Mixotrophic Production of Omega-3 Fatty Acid-rich Alga *Phaeodactylum tricornutum* on Biodiesel-derived Crude Glycerol

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Abstract

Crude glycerol is the major byproduct of the biodiesel industry. There is an abundance of this byproduct and purifying it for use in industries such as food, pharmaceutical, or cosmetic is prohibitively expensive. Developing an alternative use for crude glycerol is needed. Utilizing it as a carbon source in the fermentation of algae is one potential method for using this under-utilized byproduct.

In this research, crude glycerol is used in the mixotrophic production of the alga, *Phaeodactylum tricornutum*, which is an eicosapentaenoic acid (EPA) producing diatom. Mixotrophic growth is when cells perform autotrophic and heterotrophic modes of growth concurrently. EPA is an omega-3 polyunsaturated fatty acid that has been demonstrated to have a multitude of beneficial health effects, including maintaining human cardiovascular health, treating cancer and human depression diseases, and an anti-obesity effect.

In this study, the potential of using crude glycerol in batch mode mixotrophic culture of *P. tricornutum* was investigated. Once the mixotrophic culture was established, parameters involved in increasing the biomass and EPA production were optimized. These included nitrogen source, level of supplemental carbon dioxide, and concentration of crude glycerol. Using nitrate, 0.08 M crude glycerol, and 3% (vol/vol) carbon dioxide led to the highest biomass productivity of 0.446 g L^{-1} day^{-1} and the highest EPA productivity of 16.9 mg L^{-1} day^{-1} in batch mode culture.

The continuous culture of the mixotrophic culture was then performed following the batch culture optimization. The effects of dilution rate were observed in continuous culture with the parameters of nitrate as the nitrogen source, 0.08 M crude glycerol, and 3% (vol/vol) carbon dioxide held constant. The highest biomass productivity of 0.612 g L^{-1} day^{-1} was obtained at D = 0.24 day^{-1}. The highest EPA productivity of 16.5 mg L^{-1} day^{-1} was achieved at both D = 0.15 day^{-1} and D = 0.24 day^{-1}. The maximum specific growth rate was estimated from the washing out dilution rate and was determined to be around 0.677 day^{-1}.

Overall, it was found that crude glycerol increases the biomass and EPA productivity of *Phaeodactylum tricornutum*. Continuous culture with the use of crude glycerol can further increase these measurements. The potential for scaling up studies is demonstrated by these results and can help lead to a market for this abundant, little-used byproduct of the biodiesel industry.

Attribution

Author Kevin K. Woisard II is the major contributor and writer of the manuscript in chapter three of this thesis. Co-author Dr. Zhiyou Wen was the Committee Chair.

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Chapter 1: Introduction

1.1 Rationale and Significance

The biodiesel industry has grown rapidly in the United States and with its growth is an increasing amount of the byproduct crude glycerol. Impurities found in crude glycerol are excessively expensive to remove for use in the food, pharmaceutical, or cosmetic industry (Johnson and Taconi, 2007). New, value-added uses for this glycerol must be found.

The microalgae *Phaeodactylum tricornutum* has been shown to be able to utilize organic carbon sources (Cooksey, 1974; García et al., 2000). This species of microalgae is known to produce EPA and research has shown that providing a suitable organic carbon will increase EPA production (García et al., 2005; Sevilla et al., 2004). A number of studies have looked at using pure glycerol (García et al., 2000; García et al., 2005; Liu et al., 2009), but there is a void in studies concerning crude glycerol as a carbon source. This void provided a need for research into the effectiveness of using crude glycerol in mixotrophic culture with this omega-3 fatty acid producing microalgae.

EPA is an omega-3 fatty acid that has been demonstrated to beneficial in different aspects of human health. It contributes to a healthy cardiovascular system, with effects including a lowering of blood triglycerides and antiarrhythmic effects (Schacky and Harris, 2007). EPA can increase the response to certain types of tumor treatment (McEntee et al., 2008) and low levels of EPA can lead to a susceptibility to depression (Maes et al., 1996). Fish oil is the current major source of omega-3 fatty acids, including EPA. There are many problems associated with fish oil, including undesirable taste and a level supply with increasing demand, leading to increases in price (Pyle et al., 2008). Alternatively, microalgae can be used to as a source of omega-3 fatty acids in addition to fish oil.

The use of crude glycerol to produce omega-3 fatty acid-rich algae helps both the biodiesel industry and the fishing industry. It provides a use for the abundance of waste crude glycerol produced by the biodiesel industry. Additionally, the strain on depleted fish stocks would be reduced as demand for fish oil is reduced.

1.2 Hypothesis

Biodiesel-derived crude glycerol can be used as a suitable carbon source for the EPA-producing microalgal species *P. tricornutum*. Mixotrophic production of *P. tricornutum* will achieve a greater biomass yield and EPA productivity than photoautotrophic production.

1.3 Objectives

The objectives of this project are to:

1) Demonstrate that crude glycerol can be used as a carbon source for *P. tricornutum*,
2) Determine the optimal nitrogen source, carbon dioxide level, and crude glycerol concentration in batch mode for EPA productivity, and
3) Determine the optimal dilution rate in continuous mode in terms of EPA productivity.

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Chapter 2: Literature Review

2.1 Crude Glycerol from Biodiesel Manufacturing

Biodiesel has been in the spotlight in recent years as an alternative, renewable fuel source. There has been a trend of increasing biodiesel production in the US, with a peak of approximately 700 million gallons produced in the US in 2008. Producing biodiesel also generates crude glycerol as a byproduct that does not have any major applications currently. In general, every gallon of biodiesel generates 0.3 kg of crude glycerol. With the increase in biodiesel production, there is a large quantity of this currently underutilized waste product. Although glycerol can be commonly used in the food, pharmaceutical, and cosmetic industries, the biodiesel derived crude glycerol stream contains several impurities such as soap and methanol. Removing soap, methanol, and other impurities for use in those industries is an expensive process. Therefore, finding an economical use for this waste product is urgently needed.

2.1.1 Biodiesel Production

Biodiesel is created through a chemical reaction call transesterification (Figure 2-1). This reaction involves a catalyst, an oil or fat, and an alcohol. The most commonly used alcohol is methanol. A variety of oils and fats can be used in the reaction, with pure vegetable oils, waste vegetable oils, and rendered animal fats being used most frequently. The catalyst used in this reaction is a strong base, such as sodium hydroxide (NaOH) or potassium hydroxide (KOH). The oil or fat (i.e., triglyceride) is made up of a glycerol backbone with three long chain fatty acids attached. Through the transesterification reaction, the fatty acid chains are cleaved from the glycerol backbone.

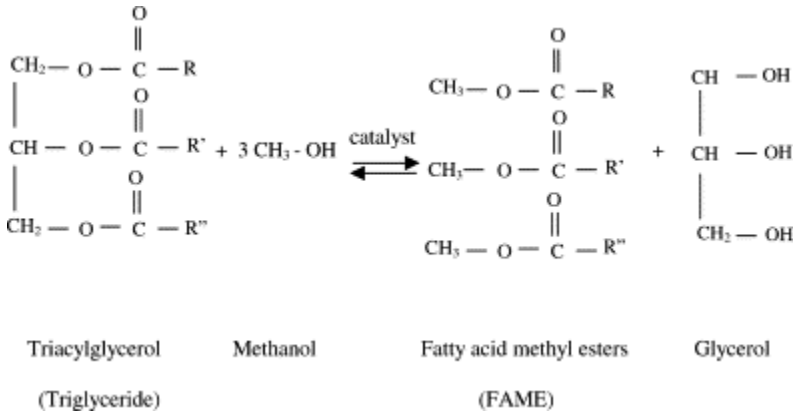

Figure 2-1. Transesterification reaction to produce biodiesel (Zhang et al., 2003).

2.1.2 Crude Glycerol Composition

Biodiesel production results in significant quantities of crude glycerol. For every tonne (1000 kg) of triglyceride utilized in biodiesel production, about 100 kg of crude glycerol is produced. Crude glycerol prices range from $0 - $70 per tonne, with most biodiesel producers considering it valueless (Miller-Klein Associates, 2006). The majority of the impurities in this crude glycerol are soaps and methanol. There are also many trace elements are present, but in general they are present in small quantities. The transesterification reaction is reversible. In order to prevent it from reversing, excess methanol is often used to favor the products side of the reaction. After the reaction, a majority of this excess methanol is found in the glycerol. Soaps, formed in side reactions between the base and free fatty acids that were initially present, are also contained within the crude glycerol stream. Some examples of the trace elements found in crude glycerol include potassium, calcium, phosphorous, and sulfur (Thompson and He, 2006).

A wide range of purities have been found in crdue glycerol stream. Gonzalez-Pajuelo et al. (2005) found crude glycerol stream generated from rapeseed oil contained as low as 65% (w/v) of glycerol. Other researchers have reported over 80% purity of "real" glycerol in other types of vegetable oils-based streams (Mu et al., 2006; Thompson and He, 2006). The impurities contained in the crude glycerol stream are mostly made up of soaps and methanol. Factors such as the type of feedstock and the method of glycerol purification play a significant role in the glycerol purity (Thompson and He, 2006).

Thompson and He (2006), as well as Schröder and Südekum (1999), have performed intensive work on identifying various element such as calcium, potassium, magnesium,

phosphorus, sulfur, sodium, lead, cadmium, mercury, and arsenic from a variety of crude glycerol samples. Cadmium, mercury, and arsenic levels in samples of crude glycerol derived from rapeseed oil were all below detectable limits (Schröder and Südekum, 1999). The other elements were found in small quantities, measurable in parts per million. For example, calcium was found to be present, on average, at 11 ppm in crude glycerol from soybean oils.

2.1.3 Uses of Glycerol

The biodiesel industry produces a significant amount of waste crude glycerol. This has caused the price of glycerol to fall drastically, leaving biodiesel refiners with limited options for the use of this glycerol. Purification of the crude glycerol for common uses in the pharmaceutical, food, and cosmetic industries is relatively expensive (Johnson and Taconi, 2007). It is for this reason that researchers have explored other routes for utilization of this waste product. Some basic methods of utilizing crude glycerol include burning for energy production (Johnson and Taconi, 2007), composting (Crooks, 2007), and anaerobic digestion for biogas production (Holm-Nielsen et al., 2008).

Crude glycerol has also been considered as an additive to animal feeds for animals such as chickens, dairy cows, and pigs (Pyle, 2008). Chickens fed diets containing 2.5 or 5% glycerol resulted in higher breast yields than the chickens with no glycerol in their diet. Although there still remained a concern about the residual methanol within the glycerol, glycerol has been shown to be a useful additive to feed for broiler chickens (Cerrate et al., 2006). In dairy cows, Defrain et al. (2004) studied whether crude glycerol would prevent ketosis, an elevation of ketones in the blood. No preventive effects were found in their study. The authors also observed that the crude glycerol did not have an effect on the dairy cows' postpartum lactation performance, but did cause changes to be observed in their ruminal profiles (Defrain et al., 2004). The ratio of metabolizable energy to digestible energy for crude glycerol is comparable to two other pig feed stocks, soybean oil and corn (Lammers et al., 2008). Studies have found crude glycerol could be used quite successfully to feed growing pigs, although again, there was concern for the impurities that may be present (Lammers et al., 2008).

Crude glycerol can be used as feedstock for making various value-added products. For example, it can be converted to acetol through a dehydration reaction, and then further converted into propylene glycol through a hydrogenation reaction (Johnson and Taconi, 2007). Pagliaro et

al. (2007) showed that crude glycerol could be converted into antifreeze at the biodiesel production sites. A number of studies have looked at the possibility of using glycerol as a carbon source for EPA-producing algal species. García et al. (2000 and 2005) found that the algal species *Phaeodactylum tricornutum* could be successfully cultured in mixotrophic systems.

2.2 Omega-3 Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids are unsaturated fatty acids that contain a carbon-carbon double bond located at the third carbon atom from the methyl end of the fatty acid chain. Some omega-3 fatty acids that have been identified as nutritionally important are α-linolenic acid (ALA, 18:3), docosahexaenoic acid (DHA, 22:6), and eicosapentaenoic acid (EPA, 20:5). Figure 2-2 shows the chemical structure of these fatty acids. Research has suggested that these omega-3 fatty acids have a number of health benefits.

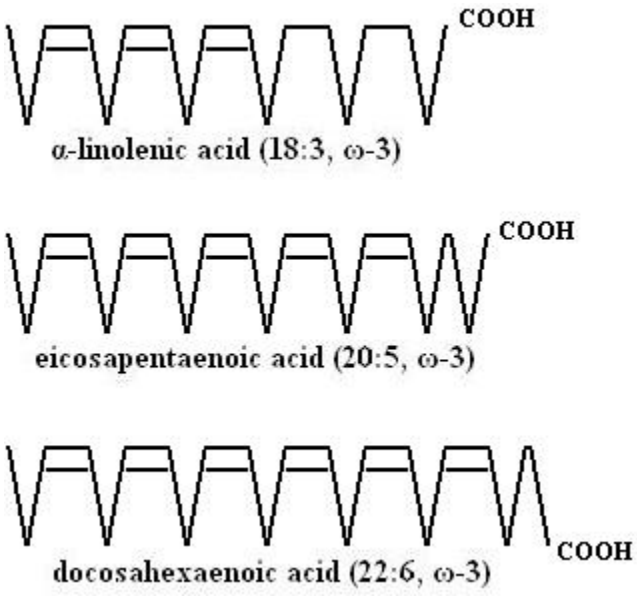

Figure 2-2. The chemical structures of ALA, EPA, and DHA.

2.2.1 Health Benefits of Omega-3 Polyunsaturated Fatty Acids

Cardiovascular Benefits

There is mounting evidence that omega-3 fatty acids have beneficial effects on the cardiovascular system. An intake of 1 g/day of EPA and DHA has been recommended by

national cardiac societies for secondary prevention, cardiovascular prevention, treatment post-myocardial infarction, and prevention of sudden cardiac death (Schacky and Harris, 2007). Changes in cellular function have been observed with the inclusion of EPA and DHA into the cell membrane. These changes include a lowering of blood triglycerides and antiarrhythmic effects (Schacky and Harris, 2007). Protection from cardiovascular events by omega-3 fatty acids has been observed with people consuming one serving of fish per month. The benefits have been seen to increase with intakes up to five or more servings per week (Gebauer et al., 2006).

Cancer

An increased intake of EPA has been found to reduce the production of inflammatory cytokines such as interleukin 1, 2, 6, and tumor necrosis factor (La Gaurdia et al., 2005). Researchers found that increased tissue levels of EPA heightened the response to hormone ablation therapy in prostate cancer cells (McEntee et al., 2008). It has been observed that tumors exposed to omega-3 fatty acids decrease in vascularity, which leads to a delay in growth (Otto et al., 2008). Omega-3 fatty acids have been shown to demonstrate antiproliferative effects in breast carcinomas (Sun et al., 2008). Mice implanted with human breast tumors that consumed canola oil, which is high in ALA, exhibited slowed tumor growth and an overall normal weight gain (Hardman, 2007).

Depression

A link between over consumption of omega-6 fatty acids, such as linoleic acid and arachidonic acid, and depression has been established (Parker et al., 2006). This over consumption changes the ratio of omega-6/omega-3 fatty acid content in diet. Ali et al. (2009) also showed that diets low in DHA and EPA can result in depression. In an earlier study, researchers found that human subjects with major depression had higher ratios of omega-6/omega-3 fatty acids and lower levels of EPA in comparison to normal subjects (Maes et al., 1996). Women exhibiting borderline personality disorder treated with a dosage of around one gram of EPA per day were seen to experience a reduction in depression levels (Zanarini and Frankenburg, 2003).

Obesity

Omega-3 fatty acids were found to have a low incorporation into fat deposits in a study on rats (Herzberg and Sheppard, 1989). This outcome is thought to be resultant of a limited tolerance or ability of adipose tissue to store long-chain unsaturated fatty acids (Mitsuyoshi et al., 1991). A study on rats that looked at diets containing stearic acid (4.7%) plus linoleic acid (0.3%), linoleic acid (5%), or EPA (4.7%) plus linoleic acid (0.3%) found that EPA had higher ketogenicity than the other fatty acids (Mitsuyoshi et al., 1991).

Polyunsaturated fatty acids (PUFAs) are thought to have several aspects that lead to an anti-obesity effect. PUFAs influence the balance between energy intake and energy expenditure, lipid metabolism, the status of adipocytes (fat cells), and the neuroendocrine system (Li et al., 2008). There is evidence that DHA and EPA suppress a transcription factor (SREBP-1) that regulates expression of fatty acid synthase, which is an enzyme involved in the synthesis of fatty acids (Li et al., 2008). Insulin resistance has been linked to obesity. Research has suggested that EPA causes an improvement in insulin sensitivity (Lorente-Cebrián et al., 2006). Studies have suggested that PUFAs may enhance thermogenesis in adipose tissue, which leads to a loss in body weight and/or fat mass (Li et al., 2008).

2.2.2 Biosynthesis of Omega-3 Polyunsaturated Fatty Acids

There are two steps in which omega-3 fatty acids are synthesized. *De novo* synthesis is the first step, in which acetate is synthesized into short chain fatty acids, namely stearic acid (C18:0) (Wen and Chen, 2010). Longer chain polyunsaturated fatty acids are then synthesized by a series of desaturation and elongation reactions shown in Figure 2-3. Although higher plants and animals typically do not have the ability to produce fatty acid chains longer than C18, several species of microorganisms are able to produce them. Humans, as well as some other organisms, are able to synthesize EPA or DHA from ALA obtained through the diet, but the conversion rates are quite low (Innis, 2007). Therefore, large quantities of dietary long chain omega-3 fatty acids are needed to gain the related health benefits.

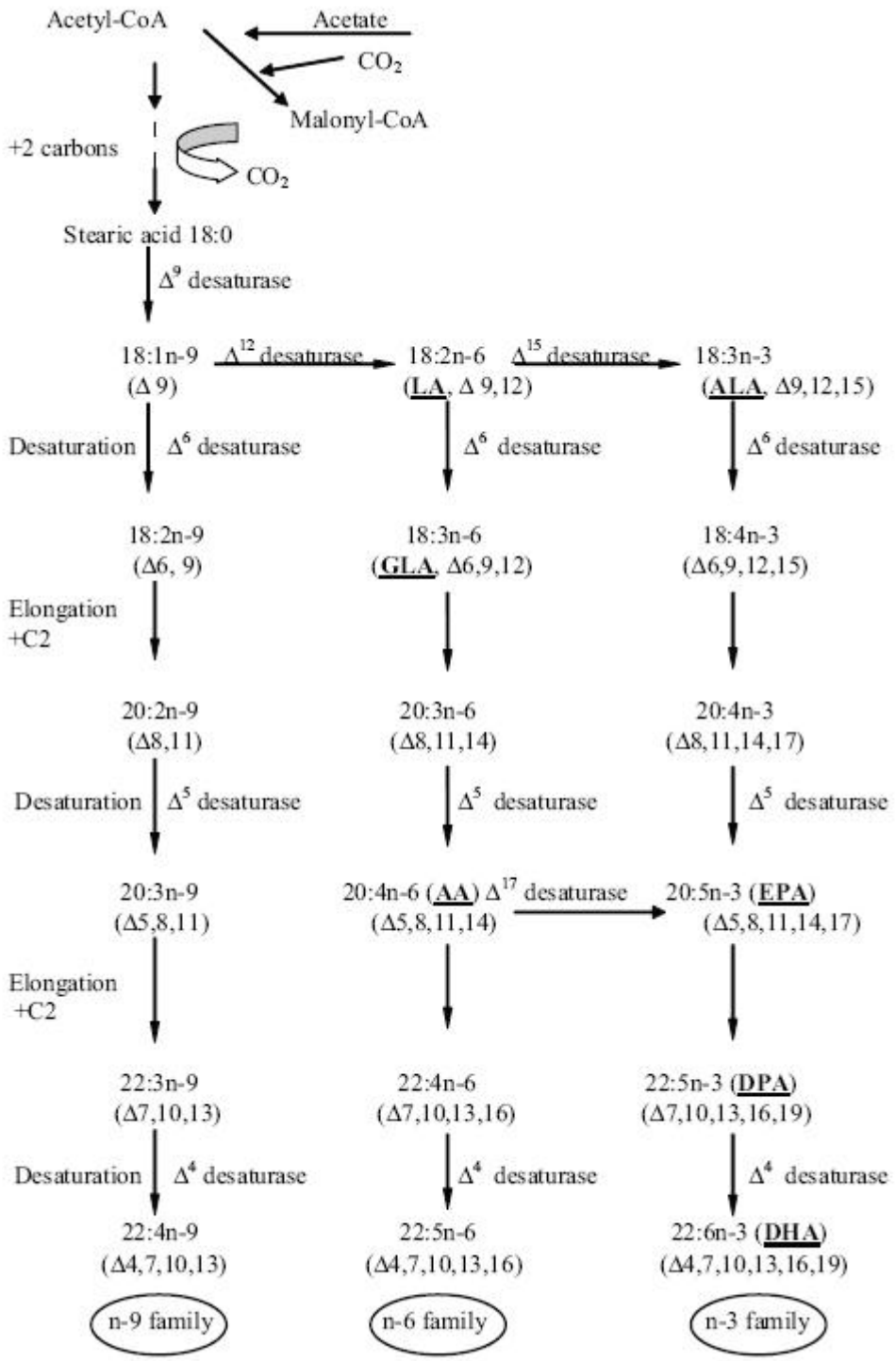

Figure 2-3. Biosynthesis pathways for polyunsaturated fatty acids (Wen and Chen, 2010).

2.2.3 Sources of Omega-3 Polyunsaturated Fatty Acids

Traditional Sources

To date, fish oil has been the most common source of omega-3 fatty acids. Around a million tons of fish oil is produced each year worldwide (ICIS, 2003). Omega-3 fatty acid containing fish oils vary from $3.50-$4.50 per kg, whereas the price for cholesterol-free fish oils is $4.50-$5.00 per kg (ICIS, 2003). While organizations such as the Dietary Guidelines Advisory Committee and the American Heart Association recommend consuming a moderate amount of fatty fish each week, there are some issues with fish oil products. Fish oil has what is typically perceived as an unpleasant taste and odor. Toxic impurities such as mercury, methylmercury, dioxins, and polychlorinated biphenyls can accumulate in fatty fish (Hooper et al., 2006). These impurities must be removed from the fish oil because they are fat-soluble and will accumulate in the human body (Marik and Varon, 2009). Fish oil is a natural resource and its sustainability is another problem to consider. Indeed, in the past 20 years, the global demand of fish oil has continued to increase while the production has been level, which drives the price of fish oil to steadily increase (Pyle et al., 2008). Therefore, alternative sources of omega-3 fatty acids are urgently needed.

Alternative Sources

There are many known species of microalgae that produce omega-3 fatty acids. Species in the genera *Phaeodactylum*, *Nitzschia*, and *Monodus* are producers of EPA. Researchers have found *P. tricornutum* to produce EPA as 30-40% of its total fatty acids under specific culture conditions (Yongmanitchai and Ward, 1991). *Nitzschia lavis* has been found to be able to produce EPA under heterotrophic conditions, while *Monodus* spp. require light (Wen, 2001; Ward and Singh, 2005). DHA-producing species of algae include *Crypthecodinium cohnii* and *Schizochytrium* spp. Both species are able to produce DHA under heterotrophic conditions. *C. cohnii* as a DHA-producer has been studied extensively (Ward and Singh, 2005). It is used by the company Martek Biosciences to commercially produce DHA. Biomass from *Schizochytrium* spp. is commercially sold as a DHA supplement for fish feed.

Some research has been conducted on the possibility of engineering plants to become sources of omega-3 fatty acids. The plant *Arabidopsis thalina* has been used by researchers to produce EPA and DHA (Robert et al., 2005). This plant was selected for its notable amounts of

linoleic and alpha-linoleic acids in its seed oil, which are used in long chain polyunsaturated fatty acid synthesis. Robert et al. (2005) successfully expressed genes required for the process of elongation and desaturation of fatty acids in *A. thalina*. Both EPA and DHA were expressed in the seed oils.

Higher animals have been looked at as well as an alternative source of omega-3 fatty acids. Researchers were able to create a transgenic pig that was capable of producing high levels of omega-3 fatty acids. Lai et al. (2006) used an omega-3 fatty acid desaturase gene found in a roundworm and successfully expressed it in live pigs. The gene allowed the pigs to convert omega-6 into omega-3 fatty acids, significantly reducing the ratio of omega-6/omega-3 fatty acids (Lai et al., 2006). Although this early research has been successful, any genetically engineered plant or animal intended for consumers will be met with controversy.

2.3 Algal Culture Systems

In general, there are three main ways of growing algae: photoautotrophic, heterotrophic and mixotrophic culture systems. Photoautotrophic algal species use light and CO_2 as their energy and carbon source, respectively. Heterotrophic algal species need an organic carbon source for their growth, and can grow in dark conditions. Mixotrophic algal species are able to use an organic carbon source, but also need light for their growth.

2.3.1 Photoautotrophic Culture System

Photoautotrophic algae growth can be performed in either open ponds or enclosed photobioreactors. In general, an open pond is difficult to maintain optimal algal growth conditions. It is essentially impossible to prevent a contamination by a different species of algae, bacteria, and predatory protozoa (Chen, 1996). Pond conditions tend to only result in dilute concentrations of algae, thus making recovery of the biomass particularly expensive (Grima et al., 2003). These factors limit the potential species that can be productively grown in this manner.

Enclosed photobioreactors are able to avoid many of the problems found in open pond systems. They are closed systems and contamination problems are far less severe, if not completely avoided. There are various designs of photobioreactors, with the tubular design being the most popular one. These systems usually are a series of transparent plastic tubes through

which the algae culture solution is circulated. This design creates a large surface-to-volume ratio and the reactors are typically placed outside for exposure to natural light (Wen and Chen, 2010). Regardless of how these reactors are illuminated, there are still problems with consistent light intensity. The algae itself can cause problems, in that the algae can build up as a film on the plastic surfaces blocking light and light penetration into the system is inversely proportional to the cell concentration (Wen and Chen, 2010; Chen & Johns, 1995). Other disadvantages include high capital costs and difficulty to scale-up.

2.3.2 Heterotrophic Culture System

In heterotrophic culture systems, algae use sugars, organic acids, or some other form of organic carbon as their energy and carbon source. Since light is not required, this type of culture system avoids any issues with light limitation problems. Some high cell density culture strategies, such as fed-batch, chemostat, or perfusion culture can be used to increase cell density in heterotrophic systems (Wen and Chen, 2010). When selecting a microbe for production purposes in a heterotrophic culture system, certain traits are more desirable than others. The microbe should be able to rapidly adapt to a new environment, grow on inexpensive and easily sterilized media, divide and metabolize without any light source, and withstand any hydrodynamic stresses caused by the fermentor and peripheral equipment (Wen and Chen, 2010).

2.3.3 Mixotrophic Culture System

In mixotrophic culture systems, conditions allow algae to carry out both photosynthesis and cellular respiration. Utilizing both types of metabolisms provides a couple of advantages. Algae using both types of metabolisms tend to exhibit an enhanced potential for producing omega-3 fatty acids such as EPA (Liu et al., 2009). Some species of algae require photosynthetic pigments (e.g., phycocyanin, carotenoid, and chlorophyll), but are unable to synthesize these compounds heterotrophically (Wen and Chen, 2010). Mixotrophic culture systems provide these species of algae with conditions that both facilitate synthesization of these photosynthetic pigments and increase omega-3 fatty acid production by providing an organic carbon source (Liu et al., 2009). The EPA productivity of *P. tricornutum* has been shown to significantly increase in mixotrophic cultures with glycerol as the carbon source. Garcia et al. (2005) found that by using

P. tricornutum, an EPA productivity of 43.13 mg L^{-1} day^{-1} could be obtained using 0.1 M of glycerol and periodic supplementation of 0.01 M urea. This value was 13 times greater than the EPA productivity under photoautotrophic culture conditions.

2.4 *Phaeodactylum tricornutum* as an EPA producer

2.4.1 Discovery and Isolation of *P. tricornutum*

This species of algae was first described by K. H. Bohlin in 1897 (De Martino et al., 2007). It was observed in samples collected near Plymouth, UK and in Baltic rock pools. There are several other occurrences in which *P. tricornutum* has been isolated – in varying places around the world. The earliest strain, that is still being kept, was isolated in 1908 off of Plymouth, UK (De Martino et al., 2007).

2.4.2 *P. tricornutum* as an Aquaculture Feed

P. tricornutum has been studied as a source of nutrition for *Artemia* sp. (brine shrimp) (Fábrega et al., 1998), *Crassostrea virginica* (eastern oyster) (Epifanio et al., 1981), and *Sparus aurata* (seabream) (Atalah et al., 2007).

Fábregas et al. (1998) used 36 combinations of six renewal rates and six concentrations of nutrients in semicontinuous cultures to test the effect on the biochemical composition of *P. tricornutum*. These different cultures of *P. tricornutum* were then evaluated as a source of nutrition for the microcrustacean *Artemia*. The authors reported that the reproductive parameters of *Artemia* are the most sensitive index for evaluation of the nutritional value in its diet (Fábregas et al., 1998). It was concluded that the nutritional value of the microalgae is largely affected by culture conditions and this in turn can be used to alter the biochemical composition of the microcrustacean.

One year old, hatchery-reared oysters (*C. virginica*) were given diets consisting of the algae *T. pseudonana*, *P. tricornutum*, or a mixture of these two species (Epifanio et al. 1981). It was found that oysters had the greatest growth yield when fed *T. pseudonana* but declined with the addition of *P. tricornutum*. Diets containing any more than 50% (w/w) *P. tricornutum* resulted in no growth, but weight loss over the course of five weeks of feeding trials. It was noted that *P. tricornutum* was lacking in some amino acids (tryptophan was completely absent) and fatty acids thought to be important in the diets of marine animals. However, this was not a

definitive reason for the poor nutritional value of *P. tricornutum* for this species of oyster, because other species of diatoms have similar deficiencies while still being considered acceptable for bivalves (Epiffano et al., 1981).

Atalah et al. (2007) used *P. tricornutum* as an alternative to fish oil supplementation in the diet of seabream (*S. aurata*) postlarvae. Fish oil is typically used as an energy source and a source of polyunsaturated fatty acids (PUFAs) in fish diets. *P. tricornutum* was investigated as a substitution to fish oil because it is known to be good source of PUFAs, particularly EPA. The seabream postlarvae fed a diet of *P. tricornutum* had lower survival rates than those fed diets containing fish oil or the algae *Cryptecodinum cohnii* (Atalah et al., 2007). The lower survival rates could possibly be related to cornutate (horn-like) processes found in the valves of *P. tricornutum* (Atalah et al., 2007). Epithelial degeneration was observed in *S. aurata* fed *P. tricornutum* and these cornutate processes could have been the cause (Atalah et al., 2007).

2.4.3 Culture Conditions for *P. tricornutum*

P. tricornutum has been intensively studied for its ability to produce EPA. Many studies have focused on optimizing culture conditions. Parameters such as carbon source, nitrogen source, salinity, phosphate level, temperature, CO_2 level, and other factors including levels of vitamin B_{12} and silica have been researched for their effects of EPA production in *P. tricornutum*.

P. tricornutum is able to use several difference sources of nitrogen. Some common sources that have been studied are nitrate, urea, and ammonium. Yongmanitchai and Ward (1991) looked at these three nitrogen sources and found that nitrate and urea resulted in high biomass production, while lower biomass values were observed with ammonium. It was thought that the pH reduction caused by ammonium ion assimilation resulted in the lower biomass values. Using nitrogen sources at an equimolar concentration of 11.8 mmol of nitrogen per liter, an EPA yield of 63 and 107 mg/L was achieved with nitrate and urea, respectively (Yongmanitchai and Ward, 1991).

Providing a small increase of carbon dioxide to an algal culture is typically expected to increase the biomass concentration with autotrophic cells. Yongmanitchai and Ward (1991) found that raising the CO_2 concentration in the air supply caused an increase in lipid content in

P. tricornutum cells. Supplementing with 1% CO_2 resulted in the highest biomass and EPA yield.

2.4.4 Mixotrophic Growth of *P. tricornutum*

Carbon sources have been looked at with the goal of improving growth rate and fatty acid content. Certain organic carbon sources are more conducive than others to these goals and vary among species of microalgae. Examples of previously researched organic carbon sources are glucose, fructose, lactose, mannose, acetate, and glycerol (García et al., 2005; García et al., 2006). Some species of microalgae have been observed to have a higher growth rate when grown mixotrophically rather than photoautotrophically. Providing an organic carbon source to *P. tricornutum* significantly increased respiration rate while reducing the net photosynthetic O_2 evolution (Liu et al., 2009). Although *P. tricornutum* was unable to grow heterotrophically on any of several studied carbon sources such as glucose, fructose, and glycerol (García et al., 2006), its ability to produce energy from organic carbon sources under mixotrophic conditions decreased the cells' dependence on light (Liu et al., 2009).

Liu et al. (2009) compared the organic carbon sources glucose, glycerol, and acetate. Of the three, glycerol was the most effective at raising the respiration rate of *P. tricornutum*. García et al. (2005) investigated at the growth performance of *P. tricornutum* on a number of carbon sources such as acetate, starch, lactic acid, glycine, glucose, and glycerol and found that in batch mode glycerol at 0.1 M resulted in the highest biomass concentration (2.99 g/L) and EPA productivity (56 mg L^{-1} day^{-1}). Glycerol, fructose, glucose, lactose, and mannose were studied for their potential to enhance the growth of *P. tricornutum* by García et al. (2006). It was found that lactose and mannose had a minimal effect on growth. Glycerol with a concentration of 0.1 M achieved the highest biomass concentration of 7.04 g/L in fed-batch culture, followed by a biomass concentration of 3.5 g/L from fructose at 0.02 M in fed-batch culture (García et al., 2006). Based on various reports, glycerol appears to be the most suitable organic carbon source for mixotrophic growth of *P. tricornutum*.

2.4.5 Genomic Information on *P. tricornutum*

The genome of *P. tricornutum* has been studied because of diatoms' role as global primary producers. Diatoms are estimated to be responsible for approximately 20% of the

primary productivity on Earth (Bowler et al., 2008). This species in particular was studied because of its ability to be genetically transformed, its short generation time, and its ease of culture (Scala et al., 2002). Using a method based on flow cytometry, Scala et al. (2002) estimated that its genome size is somewhere between 12 and 20 megabases (Mb). This would be a genome size similar to that of *S. cerevisiae*, which has 12 Mb in a haploid set of chromosomes. Recent study on genome sequence of *P. tricornutum* showed that the species contain approximately 27.4 Mb and share 57% of it genes with *T. pseudonana* (Bowler et al., 2008).

Many of gene sequences of *P. tricornutum* bear more resemblance to animal rather than its plant counterparts (Scala et al., 2002). Research has indicated that a substantial number of *P. tricornutum* predicted genes may have been horizontally transferred between diatoms and bacteria (Bowler et al., 2008). Some of these shared genes are thought to encode for novel metabolic capacities, such as organic carbon and nitrogen utilization (Bower et al., 2008). The diatom genome is believed to have diverse sources, including genes originating from cyanobacteria, proteobacteria, and archaea.

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Chapter 3: Mixotrophic Culture of the Microalgae *Phaeodactylum tricornutum* on Biodiesel-derived Crude Glycerol for Producing Eicosapentaenoic Acid

3.1 Abstract

Crude glycerol is the major by-product of the biodiesel industry and it was looked at in this research for use in increasing eicosapentaenoic acid (EPA) production in *Phaeodactylum tricornutum*. Research in past has shown pure glycerol to be effective in this pursuit. This work considers the effects of the source of nitrogen, level of supplemental carbon dioxide, and concentration of crude glycerol in batch mode production of EPA, as well as dilution rate in continuous mode. Dilution rate is an important operational parameter in continuous culture. It is calculated by dividing the flow rate of the media by the volume of the media within the reactor. The maximum specific growth rate was estimated from the washing out dilution rate at 0.677 day^{-1}. The highest biomass productivity of 0.612 g L^{-1} day^{-1} was observed at a dilution rate of 0.24. The highest EPA productivity of 16.9 mg L^{-1} day^{-1} was obtained in batch mode culture with 0.08 M crude glycerol and 3% (vol/vol) supplemental CO_2, closely followed by 16.5 mg L^{-1} day^{-1} at dilution rates of 0.15 and 0.24 day^{-1} in continuous mode culture. Greater biomass productivities were seen in continuous mode culture (dilution rate, D = 0.1, 0.15, 0.24 day^{-1}) than were obtained in any of the batch mode cultures. This research demonstrates the viability of biodiesel-derived crude glycerol in mixotrophic production of EPA.

3.2 Introduction

Biodiesel has become increasing attractive as an alternative fuel source in the past few years. Biodiesel production in the United States has reached its highest point in 2008, with 691 million gallons being produced; a decline to 490 million gallons was seen in 2009, but overall production is still historically high (NBB, 2010). A major byproduct that results from the biodiesel production process is crude glycerol. For every 9 kg of biodiesel produced, approximately 1 kg of crude glycerol is formed (Dasari et al., 2005). A number of impurities are present in crude glycerol and purification is cost prohibitive. As the biodiesel industry continues to grow, so does an abundance of waste crude glycerol and the need for a means of utilization.

A number of methods have been considered for the manufacture of value-added products from crude glycerol. Products such as propylene glycol (Dasari et al., 2005) and acetol (Chiu et al., 2006) can be made through thermochemical processes. Through fermentation processes,

products like 1,3 propanediol (Gonzalez-Pajuelo et al., 2006; Zheng et al., 2006), ethanol (Dharmadi et al., 2006), lipids (Papanikolaou and Aggelis, 2002; Meesters et al., 1996), and pigments (Narayan et al., 2005) can be produced. Hydrogen gas can be produced from glycerol through anaerobic fermentation by *E. coli* (Dharmadi et al., 2006) and through photofermentation by *Rhodopseudomonas palustris* (Sabourin-Provost and Hallenbeck, 2009).

Previous research by García et al. (2000; 2005; 2006) has shown that pure glycerol can be used in the mixotrophic production of EPA by *Phaeodactylum tricornutum*, but there is no research utilizing crude glycerol for this purpose. Research has been performed showing crude glycerol can be acceptably and inexpensively purified for use in culturing the omega-3 polyunsaturated fatty acid producing alga *Schizochytrium limacinum* (Pyle et al., 2008). EPA is an omega-3 fatty acid shown to have a number of uses in promoting human health, including a role in treating cardiovascular disease and cancer (Schacky and Harris, 2007; McEntee et al., 2008). Currently, the most common source of omega-3 fatty acids is fish oil. Fish oil faces problems with undesirable taste and odor, shelf life, impurities, and a limited supply (Barclay et al., 1994). Research by Pyle et al. (2008) has demonstrated that crude glycerol could be used in algal fermentation to produce omega-3 fatty acids while avoiding the heavy metal impurities seen in fish oil. EPA produced by *Phaeodactylum tricornutum* can be extracted and incorporated in products such as food and pharmaceuticals. Using algae as an alternative source of omega-3 fatty acids also would serve to reduce strain on fish stocks. Developing an algal method to provide large quantities of EPA requires a detailed study. The aim of this research was to investigate the EPA productivity of *Phaeodactylum tricornutum* in mixotrophic culture.

3.3 Materials and Methods

3.3.1 Algal Strain, Medium, and Subculture Conditions

The alga *Phaeodactylum tricornutum* (UTEX 640) was used. The algal cells were maintained in f/2 medium containing artificial sweater supplemented with (per liter) 0.075 g $NaNO_3$, 0.005 g $NaH_2PO_4 \cdot H_2O$, 0.03 g $Na_2SiO_3 \cdot 9H_2O$, 1 mL of trace metal solution containing iron, copper, sodium, zinc, cobalt, and manganese, and 0.5 mL of vitamin solution containing thiamine HCl, biotin, and cyanocobalamin (Guillard, 1975; Guillard and Ryther, 1962). Artificial seawater contained (per liter) 18 g NaCl, 2.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, 1.0 g Trizma base, 0.027 g/L NH_4Cl, 1.35 x 10^{-4} g vitamin B_{12}, 1

mL chelated iron solution, and 10 mL PII metal solution containing boron, cobalt, manganese, zinc, and molybdenum (Starr and Zeikus, 1993). The medium was autoclaved at 121°C for 15 min. The cells were incubated in 250 mL Erlenmeyer flasks with 50 mL of medium. The flasks were kept on a static shelf with an ambient temperature of 23°C on a static shelf. The illumination was continuously provided by 40-W cool white plus fluorescent lights at 125 μmol s^{-1} m^{-2} measured with an LI-250A light meter and Quantum Q40477 sensor (Li-Cor Biosciences, Lincoln, NE, USA). The subcultured cells were used as inoculum in the study of mixotrophic culture using biodiesel derived crude glycerol.

3.3.2 Bubble Column Culture System

Mixotrophic algal culture was performed in glass bubble columns that were 50 cm in length and 37 mm in inner diameter. The bottoms of the columns were cone-shaped. The medium for algal culture was f/2 medium supplemented with different concentrations of biodiesel derived glycerol. The medium was autoclaved at 121°C for 15 min. Compressed air (about 1 vvm, volume gas per volume liquid per minute) was sparged into the bottom of the columns through sterilized air filters. To investigate the effects of CO_2 addition on the algal growth performance, pure compressed CO_2 was mixed with compressed air (at different ratios) through gas flow meters, and mixed gases were then introduced into the bubble columns. The culture system was maintained at 20±1°C with continuous illumination at 125 μmol s^{-1} m^{-2} through cool white plus fluorescent lights. The working volume of the reactor was controlled at 400 mL.

In the continuous culture, a medium containing f/2 medium with 0.08 M crude glycerol was used in initial batch culture. After 6 days of batch culture, feed medium was added to the reactor at various dilution rates. The feed medium was identical to the medium used in the initial batch culture. At the same time, equal volumes of cell suspension were withdrawn from the reactor. Samples were taken from the reactor on a daily basis to measure the cell density. The steady-state under each operation condition was considered to have been established after at least five consecutive samples with less than 5% variation of cell density were achieved. Figure 3-1 shows a comparison of the inputs and outputs of both of the modes of operation studied.

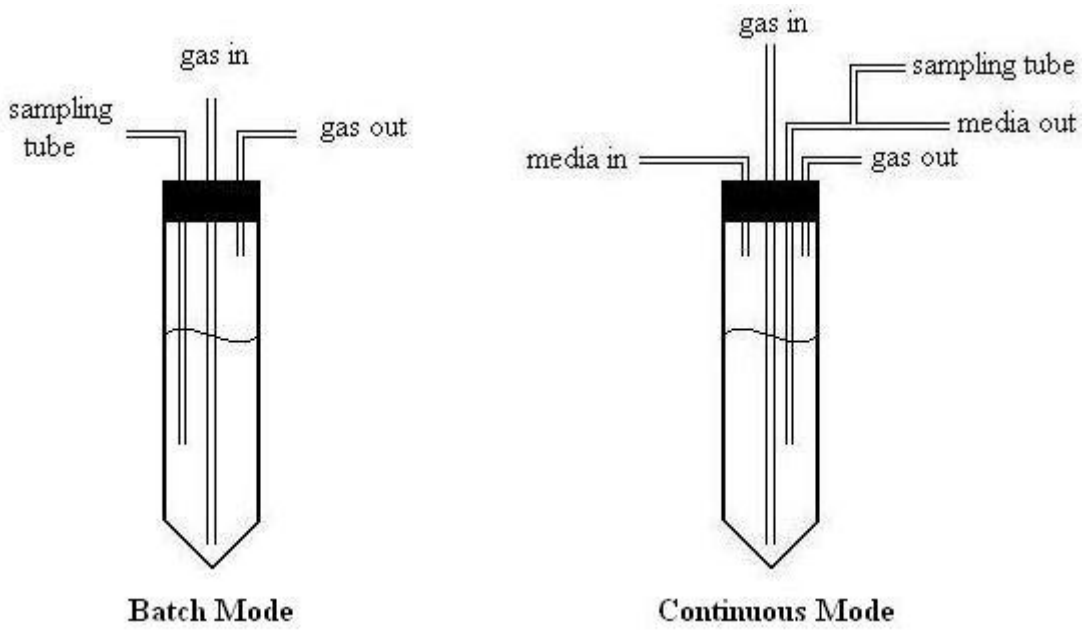

Figure 3-1. Comparison of batch versus continuous modes of operation.

3.3.3 Preparation of Crude Glycerol-containing Medium

The crude glycerol was obtained from Virginia Biodiesel Refinery (West Point, VA) that produces biodiesel from a 50:50 (w/w) mixture of soybean oil and chicken fat. The procedures for soap removal from crude glycerol and the subsequent preparation of glycerol-containing medium are summarized as follows: (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 lowered to around 3 with sulfuric acid to convert soap into free fatty acids that precipitated from the liquid, (iii) the precipitated solids formed an upper phase after the liquid was kept static for 30 minutes, and (iv) the free fatty acids in the upper phase were removed from the crude glycerol phase by the means of a separation funnel. This crude glycerol was then added to the f/2 medium at the desired concentration.

3.3.4 Analyses

Biomass concentration

To determine the cell biomass concentration, a relationship between the biomass concentration and optical density (OD at 440 nm) of the cell culture solution was experimentally established as,

$$y = 1.9029x - 0.0062 \quad (1)$$

where x is the density of the cells (g/L) and y is the OD. Afterward, a one mL cell culture sample was taken from the bubble columns, measured for its OD value, and converted into biomass concentration using the above equation.

Fatty Acid Analyses

The cell culture solution harvested at the stationary phase of batch culture or the steady state of continuous culture was centrifuged at 6000 rpm for 5 minutes. The cell pellets washed twice and then freeze-dried. The preparation of fatty acid methyl esters (FAMEs) from the freeze dried cells and the analyses of fatty acid composition were the same as those described previously (Pyle et al., 2008).

Specific Growth Rate

The specific growth rate during batch mode was calculated by taking the natural log of the cell concentration and plotting it over time. Slope, which represents the growth rate, was calculated for the exponential portion of the growth curve.

3.4 Results

3.4.1 Batch Mode Culture

3.4.1.1 Effect of Nitrogen Source

The effects of the nitrogen source on the growth and fatty acid production of *P. tricornutum* were studied. The nitrogen source in the f/2 medium was adjusted to contain ammonium chloride, sodium nitrate, and urea. The concentration of each nitrogen source was 11.8 mM of nitrogen. Cell growth performance in nitrogen-free medium was also tested. Cultures were allowed to grow until stationary phase was reached. An overview of cell growth and fatty acid production are shown in Table 3-1.

Compared with nitrogen-containing medium, the nitrogen-free medium resulted in rather poor biomass yield (1.05 g/L), specific growth rate (0.122 day^{-1}) and biomass productivity (0.087 g/L·day). The poor biomass yield and productivity were expected, because of the role of nitrogen in protein synthesis. Ammonium chloride also had a poor biomass yield (1.59 g/L) and productivity (0.133 g/L·day), which was likely related to the resulting drop in pH from the

ammonium ions (Figure 3-2). Sodium nitrate and urea had the best biomass yields (3.50 and 3.01 g/L) and productivities (0.184 and 0.158 g/L·day), with sodium nitrate outperforming urea. In terms of fatty acid production, nitrogen-free medium resulted in highest total fatty acid (TFA) content (297 mg TFA/g biomass); however, the TFA yield (311 mg TFA/L culture) was lower than those from the nitrate (361 mg/L) and urea (416 mg/L) containing mediums. The EPA production from the nitrogen free medium (2.10 g/L·day) was lower than those of the nitrogen-containing medium. Among the three nitrogen sources tested, ammonium chloride resulted in the highest TFA content (158 mg/g), but lowest TFA yield (246 mg/L) due to the poor growth from this nitrogen source. The EPA yield (86.5 mg/L) and productivity (4.55 g/L·day) from the sodium nitrate medium was the highest. Collectively, it was found that nitrate was the best of the nitrogen sources for *P. tricornutum* in terms of cell growth and fatty acid production performance, this nitrogen source was used in the following experiments.

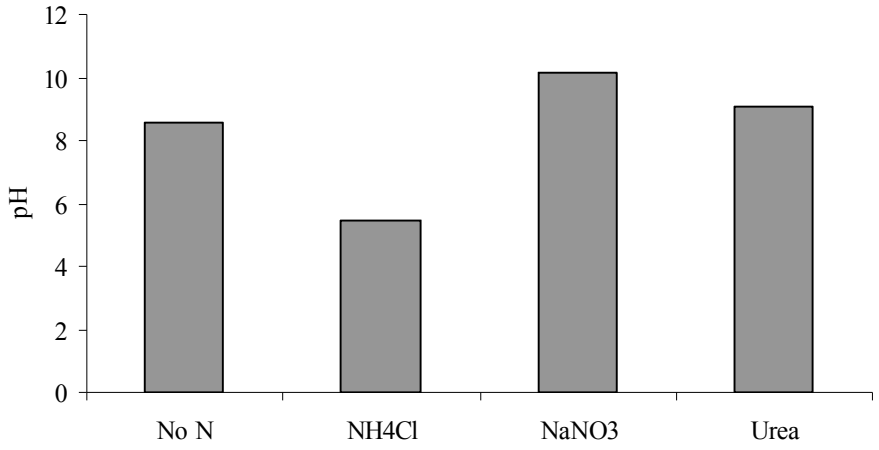

Figure 3-2. pH with respect to nitrogen source.

Table 3-1. Overview of biomass, EPA, and total fatty acid (TFA) values for nitrogen tests (data are presented as mean ± standard deviation of three replicates).

		No Nitrogen	NH_4Cl	$NaNO_3$	Urea
Biomass	Maximum biomass (g/L)	1.05±0.01	1.59±0.27	3.50±0.29	3.01±0.62
	Specific growth rate (day^{-1})	0.122	0.213	0.339	0.292
	Productivity (g/L·day)	0.087±0.001	0.133±0.022	0.184±0.015	0.158±0.033
TFA	Content (mg/g)	297.17±29.41	158.06±36.44	102.64±8.44	134.15±31.44
	Yield (mg/L)	311.19±28.05	245.63±31.02	360.78±56.56	416.28±166.14
	Productivity (mg/L·day)	25.93±2.34	20.47±2.59	18.99±2.98	21.91±8.74
EPA	Content (mg/g)	24.00±1.44	26.22±4.36	24.75±1.39	22.19±1.21
	Yield (mg/L)	25.14±1.29	42.49±13.88	86.49±5.91	67.13±16.41
	Productivity (mg/L·day)	2.10±0.11	3.54±1.16	4.55±0.31	3.53±0.86

3.4.1.2 Effect of Crude Glycerol Concentration

The effects of crude glycerol concentration on the mixotrophic culture of the *P. tricornutum* was tested at concentrations of 0, 0.04, 0.08, and 0.12 M. Figure 3-3 shows that the biomass yield increased with the tested concentrations of crude glycerol. The specific growth rates at 0.04 M, 0.08 M, and 0.12 M of crude glycerol were all very similar (0.504 – 0.523 day^{-1}) (Figure 3-4). Substrate inhibition can occur after the carbon source is increased to a certain concentration, but this was not observed with the concentrations of crude glycerol tested. Table 3-2 shows that fatty acid composition and EPA production of the mixotrophic *P. tricornutum* at different crude glycerol concentrations. The major fatty acid contained in the cells were palmitic acid (C16:0), palmitoleic acid (C16:1), and eicosapentanoic acid (C20:5) with minor amount of myristic acid (C14:0) and oleic (C18:1). For different crude glycerol concentrations, the fatty acid ratios were relatively stable. TFA and EPA content were seen to increase with crude glycerol concentration. EPA productivity also increased as crude glycerol concentration was increased.

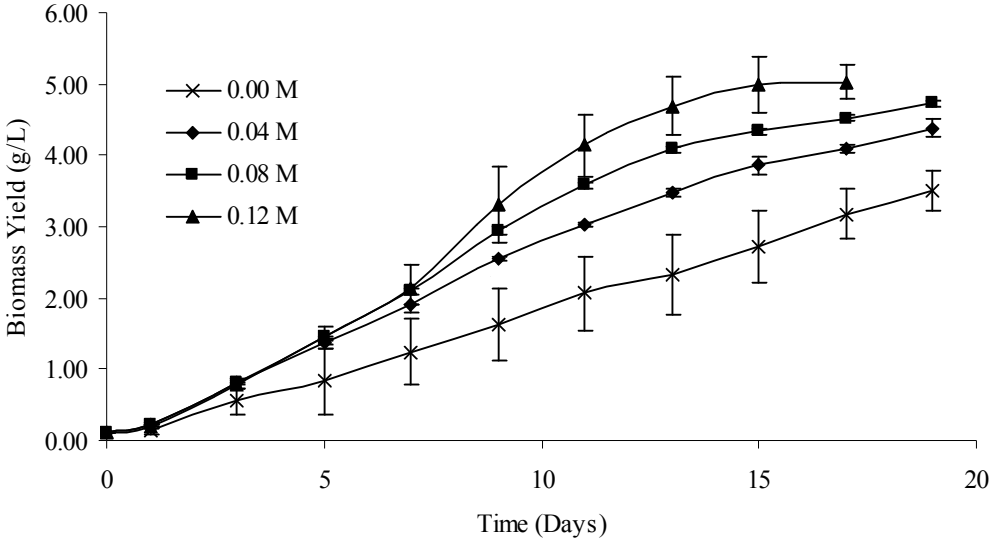

Figure 3-3. Biomass yield over time for tested glycerol concentrations.

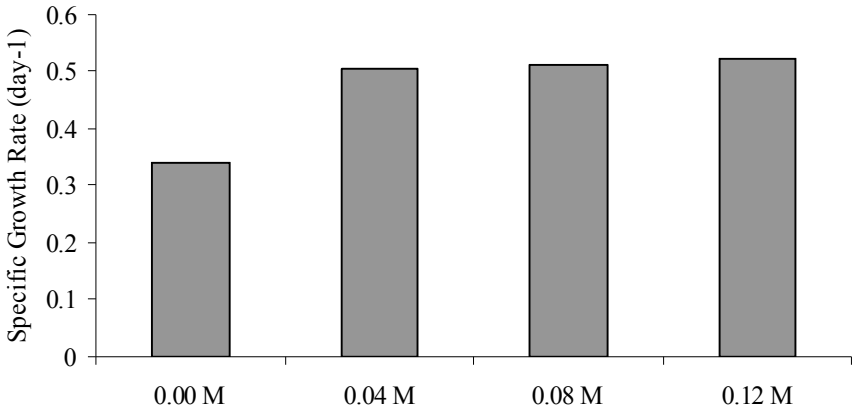

Figure 3-4. Specific growth rate versus crude glycerol concentration.

Table 3-2. Fatty acid composition, TFA content, EPA content, yield, and productivity for glycerol tests (data are presented as mean ± standard deviation of three replicates).

		Concentration			
Fatty acid	Unit	0.00 M	0.04 M	0.08 M	0.12 M
C14:0	%TFA	7.47±0.58	10.08±0.12	11.28±0.24	13.73±1.50
C16:0	%TFA	22.48±0.23	21.78±0.42	20.60±0.15	19.12±0.74
C16:1	%TFA	41.63±2.73	40.35±0.45	39.40±0.42	37.99±3.58
C18:1	%TFA	4.18±0.43	5.51±0.16	7.03±0.13	8.39±0.22
C20:5	%TFA	24.24±2.72	22.28±0.59	21.69±0.28	20.77±2.26
TFA content	mg/g	102.64±8.44	119.32±5.87	152.17±4.42	167.70±22.12
EPA content	mg/g	24.75±1.39	26.57±1.24	33.01±0.93	34.50±0.92
EPA yield	mg/L	86.49±5.91	119.57±5.09	155.99±3.44	173.57±11.87
EPA productivity	mg/L·day	4.55±0.31	6.29±0.27	8.21±0.18	10.20±0.70

3.4.1.3 Effect of Carbon Dioxide Level

CO_2 plays an important role in the mixotrophic culture of *P. tricornutum*. Providing increased carbon dioxide levels to autotrophically grown algae, such as *P. tricornutum* and *Chlorella fusea*, has been observed to increase lipid content (Yongmanitchai and Ward, 1991). However, the resulting pH drop from CO_2 dissolving into water to form carbonic acid must be taken into account as algae that require neutral or basic conditions will experience poor growth performance at acidic pH levels.

In this study, four levels of CO_2 were tested with *P. tricornutum* grown in batch mode. As shown in Figure 3-5, the highest biomass yield was seen with 3% CO_2 supplementation, followed by 0%, 6%, and 10% CO_2 supplementation. Notably, the addition of 3% and 6% CO_2 resulted in similar or greater biomass yields in nearly half the time required by 0% CO_2. The specific growth rate of *P. tricornutum* also reached the highest level at 3% CO_2 addition (Figure 3-6). The effect of carbon dioxide level on culture pH is shown in Figure 3-6. The decreasing specific growth rate at 6% and 10% CO_2 may be due to the pH drop in the medium (Figures 3-6 and 3-7).

Table 3-3 shows a summary of fatty acid analysis data. EPA was present in a consistent ratio among treatments, except for 10% CO_2, which caused a small decline. The level of CO_2 addition caused significant fluctuations in the %TFA of myristic acid (C14:0), palmitic acid (C16:0), and palmitoleic acid (C16:1). Myristic acid content increased from 0-3%, but decreased

with additional CO_2 supplementation. Palmitic acid content was seen to decrease from 0-3%, then increase with further addition of CO_2. Palmitoleic acid content behaved similarly to palmitic acid content, with the same decrease from 0-3% and increasing with additional CO_2. The TFA and EPA contents were fairly similar between treatments. Where the level of CO_2 made noticeable changes was in the EPA yield and EPA productivity. A level of 3% CO_2 resulted in the best performance in these two measurements. Supplementing with 3% and 6% CO_2 caused an increase in EPA productivity when compared to the 0% CO_2 addition. Increasing the supplemented CO_2 to 10% caused mostly undesirable effects.

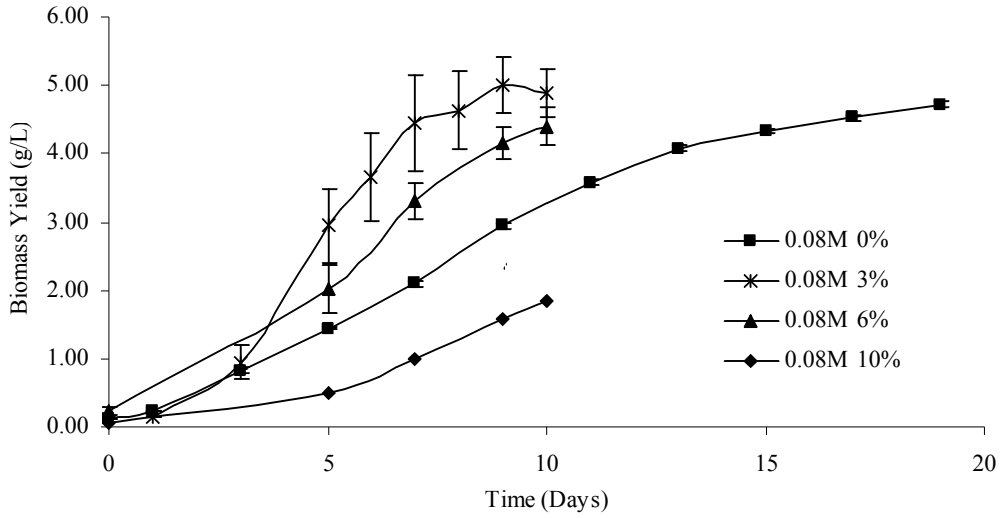

Figure 3-5. Biomass yield over time for tested carbon dioxide levels.

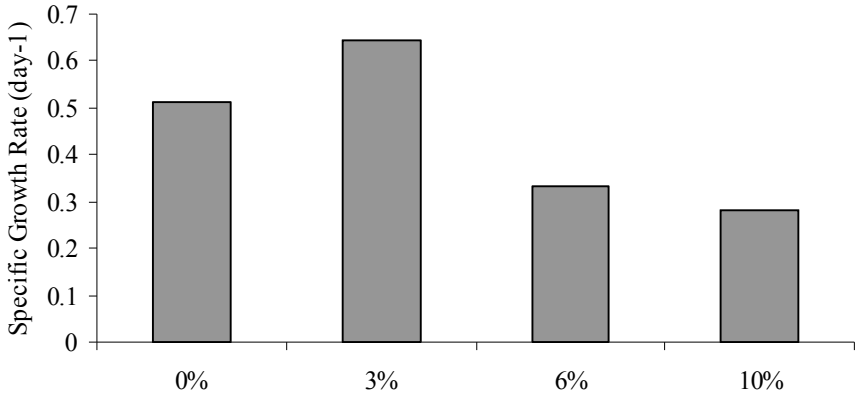

Figure 3-6. Specific growth rate versus carbon dioxide levels.

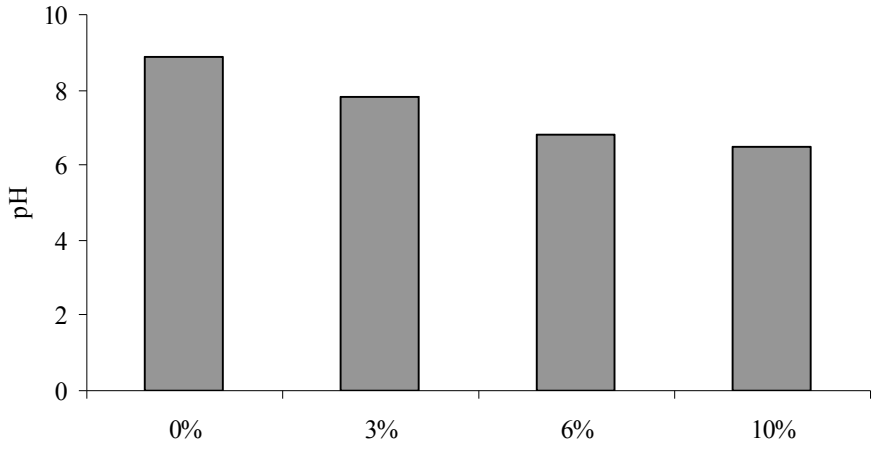

Figure 3-7. pH with respect to carbon dioxide level.

Table 3-3. Fatty acid composition, TFA content, EPA content, yield, and productivity for carbon dioxide tests (data are presented as mean ± standard deviation of three replicates).

		Level			
Fatty acid	Unit	0%	3%	6%	10% [a]
C14:0	%TFA	11.28±0.24	21.41±0.49	6.86±0.29	6.05
C16:0	%TFA	20.60±0.15	15.41±0.77	25.36±0.44	28.53
C16:1	%TFA	39.40±0.42	33.09±0.19	44.43±0.97	46.3
C18:1	%TFA	7.03±0.13	9.37±0.29	2.74±0.63	1.57
C20:5	%TFA	21.69±0.28	20.71±0.20	20.61±1.20	17.54
TFA content	mg/g	152.17±4.42	161.30±5.31	159.88±11.28	164.47
EPA content	mg/g	33.01±0.93	33.40±0.78	32.87±1.13	28.85
EPA yield	mg/L	155.99±3.44	169.34±13.08	144.92±14.20	53.61
EPA productivity	mg/L·day	8.21±0.18	16.93±1.31	14.49±1.42	5.36

a. Only one test column of the treatment provided meaningful data.

3.4.2 Continuous Mode Culture

The batch experiments were used as a basis for parameters selected in continuous culture. It was determined the continuous culture would be run with 0.08M crude glycerol, 3% CO_2, and sodium nitrate as the nitrogen source. Several dilution rates were investigated under these fixed conditions.

The biomass yields and productivities are shown in Figure 3-8. The maximum biomass productivity was observed at a dilution rate of 0.24 day^{-1}. The maximum specific growth rate was determined to be around 0.677 day^{-1} and was estimated from the washing out dilution rate.

Fatty acid analysis for continuous culture data is summarized in Table 3-4. There were consistent trends in the increase of palmitic acid (C16:0) content and the decrease of palmitoleic acid (C16:1) content as the dilution rate increased. EPA content was relatively stable for the tested dilution rates, other than the 0.1 day^{-1} setting, which was slightly lower. Figure 3-9 shows the TFA yields and productivities. A dilution rate of 0.24 day^{-1} led to the maximum observed TFA productivity. The EPA yields and productivities are shown in Figure 3-10. With respect to EPA productivity, dilution rates 0.15 and 0.24 day^{-1} resulted in the highest and the values were nearly equal. In Figures 3-8, 3-9, and 3-10, the same trend of decreased yield with corresponding increase in dilution rate was observed. This trend was expected, as there are less algal cells present with correspondingly higher flow rates of the growth medium.

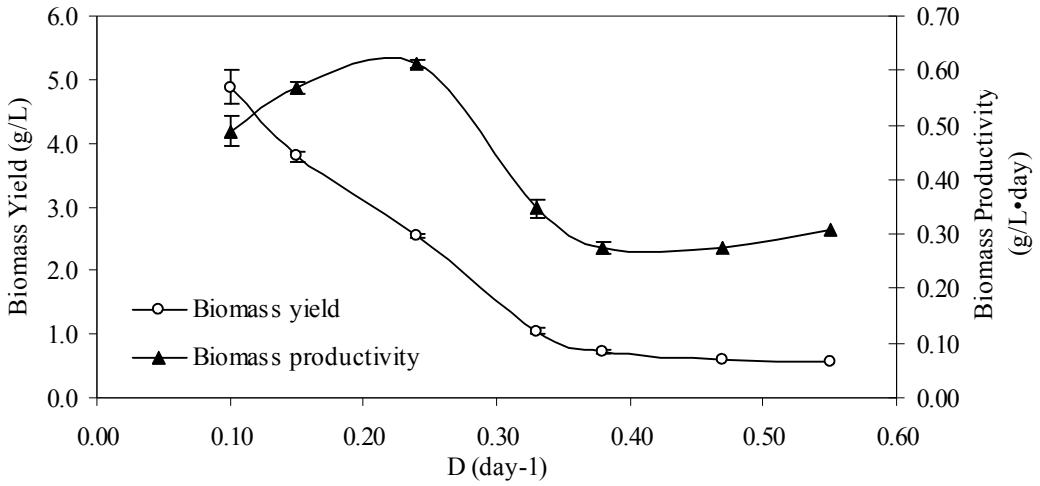

Figure 3-8. Cell growth of the continuous culture *P. tricornutum* on crude glycerol.

Table 3-4. Fatty acid composition, TFA content, EPA content, yield, and productivity for glycerol tests (data are presented as mean ± standard deviation of five replicates).

		Dilution rate (day^{-1})				
Fatty acid	Unit	0.1	0.15	0.24	0.33	0.38
C14:0	%TFA	23.77±0.62	23.35±0.75	23.72±0.61	22.68±0.82	19.85±0.47
C16:0	%TFA	11.24±0.20	11.32±0.54	12.75±0.63	16.76±1.84	20.10±0.46
C16:1	%TFA	29.90±0.27	25.14±1.20	25.85±1.10	19.01±0.72	19.38±0.51
C18:1	%TFA	6.52±0.32	4.61±0.41	3.76±0.23	4.16±0.96	4.43±0.10
C20:5	%TFA	28.57±0.71	35.58±1.63	33.92±1.32	37.38±3.45	36.24±0.59
TFA content	mg/g	89.84±3.97	81.40±4.20	79.38±3.37	53.30±3.84	51.69±6.74
EPA content	mg/g	25.67±1.41	28.93±1.24	26.91±1.11	19.90±2.00	18.76±2.71
EPA yield	mg/L	125.46±12.41	109.67±4.93	68.59±3.10	20.95±2.85	13.48±1.56
EPA productivity	mg/L·day	12.55±1.24	16.45±0.74	16.46±0.74	6.92±0.94	5.12±0.59

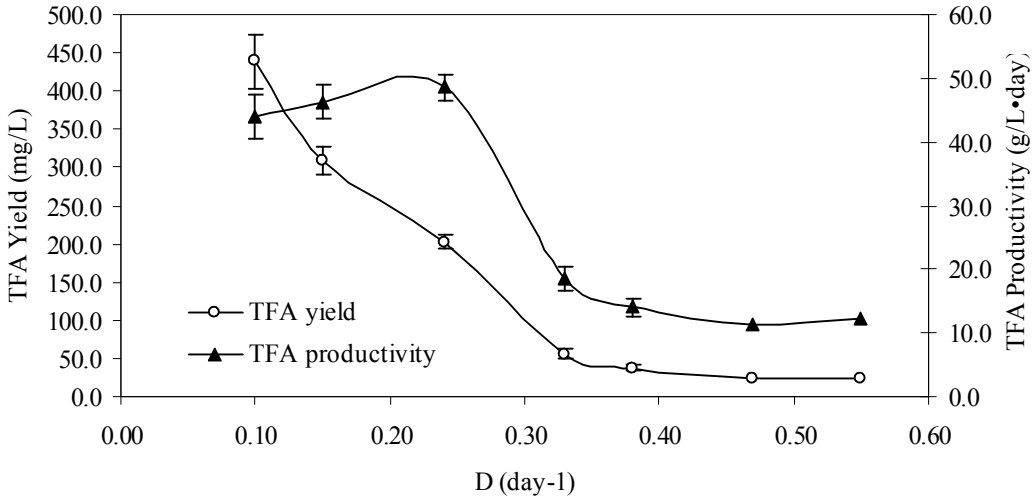

Figure 3-9. TFA production of the continuous culture *P. tricornutum* on crude glycerol.

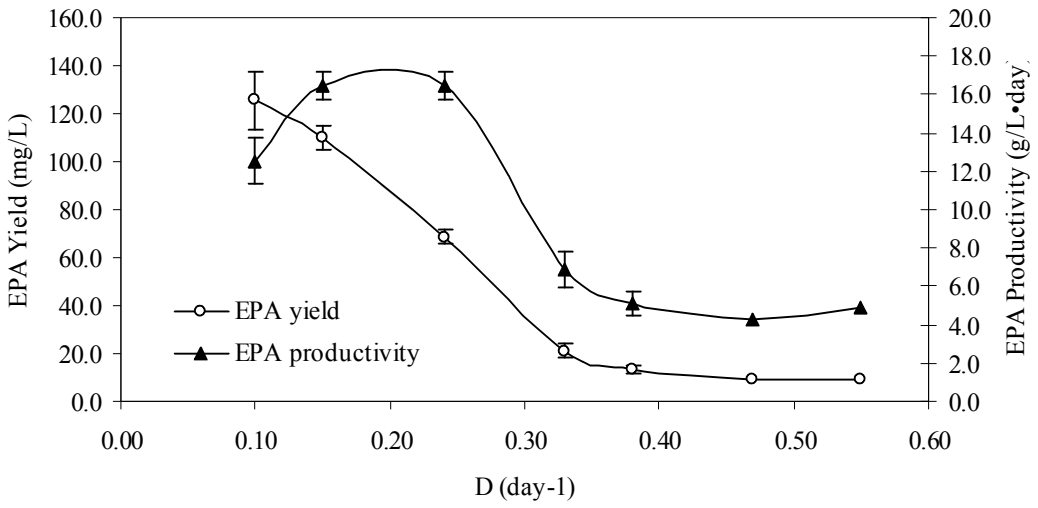

Figure 3-10. EPA production of the continuous culture *P. tricornutum* on crude glycerol.

3.5 Discussion

The microalga *Phaeodactylum tricornutum* has shown improved growth yields when grown mixotrophically (Fábregas et al. 1997; García et al. 2006; Liu et al., 2009). A variety of organic carbon sources can support the mixotrophic growth, including soluble fractions of potato (Fábregas et al. 1997), sugars such as glucose, fructose, and mannose (García et al. 2006), and glycerol (García et al. 2000; Liu et al., 2009). However, there have been no reports using biodiesel-derived crude glycerol for the mixotrophic growth of *P. tricornutum*.

Different types of nitrogen sources are known to have varying effects on microalgal growth. Not all research has reached the same conclusion on what nitrogen source is the most effective. For example, García et al. (2000) found that ammonium as the nitrogen source supported very high biomass concentrations (16.2 g/L) of *P. tricornutum* in fed batch cultures with 0.1M glycerol, whereas Yongmanitchai and Ward (1991) observed no growth at all using ammonium with *P. tricornutum*. In a study looking at sodium nitrate, ammonium chloride, and urea, each at a concentration of 11.8 mM, the highest EPA yield was 107.3 mg/L with urea (Yongmanitchai and Ward, 1991). In this research, it was found that of the nitrogen sources studied, sodium nitrate had the greatest results in terms of biomass and EPA. The average EPA yield was 86.5 mg/L, somewhat lower than the value observed by Yongmanitchai and Ward (1991), but the biomass yield was higher (3.50 g/L) than the corresponding 2.8 g/L. These differences might be attributable to variations in the growth medium used, the addition of CO_2,

and a different photoperiod. The growth mediums used were fairly similar in composition, but most notably the Mann and Myers medium used by Yongmanitchai and Ward (1991) contained 5 g/L NaCl compared to the 18 g/L NaCl in the f/2 medium. Yongmanitchai and Ward (1991) also supplied 5% (vol/vol) CO_2 and used a photoperiod of 16 h of light and 8 h of dark. No CO2 was supplied in this experiment during the nitrogen tests and a 24 h light photoperiod was used.

Studies using "pure" glycerol for mixotrophic culture of *P. tricornutum* have reported increased biomass yields and EPA yields. For example, in one study using 1 L air-sparged flasks, a biomass yield double that of the photoautotrophic control (2.99 g/L) was achieved with a glycerol concentration of 0.1 M (García et al., 2005). In another study, mixotrophic culture of *P. tricornutum* in 0.1 M glycerol was reported to have increased the biomass yield 371% (3.5 to 13 g/L) in a 60 L bubble column and 155% (0.460 to 0.713 g/L) in 250 mL flasks, respectively, when compared with the photoautotrophic cultures (Sevilla et al., 2004; Liu et al., 2009). In this work, it was observed that 0.12 M crude glycerol increased the biomass yield 144% (3.50 to 5.03 g/L) over the photoautotrophic control when cells were grown in 300-ml bubble columns. The EPA productivity for both 0.08 M and 0.12 M crude glycerol was similar to that found in the first growth stage (9.2 mg/L·day) of the 0.1 M fed-batch bubble column (Sevilla et al., 2004).

Research has demonstrated that supplemental CO_2 in the air supply increased the fatty acid content of photoautotrophically grown *Chlorella fusea* (Yongmanitchai and Ward, 1991). Yongmanitchai and Ward (1991) found that increasing supplemental CO_2 up to 5% (vol/vol) in photoautotrophic *P. tricornutum* cultures led to the greatest TFA and EPA contents and decreased TFA and EPA measurements with any further CO_2 supplementation. The researchers also observed that 1% (vol/vol) CO_2 supplementation resulted in the highest biomass (2.5 g/L) and EPA (87.5 mg/L) yields Yongmanitchai and Ward (1991). In this work, it was found that the addition of CO_2 was found to be beneficial under mixotrophic culture conditions with crude glycerol. Supplementation with 3% (vol/vol) CO_2 doubled the EPA productivity over that of the control and led to the highest EPA production among different CO_2 levels tested. It also resulted in an increased TFA content and EPA yield over the control, as suggested by previous research with *C. fusea* and *P. tricornutum* (Yongmanitchai and Ward, 1991).

Continuous culturing methods tend to be desirable in large-scale operations to improve the productivity of the end-product (i.e., EPA in this study), particularly when the growth medium is inexpensive. There is far less downtime than that associated with a batch culturing

method. By being able to harvest cells continuously, high biomass and desired-product productivities are achieved. Once a continuously operated reactor has reached steady-state, the output tends to be more stable than other culture methods. The most common parameter for controlling the continuous culture is dilution rate, which is the same value as specific growth rate at steady state. In this study, a dilution rate of 0.24 day^{-1} had the highest biomass productivity among all the dilution rates studied. The highest EPA productivity was obtained at dilution rates of 0.15 and 0.24 day^{-1}. The values achieved (16.5 mg/L·day) were higher than most of those seen in batch mode columns and were only matched by those supplemented with 3% (vol/vol) and 6% (vol/vol) CO_2 (16.9 and 14.5 mg/L·day, respectively).

The maximum specific growth rate of the *P. tricornutum* mixotrophic culture was estimated from the washing out dilution rate and was determined to be around 0.677 day^{-1}. This value is similar to the μ_{max} of 0.647 day^{-1} reported for a 75 mL photoautotrophic tube culture (Yongmanitchai and Ward, 1992). A 5.6 L chemostat with a photoautotrohpically grown culture used by Yongmanitchai and Ward (1992) had biomass and EPA productivities of 0.327 g/L·day and 15.1 mg/L·day at a dilution rate of 0.15 day^{-1}. These values were respectively increased to 0.51 g/L·day and 25.1 mg/L·day when the dilution rate was increased to 0.30 day^{-1} (Yongmanitchai and Ward, 1992). The EPA productivity at a dilution rate of 0.15 day^{-1}, found by Yongmanitchai and Ward (1992), was comparable to the EPA productivity found with the same dilution rate in this research. However, a significantly higher biomass productivity (0.569 g/L·day) was observed in this research. With a dilution rate of 0.24 day^{-1}, the EPA productivity was notably less than that achieved by Yongmanitchai and Ward (1992) at 0.30 day^{-1}, but the biomass productivity of this experiment was greater (0.612 g/L·day).

3.6 Conclusion

The batch mode experiments demonstrated the feasibility of using crude glycerol in a mixotrophic culture of *Phaeodactylum tricornutum*. Of the nitrogen sources tested, nitrate led to the highest EPA productivity. Combining this nitrogen source with supplemental CO_2 and crude glycerol caused further increases in EPA productivity. Continuous mode culture of mixotrophically grown *P. tricornutum* was more effective than batch mode culture for producing the omega-3 fatty acid EPA. The EPA productivity seen at a dilution rate of 0.24 day^{-1} was equivalent to or greater than any productivity seen in batch mode, while minimizing the

downtime seen with batch mode production. Using crude glycerol in the mixotrophic culture of *P. tricornutum* creates a value-added product from what is currently an abundant waste product with little value. With further study to optimize culture conditions and increase the scale of production, the EPA productivity could be further improved. This research provides insight into one method of economically utilizing crude glycerol, benefiting the biodiesel industry and providing a nutraceutical product.

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Chapter 4: Conclusions and Recommendations for Future Research

The work presented herein shows the possibility of mixotrophic culture of *Phaeodactylum tricornutum* using crude glycerol as a carbon source. This leads to an increased biomass and EPA productivity over a photoautotrophic culture.

It was proved in this research that crude glycerol can successfully be used to mixotrophically culture *Phaeodactylum tricornutum*. It was found that the nitrogen source, level of supplemental CO_2, and concentration of crude glycerol all have an effect on biomass and EPA productivity. A combination of nitrate, 0.08 M crude glycerol, and 3% (vol/vol) supplemental CO_2 led to the highest biomass (0.445 g L^{-1} day^{-1}) and EPA (16.9 mg L^{-1} day^{-1}) yields in batch mode culture. In continuous culture mode, the biomass yield was further increased (0.612 g L^{-1} day^{-1} at D = 0.24 day^{-1}) over batch mode, but the EPA productivity remained relatively the same (16.5 mg L^{-1} day^{-1}) as the best performance in batch mode.

Future work on this subject includes optimization and scale up. Varied concentrations of crude glycerol should be tested in continuous culture mode. Light-dark cycles, increased light intensity, manual control of the pH, and optimization of the growth medium should also be tested. Finally, further research should include scaling up the optimized continuous culture process to pilot scale.