Producing Omega-3 Polyunsaturated Fatty Acids from Biodiesel Waste Glycerol by Microalgae Fermentation

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Abstract

Crude glycerol is a major byproduct if the biodiesel industry. Biodiesel manufacturers are currently facing the challenges of appropriate disposal of this waste material. Crude glycerol is expensive to purify for use in food, cosmetic, and pharmaceutical industries and therefore, alternative methods for use of this crude glycerol are needed. A promising alternative is to use this crude glycerol as a carbon source for microalgae fermentation.

In this project, we investigated the use of crude glycerol as a less expensive substrate for the fermentation of the microalgae *Schizochytrium limacinum* and *Pythium irregulare* which are prolific producers of omega-3 polyunsaturated fatty acids. Omega-3 fatty acids have many beneficially effects on treating human diseases such as cardiovascular diseases, cancers, and neurological disorders. In addition, the omega-3 fatty acids docosahexaenoic acid (DHA) has been shown to be an important factor in infant brain and eye development.

The first part of this study focused on the continuous fermentation of *S. limacinum*, a prolific producer of DHA. The objective of this study was to examine the algal cellular physiology and maximize its DHA productivity. Two important parameters used in continuous fermentation were studied: dilution rate (D) and feed glycerol concentration (S_0). The highest biomass productivity of 3.88 g/L-day was obtained at D = 0.3 day^{-1} and S_0 = 60 g/L, while the highest DHA productivity (0.52 g/L-day) was obtained at D = 0.3 day^{-1} and S_0 = 90 g/L. The cells had a true growth yield of 0.283 g/g, a maximum specific growth rate of 0.692 day^{-1}, and a maintenance coefficient of 0.2216 day^{-1}.

The second part of this study focused on morphology issues with *P. irregulare*, a prolific producer of eicosapentaenoic acid (EPA). *P. irregulare* has a filamentous morphology, which can make fermentation difficult. The mycelium can stick to the agitation blades resulting in mechanical problems. In addition, this filamentous morphology prevents adequate amounts of oxygen from reaching some cells resulting in decreased productivities. The focus of this research was to control the fermentation conditions to make the algae grow in small pellets, a

morphology more suitable for fermentation. In flask culture studies, pellets were formed at an agitation speed of 110 rpm in both regular and baffled flasks. Baffled flasks resulted in pellet formation at 90 and 130 rpm as well. Fermentation studies resulted in pellet formation at agitation speeds of 150 and 300 rpm. Pellets were better able to form when a baffle was not in place. In addition, agitation speed influenced pellet size, with smaller pellets forming at the higher agitation speed.

Overall, this study showed that crude glycerol can be used as a carbon source for the continuous fermentation of *S. limacinum* with high DHA productivity and the morphology of *P. irregulare* could be controlled by manipulating culture conditions, mainly agitation speed. These results show the potential for scale-up studies for both algal species.

Attribution

Author Shannon E. Ethier 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. David Vaughan was the Co-Committee Chair. Kevin Woisard was a graduate student of Dr. Zhiyou Wen.

All photographs by author Shannon E. Ethier.

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Chapter 1: Overview and motivation

1.1 Rational and Significance

With the increasing price of crude oil worldwide, the biodiesel industry has grown rapidly over the past few years. A major byproduct of biodiesel production is crude glycerol. This crude glycerol contains impurities, such as soap and methanol, making it expensive to purify for pharmaceutical, food, and cosmetic industries (Johnson and Taconi, 2007). Therefore, new uses need to be found for this crude glycerol.

Previous research has shown that this crude glycerol can be used as a carbon source for the microalga *Schizochytrium limacinum* and the oomycete *Pythium irregulare* (Pyle et al., 2008; Athalye et al., 2009). *S. limacinum* is a heterotrophic producer of docosahexaenoic acid (DHA). The dry cell weight of this species can be up to 50% fatty acids and approximately 30% of the total fatty acids are DHA (Morita et al. 2006). *P. irregulare* is an oomycete that can produce high levels of eicosapentaenoic acid (EPA) (Stinson et al., 1991). This species has a filamentous morphology and can grow in mycelial clumps or pellets. *P. irregulare* tends to grow in mycelial clumps, which can make fermentation difficult (O'Brien et al., 1993). Previous research with other species has shown that this morphology can be controlled by medium conditions such as pH and temperature (Liao et al., 2007). Using crude glycerol as a carbon source for both of these omega-3 fatty acid-producing species would provide an economical alternative for use of this waste product.

Omega-3 fatty acids are essential fatty acids that have many health benefits. Omega-3 fatty acids have also been shown to promote good cardiovascular health and can help reduce the effects of neurological disorders such as Alzheimer's disease (Simopoulos, 2002). In particular, DHA is an important fatty acid for proper brain and eye development in infants and young children (Innis, 2007). Currently, the major source of omega-3 fatty acids is fish oil, which has many problems such as undesirable taste and odor and varying qualities. In addition, the demand for omega-3 fatty acids has put a strain on the fishing industry (Certik and Shimizu, 1999). Therefore, an alternative source of omega-3 fatty acids is needed.

Using biodiesel-derived crude glycerol as a carbon source for omega-3 fatty acid-producing microalgae solves two problems. First, it provides biodiesel producers with a method

of disposal for their waste glycerol. Second, it provides a sustainable source of omega-3 fatty acids, reducing the strain on the fishing industry.

1.2 Hypothesis

Biodiesel-derived crude glycerol can be used as a carbon source for the microalgae *Schizochitrium limacinum* as a DHA producer and for the oomycete *Pythium irregulare* as an EPA producer. A continuous culture of *S. limacinum* can be used to investigate the cell growth kinetics of the algal cells. While the morphology control of *P. irregulare* is crucial for developing a cost-effective fermentation process. The morphology of *P. irregulare* can also be controlled in flask culture by manipulating pH, temperature, agitation speed, and flask type. In addition, these culture conditions can be used to obtain pellet formation in batch fermentation.

1.3 Objectives

The objectives of this project are to:

1) Investigate the growth kinetics of *S. limacinum* and maximize its DHA production using continuous culture fermentation, and

2) Control the morphology of *P. irregulare* by controlling flask culture conditions (pH, temperature, shaker speed, and flask type).

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Chapter 2: Literature Review

2.1 Crude Glycerol from Biodiesel Manufacturing

Biodiesel as a renewable energy source is an excellent alternative to petroleum diesel (Zhang et al., 2003). In recent years, biodiesel production has grown rapidly with increasing prices of crude oil worldwide (Johnson and Taconi, 2007). Crude glycerol is a major byproduct created during the biodiesel production process, for every gallon of biodiesel produced 0.3 kg of crude glycerol is created. Crude glycerol contains many impurities such as soap, methanol, and other substances such as calcium, magnesium, phosphorous, and/or sulfur (Thompson and He, 2006). These impurities make the glycerol too expensive to purify for use in pharmaceutical, food, and cosmetic industries. Therefore, developing new ways for use of this crude glycerol is imperative (Johnson and Taconi, 2007).

2.1.1 Biodiesel Production

Biodiesel is produced through a transesterification process between triglycerides and an alcohol, usually methanol, using an acidic, basic, or enzymatic catalyst (Figure 2-1). Typical triglyceride sources include vegetable oils, waste cooking oils, and animal fats. Alkali catalysts such as sodium hydroxide or potassium hydroxide are commonly used (Ma and Hanna, 1999). Triglycerides are composed of three long chain fatty acids connected to a glycerol backbone. When the triglycerides react with methanol the fatty acid chains are released (Zhang et al., 2003). Since the reaction is reversible, excess methanol is typically used to shift the equilibrium to the products side. The reaction forms methyl esters (biodiesel) and a crude glycerol byproduct (Ma and Hanna, 1999).

$$CH_2 - O - C - R$$
$$CH_2 - O - C - R'$$
$$CH_2 - O - C - R''$$

Triglyceride $+$ $3CH_3-OH$ $\rightleftharpoons$

$$CH_3 - O - C - R$$
$$CH_3 - O - C - R'$$
$$CH_3 - O - C - R''$$

Fatty acid methyl esters $+$

$$CH - OH$$
$$CH - OH$$
$$CH_2 - OH$$
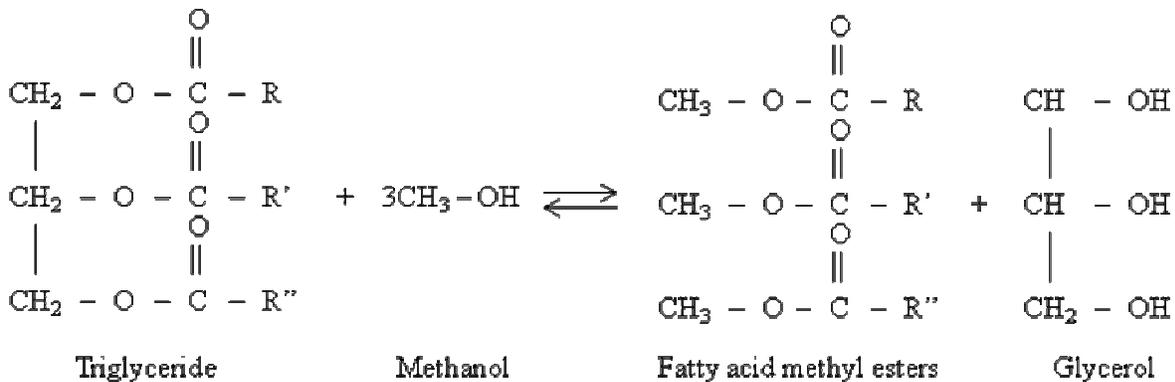
Methanol   Glycerol

Figure 2- 1. Transesterification reaction to produce biodiesel (adapted from Zhang et al., 2003; fair use).

2.1.2 Composition of Crude Glycerol

The crude glycerol formed during biodiesel production contains many impurities such as methanol, soap, and various elements. Biodiesel producers use excess methanol to ensure completion of the transesterification reaction. Up to 80% of this excess methanol can end up in the crude glycerol (Thompson and He, 2006). The rest of this excess methanol ends up in the biodiesel produced (Chiu et al., 2005). The crude glycerol also contains soap formed from side reactions and trace elements such as calcium, potassium, magnesium, sodium, phosphorous, and sulfur (Thompson and He, 2006).

Glycerol makes up about 60% to 80% (w/w) of the crude glycerol stream with the remaining weight being methanol and soaps. Thompson and He (2006) showed that the quality of the crude glycerol stream depended highly on the feedstock used in biodiesel production. Waste vegetable oil had the highest glycerol purity (76.6%) while crambe oil had the lowest (62.5%) (Thompson and He, 2006).

Schröder and Südekum (1999) conducted research on the composition of crude glycerol with rapeseed oil as the feedstock. They found glycerol concentrations from 63.3 to 85.3% (w/w) with methanol concentrations between 0.4 and 26.7% (w/w). Phosphorous, potassium, and sodium were also present in this crude glycerol while cadmium, mercury, and arsenic were all under detectable limits (Schröder and Südekum, 1999).

2.1.3 Uses of Crude Glycerol

One simple method for crude glycerol disposal is combustion. This method, though, is not economically ideal for large-scale biodiesel producers since it is not profitable (Johnson and

Taconi, 2007). Another simple method is to use the crude glycerol as an extra carbon source in anaerobic digestion to increase biogas yield. Caution has to be taken with this method though, as a high glycerol concentration can cause organic overloading (Holm-Nielsen et al., 2008). Another method for crude glycerol disposal is to compost it, but composting is not economically feasible for commercial biodiesel producers (Crooks, 2007).

Using crude glycerol as an animal feed supplement has been studied in dairy cows, pigs, and chickens. In a previous study, researchers tested whether or not feeding crude glycerol to dairy cows could prevent ketosis, an elevation of ketones in the blood of dairy cows. The research showed that the crude glycerol feed did not affect dairy cow ketosis, but did affect the propionate and butyrate ruminal profiles of the dairy cows. These cows had increased propionate and butyrate levels in the rumen, which caused a decreased ratio of acetate to propionate (DeFrain et al., 2004). In another study, glycerol was fed to lactating dairy cows to replace corn grain. Glycerol fed cows produced similar amounts of milk when compared to corn fed cows. The composition of the glycerol-fed dairy cow milk was also similar to the composition of the milk from corn fed dairy cows (Donkin et al., 2009).

Crude glycerol has also been used as an energy source for pigs. Researchers found that the ratio of metabolized energy to digestible energy was approximately 96%, which was similar to common pig feedstocks such as corn and soybean oil (Lammers et al., 2007). From further research, up to 10% of a growing pig's diet can consist of crude glycerol without detrimental effects to meat quality. Although methanol concentrations in the diet were a concern, the study showed that the methanol concentrations in crude glycerol did not negatively affect the pigs (Lammers et al., 2008).

Crude glycerol was also fed to broiler chickens (Cerrate et al., 2006). Chickens diets containing 2.5 and 5% crude glycerol led to higher breast yields than chickens fed a control diet. However, there are concerns about residual methanol in the feed (Cerrate et al., 2006). In another study, crude glycerol was fed to laying hens and was found to be a rich source of energy. Up to 6% of the laying hens' diets could consist of crude glycerol without any detrimental effects to the eggs produced (Swiatkiewicz and Koreleski, 2009).

Researchers are also studying ways to use this crude glycerol to make higher value products. Some of these products include propylene glycol (Chiu et al., 2006a), acetol (Chiu et al., 2006b), and 1,3-propanediol (González-Pajuelo et al., 2005). A cost-effective option for use

of this glycerol is to use a less refined form as a carbon source for microorganisms (Yokochi et al., 1998). Past research has shown that crude glycerol can be used as the sole carbon source for *Clostridium pasteurianum* to produce solvents, particularly butanol (Taconi et al., 2009). Researchers have also shown that the DHA-producing algal species *Schizochytrium limacinum* and the EPA-producing algal species *Pythium irregulare* can use crude glycerol as a carbon source with little refining (Pyle et al., 2008; Athalye et al., 2009).

2.2 Omega-3 Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids are fatty acids that contain multiple double bonds, with the last double bond occurring at the third carbon from the methyl end of the fatty acid chain. Two important omega-3 fatty acids are eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Wen and Chen, 2003). The chemical structures of these fatty acids can be seen in Figure 2-2.
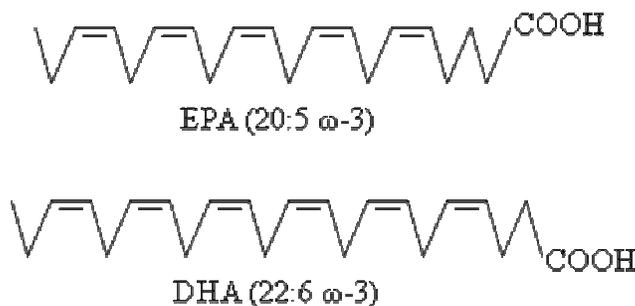
Figure 2- 2. Chemical structures of EPA and DHA (adapted from Wen and Chen, 2003; fair use).

Omega-3 fatty acids play an important role in human health. They control the expression of specific genes (Sessler and Ntambi, 1998), which affects processes in the body such as cholesterol transport and fatty acid biosynthesis. In addition, omega-3 fatty acids regulate the structure, permeability, and various other aspects of the cell membrane. They are required for every organ of the body for normal function to occur. Humans and most animals cannot synthesize omega-3 fatty acids and therefore, they must be obtained through the diet (Certik and Shimizu, 1999).

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2.2.1 Health Benefits of Omega-3 Polyunsaturated Fatty Acids

The human body is designed to live on a diet with a balance between omega-3 and omega-6 fatty acids, approximately a ratio of 1:1 (Simopoulos, 2002). The typical American diet, though, has a ratio of omega-6 to omega-3 fatty acids of 14:1 (Marik and Varon, 2009). Therefore, omega-3 fatty acids are an essential part of the American diet.

Infant brain and eye development

DHA is an important fatty acid for infant brain and eye development. A dietary deficiency in omega-3 fatty acids can cause a decrease of DHA in retina phospholipids and brain grey matter. This decrease can impair the development of these organs. Infants born with low levels of DHA in the blood tend to have lower visual and neural maturation than children born with normal DHA levels (Innis, 2007). Makrides et al. (1995) conducted a study, which found that infants fed DHA supplemented formulas and breast-fed babies had better visual acuity than infants fed a standard formula (Makrides et al., 1995). In addition, low levels of DHA can impair neurogenesis and disrupt the dopamine and serotonin metabolisms in the brain (Innis, 2007). Low levels of DHA in the brain can cause docosapentaenoic acid (DPA, 22:5 n-3) levels to increase. These high levels of DPA can lead to cognitive and behavior problems in children (Kim, 2008). Toddlers who had high DHA levels at birth had longer attention spans than toddlers who were born with low DHA levels (Kannass et al., 2009). A dietary deficiency of DHA during infancy can affect the child in future years. Four-year-old children who did not received DHA and ARA supplemented formula during their first 17 weeks of life had poorer visual acuity and verbal IQs that breast-fed children or children who received DHA and ARA supplementation (Birch et al., 2007).

Cardiovascular benefits

Consumption of omega-3 fatty acids can help reduce the incidences of cardiovascular events such as arrhythmias, inflammation, hypertension, and atherothrombosis (Masson et al., 2007; Marik and Varon, 2009). Omega-3 fatty acids can help lower a person's risk of cardiovascular disease by lowering heart rate and blood pressure. In addition, omega-3 fatty acids can reduce triglycerides in blood serum, the formation of blood clots, and irregular heartbeats (Hooper et al., 2006). EPA and DHA are precursors to certain eicosanoids such as

prostaglandins, thromboxanes, and leukotrienes. These eicosanoids are responsible for some of the cardiovascular benefits of omega-3 fatty acid consumption. They are anti-inflammatory, antithrombotic, and antiarrhythmic compounds (Covington, 2004). Omega-3 fatty acids are found in cardiac cell membranes, which may help protect the body from cardiac events such as arrhythmias (Masson et al., 2007). Previous studies have shown that people whose diets consist mainly of fish high in omega-3 fatty acids have a lower risk of coronary artery disease. Supplementing one's diet with omega-3 fatty acids for a year or more significantly decreases the risk of cardiovascular death and nonfatal cardiovascular events (Marik and Varon, 2009). To have a cardioprotective effect, people should consume approximately 1 g of omega-3 fatty acids each day. Higher doses of omega-3 fatty acids (2 – 4 g/day) are necessary for people who want to reduce their triglyceride levels (Covington, 2004).

Cancer

DHA may also have a positive effect on the prevention and treatment of cancer. DHA contains five methylene groups making it susceptible to oxidization. The oxidation products are connected with apoptosis in cancer cells. DHA is in high levels in healthy cells protecting them from apoptosis, while DHA is lower in unhealthy cells inducing apoptosis (Siddiqui et al., 2008). Increased levels of DHA may help increase the effectiveness of chemotherapy and reduce the side effects of chemotherapy treatment (Hardman, 2002). Lower incidences of certain types of cancer such as breast, prostate, and colon, have been reported in people whose diets are rich in omega-3 fatty acids (MacLean et al., 2006). Since fish tend to have high levels of omega-3 fatty acids, people in countries where fish is a large part of their diets have lower incidences of these cancers. In addition, researchers have found that immigrants from these countries to countries consuming less fish had a higher incidence of cancer (Berquin et al., 2007).

Research has shown that omega-3 fatty acids can regulate the expression of Enhancer of Zeste Homologue 2 (EZH2), a protein that is over-expressed in patients with breast cancer and prostate cancer. By regulating this protein, omega-3 fatty acids can inhibit the proliferation of cancer cells (Dimri et al., 2010). Other studies have shown that diets rich in omega-3 fatty acids can have anti-inflammatory and anti-carcinogenic effects on the colon. Omega-3 fatty acids are believed to inhibit pro-inflammatory products derived from omega-6 fatty acids (Daniel et al., 2009).

Psychiatric benefits

Low levels of DHA are associated with neurodegenerative disorders such as Alzheimer's disease and generalized peroxisomal disorders (Kim, 2008). From epidemiological studies, low levels of DHA in blood serum and brain membranes have been found in patients suffering from Alzheimer's disease (Lim et al., 2005). In behavioral animal studies, animals fed diets supplemented with omega-3 fatty acids learned faster and had better memory than animals on a standard diet (Morris et al., 2003).

Low levels of DHA and EPA have also been connected with depression in healthy individuals (Ali et al., 2009). In animal studies, a deficiency in omega-3 fatty acids led to decreased levels of serotonin and dopamine, two important neurotransmitters that can affect mood. Epidemiological studies have shown a correlation between depression and suicide with dietary deficiencies of omega-3 fatty acids (Hibbeln, 2009). In addition, one study found a strong correlation between low omega-3 fatty acid levels and depression in patients with stable coronary heart disease (Ali et al., 2009).

Omega-3 fatty acids are also believed to be beneficial in bipolar disorder, a manic depressive illness. Current drugs used to treat bipolar disorder inhibit signal transduction. These drugs tend to have adverse side effects and high toxicity (Stoll et al., 1999). Some of the adverse effects of current treatment include weight gain, gastrointestinal upset, increases in triglyceride levels, and thyroid effects (Clayton et al., 2009). Omega-3 fatty acids are believed to inhibit the same signal transduction pathways as traditional treatments without the adverse side effects (Stoll et al., 1999). In one study, children suffering from juvenile bipolar disorder were given an omega-3 fatty acid supplement of DHA and EPA for six weeks. The study found that after supplementation, the children had lower clinical ratings of mania and depression (Clayton et al., 2009).

Obesity

Obesity is a growing problem throughout the developed world (Buckley and Howe, 2009). Omega-3 fatty acids may be able to alleviate and/or prevent obesity. Omega-3 fatty acids can help prevent obesity by effecting lipid metabolism. They can reduce lipid uptake by suppressing lipoprotein lipase, an enzyme that hydrolyzes triglycerides. Omega-3 fatty acids,

specifically EPA, promote oxidation of mitochondrial fatty acids. In addition, omega-3 fatty acids can decrease lipid synthesis by inhibiting fatty acid synthase, which is involved in the synthesis of fatty acids (Li et al., 2008). Previous research has shown that the omega-3 fatty acid EPA has the potential to lower lipid concentrations in blood serum (Mitsuyoshi et al., 1991). Animal studies have shown that supplementing a high-fat obesity-inducing diet with omega-3 fatty acids can reduce body fat accumulation. In other animal studies, scientists have shown that supplementing an obese mouse's diet with omega-3 fatty acids caused a loss in body weight (Buckley and Howe, 2009).

Arthritis

Rheumatoid arthritis is a debilitating inflammatory disease (James and Cleland, 1997). The symptoms of rheumatoid arthritis can be reduced or alleviated by consumption of omega-3 fatty acids. Approximately 3 g of omega-3 fatty acids per day can reduce joint tenderness and morning stiffness in patients suffering from rheumatoid arthritis (Covington, 2004). Adding omega-3 fatty acids to the diet of rheumatoid arthritis patients can increase the efficacy of anti-inflammatory drugs. Omega-3 fatty acids also have the potential to reduce the toxicity of anti-inflammatory and antirheumatic drugs (James and Cleland, 1997).

2.2.2 Biosynthesis of Omega-3 Polyunsaturated Fatty Acids

The biosynthesis of omega-3 fatty acids begins with *de novo* synthesis of short chain fatty acids, typically oleic acid, from acetate. Most organisms are capable of *de novo* fatty acid synthesis. Next, the oleic acid is subjected to a series of desaturation and elongation reactions to form longer chain fatty acids as can be seen in Figure 2-3. Many microorganisms have the proper enzymatic pathways to produce omega-3 fatty acids, while higher plants and animals lack this ability (Wen and Chen, 2003). Therefore, dietary sources are needed to obtain omega-3 fatty acids.

Acetate

$\downarrow$

Oleic Acid (18:1, ω-9)

$\downarrow$ desaturase

Linoleic Acid (18:2, ω-6)

desaturase $\swarrow$ $\searrow$ desaturase

Gamma-Linolenic Acid (18:3, ω-6)   Alpha-Linolenic Acid (18:3, ω-3)

elongation $\downarrow$ $\downarrow$ desaturase

Dihomo-Gamma-Linolenic Acid (20:3, ω-6)   Stearidonic Acid (18:4, ω-3)

desaturase $\downarrow$ $\downarrow$ elongation

Arachidonic Acid (20:4, ω-6)   Eicosatetraenoic Acid (20:4, ω-3)

desaturase $\searrow$ $\swarrow$ desaturase

Eicosapentaenoic Acid (20:5, ω-3)

$\downarrow$ elongation

Docosapentaenoic Acid (22:5, ω-3)

$\downarrow$ desaturase

Docosahexaenoic Acid (22:6, ω-3)

Figure 2- 3. The biosynthetic pathways for omega-3 polyunsaturated fatty acids (adapted from Wen and Chen, 2003).

2.2.3 Sources of Omega-3 Polyunsaturated Fatty Acids

Traditional Sources

Currently, the most common source of EPA and DHA is fish oil. Unfortunately, there are several limitations with fish oil as an omega-3 source such as its undesirable taste and odor. In addition, impurities must be removed from the fish oil before human consumption such as cholesterol and toxic impurities such as mercury (Certik and Shimizu, 1999). Other toxic compounds including methylmercury, dioxins, and polychlorinated biphenyls may also be found in fatty fish (Hooper et al., 2006). These toxins are fat-soluble and can accumulate in the body over time (Marik and Varon, 2009). Consumption of these toxicants can lead to increased risks of cancer and neurological damage (Hooper et al., 2006). There are large variations in the quality of fish oil as well. The amount of omega-3 fatty acids in fish oil is dependent on many factors such as the season, the type of fish, where the fish is harvested, and food availability (Certik and Shimizu, 1999). Fish oil commonly contains 9 to 27% EPA (Cantrell and Walker, 2009). The demand for omega-3 fatty acids has also put a strain on the fishing industry (Pyle et al., 2008). Flaxseed, canola oil, and walnuts are also dietary sources of omega-3 fatty acids, but contain mainly α-linolenic acid (ALA) with little DHA and EPA (Covington, 2004). Therefore, alternative sources of omega-3 fatty acids, specifically DHA and EPA, are needed.

Alternative Sources

Using microalgae to produce omega-3 fatty acids eliminates many of the undesirable factors of traditional sources. In addition, microalgae can supply omega-3 fatty acids at high concentrations. Microalgae can also be grown on low to no-cost nutrients, which make them an economically viable source of omega-3 fatty acids (Certik and Shimizu, 1999). Species of *Crypthecodinium*, *Thraustochytrium*, and *Schizochytrium* are rich the omega-3 fatty acid DHA, while species of *Phaeodactylum*, *Monodus*, and *Pythium* are rich in EPA. *Crypthecodinium cohnii* is a heterotrophic algal species that is currently used to produce the DHA used in many infant formulas (Ward and Singh, 2005) Previous research has shown that approximately 50% *Thraustochytrium aureum*'s total fatty acids is DHA. The total fatty acid content of *T. aureum*, though, is greatly influenced by medium composition and can range from 0.3-16% of the biomass (Bajpai et al., 1991). *Schizochytrium limacinum* is another heterotrophic algal species

that can produce high yields of DHA. *S. limacinum* has also been shown to produce these high DHA yields under various culture conditions (Yokochi et al., 1998).

Phaeodactylum tricornutum is a high EPA-producing algal species with EPA comprising 30-40% of its total fatty acids when grown using optimum culture conditions. *P. tricornutum* is sensitive to temperature, which must be kept between 21.5 and 23°C to obtain high EPA yields (Yongmanitchai and Ward, 1991). *Monodus* species are photoautotrophic algae that can produce high levels of EPA, but the dependence on light results in low cell densities making them unfavorable species to use in the industrial production of EPA (Ward and Singh, 2005). *Pythium irregulare* is a heterotrophic producer of EPA that can grow on various substrates and at different culture conditions (Cheng et al., 1999).

Transgenic plants have also been considered as an alternative source of omega-3 fatty acids. Robert et al. (2005) were able to express a set of genes related to the elongation and desaturation of fatty acids in *Arabidopsis thaliana*. The expression of these genes resulted in the synthesis of EPA and DHA, which were found in the seed oils (Robert et al., 2005). In another study, researchers were also able to express EPA in *Arabidopsis thaliana*. They used a set of elongation and desaturation genes from three different species (*Isochrysis galbana*, *Euglena gracilis*, and *Mortierella alpina*) to transform *Arabidopsis thaliana*. In this case the fatty acids were expressed in the leaves of the plant (Qi et al., 2004).

Transgenic animals are also being considered as an alternative source of omega-3 fatty acids. Generally, meat products are low in omega-3 fatty acids, but recent studies have shown that transgenic animals can produce high levels of omega-3 fatty acids present in the meat. Lai et al. (2006) have shown that transgenic pigs containing the *fat-1* gene can convert omega-6 fatty acids to omega-3 fatty acids. The *fat-1* gene is an omega-3 fatty acid desaturase gene found in the roundworm *Caenorhabditis elegans*. The transgenic pigs, when compared to their wild-type littermates, showed a substantially lower ratio of omega-6 to omega-3 fatty acids (Lai et al., 2006). Transgenic plants and animals are two interesting alternative sources of omega-3 fatty acids, but are still in the early stages of development. In addition, consumers are apprehensive about transgenic plants and animals.

2.3 Schizochytrium limacinum as a DHA producer

S. limacinum, a marine microorganism, was first discovered in 1994 in the west Pacific Ocean in a mangrove area off of the Yap Islands of Micronesia (Honda et al., 1998). It is a heterotrophic producer of DHA. Up to 50% of this species' dry cell weight can comprise of fatty acids; approximately 30% of the total fatty acids are DHA (Morita et al., 2006). Past studies have shown that *S. limacinum* can produce approximately four grams of DHA for every liter of media, which is higher than other species studied (Yokochi et al., 1998). Due to its ability to produce large amounts of DHA many studies on media composition and growth conditions have been conducted. Yokochi et al. (1998) studied how *S. limacinum* grew on various carbon and nitrogen sources. They found that *S. limacinum* could grow (10 g/L or higher cell biomass) and produce high levels of DHA (0.25 g/L and higher) using glucose, fructose, glycerol, oleic acid, or linseed oil as a carbon source. In addition, they showed that yeast extract and corn steep liquor could be used as nitrogen sources for *S. limacinum* (Yokochi et al., 1998). In another study, researchers showed that *S. limacinum* can grow (8 g/L cell biomass) and produce DHA (1.5 g/L) using crude glycerol as a carbon source and corn steep solids as a nitrogen source (Pyle, 2008).

S. limacinum has also been shown to tolerate wide ranges of salinity: between 50 to 200% the salinity in seawater (Yokochi et al., 1998). *S. limacinum* can also tolerate variations in medium pH. Researchers have shown that biomass yield and DHA productivity were not affected by initial pH when the initial pH was between 5 and 8 (Chin et al., 2006). Studies have also shown that *S. limacinum* grows well at room temperature with an optimum temperature range between 20 and 30°C (Yokochi et al., 1998). These results show that *S. limacinum* has the ability to grow at a variety of culture conditions, which can be optimized for continuous culture fermentation.

2.4 *Pythium irregulare* as an EPA producer

Pythium irregulare is a water mold, which produces EPA. Previous research has shown that optimum EPA production occurs at a temperature of 12°C using glucose as the carbon source (Stinson et al., 1991). *P. irregulare* has the potential to grow on other substrates as well such as crude soybean oil, sucrose waste streams, soymeal waste streams, and crude glycerol (Cheng et al., 1999; Athalye et al., 2009). O'Brien et al. have shown that *P. irregulare* has a

significantly increased EPA content when grown using sweet whey permeate, a byproduct of the dairy industry, when compared to EPA content using glucose as the carbon source (O'Brien et al., 1993). Although maximum EPA production occurs at 12°C, *P. irregulare* can grow and produce EPA at room temperature making it an economical and convenient choice for EPA production in industry (Cheng et al., 1999).

Researchers have also studied the effect of initial pH on the growth and EPA production of *P. irregulare*. In a study by Stinson et al. (1991), initial pH was studied between 5 and 8. They found that growth was unaffected by the initial pH of the medium, but EPA production was significantly affected. EPA productivity was optimized when the pH was between 6 and 7 and significantly inhibited at an initial pH of 8 (Stinson et al., 1991).

This species, though, has a filamentous morphology. *P. irregulare* can grow in mycelial clumps or pellets, but tends to grow in mycelial clumps, which can make fermentation difficult (O'Brien et al., 2003). Previous research with other filamentous species has shown that changing culture conditions such as pH and temperature can control this morphology (Liao et al., 2007). In addition, growing filamentous species in baffled flasks creates a higher shear force, which can result in pellet formation (Teng et al., 2009).

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Chapter 3: Continuous Culture of the Microalgae *Schizochytrium limacinum* on Biodiesel-derived Crude Glycerol for Producing Docosahexaenoic Acid

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3.1 Abstract

Crude glycerol is a major byproduct of the biodiesel industry; previous research has proved the feasibility of producing docosahexaenoic acid (DHA, 22:6 n-3) through fermentation of the alga *Schizochytrium limacinum* on crude glycerol. The objective of this work is to investigate the algal cellular physiology and its DHA production potential through a continuous culture. Steady state biomass yield, biomass productivity, growth yield on glycerol, specific glycerol consumption rate, and fatty acid profile were investigated within the range of dilution rate (D) from 0.2 to 0.6 day^{-1}, and the range of feed crude glycerol concentration (S_0) from 15 to 120 g/L. The maximum specific growth rate was determined as 0.692 day^{-1}. The cells had a true growth yield of 0.283 g/g but with a relatively high maintenance coefficient (0.2216 day^{-1}). The highest biomass productivity of 3.88 g/L-day was obtained at D = 0.3 day^{-1} and S_0 = 60 g/L, while the highest DHA productivity (0.52 g/L-day) was obtained at D = 0.3 day^{-1} and S_0 = 90 g/L due to the higher DHA content at S_0 = 90 g/L. The biomass and DHA productivity of the continuous culture was comparable to those of batch culture, while lower than the fed-batch culture, mainly because of the lower DHA content obtained by the continuous culture. Overall, the results show that continuous culture is a powerful tool to investigate the cell growth kinetics and physiological behaviors of the algae growing on biodiesel-derived crude glycerol.

3.2 Introduction

Biodiesel as an alternative fuel has attracted increasing attention worldwide in the past few years. In the United States, for example, annual biodiesel production reached a historical high of 691 million gallons in 2008, and although the production decreased to 490 million gallons in 2009, the overall production capacity still maintains historically high (NBB, 2010). During the biodiesel manufacturing process, one of the major byproducts is crude glycerol (Johnson and Taconi, 2007). For every liter of biodiesel manufactured, 0.08 kg of crude glycerol

is generated (Thompson and He, 2006). This glycerol contains many impurities such as soap and methanol, and thus, it is prohibitively expensive to convert and purify the crude glycerol into material that can be used in the food, cosmetics, or pharmaceutical industries. With the rapid growth in biodiesel production, the market is flooded with crude glycerol; as a result, biodiesel producers must seek new uses for this waste stream.

Various methods have been explored for utilizing crude glycerol, including combustion (Johnson and Taconi, 2007), anaerobic digestion (Fountoulakis and Manios, 2009; Holm-Nielsen et al., 2008), or animal feed (Cerrate et al., 2006; Lammers et al., 2007; Lammers et al., 2008). Crude glycerol can also be used as a raw material for conversion into valued-added products such as propylene glycol (Dasari et al., 2005) and acetol (Chiu et al., 2006) through thermochemical methods or 1,3 propanediol (Gonzalez-Pajuelo et al., 2006; Zheng et al., 2006), lipid (Meesters et al., 1996; Papanikolaou and Aggelis, 2002), and pigment (Narayan et al., 2005) through biological methods. Crude glycerol was also used as a feedstock for producing hydrogen gas through photofermentation (Sabourin-Provost and Hallenbeck, 2009) or gasification (Yoon et al., 2010) processes.

Recently, a process using crude glycerol as a substrate for algal fermentation has been developed in our laboratory. The microalga *Schizochytrium limacinum* can produce significant amounts of total lipid as well as docosahexaenoic acid (DHA, C22:6 n-3) when growing in a crude glycerol-containing medium (Chi et al., 2007). The total lipid can be used as a source of biodiesel production (Johnson and Wen, 2009) while DHA, as one of important omega-3 polyunsaturated fatty acids, has various beneficial effects on human health (Nettleton, 1995). The DHA-rich algal biomass can also be used as an essential feed supplement during the culture and production of marine fish (New and Wijkström, 2002). Currently, fish oil is widely used as a traditional source of omega-3 fatty acids, but it is facing challenges such as odor/taste problems, heavy metal contamination, and limited supply (Barclay et al., 1994). Our research has shown that the crude glycerol-derived algae had no heavy metal contaminations; the overall nutritional quality of the algae was similar to that of commercial algae (Pyle et al., 2008). In order to develop a mass glycerol-based algal culture to supply a large amount of biomass, an in-depth study of the cell growth kinetics, substrate utilization, and DHA (as well as total lipid) production profile of this algal species is needed. This information is crucial to the future

optimization of a mass algal production process. The aim of the present work was to quantitatively determine these parameters through a continuous algal culture.

3.3 Materials and methods

3.3.1 Algal strain, medium, and subculture conditions

The algal species *Schizochytrium limacinum* SR21 (ATCC MYA-1381) was used. The cells were maintained in 250-mL Erlenmeyer flasks each containing 50 mL of medium, and incubated at 25°C in an orbital shaker set to 170 rpm. The medium for the seed culture was artificial seawater containing 10 g/L glucose, 1 g/L yeast extract, and 1 g/L peptone. The artificial seawater contained (per liter) 18 g NaCl, 2.6 g $MgSO_4 \bullet 7H_2O$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3 g $CaCl_2 \bullet 2H_2O$, 0.05 g KH_2PO_4, 1.0 g Trizma base, 0.027 g/L NH_4Cl, $1.35 \times 10\text{-}4$ g vitamin B12, 3 mL chelated iron solution, and 10 mL PII metal solution containing boron, cobalt, manganese, zinc, and molybdenum (Starr and Zeikus, 1993). The pH of the medium was adjusted to 7.5-8.0 before being autoclaved at 121°C for 15 min. The flask cultures were used as inoculums for the fermenter culture.

3.3.2 Continuous culture conditions

Continuous cultures were performed in a 7.5-L New Brunswick Bioflo 110 fermenter with working volume of 4.5 L at 25 °C. Agitation was provided by three turbine impellers. During the cultivation, agitation speed was varied to maintain the dissolved oxygen (DO) level above 50% of saturation. Compressed air (about 0.1 vvm) was sparged into the culture through a sterilized air filter. The medium pH was controlled within the range of 6.5-7.5. A medium containing artificial seawater with 90 g/L crude glycerol and 5 g/L corn steep solid was used in initial batch cultures. After 3 days of batch culture, feed medium was added to the fermenter at various dilution rates (with a feed crude glycerol concentration of 90 g/L) or at various crude glycerol concentrations (with a dilution rate of 0.3 day^{-1}). At the same time, equal volumes of cell suspension were withdrawn from the fermenter. The composition of the feed medium was the same as that for the initial batch cultures except different concentrations of crude glycerol were used. Samples were taken from the fermenter on a daily basis for measuring the cell dry weight. The steady state under each operation condition was considered to have been established after at least three volume changes (the total volume of liquid flowing through the fermenter),

with a variation of cell dry weight less than 5%. The light/photosynthesis contribution for the algal growth was considered negligible in the continuous culture since an opaque heating blanket was wrapped around the glass vessel, and the cell density was high, and thus, the mutual shading effect was severe.

3.3.3 Preparation of crude glycerol medium

The crude glycerol was obtained from Virginia Biodiesel Refinery (West Point, VA). The plant used a 50:50 (w/w) chicken fat and soybean oil mixture for making biodiesel. The following procedures were used to remove soap from crude glycerol: (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 sulfuric acid to convert soap into free fatty acids that precipitated from the liquid, (iii) the fatty acid-precipitated liquid was kept static for 30 minutes to allow free fatty acid and glycerol to separate into two phases, (iv), the free fatty acid phase (upper phase) was removed from the crude glycerol phase through a separation funnel, and (v) other medium compositions (seawater salts, corn steep solids, etc.) were added to the glycerol solution to adjust to the desired levels. This glycerol-containing medium was then autoclaved at 121°C for 15 min, previous research has shown that autoclaving can drive off methanol from the medium (Pyle et al., 2008).

3.3.4 Analyses

A 10-mL cell suspension sample was taken daily from the fermenter and centrifuged at 8000 rpm for 5 minutes. The solid (cell pellets) was rinsed with distilled water, and freeze-dried to obtain the cell dry weight. When the culture reached steady-state, the freeze-dried algal samples were further analyzed for fatty acid composition using the method reported previously (Pyle et al., 2008); while the residual glycerol concentration in the supernatant was measured using a Roche glycerol assay kit (R-Biopharm Inc, Marshall, MI).

3.4 Results
3.4.1 Effects of dilution rate on cell growth and DHA production

Continuous cultures of *S. limacinum* were first investigated at different dilution rates (D) with a feed crude glycerol concentration of 90 g/L (70.91 g/L true glycerol). As shown in Figure

3-1A, the steady-state biomass yield decreased with increasing D from 0.2 to 0.6 day^{-1}, while the highest biomass productivity (ca. 3.46 g/L-day) was obtained at D = 0.3 day^{-1}. The cells were washed out when the dilution rate was further increased to around 0.7 day^{-1}.

Within the dilution rates investigated, the residual glycerol concentration increased with the increasing dilution rate (data not shown). Here, we assume that the growth of *S. limacinum* follows the Monod equation, i.e.,

$$\mu = D = \frac{\mu_m \cdot S}{K_s + S} \quad (1)$$

Where μ is specific growth rate, μ_m is the maximum specific growth rate, S is the limiting substrate concentration, K_s is the half-saturation constant; by inverting eq. (1), we can obtain:

$$\frac{1}{D} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m} \cdot \frac{1}{S} \quad (2)$$

By plotting the 1/D *vs* 1/S curve (Figure 3-2), the value of μ_m and K_s were determined to be 0.692 day^{-1} and 25.87 g/L, respectively.

Figure 3-1B shows that within the range of dilution rate tested, both the yield coefficient on glycerol ($Y_{x/s}$) and the specific glycerol consumption rate (q_s) increased with dilution rate. Such a trend was considered due to the maintenance activities of the algal cells at different dilution rates (i.e., specific growth rate). The dependency of $Y_{x/s}$ on dilution rate can be expressed as

$$\frac{1}{Y_{x/s}} = \frac{1}{Y_g} + \frac{m}{\mu} = \frac{1}{Y_g} + \frac{m}{D} \quad (3)$$

Where Y_g is the true cell growth yield and m is the maintenance coefficient. By linear regression of $1/Y_{x/s}$ *vs* 1/D (Figure 3-3), the values of Y_g and m were estimated as 0.283g/g and 0.2216 day^{-1}, respectively.

The fatty acid composition of *S. limacinum* under different dilution rates is presented in Table 3-1. The algae had a relatively simple fatty acid profile with palmitic acid (C16:0) and DHA being the major fatty acids, and myristic acid (C14:0), stearic acid (C18:0) and docosapentaenoic acid (C22:5) being the minor fatty acids. The percentage of each individual fatty acid (% TFA, total fatty acid) was relatively stable, while the cellular content of TFA and DHA decreased significantly when dilution reached to 0.6 day^{-1}. As far as DHA production is concerned, Figure 3-1C shows that the highest DHA yield and DHA productivity were obtained

at a dilution rate of 0.3 day^{-1}. The TFA yield and productivity with this dilution rate had a similar trend to those of DHA yield and productivity (Figure 3-1D).
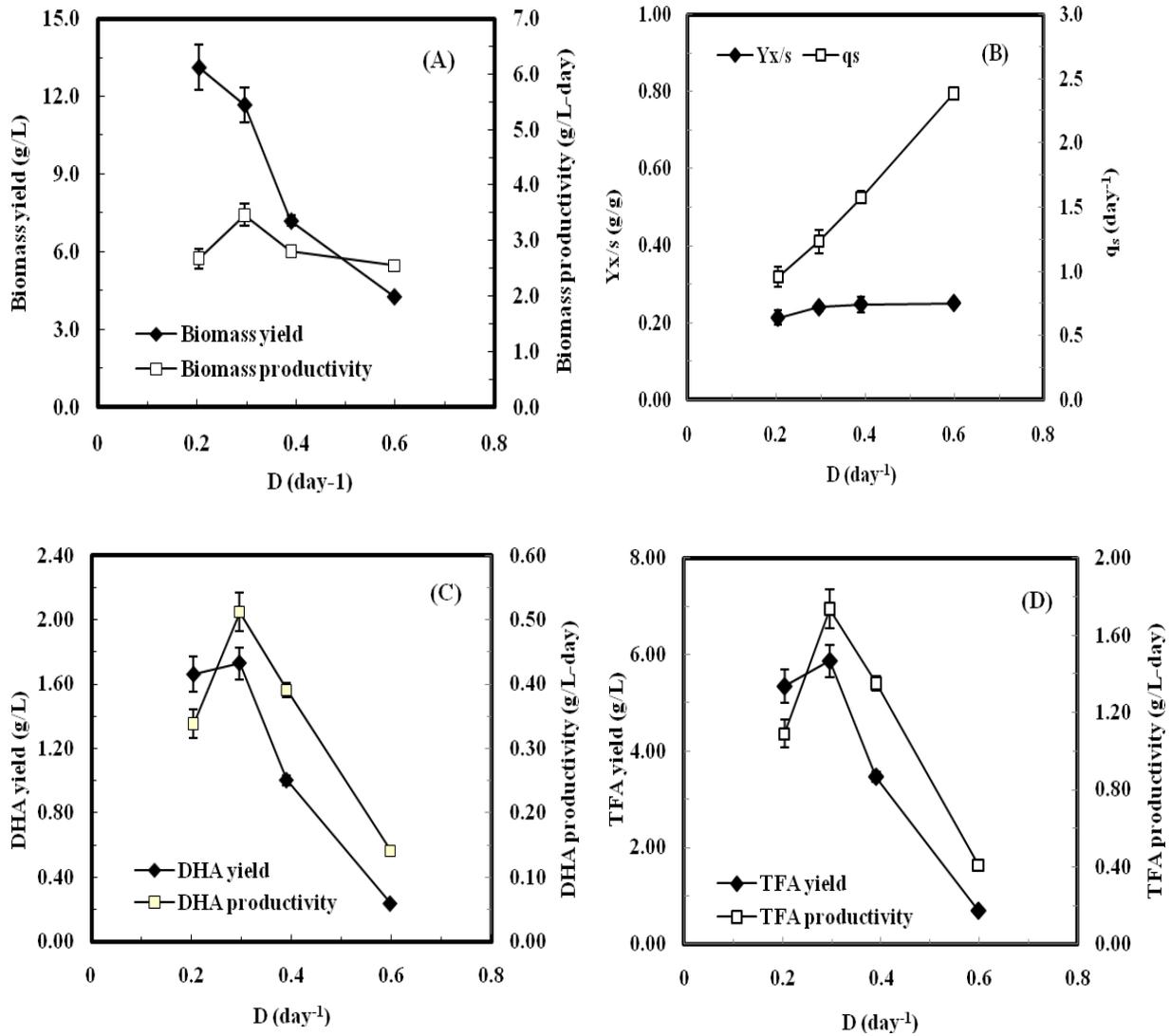
Figure 3- 1. Cell growth, substrate utilization, DHA and TFA production of the continuous culture *S. limacinum* on crude glycerol with different dilution rates (D) (S$_0$ = 90 g/L). (A). biomass yield and productivity. (B) cell growth yield (Y$_{x/s}$) and specific substrate utilization rate (q$_s$). (C) DHA yield and productivity, and (D) TFA (total fatty acid) yield and productivity. Data are means of three consecutive samples at the steady state (after at least three volume changes), and error bars show standard deviations.
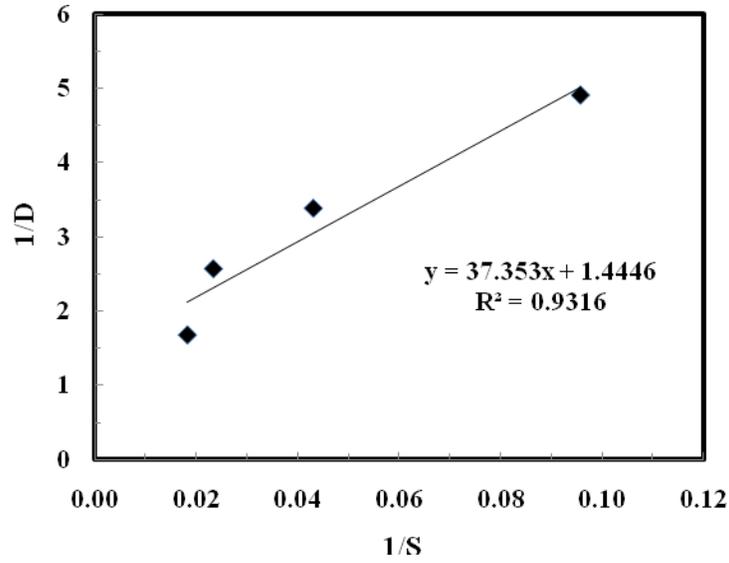
Figure 3- 2. Correlation of 1/D *vs* 1/S for estimating μ_m and K_s values.
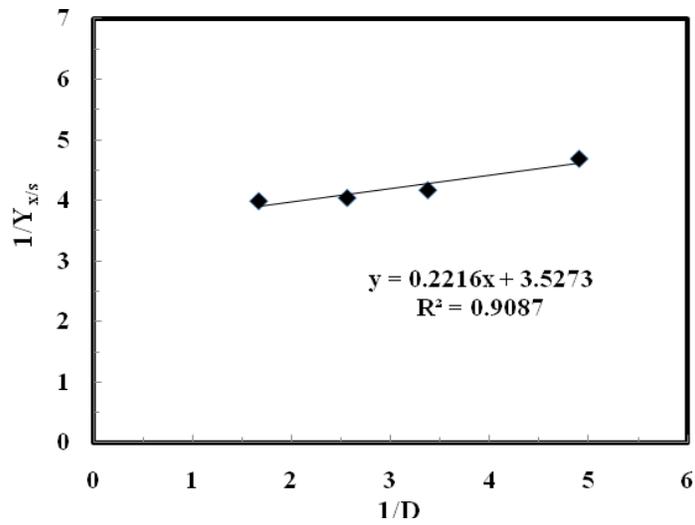
Figure 3- 3. Determination of the maintenance coefficient (m) and true growth yield coefficient (Y_g) of *S limacinum* for growth on crude glycerol in continuous culture.

Table 3- 1. Fatty acid composition (%TFA, total fatty acid,) and TFA and DHA contents (mg/g DW) of *S. limacinum* at different dilution rates (D) (feed glycerol concentration, S_0, was set at 90 g/L)

Fatty acid	Unit	D (day^{-1})			
		0.2	0.3	0.4	0.6
C14:0	%TFA	3.28 ± 0.02	3.96 ± 0.16	4.13 ± 0.09	3.25 ± 0.21
C16:0	%TFA	57.87 ± 0.44	54.61 ± 0.32	60.46 ± 1.80	53.66 ± 2.62
C18:0	%TFA	1.42 ± 0.11	3.86 ± 0.17	1.37 ± 0.10	3.57 ± 0.31
C22:5	%TFA	6.38 ± 0.10	6.47 ± 0.19	5.39 ± 0.36	4.47 ± 0.17
C22:6	%TFA	31.05 ± 0.47	31.09 ± 1.04	28.64 ± 1.48	35.05 ± 1.34
TFA content	mg/g	407.17 ± 9.67	502.5 ± 6.57	481.78 ± 15.90	159.87 ± 8.12
DHA content	mg/g	126.45 ± 4.72	148.03 ± 2.85	139.34 ± 7.91	55.38 ± 2.88

3.4.2 Effects of feed glycerol concentration on cell growth and DHA production

The physiological responses of *S. limacinum* to the change of feed glycerol concentration (S_0) were investigated with a dilution rate of 0.3 day^{-1}. Figure 3-4A shows that the trend of biomass yield and productivity with S_0 were the same, i.e., both the biomass yield and productivity increased with increasing S_0 from 15 to 60 g/L, and then decreased when S_0 exceeded 60 g/L. At S_0=15 and 30 g/L, the residual glycerol concentration was close to zero, while when S_0 exceeded 30 g/L, certain amounts of residual glycerol existed in the reactor (data not shown). The changes of $Y_{x/s}$ and q_s with S_0 are shown in Figure 3-4B. $Y_{x/s}$ decreased with the increasing S_0, indicating a more efficient glycerol utilization at lower S_0 levels. Since the q_s is the quotient of specific growth rate over $Y_{x/s}$, and the specific growth rate (i.e. dilution rate) were kept constant, the change of q_s vs S_0 showed a trend opposite that of $Y_{x/s}$ vs S_0 (Figure 3-4B).

Table 3-2 shows that fatty acid composition of *S. limacinum* at different S_0 levels. Overall, the percentage of each fatty acid (% TFA) was maintained stable except that the percentage of C18:0 fluctuated with S_0. The TFA content increased with S_0, with increasing S_0 from 15 to 90 g/L, but decreased when S_0 reached 120 g/L. The DHA content with S_0 had a

similar trend with that of TFA. Figure 3-4C shows that the trend of DHA yield with S_0 was the same as that of DHA productivity; the highest DHA yield and productivity were obtained at S_0=90 g/L. With respect to the TFA production, Figure 3-4D shows that the trend of TFA yield and productivity with S_0 were similar to the DHA yield and productivity with S_0=90 g/L being the optimal level.
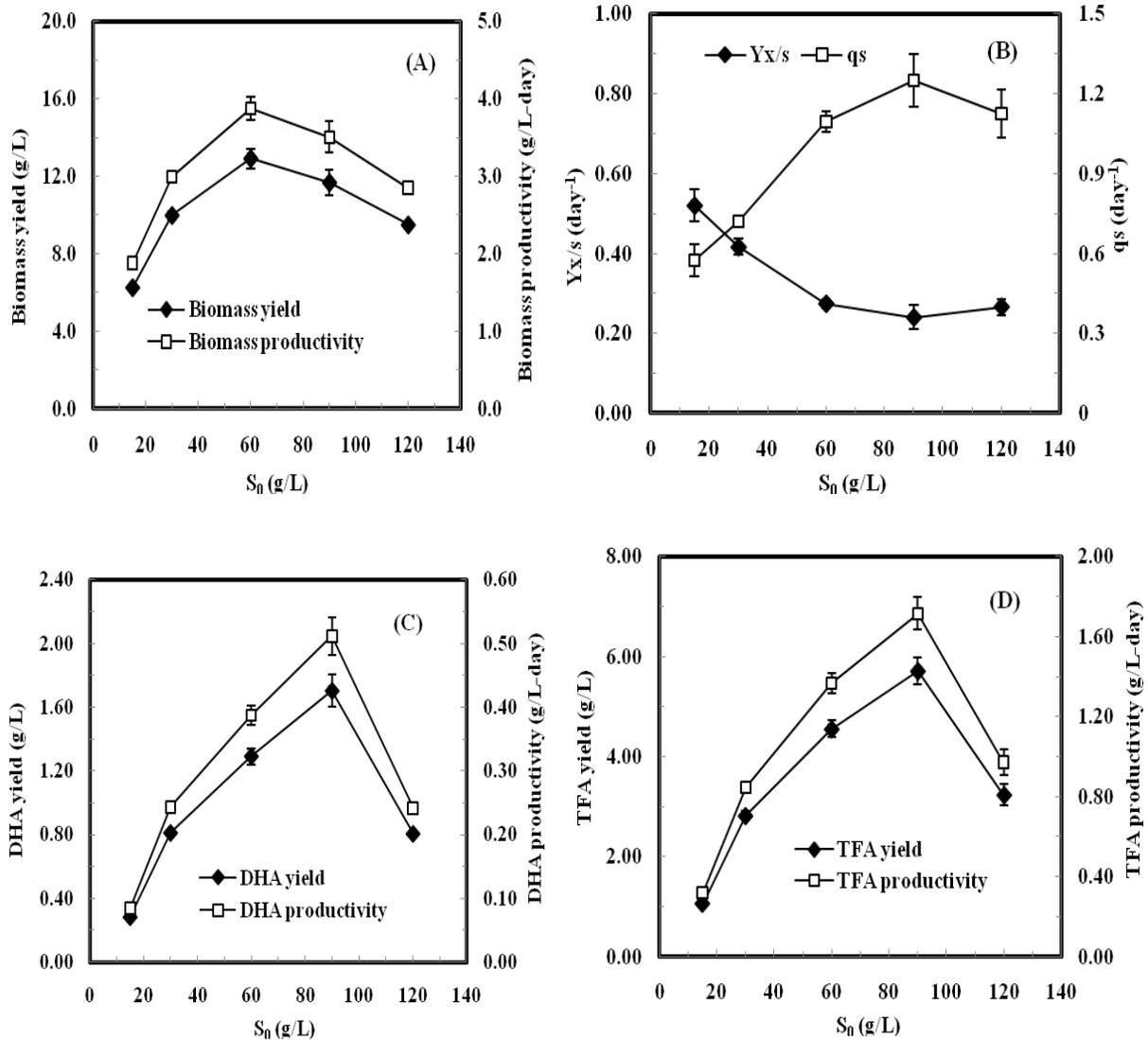
Figure 3- 4. Cell growth, substrate utilization, DHA and TFA production of the continuous culture *S. limacinum* on crude glycerol with different feed crude glycerol concentrations (S_0) (D = 0.3 day^{-1}). (A). biomass yield and productivity. (B) cell growth yield ($Y_{x/s}$) and specific substrate utilization rate (q_s). (C) DHA yield and productivity, and (D) TFA (total fatty acid) yield and productivity. Data are means of three consecutive samples at the steady state (after at least three volume changes), and error bars show standard deviations.

Table 3- 2. Fatty acid composition (% total fatty acid, TFA) and TFA and DHA contents (mg/g DW) of *S. limacinum* at different feed crude glycerol concentrations (S_0) (D was set at 0.3 day^{-1})

Fatty acid	Unit	Feed crude glycerol concentration (g/L)				
		15	30	60	90	120
C14:0	%TFA	2.70 ± 0.06	3.81 ± 0.05	3.88 ± 0.03	3.96 ± 0.16	3.18 ± 0.02
C16:0	%TFA	53.10 ± 0.31	57.45 ± 0.35	56.29 ± 0.38	54.61 ± 0.32	57.37 ± 0.15
C18:0	%TFA	12.10 ± 0.07	3.68 ± 0.27	4.95 ± 0.36	3.86 ± 0.17	9.65 ± 0.63
C22:5	%TFA	5.71 ± 0.13	6.41 ± 0.06	6.51 ± 0.18	6.47 ± 0.19	4.95 ± 0.13
C22:6	%TFA	26.39 ± 0.28	28.65 ± 0.21	28.37 ± 0.80	31.09 ± 1.04	24.86 ± 0.33
TFA content	mg/g	170.27±11.01	282.15±8.45	352.56 ±13.26	502.5±6.57	340.88±12.13
DHA content	mg/g	44.96 ± 1.98	81.20 ± 2.54	100.02 ± 4.49	148.03±2.85	84.79 ± 4.61

3.4.3 Comparison of DHA production with different culture methods

An overall comparison of cell growth and DHA production obtained by different culture methods is given in Table 3-3. The biomass yield of the continuous culture was much lower than the batch and fed-batch culture, due to the "dilution" effect as fresh medium was continuously fed to the fermenter. The biomass productivity of the continuous culture was higher than that of batch culture, but lower than the fed-batch culture. The growth yield coefficient on crude glycerol shows that the continuous culture and batch culture had a similar efficiency for utilizing crude glycerol. Table 3-3 also shows that the DHA content and DHA yield of the algae biomass were lower than both the batch culture and fed-batch culture. In terms of DHA productivity, however, the three-culture modes had a similar level, without significant differences ($P > 0.05$).

Table 3- 3. Comparison of cell growth and DHA production of *S. limacinum* using different culture methods with crude glycerol as a substrate.

Parameter	Unit	Culture methods		
		Batch	Fed-batch [a, b, c]	Continuous [d]
Maximum specific growth rate	day^{-1}	0.685	Not reported	0.692
Maximum biomass yield	g/L	18.04 ± 1.02	37.90	11.78 ± 0.86
Maximum biomass productivity	g/L-day	3.06 ± 0.07	3.25	3.48 ± 0.20
Overall $Y_{x/s}$	g/g	0.28 ± 0.02	Not reported	0.26± 0.01
DHA content	mg/g DW	170.4 ± 11.2	173	148.2 ± 2.9
DHA yield	mg/L	3.07 ± 0.19	6.56	1.74± 0.10
DHA productivity	g/L-day	0.51± 0.04	0.56	0.52 ± 0.03
Reference		(Chi et al., 2007)	(Chi et al., 2009)	This work

[a.] A two-stage DO shifting strategy was used, which DO was shifted from 50% in fermenter culture to flask culture at 40th hour culture time.
[b.] The standard deviations were not reported.
[c.] Pure glycerol was used.
The data listed was corresponding to D = 0.3 day^{-1}, and S_0 = 90 g/L

3.5 Discussion

The oleaginous alga *Schizochytrium limacinum* has capability of producing high level of DHA using a variety of substrates such as glucose, glycerol, and fructose (Yokochi et al., 1998), as well complex carbon sources such as sweet sorghum juice (Liang et al., 2010). Our previous study also showed the feasibility of growing *S. limacinum* using biodiesel-derived crude glycerol (Wen et al., 2009). The DHA production level obtained from crude glycerol culture was comparable to those reported using glucose or pure glycerol (Chi et al., 2007). In addition, the algal biomass derived from crude glycerol contained no heavy metals and had a nutritional quality similar to commercial algae (Pyle et al., 2008). A crude glycerol-based continuous culture provides quantitative information of the physiological behavior of this species.

In the study of the effects on dilution rate on the cell growth, the cells were washed out when the dilution rate was increased from 0.6 to 0.7 day^{-1}, which was in agreement with maximum specific growth rate (0.692 day^{-1}) determined from the Monod kinetic model. Compared to other continuous alga cultures such as *Nitzschia laevis* on glucose (Wen and Chen,

2002) and *Chlamydomonas reinhardtii* on acetate (Chen and Johns, 1994), the contribution of maintenance energy (m = 0.2216 day^{-1}) to the growth yield (Y_g = 0.283 g/g) of *S. limacinum* on crude glycerol was rather large, indicating less efficiency of crude glycerol utilization for cell growth.

The phenomenon that high residual glycerol concentration occurred at higher dilution rates and higher S_0 levels were also observed in the continuous culture of other microorganisms. For example, when the diatom *Nitzschia laevis* was grown at higher dilution rate (D > 0.3 day^{-1}) or higher feed glucose region (S_0 > 20 g/L), the steady-state residual glucose was higher (Wen and Chen, 2002). Similarly, at the respire-fermentative region (i.e., higher dilution rate) of the yeast *Saccharomyces cerevisiae*, the steady-state sugar concentration was usually high (de Kock et al., 2000; Diderich et al., 1999).

The continuous culture is also a better approach to investigate the fatty acid composition of the algae biomass. In a batch culture process, the fatty acids, particularly unsaturated fatty acid, is strongly correlated with the "age" of the cells (Wen et al., 2002). Fatty acids accumulated at the stationary phase of a batch culture, but decreased rapidly when the cells transit from stationary phase to the death phase (Wen et al., 2002). As a result, precisely identifying an optimal harvest time when the fatty acid content reaches the highest level is difficult. Compared to the batch and fed-batch cultures, the continuous culture provides a stable fatty acid profile at a fixed operational condition (dilution rate and S_0). Indeed, the fatty acid profile (particularly the TFA and DHA content) of the steady-state algal biomass determined in this work was very stable, with less fluctuation compared with the batch culture processes (Chi et al., 2007; Pyle et al., 2008).

Continuous culture usually gives a high biomass and end-product productivity in the fermentation process. The results obtained in this work show that the biomass productivity was higher than that of batch and fed-batch cultures. However, the DHA productivity of the continuous culture did not show any improvement compared with the batch culture, and even lower than the fed-batch culture. The major reason was due to the lower DHA content in the continuous culture. In an earlier study of batch culture (Chi et al., 2007 and Pyle et al., 2008), the crude glycerol was adjusted to pH 3 and then centrifuged to completely remove the soap. In the current study, however, the pH-adjusted crude glycerol solution was simply left stationary to separate the soap by gravity; as a result, there was still a certain amount of emulsified soap

residues left in the solution. The difference in crude glycerol pretreatment procedure was believed to be the main reason for the relatively low DHA as the existence of soap has proved inhibitory for DHA synthesis in the algal culture (Pyle et al., 2008). The presence of soap may also be the reason contributing to the high maintenance coefficient as compared with other species (Chen and Johns, 1994; Wen and Chen, 2002).

3.6 Conclusions

The results indicate the continuous culture is an effective method to investigate the growth kinetics, substrate utilization, and DHA production of *S. limacinum* on biodiesel-derived crude glycerol. The dilution rate and feed glycerol concentration are two important parameters influencing the cell growth and DHA production performance. Compared with the batch culture and fed-batch culture of the algae; however, the DHA content and the consequent DHA productivity obtained from the continuous culture were still low. This result may be due to the incomplete removal of soap residues, which are inhibitory to cell growth. With further optimization and scaling-up of the algal culture process, the glycerol-derived algae have a great potential to benefit the nutraceuticals and biodiesel industries.

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Chapter 4: Optimization of Culture Conditions to Control the Morphology of *Pythium irregulare*

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4.1 Abstract

Previous research has shown that *Pythium irregulare* can grow and produce eicosapentaenoic acid (EPA, 20:5 n-3) using crude glycerol, a byproduct of biodiesel production, as a carbon source. The objective of this research was to investigate the morphology of *P. irregulare* by changing culture conditions using flask culture and fermenter cultures. In the flask culture the following parameters were examined: initial medium pH, temperature, shaker speed, and the flask type. The initial pH ranged from 7-9, temperature ranged from 18-30°C, and shaker speed ranged from 60-250 rpm. Two types of flasks were studied: regular Erlenmeyer flasks and baffled flasks. The optimum culture conditions for pellet formation of *P. irregulare* were determined to be an initial medium pH of 7, a temperature of 25°C, a shaker speed of 110 rpm, and using baffled flasks; shaking speed and flask type were determined to be the most influential factors for pellet formation. In the fermenter culture, agitation speed and the presence of a baffle were examined. The presence of the baffle in the fermenter caused the cells to form large pieces of mycelium, while agitation speed greatly influenced the pellet sizes. A 300 rpm agitation rate generated smaller size pellets than the 150 rpm agitation rate. Overall, the results show that the morphology of *P. irregulare* can be controlled by manipulating the culture conditions with agitation speed being the most influential factor.

4.2 Introduction

The biodiesel industry has grown rapidly in recent years as a result of the rising cost of crude oil. The annual biodiesel production in the United States reached a historical high in 2008 of 691 million gallons. In 2009, the production decreased to 490 million gallons, but still remains historically high (NBB, 2010). One of the major byproducts of biodiesel production is crude glycerol (Johnson and Taconi, 2007). For every gallon of biodiesel produced, 0.3 kg of crude glycerol is created (Thompson and He, 2006). This crude glycerol contains many impurities, such as soap and methanol, making it expensive to purify for use in pharmaceutical,

food, and cosmetic industries (Johnson and Taconi, 2007). Therefore, other options are needed to utilize this crude glycerol.

Several methods have been considered for the utilization of crude glycerol such as combustion (Johnson and Taconi, 2007) and anaerobic digestion (Holm-Nielsen et al., 2008). Researchers have also considered using crude glycerol as an animal feed supplement for cows (DeFrain et al., 2004), pigs (Lammers et al., 2007), and chickens (Cerrate et al., 2006). Others have proposed that crude glycerol can be used as a raw material for thermochemical conversion into value-added products such as propylene glycol (Dasari et al., 2005) and acetol (Chiu et al., 2006). Biological conversion methods have also been considered to generate lipids (Meesters et al., 1996; Papanikolaou and Aggelis, 2002), pigments (Narayan et al., 2005), and 1,3-propanediol (Gonzalez-Pajuelo et al., 2006; Zheng et al., 2006).

Recently, research has shown that crude glycerol can be used as a carbon source for *Pythium irregulare* for producing eicosapentaenoic acid (EPA, C20:5 n-3) (Athalye et al., 2009). *P. irregulare* is an oomycete, a water mold, which can accumulate high levels of lipids, particularly EPA (Stinson et al., 2009). EPA is an omega-3 fatty acid that is important to human health. EPA promotes good cardiovascular health and can help alleviate the symptoms of inflammatory diseases such as rheumatoid arthritis (Covington, 2004). In addition, EPA can help alleviate the symptoms of neurological disorders such as depression (Ali et al., 2009) and bipolar disorder (Stoll et al., 1999). Currently, the most common source of EPA is fish oil. Fish oil can be unappealing to consumers due to its undesirable taste and odor. In addition, the quality of fish oil is dependent on many factors such as the season and feed availability for the fish (Certik and Shimizu, 1999). The demand for omega-3 fatty acids has also put a strain on the fishing industry due to the limited supply of fish (Barclay et al., 2004). For these reasons, an alternative source of EPA is needed.

Previous researchers have shown that *P. irregulare* can grow on a variety of carbon sources such as crude soybean oil, sucrose waste streams, soymeal waste streams (Cheng et al., 1999), and crude glycerol (Athalye et al., 2009). An advantage for using *P. irregulare* is its ability to grow and produce EPA at room temperature (Cheng et al., 1999). A disadvantage to using *P. irregulare* to produce EPA is its morphology. This species has a filamentous morphology and tends to grow in mycelial clumps, which can make fermentation difficult. It can, however, grow in pellets in some culture conditions (O'Brien et al., 2003). Previous

research with filamentous fungi has shown that the morphology can be controlled by changing culture conditions such as pH, temperature, and shaking speed (Liao et al., 2007; Cui et al., 1997). Other research has shown that filamentous species can form pellets in baffled flasks due to the higher shear force created by the baffles (Teng et al., 2009). Therefore, the morphology of *P. irregulare* may be controlled by changing these culture conditions. The objective of this research is to explore the possibility of controlling the cell morphology by controlling different culture conditions.

4.3 Materials and methods

4.3.1 Algal strain, medium, and subculture conditions

The oomycete *P. irregulare* (ATCC 10951) was used in this experiment. The algae was grown on agar plates containing 50 mL of medium consisting of 20 g/L glucose, 5 g/L yeast extract, and 20 g/L agar. The pH of the medium was adjusted to approximately 7 before being autoclaved at 121°C for 15 min. The plates were inoculated and incubated for five days at 25°C. After incubation, the cells were dislodged using distilled water and glass beads. This spore suspension was used as an inoculum in further experiments.

4.3.2 Flask culture conditions

Cells were grown in 250-mL Erlenmeyer flasks each containing 50 mL of medium. The medium contained 20 g/L glucose and 5 g/L yeast extract. The medium was autoclaved at 121°C for 15 min. The effect of different culture conditions on the morphology of *P. irregulare* was studied by varying medium pH, incubation temperature, shaking speed, and the type of flask used. The medium pHs were set at 5, 7, and 9; the incubation temperatures were 18, 25, and 30°C; and the shaker speeds were 60, 90, 110, 130, 180, and 250 rpm. Two different types of flasks were used in this experiment: regular and baffled flasks. Table 4-1 shows the various culture conditions used for regular flasks and Table 4-2 shows the various culture conditions used for baffled flasks. Each flask was inoculated with 2 mL of the prepared seed.

Table 4- 1. The different culture conditions tested for *P. irregulare* using regular flasks.

pH	Temperature (°C)	Shaking speed (rpm)
5	18	110
5	25	110
5	25	180
5	30	180
5	30	250
7	18	110
7	25	60
7	25	90
7	25	110
7	25	130
7	25	180
7	30	180
7	30	250
9	18	110
9	25	110
9	25	180
9	30	180
9	30	250

Table 4- 2. The different culture conditions tested for *P. irregulare* using baffled flasks.

pH	Temperature (°C)	Shaking speed (rpm)
5	18	110
5	25	110
5	25	180
5	30	180
5	30	250
7	18	110
7	25	60
7	25	90
7	25	110
7	25	130
7	25	180
7	30	180
7	30	250
9	18	110
9	25	110
9	25	180
9	30	180

9	30	250

The cell morphology was investigated for each culture condition. After the optimum culture conditions (pH, temperature, shaking speed, and flask type) were determined for pellet formation, the carbon source in the medium was switched to crude glycerol (at 30 g/L). As a control, media containing glucose (20 g/L) and pure glycerol (30 g/L) were used. The yeast extract in this second run was changed to 10 g/L. The pH of the medium was adjusted to 7 and baffled flasks were used. The medium was autoclaved at 121°C for 15 min. Each flask was inoculated with 2 mL of the prepared seed and incubated at 25°C. Two shaker speeds were used: 90 and 110 rpm.

4.3.3 Fermenter culture conditions

After optimum flask culture conditions were determined for pellet formation, batch cultures were performed in a 7.5-L New Brunswick Bioflo 110 fermenter with working volume of 2.5 L at approximately 25 °C. Agitation was provided by two turbine impellers. Compressed air (about 0.1 vvm) was sparged into the culture through a sterilized air filter. A medium containing 30 g/L crude glycerol and 10 g/L yeast extract was used for the batch cultures. Two operating conditions were examined during the fermentation: the presence of a baffle and the stir rate. Three batch runs were performed using the fermenter. First, the cells were grown using a stir rate of 150 rpm and a baffle was placed inside the fermenter. For the second run, the baffle was removed and the stir rate remained at 150 rpm. For the third run, the baffle was removed and the stir rate was increased to 300 rpm.

4.3.4 Crude glycerol preparation

The crude glycerol was obtained from Virginia Biodiesel Refinery (West Point, VA). The plant used a 50:50 (w/w) chicken fat and soybean oil mixture for making the biodiesel. The following procedure was used to remove the soap from the crude glycerol: (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 sulfuric acid to convert the soap into free fatty acids that precipitated from the liquid, (iii) the liquid was centrifuged at 8000 rpm for 5 min to separate the

free fatty acid phase from the glycerol phase, and (iv) the free fatty acid phase (upper phase) was removed from the crude glycerol using a separation funnel.

4.3.5 Analyses

For both the flask culture and fermenter culture, the entire algal biomass was harvested and washed with distilled water. A filtration unit was used to separate the supernatant from the biomass. The cell pellets were then frozen and freeze-dried to obtain the cell dry weight. The freeze-dried algal samples were further analyzed for fatty acid composition using the method reported previously (Pyle et al., 2008).

4.4 Results

4.4.1 Flask culture results

Morphology

Flask cultures of *P. irregulare* were first investigated to determine the culture conditions that result in the formation of pellets. This investigation found that medium pH and incubation temperature did not affect the cell morphology, while the shaking speed and flask type affected the cell morphology. Table 4-3 shows the cell morphology at different culture conditions using regular flask cultures; and Table 4-4 shows the cell morphology at different culture conditions using baffled flask cultures. An initial pH of 9 was inhibitory to cell growth in most cases. A shaking speed of 110 rpm resulted in pellet morphology in both flask types while 90 and 130 rpm resulted in pellet formation in baffled flasks only.

Figure 4-1 shows *P. irregulare* growing in regular flasks with clump morphology (Figure 4-1A) and pellet morphology (Figure 4-1B). Figure 4-2 shows the morphology of *P. irregulare* growing in baffled flasks.

Table 4- 3. Morphology of *P. irregulare* under different culture conditions using regular flasks.

pH	Temperature (°C)	Shaking speed (rpm)	Morphology
5	18	110	clump
5	25	110	clump
5	25	180	clump
5	30	180	clump
5	30	250	clump
7	18	110	clump
7	25	60	clump
7	25	90	clump
7	25	110	pellet
7	25	130	clump
7	25	180	clump
7	30	180	clump
7	30	250	clump
9	18	110	clump
9	25	110	clump
9	25	180	clump
9	30	180	no growth
9	30	250	clump

Table 4- 4. Morphology of *P. irregulare* under different culture conditions using baffled flasks.

pH	Temperature (°C)	Shaking speed (rpm)	Morphology
5	18	110	clump
5	25	110	pellet
5	25	180	clump
5	30	180	clump
5	30	250	clump
7	18	110	clump
7	25	60	clump
7	25	90	pellet
7	25	110	pellet
7	25	130	pellet
7	25	180	clump
7	30	180	clump
7	30	250	clump
9	18	110	no growth
9	25	110	clump
9	25	180	clump

9	30	180	no growth
9	30	250	no growth
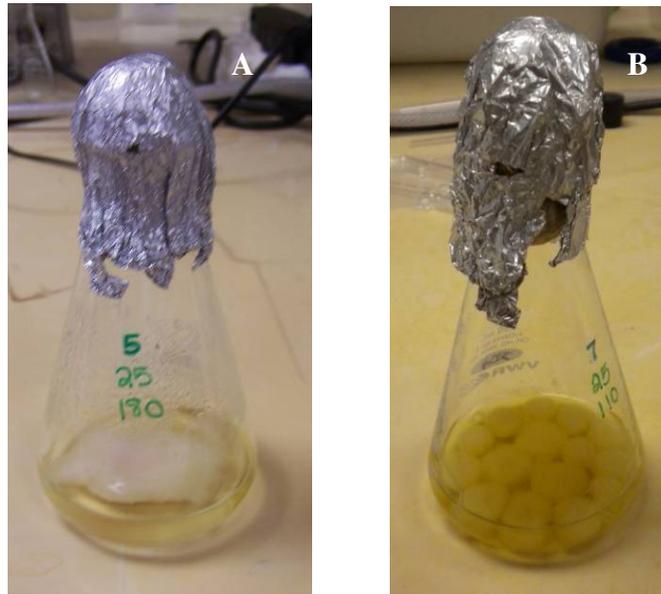
Figure 4- 1. Morphology of *P. irregulare* growing in regular flasks. (**A**) clump morphology. (**B**) pellet morphology.
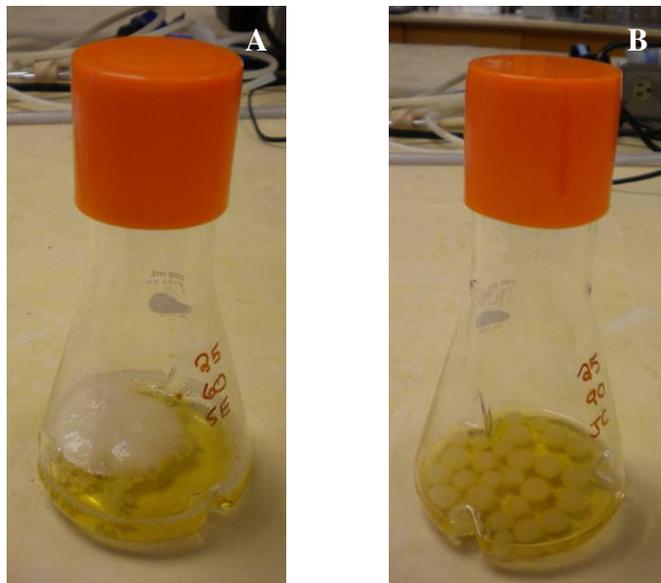
Figure 4- 2. Morphology of *P. irregulare* growing in baffled flasks. (**A**) clump morphology. (**B**) pellet morphology.

Cell growth and fatty acid production

Tables 4-5 and 4-6 show that cell growth was highly affected by the culture conditions. At some culture conditions, one or two of the flasks showed no cell growth (which is indicated by no standard deviation), which is most likely due to the small inoculum size.

Table 4- 5. Biomass yields of *P. irregulare* at different culture conditions using regular flasks.

pH	Temperature (°C)	Shaking speed (rpm)	Biomass yield (g/L)
5	18	110	9.50 ± 0.11
5	25	110	5.54
5	25	180	2.30 ± 0.76
5	30	180	5.28
5	30	250	6.45 ± 0.56
7	18	110	9.91
7	25	60	6.45 ± 0.49
7	25	90	6.13 ± 0.51
7	25	110	6.65 ± 0.25
7	25	130	7.71 ± 0.08
7	25	180	7.13 ± 0.13
7	30	180	6.58 ± 0.47
7	30	250	7.15 ± 0.81
9	18	110	4.87
9	25	110	0.10
9	25	180	0.43 ± 0.07
9	30	180	0
9	30	250	3.51

Table 4- 6. Biomass yields of *P. irregulare* at different culture conditions using baffled flasks.

pH	Temperature (°C)	Shaking speed (rpm)	Biomass yield (g/L)
5	18	110	1.82
5	25	110	2.51 ± 0.71
5	25	180	1.41
5	30	180	1.43 ± 0.46
5	30	250	0.60
7	18	110	2.98 ± 0.42
7	25	60	5.25 ± 0.22
7	25	90	5.31 ± 0.27
7	25	110	5.69 ± 0.04
7	25	130	6.96 ± 0.84
7	25	180	1.03 ± 0.26
7	30	180	0.16 ± 0.06
7	30	250	1.52 ± 0.89
9	18	110	0

9	25	110	1.28
9	25	180	0.45
9	30	180	0
9	30	250	0.08

The initial pH of the medium strongly affected the growth of *P. irregulare* as can be seen in Figure 4-3. A high pH (9) significantly inhibited cell growth. Dry cell weights were low and in some cases non-existent when the medium pH was 9. A lower pH of 5 had less of an inhibitory effect on the growth of *P. irregulare*, but still resulted in decreased biomass yields. Therefore, a pH of 7 was determined to be the optimum pH for cell growth. In addition, *P. irregulare* cells grown in baffled flasks produced less biomass than cells grown in regular flasks. In particular, cell growth in baffled flasks decreased drastically at higher shaker speeds. This decrease in biomass productivity is mostly likely due to the shear force produced by the baffles resulting in some cell death. Shaker speed did not appear to have an effect on cell growth in regular flasks.

Figure 4-3 shows that temperature did not have a significant effect on biomass yield. Cells grown at 18°C had higher biomass yields when grown in regular flasks, but took longer to grow, resulting in lower biomass productivities.
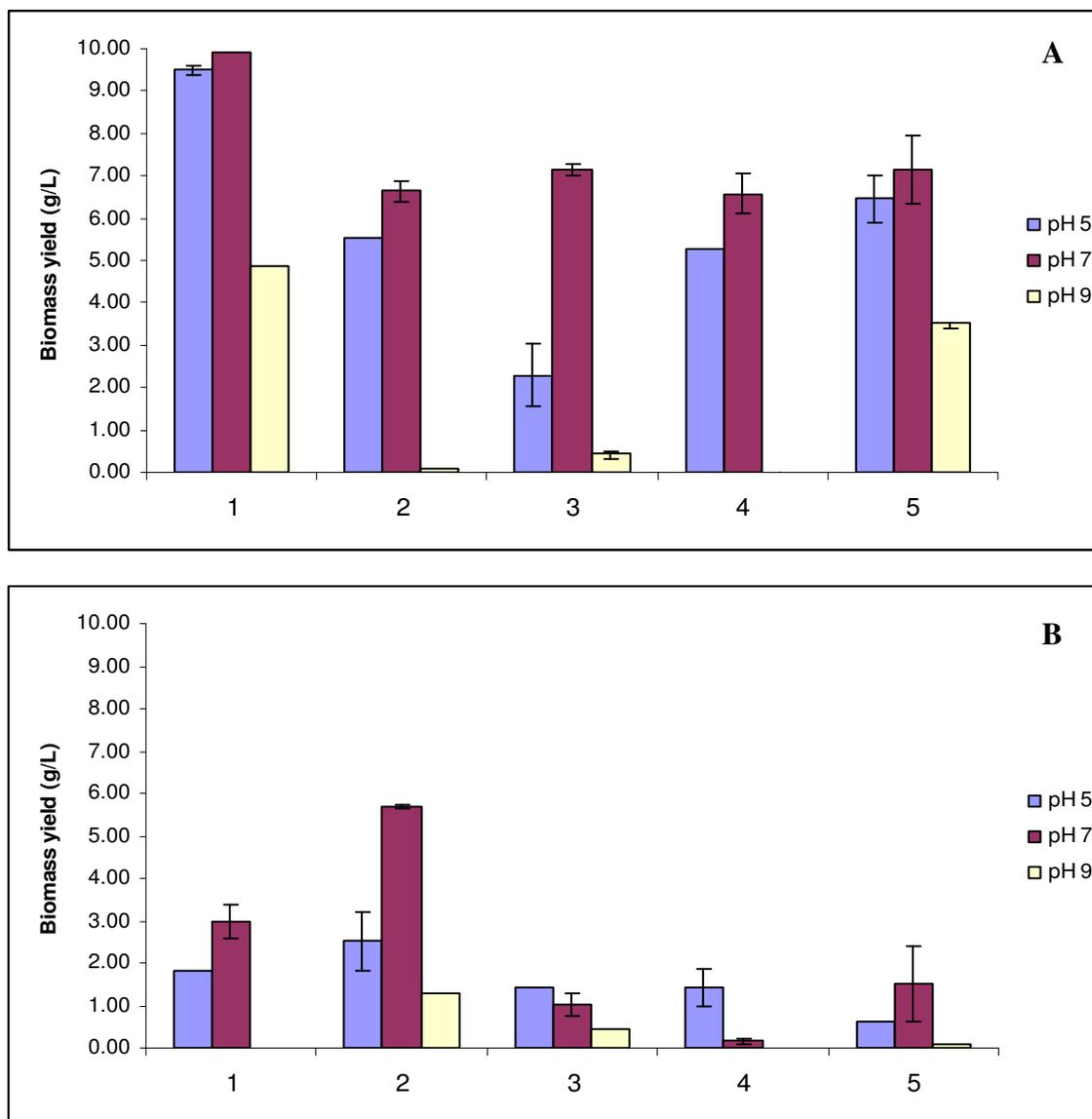
Figure 4- 3. Biomass yield (g/L) with respect to pH at various temperatures and shaking speeds: (1) 18°C and 110 rpm, (2) 25°C and 110 rpm, (3) 25°C and 180 rpm, (4) 30°C and 180 rpm, and (5) 30°C and 250 rpm. (**A**) Regular flasks. (**B**) Baffled flasks.

EPA and total fatty acid (TFA) contents were examined for only specific culture conditions as shown in Tables 4-7 and 4-8. EPA and TFA were measured for all cells grown at 18°C since previous research has shown that low temperatures result in higher EPA and TFA contents (Stinson et al., 1991). EPA and TFA were also measured for all cases with a pH of 7 and a temperature of 25°C since a pH of 7 was determined to be the optimum pH for cell growth and 25°C is room temperature. Tables 4-7 and 4-8 show the EPA and TFA contents measured

for these culture conditions. The biomass grown in baffled flasks had much lower TFA contents than the biomass grown in regular flasks. The EPA contents of the biomass grown in baffled flasks tend to be slightly lower than the EPA contents of the biomass grown in regular flasks. These results show that the baffled flasks are inhibitory to fatty acid production.

Table 4- 7. EPA and TFA content of *P. irregulare* at different culture conditions using regular flasks.

pH	Temperature (°C)	Shaking speed (rpm)	EPA content (mg/g)	TFA content (mg/g)
5	18	110	4.56 ± 0.08	76.63 ± 9.53
7	18	110	8.67	129.15
7	25	60	7.53 ± 0.12	108.97 ± 6.77
7	25	90	6.32 ± 0.58	59.17 ± 12.50
7	25	110	9.61 ± 0.90	146.16 ± 6.43
7	25	130	12.00 ± 0.11	103.69 ± 5.57
7	25	180	8.41 ± 1.09	90.98 ± 10.94
9	18	110	6.02	35.39

Table 4- 8. EPA and TFA content of *P. irregulare* at different culture conditions using baffled flasks.

pH	Temperature (°C)	Shaking speed (rpm)	EPA content (mg/g)	TFA content (mg/g)
5	18	110	5.00	25.99
7	18	110	7.39 ± 0.73	35.99 ± 3.18
7	25	60	8.15 ± 0.84	98.46 ± 11.93
7	25	90	5.71 ± 0.75	54.32 ± 12.50
7	25	110	5.61 ± 0.37	36.37 ± 5.55
7	25	130	6.23 ± 1.03	43.27 ± 2.03
7	25	180	5.63 ± 0.81	34.52 ± 0.92

4.4.2 Fermenter culture results

Three fermenter runs were performed, all of which resulted in pellet formation. The first fermenter run, using a baffle and an agitation speed of 150 rpm resulted in combination of clump and pellet formation (Figure 4-4). The *P. irregulare* cells attached to the baffle forming a clump morphology, while the cells suspended in the liquid formed pellets. The biomass yield for this fermenter run was 0.987 g/L.

Figure 4- 4. Batch fermentation of *P. irregulare* with a baffle and an agitation speed of 150 rpm.

In order to determine the effects of the baffle on the cell morphology, the baffle was removed for the second run and the agitation speed remained at 150 rpm. The cells in the second run mostly formed pellets with very little clumping (Figure 4-5A). The pellets were fairly uniform in size (Figure 4-5B). The biomass yield for this fermenter run was 0.507 g/L.

Figure 4- 5. Batch fermentation of *P. irregulare* without a baffle and an agitation speed of 150 rpm. (**A**) fermentor. (**B**) pellets.

Finally, the effects of agitation speed on the cell morphology were examined. For the third run, the baffle remained removed and the agitation speed was increased to 300 rpm. For this case, the cells formed in small pellets with little to no clumping (Figure 4-6A). The pellets

were fairly uniform in size (Figure 4-6B). From a random sample of 15 pellets, the average diameter was 1.40 ± 0.34 mm. The biomass yield for this fermenter run was 0.875 g/L.
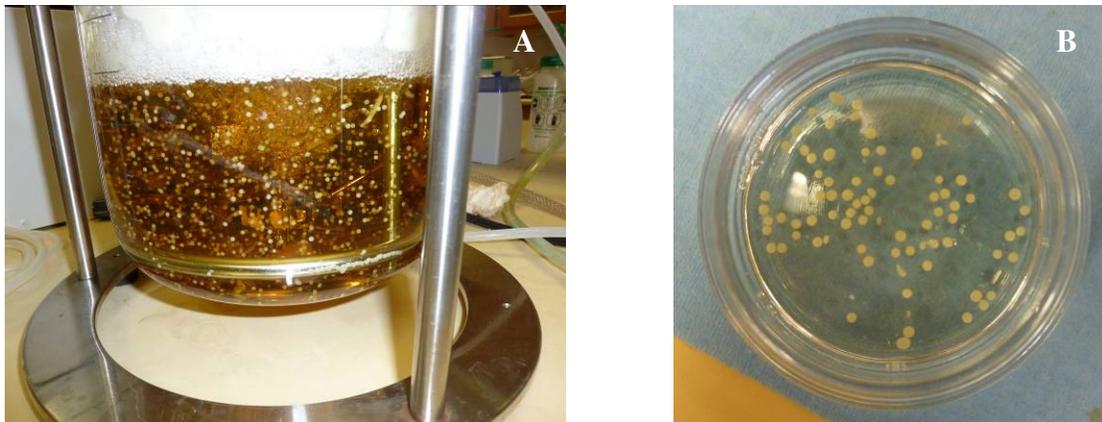
Figure 4- 6. Batch fermentation of *P. irregulare* without a baffle and an agitation speed of 300 rpm. (**A**) fermentor. (**B**) pellets.

For all three fermenter runs, the biomass yields were low when compared to yields obtained from flask cultures. The shear force created by the agitation blades may have resulted in some cell death leading to these decreased biomass yields. The carbon source used in flask cultures was glucose while the carbon source used in the fermenter runs was crude glycerol. This change in carbon source could also have caused these low biomass yields.

4.5 Discussion

Pythium irregulare has the ability to produce high levels of EPA using a variety of carbon sources such as crude soybean oil, sucrose waste streams, and soymeal waste streams (Cheng et al., 1999). Previous work in our lab showed the feasibility of growing *P. irregulare* on biodiesel-derived crude glycerol. *P. irregulare* was able to grow using crude glycerol as a carbon source with EPA production comparable to cells grown on a glucose medium (Athalye et al., 2009).

In the flask study of the effects of culture conditions on the morphology of *P. irregulare*, pellet formation depended on flask type and agitation speed. Baffled flasks inducing pellet formation is consistent with other studies; pellet formation occurred using baffled flasks, but not regular flasks at an agitation speed of 200 rpm for the filamentous fungus *Rhizopus chinensis* (Teng et al., 2009). Pellet formation occurred in this study at low agitation rates, which is

consistent with other studies. Yang and Liau (1998) found that the optimum agitation rate for the fungus *Ganoderma lucidum* was 100 rpm and that high agitation speeds had detrimental effects on pellet formation (Yang, and Liau, 1998). This agitation rate is similar to the agitation rate (110 rpm) that was optimal for pellet formation for *P. irregulare*.

When compared to other research using the *P. irregulare* species, the fatty acid data obtained in this study was low. Previous research in our lab showed that the DHA and TFA content of *P. irregulare* grown on crude glycerol could reach 15.31 and 128.79 mg/g respectively (Athalye et al., 2009). The lower fatty acid contents in this study may be due to the different culture conditions used to induce pellet formation.

In the fermenter study of the morphology of *P. irregulare*, pellet formation occurred in all trials, and agitation speed had an effect on pellet size, with higher agitation speeds resulting in smaller pellet diameters. These results are consistent with the results of Cui et al., (1997) who found that pellet diameter decreased with increasing agitation speed for the filamentous fungus *Aspergillus awamori* (Cui et al., 2007).

4.6 Conclusions

The results of this study indicate that the morphology of *P. irregulare* can be controlled by manipulating culture conditions. In flask culture growth, the type of flask and the shaker speed were important factors in pellet formation of *P. irregulare*. For the fermenter culture, agitation speed was found to be an influential factor in pellet formation and pellet size. These findings are consistent with the results of other morphology studies. Biomass yield and fatty acid content tended to be lower in cultures obtaining pellet formation. Further optimization is needed to determine the optimum conditions for pellet formation with the highest biomass yield and fatty acid production.

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Chapter 5: Conclusions and Recommendations for Future Research

The research presented in this thesis shows that optimization of culture conditions can be used to maximize productivities and control cellular morphology. In addition, this thesis shows that crude glycerol, a byproduct of biodiesel production, can be used as a carbon source in continuous culture fermentation of *Schizochytrium limacinum* and morphology studies of *Pythium irregulare*.

The research in Chapter 3 showed that continuous culture fermentation could be used to study the growth kinetics, substrate utilization, and DHA production of *S. limacinum* grown on biodiesel-derived crude glycerol. Two important parameters of continuous culture fermentation were studied: dilution rate (D) and feed glycerol concentration (S_0). Both of these parameters affect the cell growth and DHA production. DHA productivity was optimized (0.52 g/L-day) with D = 0.3 day^{-1} and S_0 = 90 while biomass productivity was optimized (3.88 g/L-day) at D = 0.3 day^{-1} and S_0 = 60 g/L. The DHA content of algae, though, was low when compared with batch culture and fed-batch culture methods.

The research in Chapter 4 showed that the morphology of *P. irregulare* can be controlled by manipulating culture conditions. Of the culture conditions studied (pH, temperature, agitation speed, and flask type), agitation speed and flask type had the greatest effect on the morphology of *P. irregulare*. The algal cells were able to form pellets using a shaking speed of 110 rpm in both regular and baffled flasks. Pellet formation also occurred in baffled flasks for the shaking speeds 90 and 130 rpm. Batch fermentation studies showed that agitation speed is an important parameter for pellet formation and size. As agitation speed increased, pellet diameter decreased.

In summary, the results presented in this thesis show the feasibility of using crude glycerol as a carbon source for the continuous culture fermentation of *S. limacinum* and culture conditions can be manipulated to induce pellet formation in *P. irregulare*. Recommendations for future work include further optimization and process scale-up. The continuous culture parameters for *S. limacinum* fermentation need to be further optimized in order to obtain algal DHA productivities similar to batch and fed-batch culture methods. In addition, complete removal of soap from the crude glycerol should be studied for continuous culture fermentation of *S. limacinum*. Future work should also include process scale-up of this continuous culture fermentation to pilot size. Further optimization of culture conditions should also be conducted

for *P. irregulare* in order to obtain pellet formation while optimizing biomass and fatty acid production. Process scale-up should also be considered with *P. irregulare* to determine the effects of scale-up on the morphology of this species.