Use of Biodiesel-Derived Crude Glycerol for the Production of Omega-3 Polyunsaturated Fatty Acids by the Microalga *Schizochytrium limacinum*

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Thesis submitted to the Faculty of
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science

In

Biological Systems Engineering

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April 18, 2008

Blacksburg, VA

keywords: biodiesel, crude glycerol, docosahexaenoic acid, algal fermentation

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Abstract

Crude glycerol is the major byproduct of the biodiesel industry. In general, for every 100 pounds of biodiesel produced, approximately 10 pounds of crude glycerol are created. Because this glycerol is expensive to purify for use in food, pharmaceutical, or cosmetics industries, biodiesel producers must seek alternative methods for its disposal.

Using crude glycerol as a carbon source for fermentation is a promising alternative use for this waste material. In this project, we propose to use crude glycerol in the fermentation of the microalga *Schizochytrium limacinum*, which is a prolific producer of docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid with proven beneficial effects on treating human diseases such as cardiovascular diseases, cancers, and Alzheimer's.

The first part of this study provided "proof-of-concept" that *Schizochytrium limacinum* was capable of utilizing crude glycerol while producing DHA. The results show that pretreated crude glycerol was a viable carbon source for algal growth and DHA production, with 75 g/L to 100 g/L of glycerol being the optimum concentration range. In addition to glycerol concentration, temperature, trace metal levels, ammonium acetate concentration, and NH_4Cl concentration also had significant effects ($P < 0.1$) on algal DHA production. Optimizing these factors using response surface methodology led to a DHA yield of 4.91 g/L and a DHA productivity of 0.82 g/L-day.

The second part of this study investigated the effects of impurities within the glycerol on DHA production and algal biomass composition, with a goal of ensuring that the algal biomass produced from crude glycerol can be safely utilized as an ingredient in omega-3 fortified foods or animal feed additives. Crude glycerol samples obtained from different biodiesel producers were tested. All the samples contained methanol, soaps, and various elements including calcium, phosphorus, potassium, silicon, sodium, and zinc. Both methanol and soaps in the glycerol

negatively influence algal DHA production. The two impurities can be removed from the culture medium by evaporation through autoclaving (for methanol) or by precipitation through pH adjustment (for soap). The results showed that the crude glycerol-derived algal biomass contained 45-50% lipid, 14-20% protein, and 25% carbohydrate, with 8-13% ash content. Palmitic acid (C16:0) and DHA (C22:6) were the two major fatty acids in the algal lipid. ICP analysis showed that that boron, calcium, copper, iron, magnesium, phosphorus, potassium, silicon, sodium, and sulfur were present in the algal biomass, but no heavy metals (such as mercury) were detected. The crude glycerol-derived algal biomass had a high level of DHA and a nutritional profile similar to glucose-derived commercial algal biomass, suggesting a great potential for using crude-glycerol derived algae in omega-3 fortified food or feed.

Overall, this study shows that crude glycerol can be used as a safe and effective substrate for algal culture to produce high levels of omega-3 fatty acids. With the rapid expansion of the biodiesel industry, there is an urgent need to develop new markets for large quantities of crude glycerol. This research provides an exciting opportunity to utilize a large quantity of this low grade glycerol.

Attribution

Author Denver J. Pyle is the major contributor and writer of the manuscripts in chapter three and chapter four of this thesis. Co-author Dr. Zhiyou Wen was the Committee Chair. Co-author Dr. Rafael Garcia served as a committee member. Co-authors Dr. Zhanyou Chi, Dr. Craig Frear, and Dr. Shulin Chen were colleagues of Dr. Zhiyou Wen while at Washington State University and contributed statistical analyses and constructive criticism.

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Acknowledgements

I would like to thank my advisor, Dr. Zhiyou Wen, for his guidance and support throughout this research. I would also like to thank my committee members, Dr. Rafael Garcia, for his guidance and instruction while I performed research at USDA-ARS, and Dr. Kumar Mallikarjunan, for his guidance during my academic career.

I am grateful to all of the members of my lab for their help and companionship while performing this research. Special thanks go to Sneha Athalye and Meg Orders who are working on similar projects regarding omega-3 fatty acids. Also, I would like to thank Rob DiCiccio at the USDA-ARS Eastern Regional Research Center for working with me during the summer of 2007.

Most importantly, I would like to thank my family. My mother, sister, grandmother, and my aunts and uncles have all supported me wholeheartedly throughout my academic career.

Publications and Presentations Derived from this Thesis

Publications:

Chi, Z., D. Pyle, Z. Wen, C. Frear, and S. Chen. 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Process Biochemistry* 42(11): 1537-1545.

Pyle, D. R. Garcia, and Z. Wen. 2008. Producing docosahexaenoic acid-rich algae from biodiesel derived-crude glycerol: effects of impurities on DHA production and algal biomass composition. *Journal of Agriculture and Food Chemistry.* Under Review.

Proceedings/Abstracts:

Pyle, D. and Z. Wen. 2008. Production of omega-3 polyunsaturated fatty acids from rendered animal proteins and biodiesel-waste glycerol by microalgal fermentation. Submitted. St. Joseph, Michigan: ASABE.

Pyle, D. and Z. Wen. 2007. Production of omega-3 polyunsaturated fatty acid from biodiesel-waste glycerol by microalgal fermentation. ASABE Paper No. 077028. St. Joseph, Michigan: ASABE

Presentations:

Pyle, D. and Z. Wen. March 2008. Production of omega-3 fatty acid-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. Location: Blacksburg, VA. Virginia Tech GSA Graduate Research Symposium.

Pyle, D. and Z. Wen. March 2007. Production of omega-3 fatty acid-rich algae from biodiesel waste for use as animal feed supplements. Location: Blacksburg, VA. Virginia Tech GSA Graduate Research Symposium.

Wen Z., D. Pyle, M. Orders, P. Mallikarjunan, S. Duncan. February 2007. Producing omega-3 fatty acid dairy products from glycerol byproducts derived from biodiesel industry. Location: Blacksburg, VA. Virginia Tech Dean's Environmental Forum.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Rationale and Significance

Glycerol is the major byproduct of the biodiesel production process. As the biodiesel industry is rapidly expanding in America, a glut of crude glycerol is being created. Because it is prohibitively expensive to purify this glycerol into material that can be used in the pharmaceutical, food, or cosmetics industries, biodiesel producers only receive 2.5-5 cents/lb for this glycerol (Johnson and Taconi, 2007). Therefore, producers must seek new, value-added uses for this glycerol.

It has been shown that glycerol can be used as a carbon source by the algal species *Schizochytrium limacinum* (Yokochi et al., 1998). This species is recognized as a prolific producer of docosahexaenoic acid (DHA) under heterotrophic culture conditions (Nakahara et al., 1996; Yaguchi et al., 1997). This leads us to investigate the possibility of using biodiesel-derived crude glycerol to culture this omega-3 fatty acid producing algal species. To the best of our knowledge, no similar work has been conducted. Using this crude glycerol as a carbon source for microalgal fermentation to produce high-value omega-3 products will provide a new way to utilize this waste product.

DHA is an important omega-3 fatty acid that is known to have various beneficial effects in human health. It has been shown to fight the effects of Alzheiemer's and other neurological diseases and it has been shown to contribute to good cardiovascular health (Simopoulos, 2002). It is also important in infant brain and retinal development (Innis, 2007). Therefore, there is increasing interest in using this fatty acid as a supplement in human foods or as an ingredient in animal feeds. In fact, over 90% of all infant formula sales in the United States are of formula fortified with omega-3 fatty acids (Gerdes, 2008).

Currently, the main source of omega-3 fatty acids is fish oil. Algal biomass is superior to fish oil because it does not have a fishy taste and it can be used as an ingredient in vegetarian foods (Gerdes, 2008). Furthermore, overfishing is depleting fish stocks and is causing a rapid increase in the price of fish oil (New and Wijkström, 2002). This rapid rise in price is causing certain industries to seek new sustainable sources of DHA. For example, in order to produce

omega-3 rich fish, the aquaculture industry is seeking new sources of DHA-rich ingredients for use in fish feed.

Utilizing crude glycerol to produce algal biomass high in DHA presents a win-win situation for the biodiesel industry and the aquaculture industry. A useful method for the disposal of crude glycerol will be created while producing a safe and sustainable source of an important omega-3 fatty acid that can be used as a feed supplement in aquaculture operations.

1.2 Hypothesis

Crude glycerol from the biodiesel industry can be used as a suitable carbon source for the DHA-producing microalgal species *Schizochytrium limacinum*. Furthermore, the algal biomass will have a nutritional quality equal to that of biomass grown using glucose as a carbon source.

1.3 Objectives

The specific objectives of this project are to:

1) demonstrate that crude glycerol can be used as a carbon source for *S. limacinum*,
2) investigate the effects of impurities within the glycerol on algal DHA production,
3) optimize culture conditions to maximize algal DHA production when using crude glycerol as a carbon source in flask culture, and
4) characterize the algal biomass produced from crude glycerol in terms of nutritional quality.

1.4 References

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Chapter 2: Literature Review

2.1 Waste Glycerol from Biodiesel Manufacturing

The United States has been experiencing historic highs in energy prices and is now showing the desire to use greener fuels to become energy independent. Biodiesel as an alternative fuel has attracted increasing attention in recent years. While biodiesel is currently produced around the country, the process produces waste glycerol. If this waste stream can be utilized economically, the biodiesel production process will become more profitable and more prevalent.

2.1.1 Biodiesel Production

Biodiesel is made through a catalyzed chemical reaction (transesterification) between oils or fats and an alcohol (usually methanol) (Figure 2-1). Common feedstocks are pure vegetable oil (e.g., soybean, canola, sunflower), rendered animal fats, or waste vegetable oils. Strong bases such as sodium hydroxide (NaOH) or potassium hydroxide (KOH) are commonly used as catalysts. The glycerol backbone of the triglyceride remains as a waste product after the reaction is completed.

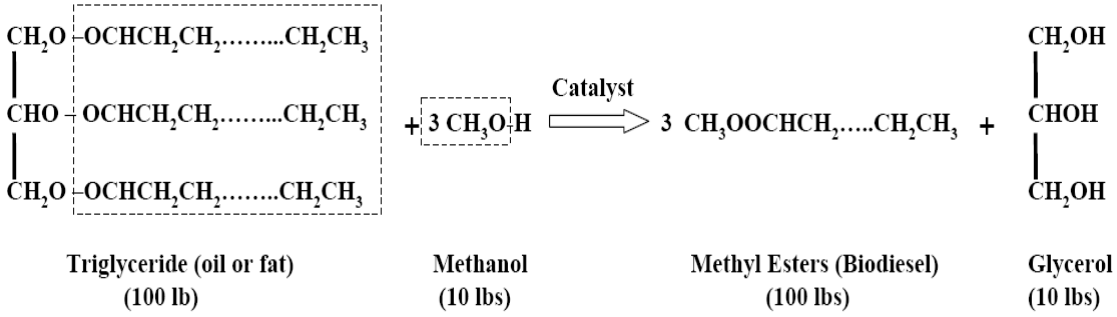

Figure 2-1. Transesterification reaction to produce biodiesel.

The National Biodiesel Board has projected an annual production of biodiesel in 2007 of 450 million gallons, a sharp increase from less than 100 million gallons prior to 2005 (NBB, 2008). As biodiesel production skyrockets, the market is being flooded with crude glycerol. Indeed, crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006 (Johnson and Taconi, 2007; Yazdani and Gonzalez, 2007) because the current U.S. demand for

glycerol is not large enough for all of this excess glycerol. It is clear that new uses for biodiesel-derived crude glycerol are needed.

2.1.2 Crude Glycerol Composition

The crude glycerol produced during the biodiesel production process is impure and of little economic value. The impurities include methanol and soaps. Biodiesel producers use excess methanol to drive the chemical transesterification and do not recover all of the methanol. Therefore, it is present in the glycerol layer. Also, free fatty acids present in the initial feedstock can react with the base to form soaps that are soluble in the glycerol layer. In addition to methanol and soaps, crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulfur (Thompson and He, 2006).

It has been reported that glycerol makes up anywhere from 65% to 85% (w/w) of the crude glycerol streams (Gonzalez-Pajuelo et al., 2005; Mu et al., 2006). The remaining weight in the crude glycerol streams is mainly methanol and soaps (Thompson and He, 2006). The wide range of the purity values can be attributed to different glycerol purification methods used by the biodiesel producers and the different feedstocks used in biodiesel production. For example, Thompson and He (2006) have characterized the glycerol produced from various biodiesel feedstocks. It was found that the crude glycerol from any feedstock is generally between 60 and 70 % (wt) glycerol. Mustard seed feedstocks had a lower level (62%) of glycerol, while soy oil feedstock had 67.8 % glycerol and waste vegetable had the highest level (76.6 %) of glycerol.

Thompson and He (2006) also investigated the elemental composition of crude glycerol. The elements present in the glycerol produced from most feedstocks (such as mustard seeds, canola, soybean, and waste vegetable oil) were similar. Calcium was in the range of 10-20 ppm, magnesium was 3-7 ppm, phosphorous was 10-60 ppm, and sulfur was 14-21 ppm. It should be noted that when crambe, an oilseed crop, was used as feedstock, the crude glycerol contained the same elements, but at vastly different concentrations. Schröder and Südekum (1999) have also reported the elemental composition of crude glycerol from rapeseed oil feedstock. Phosphorous was found to be between 1.05 % and 2.36 % (w/w) of the crude glycerol. Potassium was between 2.20 % and 2.33%, while sodium was between 0.09% and 0.11%. Cadmium, mercury, and arsenic were all below detectable limits.

2.1.3 Glycerol Uses

Because there is a glut of this impure glycerol, there have been many investigations into alternative uses for it. Combustion, composting, animal-feeding, thermo-chemical conversions and biological conversion methods for glycerol usage and disposal have all been proposed.

Some simple methods for the usage of glycerol have been proposed. For example, Johnson and Taconi (2007) reported that combustion of crude glycerol is a method that has been used for disposal. However, this method is not economical for large producers of biodiesel. It has also been suggested that glycerol can be composted (Brown, 2007) or used to increase the biogas production of anaerobic digesters (Holm-Nielsen et al., 2008).

DeFrain et al. (2004) attempted to feed biodiesel-derived glycerol to dairy cows in order to prevent ketosis, but found that it was not useful. Also, Lammers et al. (2008) studied supplementing the diet of growing pigs with crude glycerol. This study found that the metabolizable to digestible energy ratio of glycerol is similar to corn or soybean oil when fed to pigs. Therefore, the study concludes that "crude glycerol can be used as an excellent source of energy for growing pigs", but also cautions that little is known about what the impacts of impurities in the glycerol may be. Furthermore, Cerrate et al. (2006) have had some success with feeding glycerol to broiler chickens. Birds fed 2.5 % of 5% glycerine diets had higher breast yield than the control group, but the authors caution that there is still concern about methanol impurities within the glycerol.

These simple methods may be useful in disposing of excess glycerol, but higher-value processes for glycerol utilization should also be investigated. For example, glycerol can be thermochemically converted into propylene glycol (Alhanash et al., 2008; Dasari et al., 2005), acetol (Chiu et al., 2006), or a variety of other products (Johnson and Taconi, 2007).

Glycerol can also serve as a feedstock in fermentation processes. For example, Lee et al. (2000) have used glycerol in the fermentation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid. Glycerol has also been used as a carbon source in the fermentation of *E. coli*. This leads to a mixture of products such as ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi et al., 2006).

Furthermore, Cortright et al. (2002) have described an aqueous phase reforming process that will transform glycerol into hydrogen that could be used as a fuel. Virent Energy Systems is

currently trying to commercialize this technology and claim that sodium hydroxide, methanol, and high pH levels within crude glycerol help the process (Nilles, 2005).

Overall, there has been much research into using thermochemical processes or using various microorganisms to create valuable industrial products using glycerol as a building block. Given the current glut of glycerol in the American market, due to the booming biodiesel industry, all of these avenues should continue to be investigated and commercialized if feasible.

2.2 Omega-3 Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids (omega-3 fatty acids) are fatty acids containing two or more double bonds, with the last double bond located at the 3rd carbon atom from the methyl end of the fatty acid chain. α – linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) are the most common omega-3 fatty acids. The chemical structures of these essential fatty acids are shown in Figure 2-2. These fatty acids have a host of health benefits that have led to much research about their biosynthesis, natural sources, and production.

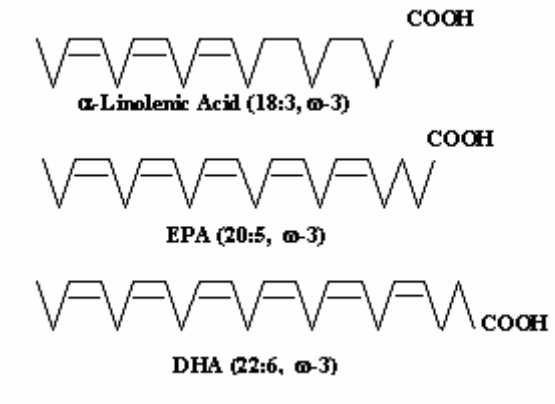

Figure 2-2. Chemical structures of α-linolenic acid, EPA, and DHA.

2.2.1 Health Benefits of Omega-3 Fatty Acids

Omega-3 fatty acids have proven beneficial effects in the prevention of cardiovascular diseases, cancers, Alzheimer's and schizophrenia. In particular, DHA plays an important role in infant brain and retinal development. As essential fats for humans, omega-3 fatty acids need to be added to the diet in order to maintain the body and mind in peak condition.

Cardiovascular Diseases

Omega-3 fatty acids have been shown to have beneficial effects on the cardiovascular system. The risks of suffering from diseases such as diabetes, obesity, asthma, and others have been shown to decrease with increasing omega-3 consumption (Simopoulos, 2002). Recent studies have shown that omega-3 fatty acids may be incorporated into cardiac cell membranes, leading to cardioprotective effects against several diseases (Masson et al., 2007). These effects can be maintained by "occasional fish consumption (e.g., 1-2 times per week)" as the washout period for omega-3 fatty acids is relatively long. Heart health can be maintained by a modest dose of 1g/day of omega-3 fatty acids (Masson et al., 2007).

Schacky and Harris (2007) have proposed using an "omega-3 index" as an indication of overall heart health. Their research has shown that measuring the combined percentage of DHA and EPA out of the total fatty acids in red blood cell membranes is as accurate as the currently used low-density lipoprotein cholesterol measurement for heart health. Patients with higher omega-3 indices are less likely to experience a sudden cardiac death.

Cancers

The beneficial effects of omega-3 fatty acids towards cancers were first associated with the fact that a low incidence of breast cancer occurred among Eskimos whose diet contained high levels of omega-3 fatty acids due to fish consumption (Nettleton, 1995). Many studies have since been undertaken to further explore the beneficial effects of omega-3 fatty acids on the treatment of various cancers.

Overall, omega-3 fatty acids clearly have beneficial effects against several cancers. The mechanisms that lead to these effects are not fully understood, but it is clear that omega-3 fatty acids can be useful in fighting various cancers. For example, Hering et al (2007) found that the proliferation of pancreatic cancer cells is inhibited after treatment with omega-3 fatty acids. It is thought that the omega-3 fatty acids may restore certain regulatory processes within the cells. With these processes restored, the cells have "restored apoptosis" (i.e. the cells are no longer chemoresistant) and can be fought by the immune system or traditional cancer treatments (Hering et al., 2007).

Omega-3 fatty acids can also help prevent colorectal cancer (Nowak et al., 2007). Mice treated with high levels of omega-3 fatty acids showed "reduced colon inflammation and

tumorigenesis." Therefore, it is recommended that humans consume omega-3 fatty acids to prevent colon cancer instead of using anti-inflammatory drugs that have long-term side effects.

In terms of breast cancer, Hardman (2007) has shown that when mice implanted with human breast tumors consumed canola oil (high in ALA), tumor growth was slowed and overall weight gain remained normal. Mice consuming corn oil suffered the full effects of cancer.

Psychiatric Effects: Alzheimer's and Schizophrenia

Alzheimer's is a devastating disease affecting the elderly. While no clinical trials have been performed on human subjects, it has been shown that withholding DHA from the diets of rats led to a deficit in higher order learning ability (Catalan et al., 2002 and Hashimoto et al., 2002), similar to the effects of Alzheimer's. Furthermore, Little et al. (2007) found that aged rats given a daily dose of 10 mg of DHA experienced less age-related impairment in long-term potentiation than control rats. This effect was attributed to changes in brain membrane fluidity caused by the DHA.

Besides having effects on aging minds, omega-3 fatty acids have been shown to have other effects on the brain. Branchey and Branchey (2008) found that substance abusers that were treated daily with 3 grams of omega-3 fatty acids for 3 months became less angry or aggressive than patients given a placebo. This result could be due to modification of serotonergic neurotransmitters. Increasing the serotonin level in the brain eventually resulted in less aggressiveness in patients (Branchey and Branchey, 2008).

Studies have also shown that omega-3 fatty acids have beneficial effects on treating human schizophrenia. For example, Peet and Stokes (2005) state that "five of six double-blind, placebo-controlled trials in schizophrenia" reported therapeutic benefits from omega-3 fatty acid supplementation. These beneficial effects are due to alterations in the fatty acid composition of brain membranes that lead to changes in "several intra-neural signal transduction systems" (Peet and Horrobin, 2002).

Infant brain and vision development

DHA plays an important role in the development of the brain and vision in infants. Makrides et al. (1995) showed that infants fed with DHA-supplemented formula exhibited improved visual acuity over infants fed with standard formula. However, infants that were

breastfed exhibited even higher performance levels, perhaps due to some unknown ingredients in the maternal milk.

The beneficial effects of these dietary omega-3 fatty acids will continue for years after infancy. Four-year old children given DHA and ARA-supplemented formula during the first 17 weeks of life were shown to have "visual acuity and Verbal IQ scores that did not differ significantly from breast-fed children." Children who received formula with no supplementation had significantly lower scores, showing the omega-3 fatty acid supplementation is of utmost importance at the early stages of life (Birch et al., 2007).

2.2.2 Biosynthesis of Omega-3 Fatty Acids

The synthesis of omega-3 fatty acids occurs in two stages. The first stage is *de novo* synthesis of acetate to short chain fatty acids. In most cases, the resultant short chain fatty acid is oleic acid (C18:1, ω-9) (Wen, 2001).

After oleic acid is synthesized, longer chain polyunsaturated fatty acids are created by a series of desaturation and elongation reactions shown in Figure 2-3. Several microorganisms posses these pathways and have the ability to naturally produce omega-3 fatty acids. Generally, higher plants and animals do not posses these pathways. Some organisms, including humans, do have the ability to make EPA or DHA from dietary ALA, but the conversion rates are very low (Innis, 2007). Thus, these omega-3 fatty acids need to be taken in from dietary sources.

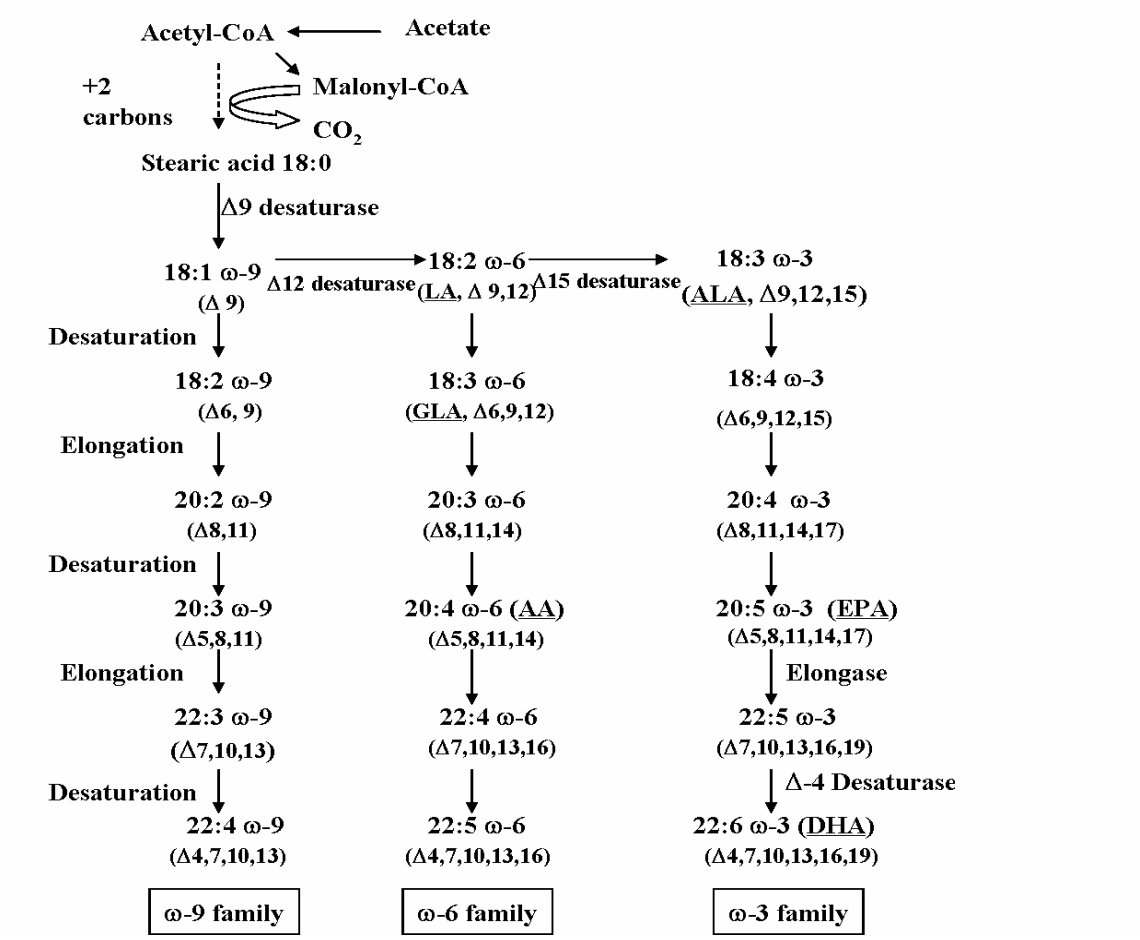

Figure 2-3. Biosynthetic pathways for formation of long chain fatty acids (Wen, 2001).

2.3 Sources of Omega-3 Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids have a multitude of health benefits and should be included in the diets of humans. In fact, the American Dietetic Association and Dietitians of Canada officially recommend that 20 to 35% of daily energy should come from dietary fat, with an emphasis on consuming omega-3 fatty acids (Kris-Etherton and Innis, 2007). These fatty acids can be obtained from a variety of sources.

2.3.1 Traditional Sources

The major source of omega-3 fatty acids available on the market is fish oil, which is usually consumed in the form of cooked fish, fish oil capsules, or food with fish oil additives (Alonso and Maroto, 2000). In fact, the American Heart Association recommends that healthy consumers eat two fatty fish meals per week and encourages patients with coronary heart disease

to consume 1 g/day of EPA and DHA (Kris-Etherton et al., 2002). Furthermore, in the aquaculture industry, fish oils also represent the major form of omega-3 fatty acids that are used in dietary formulations for farm-raised marine fish.

It should be noted that fish oil as an omega-3 fatty acid source has several limitations. First, there is concern about consumer acceptance of fish oils or fish oil capsules because of particular taste and odor. For example, Kris-Etherton et al. (2002) reported that consuming over 1 g/day of fish oil-derived omega-3 fatty acids will likely lead to a fishy aftertaste and possibly to moderate gastrointestinal upset in a percentage of the population. There are also concerns about heavy metal contamination of fish and fish oils. The Environmental Protection Agency and the Food and Drug Administration recommend that women who are pregnant or may become pregnant, nursing mothers, and infants avoid eating fish and shellfish that may be high in mercury (EPA, 2004). Table 2-1 shows the amount of mercury and omega-3 fatty acids consumed when eating common seafood products.

Table 2-1. Levels of mercury and omega-3 fatty acids in common seafood dishes (AHA, 2008).

Seafood	Mean Mercury Level (ppm)	Omega-3 Fatty Acid Content (g/3 oz. serving)
Canned tuna (light)	0.12	0.26-0.73
Shrimp	< 0.01	0.27
Pollock	0.06	0.46
Salmon	0.01	0.68-1.83
Cod	0.11	0.13-0.24
Catfish	0.05	0.15-0.20
Clams	<0.01	0.24
Flounder or Sole	0.05	0.43
Crabs	0.06	0.34-0.40
Scallops	0.05	0.17
Lobster	0.31	0.07-0.41
Grouper	0.55	0.21
Halibut	0.26	0.40-1.00
Oysters	<0.01	0.37-1.17
Mahi Mahi	0.19	0.12
Herring	0.04	1.71-1.81

Another concern with using fish oil is the sustainability of this natural source, which is of particular concern to the aquaculture industry. Since 1984 production of fish oil has remained stable, with an average annual production of around 1.3 million tons, but demand for fish oil has

substantially increased, leading to rapid upward price shifts for this commodity. At present, approximately 50 % of this fish oil is used by the aquaculture industry (Tidwell and Allan 2001). The Food and Agriculture Organization of the UN predicts that global fish oil demand in 2015 will be 145% of historical global production capacity and will only continue to grow (New and Wijkström, 2002).

2.3.2 Alternative Sources

Because of the concerns associated with the use and consumption of fish and fish oil as sources of omega-3 fatty acids, there has been extensive research into developing alternative sources of these important fatty acids.

Fish do not naturally produce omega-3 fatty acids *de novo*. Microorganisms such as algae or fungi are the primary producers of omega-3 fatty acids because they have the necessary biosynthetic pathways. These microorganisms have been extensively studied as potential sources of fatty acids. The fatty acids from these microbial sources can be extracted and used as ingredients in omega-3 fortified foods (Simopoulos, 1999) or the organisms can be used directly as feed additives for animals such as laying hens (Chin et. al, 2006) or farm-raised fish (Harel et al., 2002). Recent studies have also investigated the development of genetically altered higher plants and animals to produce omega-3 fatty acids. Currently, microbial production of omega-3 fatty acids is still the preferred alternative omega-3 source, although research on developing transgenic plants or animals for omega-3 production will continue.

Microorganisms

Many microalgae are capable of producing high level of omega-3 fatty acids. Species such as *Nitzschia* spp., *Nannochloropsis* spp., *Navicula* spp., *Phaeodactylum* spp., and *Porphyridium* spp. have been studied for EPA production. A majority of these algal species are autotrophic and need to be cultured in photobioreactors (Ward and Singh, 2005). The costs associated with these growing conditions are not favorable for industrial use of these algae. Some algal species, such as *Nitzschia lavis*, can produce EPA under heterotrophic conditions (Wen, 2001). Among DHA-producing algae, *Crypthecodinium cohnii* and *Schizochytrium* spp. are commonly used species capable of producing high levels of DHA under heterotrophic conditions. *C. cohnii* has been studied as a producer of DHA by several research groups (Ward

and Singh, 2005) and is the microorganism used by Martek Biosciences to commercially produce DHA (Martek, 2008). *Schizochytrium* spp. have also been identified and studied as producers of DHA. Biomass from these algae is currently sold as a DHA supplement for fish feed by Aquafauna Bio-Marine Inc. (Hawthorne, CA; http://www.aquafauna.com/Profiles-AlgaMac-3000.htm) and Advanced BioNutrition Corp. (Advanced BioNutrition Corp. (Columbia, MD; http://www.advancedbionutrition.com/html/abn_dha.pdf).

Fungi are another group of microorganisms capable of producing high levels of omega-3 fatty acids. In fact, *Mucor circinelloides* has been used commercially by JE Sturge of the United Kingdom, but prices of plant oils and a small market demand made it uneconomical (Ward and Singh, 2005). *Mortierella alpina* has been identified and studied as a producer of both ARA and EPA. It has been reported that high ARA yields, ranging from 4.5 g/L to 11.1 g/L, have been achieved by this organism (Ward and Singh, 2005). Fungal species like *Pythium*, *Thraustochytrium*, and *Entomophthora* contain omega-3 fatty acids anywhere from 1% to 6.4% ("fresh weight basis") (Gill and Valivety, 1997).

Transgenic Plants

Research has been undertaken to genetically engineer land plants to produce omega-3 fatty acids. Oilseed crop plants, such as soy, canola, palm, peanut, and others are the main source of oils and fats in the human diet, but they are all high in 18-carbon omega-6 fatty acids (Damude and Kinney, 2007). Therefore, initial research efforts have been undertaken to manipulate the natural fatty acid metabolic pathways of these plants, so that the plant fatty acid profiles can be changed. For example, Robert et al. (2005) have used the model plant *Arabidopsis thalina* to produce ARA, EPA, and DHA. This plant was chosen because its seed oil contains significant amounts of linoleic and alpha-linoleic acid precursors that are necessary for long chain-polyunsaturated fatty acid synthesis. By expressing genes for certain elongases and desaturases, "the first successful transgenic assembly of a complete and effective DHA biosynthetic pathway in seeds" was accomplished.

The introduction of entirely new metabolic pathways through advanced gene expression technology has also been pursued (Damude and Kinney, 2007). Using pathways from omega-3-producing microorganisms provides an alternative method for production of EPA or DHA by oilseed crop plants. Using transgenic plants provides an interesting alternative source for

producing omega-3 fatty acids, but microbial based omega-3 sources remain the most feasible and reliable source in the near future.

Transgenic Animals

Traditionally, the only way to increase the omega-3 content of animal meat has been to supplement the diet with omega-3 sources (flaxseed oil, fish oil, etc.). However, a recent study has reported the development of transgenic animals capable of producing high levels of omega-3 fatty acids. Lai et al. (2007) report that a gene encoding a n-3 fatty acid desaturase can be implanted into pigs, allowing piglets to convert n-6 fatty acids to n-3 fatty acids. The gene was only successfully expressed in 6 of 10 live piglets born, but these piglets showed a significantly reduced ratio of omega-6 to omega-3 fatty acids. It should be noted that even though this research is promising, technical problems with the cloning of animals and consumer concerns will be hurdles to overcome before this avenue can be commercially implemented.

2.4 The microalga *Schizochytrium limacinum* as a DHA producer

Among the microalgae identified as producers of omega-3 fatty acids, *Schizochytrium limacinum* (ATCC MYA-1381) is a prolific producer of DHA. This algal species has been extensively studied for its DHA production potential.

2.4.1 Discovery and Isolation

The alga was first screened from a seawater sample from a mangrove area in the Yap Islands of Micronesia in 1994. Isolation and studies of the organism showed that it differed from previous *Schizochytrium* species in "its limaciform amoeboid cells, the size of zoospores…and the assimilation profile of carbon sources." (Honda et al.,1998). Further studies showed that this strain was a prolific producer of docosahexaenoic acid under heterotrophic culture conditions (Nakahara et al., 1996; Yaguchi et al., 1997).

2.4.2 Culture Conditions

The fact that this species is a prolific producer of DHA led to much research investigating various culture conditions in an attempt to optimize the production of DHA by this species. Carbon source, nitrogen source, temperature, salinity, and other factors have been investigated.

Glucose has conventionally been used as the carbon source for heterotrophic culture of this species. Yokochi et al. (1998) investigated several alternative carbon and nitrogen sources for this algal culture. It was found that glucose, fructose, glycerol, and oleic acid led to high levels of cell growth (over 10 g/L of cell biomass) and DHA production (over 0.5 g/L of DHA), while saccharose, lactose, maltose, starch, and linseed oil resulted in low levels of DHA production. Of the various nitrogen sources tested, corn steep liquor or yeast extract led to the highest DHA production levels (over 0.5 g/L). From this work, it is clear that various carbon sources (including glycerol) or nitrogen sources can be used to support algal growth and DHA production. In terms of medium salinity and culture temperature, it was found that maximum cell dry weight can be achieved in mediums ranging from 50% to 200% of normal seawater concentration. The algae species grew well between 20°C and 30°C. Major growth inhibition occurred at temperature extremes on both ends of the spectrum (Yokochi et al., 1998).

The above results clearly show that *Schizochytrium limacinum* is an ideal species for DHA production. While this species has been previously studied, there is still ample room for optimization of culture conditions and for testing of alternative nutrient sources. The fact that this species is capable of using glycerol as a carbon source leads to exploration into the feasibility of using biodiesel-derived crude glycerol for growing this alga. As the market is currently flooded with a glut of crude glycerol from biodiesel plants, using this glycerol as a carbon source for *Schizochytrium limacinum* provides an important outlet for this waste product. This effort will help yield a more affordable strategy for producing omega-3 fatty acids from the alga *Schizochytrium limacinum* while economically helping the rapidly-growing biodiesel industry.

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Chapter 3:
A feasibility study of producing docosahexaenoic acid from biodiesel-waste glycerol by *Schizochytrium limacinum*

3.1 Abstract

Crude glycerol is the primary by-product in the biodiesel industry, which is too costly to be purified into to higher quality products used in the health and cosmetics industries. This work investigated the potential of using the crude glycerol to produce docosahexaenoic acid (DHA, 22:6 n-3) through fermentation of the microalga *Schizochytrium limacinum*. The results showed that crude glycerol supported alga growth and DHA production, with 75 to 100 g/L concentration being the optimal range. Among other medium and environmental factors influencing DHA production, temperature, trace metal (PI) solution concentration, ammonium acetate, and NH_4Cl had significant effects ($P < 0.1$). Their optimal values were determined 30 mL/L of PI, 0.04 g/L of NH_4Cl, 1.0 g/L of ammonium acetate, and 19.2°C. A highest DHA yield of 4.91 g/L with 22.1 g/L cell dry weight was obtained. The results suggested that biodiesel-derived crude glycerol is a promising feedstock for production of DHA from heterotrophic algal culture.

3.2 Introduction

Biodiesel as an alternative fuel has attracted increasing interest worldwide in recent years. In the United States, for example, the biodiesel production capacity has increased sharply from less than 100 million gallon/year in 2005 to an expected 580 million gallon/year in 2007 (NBB, 2008). During the biodiesel production process, oils/fats (triglycerides) are mixed with methyl alcohol and alkaline catalysts to produce esters of free fatty acids, with glycerol as a primary by-product. In general, production of 100 kg of biodiesel yields approximately 10 kg of glycerol (Van Gerpen et al., 2004), which is impure and of low economic value. With the even-growing production of biodiesel and by-product glycerol, it has been suggested that the open market value of crude glycerol may eventually stabilize at the price of $0.05/lb, but the cost to refine this crude glycerol will be approximately $0.20/lb (DOE, 2004). Considering the fact that the projected production volume of crude glycerol will exceed the present commercial demand for purified glycerol, and that purification for sales of pharmaceutical-grade glycerol will not be

a viable option for the biodiesel industry; some alternative uses for the glycerol will need to be found.

Converting glycerol into value-added products provides an alternative for glycerol disposal and for its surplus problems. Through thermo-chemical processes, glycerol can be converted into propylene glycol (Dasari et al., 2005) and acetol (Chiu et al., 2006). It can also serve as carbon source in fermentation processes to produce various products such as 1,3 propanediol (Gonzalez-Pajuelo et al., 2006; Zheng et al., 2006), lipids (Papanikolaou and Aggelis, 2002; Meesters et al., 1996), or pigments (Narayan et al., 2005). Anaerobic fermentation of glycerol by *E. coli* also generates a mixture of products such as ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi et al., 2006).

In this work, crude glycerol was targeted for production of docosahexaenoic acid (DHA, 22:6 n-3) through heterotrophic microalgal fermentation. DHA is an important omega-3 polyunsaturated fatty acid (ω-3 PUFA) with medically established therapeutic capabilities against cardiovascular diseases, cancers, schizophrenia, and Alzheimer's (Simopoulos,1999; Ursin, 2003). It is also an essential nutrient during early human development (Innis, 1994; Makrides and Gibson, 1995). Currently, fish oil is the major commercial source of ω-3 PUFA. However, there are some limitations for fish oil use such as peculiar taste, odor, stability problems, and resource sustainability of fish stocks (Barclay et al., 1994). As marine algae is the primary producer of ω-3 PUFA in the natural environment, replacing fish oil with ω-3 PUFA enriched algae provides an excellent opportunity to develop alternative ω-3 PUFA (Ward and Singh, 2005). The ω-3 PUFA can be extracted from the algae biomass and used in infant formulae, food, and pharmaceuticals; or the algae biomass can be used directly as a fish feed additive in the aquaculture industry (Harel et al., 2002). The alga *Schizochytrium limacinum* has been reported as an excellent DHA producer (Yokochi et al., 1998; Chin et al., 2006; Morita et al., 2006; Zhu et al., 2007), as well as being capable of producing a high level of DHA when growing on glucose or glycerol (Yokochi et al., 1998). When growing on glucose, its zoospores were quickly induced and lipid bodies formed during the synchronous growth (Morita et al., 2006). However, growing the alga on biodiesel-derived crude glycerol was not investigated. The objective of this work was to investigate the potential of using this alga to produce DHA from biodiesel industry-derived crude glycerol.

3.3 Materials and methods

3.3.1 Algal strain, medium and culture conditions

The alga *Schizochytrium limacinum* SR21 (ATCC MYA-1381) was used in the studies. The cells were maintained in ATCC 790 By+ medium containing 5 g/L glucose, 1.0 g/L yeast extract and 1.0 g/L peptone in an artificial seawater. The artificial seawater culture solution consisted of (per liter) 18 g NaCl, 2.44 g $MgSO_4$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, 1 g Tris buffer (Sigma Co.), 0.027 g NH_4Cl, 15.0 x 10^{-8} g vitamin B_{12}, 10 mL/L PI metal solution, and 3 mL/L chelated iron solution (Starr and Zeikus, 1993). The pH was adjusted to 8.0 before autoclaving the medium at 121°C for 15 min. The cells were grown in 250-mL Erlenmeyer flasks each containing 50 mL of medium and incubated at 20°C in an orbital shaker set to170 rpm. Subcultured cells were used as inoculum for future studies. The inoculum size was 10% of the total liquid volume in each flask. In later cell growth and DHA production studies, glucose was replaced as the carbon source by either laboratory grade glycerol or pretreated glycerol obtained from biodiesel industry. All the other components were the same as those used in the subculture. For each experimental condition, three replicates were used, and the standard deviation was calculated.

3.3.2 Crude glycerol pretreatment

Crude glycerol was obtained from both the Virginia Biodiesel Refinery (West Point, VA, USA) and Seattle Biodiesel (Seattle, WA, USA). Both refineries used alkali-catalyzed transesterification to produce the biodiesel from soybean oil. It was found that when crude glycerol was directly mixed with artificial seawater, soaps precipitated from the liquid, which later proved detrimental to cell growth (data not shown). To avoid this phenomenon, the crude glycerol was pretreated prior to use in the algal culture. The pretreatment protocol was as follows: (1) the glycerin was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, (2) the pH of the fluid was adjusted from 12 to around 4 with hydrochloric acid to convert the soluble soap into insoluble free fatty acids which precipitated from the liquid, (3) precipitated solids, which floated to the top of the liquid, were separated from the crude glycerol solution by centrifugation at 5000 rpm, and (4) after separation, other nutrients (mineral

salts, nitrogen source, etc.), with additional water were added to adjust the nutrient level (including glycerol) to a desired level.

3.3.3 Analysis

To determine cell dry weight, 5 mL cell suspension sample was transferred to a pre-weighed centrifuge tube and centrifuged at 3444 g for 5 min. The cell pellet was then washed twice with distilled water, and then dried at 80^{o}C to constant weight. Glucose concentration was determined by the 3,5-dinitrosalicylic method (Miller, 1959). Glycerol and methanol concentration was determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc. Columbia, MD) with a pulsed refractive index detector. An Aminex HPX-87H (Bio-Rad, Sunnyvale, CA) column was used with 0.1% (v/v) H_2SO_4 solution as mobile phase. The flow rate was controlled at 0.6 mL/min, and the column temperature was 65^{o}C.

Algal cells were harvested and freeze-dried for fatty acid analysis. Fatty acid methyl esters (FAME) were prepared from dried algal biomass according to the protocol developed by Indarti et al. (2005). In short, the method involves a 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) being added into a tube containing ~20 mg of dried cell biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The tubes were heated in a water bath at 90 ºC for 40 min, and then later cooled down to room temperature, at which point 1 mL of distilled water was added. The liquid in the tubes were thoroughly mixed through a vortex for 1 min, and then settled for separation of the two phases. The lower phase containing the FAME was transferred to a clean vial and dried with anhydrous Na_2SO_4. One-half mL dried solutions were transferred into a vial and analyzed using gas chromatography.

A Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Inc. Columbia, MD) was used for FAME analysis. The GC was equipped with a flame-ionization detector and a SGE Sol Gel-Wax^{TM} capillary column (30m×0.25mm×0.25um). The injector was kept at 250^{o}C, with an injection volume of 1µl by split injection mode (ratio: 10:1). The profile of the column temperature was as follows: 80^{o}C for 0.5 min; raised to 175^{o}C at 30^{o}C/min; raised to 260 ^{o}C at 5^{o}C/min; maintained for 6 min; raised to 280^{o}C at 30^{o}C/min; maintained for 1 min. Helium was used as the carrier gas. The detector temperature was kept at 300^{o}C. The fatty acids of the algae sample were identified by comparing the retention times with those of standard fatty acids (Sigma, MO). To quantify the fatty acids, first, the response factor of each fatty acid was determined by GC-running the FAMEs of the fatty acid and the internal standard at equal

amount, and comparing the peak area of the fatty acid to that of the internal standard (C17:0). Here, we set response factor of internal standard (17:0) as 1, the response factors of the representative fatty acids were determined as 0.99 for C14:0, 0.99 for C16:0, 0.97 for C18:0, 0.87 for C22:5 and 0.87 for C22:6. Based on these response factors, the fatty acids of the algal sample were quantified by comparing their peak area with that of the internal standard (C17:0), and correcting by their response factors.

3.3.4 Experimental design and data analysis

Plackett-Burman design

A Plackett-Burman design was used to screen the factors having significant effects on the DHA production from crude glycerol. The variables to be evaluated are listed in Table 3-1, including various medium components, pH, and temperature. Each independent variable was investigated at a high (+) and a low (−) level. The low levels (−) of medium components were taken as their concentrations in ATCC 790 By+ medium. The high levels (+) were empirically set based on previous experience. The design matrix and data analysis were similar as previously reported by Wen and Chen (2001). In summary, there were 20-runs of experiment, with 15 variables and 4 dummy variables (D1~D4) (Table 3-2). The effect of each variable (E_i) on response was determined by subtracting the average response of the low level from the high level. The effects of dummy variables reflect the standard error of the experiments, which can be used to derive the significance level (Wen and Chen, 2001). In this work, the software *Design-Expert* (Stat-Ease Inc. Minneapolis, MN) was used for analyzing the significance level through an *F*-test. Here, only $P<0.10$ were accepted as significant factors, which were further optimized in the following central composite design.

Table 3-1. Variables to be screened in Plackett-Burman design.

	Variables	Unit	Low (-)	High (+)
A	NaCl	g/L	9	18
B	KH_2PO_4	g/L	1	4
C	Corn steep liquor	g/L	5	10
D	Ammonium acetate	g/L	0.6	1.2
E	$MgSO_4.7H_2O$	g/L	2.4	4.8
F	KCl	g/L	0.6	1.2
G	$NaNO_3$	g/L	1	2
H	$CaCl_2.2H_2O$	g/L	0.3	0.6
I	Tris Buffer	mL/L	10	20
J	NH_4Cl	g/L	0.027	0.054
K	Vitamin B12	mL/L	1	2
L	PI metal solution	mL/L	10	20
M	Chelated iron solution	mL/L	3	6
N	Initial medium pH	-	6.00	8.00
O	Temperature	ºC	20	28

Table 3-2. The Plackett-Burman design of the variables (Table 3-1) with cell dry weight and DHA yield as responses.

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	D1	D2	D3	D4	Cell dry weight (g/L)	DHA yield (g/L)
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	23.6	4.29
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	27.3	5.01
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	25.0	4.50
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	7.1	0.88
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	25.6	4.89
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	3.4	0.05
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	24.4	4.18
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	12.6	2.31
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	10.5	1.68
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	9.4	1.42
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	20.2	2.89
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	24.6	4.54
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	23.8	4.29
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	7.6	0.99
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	16.6	2.34
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	24.7	4.33
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	3.4	0.08
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	3.6	0.05
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	22.2	4.11
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	24.4	4.64

Central composite design

Once the significant factors were identified in the Plackett-Burman design, a central composite design was used to optimize their levels. The design matrix was a 2^4 full factor design combined with 6 central points, and 8 axial points where one variable was set at an extreme level while other variables were set at their central point level (Table 3-3). The coded and real values of each parameter are shown in Table 3-4. Based on experimental results obtained in Table 3-3, the responses (cell dry weight and DHA yield) were correlated as functions of variables by a second-order polynomial equation, i.e.,

$$Y= \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_i x_i^2 + \Sigma\beta_{ij} x_i x_j \qquad (3\text{-}1)$$

where *Y* is the predicted response, β are the coefficients of the equation, and *xi* and *xj* are the coded levels of variables *i* and *j*, respectively. The software *Design-Expert* (Stat-Ease Inc. Minneapolis, MN) was used for this correlation through non-liner regression. The *F*-test was used to evaluate the significance of the models. The biomass and DHA productivity were not used as responses because the two parameters could be well represented by cell dry weight (for biomass productivity) and DHA yield (for DHA productivity) at constant sampling time.

Table 3-3.The central composite design of the significant variables (in coded levels) with cell dry weight and DHA yield as responses

Run	Temp	Ammonium acetate	NH_4Cl	PI	Cell dry weight (g/L)	DHA yield (g/L)
1	-1	-1	-1	-1	14.6	3.30
2	-1	-1	-1	1	15.1	3.33
3	-1	-1	1	-1	15.0	3.18
4	-1	-1	1	1	15.0	3.44
5	-1	1	-1	-1	19.8	4.55
6	-1	1	-1	1	19.1	4.50
7	-1	1	1	-1	20.0	4.73
8	-1	1	1	1	20.4	4.78
9	1	-1	-1	-1	22.9	4.88
10	1	-1	-1	1	21.1	4.22
11	1	-1	1	-1	22.5	4.64
12	1	-1	1	1	22.9	4.75
13	1	1	-1	-1	22.3	4.52
14	1	1	-1	1	24.2	5.26
15	1	1	1	-1	23.2	4.73
16	1	1	1	1	22.0	4.62
17	-2	0	0	0	15.0	2.77
18	2	0	0	0	22.5	3.72
19	0	-2	0	0	23.4	4.57
20	0	2	0	0	24.5	4.37
21	0	0	-2	0	24.1	4.78
22	0	0	2	0	23.4	4.64
23	0	0	0	-2	23.9	5.01
24	0	0	0	2	23.5	4.87
25	0	0	0	0	22.7	4.88
26	0	0	0	0	21.6	4.79
27	0	0	0	0	22.1	5.00
28	0	0	0	0	21.9	4.95
29	0	0	0	0	21.0	4.80
30	0	0	0	0	20.4	4.42

Table 3-4. The coded levels and real values of the independent variables in the central composite design

Variables	Unit	-2	-1	0	+1	+2
Temperature	^oC	14	17	20	23	26
Ammonium acetate	g/L	0.2	0.4	0.6	0.8	1
NH_4Cl	g/L	0.005	0.016	0.027	0.038	0.049
PI metal solution	mL/L	10	15	20	25	30

3.4. Results and discussion

3.4.1 Characteristics of cell growth, nutrient consumption, and fatty acid profile

Figure 3-1 shows the time courses of cell growth and substrate consumption of the algal cultures with 9% (90g/L) of glucose, pure glycerol and crude glycerol being used in the medium, respectively. As shown in Figure 3-1A, the cells grew very well in crude glycerol compared with glucose and pure glycerol. The cell dry weight reached the highest level in day 5 - 6. For all three cultures, there were still relatively high levels of residual glucose or glycerol when the cells ceased growth (Figure 3-1B). The reason may be that the nitrogen sources used here (1g/L tryptone and 1g/L yeast extract) were lower than that reported by Yokochi et al. (1998) (20 g/L corn steep liquor). Thus, nitrogen may be the limiting factor when 90 g/L to 75 g/L glucose or glycerol were used. Cell growth kinetics and DHA production by *S. limacinum* on different substrates were further compared (Table 3-5). It was found that the specific cell growth rate, maximum cell dry weight, and biomass productivity of the crude glycerol-culture was higher than that of the pure glycerol-culture. The growth yield on glycerol ($Y_{x/s}$) was lower than that on glucose, suggesting the algal cells could utilize glucose more efficiently for their growth. The DHA content of the three cultures were similar, however, due to the low biomass in pure glycerol culture, the DHA yield and productivity from the pure glycerol-algal culture were lower than the other two cases.

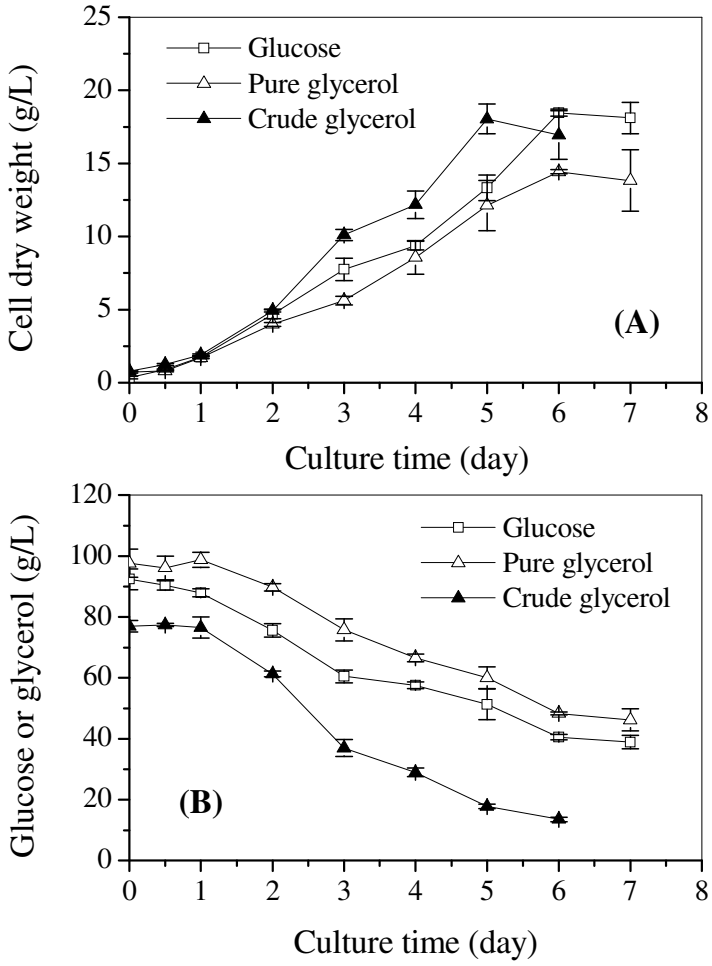


Figure 3-1. Time course of cell growth (A) and substrate consumption (B) of *S. limacinum* with glucose, pure glycerol, and crude glyerol as substrate.

Table 3-5. Cell growth, substrate consumption, and DHA production of *S. limacinum* using glucose, pure glycerol, and crude glycerol as a substrate.

Parameters [a]	Substrate		
	Crude glycerol	Pure glycerol	Glucose
Specific growth rate, μ (day^{-1})	0.685 ± 0.042	0.562 ± 0.009	0.575 ± 0.012
Maximum cell dry weight, X_{max} (g/L)	18.04 ± 1.02	14.43 ± 0.14	18.47 ± 0.25
Biomass productivity (g/L-day)	3.06 ± 0.17	2.41 ± 0.05	3.08 ± 0.06
Growth yield, $Y_{x/s}$ (g/g)	0.284 ± 0.016	0.280 ± 0.003	0.356 ± 0.005
DHA content (mg/g DW)	170.45 ± 11.27	171.37 ± 0.96	165.74 ± 7.82
DHA yield (g/L)	3.07 ± 0.19	2.47 ± 0.03	3.05 ± 0.14
DHA productivity (g/L-day)	0.51 ± 0.04	0.42 ± 0.01	0.51 ± 0.03

[a.] Data are expressed as mean ± SD of three replicates

The fatty acid profile of *S. limacinum* is shown in Table 3-6. As shown in the table, fatty acid compositions obtained from the three algal cultures are very similar. The two fatty acids, C16:0 and C22:6 (DHA) accounted for about 90% of total fatty acid (TFA) content. The alga also contained small amounts of C14:0, C18:0 and C22:5 (DPA). The total fatty acid content was about half of the cell dry weight. The fatty acid profile was similar as those reported previously for this alga culture (Yokochi et al., 1998; Nakahara et al., 1996).

Table 3-6. Fatty acid compositions (% of total fatty acid, TFA) and total fatty acids (TFA) content of S. *limacinum* with glucose, pure glycerol, and crude glycerol as substrate [a]

		Substrate		
Fatty acid	Unit	Crude glycerol	Pure glycerol	Glucose
14:0	%TFA	5.16±0.04	4.29±0.05	4.74±0.19
16:0	%TFA	54.70±0.41	52.45±3.38	55.54±1.79
18:0	%TFA	1.04±0.03	0.47±0.06	0.98±0.02
22:5 (n-6)	%TFA	5.48±0.10	4.97±0.04	5.55±0.20
22:6 (n-3)	%TFA	33.62±0.34	37.82±3.43	33.20±1.35
TFA	mg/g DW	505.69±45.14	447.93±44.14	499.71±31.37

a. Data are expressed as mean ± SD of three replicates.

It has been reported that *S. limacinum* was capable of growing on a variety of carbon sources such as glucose, fructose, glycerol, oleic acid and linseed oil (Yokochi et al., 1998). The alga demonstrated good growth and DHA production with 9% (90 g/L) solutions of glucose or glycerol (Yokochi et al., 1998). When 90 g/L of crude glycerol was used, the "real" glycerol concentration in the medium was about 75 g/l, suggesting that around 16% of impurities (e.g., soap, free fatty acids, excess methanol, un-reacted triglycerides, diglycerides, or monoglycerides) were contained in the crude glycerol.

It was found that methanol accounted for ~12% of the crude glycerol used in this work. When 90 g/L crude glycerol was used, there was approximately 10.8 g/L methanol and 75 g/L "real" glycerol. An additional experiment was performed to test if methanol "contributed" to the best results shown for the crude glycerol extract. Different levels of methanol (0 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L and 50 g/L) were added to the medium containing 75 g/L of pure glycerol; the cell growth of these glycerol-methanol combined cultures was investigated. As shown in Table 3-7, the cell growth was not significantly different (P>0.1) in the methanol range from 0 to 20 g/L, but reduced significantly when methanol concentration reached 50 g/L. It was also found that the alga could not grow when methanol was used as the sole carbon source (data not shown). These results suggest that methanol does not contribute to the cell growth enhancement when cruder glycerol (at 90 g/L) was used as carbon source for algal growth.

Table 3-7. Effects of methanol concentration cell growth of *S. limacinum* [a]

Methanol concentration (g/L)	Cell dry weight (g/L)
0	17.52 ± 0.34
5	18.48 ± 0.49
10	17.28 ± 0.68
15	19.68 ± 1.35
20	18.01 ± 0.40
50	10.40 ± 1.08

[a.] 75 g/L of glycerol was used as carbon source, Data are expressed as mean ± SD of three replicates.

Here, the high algal biomass obtained in the crude glycerol-containing medium might be due to the residual soap contained in crude glycerol. As described in Section 2.3.2 , when crude glycerol (at 90 g/L) was mixed with seawater, soap precipitated from the liquid, which later proved detrimental to cell growth. However, our ongoing experiments show that crude glycerol extract still contains a small amount of soap residual after the pH adjustment. Instead of being detrimental to algal cells, this small amount of soap has beneficial effects on cell growth (data not shown).

As the carbon source, crude glycerol from the biodiesel industry was also used for production of lipids by *Yarrowia lipolytica* (Papanikolaou and Aggelis, 2002) and *Cryptococcus curvatus* (Meesters et. al, 1996). Although a lipid production level was obtained, the major fatty acids of the accumulated lipids were C18:0 (stearic acid) and C18:1 (oleic acid) (Meesters et al., 1996). The results obtained in this work indicate that crude glycerol is a potential good carbon source for algal DHA production. In the following sections, the algal culture conditions including crude glycerol concentration, other medium nutrients, pH and temperature were optimized to achieve a high level of cell growth and DHA production from crude glycerol.

3.4.2 Effects of crude glycerin concentration

The effects of crude glycerol concentration on cell growth and DHA production were investigated by including different levels of crude glycerol (ranging from 75 g/L to 150 g/L) into

artificial seawater, as described in Section 2.3.4. The complex nitrogen sources used in this study were tryptone (1 g/L) and yeast extract (1 g/L).

As shown in Table 3-8, due to the impurities contained in the crude glycerol, the "real" glycerol concentrations were about 15% lower than the corresponding crude glycerol levels. The cell dry weight and biomass productivity were at high levels when crude glycerol was within the range from 75 g/L to 100 g/L, with algal growth being inhibited when the crude glycerol concentration exceeded 100 g/L. Within the glycerol range investigated, DHA content was relatively stable, while DHA yield and productivity had a similar trend as that of cell dry weight.

Table 3-8. Effects of crude glycerol concentration on cell growth and DHA production by *S. limacinum* [a]

Crude glycerol (g/L)	"Real" glycerol (g/L)	Cell dry weight (g/L)	Biomass productivity (g/L-day)	DHA yield (g/L)	DHA productivity (g/L-day)
75	64	18.7± 1.2	3.11±0.20	3.05±0.28	0.50±0.05
100	85	18.6 ± 0.9	3.10±0.15	2.71±0.35	0.44±0.06
125	106	7.7 ± 0.5	1.28±0.08	1.43±0.22	0.23±0.04
150	128	6.3 ± 0.6	1.05±0.10	0.96±0.17	0.16±0.03

[a] Cell dry weight and DHA yield are expressed as mean ± SD of three replicates

It has been reported that when *S. limacinum* was grown in glycerol-containing medium, the optimal glycerol concentration on cell growth and DHA production was within 90 g/L to 120 g/L; with the cells being inhibited when glycerol exceeded 120 g/L (Yokochi et al., 1998). In this study, however, the inhibition occurred at a lower glycerol level (100 g/L of crude glycerol, ca. 85 g/L real glycerol) and the maximum cell dry weight and DHA yield was lower than those reported by Yokochi et al. (1998). The reason might be that 100% seawater salts were used in this work, while 50% seawater salt was used by Yokochi's work. The low salt level provided a lower osmotic pressure and thus, allowed more glycerol to be added. Based on the above speculations, other factors influencing algal DHA production including seawater salts were optimized in the following work, with the crude glycerol level being maintained at 100 g/L.

3.4.3 Optimization of medium components and environmental factors for algal DHA production

Screening the significant factors

Experiments were initiated for screening the significant factors according to the Plackett-Burman design. Algal cells were grown under 20 different conditions, with cell dry weight and DHA yield being taken as the responses for each run (Table 3-2). With the experimental data, the effects of the variables on the responses, and their associated *F*-test and significance level (*p*) were calculated (Table 3-9). Based on the effects of dummy variables, the standard error of the PB design was determined as 1.23 g/L for cell dry weight, and 0.21 g/L for DHA yield. Here, the effects with $p<0.10$ were accepted as significant. It was found that among the 15 factors investigated, trace metal (PI) solution, NH_4Cl, ammonium acetate, and temperature had significant effects on cell weight or DHA yield (Table 3-9).

Table 3-9.The effects of variables on cell dry weight and DHA yield and associated statistical tests

	Cell dry weight		DHA yield	
Variables	Effect	*p*-level	Effect	*p*-level
NaCl	2.26	0.1407	0.32	0.2046
KH_2PO_4	-0.02	0.9878	0.01	0.9746
Corn steep liquor	0.72	0.5905	-0.06	0.7826
Ammonium acetate	-4.80	**0.0176**	-1.01	**0.0083**
$MgSO_4$. $7H_2O$	-1.42	0.3135	-0.26	0.2723
KCl	-1.78	0.2222	-0.21	0.3775
$NaNO_3$	-0.84	0.5330	-0.22	0.3571
$CaCl_2$• $2H_2O$	1.58	0.2692	0.23	0.3377
Tris Buffer	1.70	0.2399	0.43	0.1055
NH_4Cl	-2.68	**0.0954**	-0.43	0.1066
Vitamin B_{12}	0.20	0.8790	-0.01	0.9819
PI metal solution	2.72	**0.0920**	0.45	**0.0956**
Chelated iron solution	1.00	0.4627	0.17	0.4450
pH	-0.60	0.6519	-0.01	0.9529
Temperature	-15.12	**0.0003**	-3.22	**0.0001**

Temperature was the most significant factor influencing algal growth and DHA production. Low temperature (20^{o}C) favored the DHA production, which was similar to those reported in other algal culture such as *Crypthecodinium cohnii* (Jiang and Chen, 2000) and *Nitzschia laevis* (Wen and Chen, 2001). It has been reported that high DHA content might be due to the increased membrane fluidity as cells respond to the low temperature conditions (Richmond, 1986). Also, low temperature led to an increased availability of intracellular molecular oxygen, which facilitated the oxygen-dependent enzymes in the desaturation and elongation of DHA (Higashiyama et al., 1999; Singh and Ward, 1997). In addition to temperature, it was also found that high levels of ammonium acetate significantly influenced cell growth and DHA production in a negative manner (Table 3-9). For NH_4Cl and PI metal solution, although the *p*-levels of their effects were within the significance range ($P=0.09<0.10$) and their effects were not as "notable" as that of temperature, which had a $P<0.001$ (Table 3-9).

Based on the results in Table 3-9, PI metal solution, NH_4Cl, ammonium acetate, and temperature were identified as the significant factors influencing algal growth and DHA production. Those parameters were further optimized through a central composite design.

Optimizing the significant factors

In the central composite design, the values of central points and the ranges of levels of the four significant factors were chosen based on the previous Plackett-Burman design results (Table 3-4). The concentrations of non-significant medium components as identified in Table 3-9 were set at lower levels while medium pH was set at 8.0 (Table 3-1).

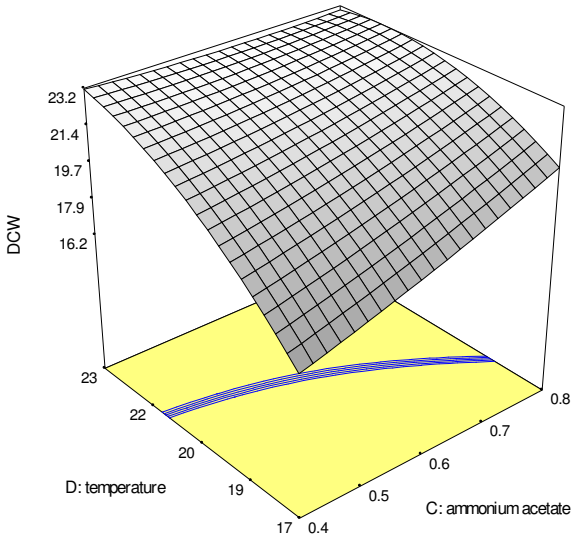
As shown in Table 3-3, the central point in the central composite design was repeated six times; and the standard deviation of the six replicates was used to determine the experimental errors for cell dry weight as 0.82 g/L, and for DHA yield as 0.21 g/L. The experimental data of cell dry weight and DHA yield in Table 3-3 were correlated as functions of the four variables by a second-order polynomial equation (Eq.3-1) using the software *Design-Expert*. The values of the coefficients in Eq. 3-1 and their *P*-values are listed in Table 3-10. The optimal value of the four parameter were derived by the software *Design-Expert* as 10 mL/L of PI, 0.03 g/L of NH_4Cl, 0.20 g/L of ammonium acetate, 26.0^oC for maximum cell dry weight; and 30 mL/L of PI, 0.04 g/L of NH_4Cl, 1.0 g/L of ammonium acetate, and 19.2^oC for maximum DHA yield.

Table 3-10. The values of coefficients in the second-order polynomial (Eq. 3-1) and the associated statistical test for cell dry weight and DHA yield [a]

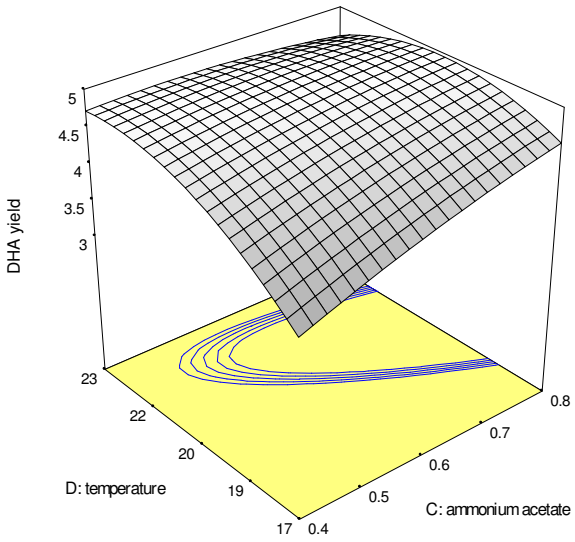
		Cell dry weight		DHA yield	
Coefficient	Variable	Estimate	*P*- level	Estimate	*P*-level
β_0	Constant	21.617		4.81	
β_1	[PI]	-0.054	0.890	0.004	0.959
β_2	$[NH_4Cl]$	0.021	0.957	0.001	0.986
β_3	$[NH_4AC]$	1.004	0.019	0.230	0.006
β_4	[T]	2.379	0.000	0.320	0.000
β_{11}	$[PI]^2$	0.099	0.786	0.033	0.629
β_{22}	$[NH_4Cl]^2$	0.111	0.760	-0.024	0.720
β_{33}	$[NH_4AC]^2$	0.161	0.659	-0.084	0.226
β_{44}	$[T]^2$	-1.139	0.006	-0.39	< 0.0001
β_{12}	$[PI] \cdot [NH_4Cl]$	-0.019	0.969	0.016	0.861
β_{13}	$[PI] \cdot [NH_4AC]$	0.081	0.865	0.056	0.535
β_{14}	$[PI] \cdot [T]$	-0.056	0.906	-0.013	0.883
β_{23}	$[NH_4Cl] \cdot [NH_4AC]$	-0.094	0.844	-0.016	0.861
β_{24}	$[NH_4Cl] \cdot [T]$	-0.106	0.824	-0.037	0.680
β_{34}	$[NH_4AC] \cdot [T]$	-1.081	0.036	-0.290	0.005
Model			0.003		0.001

[a] [PI]: Concentration of PI metal solution; $[NH_4Cl]$: NH_4Cl concentration; $[NH_4AC]$: ammonium acetate concentration; [T]: temperature level.

Three-dimensional surface responses were further plotted to illustrate the relationships between the responses and the variables. Because the statistical analysis indicates that temperature and ammonium acetate concentration had more significantl effects on the responses than NH_4Cl and PI metal solution (*P*-level, Table 3-10), the responses (cell dry weight and DHA yield) were plotted as the functions of temperature and ammonium acetate concentration. As shown in Figure 3-2, at high temperatures, the increase of ammonium acetate led to a decrease in the cell dry weight and DHA yield, but at low temperatures, both cell dry weight and DHA yield increased with increasing ammonium acetate concentration.



(A)



(B)

Figure 3-2. Three-dimensional response surface plot of cell dry weight in g/L (A) and DHA yield in g/L (B) as a function of ammonium acetate (g/L) and temperature (°C).

Verification of optimized culture conditions

The optimal conditions determined from the central composite design were further verified by comparing the experimental data obtained at these conditions with that predicted from central composite design (Eq.3-1 and Table 3-9). Two verification experiments were performed: one was at the optimal culture condition for cell dry weight; the other was that for DHA yield (Table 3-11). It was found that the cell dry weight prediction had around 11-14% deviation, while the deviation of DHA yield between the experimental and predicted data were less than 5% (Table 3-11), suggesting the experimental designs used in this work are effective for optimizing algal culture conditions for producing DHA from biodiesel-derived crude glycerol. Based on the experimental data of DW and DHA yield, the biomass and DHA productivity at the two optimal conditions were calculated (Table 3-11).

Table 3-11. Comparison of experimental and predicted data of cell dry weight (DW) and DHA yield at optimal culture conditions.

Culture conditions		DW (g/L)	DHA yield (g/L)	Biomass productivity (g/L-day)	DHA productivity (g/L-day)
Optimized conditions for cell dry weight	Predicted value	26.4	4.68	-	-
	Experiment data [a]	23.5±1.25	4.81±0.41	3.92±0.20	0.80±0.03
	Error	-11.0%	+2.7%	-	-
Optimized conditions for DHA yield	Predicted value	25.8	5.18	-	-
	Experiment data [a]	22.1±0.6	4.91±0.30	3.68±0.10	0.82±0.05
	Error	-14.3%	-4.3%	-	-

[a] The experimental data are mean ± SD of three replicates

In summary, the above results demonstrate that it is feasible to use biodiesel-derived crude glycerol for algal DHA production. A similar growth rate and cell density was obtained when using glucose and glycerol. For a typical heterotrophic algal culture, however, glucose is usually expected as an easier consumed carbon source versus other substrates, and oxygen is the main factor determining the yield of culture. To test if the similar results from glucose-based and glycerol-based cultures were caused by oxygen-limitation; a further experiment was performed in flask cultures by loading the flasks with different volumes of medium, and comparing the

algal growth. The hypothesis is that if oxygen in the flask is the limiting factor, the cell growth will be reduced in the high volume loading flasks. The medium composition and agitation speed were the same for different flasks; 90 g/L of glucose was used as a carbon source. It was found that within the range of liquid loading from 25 ml to 100 ml per flask, the cell growth remained at a relatively stable level (Table 3-12); the differences between different liquid loadings were insignificant (P>0.1). The results indicate that for the experimental conditions used in this work, the oxygen supply is not a limiting factor. The similar algal growth in glucose- and glycerol-medium was considered to be due to the specific characteristics of the algal species used in this study. Indeed, *S. Limacinum* has been reported a good DHA producer when growing in glucose or glycerol, the two carbon sources provides a similar algal growth and DHA production (Yokochi et al., 1998).

Table 3-12. Effects of different liquid volume loading in each flask on algal growth of *S. limacinum* [a]

Liquid loading (mL/flask)	Cell dry weight (g/L)
25	15.80±0.60
50	17.02±1.40
75	16.32±1.40
100	16.78±0.10

[a.] 250-mL Erlenmeyer flasks were used for algal culture. Data are expressed as mean ± SD of three replicates.

3.5 Conclusions

The work presented here showed the potential of using less expensive crude glycerol from the biodiesel industry to produce DHA by microalgal culture. The optimal crude glycerol content for algal DHA production was around 100 g/L. Using statistically based experimental designs, it was found that temperature and ammonium acetate were factors significantly influencing the algal DHA production from crude glycerol. The optimal level of these two factors for DHA yield were determined as 19.2^{o}C and 1.0 g/L ammonium acetate. Under the optimal culture conditions, DHA content in the algae biomass was more than 20%; with a yield of DHA 4.91 g/L. Successful application of this process could have a significant impact on the

biodiesel industry as it helps to solve the problem of waste glycerol disposal in the biodiesel industry, while simultaneously produces a valuable nutraceutical.

3.6 Acknowledgements

We appreciate the financial support from Washington Technology Center, American Premix Technologies, Inc., and Virginia Agricultural Council. The authors acknowledge Center for Multiphase Environmental Research (WSU) for making the equipment available to this research; Dr. Chuanbin Liu and Mr. Bo Hu for their technical assistance.

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Chapter 4:

Producing docosahexaenoic acid-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition

4.1 Abstract

Crude glycerol is the primary byproduct of the biodiesel industry. Producing docosahexaenoic acid (DHA, 22:6 n-3) through fermentation of the alga *Schizochytrium limacinum* on crude glycerol provides a unique opportunity to utilize a large quantity of this byproduct. The objective of this work is to investigate the effects of impurities contained in the crude glycerol on DHA production and algal biomass composition. Crude glycerol streams were obtained from different biodiesel refineries. All the glycerol samples contained methanol, soaps, and various elements including calcium, phosphorus, potassium, silicon, sodium, and zinc. Both methanol and soap were found to negatively influence algal DHA production; these two impurities can be removed from culture medium by evaporation through autoclaving (for methanol) and by precipitation through pH adjustment (for soap). The glycerol-derived algal biomass contained 45-50% lipid, 14-20% protein, and 25% carbohydrate, with 8-13% ash content. Palmitic acid (C16:0) and DHA were the two major fatty acids in the algal lipid. The algal biomass was rich in lysine and cysteine, relative to many common feedstuffs. Elemental analysis by Inductively Coupled Plasma showed that boron, calcium, copper, iron, magnesium, phosphorus, potassium, silicon, sodium, and sulfur were present in the biomass, while no heavy metals (such as mercury) were detected in the algal biomass. Overall, the results show that crude glycerol was a suitable carbon source for algal fermentation. The crude glycerol-derived algal biomass had a high level of DHA and a nutritional profile similar to commercial algal biomass, suggesting a great potential for using crude-glycerol derived algae in omega-3 fortified food or feed.

4.2 Introduction

In recent years, biodiesel has become an important source of alternative fuel for the United States. The National Biodiesel Board has projected an annual production of biodiesel in 2007 of 450 million gallons, a sharp increase from less than 100 million gallons prior to 2005

(NBB, 2008). The major byproduct of biodiesel production is crude glycerol. In general, for every gallon of biodiesel produced, 0.3 kilograms of glycerol are generated (Thompson and He, 2006; Papanikolaou and Aggelis, 2002). As biodiesel production skyrockets, the market is being flooded with crude glycerol. Indeed, crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006 (Johnson and Taconi, 2007; Yazdani and Gonzalez, 2007) because the current U.S. demand for glycerol is not large enough for all of this excess glycerol. It is clear that new uses for biodiesel-derived crude glycerol are needed.

Several avenues for utilizing this crude glycerol have been investigated. For example, glycerol can be thermochemically converted into propylene glycol (Chiu et al., 2006) or acetol (Chin et al., 2006a). It can also be used in fermentation processes to produce 1,3-propanediol (Gonzalez-Pajuelo et al., 2005), lipids (Papanikolaou and Aggelis, 2002; Narayan et al., 2005; Meesters et al., 1996), pigments (Narayan et al., 2005), and a mixture of succinic acid, butanol, ethanol, and hydrogen (Dharmadi et al., 2006). Recently, research conducted in our laboratory has shown that crude glycerol can be used to produce docosahexaenoic acid (DHA, 22:5 n-3) through fermentation of the alga *Schizochytrium limacinum* (Chi et al., 2007).

DHA is an important omega-3 polyunsaturated fatty acid (n-3 PUFA) that has been shown to have beneficial effects on preventing human cardiovascular diseases, cancer, schizophrenia, and Alzheimer's (Simopoulos, 1999). DHA also plays an important role in infant brain and retinal development (Innis, 2007). In the aquaculture industry, omega-3 fatty acids are essential nutrients for cultured marine fish. The major commercial source of omega-3 fatty acids is fish oil, which faces challenges such as odor/taste problems, heavy metal contamination, and limited supply (Barclay et al., 1994). Currently, the aquaculture industry is experiencing rapid increases in fish oil price due to flat supply and increased global demand for this commodity. In fact, the Food and Agriculture Organization (FAO) of the UN predicts that fish oil demand in 2015 will be 145% of historical global production capacity (New and Wijkström, 2002). The inability to expand fish oil production makes development of fish oil alternatives imperative.

Developing DHA-containing microalgae from biodiesel waste glycerol is an excellent opportunity to provide alternative omega-3 sources. The fatty acids can be extracted from algae and used in fortified foods, or the biomass can be used directly as a feed additive in various animal industries such as aquaculture (Harel et al., 2002) or poultry (Chin et al., 2006b). In

addition, potential environmental impacts associated with crude glycerol disposal could be minimized.

While it has been shown that crude glycerol from the biodiesel industry can support the growth and DHA production of *S. limacinum* (Chi et al., 2007), a thorough chemical characterization of the algal biomass is needed before the algae-derived omega-3 fatty acids are used as food or feed additives. In general, the composition of crude glycerol varies from plant to plant; it contains methanol and soap as the two major impurities, with various elements such as calcium, potassium, phosphorus, magnesium, sulfur, and sodium present. Although the biodiesel manufacturing process does not involve any heavy metals, the oil feedstock may contain trace amounts of heavy metals, which may eventually end up in algal cells. The objective of this work is to investigate the effects of these impurities on algal DHA production and the "quality" of algal biomass in terms of its elemental composition and nutritional levels. Ensuring that algal biomass can be easily produced and can be safely utilized will open new markets for biodiesel-derived crude glycerol.

4.3 Materials and Methods

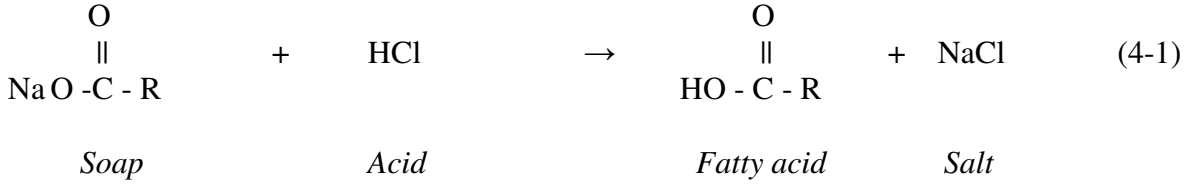
4.3.1 Algal strain, medium, and culture conditions

Schizochytrium limacinum SR-21 (ATCC MYA-1381) was used. The cells were maintained in a medium containing 10 g/L glucose, 1.0 g/L yeast extract, and 1.0 g/L peptone in artificial seawater. The artificial seawater contained (per liter) 18 g NaCl, 2.44 g $MgSO_4 \cdot 7H_2O$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, 1.0 g Tris buffer (Sigma Co.), 0.027 g NH_4Cl, 15.0 x 10^{-8} g vitamin B_{12}, 3 mL chelated iron solution, and 10 mL trace element solution including boron, cobalt, manganese, zinc, and molybdenum (Starr & Zeikus, 1993). The pH was adjusted to 7.5-8.0 before autoclaving the media at 121°C for 15 minutes. The cells were grown in 250-mL Erlenmeyer flasks, each containing 50 mL of medium, and incubated at 20°C in an orbital shaker set to 170 rpm. Subcultured cells were used as inoculum. The inoculum size was 10% of the total liquid volume in each flask. In the study of DHA production from crude glycerol, all the medium components were the same as those used in the subculture except that different concentrations of crude glycerol were used to replace glucose.

4.3.2 Crude glycerol sources and soap removal procedures

Different crude glycerol streams were tested, including (G1) glycerol derived from soybean oil by Virginia Biodiesel Refinery (West Point, VA), (G2) glycerol from a 50:50 (w/w) chicken fat and soybean oil mixture by Virginia Biodiesel, and (G3) glycerol from canola oil by Seattle Biodiesel LLC (Seattle, WA). Both the biodiesel plants used alkali-catalyzed transesterification of oil with methanol to produce the biodiesel. Virginia Biodiesel used KOH as catalyst, while Seattle Biodiesel used NaOH as catalyst.

All the crude glycerol samples contained soaps which were formed from a side reaction. The soaps can be split into free fatty acids (FFAs) and salt by adding a strong acid to the glycerol to neutralize the catalyst, i.e.,



Depending on the experimental conditions, soaps either remained in, or were removed from, the algal culture medium. To prepare soap-free medium, the following procedures were used: (i) the glycerol was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, (ii) the pH of the fluid was adjusted to 3 with hydrochloric acid to convert soap into free fatty acids that precipitated from the liquid, (iii) precipitated free fatty acids were separated from the crude glycerol solution by centrifugation at 5000 rpm, and (iv) other nutrients (mineral salts, nitrogen source, etc.) were added to the glycerol solution to adjust to the desired levels.

4.3.3 Analysis

Cell Dry Weight, Glycerol and Methanol Concentrations

To determine cell dry weight, 5 mL cell suspension samples were transferred to a centrifuge tube and centrifuged at 3444 g for 8 minutes. The cell pellet was then washed with distilled water and centrifuged again. The pellet and liquid were then filtered through preweighed filter paper (VWR, Glass Microfibre 691) and the algal biomass remaining on the filter paper was dried at 95^{o}C to constant weight. Glycerol and methanol concentrations were determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc. Columbia, MD). The detailed procedures were described previously (Chi et al., 2007).

Proximate Analysis

Algal biomass was freeze dried prior to proximate analysis. The lipids from the algal biomass was extracted and quantified by the Bligh and Dyer method (Bligh and Dyer, 1959). The crude protein content was estimated by measuring the Kjeldahl nitrogen content and multiplying the result by 6.25. The ash content was determined by heating the sample at 550^{o}C overnight and then weighing the remaining matter. The carbohydrate was then calculated by subtraction.

Fatty acid analysis

Fatty acid compositions of the lyophilized algal biomass and of free fatty acid from the crude glycerol were determined. Fatty acids methyl esters (FAME) were prepared by direct-methylation with 5% methanolic HCl (Schreiner, 2006; Christie, 2003; Ulberth and Henninger, 1992), and determined by a Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame-ionization detector and a SGE SolGel-Wax^{TM} capillary column (30m×0.25mm×0.25um). Helium was used carrier gas. The temperature settings for injector, column, and detector were described previously (Chi et al., 2007). The fatty acids were identified by comparing the retention times with those of standard fatty acids (Nu-Chek Prep Inc., MN), and quantified by comparing their peak area with that of the internal standard (C17:0) (Chi et al., 2007).

Amino acid analysis

Freeze-dried algal biomass (~25 mg) was hydrolyzed in triplicate using a PicoTag workstation (Waters Corporation, Milford, MA) according to the manufacturer's directions. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as 'mixture 1' by van Wandelen and Cohen (1997), with α-aminobutyric acid as an internal standard. Separate analyses were performed for cysti(e)ne, using the method described by Finely (1985) to quantitatively oxidize cystiene and cystine to cysteic acid prior to hydrolysis; these samples were then analyzed in the same manner as the other samples.

Elemental analysis

Elemental composition of the crude glycerol and algal biomass were determined by an Inductively Coupled Plasma Semi-Quantitative Scan of 69 elements according to EPA Method SW-846 6010B (SuperScan 69 performed by Prochem Analytical Inc., Elliston, VA). The elements and their detection limits are listed in Table 4-1. In addition, EPA Method SW-846 7471A was further used to detect any trace amount of mercury possibly contained in the algal biomass; the detection limit for this measurement was 0.025 ppm (25 ppb).

Table 4-1. Detectable limits of various elements with ICP analysis.

Element	Detection Limit (ppm)	Element	Detection Limit (ppm)
Aluminum	10	Nickel	5
antimony	20	Niobium	50
arsenic	50	Osmium	10
barium	5	Palladium	50
berylium	2	phosphorus	50
bismuth	10	Platinum	50
boron	10	Potassium	100
cadmium	2	praseodymium	50
calcium	50	Rhenium	50
cerium	50	Rhodium	100
chromium	2	Ruthenium	50
cobalt	5	Samarium	50
copper	2	Scandium	10
dysprosium	10	Selenium	50
erbium	10	Silicon	10
europium	10	Silver	2
gadolinium	20	Sodium	50
gallium	20	Strontium	10
germanium	50	Sulfur	100
Gold	20	Tantalum	50
hafnium	50	Tellurium	100
holminum	50	Terbium	50
indium	50	Thallium	50
iodine	200	Thorium	50
iridium	50	Thulium	50
Iron	10	Tin	10
lanthanum	50	Titanium	2
Lead	20	Tungsten	50
lithium	10	Uranium	100
lutetium	10	Vanadium	5
magnesium	50	Ytterbium	10
manganese	2	Ytrtrium	2
mercury*	50	Zinc	2
molybdenum	10	Zirconium	10
neodymium	20		

* EPA method SW-846 7471A was also used to detect trace amounts of mercury with a detection limit of 0.025 ppm

4.4 Results and Discussion

4.4.1 Characterization of crude glycerol

The crude glycerol used in this work had a dark brown color with a high pH level (11-12). Because the biodiesel refineries used excess methanol in order to drive the transesterification towards a maximum biodiesel yield, the crude glycerol contained methanol. Soap was also found in the crude glycerol due to a side-reaction. Considering the difficulty to determine the exact amount of soap dissolved in the crude glycerol solution, we used free fatty acid precipitated from the crude glycerol (Equation 4-1) to roughly estimate the amount of the soap residue.

As shown in Table 4-2, the glycerol G1 and G2 contained 12-14% methanol and 23-25% soap, while crude glycerol from Seattle Biodiesel (G3) had less soap and more methanol; indicating a more complete transesterification, but more methanol being used by this refinery. The glycerol contents in these crude streams were around 55-58%. Glycerol purity in crude glycerol streams have been reported in a wide range from 65% (Gonzalez-Pajuelo et al., 2005) to 85 % (Mu et al., 2006), which probably results from different glycerol purification procedures used by biodiesel plants.

Table 4-2. Composition of methanol and soap residues in crude glycerol streams

		Crude glycerol streams		
Composition	Unit	G1	G2	G3
Methanol	(%, w/w)	12.05 ± 0.26	13.13 ± 2.89	27.74 ± 2.07
Soap	(%, w/w)	25.17 ± 1.70	23.24 ± 4.49	15.28 ± 3.27
Glycerol	(%, w/w)	58.51 ± 1.00	56.66 ± 5.08	55.33 ± 0.98

Data are means of three replicates ± standard deviations.

The fatty acid compositions of the soap residues were also determined. As shown in Table 4-3, for all three soaps residues, oleic acid (18:1) and linoleic acid (18:2) were the two major fatty acids. G1 and G3 had a very similar fatty acid profile with high levels of linoleic acid, while G2 had a relatively higher portion of oleic acid (18:1). These fatty acid profiles were an indication of different feedstocks used in the biodiesel production.

Table 4-3. Fatty acid composition (% of total fatty acid, TFA) of the soaps.

		Soap source		
Fatty acid	Unit	Soap from G1	Soap from G2	Soap from G3
16:0	% TFA	11.61 ± 1.05	19.15 ± 2.82	11.14 ± 0.00
16:1	% TFA	ND	2.70 ± 0.24	ND
18:0	% TFA	4.01 ± 0.89	3.07 ± 2.66	3.61 ± 0.04
18:1	% TFA	22.97 ± 0.39	30.87 ± 3.59	23.53 ± 0.08
18:2	% TFA	54.35 ± 1.47	39.58 ± 1.43	54.46 ± 0.16
18:3 (n-3)	% TFA	7.05 ± 0.11	4.63 ± 0.34	7.25 ± 0.20
TFA	mg/g DW	866.46 ± 73.53	830.04 ± 59.93	895.94 ± 19.49

ND: not detected
Data are means of three replicates ± standard deviations.

Table 4-4 shows ICP elemental analysis of the crude glycerol. Potassium was present at high levels in G1 and G2 while sodium was the major element in G3. This was attributed to the fact that G1 and G2 came from Virginia Biodiesel using KOH as a catalyst while G3 was provided by Seattle Biodiesel utilizing NaOH as a catalyst. Trace amounts of boron, calcium, phosphorus, silicon and zinc were also found in the crude glycerol samples (Table 4-3). No heavy metals (such as mercury) were found in any of these samples, suggesting crude glycerol can be used as a safe substrate for algal DHA production.

Table 4-4. Elemental composition of crude glycerol streams as detected by ICP analysis (Elements analyzed in Table 4-1, but not included in this table were not detected in any sample).

	Crude glycerol		
Element (ppm)	G1	G2	G3
Boron	BDL	BDL	11.50 ± 0.75
Calcium	BDL	140 ± 36	BDL
Phosphorus	BDL	480 ± 104	BDL
Potassium	31250 ± 212	27300 ± 5515	BDL
Silicon	62 ± 23	29.80 ± 6.36	17.30 ± 2.83
Sodium	214 ± 45	248 ± 45	12550± 2757
Zinc	2.50 ± 0.25	2.30 ± 0.15	2.30 ± 0.15

Data are means of three replicates ± standard deviations. (BDL: Below Detection Limit)

The above results show that the composition of crude glycerol streams varied, presumably depending on the feedstocks and biodiesel production processes employed by the refineries. However, all batches of crude glycerol consisted of methanol and soaps as the major impurities. The effects of these two components on DHA production by *S. limacinum* were further investigated.

4.4.2 Effects of methanol on algal DHA production

It has been reported that ethanol is beneficial for the growth of some microalgae producing DHA (de Swaaf et al., 2003). This leads us to explore the *possible* beneficial effects of methanol on growth and DHA production of *S. limacinum*. The algae were grown in a medium containing pure glycerol (70 g/L) with different concentrations of methanol. Considering the fact that methanol evaporates at 65^{o}C, the autoclave process (at 121^{o}C for 15 min) resulted in a significant loss of methanol. We did not autoclave the methanol in the media; instead, the autoclaved media was spiked with varying levels of methanol, sterilized by passing through a 0.2 μm filter.

As shown in Table 4-5, the maximum cell dry weight, biomass productivity, and cell yield decreased as methanol concentration increased from 0 to 20 g/L. In terms of fatty acid composition of the algal biomass, it was found that that the proportion of DHA in total fatty acids maintained a relatively constant level, however, the total fatty acid content decreases with methanol concentration (Table 4-6). This fatty acid profile, together with the cell growth performance, resulted in a decreased DHA production level at higher methanol concentrations.

Table 4-5. Effects of methanol concentration on growth and DHA production of *S. limacinum* (grown in 70 g/L of pure glycerol).

Parameter	Unit	Methanol concentration (g/L)			
		0	10	15	20
Specific growth rate, μ	day^{-1}	0.59 ± 0.01	0.50 ± 0.03	0.52 ± 0.03	0.32 ± 0.01
Max. cell dry weight	g/L	11.49 ± 0.49	10.31 ± 0.07	8.36 ± 0.31	5.63 ± 0.47
Biomass productivity	g/L-day	1.92 ± 0.08	1.72 ± 0.01	1.39 ± 0.05	0.94 ± 0.08
Growth Yield	g/g	0.23 ± 0.01	0.23 ± 0.01	0.18 ± 0.01	0.13 ± 0.01
DHA content	mg/g DW	146.4 ± 6.9	158.5 ± 17.7	118.7 ± 14.9	124.6 ± 2.2
DHA yield	g/L	1.68 ± 0.11	1.63 ± 0.18	0.99 ± 0.13	0.70 ± 0.06
DHA productivity	g/L-day	0.28 ± 0.02	0.27 ± 0.03	0.17 ± 0.02	0.12 ± 0.01

Data are means of three replicates ± standard deviations.

Table 4-6. Fatty acid composition (% of total fatty acid, TFA) and total fatty acids content of *S. limacinum* grown on 70 g/L of pure glycerol spiked with various amounts of methanol.

Fatty Acid	Methanol Concentration (g/L)			
	0	10	15	20
C14:0 (% TFA)	4.59 ± 0.14	4.51 ± 0.07	5.03 ± 0.13	4.85 ± 0.25
C16:0 (% TFA)	54.70 ± 1.95	54.99 ± 0.14	55.13 ± 0.56	53.77 ± 0.64
C18:0 (% TFA)	0.97 ± 0.10	0.98 ± 0.00	1.01 ± 0.02	1.07 ± 0.02
C22:5 (% TFA)	5.45 ± 0.12	5.50 ± 0.01	5.36 ± 0.09	5.50 ± 0.11
C22:6 (% TFA)	34.28 ± 2.27	34.02 ± 0.07	33.48 ± 0.56	34.81 ± 0.63
TFA (mg/g DW)	427.45 ± 17.46	465.78 ± 53.00	354.25 ± 40.59	357.94 ± 6.73

Data are mean ± standard deviation of two replicates.

The above results clearly indicate the negative effects of methanol on growth and DHA production of *S. limacinum*. Fortunately, under the typical crude glycerol levels used for algal culture (e.g., 60-90 g/L), autoclaving the media can evaporate methanol contained in the original glycerol stream; this allows the algae to thrive on the methanol-free crude glycerol.

4.4.3 Effects of soap on algal DHA production

The effects of soap on algal growth and DHA production were investigated by growing the algal cells in different levels of crude glycerol. The soap residues in the crude glycerol either remained in the medium (soap-containing); or were precipitated into free fatty acids and then

removed from the medium (soap-free). The glycerol G1 was used as representative of all the crude glycerol samples.

The growth kinetics of *S. limacinum* on soap-containing and soap-free medium were compared. As shown in Figures 4-1A-4-1C, in the lower range of crude glycerol concentrations (10 and 20 g/L), the algal growth on soap-free and soap-containing medium was similar. When crude glycerol concentration increased to 40 g/L, the specific growth rates of the two cultures were still similar (Figure 4-1A), but the maximum cell dry weight and biomass productivity for soap-containing culture were lower than those for soap-free cultures (Figures 4-1B and 4-1C). At 60 g/L glycerol concentration, the growth in soap-containing medium was significantly inhibited. It was found that when crude glycerol concentration exceeded 60 g/L, the algal cells in soap-containing medium were not viable after one day of culture (data not shown).

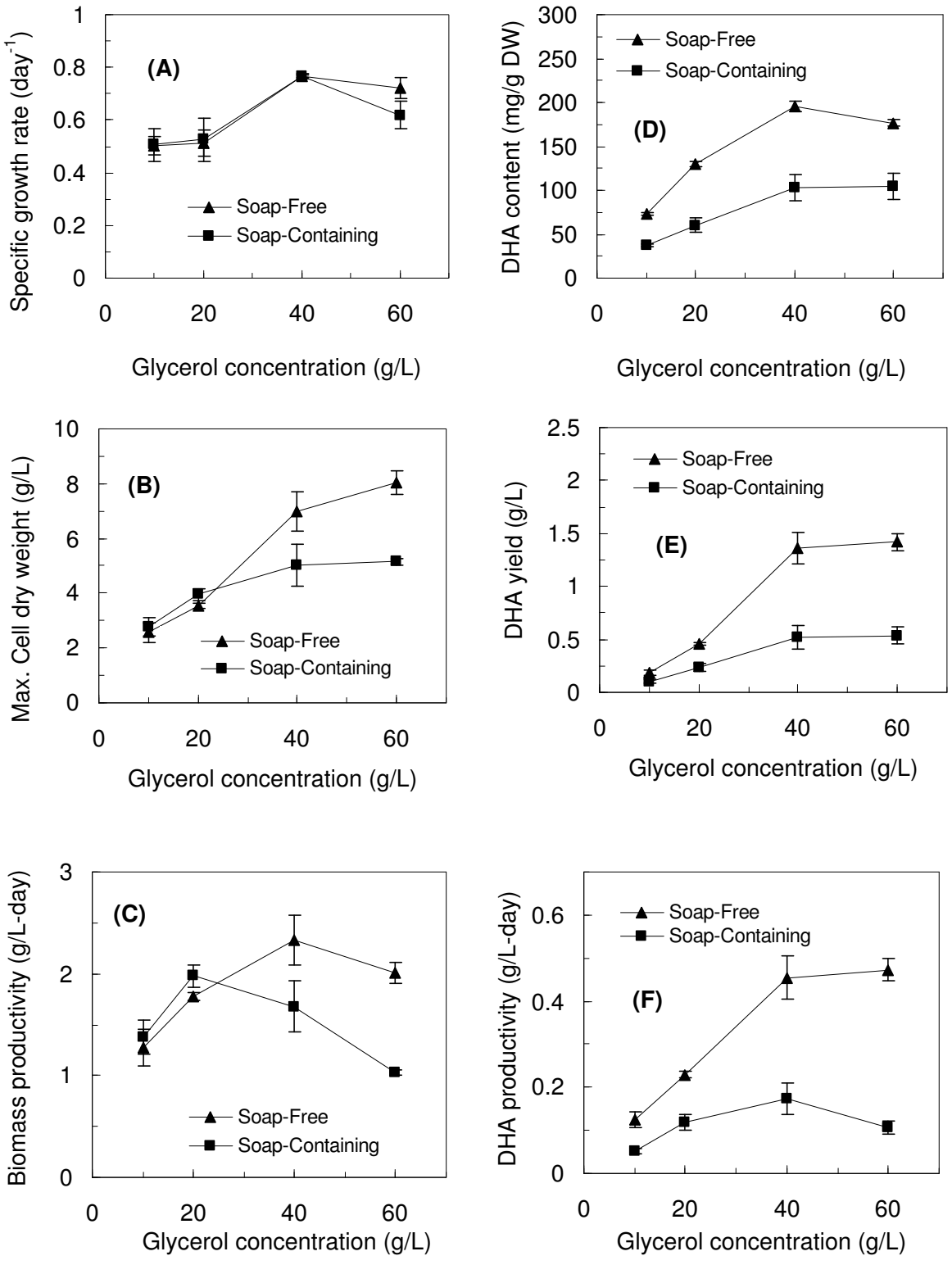


Figure 4-1. Cell growth performance (A, B, C) and DHA production (D, E, F) by *S. limacinum* on soap-free and soap-containing medium with different crude glycerol concentrations (10, 20, 40, and 60 g/L).

The soap also had significant effects on fatty acid composition. As shown in Table 4-7, in soap-free medium, the proportion of C18 fatty acids was negligible (~1% of TFA), while soap-containing medium resulted in significant proportions of C18s fatty acids (25-35% of TFA). Considering the fact that C18s were the major fatty acids contained in soaps (Table 4-3), the difference in fatty acid composition between the soap-free and soap-containing algal biomass indicate that the C18s from soap are absorbed by, or bound tightly to, the algal cells (Figure 4-2 and Table 4-7). However, the proportion of long chain fatty acids (C22:5 and C22:6) in the soap-containing algae was lower than those in the soap-free algae (Table 4-7), suggesting that those absorbed/bound C18s were not further elongated by the algal cells.

These fatty acid profiles agree with results reported by Hauvermale et al. (Hauvermale et al., 2006). These authors used radiolabeled C16:0, C18:1, or C18:3 fatty acids to feed *Schizochytrium* sp. and found that none of the labels appeared in long-chain PUFA (Hauvermale et al., 2006). Table 4-7 also shows that at 10 g/L of crude glycerol, the algae in soap-containing medium had more total fatty acids (TFAs) than algae in soap-free medium; when crude glycerol concentrations exceeded 20 g/L, the TFAs content of soap-free algae became more than that of soap-containing algae (Table 4-7). This is probably due to growth inhibition at higher soap levels (Figures 4-1A-4-1C).

By combining the soap effects on both cell growth (Figures 4-1A-4-1C) and fatty acid profile (Table 4-7) of *S. limacinum*, it was found that under different crude glycerol levels, DHA content, yield and productivity from the soap-containing algae were lower than those obtained from the soap-free algae (Figures 4-1D-4-1F). The results clearly indicated a negative effect of soap on algal DHA production. This effect is more pronounced at higher levels.

The inhibitory effect of soap on *S. limacinum* agreed with other reports in which the growth of various algal species was inhibited by surfactants including soap (Yamane et al., 1984; Ukeles, 1965). This inhibition was caused by the complex interaction between the cell wall/membrane and the surfactants (Ukeles, 1965). To elucidate the mechanism of soap effects on *S. limacinum,* further study of the interaction between soaps and cell membrane of this species are needed.

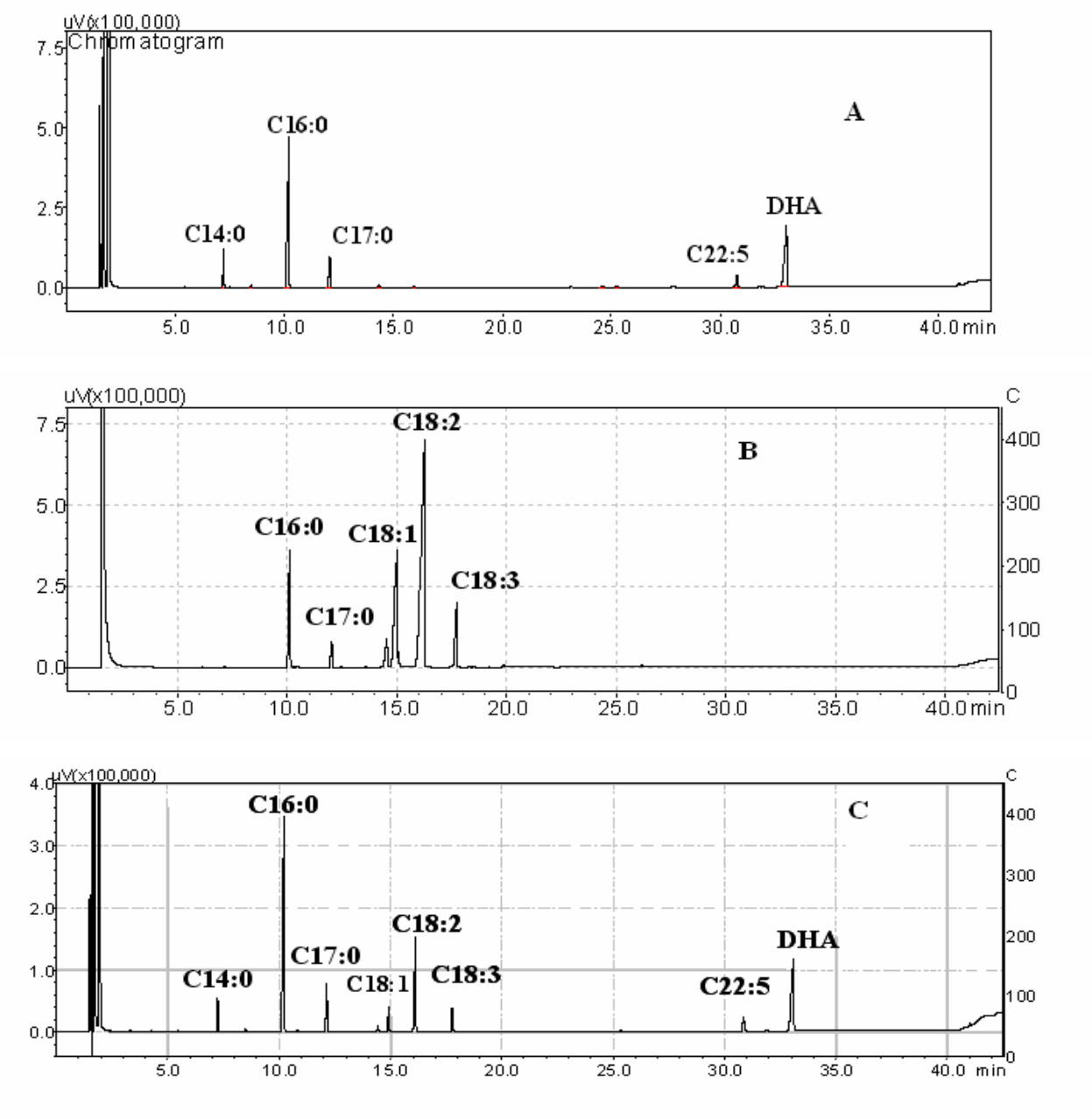


Figure 4-2. Typical fatty acid profile of (A) *S. limacinum* grown on purified crude glycerol, (B) crude glycerol soap, and (C) algae grown with soap remaining in media.

Table 4-7. Fatty acid composition (% of total fatty acid, TFA) of the algal biomass grown in soap-free and soap-containing medium with different crude glycerol concentration loading

		Glycerol concentration (g/L)			
Fatty Acid	Unit	10	20	40	60
Algae on soap-free medium					
C14:0	% TFA	2.06 ± 0.18	3.32 ± 0.24	4.21 ± 0.65	4.77 ± 0.05
C16:0	% TFA	35.50 ± 1.28	44.11 ± 0.53	49.80 ± 3.76	52.64 ± 0.37
C18:0	% TFA	0.81 ± 0.04	0.93 ± 0.05	0.97 ± 0.05	1.04 ± 0.02
C22:5	% TFA	8.58 ± 0.22	7.35 ± 0.27	6.29 ± 0.77	5.66 ± 0.05
C22:6	% TFA	53.05 ± 1.30	44.30 ± 0.53	38.73 ± 3.69	35.89 ± 0.38
TFA	mg/g DW	138.1 ± 2.6	292.2 ± 5. 9	503.9 ± 7.2	492.3 ± 12.6
Algae on soap-containing medium					
C14:0	% TFA	2.59 ± 0.98	3.14 ± 0.32	2.68 ± 0.25	2.99 ± 0.09
C16:0	% TFA	40.40 ± 12.73	42.35 ± 4.28	38.35 ± 3.04	39.35 ± 0.84
C18:0	% TFA	2.33 ± 0.28	1.91 ± 0.50	2.79 ± 1.48	1.10 ± 0.08
C18:1	% TFA	9.17 ± 1.72	7.43 ± 1.07	6.65 ± 1.00	3.93 ± 0.53
C18:2	% TFA	21.50 ± 6.11	17.68 ± 3.21	18.77 ± 3.28	19.23 ± 1.89
C18:3	% TFA	2.81 ± 0.91	2.72 ± 0.61	3.37 ± 0.69	4.85 ± 0.35
C22:5	% TFA	2.93 ± 0.66	3.55 ± 0.36	3.88 ± 0.49	4.07 ± 0.34
C22:6	% TFA	18.26 ± 4.94	21.23 ± 2.03	23.50 ± 2.94	24.49 ± 2.04
TFA	mg/g DW	199.9 ± 62.9	282.9 ± 23.1	446.2 ± 92.9	427.3 ± 71.2

Data are means of three replicates ± standard deviations.

4.4.4 Characterization of algal biomass composition

To ensure that crude glycerol derived algal biomass is suitable for use in omega-3 fortified food or animal feed, a thorough chemical characterization of the algal biomass was performed. Because soap residue had shown negative effects on algal growth and DHA production (Figure 4-1), the algae were grown in soap-free medium. Algae derived from all three crude glycerol samples were evaluated. Algae grown on glucose were also evaluated as a control.

Table 4-8 shows the proximate analysis of the algal biomass. Overall, the algae grown on the three crude glycerol samples had similar nutritional profiles, except that G2 had a relatively high portion of lipid and low portion of protein. The ash content of G1 was higher than those of G2 and G3. Compared with the glucose-derived algae, the glycerol-derived algae had similar lipid and protein contents, but less carbohydrate; the ash content of glycerol algae was higher.

Table 4-8. Proximate analysis of freeze dried algal biomass grown on glucose and different crude glycerol streams (soap-free). (75 g/L of carbon source was used).

	Carbon source			
Composition (%, w/w)	Glucose	G1	G2	G3
Lipid	44.61 ± 3.91	43.24 ± 1.28	50.57 ± 1.32	46.71 ± 1.01
Protein	13.05 ± 0.10	16.22 ± 0.00	13.90 ± 0.00	20.78 ± 0.66
Carbohydrate	33.38 ± 3.91	25.69 ± 1.38	23.93 ± 1.34	23.02 ± 1.48
Ash	7.99 ± 0.14	13.88 ± 0.52	10.62 ± 0.19	8.51 ± 0.86
Moisture	0.97 ± 0.00	0.97 ± 0.01	0.98 ± 0.00	0.97 ± 0.00

Data are means of three replicates ± standard deviations.

Table 4-9 shows the fatty acid profiles of the algae. All of the carbon sources yield similar fatty acid compositions. C16:0 and C22:6 (DHA) accounted for about 90% of the total fatty acid (TFA). The alga also contained small amounts of C14:0, C18:0 and C22:5 (DPA).

Table 4-9. Fatty acid composition of algal biomass grown on glucose and different crude glycerol streams (soap-free). 75 g/L of carbon source was used.

Fatty Acid	Carbon source			
	Glucose	G1	G2	G3
	mg/g dry biomass			
C14:0	33.7 ± 1.0	28.8 ± 0.6	29.2 ± 0.3	31.1 ± 2.38
C16:0	271.2 ± 17.6	263.0 ± 1.5	287.5 ± 2.2	276.1 ± 2.2
C18:0	3.2 ± 0.5	3.3 ± 0.1	4.2 ± 0.1	4.6 ± 1.1
C22:5	8.3 ± 2.7	9.8 ± 1.2	14.3 ± 1.2	11.7 ± 0.8
C22:6	119.4 ± 13.4	117.7 ± 3.1	156.9 ± 1.2	121.2 ± 2.6
	mass % of total fatty acids			
C14:0	7.7 ± 0.2	6.8 ± 0.1	5.9 ± 0.1	7.0 ± 0.5
C16:0	62.2 ± 4.0	62.2 ± 0.4	58.4± 0.4	62.1 ± 0.5
C18:0	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	1.0 ± 0.2
C22:5	1.9 ± 0.6	2.3 ± 0.2	2.9 ± 0.2	2.6 ± 0.2
C22:6	27.4 ± 3.1	27.8 ± 0.7	31.9 ± 0.2	27.3 ± 0.6

Data are means of three replicates ± standard deviations.

The amino acid composition of algal biomass (Table 4-10, top half) is rich in cysteine (4.6-5.2 mg/g) and lysine (7.1-8.5 mg/g) relative to many common feedstuffs; lysine and cysteine are frequently the first or second limiting amino acids in feedstuffs for a given species (Kim et al., 2001; Greenwood and Titgemeyer, 2000; Webel and Baker, 1999). Although there are significant differences in the concentrations of many amino acids between the algae grown on different carbon sources, this is largely due to differences in overall concentration of protein

(Table 4-8). Comparison of the amino acid composition in terms of percent of total protein (Table 4-10, bottom half) reveals that the composition of the protein in each algal culture is very similar.

Table 4-10. Amino acid composition of algal biomass grown on glucose and different crude glycerol streams (soap-free). (75 g/L of carbon source was used.)

	Carbon Source			
	Glucose	G1	G2	G3
Amino Acid	mg/g dry biomass			
asx *	10.4 ± 0.2	12.1 ± 1.4	10.7 ± 0.4	10.4 ± 0.2
Ser	5.3 ± 0.1	6.2 ± 0.7	5.5 ± 0.3	5.3 ± 0.1
glx *	17.2 ± 0.3	20.5 ± 2.2	18.8 ± 0.8	17.2 ± 0.3
Gly	4.4 ± 0.1	5.3 ± 0.7	4.8 ± 0.2	4.4 ± 0.1
His	2.4 ± 0.1	2.9 ± 0.4	2.5 ± 0.1	2.4 ± 0.1
NH_3**	2.2 ± 0.0	2.6 ± 0.1	2.3 ± 0.1	2.2 ± 0.0
Arg	8.4 ± 0.8	9.4 ± 0.1	7.7 ± 0.3	8.4 ± 0.8
Thr	5.4 ± 0.0	6.2 ± 0.7	5.6 ± 0.2	5.4 ± 0.0
Ala	6.2 ± 0.1	7.5 ± 0.9	6.7 ± 0.3	6.2 ± 0.1
Pro	4.2 ± 0.0	5.0 ± 0.6	4.4 ± 0.2	4.2 ± 0.0
Tyr	4.3 ± 0.3	5.1 ± 0.9	5.4 ± 0.2	4.3 ± 0.3
Val	5.8 ± 0.2	7.0 ± 0.8	6.3 ± 0.3	5.8 ± 0.2
Cys	5.2 ± 0.1	4.6 ± 1.6	5.1 ± 0.5	5.2 ± 0.1
Met	1.3 ± 1.4	3.9 ± 0.2	3.2 ± 0.3	1.3 ± 1.4
Ile	4.6 ± 0.1	5.9 ± 0.7	5.3 ± 0.2	4.6 ± 0.1
Leu	8.1 ± 0.1	9.8 ± 1.2	8.7 ± 0.4	8.1 ± 0.1
Lys	7.1 ± 0.2	8.5 ± 1.1	7.5 ± 0.4	7.1 ± 0.2
Phe	5.0 ± 0.1	6.1 ± 0.7	5.4 ± 0.2	5.0 ± 0.1
	mass % of protein			
asx *	9.6 ± 0.2	9.4 ± 1.1	9.3 ± 0.4	9.3 ± 0.3
Ser	4.9 ± 0.1	4.8 ± 0.5	4.7 ± 0.2	4.7 ± 0.2
glx *	16.0 ± 0.3	15.9 ± 1.7	16.2 ± 0.7	15.3 ± 0.5
Gly	4.1 ± 0.1	4.1 ± 0.5	4.1 ± 0.2	4.1 ± 0.1
His	2.2 ± 0.1	2.2 ± 0.3	2.2 ± 0.1	2.2 ± 0.1
NH_3**	2.1 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	1.9 ± 0.2
Arg	7.8 ± 0.8	7.3 ± 0.1	6.7 ± 0.3	7.5 ± 1.1
Thr	5.1 ± 0.0	4.8 ± 0.5	4.8 ± 0.2	4.7 ± 0.2
Ala	5.8 ± 0.1	5.8 ± 0.7	5.8 ± 0.3	5.9 ± 0.2
Pro	3.9 ± 0.0	3.9 ± 0.5	3.8 ± 0.2	3.9 ± 0.2
Tyr	4.0 ± 0.3	4.0 ± 0.7	4.7 ± 0.2	3.9 ± 0.1
Val	5.4 ± 0.1	5.5 ± 0.6	5.5 ± 0.2	5.5 ± 0.2
Cys	4.8 ± 0.1	3.6 ± 1.2	4.4 ± 0.4	4.0 ± 0.1
Met	1.2 ± 1.3	3.0 ± 0.1	2.7 ± 0.2	3.4 ± 1.3
Ile	4.3 ± 0.1	4.6 ± 0.5	4.6 ± 0.2	4.6 ± 0.2
Leu	7.5 ± 0.1	7.6 ± 0.9	7.5 ± 0.3	7.8 ± 0.3
Lys	6.6 ± 0.1	6.6 ± 0.8	6.5 ± 0.3	6.6 ± 0.2
Phe	4.6 ± 0.1	4.7 ± 0.6	4.7 ± 0.2	4.8 ± 0.2

*glx = glu + gln ; asx = asp + asn

**NH_3 resulted from the deamination of asparagine and glutamine during the analysis process

Data are means of three replicates ± standard deviations.

Table 4-11 shows the results of ICP elemental analysis of algal biomass. All samples have essentially the same elemental profile. The biomass from G1 and G2 had higher potassium content than the biomass from G3 and glucose, probably due to the higher amount of potassium contained in the original G1 and G2 streams (Table 4-4). Although the stream G3 had a higher sodium content than G1 and G2 (Table 4-4), the algal biomass from these glycerol samples was not significantly different (Table 4-11), due to the fact that the artificial seawater medium contained a high level of sodium. All the other elements contained in the algal biomass were considered resultant from the seawater medium solution, instead of from the crude glycerol. It should be noted that no heavy metals were detected by the ICP analysis. Even specialized testing, using EPA method SW-846 7471A with a reporting limit of 0.025 ppm, did not detect any mercury in the algal biomass. Concerns about heavy metal contamination have limited the usage of fish oil products in food or animal feed, but these results alleviate any concerns about heavy metal contamination of algal biomass.

Table 4-11. ICP elemental analysis of algal biomass grown on glucose and different crude glycerol streams (soap-free). (75 g/L of carbon source was used; Elements analyzed in Table 4-1, but not included in this table were not detected in any sample).

	Carbon Source			
Element (ppm)	Glucose	G1	G2	G3
Boron	17.2 ± 5.3	23.7 ± 4.5	15.4 ±1.6	14.7±0.95
Calcium	506 ± 19	603 ± 55	647 ±43	592±102
Copper	6.5 ± 0.9	BDL	4.9 ± 1.8	BDL
Iron	69.0 ± 4.2	102±4.7	95.8 ± 8.2	94.5±5.9
Magnesium	1825 ± 155	2050±335	1715 ±205	1380±152
Phosphorus	2595 ± 355	3740±63	4290 ± 114	3610±124
Potassium	5503 ± 998	18600±1700	12450 ± 1150	8310±560
Silicon	16.1 ± 3.2	26.5±3.95	19.6 ± 0.4	10.6±1.74
Sodium	156650 ± 950	26400±1250	14750 ± 1750	17900±1932
Sulfur	6145 ± 747	6530±605	7900 ± 600	5320±386

Data are means of three replicates ± standard deviations.
BDL: Below Detection Limit

In summary, the above compositional analysis indicates that the algal biomass grown in crude glycerol had a similar composition to the algae grown in glucose. Currently, *Schizochytrium* algae are available from commercial producers such as Aquafauna Bio-Marine

Inc. (Hawthorne, CA; http://www.aquafauna.com/Profiles-AlgaMac-3000.htm) and Advanced BioNutrition Corp. (Columbia, MD; http://www.advancedbionutrition.com/html/abn_dha.pdf). The algae produced from biodiesel-derived glycerol had a composition similar to the commercial algae. Different sources of crude glycerol did not cause significant variation of algal biomass composition. The results indicate that producing omega-3 algae from crude glycerol is a feasible and durable option for utilizing this glycerol. The algal biomass is a safe option for use as an ingredient in omega-3 fortified food or animal feed.

4.5 Acknowledgements

The authors gratefully acknowledge Virginia Tech Institute for Critical Technology and Applied Science (ICTAS), Virginia Agricultural Council, U.S. Poultry and Egg Association, Fats and Proteins Research Foundation, and Virginia Sea Grant for their financial support of this project. Dr. Steven Craig at the Virginia Tech Aquaculture Center performed proximate analysis.

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Chapter 5: Conclusions and Recommendations for Future Work

The work presented in this thesis leads to a deeper understanding of using crude glycerol from the biodiesel industry as a carbon source for microalgal fermentation to produce omega-3 fatty acids.

The work conducted in Chapter 3 provides a "proof-of-concept" that the alga *Schizochytrium limacinum* is capable of utilizing crude glycerol as a carbon source for DHA production. The results show that 75 g/L to 100 g/L of glycerol is the optimum range for algal growth and DHA production. Algal DHA production is also significantly ($P < 0.1$) influenced by culture temperature, the levels of trace metals in the medium, ammonium acetate concentration, and NH_4Cl concentration. A DHA yield of 4.91 g/L with 22.1 g/L biomass concentration was obtained when the culture conditions were at the optimal levels.

The work conducted in Chapter 4 built upon the previous feasibility study. The aim was to ensure that the algal biomass could be safely utilized as an animal feed additive. The study showed that both methanol and soaps in the glycerol negatively influence algal DHA production, but these impurities can be easily removed from the culture medium by evaporation through autoclaving (for methanol) or by precipitation through pH adjustment (for soap). The glycerol-derived algal biomass had similar proximate characteristics and fatty acid/amino acid profiles as those of commercial algal biomass, which are obtained from glucose-based culture. Furthermore, ICP analysis showed that no heavy metals were present in the algal biomass. Based on the above results, it was concluded that that crude glycerol is a suitable and safe carbon source for algal fermentation.

In summary, the results presented in this thesis show that pretreated crude glycerol from the biodiesel industry can be used as a carbon source for the DHA-producing microalga *Schizochytrium limacinum*. Further work needs to be focused on developing optimized culture protocols in fermenter operations and scaling-up the optimized algal fermentation process to pilot scale. Eventually, an economic evaluation of the process is needed. Another aspect as a continuation of this study will be the development of omega-3 fortified food and animal feeds from this crude glycerol-derived algal biomass, and this work is under way.