOPS; pH 8.0-10.0 CAPS. For assays that determined the optimal temperature, the following temperatures were used: 37 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C, and 95 °C. When evaluating substrate preference, the following pNP esters were used at a final concentration of 1 mM: \( pNP\)-butyrate (C\(_4\)), \( pNP\)-octanoate (C\(_8\)), \( pNP\)-decanoate (C\(_{10}\)), \( pNP\)-dodecanoate (C\(_{12}\)), \( pNP\)-myristate (C\(_{14}\)), \( pNP\)-palmitate (C\(_{16}\)), and \( pNP\)-stearate (C\(_{18}\)). To measure the effect of metal ions and a chelating agent on the catalytic activity, the following were used at a final concentration of 1 mM: CaCl\(_2\), CuSO\(_4\), EDTA, KCl, MgCl\(_2\), and ZnCl\(_2\). For assays that examined the effect of detergents and an oxidizing agent on the enzyme activity, detergents Triton, Tween-20, Tween-80, CHAPS, and the oxidizing agent hydrogen peroxide were used at final concentrations of 0.1 % (w/v) and 1 % (w/v). The effect of SDS on the enzyme activity was also evaluated at final concentrations of 1 mM and 5 mM. To investigate the effect of organic solvents on the enzyme activity, the following organic solvents were used at a final concentration of 10 % (v/v): acetone, ethanol, isopropanol, methanol, and n-butanol. Thermostability was measured after incubation of Msed_1072Nt and Msed_1072Ct (0.02 mg/ml) at 70 °C and 90 °C, respectively, at various intervals of time.
A reaction mixture (500 uL) consisting of 50 mM MOPS buffer (pH 7.0), 0.025 %
Tween-20, 10 g tri-palmitate, and 78 ug histidine-tagged enzyme (Msed_1072Nt and
Msed_1072Ct) was incubated at 65 °C for 3 h. After incubation, the reaction products were
converted to fatty acid methyl esters as described by Bannon et al. (1982). The hexane layer
was directly analyzed with a GC HP5890 series II equipped with a FID (Hewlett Packard,
refurbished by Primera Scientific LLC, Princeton, New Jersey) and a 7673A autosampler
(Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC, Princeton, New
Jersey). Separation was achieved in an Rtx-2330 capillary column (Hewlett Packard). The
temperature was programmed to include an initial 3 min at 60 °C, and then was increased to
230 °C at a rate of 4 °C/min. Both injector and detector temperatures were set at 265 °C.
Injections were performed under the splitless mode (Sparkman et al. 2011). Data acquisition
and analysis were completed using the GC Chemstation Rev. A.08.03 (847) software.
Calculations for total FAs were completed using the relative response factor (RRF) method
(Sparkman et al. 2011) and area ratios were compared to the internal standard (KEL-FIM-
FAME-5 Mixture, Matreya, LLC, Pennsylvania, USA).
3.3 RESULTS AND DISCUSSION

Amino Acid Sequence Comparison

The BLASTP tool was used to find identities of Msed_1072 among the non-redundant protein sequence data deposited at the National Center for Biotechnology Information database. The deduced amino acid sequence for the *M. sedula* carboxylesterase gene (*Msed_1072*) showed 46%, 47%, and 47% identity with those from a metagenomic DNA library, *Sulfolobus tokodaii* strain 7, and *Archaeoglobus fulgidus* DSM 4304, respectively (Figure 1). The amino acid sequence of the *M. sedula* carboxylesterase was the most similar (65% identity) to that of a putative alpha/beta hydrolase from *Metallosphaera cuprina* Ar-4 (Liu et al. 2011). The Msed_1072 sequence has 53% identity to a putative 308 amino acid alpha/beta hydrolase from *Sulfolobus islandicus* M.14.25 (Reno et al. 2009) and 52% identity to a 311 amino acid lipase from *Sulfolobus solfataricus* P2 (She et al. 2001). In addition, sequence alignment revealed that the *M. sedula* carboxylesterase contains the typical catalytic triad composed of Ser-Asp-His and the consensus sequence (Gly-X-Ser-X-Gly) around the active-site serine (Figure 3-1) (Jaeger et al. 1994). Sequence alignment also showed that the amino acid residues Gly-78 and Gly-79 (within the conserved sequence motif HGGG, that is observed in the hormone-sensitive lipase (HSL) family) and Ala-151 form the oxyanion hole in the Msed_1072 sequence (De Simone et al. 2001).
Expression and Purification of Recombinant Carboxylesterase from *M. sedula*

The carboxylesterase gene variants were heterogeneously expressed in *E. coli* BL-21 (DE3)pLysS cells under the control of the T7 promoter to allow for a high level of expression. The expression of the targeted protein was induced by α–lactose monohydrate while culturing in autoinduction media (Studier 2005). Cells from a two liter culture were incubated for 16 h at 37 °C, harvested, and lysed by the addition B-PER® bacterial protein extraction reagent followed by passage through a French pressure cell. The cell-free supernatant was heat-treated (to remove native proteins), centrifuged, and filtered through a 0.45 μM membrane. A histidine-tag on the targeted proteins (Msed_1072Nt and Msed_1072Ct) allowed for a single-step purification using nickel affinity chromatography. Msed_1072 (untagged enzyme) was purified by a two-step procedure utilizing anion exchange chromatography followed by cation exchange chromatography. Initially, between the purification steps for Msed_1072, the targeted protein was dialyzed in the appropriate start buffer with a pH 4.6. This pH was selected for dialysis based upon the isoelectric point of Msed_1072; however, at this low pH for the start buffer, the partially purified Msed_1072 formed aggregates. Thus, the purification buffer pH levels were increased to 6.0 with the expectation that Msed_1072 would be collected in the flow-through while the *E. coli* native proteins would bind the column. Detergents, 0.025 % Tween-20 and 0.1 % CHAPS, were also added to the purification buffers to avoid aggregation of the carboxylesterase. After column purification, enzyme purity was confirmed with a 12.5 % SDS-PAGE gel (Figure 3-2). The purified carboxylesterase variants were stored at 4 °C and were used to characterize
the enzyme.

Substrate Specificity

The substrate specificity of each purified carboxylesterase variant was investigated using several pNP-esters as substrates for the enzyme assays (Figure 3-3). The highest specific activity was observed with pNP-octanoate for all three enzyme variants (Msed_1072Nt, Msed_1072Ct, and Msed_1072). This result suggests that the presence of the histidine-tag does not change the substrate preference. It is also similar to that of a well-studied thermostable esterase Thermotoga maritima EstA (Levisson et al. 2009a). EstA demonstrated an optimal activity towards pNP-acetate and pNP-octanoate. When EstA hydrolyzed pNP-octanoate, the catalytic activity ($k_{\text{cat}}$) was 37 and the catalytic efficiency ($k_{\text{cat}}/K_m$) was 1370. Until recently, most hyperthermophilic carboxylic ester hydrolases were characterized as esterases, preferring medium chain (acyl chain length of 6) p-nitrophenyl substrates (Atomi et al. 2004; Levisson et al. 2009b). The location of the histidine-tag, however, does influence the specific activity. The C-terminal histidine-tagged protein had activity towards medium-chain substrates (C$_8$-C$_{12}$) comparable to that of the untagged protein, whereas, the N-terminal histidine-tagged protein had less comparable activity for this range of substrates. All enzyme variants had higher activity toward pNP-esters of carboxylic acids with medium chain length (C$_8$-C$_{12}$) when compared to those of longer chain length (C$_{14}$-C$_{18}$). In addition, the apparent rate of hydrolysis decreased as the carbon chain length increased. Interestingly, the carboxylesterase variant with a polyhistidine-tag fused to the N-
terminal region demonstrated hydrolytic activity within a broader specific specificity range using pNP-esters as a substrate (C₄-C₁₈), albeit at a lower rate with long chain esters. It is important to note that at high temperatures, such as 95°C, there is an increase in breakdown of pNP-esters with acyl chains of short length (C₄) and long length (C₁₆ and C₁₈). For this reason, 4-methylumbelliferone was used to measure Msed_1072 and Msed_1072Ct ability to hydrolyze carbon chains of these lengths (Sehgal et al. 2001). The activity of Msed_1072 and Msed_1072Ct towards these substrates was undetectable (data not shown). Based on the conserved domain program on NCBI, the substrate binding pocket is located in the N-terminus of the protein. An explanation could be the presence of the poly-histidine affinity tag on the N-terminus influenced the conformation of the substrate binding pocket. Utilizing another substrate (triacylglycerol), which was tested by GC-FID, the tagged esterase variants were able to hydrolyze a long-chain substrate (C₁₆). Msed_1072Nt had a total fat of 2.86 mg/g and Msed_1072Ct had a total fat of 45.78 mg/g (data not shown). Generally, the algal fatty acid synthesis pathway produces a carbon chain length of 14 to 18, saturated or mono-unsaturated fatty acid (Harwood 1998). For algae-based biofuel production, these results suggest that recombinant Msed_1072 is a carboxylesterase that will enhance the free fatty acid pool by releasing the fatty acids previously sequestered during cell growth as part of algal lipid bodies.
Influence of Temperature and pH on Enzyme Activity

The activity response of each esterase variant was determined by monitoring the hydrolysis of pNP-octanoate over a temperature range of 37 °C to 95 °C using the standard spectrophotometric assay as described in Methods and Materials. The results (Figure 3-4) indicate that all enzyme variants were active over a broad temperature range, with the highest hydrolytic activities measured at 70 °C (Msed_1072Nt), 95 °C (Msed_1072Ct), and 95 °C (Msed_1072). For the temperature (75 °C) consistent with what is typically found in the habitat of M. sedula, all variants of the enzyme exhibited catalytic activity. Thermostability for Msed_1072Nt and Msed_1072Ct was also examined as described in Methods and Materials. At 70 °C, Msed_1072Nt was not thermostable (data not shown), but the half-life of Msed_1072Ct at 90 °C was ~ 28 min (data not shown). The unstable characteristics of the N-terminal tagged protein may be due to the position of the poly-histidine tag. Mandrich and colleagues reported that the NH2-terminal region contributes to enzyme activity and stability in esterases of the HSL family (Mandrich et al. 2005).

The effect of pH on the activity of each Msed_1072 variant was investigated using pNP-octanoate (Figure 3-5). Activity was measured within a pH range of 4 to 10. A broad pH range for activity was observed for all variants of the enzyme. Nevertheless, Msed_1072Nt, Msed_1072Ct, and Msed_1072 each displayed the highest activity at neutral pH, with an optimum pH of 7. This result suggests that Msed_1072 is likely an intracellular protein. Although M. sedula thrives in an acidic environment (pH 1 - 4), the organism is able to maintain a neutral internal environment with membrane localized proton pumps, a feature
found in many microorganisms living in extreme pH conditions.

Effects of Detergents/Oxidizing Agent, Metal Ions/Chelating Agent, and Organic Solvents

The stability of enzyme activity in the presence of detergents and oxidizing agents is generally considered a desired feature of industrial enzymes. Our results demonstrated that the position of the poly-histidine tag likely determines the stability of the Msed_1072 variants when they are incubated with a surfactant or an oxidizing agent. Enzyme activity toward pNP-octanoate was evaluated for Msed_1072Nt, Msed_1072Ct, and Msed_1072 in the presence of nonionic (Tween20, Tween80, and Triton-X100), zwitterionic (CHAPS), and ionic (SDS) detergents as well as H$_2$O$_2$ (oxidizing agent). These results are summarized in Table 3-1. In the presence of nonionic detergents, activity for both tagged enzyme variants was inhibited as compared to the no-detergent control. In general, relative enzyme activity decreased as the concentration of these detergents increased, which was also observed with the ionic detergent. The exception was Msed_1072Nt activity, which was not affected by the presence of 1.0 % Tween-20. Msed_1072Nt and Msed_1072Ct retained 32 % and 100 % activity, respectively, in the presence of 1 mM sodium dodecyl sulfate (SDS); however, there was no activity observed for either Msed_1072 variants in the presence 5 mM SDS (data not shown). The addition of 0.1 % Zwitterionic 3-[(3-cholamidopropyl) dimethyl- ammonio]-1-propanesulfonate (CHAPS) had a slight activating effect on both variants of the enzyme. With regards to the enzyme assay, the addition of a detergent (0.025 % Tween-20) to the buffer was critical. When a small amount of this non-ionic surfactant was added, it likely
decreased formation of substrate micelles as described for *Bacillus stearothermophilus* NCA2184 carboxylesterase (Matsunaga et al. 1974). Reduced micelle formation then increased enzyme accessibility to the substrate and observed enzyme activity improved. Supplementation with 0.1 % H$_2$O$_2$ resulted in a significant reduction of activity for Msed_1072Ct; however, Msed_1072 activity was not affected significantly. It should be noted that the activity of the enzymes (Msed_1072Ct and Msed_1072) were measured in 1.0 % H$_2$O$_2$; however, due to breakdown of the substrate (pNP-octanoate) at that concentration, an accurate determination of the enzyme activity could not be made.

Enzyme activity for each Msed_1072 variant was also evaluated when the enzymes were pre-incubated with and tested for activity in the presence of either a metal ion or a chelating agent (Figure 3-6). As compared to the control reactions with no added metal ion, the presence of either Cu$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ reduced enzyme activity for each enzyme variant. Reduced activity was most significant for Cu$^{2+}$ and Zn$^{2+}$, suggesting that these ions are potent enzyme inhibitors. Ca$^{2+}$ had no significant impact on the enzyme activity of Msed_1072 and Msed_1072Ct, but reduced the activity of Msed_1072Nt. Interestingly, magnesium ion (Mg$^{2+}$) stimulated the activity of Msed_1072Ct, reduced the activity of Msed_1072Nt, and had no significant impact on Msed_1072. Potassium ion (K$^+$) significantly reduced the activity of Msed_1072Nt and Msed_1072Ct, while having no significant impact on the activity of Msed_1072. There was also no effect on enzyme activity upon incubation with the metal chelator EDTA for either variant of the enzyme (Msed_1072Nt and Msed_1072Ct), but the activity of Msed_1072 was slightly stimulated in the presence of the chelator. Our findings suggest that Msed_1072 is not a metalloenzyme, in
agreement with the fact that lipases and esterases generally do not require cofactors for catalysis (Jaeger and Reetz 1998).

Enzyme activity toward pNP-octanoate was evaluated for Msed_1072Nt and Msed_1072Ct in the presence of various organic solvents, and the results are summarized in Table 3-2. While esterase activity of Msed_1072Nt was increased with the addition of ethanol, this polar solvent had an inhibitory effect on Msed_1072Ct. The presence of n-butanol had a significant inhibitory effect on both enzyme variants, suggesting that this solvent may be toxic to the enzyme. Supplementation with acetone and isopropanol each resulted in reduced activity for Msed_1072Ct; however, Msed_1072Nt activity was not affected significantly. Again, our research findings revealed that the recombinant carboxylesterase can tolerate exposure to organic solvents, and this tolerance depends on the position of the poly-histidine tag. For example, although only Msed_1072Nt demonstrated stability towards ethanol and 2-propanol, the activities of both tagged variants were significantly enhanced by methanol. There are only a few reports showing stability of native lipases, isolated from non-solvent-tolerant microorganisms, in the presence of an organic solvent. For example, a novel enantioselective lipase produced by *Acinetobacter* species SY-01 had a relative activity value of 100% after 1 hour at 50 °C in methanol (Han et al. 2003). In the case of increased activity of Msed_1072Nt and Msed_1072Ct in the presence of methanol, a likely explanation is that methanol provides enhanced access of the enzyme to the substrate. The ability of Msed_1072 variants to hydrolyze fatty acid esters in the presence of organic solvents does provide strong evidence that this carboxylesterase could be used for high temperature organic syntheses (60 °C - 95 °C).
3.4 CONCLUSION

This is the first report of the cloning, functional, and biochemical characterization of a thermostable carboxylesterase enzyme from *Metallosphaera sedula* DSM5348 for the purpose of microalgae based biofuel production. Although the fusion of the protein with a poly-histidine tag did not shift substrate specificity to carboxylic acids of longer chain length (C_{12} - C_{18}), it did provide insight on which carboxylesterase variation can hydrolyze fatty acid esters in the presence of various chemicals that can be beneficial to industrial processes. To achieve the goal of increasing the enzyme’s affinity towards longer acyl chains, future experiments should focus on altering the acyl binding pocket via site-directed and saturation mutagenesis experiments (Manco et al. 2001).

3.5 ACKNOWLEDGEMENTS

The authors thank Drs. Lisa Dean and Stephanie Mixson for their help with the GC-FID protocol and analysis. This work was supported by the National Science Foundation EFRI program under Grants EFRI 093772 and 1332341.
3.6 REFERENCES

http://dx.doi.org/10.1111/j.1742-4658.2012.08687.x


Manco G, Mandrich L, and Rossi M (2001) Residues at the active site of the esterase 2 from *Alicyclobacillus acidocaldarius* involved in substrate specificity and catalytic activity at high temperature. *Journal of Biological Chemistry*, 276: 37482-37490. [http://dx.doi.org/10.1074/jbc.M103017200](http://dx.doi.org/10.1074/jbc.M103017200)


Prior to the standard activity assay, the enzyme variants were pre-incubated for 5 min at 65 °C in the reaction mixture, containing 50 mM MOPS buffer, pH 7.0, 0.025 % Tween-20, and one detergent. The reaction was initiated by the addition of 1 mM pNP-octanoate. Hydrolysis of pNP-octanoate was carried out at 70 °C and 95 °C for Msed_1072Nt and Msed_1072Ct, respectively. The reaction was terminated by the addition of 97.5 µl of chilled 0.25 M sodium carbonate. Enzyme activity determined in the absence of a detergent was defined as 100% activity.
Table 3-2 Effect of organic solvents on the activity of *M. sedula* carboxylesterase variants.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative activity (%)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Msed_1072Nt</td>
<td>Msed_1072Ct</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Methanol</td>
<td>189</td>
<td>137</td>
</tr>
<tr>
<td>Ethanol</td>
<td>158</td>
<td>60</td>
</tr>
<tr>
<td>Acetone</td>
<td>88</td>
<td>47</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>100</td>
<td>39</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>28</td>
<td>12</td>
</tr>
</tbody>
</table>

Prior to the standard activity assay, the enzyme variants were pre-incubated for 5 min at 65 °C in the reaction mixture, containing 50 mM MOPS buffer, pH 7.0, 0.025 % Tween-20, and an organic solvent. The reaction was initiated by the addition of 1 mM *p*NP-octanoate. Hydrolysis of *p*NP-octanoate was carried out at 70 °C and 95 °C for Msed_1072Nt and Msed_1072Ct, respectively. The reaction was terminated by the addition of 97.5 µl of chilled 0.25 M sodium carbonate. Enzyme activity determined in the absence of an organic solvent was defined as 100 % activity.
Figure 3-1 Alignment of amino acid sequences of Msed_1072 (this study), a hyperthermophilic esterase (EstE1) from a metagenomic DNA library [18], a thermostable esterase (Sto-Est) from *Sulfolobus tokodaii* strain 7 [19], and a thermosable esterase (Est-AF) from *Archaeoglobus fulgidus* DSM 4304 [20]. The putative catalytic triad (Ser150, Asp244, His274) on Msed_1072 deduced from the alignment is indicated by an arrow.
**Figure 3-2** SDS-PAGE of purified *M. sedula* carboxylesterase variants. L: Molecular Weight Marker (in kDa), Lane 1: Purified Msed_1072 (1 ug), Lane 2: Purified Msed_1072Nt (1 ug), Lane 3: Purified Msed_1072Ct (1 ug).
The specific activities of purified Msed_1072 (gray bar), Msed_1072Nt (white bars), and Msed_1072Ct (black bars) toward pNP-esters with different acyl chain lengths were determined under standard conditions at optimal temperatures of 70 °C (Msed_1072Nt), 95 °C (Msed_1072), and 95 °C (Msed_1072Ct). The highest specific activities were observed using pNP-octanoate as the substrate, and they were as follows: Msed_1072Nt (4079 U/mg), Msed_1072Ct (19089 U/mg), and Msed_1072 (19961 U/mg). Error bars represent standard error.
Figure 3-4 Temperature profile of purified Msed_1072 (gray bar), Msed_1072Nt (white bars), and Msed_1072Ct (black bars). Enzyme activity of each esterase variant was determined over a temperature range from 37 °C to 95 °C, using pNP-octanoate as the substrate. The reaction mixture consisted of 50 mM MOPS buffer, pH 7.0, 0.025 % Tween-20, and 1 mM pNP-octanoate. Error bars represent standard error.
Effect of pH on the activity of purified Msed_1072 (gray bar), Msed_1072Nt (white bars), and Msed_1072Ct (black bars) was determined over a pH range from 4 to 10, at the optimal temperature for each esterase variant. Assays contained 1 mM pNP-octanoate, 0.025 % Tween-20, and 50 mM of the following buffers: pH 4.0 - 5.0, sodium acetate; pH 6.0, MES; pH 7.0, MOPS; pH 8.0 - 10.0, CAPS. Error bars represent standard error.
Enzyme activity after pre-incubation with and in the presence of either metal ion or chelating agent supplementation. Prior to the standard activity assay, each enzyme variant was pre-incubated for 5 min at 65 °C in the reaction mixture, containing 50 mM MOPS buffer, pH 7.0, 0.025 % Tween-20, and either a metal ion or chelating agent. The reaction was initiated by the addition of 1mM pNP-octanoate. Hydrolysis of pNP-octanoate was carried out at 70 °C, 95 °C, and 95 °C for Msed_1072Nt, Msed_1072Ct, and Msed_1072, respectively. The reaction was terminated by the addition of 97.5 µl of chilled 0.25 M sodium carbonate. Enzyme activity determined in the absence of metals or chelator was defined as 100 % activity.
CHAPTER 4

Recombinant expression and biochemical characterization of a thermostable esterase from *Sulfolobus solfataricus* P2 to augment production of microalgal-derived biofuel

Rushyannah Killens-Cade¹, Christine MacInnes², Amy Grunden¹

¹Department of Plant and Microbial Biology, North Carolina State University, Raleigh, USA 27695
²Department of Biology, North Carolina State University, Raleigh, USA 27695
ABSTRACT

With the dwindling supply of fossil fuels, there has been a push towards creating renewable biofuels, and algae have emerged as a promising alternative. However, for algae to be a viable alternative, the process of creating biofuels must be more efficient. One way to increase efficiency is to transform algae with thermostable lipases/esterases that will cleave free fatty acids to the desired length of 10-12 carbons for diesel and jet fuel production. The research project described here was focused on the recombinant expression of the *Sulfolobus solfataricus* P2 gene *LipP-1Nt*, which encodes for a thermostable esterase in an effort to biochemically characterize the lipase for its application in algal biofuel production. The target esterase gene was amplified using genomic DNA from *S. solfataricus* as well as molecular biology techniques to generate an expression vector for recombinant protein production in the bacterium *Escherichia coli*. The vectors used for the recombinant protein expression coded for an N-terminal hexahistidine (His$_6$)-tag, which enabled protein purification via metal affinity chromatography. After the N-terminal his-tagged protein was purified, temperature range, pH range, and substrate affinity assays were completed to determine the conditions under which the esterase has optimal activity.
4.1 INTRODUCTION

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are enzymes that catalyze the synthesis of ester compounds and hydrolyze triglycerides (Jaeger and Eggert 2002). As hydrolases, these lipolytic enzymes act on the ester bonds of the triglycerides to liberate fatty acids and glycerols. Although both enzyme types share structural characteristics, the fundamental difference between esterases and (true) lipases is that the former acts on shorter chain triglycerides (shorter than 10 carbon atoms) which are water-soluble, whereas the latter hydrolyzes longer chain triglycerides (Gilham and Lehner 2005; Jaeger et al. 1999). Based on their regio- and stereo-specific properties, cofactor-independent activity, broad substrate specificity, and stability in the presence of organic solvents, ester-hydrolyzing enzymes are attractive biocatalysts (Jaeger et al. 1999). As such, they are used widely in a number of industrial applications, including medical biotechnology, resin removal from pulp, detergent production, organic synthesis, flavor and aroma synthesis, food related processes, and biofuel production (Panda and Gowrishankar 2005; Salameh and Wiegel 2007).

The increasing consumption of energy, population growth, and environmental concerns have resulted in an urgent need to explore more sustainable energy sources. Lipid-producing microalgae are emerging as a leading platform for producing alternative sustainable energy, mainly because of desirable characteristics such as rapid growth rate, production of valuable co-products, and cultivation in various marine and freshwater environments that do not compete with arable crop lands. (Menetrez 2012; Pienkos and Darzins 2009; Guschina and Harwood 2006). Their adaptability to a range of growth
environments may result in the ability to produce lipids of diverse compositions (Guschina and Harwood 2006). These include membrane lipids as well as lipids involved in carbon and energy storage such as triacylglycerides (TAG), which are neutral lipids that consist of three fatty acids esterified to glycerol. In addition to having a diversified lipid composition that may aid survival in harsh environments some algae may also alter lipid biosynthetic pathways leading to the accumulation of TAG during unfavorable environmental or stress conditions (Hu et al. 2008).

Recent genetic and metabolic engineering research has focused on increasing lipid yields in microalgae by targeting the carbon flux through the fatty acid synthesis pathway. However, for microalgae to be a cost effective and reliable alternative energy source, the process for the direct conversion of lipidic biomass feedstock to combustible fuel must become more efficient. Specifically, there is a need to save energy during the conversion process and avoid extensive degumming. A synthetic biology approach which targets increasing the yield and quality of algal feedstock is one way of overcoming these difficulties. By transforming algae to express thermostable lipases/esterases, fatty acid release from TAGs that are stored in lipid bodies can be achieved during the high temperature conversion of lipids to fuel. Having enzymes that are active during this process can reduce the time required for lipid to fuel conversion, thereby saving energy and improving cost effectiveness.

For our microalgae based biofuel system, we selected a homolog of an *E. coli* acyl-ACP thioesterase, TesA. TesA, expressed in an *E. coli* expression system, has been shown to hydrolyze long chain fatty acyl-ACPs (products of the fatty acid synthesis pathway),
resulting in relief of feedback inhibition and increased production of total free fatty acids (FFAs) (Liu et al. 2010, Voelker and Davies 1994). It also has been shown that TesA has a wide substrate specificity and is able to hydrolyze FFAs to produce long-chain fatty acyl-ACPs containing 12 to 18 carbons (Barnes and Wakil 1968). This hydrolysis profile works to the advantage of biofuel systems because aromatic hydrocarbons with 12 to 14 carbon atoms are ideal for production of petroleum derived jet fuels.

To evaluate its application for algae transformation and subsequent biofuel production, we have recombinantly expressed and characterized a putative lipase (LipP-1Nt) from the thermoacidophilic archaeon *Sulfolobus solfataricus* P2 (optimal growth at 80 °C and a pH range of 2.0 to 4.0) which was isolated from a sulfur-rich volcanic spring in Yellowstone National Park (She et. al 2001). *S. solfataricus* LipP-1Nt was originally targeted for analysis based on its similarity to *E. coli* TesA (24% similarity; conserved catalytic triad amino acids Ser-Asp-His) was produced recombinantly in *E. coli* fused with an N-terminal hexahistidine (His\textsubscript{6})-tag to aid in protein purification. It is important to note that Kim and Lee (2004) published research findings outlining the cloning and expression of three ORFs (SS02493, SSO2517, SSO2521) from *S. solfataricus*, coding for putative lipases/esterases. However, the authors published on the characterization of an esterase, called Est3, resulting from the cloning of SSO2493, erroneously attributing the sequence of SSO2517 to Est3, and the protein sequence of SSO2493 was named Est2. Nevertheless, Kim and Lee (2004) successfully reported the characterization of SSO2517 from *S. solfataricus* P2.
4.2 MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Reagents

The genomic DNA of *Sulfolobus solfataricus* P2 was kindly provided by Dr. Robert Kelly of NCSU. The *Escherichia coli* XL1-Blue strain (Novagen, EMD Biosciences) was used for cloning and maintaining the recombinant plasmid. *E. coli* strain BL-21(DE3)LysS (Novagen, EMD Biosciences) was used for over-expression of the recombinant protein. The expression plasmid pET28a was obtained from Novagen, EMD Biosciences. Restriction endonucleases, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs. Synthetic oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). The PCR products were purified using a QIAquick PCR purification kit (Qiagen). The plasmid was purified using a QIAprep spin miniprep kit (Qiagen). All assay substrates were purchased from Sigma-Aldrich. A 5 ml HisTrap Nickel Sepharose™ chromatography column was utilized for the enzyme purification step (GE Healthcare Life Sciences).

Cloning of a Thermoactive Esterase from *Sulfolobus solfataricus*

The *LipP-1Nt* gene was amplified using the forward primer: 5’-AAAAGAATTCATATTTCTGGAGACATATGCCCTAGAC-3’, with an *EcoRI* site (bold), and the reverse primer: 5’-
GAGCTGGAAGAATAACAGCTTTCCATAGTCTTAATTTGTTGTCGACTCTTT-3’, with a SalI site (bold) and Phusion® high fidelity DNA polymerase in a 50 µl PCR reaction solution containing 10 µl 5x Phusion® HF buffer, 1 µl dNTPs (10 mM), 0.5 µl forward primer (40 uM), 0.63 µl reverse primer (40 uM), 0.5 µl Phusion® DNA polymerase, and 2 µl S. solfataricus genomic DNA (50 ng/µl). The PCR reactions were carried out using a thermal cycler (Bio-Rad) under the following conditions: an initial denaturing step at 98 °C for 30 sec; 30 cycles at 98 °C for 10 sec (denaturing), annealing at 64 °C for 30 sec, and extension at 72 °C for 30 sec; a final extension cycle of 72 °C for 7 min, and preservation at 4°C. The amplicons were purified and visualized using a 1 % (w/v) agarose gel. The purified DNA products were subjected to a double digestion, over-night, with EcoRI and SalI-HF to render sticky ends needed for cloning. For construction of pET28a-LipP-1Nt, the plasmid was digested with EcoRI and SalI-HF, gel purified, and ligated to the compatible sticky end DNA molecule of LipP-1Nt. CaCl₂-competent cells of E. coli XL1-Blue were transformed with the ligated reaction and plated on solid LB agar media supplemented with kanamycin (50 µg/ml) for selection of pET28a-LipP-1Nt. A positive clone of each construct was sequenced by Eurofins MWG Operon (Huntsville, AL) to ensure that no mutations were generated during the amplification process. The sequence information was analyzed by using MacVector (Accelrys) computer software.
Over-expression of Recombinant Thermoactive Esterase LipP-1Nt

For the over-expression of *S. solfataricus* esterase, each construct and the rare arginine, leucine, and isoleucine tRNA encoding plasmid pRIL were used to transform *E. coli* BL21(DE3)pLysS cells. For the control expression samples, the plasmids without the *LipP-1Nt* gene were also used to transform *E. coli* BL21(DE3)pLysS. The transformants were plated and selected on LB agar plates containing the appropriate antibiotics after incubation overnight at 37 °C. The *E. coli* BL21(DE3)pLysS cells harboring the recombinant plasmids were used to inoculate a 2 L culture of auto-induction media (Studier 2005) supplemented with the appropriate antibiotics for large scale expression of the protein. The cells were incubated with shaking at 200 rpm for 16 h at 37 °C. Cells were harvested by centrifugation (9500 rpm, 50 min, 4 °C) and stored at -20 °C prior to preparing cell lysates. The expression of the recombinant protein was evaluated by SDS-PAGE using a 12.5 % polyacrylamide gel with the Bio-Rad Mini-PROTEAN® apparatus and visualized using Coomassie brilliant blue R250. The molecular mass of the recombinant esterase was determined to be approximately 38.3 kDa by SDS-PAGE. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).
Recombinant Thermoactive Esterase Purification

The *E. coli* BL21(DE3)pLysS cell pellets containing the LipP-1Nt protein were resuspended and lysed for 10 min with 10 mL B-PER® bacterial protein extraction reagent (Pierce, Rockford, IL) containing 1 mM benzamidine-HCl. The use of the nonionic detergent in the initial lysing step was critical for the solubility of the protein. The cells were further lysed by three passages of the lysate through a French pressure cell (20,000 lb/in²). Cellular debris was removed by centrifugation (15,000 rpm, 45 min, 4 °C). The supernatant was collected and heated at 70 °C for 15 min. The heat-treated supernatant was centrifuged (15,000 rpm, 15 min, 4°C) to remove denatured proteins.

The supernatant containing the solubilized his-tagged recombinant protein (LipP-1Nt) was added to a 5 ml HisTrap Ni Sepharose™ column for purification. Prior to the addition of the protein, the Ni²⁺-charged column was equilibrated with 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, and 10 mM imidazole. After binding the protein to the metal charged column, the column was washed with the equilibration buffer to remove all unbound protein. The thermostable esterase bound to the affinity column was then eluted with a linear 10 mM to 250 mM imidazole gradient. The peak fractions were visualized using 12.5 % SDS-PAGE gels. To remove the imidazole, fractions containing the recombinant proteins were pooled and dialyzed against the storage buffer, 50 mM Tris-HCl buffer (pH 7.0), for 16 h at 4 °C.
Measurement of Esterase Activity

Enzyme activity was determined by a spectrophotometric assay using 4-nitrophenyl-octanoate as substrate, which was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 M. The reaction mixture (1 ml) consisted of 50 mM MOPS buffer, pH 7.0 and 1 mM substrate. The reaction mixture was pre-heated with enzyme at 65 °C for 5 min to thermo-activate the enzyme. The reaction was initiated by the addition of substrate and allowed to proceed for 5 min at determined optimal temperatures. The enzyme reaction was stopped by adding 97.5 µl of chilled 0.25 M sodium carbonate. Controls were carried out as above, minus the addition of enzyme, to monitor the background hydrolysis of the substrate. The amount of liberated p-nitrophenol (pNP) was determined by reading absorbance of the sample at 410 nm. Esterase samples were assayed in 6 technical replicates. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per min per mg of protein; thus, activities were expressed in units per milligram of protein.

For assays conducted at different pH values, the following buffers were used at a final concentration of 50 mM: pH 4.0 and 5.0, sodium acetate; pH 6, MES; pH 7.0 MOPS; pH 8.0-10.0 CAPS. For assays that determined the optimal temperature, the following temperatures were used: 37 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C, and 95 °C. When evaluating substrate preference, the following pNP esters were used at a final concentration of 1 mM: pNP-butyrate (C₄), pNP-octanoate (C₈), pNP-decanoate (C₁₀), pNP-dodecanoate (C₁₂), pNP-myristate (C₁₄), pNP-palmitate (C₁₆), and pNP-stearate (C₁₈).
Amino Acid Sequence Comparison

The BLASTP tool was used to find identities of LipP-1Nt among the non-redundant protein sequence data deposited at the National Center for Biotechnology Information database. The deduced amino acid sequence for the *S. solfataricus* esterase gene (*LipP-1Nt*) showed 42%, 45%, and 73% identity with those from *Archaeoglobus fulgidus* DSM 4304, a metagenomic DNA library, and *Sulfolobus tokodaii* strain 7, respectively (Fig. 4-1). The amino acid sequence of the *S. solfataricus* esterase was the most similar (99% identity) to that of putative alpha/beta hydrolases from *Sulfolobus islandicus* spp. The LipP-1 sequence has 49% identity to a 301 amino acid carboxylesterase from *Metallosphaera sedula* DSM 5348 (Killens-Cade et al. 2014). In addition, sequence alignment revealed that the *S. solfataricus* esterase contains the typical catalytic triad composed of Ser-Asp-His and the consensus sequence (Gly-X-Ser-X-Gly) around the active-site serine (Figure 4-1) (Jaeger et al. 1994). Sequence alignment also showed that the amino acid residues Gly-80 and Gly-81 (within the conserved sequence motif HGGG, that is observed in the hormone-sensitive lipase (HSL) family [De Simone et al. 2001]) and Ala-152 form the oxyanion hole in the LipP-1Nt sequence.
Expression and Purification of Recombinant Esterase from *S. solfataricus*

The esterase gene variants were heterogeneously expressed in *E. coli* BL-21 (DE3)pLysS cells under the control of the T7 promoter to allow for a high level of expression. The expression of the targeted protein was induced by alpha-lactose monohydrate while culturing in autoinduction media (Studier 2005). Cells from a two liter culture were incubated for 16 h at 37 °C, harvested, and lysed by the addition B-PER® bacterial protein extraction reagent followed by passage through a French pressure cell. The cell-free supernatant was heat-treated (to remove native *E. coli* proteins), centrifuged, and filtered through a 0.45 uM membrane. A histidine-tag on the targeted protein allowed for a single-step purification using nickel affinity chromatography. After column purification, enzyme purity was confirmed with a 12.5 % SDS-PAGE gel (Figure 4-2). The purified esterase was stored at 4 °C and used to characterize the enzyme.

Substrate Specificity

The substrate specificity of the purified esterase was investigated using several *p*NP-esters as substrates for the enzyme assays (Figure 4-3). The highest specific activity was observed with *p*NP-butyrate. LipP-1Nt demonstrated higher activity toward *p*NP-esters of carboxylic acids with short chain length (C₄-C₈) when compared to those of medium-to-long chain length (C₁₀-C₁₈). Our findings suggest that LipP-1Nt is a typical esterase with broad substrate specificity. Until recently, most hyperthermophilic carboxylic ester hydrolases were
characterized as esterases, preferring medium chain (acyl chain length of 6) \( p \)-nitrophenyl substrates (Atomi et al. 2004, Levisson et al. 2009). It would be interesting to see if the absence of a poly-histidine affinity tag or a C-terminus hexahistidine (His\(_6\))-tag could influence substrate preference of LipP-1 (Lee et al. 1999). Based on the conserved domain program on NCBI, the substrate binding pocket is located in the N-terminus of the protein, suggesting that the N-terminus influences the conformation of the substrate binding pocket. Mandrich et al. (2005) demonstrated that the N-terminus of enzymes of the HSL (hormone-sensitive lipase) family is involved in substrate specificity.

**Influence of Temperature and pH on Enzyme Activity**

The activity response of the esterase was determined by monitoring the hydrolysis of \( p \)NP-butyrate and \( p \)NP-dodecanoate over a temperature range of 37 \(^\circ\)C to 95 \(^\circ\)C using the standard spectrophotometric assay as described in Methods and Materials. The results (Figure 4-4) indicate that LipP-1Nt was active over a broad temperature range when using both substrates, with the highest hydrolytic activities measured at 60 \(^\circ\)C (\( p \)NP-butyrate) and 70 \(^\circ\)C (\( p \)NP-dodecanoate). Generally, thermophilic enzymes are often barely active at low temperatures. Interestingly, LipP-1Nt demonstrated similar specific activity units at a low temperature (37 \(^\circ\)C) and high temperatures (90 \(^\circ\)C) regardless of the substrate used. This unique characteristic of LipP-1 may be due to the presence of the N-terminus polyhistidine-tag. Mandrich et al. (2005) reported that the N-terminus of enzymes of the HSL family is
involved in thermostability and thermophilicity. For the temperatures consistent with what is typically found in the habitat of *S. solfataricus*, LipP-1Nt exhibited catalytic activity.

The effect of pH on the activity of LipP-1Nt was investigated using *p*NP-butyrate and *p*NP-dodecanoate (Figure 4-5). Activity was measured within a pH range of 3 to 7. A broad pH range for activity was observed for the enzyme. Nevertheless, LipP-1Nt displayed the highest activity at an optimum pH of 6 (*p*NP-butyrate) and 7 (*p*NP-dodecanoate). This result suggests that LipP-1Nt is likely an intracellular protein. Although *S. solfataricus* thrives in an acidic environment (pH 2 - 4), the organism is able to maintain its cytoplasmic pH at about 6.5 by generating a large pH gradient across the cytoplasmic membrane (Moll and Schafer 1988).

4-4 REFERENCES


Figure 4-1 Alignment of amino acid sequences of LipP-1Nt (this study), a hyperthermophilic esterase (Est-AF) from *Archaeoglobus fulgidus* DSM 4304 (Kim et al. 2008), a hyperthermophilic esterase (Este1) from a metagenomic DNA library (Byun et al. 2006), and a thermostable esterase (Sto-Est) from *Sulfolobus tokodaii* strain 7 (Suzuki et al. 2004). The putative catalytic triad (Ser<sup>151</sup>, Asp<sup>244</sup>, His<sup>274</sup>) on LipP-1Nt deduced from the alignment is indicated by an arrow.
Figure 4-2 SDS-PAGE of purified *S. solfataricus* esterase. Lane 1: Whole cell extract, Lane 2: Heat-Treated Cell Free Extract, Lane 3: Purified LipP-1Nt (2 ug), Lane 4: Purified LipP-1Nt (5 ug), Lane 5: Molecular weight marker (in kDa).
Figure 4-3 The specific activities of purified LipP-1Nt toward \( p \)NP-esters with different acyl chain lengths were determined under standard conditions at a temperature of 70 °C. The highest specific activities were observed using \( p \)NP-butyrate (1186 U/mg) as the substrate. LipP-1Nt was able to hydrolyze \( p \)NP-dodecanoate (16 U/mg) when used as a substrate. Error bars represent standard error.
Figure 4-4 Temperature profile of purified LipP-1Nt. Enzyme activity of the esterase was determined over a temperature range from 37 °C to 95 °C, using either pNP-butyrate and pNP-dodecanoate as a substrate. The reaction mixture consisted of 50 mM MOPS buffer, pH 7.0, and 1 mM substrate. Error bars represent standard error.
Figure 4-5  Effect of pH on the activity of purified LipP-1Nt was determined over a pH range from 3 to 7, at the optimal temperature for each esterase variant. Assays contained either 1 mM pNP-butyrate or pNP-dodecanoate and 50 mM of the following buffers: pH 4.0-5.0, sodium acetate; pH 6.0, MES; pH 7.0, MOPS; pH 8.0-10.0, CAPS. Error bars represent standard error.