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Eicosapentaenoic Acid (EPA) from Porphyridium Cruentum: Increasing Growth and Productivity of the Microalgae for Pharmaceutical Products

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Eicosapentaenoic Acid (EPA) from *Porphyridium Cruentum*: Increasing Growth and Productivity of the Microalgae for Pharmaceutical Products

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ABSTRACT

One of the major nutritional requirements in our diet is an adequate intake of omega-3 specially eicosapentaenoic acid (EPA). In the present study, the effects of two temperatures (16°C & 20˚C) and light intensities (140 & 180µE/M^2.S) and four nitrate levels (0.075, 0.3, 0.5 and 0.7g/L) on the cell growth and lipid productivity of *Porphyridium cruentum,* one of the most promising oil-rich species of microalgae, were investigated. A growth comparison was carried out using pure CO_2 and 5% CO_2/air. Additionally, the ratio of the fatty acids with omega-3 and omega-6 groups at various growth conditions were compared, since an appropriate proportion of omega-6 (arachidonic acid(ARA)) to omega-3(EPA) is vital for healthy nutrition. Lower EPA production and consequently higher ARA/EPA ratio occurred when 5%CO_2/Air was utilized as CO_2 supplementation. The highest biomass productivity (143 mg/L.day) and EPA (13.08%w/w) were achieved at 20˚C, 140µE/M^2.S and 0.3g/L nitrate, while lipid content was the lowest (0.5%w/w) at this condition. The optimal growth condition to produce the lowest ARA/EPA, 2.17, was achieved at 20°C, 140µE/M^2.S (pure CO_2) and 0.5g/L nitrate.

The possible growth of *P. cruentum* in ultra-filtered swine wastewater was also evaluated in this study. *P. cruentum* was grown in different waste dilutions (total nitrogen concentrations of 12.4, 49.4, 82.4 and 115.3mg/L) to achieve the optimum nutrient condition for producing EPA. Various salinities (0-3.2%) were examined in the optimum nitrogen concentration. The data were compared to L1-medium and the results generally demonstrate better growth of *P. cruentum* in swine waste with higher lipid productivity and lipid content. The maximum lipid productivity (143mg/L) and lipid content (4.71 mg/100g biomass) was achieved in 3.2% salinity swine waste with 82.4mg/L nitrogen concentration while control medium with 2.5% salinity resulted the highest biomass productivity (3.76g/L). Markedly higher biomass productivity, lipid productivity

and lipid content were achieved from swine waste culture diluted with seawater compared to saltwater. Higher C16:0, 18:2 and C20:5 (EPA) and lower C18:0 and ARA/EPA were achieved from swine waste culture compared to the control medium. Generally, the minimum ARA/EPA(1.33) was obtained at 82.4mg/L nitrogen concentration and 3.2% salinity in swine waste diluted with seawater.

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DEDICATION

I dedicate my dissertation work especially to my family. My husband and my love Mohammad Ali Khorrami have never left my side and being there for me throughout the doctorate program. A special feeling of gratitude to my parents, Gholamhossein Asgharpour and Parivash Nejad Seddighi and to my father-in-law, Mahmoud Khorrami and my lovely mother-in-law (who will never will be missed in my heart), Nahid Rahimi for their assistance with my son, for their involvement in my efforts, and for their love.

TABLE OF CONTENTS

List of Publications

Published Papers

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To be Submitted Papers

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Referenced Conference Papers

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[4] Maryam Asgharpour, Jamie A. Hestekin. Optimization of nitrogen amount for production of EPA by the *Porphyridium cruentum*. 2014 AIChE Annual Meeting, Atlanta, GA, November 20, 2014.

[5] Maryam Asgharpour, Neda Mahmoudi, Shannon Servoss, Jamie A. Hestekin. Evaluation of gas exchange in hollow-fiber membranes for artificial lungs. 2014 AIChE Annual Meeting, Atlanta, GA, November 20, 2014.

[6] Maryam Asgharpour, Brigitte Rogers, Jamie A. Hestekin. "Effect of light intensity on EPA content from a red microalga, *Porphyridium cruentum*." Poster. 2014 ABI Fall Research Symposium, Arkansas State University – Jonesboro, USA, October 7, 2014.

[7] Maryam Asgharpour, Brigitte Rogers, Jamie A. Hestekin. "Lipid levels of *P. cruentum* at various growth conditions." Poster. 2013 ABI Fall Research Symposium, Little Rock, USA, October 15, 2013.

Articles by Others

[8] Sandefur H N, Asgharpour M, Mariott J, Hestekin J, Matlock M. (2015) Recovery of nutrients from swine wastewater using ultrafiltration: applications for microalgae cultivation in photobioreactors. Ecological Engineering. Under Review.

[9] Neda Mahmoudi, Maryam Asgharpour, Lauren Reed, Jamie Hestekin, Shannon Servoss. A new method of decreasing fouling and increasing gas transfer of polysulfone hollow-fiber membranes using peptoids. Submitted to ACS National Meeting & Exposition, San Diego, CA, March 13-17, 2016.

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[11] Neda Mahmoudi, Maryam Asgharpour, Lauren Reed, Jamie Hestekin, Shannon Servoss. Peptoid modified membranes for artificial lung applications. Poster. 2014 MAST Meeting, University of Arkansas Fayetteville, October 13, 2014.

[12] Neda Mahmoudi, Maryam Asgharpour, Jamie A. Hestekin, Shannon L. Servoss. A New Method of Decreasing Fouling of Polysulfone Hollow-Fiber Membranes Using Peptoids. 2014 AIChE Annual Meeting, Atlanta, GA, November 20, 2014.

1. INTRODUCTION

1.1 Biology of Algae

Microalgae are a group of microorganisms which have been commonly described as unicellular or multicellular mainly water residence organisms. These organisms include both eukaryotic and prokaryotic species that can live in harsh circumstances and grow rapidly because of their simple structure [1,2]. Most of microalgae can grow photo-synthetically (autotrophic) which require only inorganic compounds such as CO_2, salts and a light energy source for growth and they convert solar radiation, minerals and carbon dioxide by chloroplasts into biomass and oxygen. However, there are some microalga species which grow heterotrophically or non-photosynthetic which require an external organic source and nutrients as an energy source. Some microalgae are mixotrophic means that they are enabling to grow photo-synthetically as well as gaining exogenous organic nutrients [2-5].

Microalgae are classified based on some characteristics such as their photosynthetic pigment nature, cell wall, storage products and the number of membrane around the chloroplast and recently based on specific DNA sequence [6,7]. Eukaryotic microalgae have membrane-bound organelles (plastids, mitochondria, nuclei, Golgi bodies, and flagella) that control the cell's functions and let it to survive and reproduce. Eukaryotic cells categorized into a variety of classes based on different pigment position, life cycle, basic cellular structure and biochemical constituents which the most important ones are green algae (*Chlorophyta*), red algae (*Rhodophyta*) and diatoms (*Bacillariophyta*). However, *cyanobacteria* (blue-green algae) are prokaryotic microalgae which lack these organelles and are more similar to bacteria than algae [3,5,7,8].

Microalgae represent a large variety of species which live in varied environmental condition, not only aquatic but also terrestrial ecosystems. More than 50,000 microalgae exist, however, a limited number of about 30,000 have been identified to date [8]. The large diversity of microalgae testifies a wide variety of their applications including pharmaceutical purposes, energy sources and food crops for human consumption [1].

1.2 Cultivation Systems

Microalgae can be cultivated such as other microorganisms in three modes of batch, semi-continuous and continuous. In batch cultivation, the organisms grow in a fresh medium with sufficient nutrients until limitation of some required components such as light or nutrients. So, cells accumulate high concentrations of lipid or other metabolites under stress condition and before completing the growth process. In semi-continuous cultivation, a certain amounts of biomass harvest every few days and water and essential nutrients add to the reactor for new growth cycle. In this mode, complete restarting of the reactor with new medium and microalgae do not required and it can reduce time and cost of the process [6]. Growth and lipid production of *Nannochloropsis* and *Isochrysis* were investigated by Boussiba et al. (1987, 1988). The biomass with average 30% lipid content was obtained over a two months period [9,10]. Continuous cultivation is used generally for heterotrophic organisms and biomass is continuously harvested to maintain the growth at optimum condition to extend life cycle. Also, all reactor parameters and cell density are controlled. The best possible biomass production will be achieved in this mode, however, high concentration of lipid or carotenoid production which required stress conditions is not possible [6].

1.3 Photobioreactors

Photobioreactors (PBRs) are utilized as a culture system for phototrophic microorganisms in which specific conditions such as light intensity and temperature are carefully controlled for particular species. Bioreactors utilized for the cultivation of microalgae may be classified base on both design and operation mode. Many studies are being performed to reduce the costs of installation and operation of PBRs and therefore produce low value products such as algal lipids for different applications of pharmaceutical and biofuel productions. There are many factors such as nutrients, light intensity and temperature which need to be considered for optimal culture conditions [6,11].

Bioreactors may be open or closed systems. Open pond systems have been employed since the 1950s [12]. There are two categories for open pond systems including natural waters (such as lakes and lagoons) and artificial ponds or containers. An open pond system is a cheaper method to produce large scale algal biomass compared to closed photobioreactors. Open pond systems do not require specific land like agricultural crop productions [13]. Moreover, they have lower energy input requirement, easier cleaning and regular maintenance [14,15]. On the other hand, closed photobioreactors overcome some major problems in open pond systems such as pollution and contamination risks particularly for high-value products to use in pharmaceutical and cosmetic industries [15]. Cultivation of a single microalgal species is performed in a closed photobioreactor with lower risk of contaminations unlike open pond systems. Closed photobioreactors include flat plate, tubular and column. These photobioreactors are more appropriate for sensitive strains since potential contaminations can be control easier. However, the costs of closed systems are higher compared than open pond bioreactors [16].

1.4 Microalgae Based Systems

The microalgae are gaining more attention within the scientific community for many different applications, such as wastewater treatment [17], pharmaceuticals and medicines [18] and biofuel productions [19]. To improve the nutrient of food products, some microalgae are added to specific foods like noodles in several countries [20]. The microalgae have numerous advantages over other sources of fatty acids, such as: more oil yield per area of microalgae than the best oilseed crops, less required water for growth, the possibility to be cultivated in seawater or non-arable lands, obtaining cultivation fertilizers specially phosphorus and nitrogen from wastewaters [21], production of valuable co-products such as proteins and residual biomass of oil extraction [18], and pharmaceuticals like omega-3 fatty acid (ω3).

Production of algal lipids needs some inputs such as energy, water, nutrients and land. Water is employed to provide physical growth environment and also a medium to deriver nutrients. Some microalgal strains including *Porphyridium cruentum* can grow in saltwater, thus, the request for freshwater inputs can decrease. In addition, wastewater can be an alternative potentially viable growth medium for microalgal feedstock [22]. Another input for growth of microalgae is essential nutrients such as carbon dioxide (for photosynthetic production of biomass), phosphorous and nitrogen (for the energy related reactions). The nitrogen and phosphorous also could be prepared from wastewaters. There is a significant advantage in lipid production from algae over lipids made from land plants (such as food crops). Non-farmable lands can be employed to locate algal production facilities.

There are many different production systems and processing methods to produce and convert lipids from microalgae, but they generally include same basic steps: growth, harvest, recovery and processing. Microalgae can be cultivated in closed photobioreactors or open ponds.

These systems both require agitation for nutrient distribution and also to prevent sedimentation. In addition, microalgae need to be exposed in light and carbon dioxide for photosynthesis process which can help partially offset carbon dioxide in the atmosphere. In order to harvest microalgae, they need to be separated from their liquid culture medium, for instance, by filtering and centrifuge to remove algal cells from water. During recovery of microalgae, the extracted lipids from algal cells and the remained residue can be recovered for nutrient or may be processes as a co-product. Finally, algal lipids are processes into fuels or other useful products [23].

Microalgae have been used in fatty acid productions to produce fuels and many useful co-products. A potential application that has been the subject of intense research is the production of omega-3 which plays an important role in pharmaceutical industries. Advantages of microalgae over plants as a source of fatty acids have motivated many research groups to employ microalgae as producing omega-3 and omega-6. There are some microalgae, such as *Porphyridium cruentum*, which are rich sources of these fatty acids. These researches are mainly focused to achieve the best growth condition of the microalgae to maximize desired fatty acids. It is expected that these investigations will allow researchers more insight into the production of omega-3 and omega-6 fatty acids in commercial pharmaceutical applications [24].

1.5 Fatty Acids Especially EPA or Omega-3

Fatty acids are hydrocarbons with straight chains and a carboxyl group (COOH) at one end. The carbon next to the carboxyl group is labeled as α and the next one is β. The last position of fatty acid chain (the methyl end (CH_3)) is known as ω. The omega-3 fatty acids (ω3 or n3 fatty acids) are a main family of polyunsaturated fatty acids (PUFAs) which have a double bond

carbon at the third carbon of the methyl end so called ω-3. There are three kinds of ω-3 fatty acids in human phycology: ALA (α-linolenic acid; 18:3, n-3), EPA (eicosapentaenoic acid; 20:5, n-3) and DHA (docosahexaenoic acid; 22:6, n-3) which have the carbon chains of 18, 20 or 22 with 3, 5 or 6 double bond, respectively. ALA can be gained from some vegetables such as linseed oil, however, EPA and DHA can be obtained from marine fish oils. There is another family of long chain PUFA known as arachidonic acid (ARA, 20:4, n-6) or omega-6 [25]. ALA is a precursor for ARA, EPA and DHA which are synthesized by alternating desaturation and elongation steps. Human do not have sufficient enzymes to do these steps [26]. Moreover, these low amounts of the enzymes deteriorate with age and under some disease conditions [27]. Therefore, researchers found that these fatty acids should be considered essential for normal growth of children and animals. “Essential” nutrients mean that they cannot be synthesized sufficiently by our bodies. ARA and EPA are precursors for a group of eicosanoids. Eicosanoids are hormone-like substances containing prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) which are crucial in regulating developmental and regulatory physiology [28]. However, the two acids make functionally and structurally different eicosanoids, sometimes with antagonistic effects [25].

Adequate omega-3 fatty acid intake improves infant cognitive development and visual acuity. Recent medical studies recommend that both preterm and at term dietary need an appropriate ratio of ω-3/ω-6. Therefore, it is becoming a common practice in many countries to include them in baby formula [26]. Omega-3 or EPA plays a main role as a membrane fluidity adjuster, as the precursor of different biologically active regulators such as hormones, and in reducing plasma triacylglycerol and cholesterol levels [29]. Also, it has been demonstrated that EPA can reduce the risk of some diseases such as diabetes [30], brain disorders [31],

arteriosclerosis, coronary heart disease, inflammation and several carcinomas [32], Alzheimer's and psoriasis [33]. In addition, the properties of omega-6 fatty acids rather than omega-3 fatty acids are important to consider. Unbalanced intake of omega-6 to omega-3, mainly the insufficient uptake of omega-3, results in increased cardiovascular diseases, risk of cardiac deaths and mental illnesses. A diet with a ratio of 1:1 is ideal, while ratios higher than 10:1 are not recommended. With common intake of fast meals with high amounts of fried foods and low consumption of fresh whole meals, this ratio will be between 1:25 and 1:50 which is very unhealthy [26,34].

1.6 Algal Based Systems as a Source of EPA

The market for EPA fatty acids is growing due to their large applications in pharmaceutical and medical industries. The traditional resource of EPA for human nutrition is marine fish oils. In addition to some limitations including odor, high purification costs and oil stability, the population of fish to produce EPA may not be able to meet the demands of human population. Therefore, the search for an alternative source to produce these fatty acids has been encouraged due to the concerns on the limitations of EPA from fish [26,29]. The production of EPA in the body of fish is because of the intake of marine algae which produce EPA *de novo* (Figure 1). Therefore, developing a commercial feasible methodology to produce EPA directly from microalgae has been the subject of many intense researches. Microalgae, "single-celled eukaryotic organisms", are the primary natural producer of high-quality relative fatty acids such as EPA.

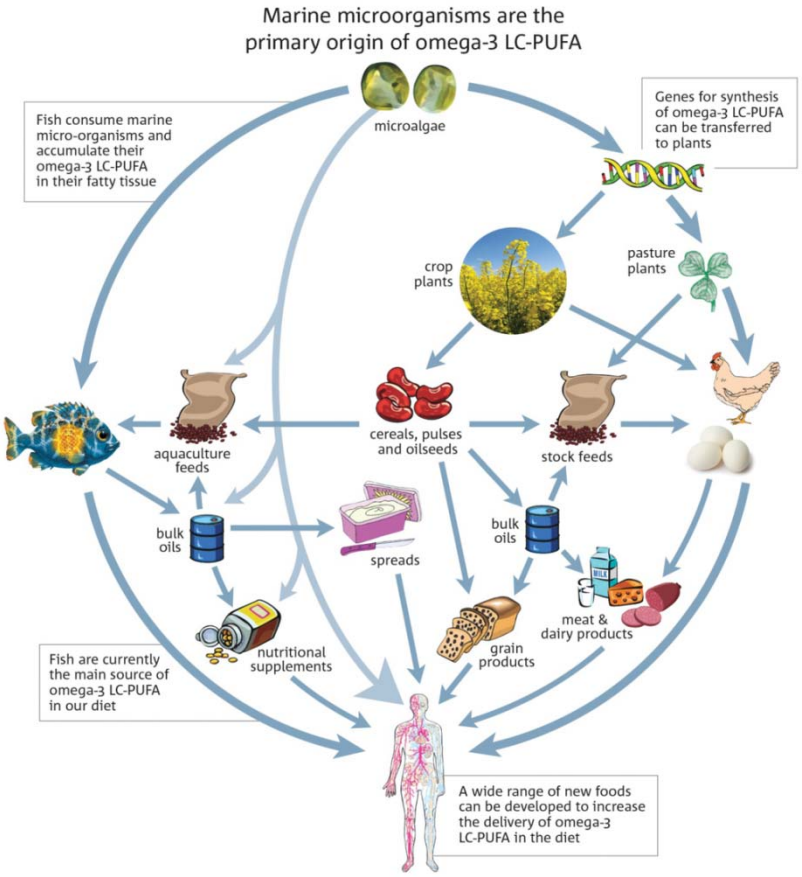

Figure 1.1 Different sources of omega-3 in human dietary cycle [35]

1.6.1 Related Works

Since the 1930s, omega-3 fatty acids have been recognized as essential nutrients for health and a normal growth. Burr and Burr at 1929 discovered essentiality of the long-chain PUFAs [36]. However, until the 1980s, knowledge about their health benefits was not improved considerably. Eventually, “qualified health claim” status for reduced risk of coronary heart disease (CHD) was given to DHA and EPA omega-3 fatty acids by the United States Food and Drug Administration on 2004 [37].

Omega-3 fatty acids have been found in a wide variety of microorganisms such as bacteria, fungi and microalgae. Microalgae have been used as an alternative source of EPA omega-3 fatty acids instead of fish oils. With the increased listed potential applications, many studies allocated to find proper microalgae to produce omega-3. Moreover, in order to optimize the productivity and finances of the process, cultivation system design is becoming more critical. The first parameter to have a successful algal biotechnology is selecting the right microalgae species with appropriate characteristics for example fatty acid and biomass productivities [38]. Therefore, primary strain selection is the most important factor to achieve maximal desired fatty acid compositions and yields. Vazhappilly and Chen (1998) investigated twenty microalgae strains in photoautotrophic flask cultures to identify those with potentials to produce EPA and DHA. They indicated that some of these microalgae such as *Monodus subterraneus*, *Phaeodactylum tricornutum*, *Chlorella minutissima* and *Porphyridium cruentum* can produce significant amounts of EPA (18–96 mg/L). Considering the individual production of EPA without DHA due to their different pharmacological and nutritional importance and to minimize problems related to downstream processing, it is desired to choose a strain that yield less DHA for EPA production. Therefore, it is very significant to select an alga strain to have less DHA to produce EPA. *Porphyridium cruentum* was one of the best potential strains for EPA production without any DHA [33]. *P. cruentum* is a red microalga which accumulates large amounts of lipids especially EPA and ARA in its cells [39]. Its protein contents and carbohydrates are 28-39% and 40-57%, respectively. The total lipid ranges may vary up to 14% of dry weight [40].

It has been indicated that the contents of EPA and other fatty acids of microalgae can be changed under different environmental and nutrition conditions including light intensity, temperature and culture medium composition [41]. Since ARA can reduce the EPA production

and make problem in EPA recovery, producing ARA during the EPA production is a critical factor. Therefore, the market for specific products, in this case the maximum ratio of EPA to ARA, is predicted to develop and diversify [42]. *P. cruentum* is one of the microalgae which produce EPA and ARA, however, the ratio of EPA and ARA contents in this microalga can be controlled using optimization of growth conditions. Consequently, researchers were interested in investigating the effects of environmental and nutrition conditions to produce EPA omega-3 fatty acids with reduced amounts of ARA.

The primary parameters affecting PUFA production are temperature and light intensity [20]. The total fatty acid and EPA contents for unicellular algae have been reported to decline with increasing temperature. However, it has been shown that the lipid contents of *P. tricornutum* cells increase at higher temperature [43]. Other research has shown that the optimum temperature for optimal EPA of *P. cruentum* should not exceed 25°C. They illustrated that a temperature of 30°C results a low concentration of EPA and high concentrations of ARA and other fatty acids [24].

Under natural growth circumstances, phototrophic algae utilize sunlight, nutrients from their environment, and CO_2 from air. Artificial production of algae should attempt to reproduce the optimum natural growth conditions [5]. Artificial lighting is one of the critical factors in commercial production of algae. Low light intensity has been reported to increase accumulation and production of PUFAs of some microalgae cells such as *Cyclotella menaghiniana*, *Nitzschia closterium* and *Euglena gracilis* [44-46]. However, light intensity effect was reversed for the red alga *P. cruentum* [47]. Also, hydrocarbon production could be reduced at lower illuminations. It has been reported that light intensities less than 30 W/m^2 inhibited the growth rate of *Botryococcus braunii*, while photo-inhibition and less attained lipid amounts occurred at light

intensities above 100 W/m2. This occurs due to the low biomass as well as fewer hydrocarbons [48].

Another main factor affecting biomass and lipid productivities is nutrient composition (such as nitrogen and phosphorous) in culture media. In general, biomass growth is usually inhibited in a nitrogen starvation situation. However, algae lipid content is often enhanced because the lipid-synthesizing enzymes are less susceptible to disorganization than carbohydrate-synthesizing enzymes in this situation, so the major amount of carbon is bound in lipids [40]. Therefore, there should be a lipid productivity peak related to nitrogen concentrations at a certain nitrogen source. Miyamoto illustrated that lipid content from *Nannochlolis* decreased about 22% with increasing nitrate concentration from 0.9 mMol/L to 9.9 mMol/L, while lipid yield as well as biomass productivity increased [49].

Freshwater green alga *Parietochloris incise* contained over 35% (of dry weight) fatty acid content with 60% (of total fatty acids) ARA under nitrogen starvation [50]. Another study showed the effect of nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* in view of their possible application as novel raw materials for biodiesel production. Results show that all the considered stress conditions in this study (temperature and nitrogen concentration) caused the accumulation of lipids as well as a reduction in microalgal growth and consequently the lipid productivity. Lipid contents of *N. oculata* and *C. vulgaris* increased from 7.9 to 15.3% and 5.9 to 16.4%, respectively, with a 75% decrease of the nitrogen level in the culture medium. Around 60% (mol/mol of total lipid) palmitic acid (C16:0) was obtained in both microalgae [51]. Effect of nitrogen source and concentration on growth rate and fatty acid composition of *Ellipsoidion sp.* was investigated by Xu et al. They presented that the microalgae growth with ammonium as a nitrogen source was faster compared to nitrate

during the pre-logarithmic phase. However, the result was reverse during the post-logarithmic phase. The algal growth was very low in the medium without nitrogen or with urea as the nitrogen source. The total lipid yield in the medium contained ammonium was higher than nitrate medium; however, the EPA yield did not vary significantly. The maximum growth rate was related to the nitrate concentration of 1.28 mmol/l. The maximum total lipid and EPA contents occurred in the medium containing 1.92 mm/l nitrate and EPA was 27.9% of total fatty acids in this concentration. With stable growth rate in ammonium concentrations from 0.64 to 2.56 mmol/l, the maximum total lipid and EPA (39.0%) were related to the ammonium concentration of 2.56 mmol/l [52]. Another research showed the effect of nitrogen deficiency on biomass and fatty acid composition in a unicellular freshwater *chlorophyte*, *Parietochloris incisa*, which accumulates high amount of arachidonic fatty acids. The arachidonic fatty acid contents increase strongly in a medium with nitrogen starvation [53]. The lipid yield of autotrophic *Scenedesmus dimorphus* and heterotrophic *Chlorella* were investigated with different source (urea and glycine/yeast extract) and three concentrations of nitrogen in the culture medium. The highest lipid yields of *S. dimorphus* and *C. protothecoides* (0.4 g/L and 5.89 g/L) was related to the medium containing 1.8 g/L urea and 2.4 g/L, respectively [54]. Shi et al. reported that urea wass the best nitrogen source compared to ammonium and nitrate for heterotrophic growth of *Chlorella protothecoides*, while ammonium was the worst nitrogen source. However, ammonium which is the main nitrogen source of wastewaters is preferred for other algal strains such as *Chlorella sp.* [55]. The effect of nitrogen deficiency on growth and lipid production of *Dunaliella salina* was investigated by Weldy and Huesemann. The results show that higher lipid contents were obtained in the nitrogen deficient culture medium compared to nitrogen sufficient medium. However, higher amounts of lipids (450 mg lipid/l) were produced under nitrogen

sufficient condition according to culture lipid concentration and lipid production because of higher biomass growth. Therefore, optimizing the growth according to lipid production is more important than lipid content (%). The lipid contents of *Dunaliella salina* was in the range of 16-44% (wt) [56].

Several attempts have been carried out to establish the nutritional requirements of *P. cruentum* and to optimize the media formulation. Roessler et al. studied the nitrogen starvation effects on *P. cruentum* growth. They found that the lack of nitrogen in culture medium can lead to the accumulation of lipids such as triacylglycerol (TAG) in microalgae cells [57]. Cohen (1986) illustrated that starvation generally resulted in a sharp reduction of content and proportion of PUFAs and also leads to the accumulation of saturated and monounsaturated fatty acids in algae cells [58]. Cohen also showed in 1990 that nitrogen starvation in *P. cruentum* caused increased amounts of TAG and ARA and consequently increased ARA/EPA ratio [42].

1.7 Algal Based Systems for Wastewater Treatment

Livestock wastewaters can cause serious ecological problems. Uncontrolled nutrient discharges (two orders of magnitude higher carbon and nutrient concentration compared to domestic wastewaters) in the livestock effluents lead to severe contaminations of groundwater resources. Therefore, the treatment of these wastewaters is receiving an interesting attention between researchers and industries [59] and the development of environmental friendly technique to treat the livestock effluents is mandatory. Conventional aerobic methods such as activated sludge processes have several limitations, for instance, intensive energy consumption for oxygen supply and the impossibility of recycling the valuable nutrients in the pig wastewater [60]. Microalgae based systems for wastewater treatment have been gaining remarkable attention

due to their potential advantages (the cost effective in situ oxygenation via photosynthesis) over the common activated sludge process [60]. In addition, swine wastewater is rich of nutrients and constitutes an excellent medium to grow microalgae, remove the nutrients, produce oxygen to attain a better quality effluent and yield valuable co-products such as bio-fuels and some pharmaceutical products such as omega-3 [61,62].

1.7.1 Related Works

Treatment of swine and dairy manure effluents was investigated using freshwater algae, *Rhizoclonium sp.* Fatty acid contents as well as their compositions from algal biomass were tested at various manure loading rates (0.2 to 1.3 g TN m^{-2} day^{-1} and 0.3 to 2.3 g TN m^{-2} day^{-1} for raw swine and dairy manure effluents, respectively). This study showed fatty acid contents of 0.6 to 1.5% of dry weight biomass and no consistent relationship to type of manure and loading rate. A consistent fatty acid composition between samples including 14:0, 16:0, 16:1 (ω7), 16:1 (ω9), 18:0, 18:1 (ω9), 18:2 (ω6), and 18:3 (ω3) was resulted. The fatty acid 16:0 from algae grown in swine waste was 43% of total fatty acids which was not changed in various manure loading rates [63].

The effect of light intensity and nitrogen deficiency on growth and chemical composition of *Spirulina sp.* in a culture containing seawater supplemented with digested pig waste was investigated by Olguin et al. (2001). Biomass concentration obtained from seawater and digested pig waste was similar to a chemically defined medium (Zarrouk medium). However, protein content of the biomass in the medium with pig waste was significantly lower than Zarrouk medium. According to their results, higher total lipid from the biomass grown in pig waste culture (28.6% and 18.0% at 66 and 144 μmol photon m^{-2} s^{-1} light intensity, respectively) was

obtained compared to chemically defined medium (8.0% and 6.4%, respectively). Nitrogen deficiency on growth of Spirulina cultivated in the seawater supplemented with digested pig waste resulted higher lipids and higher polysaccharides [64].

Another study investigated an algal-bacterial system to treatment of livestock effluents in which the microalgae produce O_2 by photosynthesis and aerobic bacteria utilize oxygen to oxidize ammonia and mineralize organic pollutants. Sequentially, CO_2 released by bacteria in this process was consumed by the microalgae and mitigates carbon dioxide emission [17]. Microalgae can utilize nutrients in the wastewater for their growth, such as, nitrogen for formation of protein and phosphorous for synthesis of nucleic acid and phospholipids. A higher nutrient uptake was obtained with CO_2 released via bacteria and it can improve nutrient removals. In addition, pH can enhance ammonium and phosphorus removals because photosynthesis process triggers a rise in pH which causes phosphorous precipitation and ammonia stripping in open systems. This research reported the limitations and potential of photosynthetic oxidation to remove nitrogen and carbon from swine wastewater using a SS activated sludge and a microalga *Chlorella sorokiniana*. Both system, activated sludge and algal-bacterial system was shown similar performance in the dilutions of four and eight times. However, the biodegradation process was inhibited severely in two times diluted and undiluted waste. The main reason for this inhibition was identified high NH_4^+ concentrations and high pH levels [65]. The algal-bacterial culture was grown in a tubular biofilm photo-bioreactor. The diluted swine wastewater with characteristics of 180, 15 and 2,000 mg/L ammonia, phosphate and COD, respectively, fed to the reactor. The photo-bioreactor showed up to 99%, 86% and 75% ammonia, phosphate and COD removal efficiencies [60]. Since most algae such as *Chlorella sorokiniana*, *Scenedesmus obliquus* used in photo synthetically oxygenated systems

showed very poor settling characteristics; biomass harvesting costs may be a problem in these systems. These limitations may be overcome by biomass immobilization and producing an effluent including highly settleable flocks [66]. Carrageenan, chitosan and alginate (biopolymers) were tested successfully to immobilize microalgae. However, there are several limitations such as their high expenses for long term operation and large scale applications [67]. Therefore, Gonzales et al. utilized the biofilm based photo-bioreactor to improve algal sedimentations and N, P and C removal efficiency [60].

The ability of three species (two *chlorophyceae* and one *cyanobacterium*) was tested for anaerobically treatment of swine manure effluents and exhaustion of inorganic nitrogen and orthophosphate. The dilutions of 0.6-3.0% on the digested manure corresponded to the $N\text{-}NH_4^+$ concentrations of 19.8-98.8 mg/L were investigated in this study. The average biomass productivities were 31, 37 and 53 mg dry wt/l for *P. bohneri*, *Chlorella sp.* and *S. obliquus*, respectively. Total $N\text{-}NH_4^+$ exhaustion, 90% $P\text{-}PO_4^{3-}$ reduction and 60-90% COD abatement were obtained for all three species at 20°C and dilution of 2.0% manure. From the results of this study, the best candidate between these three species was *Chlorella* because of its best nitrogen and phosphorous removal at the dilution of 3.0% [68].

Bio-treatment of anaerobically digested swine waste via *Phormidium sp.* was evaluated by Canizares et al.. Growth of *Phormidium* in addition to removal of total phosphorus, orthophosphate, nitrates and ammonium were investigated in dilutions of 10-50% and the results compared to Dauta's medium as a control. Complete orthophosphate removal, 87% nitrate, 68% total phosphate and 48% ammonium were obtained at 25% dilution in 2 liter graduated cylinder. However, orthophosphate, nitrate, total phosphorus and ammonium removals were 48%, 30%,

63% and 100%, respectively, in a carrousel type reactor. The results were better in both cases compared to Dauta's medium [61].

Growth of *Spirulina maxima* for tertiary treatment of aeration-stabilized swine waste was investigated by Canizares in 1993. A 50% dilution of the waste showed the best nutrient removal as well as biomass production. Protein content, lipids and crude fiber were 36%, 6% and 0.02%, respectively. The biomass production of *Spirulina* in the swine waste was better compared to mineral medium (Zarrouk's) [69]. Higher nutrient removal from aeration stabilized swine waste was obtained using immobilized *Spirulina maxima* compared with suspended cells [70].

Gantar et al. investigated the ability of *Scenedesmus quadricauda* (green alga) and *Spirulina platensis* (blue-green alga) in pig wastewater with dilutions of 10%, 20%, 30% and 50% (the wastewater included 4745 mg/L total nitrogen, 4650 mg/L NH_3-N and 17.0 mg/L P-PO_4). It is difficult to control and maintain the microalgae growth in the medium with high percentage of swine waste [71].

Kim et al resulted enhanced production of a green microalga *Scenedesmus spp.* in a medium containing 3% (v/v) fermented swine waste. A 3-fold growth rate, 2.6-fold dry weight and 2.7-fold amino acid levels were achieved after 31 days compared to control medium. However, total lipids in the microalgal culture grown in the medium contained swine waste were found to be much less than those in the control medium (0.9% and 5%, respectively). In addition, the individual fatty acid levels were lower in cells in swine medium than control media [72]. Generally, environmental stress conditions lead to more accumulations of lipid and fatty acid concentrations in cells compared to cells grown under favorable conditions. Thus, control medium shown to be less favorable to grow *Scenedesmus spp.* cells compared to the medium

contained swine waste. Healthy *Scenedesmus* cells grown under favorable growth conditions showed to produce higher ratio of C16:0 and C18:1 [73].

Hu et al. investigated growth rate of green microalgae *Chlorella sp.* and nutrient removal from digested swine waste. High protein and lipid contents of 58.8% and 26.1% of dry weight, respectively, were achieved from algal biomass. The lipid productivity of *Chlorella sp.* was 3.65 g/m2.d (1.00 × 104 L/ha.y) which is much lower than the reported algal oil yield from the artificial media (5.87 × 104 - 1.37 × 105 L/ha.y). However, it is greater than oil production from soybean (598.6 L/ha.y). Therefore, swine waste media exhibits a low-cost feedstock for biofuel production. The main fatty acids from *Chlorella sp.* were saturated C14-C18 and mono-saturated C16-C18 fatty acids (62.8% and 31.8% of total fatty acids weight, respectively). C18:0 and C18:1 were the major fatty acids with amounts of 22.5% and 19.0%, respectively. Research found that the biodiesel from *Chlorella sp.* contained 18 carbon methyl esters. In addition, C18:3 or linoleic acid which should be less than 12% in high quality biodiesel, was not detected in fatty acids obtained from *Chlorella sp.* in Hu et al. results. After lipid extraction from *Chlorella sp.* cells, the remaining biomass can be utilized as a high protein feed for animals instead of conventional protein sources. Therefore, results indicated that the algal biomass grown in digested swine waste could be used as ideal feedstock for both livestock feed and biofuel productions. [74].

Study objectives

Aim 1

A promising source of omega-3 is microalgae, which has become increasingly attractive for the commercial production of these fatty acids [33]. Several studies have screened different microalgal strains to identify those with high EPA yields [33]. Microalgae such as *Chlorella minutissima* [75], *Porphyridium cruentum* [42], *Ocharomonas danica* [76], *Phaeodactylum tricornutum* [77], and the freshwater alga *Monodus subterraneus* [43] were suggested as potential sources. *Porphyridium cruentum* has demonstrated high amounts of long-chain polyunsaturated fatty acids (PUFA) and EPA without any production of DHA. The individual production of EPA without DHA has been considered because of the different pharmacological and nutritional importance [33]. On the other hand, production of ARA during EPA production is detrimental because high concentrations of ARA can reduce EPA production and make recovery difficult [77]. Therefore, it is critical to have minimum ARA and maximum EPA, which can be controlled by growth condition optimization [78,42,20].

The implementation of an algae cultivation system requires considering several criteria such as the water supply/demand, salinity and chemistry, environmental conditions such as temperature and light and, nutrients and carbon supplementations [79]. There have been many studies which analyzed microalgae growth, lipid yield and omega-3 production from microalgae lipids. However, to date few studies have focused on collecting data from *Porphyridium cruentum*, at many different culture conditions but a same system. None of previous studies investigated all environmental and nutrient parameters investigated in this study (temperature,

light intensity, nitrate concentration and CO_2 supplementation) in one single photobioreactor. This can lead to various results from one system to another.

Previous studies investigated the effect of temperature in the range of 20-32°C. They showed that the optimum temperature for optimal yields of EPA from *P. cruentum* should not exceed 25 °C and a temperature of 30 °C resulted a low concentration of EPA and high concentrations of ARA and other fatty acids [24,80,42,47]. It has been reported that formation and accumulation of PUFAs in *P. cruentum* cells decreased at lower light intensity and higher temperatures [47]. Another research showed the effect of light intensity (4-12 klux corresponded to about 54-160 $\mu E/M^2.S$ for fluorescent lamp) on *P. cruentum* growth. However, we have investigated the effect of lower temperature range (16°C and 20°C) and higher illumination rate (up to 180 $\mu E/M^2.S$) in our research compared to previous studies.

Another main factor affecting biomass and lipid productivity is culture medium composition such as nitrogen and phosphorous concentration. Several attempts have been carried out to establish the nutritional requirements of *P. cruentum* and to optimize the media formulation. A lack of nitrogen in the culture medium can lead to the accumulation of lipids such as triacylglycerol (TAG) in microalgae cells [57]. A study showed that nitrogen starvation generally resulted in a sharp reduction of content and proportion of PUFAs and also leads to the accumulation of saturated and monounsaturated fatty acids in algae cells [58]. In addition, nitrogen starvation in *P. cruentum* caused increased amounts of TAG and ARA and consequently increased the ARA/EPA ratio [42]. Nitrogen deprivation usually inhibits biomass growth. However, lipid-synthesizing enzymes are less susceptible to disorganization than carbohydrate-synthesizing enzymes in a nitrogen-limited situation, thus the lipid count increases. Subsequently, most of the carbon will be bound in the lipids [40]. Therefore, there should be a

lipid productivity peak related to nitrogen concentrations at a certain nitrogen source. Most studies investigated the effect of nitrogen starvation on algal growth. However, a range of nitrogen concentration (0.075- 0.7 g/L) in the culture media was examined in this study to investigate the effect of nitrogen level on algal growth and EPA concentration.

The objectives of this study were to determine the optimal growth condition for *Porphyridium cruentum* in order to achieve optimum fatty acid content, biomass content, and EPA concentration over other fatty acids, particularly ARA. Since a low ratio of ARA to EPA decreases the cost of extraction and purification and also increases the process yield coefficient, this ratio was analyzed under different growth conditions. The parameters investigated were temperature, light intensity and nitrate concentration as well as CO_2 supplementation. The desired outcome was to optimize the maximum productivity of EPA rather than other fatty acid content.

Aim 2

Porphyridium cruentum can grow in saltwater, thus, the need for freshwater inputs decreases. In addition, wastewater can be used as a potentially viable growth medium for microalgal feedstock due to its high concentrations of nutrients such as nitrogen and phosphorous [22]. Since the 1960s, wastewater treatment systems using algae have been gaining attention due to their potential advantages over the common activated sludge process which requires costly sludge processing and high energy inputs for aeration [81]. It has been shown that swine waste media exhibits a low-cost feedstock for biodiesel production and other valuable co-products such as EPA [74,82].

The freshwater algae *Rhizoclonium sp.* was grown in swine wastewater at various manure loading rates (0.2 to 1.3 g TN m^{-2} day^{-1}) and a consistent fatty acid composition with a range of 0.6 to 1.5% of dry weight biomass resulted from this study [63]. Another study on the growth of *Spirulina sp.* in a culture containing seawater supplemented with digested swine waste showed a higher total lipid count than the biomass grown in swine waste culture (28.6%) compared to a chemically defined medium (8.0%). In addition, higher lipids were obtained under nitrogen deficiency condition [64]. Growth of *Spirulina maxima* in a 50% dilution of swine waste exhibited the best nutrient removal as well as biomass production with a 6% lipid content [69]. A three-fold growth rate and 2.6-fold dry weight increase were achieved for growth of a green microalga, *Scenedesmus spp.,* in a medium containing 3% (v/v) fermented swine waste. In another study, high protein and lipid contents of 58.8% and 26.1% of dry weight were obtained from growth of green microalgae *Chlorella sp.* in digested swine waste [74]. Since *P. cruentum* is a salt water alga, the concentration of salt in the culture medium is another factor affecting microalgal growth. The best concentration of sodium chloride to grow *P. cruentum* was shown to be between 0.45 and 0.8 M. In addition, the amounts of PUFAs increased with increasing NaCl concentrations from 0.8 to 1.5 M [83]. It can be seen that the literature mainly focused on nutrient removal from swine waste using microalgae, and a few have discussed the microalgae growth profile or lipid yield. There is not any research which investigates the lipid content and fatty acid profile of *Porphyridium cruentum* as a good source of biofuel and omega-3 or EPA grown in swine wastewater. In addition, the effect of salt concentration on EPA profile from *P. cruentum* needs to be considered.

The main objective of this part of study is to investigate the growth and lipid productivity of *Porphyridium cruentum* in ultra-filtered swine waste medium at 1) different nitrogen levels (0.075-0.7 g/L), and 2) different salinities (0-3.2%) in order to attain maximum fatty acid content, biomass content, and EPA level over other fatty acids, particularly ARA. The results were compared to *P. cruentum* grown in the L1-medium as a control medium at the same environmental and nutrient conditions.

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2. EICOSAPENTAENOIC ACID FROM PORPHYRIDIUM CRUENTUM: INCREASING GROWTH AND PRODUCTIVITY OF MICROALGAE FOR PHARMACEUTICAL PRODUCTS

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2.1 Abstract

An alternative source of eicosapentaenoic acid (EPA) or omega-3 could be microalgae lipids instead of fish oils. However, EPA and lipid contents extracted from microalgae vary at different growth conditions. Therefore, it is of paramount importance to optimize the growth conditions of microalgae to maximize EPA production. In this paper, the effects of temperature (16 °C and 20 °C), light intensity (140 μE m^{-2} s^{-1} and 180 μE m^{-2} s^{-1}) and nitrate level (0.075, 0.3, 0.5, and 0.7 g/L) on the cell growth, lipid productivity, and omega-6/omega-3 ratio of *Porphyridium cruentum*, one of the most promising oil-rich species of microalgae, are investigated. The ratio of the fatty acids with omega-6 and omega-3 groups at various growth conditions were compared, since an appropriate proportion of ω-6 (arachidonic acid (ARA)) to ω-3 (EPA) is vital for healthy nutrition. Lower EPA production and consequently a higher ARA/EPA ratio occurred when 5% CO_2/air was utilized as CO_2 supplementation compared to pure CO_2. The highest EPA (13.08% (w/w) of total fatty acids) and biomass productivity (143 mg L^{-1} day^{-1}) was achieved at 140 μE m^{-2} s^{-1}, 20 °C, and 0.3 g/L nitrate, while lipid content was the lowest (0.5% w/w) at this condition. The optimal condition with minimum ARA/EPA ratio (2.5) was identified at 20 °C, 140 μE m^{-2} s^{-1}, and 0.5 g/L nitrate concentration.

Keywords: eicosapentaenoic acid (EPA); fatty acids; lipids; microalgae; photo bioreactor; *Porphyridium cruentum*

2.2 Introduction

The natural products of microalgae are becoming increasingly important within the scientific community for their different applications, such as in pharmaceuticals [1] and biofuel production [2]. The advantages of microalgae are significant and include: more oil yield per area of microalgae compared to the foremost oilseed crops, less water required for growth, the possibility of cultivation in seawater or non-arable lands [3], production of valuable co-products such as proteins and residual biomass of oil extraction [1], and pharmaceutical products such as omega-3 fatty acids (ω-3). It has also been indicated that long-chain N-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), play important roles in the treatment of many diseases such as cancer, atherosclerosis, rheumatoid arthritis, Alzheimer's, and psoriasis [4,5].

EPA or ω-3 is a polyunsaturated fatty acid (PUFA) containing 20 carbons in its chain and five *cis* double bonds. One of the major nutritional requirements in our diet, adequate omega-3 fatty acid intake improves infant cognitive development and visual acuity [6]. Recent medical studies recommend that both preterm and at term infants need to receive an appropriate ratio of ω-3/ω-6. Thus, it is becoming common practice in many countries to include them in baby formula [7]. EPA plays a main role in altering membrane fluidity, as the precursor to different biologically active regulators such as hormones, and in reducing plasma triacylglycerol and cholesterol levels [8]. Also, it has been demonstrated that EPA can reduce the risk of some

diseases such as diabetes [9], brain disorders [10], arteriosclerosis, coronary heart disease, inflammation, and several carcinomas [11]. Unbalanced intake of omega-6 to omega-3, mainly the insufficient uptake of omega-3, results in increased cardiovascular diseases, risk of cardiac death, and mental illnesses. A diet with a ratio of 1:1 is ideal, while ratios higher than 10:1 are not recommended [7].

Mammals are unable to synthesize EPA due to the absence of the essential enzymes that place a double bond at the ω-3 position [12]. Currently, the richest source of EPA (up to 15% of total fatty acids) is fish oils [13,14]. However, fish oil drawbacks include peculiar taste, contamination, odor, availability, quality, oil stability, and high costs of purification and extraction [5]. Therefore, the search for an alternative source has been encouraged [8]. A promising source of EPA and DHA is microalgae, which has become increasingly attractive for the commercial production of these fatty acids [5].

The first parameter to successful algal biotechnology is selecting the right microalgae species with appropriate characteristics, for example, fatty acid content and biomass productivity [15]. Therefore, primary strain selection is the most important factor in achieving maximal desired fatty acid compositions and yields. Several studies have screened different microalgal strains to identify those with high EPA yields [5]. Microalgae such as *Chlorella minutissima* [16], *Porphyridium cruentum* [17], *Ocharomonas danica* [18], *Phaeodactylum tricornutum* [19], and the freshwater alga *Monodus subterraneus* [20] were suggested as potential sources. *Porphyridium cruentum* has demonstrated high amounts of long-chain polyunsaturated fatty acids (PUFA) and EPA. In addition, the individual production of EPA without docosahexaenoic acid (DHA) has been considered because of the different pharmacological and nutritional importance. Therefore, it is important to select an alga strain with less DHA than EPA potential.

P. cruentum is one of the best strains for EPA production, with yields exceeding 19.7 mg/L of EPA, without any production of DHA [5].

The contents of EPA and other fatty acids of microalgae can be changed under different environmental conditions, including light intensity, temperature, and nutrient composition [21]. Production of arachidonic acid (ARA, C20:4 n-6) during EPA production is detrimental because high concentrations of ARA can reduce EPA production and make recovery difficult [19,22]. Therefore, it is critical to have minimum ARA and maximum EPA, which can be controlled by growth condition optimization [13,17,23]. Total fatty acid and EPA contents for unicellular algae have been reported to decline with increasing temperature [24]. However, it has been shown that the lipid contents of *P. tricornutum* cells increase at higher temperature [20]. Other research has shown that the optimum temperature for optimal yields of EPA from *P. cruentum* should not exceed 25 °C with a temperature of 30 °C resulting in a low concentration of EPA and high concentrations of ARA and other fatty acids [25].

Phototropic algae absorbs sunlight, thus artificial lighting is a critical factor in the commercial production of algae [26]. Low light intensity has been reported to increase accumulation and production of PUFAs of some microalgae cells such as *Cyclotella menaghiniana*, *Nitzschia closterium*, and *Euglena gracilis* [27–29]. However, the light intensity effect was reversed for the red alga *P. cruentum* [30]. It has been reported that light intensities lower than 30 W/m^2 inhibited the growth rate of *Botryococcus braunii*, while photo-inhibition and lower lipid amounts occurred at light intensities above 100 W/m^2. This occurs due to the low biomass and fewer hydrocarbons [31].

Another main factor affecting biomass and lipid productivity is culture medium composition. Nitrogen deprivation usually inhibits biomass growth. However, lipid-synthesizing

enzymes are less susceptible to disorganization than carbohydrate-synthesizing enzymes in a nitrogen-limited situation, thus the lipid count increases. Subsequently, most of the carbon will be bound in the lipids [32]. Therefore, there should be a lipid productivity peak related to nitrogen concentrations at a certain nitrogen source. Miyamoto illustrated that lipid content from *Nannochlolis* decreased about 22% with increasing nitrate concentration from 0.9 mMol/L to 9.9 mMol/L, while lipid yield as well as biomass productivity increased [33]. Several attempts have been carried out to establish the nutritional requirements of *P. cruentum* and to optimize the media formulation. Roessler [34] studied the nitrogen starvation effects on *P. cruentum* growth in 1990. They found that a lack of nitrogen in the culture medium can lead to the accumulation of lipids such as triacylglycerol (TAG) in microalgae cells. Cohen [35] illustrated that starvation generally resulted in a sharp reduction of content and proportion of PUFAs and also leads to the accumulation of saturated and monounsaturated fatty acids in algae cells in 1986. Cohen [17] also showed in 1990 that nitrogen starvation in *P. cruentum* caused increased amounts of TAG and ARA and consequently increased the ARA/EPA ratio.

The purpose of this project was to determine the optimal growth conditions for *Porphyridium cruentum* in order to achieve maximum fatty acid content, biomass content, and EPA concentration over other fatty acids, particularly ARA. The parameters investigated were temperature, light intensity, and nitrate concentration as well as CO_2 supplementation. The desired outcome was to optimize the maximum productivity of EPA rather than other fatty acid content. A high content of EPA decreases the cost of extraction and purification and also increases the process yield coefficient [35].

2.3 Experimental

2.3.1 Strains and Growth Conditions

The marine microalgae *Porphyridium cruentum,* CCMP1328 isolated by the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, East Boothbay, ME, USA), was used. The cells were red and spherical with a length of 5–8 µm. *P. cruentum* cultures were maintained in a modified L1 medium containing $NaNO_3$, $NaH_2PO_4{\cdot}H_2O$, $Na_2EDTA.2H_2O$, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2.6H_2O$, $CuSO_4{\cdot}5H_2O$, $Na_2MoO_4{\cdot}2H_2O$, H_2SeO_3, $NiSO_4.6H_2O$, Na_3VO_4, K_2CrO_4, Thiamine-HCl (Vit. B_1), Biotin (Vit. H), Cyanocobalamin (Vit. B_{12}), and filtered seawater [36]. The media were modified by omitting Si ($Na_2SiO_3{\cdot}9\ H_2O$) from the original recipe. The medium was sterilized by autoclaving at 127 °C for 30 min.

P. cruentum was pre-cultured at 22 °C with natural illumination in a 500-mL glass bottle containing 250 mL of medium. Afterwards, pre-cultured algae in the early exponential growth phase were inoculated at fixed cell numbers of around 5000 cells per mL. Growth experiments were carried out axenically for approximately 30 days at two different temperatures (20 °C and 16 °C) and illuminations (140 µE m^{-2} s^{-1} and 180 µE m^{-2} s^{-1}) for 13/11 hours light/dark cycle. In addition, after optimizing light and temperature, four nitrate concentrations in the growth media including 0.075 (original recipe of L1-medium), 0.3, 0.5, and 0.7 g$NaNO_3$/L media were investigated in the best light intensity and temperature to see the effect of nitrogen levels. pH values were monitored daily during the experiments and the required pH was achieved by bubbling (approximately 5 psi) pure CO_2 through a small hole at the bottom of the column. CO_2 was bubbled periodically every couple of days for several seconds to reach the targeted pH. In

addition, one extra experiment was performed with a ratio of 5% CO_2 in the air to adjust the pH to investigate the effect of air in growth and lipid composition.

The algal cells were harvested in their stationary growth phase. Harvesting was performed by centrifugation at 5000 rpm for 15 min. The harvested biomass cake with about 80% moisture was washed with distillated water to remove non-biological material such as mineral salt precipitates. The pellets were lyophilized and then grounded to powder prior to fatty acid extraction. Figure 1 depicts the experimental bioreactor used for this research. The photo bioreactor (ePBR v1.1 was purchased from Phenometrics (Phenometrics, Inc. Alliance Drive, Lansing, MI, USA) and the experimental parameters (such as temperature and light intensity) were controlled simultaneously by a single computer running Algal Command, Phenometrics' software.

Cell numbers were counted using a microscope with a bright-light hemacytometer. Data were statistically analyzed to obtain the standard deviation. Calculations of growth rates (μ) were done using Equation (1):

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t - t_0} \tag{1}$$

where N_t and N_0 are the cell density at time t and t_0, respectively [23]. Moreover, the biomass productivity was presented by Equation (2):

$$\text{Biomass productivity}\,(\text{mg/Lday}) = \frac{\text{Dried microalgae biomass (mg)}}{\text{Culture time (days)} \times \text{working volume (L)}} \tag{2}$$

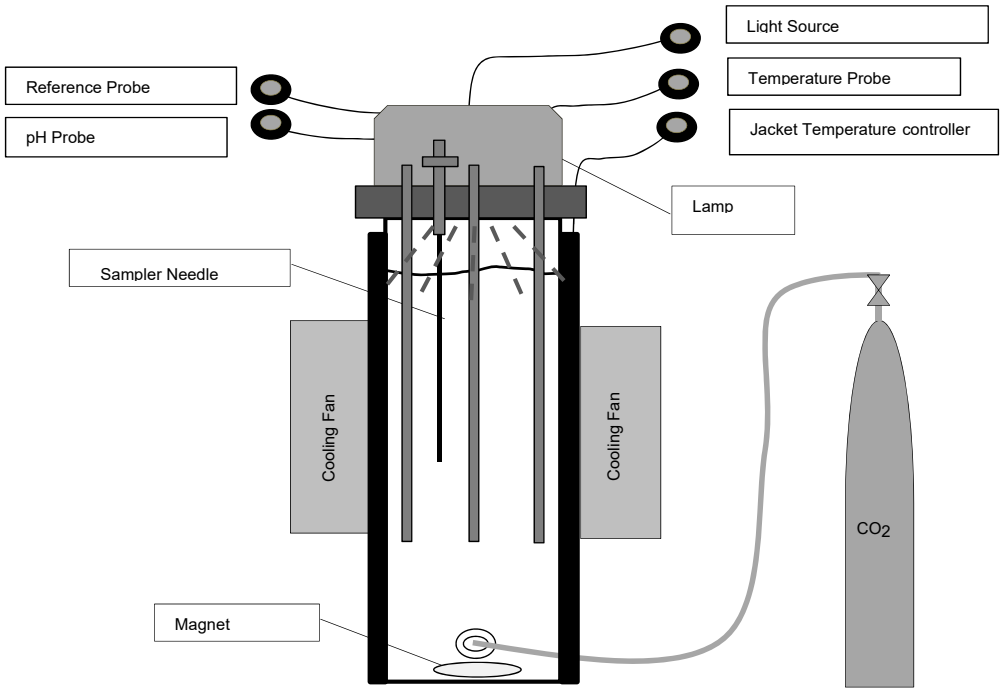

Figure 2.1 Schematic representation of the *Porphyridium cruentum* photo bioreactor.

2.3.2 Lipid Extraction

Lipids were extracted using a modified procedure of Bligh and Dyer [37]. The dried algae were homogenized in a chloroform/methanol/water solution with a volume ratio of 1:2:0.8, respectively.

Next, an equal volume of water and chloroform was added to the mixture to reach a final ratio of 2:2:1.8. This mixture was left at room temperature overnight. Afterwards, the mixture was filtered to remove the algae pellets and then allowed to resettle to obtain a two-phase mixture. The lower phase (chloroform layer) containing the lipids was isolated from the aqueous phase. The remaining aqueous phase and also the residues that remained on the filter were re-extracted with chloroform/methanol with a volume ratio of 2:1. The chloroform layers obtained from these two steps were combined together and dried to obtain the lipid content. The lipid contents were

obtained by subtracting the weight of the empty flask from the flask containing lipids. The percentage of the lipid content was determined by Equation (3) [38]:

$$\text{Lipid content } \% = \frac{\text{Weight of lipid (mg)}}{\text{Weight of dried microalgae biomass (mg)}} \times 100 \quad (3)$$

The lipid productivities were calculated using Equation (4) [39]:

$$\text{Lipid productivity } (\text{mg/Lday}) = \frac{\text{Lipid content } (\text{mg/mg}) \times \text{Dried biomass content } (\text{mg/L})}{\text{Cultivation period (days)}} \quad (4)$$

2.3.3 Lipid Transmethylations

Analysis of fatty acids through gas chromatography (GC) required extreme temperatures above the upper temperature limit of the stationary phase. For this reason, the free fatty acids were derivatized to methyl esters prior to GC analysis. First, every 10 mg of lipid sample was mixed with 1 mL of toluene and 1 mL of 1.5% sulfuric acid in dry methanol. Second, the mixture was incubated at 55 °C overnight. Third, 2 mL of saturated NaCl solution was added and vortexed. Finally, 1 mL of hexane was added followed by 1.5 mL of sodium hydrogen carbonate (2% $NaHCO_3$). The mixture was vortexed and the upper phase was taken for analysis via GC [38].

2.3.4 Gas Chromatography Analysis

The analysis of fatty acid methyl esters (FAME) was performed using a gas chromatograph GC-2014 (Shimadzu, Columbia, MD, USA), equipped with an auto-sampler and a flame ionization detector (FID). A Zebron™ZB-FFAP polar capillary column (30 m × 0.32 mm × 0.25 µm film thickness; Phenomenex, Torrance, CA, USA) was used to separate the FAME. The carrier gas was helium at a linear velocity of 35 cm/s. The column was subjected to a

temperature program for separation as follows: initial column oven temperature of 150 °C held for 3 min, heating at 1.5 °C/min to 240 °C and sustained for 15 min. The injector (FAME samples of 2 µL each and split ratio 10:1) and detector temperature were kept constant at 250 °C during the 78-min analysis. The compounds were identified and quantified by comparing the peaks with Marine Oil Test Mix (Restek Corp., Bellefonte, PA, USA) FAME standards. The provided GC data are based on duplicate measurements.

2.4 Results and Discussion

2.4.1 Light Intensity, Temperature, and CO_2 Supplementation

2.4.1.1 Cell Densities and Growth Rates

The effects of light intensity and temperature as well as CO_2 supplementation on cell densities and growth rates of *P. cruentum* were investigated. Illumination was fixed at two different intensities (140 µE m^{-2} s^{-1} and 180 µE m^{-2} s^{-1}) and a day/night light cycle of 13/11 h. The culture was harvested in the stationary phase after approximately 30 days. Figure 2 shows the effect of light intensity on the cell density of *P. cruentum* during growth at 20 °C.

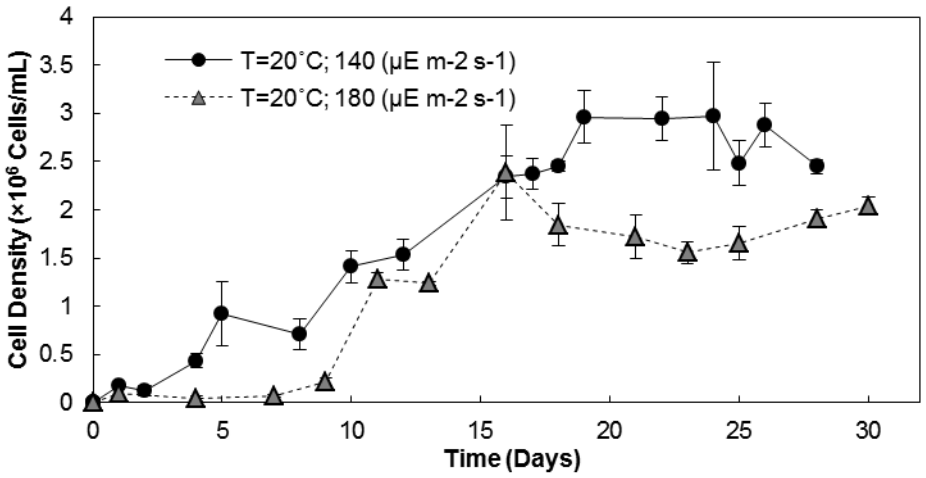

Figure 2.2 Cell density (×10^6 cell/mL) of *P. cruentum* at different light intensities of 140 µE m^{-2} s^{-1} and 180 µE m^{-2} s^{-1}.

The cell density for the light intensity of 140 µE m^{-2} s^{-1} increased during the growth time without any obvious lag phase. The maximum cell density was approximately 3.0 × 10^6 cells per mL on day 19. However, there was a lag time (about 7 days) at the growth in the light intensity of 180 µE m^{-2} s^{-1}. Nevertheless, at this light intensity the growth rate increased rapidly after seven days, reaching maximum cell numbers on day 16 (2.4 × 10^6 cells per mL). In addition, the effect of temperature on the growth at 140 µE m^{-2} s^{-1} is shown in Figure 3. Cell densities were reduced at lower temperatures with longer lag times. The maximum cell density (2.02 × 10^6 cells per mL) occurred on day 20. The results indicate that higher light intensity and lower temperature result in longer lag times and lower cell densities.

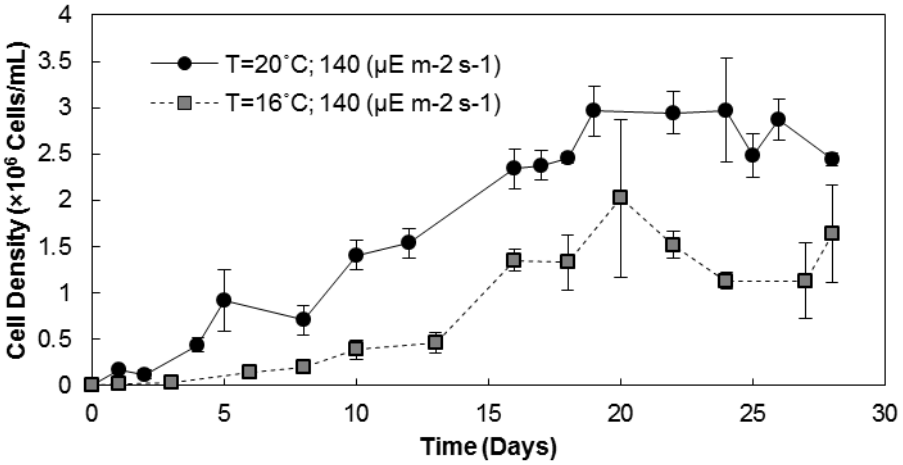

Figure 2.3 Cell density (×10^6 cell/mL) of *P. cruentum* at different growth temperatures of 16 °C and 20 °C.

The effect of culture gassing with carbon dioxide was studied by providing CO_2 and 5% CO_2 in air. Carbon dioxide caused a change in culture pH in addition to other physiological effect on cells [19]. The cell densities are shown in Figure 4. Algal growths were approximately the same for both conditions in the early growth phase but after about 12 days cell densities declined when 5% CO_2/air was used as CO_2 supplementation.

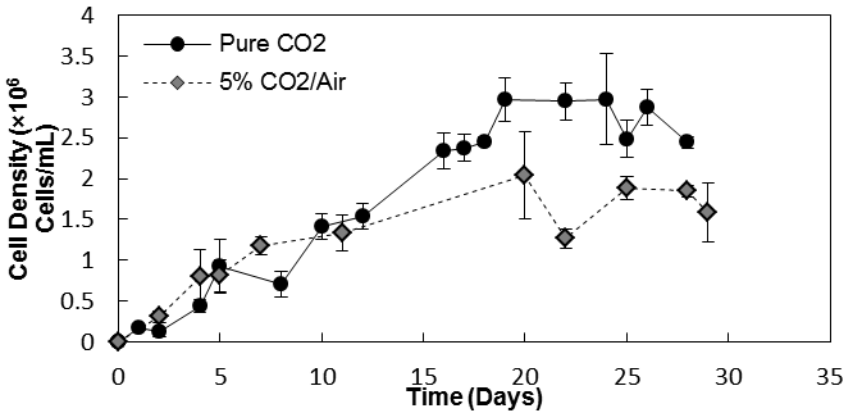

Figure 2.4 Effect of CO_2 supplementation on cell density (×10^6 cell/mL) of *P. cruentum* at optimum temperature (20 °C) and light intensity (140 µE m^{-2} s^{-1}) and nitrogen concentration (0.075 g nitrate/L).

Figure 5 presents the effect of temperature and light intensity as well as CO_2 supplementation on growth rates (μ) of *P. cruentum*. Growth rates were not significantly affected by temperature and CO_2 supplementation. Interestingly we also see very little difference between using pure carbon dioxide and a mixture that is more representative of what would be seen by flue gas. This suggests that this alga is quite amenable to using a waste carbon dioxide source.

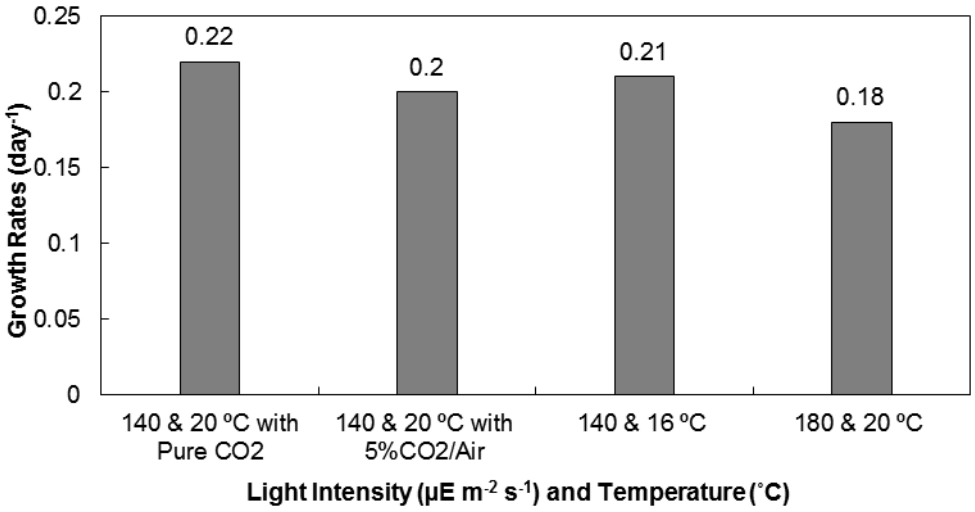

Figure 2.5 The effect of temperature and light intensity as well as CO_2 supplementation on growth rates (μ) of *P. cruentum*.

2.4.1.2 Biomass and Lipid Contents

The effects of light intensity, temperature, and CO_2 supplementation on the biomass productivities, lipid contents and lipid productivities of *P. cruentum* are shown in Figure 6. Increased lipid productivity and lipid content were observed when 5% CO_2 in air was used. However, the biomass productivity was lower compared to pure CO_2 supplementation. As seen in Figure 6, decreased temperature and enhanced light intensity resulted in a significant change in cell composition, favoring the accumulation of lipid components during the batch growth of *P.*

cruentum. In previous studies involving *Porphyridium cruentum* [32], lipid levels were measured between 9% and 14% per dry weight. However, in this study, the lipid levels of the *P. cruentum* cells were substantially lower (0.5%–1.6%). This is possibly due to the difference in strains or culture medium compositions. Lipid contents increased from 0.85% to 0.98% and 1.1% with decreased temperature (from 20 °C to 16 °C) and enhanced light intensity (from 140 $\mu E\ m^{-2}\ s^{-1}$ to 180 $\mu E\ m^{-2}\ s^{-1}$), respectively. In contrast, a decrease in the growth temperature from 20 °C to 16 °C led to a diminution in biomass productivity of *P. cruentum* from 92 mg L^{-1} day^{-1} to 63 mg L^{-1} day^{-1}. Moreover, biomass productivity reduced from 92 mg L^{-1} day^{-1} to 59 mg L^{-1} day^{-1} by increasing light intensity from 140 $\mu E\ m^{-2}\ s^{-1}$ to 180 $\mu E\ m^{-2}\ s^{-1}$. Lipid productivity considers both the biomass produced by microalgae cells and the lipid accumulations in these cells [26]. Comparing the lipid contents and biomass productivity shown in Figure 6, it can be seen that higher biomass productivities did not result in higher lipid contents. This result may be due to the stress conditions that lead to the accumulation of lipids in the cells and also to a reduction in microalgae biomass productivity. Microalgae replace cell production with lipid biosynthesis in cells under unfavorable environmental or stress conditions. The lipid productivities for different light intensities and temperatures are presented in Figure 6. Enhanced lipid productivities occurred at higher temperature (0.62 mg L^{-1} day^{-1} to 0.78 mg L^{-1} day^{-1}, for temperatures from 16 °C to 20 °C) and lower light intensity (0.65 mg L^{-1} day^{-1} to 0.78 mg L^{-1} day^{-1} for illuminations from 180 $\mu E\ m^{-2}\ s^{-1}$ to 140 $\mu E\ m^{-2}\ s^{-1}$). As presented in Figure 6, a similar trend was observed for lipid contents and productivities.

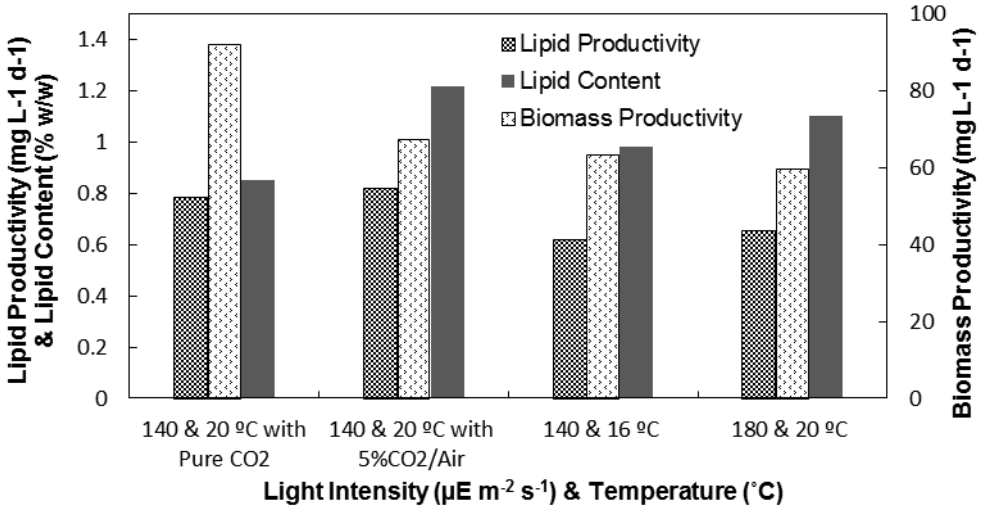

Figure 2.6 Biomass productivity, lipid contents, and lipid productivity of *P. cruentum* at different light intensities and temperatures as well as CO_2 supplementation.

2.4.1.3 Fatty Acid Compositions

The fatty acid compositions were related to growth conditions such as temperature and illumination as well as nutrient levels. As presented in Table 1, the major fatty acids from *P. cruentum* were C16:0 (palmitic acid; 25.0%–30.6%), C18:2 (linoleic acid; 12.4%–27.6%), C20:4 (ARA; 23.4%–30.5%) and C20:5 (EPA; 6.7%–13.1%). In addition, it contained low levels of C14:0 (myristic acid; 0.2%–0.3%), C14:1 (myristoleic acid; 0.1%–0.4%), C16:1 (palmitoleic acid; 0.2%–2.8%), C18:0 (stearic acid; 1.5%–3.5%), C18:1-Cis-9 (oleic acid; 1.3%–4.3%), C18:1-trans (octadecenoic acid; 0.3%–0.8%), C18:3 (linolenic acid; 0.1%–0.4%), C20:2 (eicosadienoic acid; 0.8%–2.5%), and C22:1 (erucic acid; 1.2%), as well as trace amounts of C20:0 (arachidic acid), C20:1 (11-eicoenoic acid), and C20:3 (11-14-17 eicosatrienoic acid). Unknown fatty acids were about 4.6%–7.7%. Additionally, there was no trace of C22:6 (DHA) in the *P. cruentum*. These data are in agreement with Cohen *et al.* [40]. However, Oh *et al.* [41]

exhibited considerable amounts of DHA without any EPA from *P. cruentum* cells grown under different light and dark cycles.

The effects of light intensity and temperature as well as CO_2 supplementation on the percentage of EPA and ARA in total lipid contents are presented in Figure 7. Decreasing temperature (from 20 °C to 16 °C) led to higher percentages of EPA (from 9.5% to 9.9%) and ARA (from 23.9% to 26.2%). In addition, the ratio of ARA to EPA was increased slightly from 2.5 to 2.6 at a lower temperature. An analysis of the data of Cohen *et al.* [40] reveals a similar variation in the EPA value in response to changes in temperature from 30 °C to 25 °C. A further drop in the growth temperature to 20 °C produced a reduction in the proportion of EPA. Their results also showed a decrease in ARA values with reducing the growth temperature and the culture should be maintained at 30 °C for optimal ARA production with a minimum proportion of EPA.

Table 2.1 Fatty acid compositions of *P. cruentum* strain (% of total fatty acids). ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

***T* (°C)**		**20**	**20**	**16**	**20**	**20**	**20**	**20**
Light intensity (μE m^{-2} s^{-1})		**140**	**140**	**140**	**180**	**140**	**140**	**140**
Nitrate concentrations		**0.075**	**0.075 (5% CO_2/Air)**	**0.075**	**0.075**	**0.3**	**0.5**	**0.7**
	C14:0	-	0.2%	-	-	0.2%	0.3%	0.3%
	C16:0	30.5%	25.0%	27.7%	26.6%	29.6%	27.4%	30.6%
	C18:0	2.5%	3.1%	3.0%	3.5%	1.5%	2.3%	2.9%
Saturated	C20:0	-	0.1%	-	-	0.1%	-	-
	C22:0	-	-	-	-	-	-	-
	C24:0	-	-	-	-	-	-	-
	Total	33.0%	28.3%	30.7%	30.1%	31.3%	29.9%	33.7%
	C14:1	-	-	-	-	-	0.1%	0.4%
	C16:1	1.0%	0.2%	1.0%	1.0%	0.2%	2.0%	2.8%
	C18:1 (cis-9)	3.2%	3.9%	3.8%	4.3%	1.3%	2.3%	1.6%
Mono-	C18:1 (trans)	-	0.3%	-	-	0.7%	0.4%	0.8%
unsaturated	C20:1	-	0.2%	-	-	0.1%	0.1%	-
	C22:1	1.2%	-	-	-	-	-	-
	C24:1	-	-	-	-	-	-	-
	Total	5.4%	4.5%	4.8%	5.3%	2.3%	5.0%	5.5%
	C18:2	21.0%	24.6%	22.4%	27.6%	12.8%	19.5%	12.4%
	C18:3	-	0.1%	-	-	0.1%	0.4%	-
	C20:2	0.8%	1.9%	1.1%	1.3%	2.1%	2.5%	1.7%
Poly-	C20:4 (ARA)	23.9%	26.1%	26.2%	23.4%	30.5%	25.1%	29.1%
unsaturated	C20:3	-	0.1%	-	-	0.1%	0.1%	-
	C20:5 (EPA)	9.5%	8.7%	9.9%	6.7%	13.1%	11.5%	13.1%
	C22:6 (DHA)	-	-	-	-	-	-	-
	Total	55.2%	61.3%	59.5%	59.0%	58.8%	59.1%	56.3%
Unknown FAs		6.4%	5.8%	5.1%	5.6%	7.7%	6.1%	4.6%

Cohen [17] also illustrated that the range of ARA/EPA values in the exponential phase at 25 °C was 0.33–0.63. However, this ratio increased to 0.86–2.1 at the stationary phase as ARA increased while the EPA levels decreased drastically. According to their results, *P. cruentum* contained more than 39% of total fatty acids as ARA and only 6.6%−14.2% as EPA with

ARA/EPA values of 1.7–6.1 when exponentially cultivated under the same light intensity at 30 °C. The ARA/EPA values at the stationary phase were higher, 2.3–11.5. Akimoto *et al.* [42] showed an increase in EPA and decrease in ARA and consequently reduced the ARA to EPA ratio by decreasing the temperature from 32 °C to 20 °C. We suggest that this disagreement could possibly stem from the different culture medium and other factors such as environmental and nutrient conditions that govern the fatty acid composition of the alga. In addition, none of these studies investigated a temperature below 20 °C.

Light intensity affects the fatty acid composition, especially the proportion of ARA and EPA and the ratio of ARA to EPA. The proportion of EPA increased considerably from 6.7% to 9.5% when the light intensity was reduced from 180 μE m^{-2} s^{-1} to 140 μE m^{-2} s^{-1}, while the ARA amounts slightly increased from 23.4% to 23.9% with decreased light intensity. Consequently, ARA to EPA ratio showed a reduced trend with reduction in light intensity. These results are in agreement with Akimoto *et al.* [42], who reported an enhanced ARA and EPA contents from *P. cruentum* by decreasing light intensity at a growth temperature of 30 °C.

ARA was higher when 5% CO_2/Air was used compared to pure CO_2, as shown in Figure 7. In addition, 5% CO_2/Air caused an 8.5% reduction in EPA amounts. ARA contents are higher than EPA for all four conditions. As reported in previous studies [12], lower ratios of ARA to EPA lead to easier separations of ARA from EPA. Furthermore, since EPA with a certain level of ARA is required for optimal healthy nutrition, ARA to EPA ratio is a prominent parameter. The lowest and highest amounts of this ratio (2.5 and 3.4) occurred on 140 μE m^{-2} s^{-1}, 20 °C (with pure CO_2) and 180 μE m^{-2} s^{-1}, 20 °C, respectively. Since the goal is to achieve the lowest ARA/EPA or omega-6 to omega-3 ratio, 20 °C and 140 μE m^{-2} s^{-1} (with pure CO_2) are the optimum environmental growth conditions for *P. cruentum*.

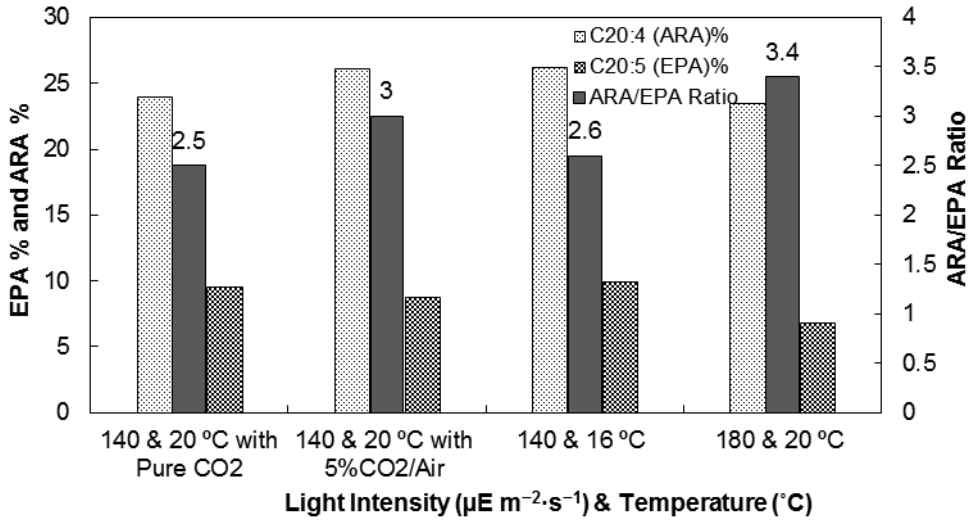

Figure 2.7 Effect of illumination and temperature on the EPA and ARA contents of *P. cruentum*.

2.4.2 Nitrate Concentration

2.4.2.1 Cell Densities and Growth Rates

The effect of nitrogen concentration on the cell growth and fatty acid compositions of *P. cruentum*, in modified L1-media containing 0.075, 0.3, 0.5, or 0.7 g/L sodium nitrate, was tested. The cell growth profile of *P. cruentum* obtained with different nitrate concentrations during growth time are shown in Figure 8. The cell density of *P. cruentum* reached the maximum amounts (0.45×10^6 cells per mL and 1.3×10^6 cells per mL) on days 14 and 23 in the medium containing 0.5 g/L and 0.7 g/L nitrate, respectively. The cell density improved significantly when the sodium nitrate concentration decreased to 0.3 g/L and 0.075 g/L (maximum values of approximately 3.0×10^6 cells per mL on day 30 and 19, respectively). In addition, smaller lag times were observed with reduced nitrogen concentrations.

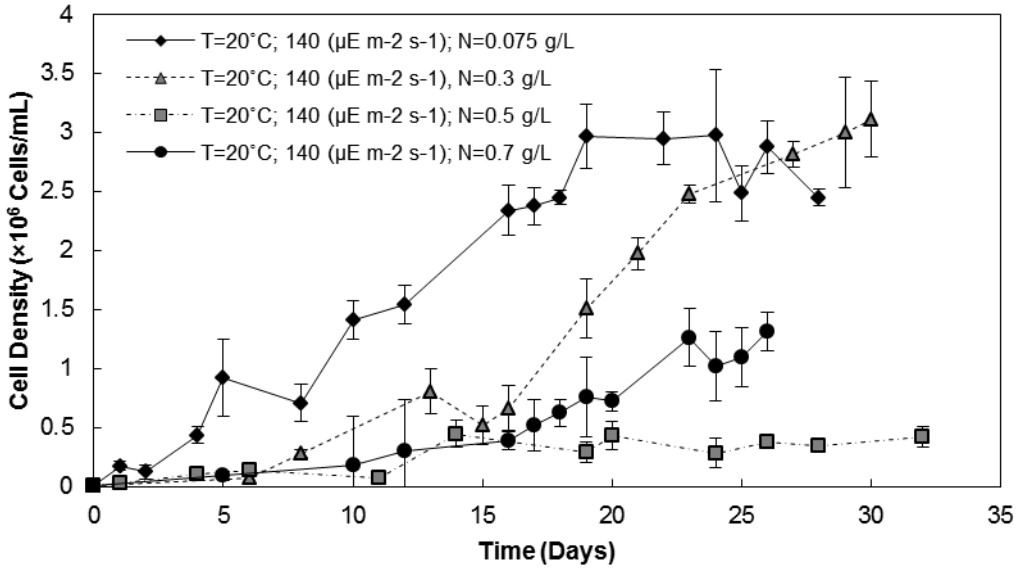

Figure 2.8 Cell density (×10^6 cell/mL) of *P. cruentum* at optimum temperature (20 °C) and light intensity (140 µE m^{-2} s^{-1}) with different growth nutrient levels (0.075, 0.3, 0.5, or 0.7 g $NaNO_3$/L).

The effect of increased nitrate concentration on growth rates (µ) of *P. cruentum* is shown in Figure 9. Growth rate amounts were calculated by Equation (1). The microalga grew most robustly in the medium containing 0.075 g/L nitrate, reaching a specific growth rate of 0.22 day^{-1}. Conversely, in the medium with 0.5 g/L nitrate, the specific growth rate was the lowest at 0.14 day^{-1}. Growth rates decreased by 14% and then 26% with an increase in nitrate concentration from 0.075 g/L to 0.3 g/L and 0.5 g/L, respectively. In addition, a higher growth rate (0.17 day^{-1}) was observed for the medium containing 0.7 g/L nitrate.

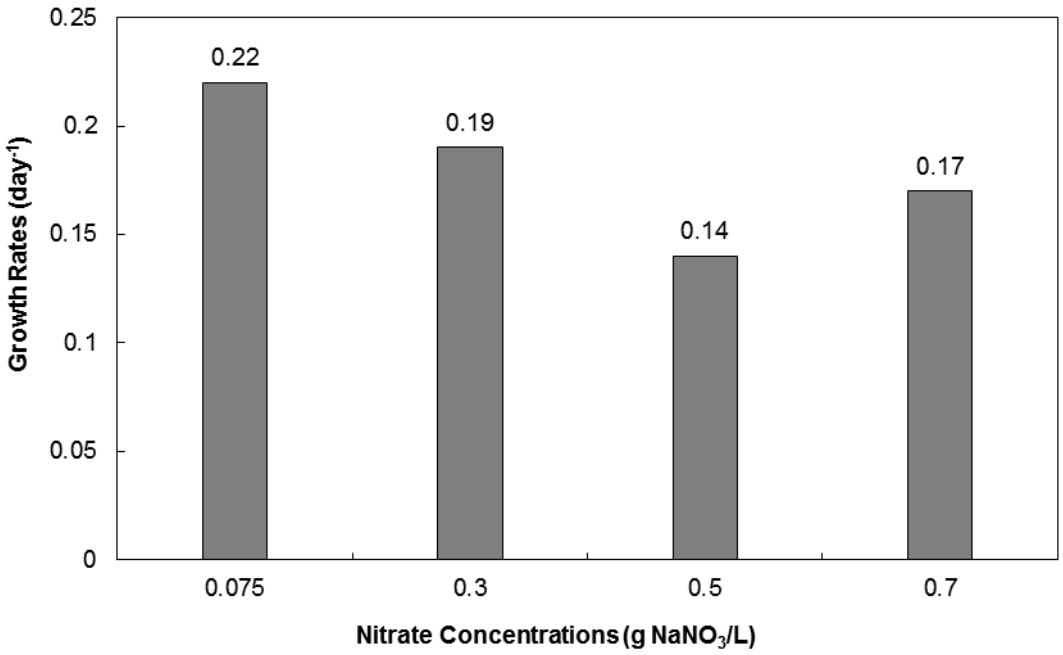

Figure 2.9 The effect of nitrate concentration on growth rates (μ) of *P. cruentum*.

2.4.2.2 Biomass and Lipid Contents

Algal biomass was harvested from the culture at the stationary phase and subjected to the Bligh and Dyer [37] method to extract lipids from cells. Figure 10 demonstrates the effect of nitrate concentration on lipid contents as well as biomass and lipid productivities of *P. cruentum* at 20 °C and 140 μE m^{-2} s^{-1}. As shown in Figure 10, the biomass obtained from the medium with a nitrate concentration of 0.3 g/L has the highest biomass productivity, 143 mg L^{-1} day^{-1}, followed by 0.075 g/L and 0.5 g/L nitrate, 79 mg L^{-1} day^{-1} and 54 mg L^{-1} day^{-1}, respectively. The smallest amount is at a nitrate level of 0.7 g/L, which is 30 mg L^{-1} day^{-1}. Figure 10 also demonstrates that lipid contents and biomass productivities have opposite trends. The lipid contents dropped about 70% with nitrate concentration of 0.3 g/L compared to 0.075 g/L and then went in the opposite direction. Clearly, maximum biomass contents do not lead to maximum lipid contents. These observations agree with those of Cohen *et al.* [43], and indicate that

carbohydrate and lipid synthesis may be enhanced at the expense of protein synthesis when growth is slowed by any stressing factor, such as nutrient variation. Figure 10 also shows that lipid productivities declined with an increase in nitrate concentrations.

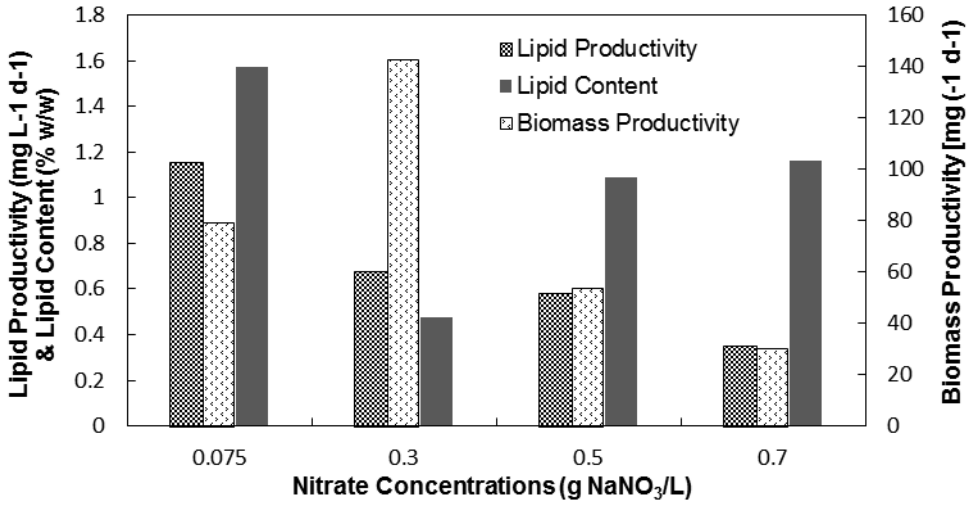

Figure 2.10 Biomass productivity, lipid contents, and lipid productivity of *P. cruentum* at optimum temperature (20 °C) and light intensity (140 µE m^{-2} s^{-1}) with different growth nutrient levels (0.075, 0.3, 0.5, or 0.7 g $NaNO_3$/L).

2.4.2.3 Fatty Acid Compositions

According to Table 1, the main fatty acids of *P. cruentum* in various nitrogen concentrations were the same as those described for different temperatures and light intensities. However, decreased amounts of 18:1 and 18:2 and enhanced 16:0, 16:1, 20:2, 20:4 (ARA), and 20:5 (EPA) were observed with increasing nitrogen concentrations. Figure 11 shows the percentage of EPA and ARA as well as their ratio (ARA/EPA) in lipids extracted from *P. cruentum* cells grown in different nitrate concentrations. The EPA percentages were approximately the same for two nitrate concentrations of 0.3 g/L and 0.7 g/L. The experimental

results showed that the highest lipid content does not induce the highest EPA amount. The EPA amounts were slightly decreased to 9.5% and 11.5% for 0.075 g/L and 0.5 g/L nitrate levels, respectively. Also, the same trend can be seen for the percentage of ARA extracted from *P. cruentum*. The highest and the lowest amounts of ARA were related to 0.3 g/L and 0.075 g/L nitrate, which were 30.5% and 24%, respectively. Our results were similar to Cohen [17], who reported a decrease in 18:1 and 18:2 in nitrogen-sufficient culture compared to those under nitrogen starvation conditions while 16:0, 16:1, and EPA increased under nitrogen sufficient conditions. In addition, our experimental results showed a gradual reduction in ARA/EPA amounts (from 2.5 to 2.17), which is in agreement with Cohen [17] (from 3.0 to 0.7). Figure 11 shows that the smallest ratio of ARA/EPA occurred when the sodium nitrate concentration in the medium was 0.5 g/L. These results suggest that the optimal trade-off between maximizing biomass and lipid productivities as well as minimizing ARA/EPA ratio occurs at 20 °C μE m^{-2} s^{-1} and 140 μE m^{-2} s^{-1} with a nitrate concentration of 0.5 g/L among the tested conditions.

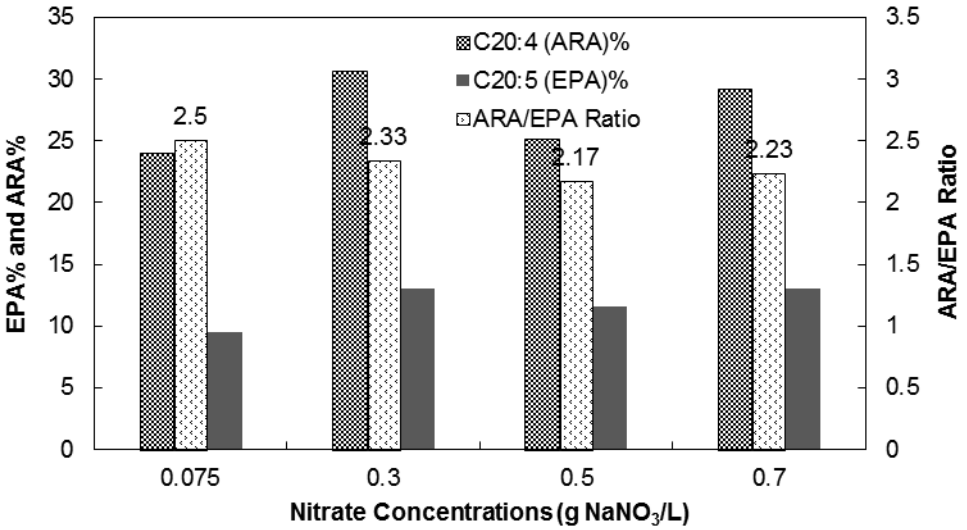

Figure 2.11 Effect of nitrogen level on EPA and ARA contents of *P. cruentum*.

2.5 Conclusions

This study demonstrates that temperature, illumination, nitrogen level, and CO_2 levels in the growth media significantly impact the cell growth, lipid content, lipid productivity, and fatty acid compositions of *P. cruentum*. The results indicate that decreased temperature and enhanced light intensity result in lower lipid and biomass productivities as well as higher lipid contents. Therefore, maximum biomass contents do not lead to maximum lipid contents. Synthesis and accumulation of higher lipid amounts occur in the cells when *P. cruentum* microalgae are placed under stress conditions imposed by physical environmental stimuli such as temperature and illumination. In addition, higher lipid productivity, lipid contents and lower biomass were observed when 5% CO_2 in air was used as CO_2 supplementation. Higher ARA and lower EPA, and consequently a higher ARA/EPA ratio, was achieved with 5% CO_2 in air compared to pure CO_2. Furthermore, the results demonstrate that 0.3 g/L nitrate developed the highest biomass, 143 mg L^{-1} day^{-1}, while the minimum amount, 30 mg L^{-1} day^{-1}, was related to the medium with a nitrate concentration of 0.7 g/L. An opposite trend was observed for lipid contents. Also, the results showed that lipid productivities declined with an increase in nitrate concentrations. The fatty acid composition showed that the major fatty acids from *P. cruentum* were C16:0, C18:2, C20:4 (ARA), and C20:5 (EPA). As lower ratios of ARA to EPA lead to easier separations of EPA from ARA and also are desirable nutritionally, this study shows that the optimal growth conditions to produce the lowest ratio, 2.17, was achieved at 20 °C and 140 μE m^{-2} s^{-1} (with pure CO_2) and 0.5 g/L nitrate.

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3. FATTY ACID COMPOSITION OF *PORPHYRIDIUM CRUENTUM* GROWN IN SWINE WASTEWATER AT MULTIPLE CONCENTRATIONS

3.1 Abstract

The high concentrations of nutrients in swine wastewater make it a potentially valuable resource for microalgae production. Microalgae can be used to produce bio-fuels and profitable pharmaceutical co-products such as omega-3 fatty acids. Additionally, the capture of nutrients by microalgae can avert uncontrolled nutrient discharge from livestock effluents to groundwater and consequently prevent serious ecological problems.

In this study, the microalgae *Porphyridium cruentum* was grown in ultra-filtered swine waste medium and the lipid and fatty acid composition from the biomass was evaluated. Different dilutions of the swine waste corresponding to total nitrogen concentrations of 12.4, 49.4, 82.4 and 115.3 mg/L were considered to achieve optimum nutrient conditions for producing eicosapentaenoic acid (EPA) or omega-3. The maximum lipid content and lipid productivity were achieved in a nitrogen concentration of 82.4 mg/L. For comparison, L1-medium was utilized as a control medium. The results showed better *P. cruentum* growth with higher lipid productivity and lipid content in the swine waste medium. Additionally, the effect of salt concentration on the accumulation of lipids in *P. cruentum* cells was investigated in order to reach higher lipid productivities and EPA content. To this end, various salinities from zero to 3.2% were examined in both the swine waste and control mediums with optimum nitrogen concentrations. The maximum biomass productivity (3.76 g/L) was obtained from the microalgae grown in control medium with 2.5% salinity while the highest lipid productivity (143

mg/L) and lipid content (4.71 mg/100 g biomass) was achieved in the swine waste medium with 3.2% salinity. The fatty acid profile was remarkably consistent among samples and consisted of C16:0, C16:1, C18:0, C18:1, C18:2, C20:4 (arachidonic acid; ARA) and 20:5 (EPA) fatty acids. Lower proportions of C16:0, 18:2 and C20:5 (EPA) were found in control medium compared to swine waste medium while C18:0 was higher in the control culture. The ARA to EPA ratio was lower for the microalgae harvested from swine waste medium than that obtained in L1-medium. The salinity of 3.2% in swine waste medium achieved the minimum ARA/EPA ratio (1.33).

Keywords

Fatty acids, Lipids, Microalgae, Photo bioreactor, *Porphyridium cruentum*, Salinity, Swine waste

3.2 Introduction

Microalgae are gaining attention due to the emergence of multiple applications, such as in wastewater treatment [1], pharmaceutical and medicine production [2] and bio-fuel productions [3]. Microalgae have a number of advantages over other sources of fatty acids. These include more oil yield per area of microalgae than the best oilseed crops, less water required for growth, the possibility of cultivation in seawater or on non-arable lands, the opportunity to remediate eutrophic waters [4], the production of valuable co-products such as proteins and residual biomass for oil extraction [2], and the production of pharmaceuticals like omega-3 fatty acids (ω3).

The production of algal lipids requires inputs including water, nutrients and land. Water provides a physical growth environment and also a medium for nutrients. Some microalgal strains including *Porphyridium cruentum* can grow in saltwater, thus, the need for freshwater inputs decreases. Essential nutrients include carbon dioxide (for photosynthetic production of biomass), phosphorous and nitrogen (for the energy related reactions). Wastewater can also be used as a potentially viable growth medium for microalgal feedstock due to its high concentrations of nutrients [5]. A significant advantage in producing lipids from algae over lipids from land plants (such as food crops) is that algal production facilities can be sited on non-farmable lands.

The fatty acids in microalgae have been used to produce many useful co-products. One particular product that has been the subject of intense research is omega-3, which humans do not have sufficient enzymes to synthesize [6]. Moreover, the low amount of the enzymes deteriorates

with age and under some disease conditions [7]. Omega-3, or eicosapentaenoic acid (EPA), can reduce the risk of diseases such as diabetes [8], brain disorders [9], coronary heart disease, Alzheimer's and psoriasis [10]. Researchers have found that these fatty acids should be considered essential for the normal growth of children and animals [11].

Omega-3 fatty acids, especially EPA (20:5, n-3), have been found in a wide variety of microorganisms such as bacteria, fungi, microalgae and fish oils. The advantages of microalgae over other sources have motivated many research groups to use microalgae to produce omega-3 and omega-6. Some strains of microalgae, such as *Porphyridium cruentum*, are naturally rich sources of these fatty acids. Researchers are mainly focused on achieving the best growth conditions of the microalgae to maximize desired fatty acids. Vazhappilly and Chen (1998) [10] investigated twenty microalgae strains in photoautotrophic flask cultures to identify ones with the potential to produce EPA and DHA (docosahexaenoic acid; 22:6, n-3). They indicated that some of these microalgae such as *Monodus subterraneus*, *Phaeodactylum tricornutum*, *Chlorella minutissima* and *Porphyridium cruentum* can produce significant amounts of EPA (18–96 mg/L). It is desired to choose a strain that yields less DHA for EPA production, as they have different pharmacological and nutritional benefits, and also to minimize downstream processing problems. Moreover, since omega-6, or ARA (arachidonic acid; 20:4, n-6), can reduce EPA production and disrupt EPA recovery, the presence of ARA during EPA production from microalgal cells is a critical factor [12,13].

The unbalanced intake of omega-6 to omega-3, mainly the insufficient uptake of omega-3, results in increased cardiovascular diseases, risk of cardiac death and mental illnesses. Diets with a ratio of 1:1 is perfect, while ratios higher than 10:1 are not recommended [6,14].

Therefore, the market for specific products, in this case the minimum ratio of ARA to EPA, is predicted to develop and diversify [15]. *P. cruentum*, a kind of red algae, is one of the best potential strains to accumulate large amounts of EPA and ARA in its cells without producing DHA. Moreover, the ratio of ARA and EPA contents in this microalga can be controlled by optimizing growth conditions [10]. Consequently, researchers are interested in continuing to investigate the effects of environmental and nutrition conditions on the production of EPA omega-3 fatty acids with reduced amounts of ARA.

It has been indicated that growth rates and fatty acid compositions of microalgae can be affected by several factors, such as growth conditions and nutrient compositions in the culture media [16]. Various culture media including L1 [17], f/2 [18], Koch [19], ASW [20] and Jones' medium [21,22] have been utilized to grow *P. cruentum*. However, the limitations in using these media include a lack of select nutrients required to increase algal production over long growth times [23]. Since the 1960s, wastewater treatment systems using algae have been gaining attention due to their potential advantages over the common activated sludge process which requires costly sludge processing and high energy inputs for aeration [24]. Furthermore, using algae in purification systems to eliminate nutrients causes no secondary contamination and the resulting biomass can be reused as a high protein feed for animals [25]. It has also been show that swine waste media exhibits a low-cost feedstock for valuable products such as biofuel production [26].

Since livestock wastewater can cause serious ecological problems from uncontrolled nutrient discharges (two orders of magnitude higher carbon and nutrient concentration compared to domestic wastewaters) in effluent [27], microalgae-based systems can be employed as a

bioremediation agent to remove excess nutrients [25]. Algae-based systems have the ability to assimilate organic carbons in addition to inorganic nutrients such as phosphorous and nitrogen from wastewater without using air. Moreover, the attained algal biomass includes high amounts of extractable lipids, such as fatty acids, which are ideal feedstocks for biodiesel production and other valuable co-products such as EPA [28].

Mulbry et al. investigated swine wastewater treatment using the freshwater algae *Rhizoclonium sp*. at various manure loading rates (0.2 to 1.3 g TN m^{-2} day^{-1}). A consistent fatty acid composition including 14:0, 16:0, 16:1 (ω7), 16:1 (ω9), 18:0, 18:1 (ω9), 18:2 (ω6), and 18:3 (ω3) with a range of 0.6 to 1.5% of dry weight biomass resulted from this study [29]. Another study on the growth of *Spirulina sp.* in a culture containing seawater supplemented with digested swine waste showed a higher total lipid count than the biomass grown in pig waste culture (28.6%) compared to a chemically defined medium (8.0%). In addition, higher lipids and polysaccharides were obtained under nitrogen deficiency condition [30]. Growth of *Spirulina maxima* in a 50% dilution of swine waste exhibited the best nutrient removal as well as biomass production with a 6% lipid content [31]. A three-fold growth rate and 2.6-fold dry weight increase were achieved for growth of a green microalga, *Scenedesmus spp.,* in a medium containing 3% (v/v) fermented swine waste. However, total lipid and fatty acid contents were found to be much less compared to biomass cultivated in the control medium (0.9% and 5%, respectively) [32]. In another study, high protein and lipid contents of 58.8% and 26.1% of dry weight were obtained from growth of green microalgae *Chlorella sp.* in digested swine waste [26].

Another factor affecting microalgal growth is the concentration of salts in the culture medium. EPA content from *Navicula sp.* increased slightly when high concentration of NaCl (1.7 M) was used in the culture medium [33]. Moreover, a 20 g/L salinity resulted in higher polyunsaturated fatty acids (PUFAs) (by more than 13%) and EPA (by 0.5%) from *Nannochloropsis sp.* compared to a salinity of 30 g/L [34]. Lee et al. showed that the best concentration of sodium chloride to grow *P. cruentum* is between 0.45 and 0.8 M. In addition, the amounts of PUFAs increased with increasing NaCl concentrations from 0.8 to 1.5 M [35].

The main objective of this study was to investigate the growth and lipid productivity of *Porphyridium cruentum* in ultra-filtered swine waste medium at 1) different nitrogen levels, and 2) different salinities in order to attain maximum fatty acid content, biomass content, and EPA level over other fatty acids, particularly ARA. The results were compared to *P. cruentum* grown in the L1-medium as a control medium at the same environmental and nutrient conditions.

3.3 Materials and Methods

3.3.1 Strain and Culture Medium

The marine microalgae *Porphyridium cruentum* (CCMP1328) were used in these experiments, and were obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, East Boothbay, ME, USA). *P. cruentum* cells are red, spherical and 5-8 μm in length. L1-medium was chosen as a control medium and maintenance, containing $NaNO_3$, $NaH_2PO_4 \cdot H_2O$, $Na_2EDTA.2H_2O$, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2.6H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, H_2SeO_3, $NiSO_4.6H_2O$, Na_3VO_4, K_2CrO_4, Thiamine-HCl (Vit. B_1), Biotin (Vit. H), Cyanocobalamin (Vit. B_{12}), and filtered seawater [36]. *P. cruentum* was pre-

cultured at 22 ˚C with natural illumination in a 500 ml glass bottle containing 250 ml of the sterilized medium (autoclaved at 127 ˚C for 30 min).

3.3.2 Swine Waste Preparation and Ultrafiltration

Swine waste samples were obtained from a manure holding lagoon located at a grow-finish swine farm in Savoy, AR (36°6′20″N 94°19′58″W). The farm is run by the University of Arkansas Extension Service. The feed sample source was an anaerobic digestion lagoon that was used to hold waste flushed from a 150 head grow-finish swine operation, which was used to house weaned pigs until they reach market weight. The facility was made up of a drop curtain style barn that housed pens with fully slatted floors for manure removal via flushing into an adjacent lagoon. Each sample was taken from the lagoon using a telescopic dipping sampler and then refrigerated for up to 24 hours prior to each ultrafiltration run. An ultrafiltration system was used to remove biological contaminants and inorganic solids. The ultrafiltration system used 1 inch hollow fiber membrane cartridges (50,000 MWCO; Koch Romicon PM50) and was operated at a transmembrane pressure of 17.5 psi. The permeate sample was taken after two hours of ultrafiltration operation in the recycle mode. After processing, the permeate samples were cultured using the IDEXX Colilert method [37] to check for the presence of *E. coli* and coliforms. In addition, the permeate samples were analyzed for total phosphorus (TP), total nitrogen (TN), total organic carbon (TOC) and ammonia-N using APHA methods [37]. After processing with ultrafiltration, the complete rejection of *E. coli* and coliforms were observed for the swine waste samples. The nutrient concentrations for the swine waste permeate used in the algae culture are given in Table 1.

Table 3.1 Water quality characteristics of the swine waste before and after processing with ultrafiltration. The swine waste was obtained from a manure holding pond at a swine farm in Savoy, AR.

Constituent	Concentration in Feed (mg/L)	Concentration in Permeate (mg/L)
Total Phosphorus	83.105	18.108
Total Nitrogen	705.06	196.98
Ammonia	539.50	189.36
Total Organic Carbon	350.00	56.65

3.3.3 Growth Experiments

Algal cultivation was performed in laboratory-scale corning sterile bottles from VWR. The inoculum volume for each sample was 100 ml, containing about 5,000 cells per ml, obtained from the pre-cultured algae in the early exponential growth phase. The effect of nitrogen concentration in swine waste was investigated first and then the effect of salinity on algal growth and fatty acid composition was studied.

Since wastewaters include several forms of nitrogen, not only nitrate, we analyzed the total nitrogen (TN) concentrations of swine wastewater before growing the algae. TN is the sum of four major forms of nitrogen (nitrate (NO_3), nitrite (NO_2), ammonia and organically bonded nitrogen). We utilized a range of nitrogen concentrations to investigate the effect on algal growth. For these experiments, the ultra-filtered swine waste was diluted using seawater to 12.4, 49.4, 82.4 and 115.3 mg/L of nitrogen concentrations. These nitrogen concentrations are corresponded to the nitrate concentrations (0.075, 0.3, 0.5 and 0.7 g nitrate/L) on first aim (Chapter 2). The nitrogen concentrations were calculated according to the following calculations for example for 0.075 g nitrate/L to 12.4 mg nitrogen/L:

$$0.075\frac{\text{g NaNO}_3}{L}\times\frac{1\ mol\ NaNO_3}{85\ g\ NaNO_3}\times\frac{1\ mol\ N}{1\ mol\ NaNO_3}\times\frac{14\ g\ N}{1\ mol\ N}=0.01235\ g\ N/L=12.4\ mg\ N/L \quad (1)$$

Filtered seawater was provided from NCMA, W Eel Pond, Woods Hole, Massachusetts, USA. The nitrogen concentration of 82.4 mg/L was picked as an optimum level and then, with the constant dilution of swine waste, the salinity was adjusted with seawater as well as salt water to study the effect on growth and lipid compositions. Sodium chloride and distillated water was used to make the salt water solutions. Salt water was used in this study in addition to seawater since in many places there is no easy access to seawater. Various salinities of 3.2%, 3.0%, 2.5%, 2.0%, 1.5%, 1.0% and 0.5% as well as zero salinity were investigated. In addition, the salinity study was performed with L1-medium without waste. The salinities of 3.2%, 2.5%, 1.5% and 0% were adjusted in L1-medium with seawater as well as salt water. All the experiments were carried out in triplicate and mean values of each sample are reported. The samples were combined in salinity study to have reasonable analysis. Finally, the resiliency of *P. cruentum* was assessed by replicating growth under optimized condition and adding 1 mL of raw swine wastewater. The raw swine waste contains native microalgae and cyanobacteria species. Growth of *P. cruentum* was observed qualitatively relative to the ultrafiltered swine medium and the control medium.

During the experiments, the containers were maintained at ambient laboratory temperatures (18-22 ℃), and illuminated using 4 fluorescent lamps under a light-dark cycle of 13:11 hours. The incident light average was 140 (130-150) μE m^{-2} s^{-1}. This condition was selected according to our previous study on optimum growth condition of *P. cruentum* [17]. Cell numbers were counted using a bright-light hemacytometer under a microscope every 3 to 4 days. The biomass was harvested after approximately 18-25 days in the stationary phase using centrifugation at 5,000 rpm for 15 min. The harvested biomass cake with about 80% moisture was washed with distillated water to remove non-biological material such as mineral salt

precipitates. The pellets were lyophilized and then grounded to powder prior to fatty acid extraction. The biomass productivities were obtained by Eq. 2:

$$\text{Biomass productivity}\,(\text{g/L}) = \frac{\text{dried microalgae biomass}\,(\text{g})}{\text{working volume}\,(\text{L})} \tag{2}$$

3.3.4 Lipid Extraction and Transmethylation

A modified method of Bligh and Dyer [38] was employed to extract lipids from algal cells. The dried biomass was homogenized in a solution of chloroform/methanol/water with a final volume ratio of 2:2:1.8, respectively. The remaining residues on the filter were extracted using chloroform/methanol with a volume ratio of 2:1. The mixture was left at room temperature overnight and then filtered to remove the algal pellet. Next, the filtered mixture was allowed to resettle to obtain a two-phase mixture. The lower chloroform layer containing the lipids was isolated from the upper layer, or aqueous phase, and then dried to obtain the lipid content. The lipid contents were obtained by subtracting the weight of the empty flask from the flask containing lipids. The percentage of the lipid content was determined by Eq. 3 [39]:

$$\text{Lipid content}\,\% = \frac{\text{weight of lipid}\,(\text{mg})}{\text{weight of dried microalgae biomass}\,(\text{mg})} \times 100 \tag{3}$$

The lipid productivities were calculated using Eq. 4 [40]:

$$\text{Lipid productivity}\,(\text{mg/L}) = \frac{\text{Lipid content}\,(\text{mg})}{\text{Working volume}\,(\text{L})} \tag{4}$$

Since fatty acid compositions were analyzed using gas chromatography (GC) and extreme temperatures higher than the upper temperature limitation of the stationary phase were required, the free fatty acids were derivatized to methyl esters before analyzing via GC. 1 ml of toluene and then 1 ml of 1.5% sulfuric acid in dry methanol was added to every 10 mg of lipid

and next incubated at 55 °C overnight. Afterward, 2 ml of saturated NaCl solution, 1 ml of hexane and 1.5 ml of sodium hydrogen carbonate (2% $NaHCO_3$) were added to the mixture and vortexed well. Finally, the upper phase was separated for GC analysis [40].

3.3.5 Gas Chromatography Analysis

The gas chromatograph to analyze fatty acid methyl esters (FAME) was GC-2014 (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and an auto-sampler. The GC column used to separate the FAME was a Zebron™ZB-FFAP polar capillary column (30 m x 0.32 mm x 0.25 μm film thickness; Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas with a linear velocity of 35 cm/s. The column was temperature programmed from 150 ˚C (held for 3 min) to 240 ˚C at 1.5 ˚C/min. Sample volumes of 2 μL were injected with a split ratio of 10:1. Detector temperature was set at 250 ˚C. The obtained peaks from the GC were compared to Marine Oil Test Mix. (Restek Corp., Bellefonte, PA) as FAME standards. The GC data are based on duplicate measurements.

3.4 Results and Discussion

3.4.1 Nitrogen Concentrations

Growth of *P. cruentum* was evaluated in diluted ultra-filtered swine wastewater. The effect of four nitrogen concentrations on lipid and fatty acid production were investigated to establish an optimum nitrogen level. *P. cruentum* was able to grow in all examined nitrogen concentrations from 12.4 to 115.3 mg/L in the culture medium. The biomass productivity increased with decreasing concentrations of nitrogen and maximum biomass (4.4 g/L) was

achieved in 12.4 mg/L (Figure 1). Our results demonstrate higher biomass productivities of *P. cruentum* in swine waste medium compared to a study of three species (two *chlorophyceae* and one *cyanobacterium*) of anaerobically treated swine manure effluents with the dilutions of 0.6-3.0% (corresponded to the 19.8-98.8 mg/L N-NH^{4+} concentrations). The average biomass productivities were 31, 37 and 53 mg dry wt/l for *P. bohneri*, *Chlorella sp.* and *S. obliquus*, respectively [41].

Figure 1 shows that the lipid productivity increased significantly from 50 to 143 mg/L when the nitrogen concentration decreased from 115.3 to 82.4 mg/L. However, lipid productivity declined to 74 and then to the minimum amount of 4.4 mg/L with decreasing the nitrogen concentrations to 49.4 and 12.4 mg/L, respectively. A similar trend was observed for lipid contents. Their amounts increased 50% when the nitrogen concentration decreased from 115.3 to 82.4 mg/L and then dropped considerably, 53% and 39%, with nitrogen concentrations of 49.4 and 12.4 mg/L, respectively. The maximum lipid content (4.7%) and lipid productivity (143 mg/L) were obtained at the nitrogen concentration of 82.4 mg/L while the biomass productivity was not the highest at this concentration. Therefore, higher growth performance does not lead to higher lipid contents and productivity. This is possibly due to membrane degradation and higher accumulation of lipids in algal cells under stressing conditions such as nutrient variations. Figure 1 shows that the lipid contents and productivity increased with decreasing nitrogen concentration and then declined considerably. This decrease in lipids is possibly due to a dual carbon and nitrogen limitation of the diluted swine waste culture medium. The result demonstrates that there is an optimum concentration for nitrogen levels to produce maximum lipid contents with reasonable biomass amounts.

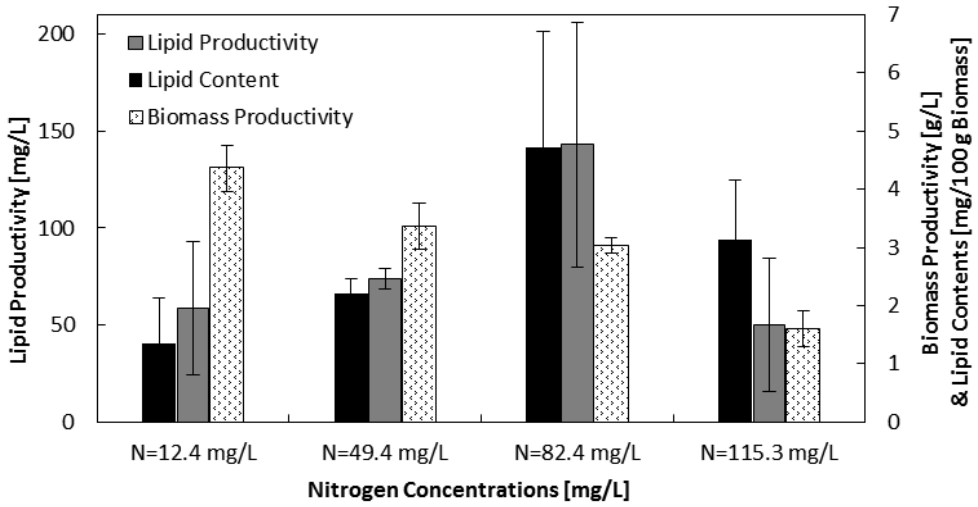

Figure 3.1 The effect of nitrogen concentration in diluted swine wastewater medium on biomass productivity as well as lipid contents and lipid productivity of *P. cruentum*

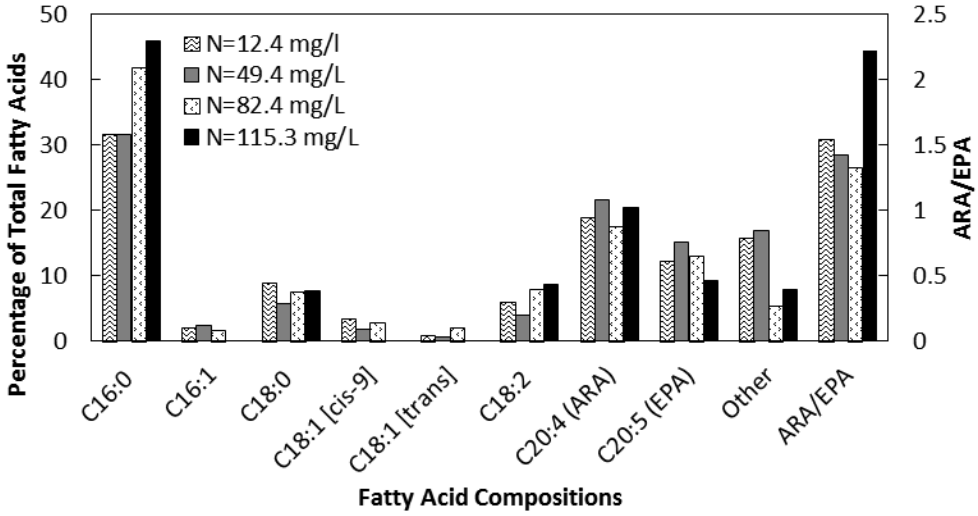

Figure 3.2 The effect of nitrogen concentration in diluted swine wastewater medium on fatty acid composition from *P. cruentum*

Fatty acid composition was also analyzed as a function of nitrogen concentration in the swine waste medium. Eight major fatty acids were presented as a percentage of the total fatty

acids (C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1-Cis-9 (oleic acid), C18:1-trans (octadecenoic acid), C18:2 (linoleic acid), C20:4 (arachidonic acid or ARA) and C20:5 (eicosapentaenoic acid or EPA)). There was no trace of C22:6 (docosahexaenoic acid, DHA) in the lipids from *P. cruentum*. The statistical analysis of the data showed that the percentage of palmitic acid (C16:0) increased by increasing the nitrogen concentration from 49.4 to 115.3 mg/L and the cetane numbers consequently increased. A higher saturated fatty acid such as C16:0 leads to higher cetane numbers which plays an important role in the quality of biodiesel. An opposite trend was observed for the EPA (C20:5) percentage. The minimum ratio of ARA to EPA (1.33) was achieved with a nitrogen concentration of 82.4 mg/L. The same results have been reported in our previous study [17]. The minimum ARA/EPA ratio was obtained at a nitrate concentration of 0.5 g nitrate/l which corresponded to 82.4 mg nitrogen/L [17]. According to Table 3.1, the ratio of ammonia per total nitrogen shows that these effects obtained from ammonia concentration as a source of nitrogen instead of nitrate. Optimizing between biomass productivity and lipid productivity as well as minimizing ARA/EPA ratio leads to the optimum nitrogen concentration of 82.4 mg/L. This optimum concentration was employed in the next experiments.

3.4.2 Salinity

Figure 3 shows the effect of salinity on biomass productivity and lipid productivity as well as lipid content from *P. cruentum* grown in swine waste medium (a and b) and control medium (c and d). The scales of four graphs are different. The numbers in parenthesis shows maximum and minimum contents for each case. The growth media contained the optimum nitrogen concentration (82.4 mg/L) and received optimum environmental conditions (18-22 °C

and the average light intensity of 130-150 μE m^{-2} s^{-1}) according to our previous study [17]. The microalgae survived under various salinities but, in general, growth diminished with decreasing salinity. The maximum biomass productivity, lipid productivity and lipid content is related to the algae grown in 3.2% and 3.0% salinity in swine waste medium diluted with seawater and saltwater, respectively. The minimum amounts occurred in the swine waste medium with a salinity of zero.

Biomass productivity and lipid productivity as well as lipid content of algal cells harvested in the swine waste diluted with seawater were markedly higher (36%, 84% and 75%, respectively) than those diluted with salt water, as shown in Figures 3 (a) and (b). The same outcome was also seen in control medium, since biomass productivity, lipid productivity and lipid contents increased for algae grown in the control medium diluted with seawater. As seen in Figure 3 (c), the algal biomass productivity in control medium diluted with seawater increased from 2.06 to 3.76 g/L when salinity decreased from 3.2% to 3.0% and then dropped to 0.163 g/L with a salinity of zero. However, the maximum lipid productivity (86.33 mg/L) and lipid contents (4.20 mg/100 g biomass) occurred in the salinity of 3.2%. The maximum biomass productivity (1.17 g/L), lipid productivity (48.67 mg/L) and lipid contents (4.15 mg/100 g biomass) from algae grown in control media diluted in salt water were recorded at the salinity of 3.2%. Figure 3 shows that the maximum biomass productivity (3.76 g/L) was obtained from the microalgae grown in control media with 2.5% salinity diluted by seawater. However, maximum lipid productivity (143 mg/L) and lipid content (4.71 mg/100 g biomass) was achieved in the swine waste medium with 3.2% salinity diluted by seawater. Generally, a comparison between growth of *P. cruentum* in swine waste medium and L1-medium concludes higher lipid contents and lipid productivities obtained from algal growth in swine waste culture.

There have been no previous studies describing the lipid or fatty acid contents of *P. cruentum* grown in swine waste medium. However, growth of *Spirulina maxima* for tertiary treatment of swine waste was studied by Canizares (1993) and the best nutrient removal and biomass production with a 6% lipid content was obtained in a 50% dilution of waste. This study showed better biomass production of *Spirulina* in the swine waste compared to mineral medium (Zarrouk's) [42]. In addition, Mulbry, (2008) investigated treatment of swine and dairy manure effluents using freshwater algae, *Rhizoclonium sp.*, and fatty acid contents of 0.6-1.5% of dry weight biomass resulted [29]. Kim et al. achieved a 2.6-fold dry weight after 31 days growth of *Scenedesmus spp.* in a medium containing 3% (v/v) fermented swine waste compared to control medium. However, total lipids in the microalgal culture grown in the medium contained swine waste were found to be much less than those in the control medium (0.9% and 5%, respectively) which is in contrast with our study [43].

The fatty acid composition of *P. cruentum* was determined and the results are shown in Figure 4. The scales of four graphs are different. The algal cells accumulated fatty acids in the range of C16:0 to C20:5. C16:0 (palmitic acid: 26%-50%) and then C20:4 or ARA (arachidonic acid: 2-24%) and C20:5 or EPA (eisocapentaenoic acid: 2-22%) were the predominant fatty acids in *P. cruentum* cells. These fatty acids were also the predominate fatty acids in *P. cruentum* reported by Cohen (1988) [13] and Durmaz et al. (2007) [44], whereas C18:1 acids were also described as the dominant fatty acids in *P. cruentum* reported by Oh et al. (2009) [18]. As shown in Figure 4 (a) for the microalgae grown in swine waste medium diluted with seawater, the minimum and maximum amounts of both C16:0 (29.4%-49.2%) and C16:1 (1.1%-2.9%) were obtained from the medium with the salinity of 1.0% and 3.0%, respectively. Moreover, the highest ARA (19.7%) and EPA (12.9%) occurred in 2.5% and 3.2% salinity while the lowest

ARA (15.1%) and EPA (7.7%) was achieved when the algae were cultivated in the seawater swine culture with 3.0% and 1.0% salinity, respectively. The minimum ARA to EPA ratio (1.33) was related to 3.2% salinity.

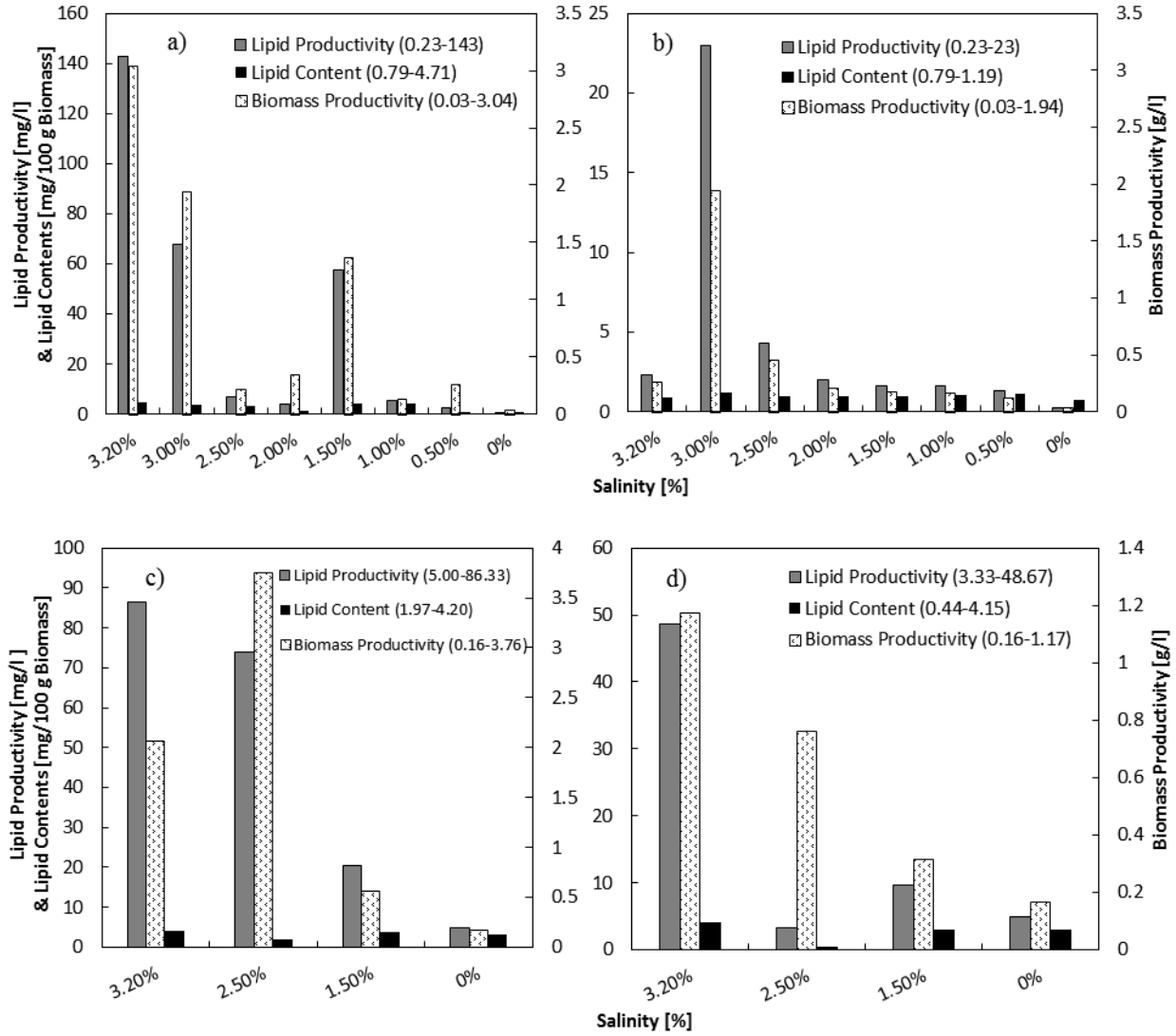

Figure 3.3 The effect of salinity on biomass productivity as well as lipid contents and lipid productivity of *P. cruentum* grown in a) swine waste medium diluted with seawater; b) swine waste medium diluted with salt water; c) L1-medium diluted with seawater; d) L1-medium diluted with salt water.

The major fatty acid trends shown in *P. cruentum* cells grown in swine waste medium diluted with salt water (Figure 4 (b)) was similar to those diluted with seawater. A comparison between Figure (a) and (b) indicate that higher percentages of ARA and EPA were in the swine waste diluted with salt water compared to seawater, whereas C16:0 was lower. The salinity of 3.0% and 1.5% led to the maximum amounts of ARA and EPA (23.25% and 16.02% of total fatty acid contents, respectively) while these fatty acids both were at the minimum (6.7% and 3.5% of total fatty acids, respectively) in the culture with salt concentration of 0.5%. In salt water swine medium, ARA to EPA ratio was in the range of 1.41-1.91 (2.5%-0.5% salinity, respectively).

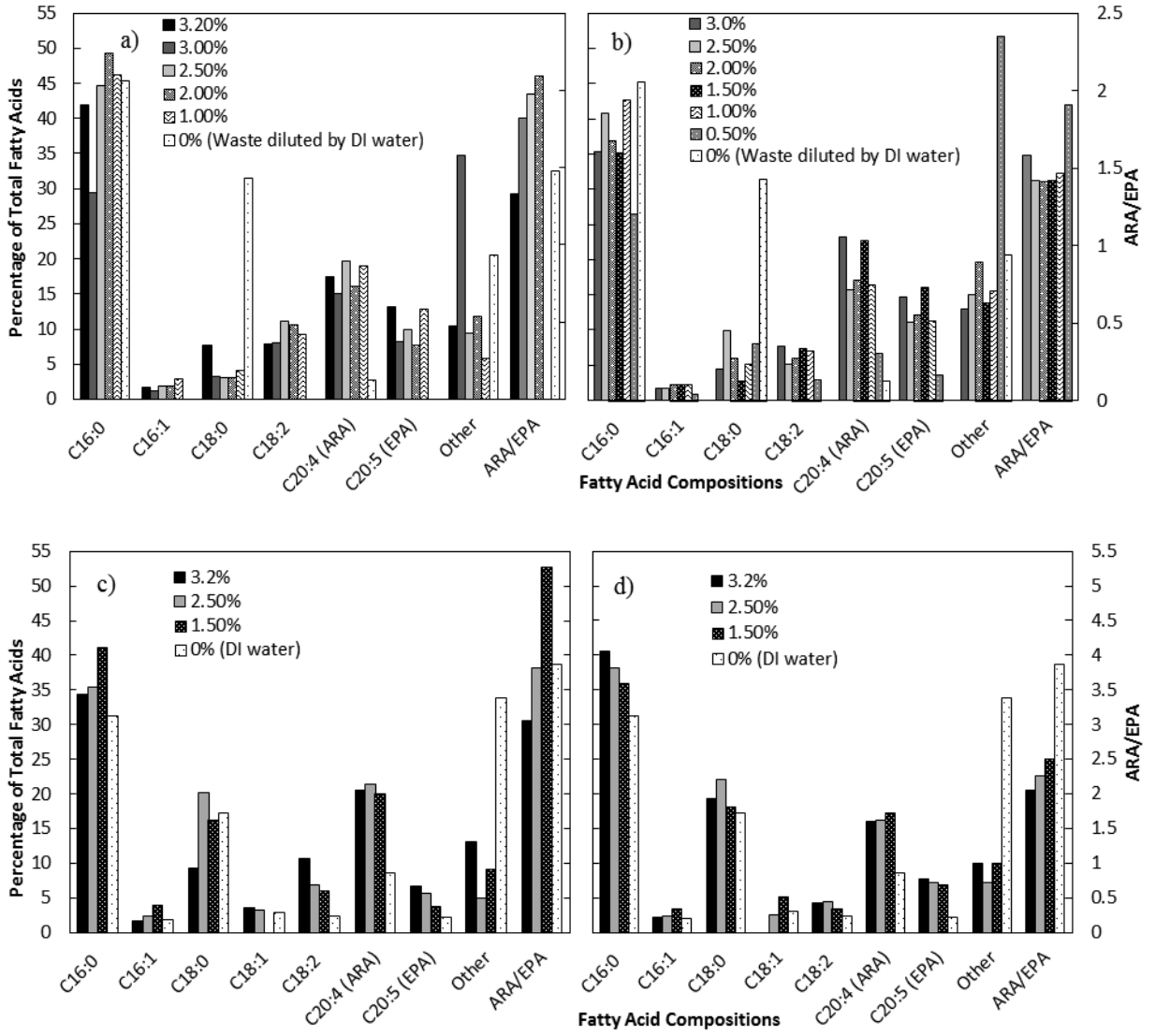

Figure 3.4 The effect of salinity on fatty acid compositions from *P. cruentum* grown in a) swine waste medium diluted with seawater; b) swine waste medium diluted with salt water; c) L1-medium diluted with seawater; d) L1-medium diluted with salt water

The fatty acid profile for microalgal grown in control medium as shown in Figure 4 (diluted with c: seawater and d: salt water) indicates the presence of C16:0, C16:1, C18:0, C18:1, C18:2, C20:4 (ARA) and 20:5 (EPA) fatty acids. In general, C16:0, 18:2 and C20:5 (EPA) levels

were lower in the control medium than swine waste, while C18:0 was higher in the control culture. The algal lipids from control medium contained C18:1 fatty acids. It was not seen in the algal lipids obtained from swine waste medium.

As shown in Figure 4 (c), control medium diluted with seawater, the maximum amounts of C16:0 and C16:1 were related to 1.5% salinity, while the minimum amounts were obtained in the salinity of 0% and 3.2%, respectively. Furthermore, the amount of C18:0 fatty acid accumulated in control culture was in the range of 9.4%-20.2% of total fatty acids (in 3.2% and 2.5% salinity), which was much higher than those values obtained from the swine culture (3.0%-7.7% of total fatty acid contents related to 2.0% and 3.0% salinity, respectively, without consideration of zero salinity in swine waste in which the amounts of C18:0 was the highest, 31.4% of total fatty acids). The amounts of C18:0 were in the range of 17.2-22.1% (in 0%-2.5% salinity) in the salt water control culture. The quantities of C16:0, C16:1 and C18:1 did not vary significantly when changing salinity source.

ARA (21.4%) and EPA (6.7%) were the highest in 2.5% and 3.2% salinity, respectively. The lowest amounts of both fatty acids were obtained in the culture medium with zero salinity. The same result for the minimum levels of EPA and ARA was observed for control medium diluted with salt water. The maximum ARA (17.2%) and EPA (7.8%) were related to 1.5% and 3.2% salinity in salt water control medium. Overall, the quantity of ARA was in lower amount in control culture diluted with salt water than seawater while the accumulation level of EPA was higher in the salt water control culture. Consequently, ARA to EPA ratio was lower in the salt water control medium compared to those in seawater control medium. The lowest amounts of this ratio were 3.05 and 2.06, which was obtained from the salinity of 3.2% in seawater and salt water control medium, respectively.

In general, lower ARA/EPA ratios and higher EPA values were achieved from *P. cruentum* grown in the swine waste medium compared to the control medium. Furthermore, all ARA/EPA ratios obtained from swine waste culture are lower compared to the minimum ratio (2.17) achieved in optimal condition attained from our previous study (at 20°C and 140 μE m^{-2} s^{-1}, pure CO_2 and 0.5 g/L nitrate) [17]. However, the optimum nitrogen concentration with the minimum ARA/EPA ratio was the same (82.4 mg/L nitrogen corresponded to a nitrate concentration of 0.5 g/L in our previous study) in both studies. Maximizing biomass productivity, lipid productivity and EPA contents while also minimizing ARA/EPA ratio require a swine waste medium diluted with a seawater salinity of 3.2%. This level led to the minimum ARA/EPA ratio (1.33) and the maximum lipid productivity (143 mg/L).

The main fatty acids gained from *P. cruentum* (Figure 4) can be utilized as a good-quality feedstock for biodiesel production since linoleic acid (C18:3), which is specified at a limit of 12% for a quality biodiesel according to EN14214 standard [45], was not detected in the *P. cruentum* in this study. In addition, a comparison between fatty acids from algal lipids grown in the swine waste medium (Figure 4 a and b) and the control medium (Figure 4 c and d) shows higher amounts of palmitic acid or C16:0 from swine waste cultures. Higher saturated fatty acids such as C16:0 lead to higher cetane numbers which plays an important role in the quality of biodiesel. Similar results were reported by Van Gerpen (1996) and Ramos et al. (2009) observing the higher cetane number with higher percentages of methyl palmitate [46,47].

Finally, when 1 mL of raw swine waste was added to the swine waste culture under optimized conditions, *P. cruentum* showed a very good growth rate until the last few days. A green algae strain was observed to outcompete *P. cruentum* in the use of nutrients in the medium. Therefore, saline culture medium could not prevent contamination with native algae or bacteria

in the raw swine waste. The lack of contamination during the growth of *P. cruentum* in the ultrafiltered swine waste medium, suggests that ultrafiltration can be utilized as an affordable technique for the removal of biological contaminations from wastewater before microalgae cultivation. Based on the results of this study, *P. cruentum* biomass grown in ultra-filtered swine waste demonstrates promising potential for use as a source of high quality omega-3, biodiesel feedstock, as well as wastewater treatment and an inexpensive source of protein for livestock feed.

3.5 Conclusions

In this study we employed a medium of ultrafiltered swine waste as a nutrient supplement to cultivate the microalgae *P. cruentum*. The results indicated that the swine waste medium was able to support the growth of this algal strain, and higher lipid productivity and lipid content was achieved in swine waste medium compared to the control medium. Based on the results, different dilutions of swine waste showed increased biomass productivity by decreasing the nitrogen concentration from 12.4 to 115.3 mg/L in the culture medium. However, the maximum lipid contents (4.7%) and lipid productivity (143 mg/L) were achieved in the nitrogen concentration of 82.4 mg/L. The experiments based on salinity shows enhanced lipid productivity (143 mg/L) and lipid content (4.71 mg/100 g biomass) in the seawater swine waste medium with 3.2% salinity. A markedly higher biomass productivity (36%) and lipid productivity (84%) as well as lipid content (75%) of algal cells harvested in the swine waste diluted with seawater was achieved compared to those in salt water. The minimum ARA to EPA ratio (1.33) was obtained at 82.4 mg/L nitrogen concentration and 3.2% salinity in seawater swine wastewater.

This study established that a process combining ultrafiltration and algae cultivation has the potential for commercialization to convert concentrated swine waste into profitable by-products while reducing environmental contaminants. However, we cannot predict the feasibility of the system in a large scale, outdoor operation. An evaluation of energy production from these different processes along with pilot scale testing is needed. Algal feedstock, however, does appear promising for future pharmaceutical and biofuel application.

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4. CONCLUSIONS AND FUTURE WORKS

4.1 Conclusions

In this work, the effect of environmental condition and nutrient composition was investigated on cell growth, lipid content and fatty acid composition of *Porphyridium cruentum*. Since an appropriate proportion of omega-6 to omega-3 is vital for healthy nutrition, the ratio of omega-6 to omega-3 (ARA/EPA) at different growth conditions was compared. The results illustrates that maximum biomass productivities do not lead to maximum lipid contents, since decreased lipid and biomass productivities as well as increased lipid contents were achieved at lower temperatures and higher light intensities. Synthesis and accumulation of higher lipid amounts occur in *P. cruentum* cells when the algae are placed under stress conditions imposed by physical environmental stimuli such as temperature and illumination. Lower lipid productivity and lipid content but higher biomass productivity and lower ARA/EPA ratio was achieved when pure CO_2 was used as CO_2 supplementation compared to 5%CO_2 in air. An analysis of results from microalgae grown at various nitrogen concentrations shows that the highest and lowest amounts of biomass was obtained from the culture medium with nitrate levels of 0.3 g/L and 0.7 g/L, respectively, while an opposite trend was observed for lipid contents. Since the lower ratio of ARA/EPA are desirable nutritionally and lead to easier separation of EPA from ARA, this study shows that the optimal growth conditions to produce the lowest ratio, 2.17, was achieved at 20°C and 140 $\mu E/M^2.S$ (with pure CO_2) and 0.5 g/L nitrate.

In the second phase of this research, the swine waste from ultrafiltration permeate was used as a great source of nutrient supplementation to cultivate the red microalgae, *P. cruentum*. As mentioned, there is not any study which focused of fatty acid profile of *P. cruentum* as a good

source of omega-3 or EPA grown in swine wastewater. The results of this study indicated that swine waste was able to support growth of algal strain *P. cruentum*. Swine waste culture led to higher lipid productivity and lipid content from the microalgae compared to the control medium. According to the salinity experiments, enhanced lipid productivity and lipid content were achieved when the microalgae were cultivated in the seawater swine waste medium with 3.2% salinity. Biomass productivities and lipid productivities as well as lipid contents were significantly increased when the swine waste medium was diluted with seawater compared to those diluted with salt water. This study demonstrated that the minimum ARA/EPA ratio was obtained from the biomass harvested at 82.4 mg/L nitrogen concentration and 3.2% salinity in seawater swine wastewater.

This study established that a process combining ultrafiltration and algae cultivation has the potential for commercialization to convert high strength swine waste into high-value products from microalgae as well as to reduce environmental contaminations. This research suggests that the costs of production decrease using a low cost medium (wastewater). Additionally, the microalgae biomass produced could be utilized as a low-cost feedstock for some valuable fatty acids and biodiesel production. After lipid extraction from the microalgae cells, the remaining biomass can be utilized as a high protein feed for animals instead of conventional protein sources.

4.2 Future Works

There are several challenges that need to be address in lipid production from *P. cruentum*. These challenges suggest a variety of research directions that need to be pursued to make algal lipid production commercially feasible. These directions would be:

1) The feasibility of the swine waste system in an outdoor, large scale operation needs to be confirmed in a pilot scale before being commercially viable. Additionally, evaluation of energy production from different processes and algal feedstock appears promising for future pharmaceutical and biofuel application.
2) The effect of higher salinities in seawater culture medium on algal lipid production needs to be investigated since the maximum growth and lipid production were obtained at the highest salinity studied in this work.
3) Another possibility area would be to compare different wastewater types such as dairy or municipal wastewater to grow microalgae and produce biofuels and pharmaceutical products.
4) Immobilization of microalgae in either polymeric or bio-polymeric surface may be another possible area since immobilized cells can be harvested easily, are easier to handle and occupy less space compared to free-cell counterparts [48]. Also immobilized cells can be more resistance to harsh environments such as pH and metal toxicity. It is suggested to grow *P. cruentum* on alginate beads as an immobilization matrix [49].
5) Different kinds of microalgae strains can be compared together in producing valuable fatty acids such as omega-3.
6) Finally, in term of wastewater treatment, there is another possible area in which an algal-bacterial system can be utilized to treatment of livestock effluents. Only a few studies on

the use of algal-bacterial systems for wastewater treatment have been reported. In this system, microalgae can utilize nutrients in the wastewater for their growth and produce O_2 by photosynthesis. On the other hand aerobic bacteria oxidize ammonia and mineral organic pollutants via produced oxygen. A higher nutrient uptake by microalgae is obtained with CO_2 released via bacteria and it can improve nutrient removals.

APPENDIX A. EXPERIMENTAL PROCEDURES AND DATA

A.1 Preparing L1-medium

The L1-medium is based on f/2 medium [1] but has additional trace metals. To prepare the medium first begin with about 950 mL of filtered natural seawater from NCMA (NCMA, East Boothbay, ME, USA). Then, add the quantity of each component according to table A.1 and bring the final volume to 1 liter by filtered natural seawater. The trace element and vitamin solutions are given in the following tables (A.2 and A.3). The media were modified by omitting Si ($Na_2SiO_3 \cdot 9\ H_2O$) from the original recipe. Then, autoclave the container contained media at 121 °C for 30 min. It may take one day to cool down to the ambient temperature. Final pH should be 8.0 to 8.2.

Table A.1 The quantity of each component in L1-medium [2,3]

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75.00 g L^{-1} dH_2O	1 mL	8.82 x 10^{-4} M
NaH_2PO_4	5.00 g L^{-1} dH_2O	1 mL	3.62 x 10^{-5} M
Trace element solution	(see recipe below)	1 mL	----
Vitamin solution	(see recipe below)	0.5 mL	----

Table A.2 Trace element solution in 1 L dH_2O [2,3]

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$Na_2EDTA.2H_2O$	------	4.36 g	1.17×10^{-5} M
$FeCl_3.6H_2O$	------	3.15 g	1.17×10^{-5} M
$MnCl_2{\cdot}4H_2O$	178.10 g L^{-1} dH_2O	1 mL	9.09×10^{-7} M
$ZnSO_4.7H_2O$	23.00 g L^{-1} dH_2O	1 mL	8.00×10^{-8} M
$CoCl_2.6H_2O$	11.90 g L^{-1} dH_2O	1 mL	5.00×10^{-8} M
$CuSO_4.5H_2O$	2.50 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
$Na_2MoO_4.2H_2O$	19.9 g L^{-1} dH_2O	1 mL	8.22×10^{-8} M
H_2SeO_3	1.29 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
$NiSO_4.6H_2O$	2.63 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
Na_3VO_4	1.84 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
K_2CrO_4	1.94 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M

Table A.3 Vitamin solution in 1 L dH_2O [3]

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
Thiamine.HCl (vit. B1)	----	200 mg	2.96×10^{-7} M
Biotin (vit. H)	0.1 g L^{-1} dH_2O	10 mL	2.05×10^{-9} M
Cyanocobalamin (vit. B12)	1.0 g L^{-1} dH_2O	1 mL	3.69×10^{-10} M

Table A.4 Mineral makeup of seawater (In order of most to least)

Element	Molecular Weight	PPM in Seawater	Molar Concentration
Chloride	35.4	18980	0.536158
Sodium	23	10561	0.459174
Magnesium	24.3	1272	0.052346
Sulfur	32	884	0.027625
Calcium	40	400	0.01
Potassium	39.1	380	0.009719
Bromine	79.9	65	0.000814
Carbon(inorganic)	12	28	0.002333
Strontium	87.6	13	0.000148
Boron	10.8	4.6	0.000426
Silicon	28.1	4	0.000142
Carbon (organic)	12	3	0.00025
Aluminum	27	1.9	0.00007
Fluorine	19	1.4	0.000074
N as nitrate	14	0.7	0.00005
Nitrogen (organic)	14	0.2	0.000014
Rubidium	85	0.2	0.0000024
Lithium	6.9	0.1	0.000015
P as Phosphate	31	0.1	0.0000032
Copper	63.5	0.09	0.0000014
Barium	137	0.05	0.00000037
Iodine	126.9	0.05	0.00000039
N as nitrite	14	0.05	0.0000036
N as ammonia	14	0.05	0.0000036
Arsenic	74.9	0.024	0.00000032
Iron	55.8	0.02	0.00000036
P as organic	31	0.016	0.00000052
Zinc	65.4	0.014	0.00000021
Manganese	54.9	0.01	0.00000018
Lead	207.2	0.005	0.000000024
Selenium	79	0.004	0.000000051
Tin	118.7	0.003	0.000000025
Cesium	132.9	0.002	0.000000015
Molybdenum	95.9	0.002	0.000000021
Uranium	238	0.0016	0.0000000067

Gallium	69.7	0.0005	0.0000000072
Nickel	58.7	0.0005	0.0000000085
Thorium	232	0.0005	0.0000000022
Cerium	140	0.0004	0.0000000029
Vanadium	50.9	0.0003	0.0000000059
Lanthanum	139.9	0.0003	0.0000000022
Yttrium	88.9	0.0003	0.0000000034
Mercury	200.6	0.0003	0.0000000015
Silver	107.9	0.0003	0.0000000028
Bismuth	209	0.0002	0.00000000096
Cobalt	58.9	0.0001	0.0000000017
Gold	197	0.000008	0.00000000004

A.2 Growth Conditions

The red marine microalgae *Porphyridium cruentum,* CCMP1328 isolated by the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, East Boothbay, ME, USA), was used. The cells were red and spherical with a length of 5–8 μm. *P. cruentum* cultures were maintained in a modified L1-medium [2,3].

- *P. cruentum* was pre-cultured at 22 °C with natural illumination in a 500 mL glass bottle containing 250 mL of medium.
- Pre-cultured algae in the early exponential growth phase were inoculated at fixed cell numbers of around 5000 cells per mL.

A.2.1 Aim 1 experimental procedures

- Growth experiments were carried out axenically for approximately 30 days in a photobioreactor (ePBR v1.1) purchased from Phenometrics (Phenometrics, Inc. Alliance Drive, Lansing, MI, USA).
- The experimental parameters (such as temperature, light intensity, pH and stirring rate) were controlled simultaneously by a single computer running Algal Command, Phenometrics' software.

Table A.5 Aim 1 experimental growth conditions

Experiments	Temperature (°C)	Light intensity (μE m^{-2} s^{-1})	Nitrate concentration (g L^{-1})	Stirring rate (rpm)	Light/dark cycle (hours)	CO_2 supplementation
A1	20	140	0.075	150	13/11	pure CO_2
A2	20	140	0.075	150	13/11	5% CO_2 in air
A3	16	140	0.075	150	13/11	pure CO_2
A4	20	180	0.075	150	13/11	pure CO_2
A5	20	140	0.3	150	13/11	pure CO_2
A6	20	140	0.5	150	13/11	pure CO_2
A7	20	140	0.7	150	13/11	pure CO_2

- pH values were monitored daily during the experiments and the required pH was achieved by bubbling (approximately 5 psi) pure CO_2 through a small hole at the bottom of the column. CO_2 was bubbled periodically every couple of days for several seconds to reach the targeted pH. In addition, one extra experiment was performed with a ratio of 5% CO_2 in the air to adjust the pH to investigate the effect of air in growth and lipid composition.
- Cell numbers were counted using a microscope with a bright-light hemacytometer. Data were statistically analyzed to obtain the standard deviation.

A.2.2 Aim 2 experimental procedures

Swine waste samples were obtained from a manure holding lagoon located at a grow-finish swine farm in Savoy, AR (36°6′20″N 94°19′58″W). Each sample was taken from an anaerobic digestion lagoon using a telescopic dipping sampler and then refrigerated for up to 24 hours prior to each ultrafiltration run. An ultrafiltration system was used to remove biological contaminants and inorganic solids. The ultrafiltration system used 1 inch hollow fiber membrane cartridges (50,000 MWCO; Koch Romicon PM50) and was operated at a transmembrane

pressure of 17.5 psi. The permeate sample was taken after two hours of ultrafiltration operation in the recycle mode. After processing, the permeate samples were cultured using the IDEXX Colilert method [4] to check for the presence of *E. coli* and coliforms. In addition, the permeate samples were analyzed for total phosphorus (TP), total nitrogen (TN), total organic carbon (TOC) and ammonia-N using APHA methods [4]. After processing with ultrafiltration, the complete rejection of *E. coli* and coliforms were observed for the swine waste samples. The nutrient concentrations for the swine waste permeate used in the algae culture are given in Table A.6 [5].

Table A.6 Water quality characteristics of the swine waste before and after processing with ultrafiltration [5].

Constituent	Concentration in Feed (mg L^{-1})	Concentration in Permeate (mg L^{-1})
Total Phosphorus	83.105	18.108
Total Nitrogen	705.06	196.98
Ammonia	539.50	189.36
Total Organic Carbon	350.00	56.65

- Algal cultivation was performed in laboratory-scale corning sterile bottles from VWR. The inoculum volume for each sample was 100 ml, containing about 5,000 cells per ml, obtained from the pre-cultured algae in the early exponential growth phase.
- Table A.7 shows all experiments in this part of study.
- A range of nitrogen concentrations was examined to investigate the effect on algal growth. For these experiments, the ultra-filtered swine waste was diluted in seawater to 12.4, 49.4, 82.4 and 115.3 mg/L of nitrogen concentrations.

- The nitrogen concentration of 82.4 mg/L was picked as an optimum level and then, with the constant dilution of swine waste, the salinity was adjusted with seawater as well as salt water to study the effect on growth and lipid compositions.
- Sodium chloride and distillated water was used to make the salt water solutions.
- Various salinities of 3.2%, 3.0%, 2.5%, 2.0%, 1.5%, 1.0% and 0.5% as well as zero salinity were investigated and the salinities were adjusted with seawater and salt water.
- In addition, the salinity study was performed with L1-medium without waste. The salinities of 3.2%, 2.5%, 1.5% and 0% were adjusted in L1-medium with seawater as well as salt water.
- Finally, the resiliency of *P. cruentum* was assessed by replicating growth under optimized condition and adding 1 mL of raw swine wastewater. Growth of *P. cruentum* was observed qualitatively relative to the ultrafiltered swine medium and the control medium.
- During the experiments, the containers were maintained at ambient laboratory temperatures (18-22 °C), and illuminated using 4 fluorescent lamps under a light-dark cycle of 13:11 hours. The incident light average was 140 (130-150) $\mu E\ m^{-2}\ s^{-1}$. This condition was selected according to our previous study on optimum growth condition of *P. cruentum* [6].

Table A.7 Aim 2 experiments

Experiments (triplicate)	Nitrogen Concentration mg N/L	Salinity	Salt source
Growth in Wastewater:			
N1	12.4 (Corresponded to 0.075 g nitrate/L)	3.2%	Seawater
N2	49.4 (0.3 g nitrate/L)	3.2%	Seawater
N3	82.4 (0.5 g nitrate/L)	3.2%	Seawater
N4	115.3 (0.7 g nitrate/L)	3.2%	Seawater
S1	82.4 (0.5 g nitrate/L)	3.0%	Seawater
S2	82.4 (0.5 g nitrate/L)	2.5%	Seawater
S3	82.4 (0.5 g nitrate/L)	2.0%	Seawater
S4	82.4 (0.5 g nitrate/L)	1.5%	Seawater
S5	82.4 (0.5 g nitrate/L)	1.0%	Seawater
S6	82.4 (0.5 g nitrate/L)	0.5%	Seawater
SW	82.4 (0.5 g nitrate/L)	3.2%	Saltwater
SW1	82.4 (0.5 g nitrate/L)	3.0%	Saltwater
SW2	82.4 (0.5 g nitrate/L)	2.5%	Saltwater
SW3	82.4 (0.5 g nitrate/L)	2.0%	Saltwater
SW4	82.4 (0.5 g nitrate/L)	1.5%	Saltwater
SW5	82.4 (0.5 g nitrate/L)	1.0%	Saltwater
SW6	82.4 (0.5 g nitrate/L)	0.5%	Saltwater
SW7	82.4 (0.5 g nitrate/L)	0%	Saltwater
Growth in Control Medium:			
L1	82.4 (0.5 g nitrate/L)	3.2%	Seawater
LS1	82.4 (0.5 g nitrate/L)	2.5%	Seawater
LS2	82.4 (0.5 g nitrate/L)	1.5%	Seawater
LSW	82.4 (0.5 g nitrate/L)	3.2%	Saltwater
LSW1	82.4 (0.5 g nitrate/L)	2.5%	Saltwater
LSW2	82.4 (0.5 g nitrate/L)	1.5%	Saltwater
LSW3	82.4 (0.5 g nitrate/L)	0%	Saltwater

A.3 Harvesting and drying

- The algal cells were harvested in their stationary growth phase.
- Harvesting was performed by centrifugation at 5000 rpm for 15 min.
- The harvested biomass cake with about 80% moisture was washed with distillated water to remove non-biological material such as mineral salt precipitates.
- The pellets were kept in -80°C for 48 hours and then dried using a lyophilizer.

- The dried biomass was grounded to powder using mortar and pestle prior to fatty acid extraction.

A.4 Lipid extraction

Lipids were extracted using a modified procedure of Bligh and Dyer [7].

The dried biomass was weighted exactly.

- The dried algae were homogenized in a chloroform/methanol/water solution with a volume ratio of 1:2:0.8, respectively (for each 100 mg: 100 µl chloroform, 200 µl methanol and 80 µl water).
- After adding each solvent cover and mix it for several seconds.
- An equal volume of water and chloroform was added to the mixture to reach a final ratio of 2:2:1.8 (for each 100 mg: 100 µl chloroform and 100 µl water).
- The mixture was left at room temperature overnight.
- The mixtures was filtered through a filter paper and funnel to remove the algae pellets and then allowed to resettle to obtain a two-phase mixture.
- The upper layer (alcoholic layer included residues) was separated from the lower phase (chloroform layer contained lipids) with glass pipet (or syringe and needle) and transferred to the first tube (tube which was contained biomass)
- The alcoholic phase and also the residues that remained on the filter were re-extracted with chloroform/methanol with a volume ratio of 2:1 (for each 100 mg: 200 µl chloroform and 100 µl methanol).
- The chloroform layers obtained from these two steps were combined together.

- Wash it by adding 0.25 volumes of 0.88% (wt/vol) potassium chloride and then 1 volume of methanol (for each 100 mg: 25 µl 0.88% (wt/vol) potassium chloride and 100 µl methanol)
- Leave it without any cover for one hour to be separated phases
- Separate the upper layer (alcoholic layer included residues) carefully with the glass pipet (or syringe and needle)
- Leave it for several hours to dry and obtain the lipid content (it may take several hours to dry and remain the lipids).
- The lipid contents were obtained by subtracting the weight of the empty flask from the flask containing lipids. The percentage of the lipid content was determined by Equation (1) [8]:

$$\text{Lipid content } \% = \frac{\text{Weight of lipid (mg)}}{\text{Weight of dried microalgae biomass (mg)}} \times 100 \quad (1)$$

- Cover and store it for transesterification

A.5 Transesterification [8]:

- Mix every 10 mg of lipid sample with 1 ml of toluene
- Add 1 ml of "1.5% of sulfuric acid in dry methanol"
- Mix well, cover and incubate at 55 °C overnight (in water bath)
- Let it to cool down for several minutes and then open the lid
- Add 2 ml of saturated NaCl solution and vortex
- Add 1 ml of hexane
- Add 1.5 ml of sodium hydrogen carbonate (2% $NaHCO_3$)
- Vortex well
- Take the upper phase for gas chromatography analysis

- Weigh the mixture and write the exact weight and the volume

A.6 Gas Chromatography Analysis

The analysis of fatty acid methyl esters (FAME) was performed using a gas chromatograph GC-2014 (Shimadzu, Columbia, MD, USA), equipped with an auto-sampler and a flame ionization detector (FID).

- A Zebron™ZB-FFAP polar capillary column (30 m × 0.32 mm × 0.25 μm film thickness; Phenomenex, Torrance, CA, USA) was used to separate the FAME.
- The carrier gas was helium at a linear velocity of 35 cm/s.
- The column was subjected to a temperature program for separation as follows: initial column oven temperature of 150 °C held for 3 min, heating at 1.5 °C/min to 240 °C and sustained for 15 min.
- The injector (FAME samples of 2 μL each and split ratio 10:1) and detector temperature were kept constant at 250 °C during the 78-min analysis.
- The compounds were identified and quantified by comparing the peaks with Marine Oil Test Mix (Restek Corp., Bellefonte, PA, USA) FAME standards. The provided GC data are based on duplicate measurements.

A.7 Experimental Data

All experimental data were listed in the following tables.

A.7.1 Aim 1 experimental data

Table A.8 Cell numbers versus growth time for Aim 1 experiments

Experiments	A1	A2	A3	A4	A5	A6	A7
Growth time (days)	Cell Numbers (Cells/mL)						
0	5000	5000	5000	5000	10000	5000	5000
1	175000		13750	97800		33300	
2	123300	308000					
3			31670				
4	437143	806666.7		50000		101100	
5	923300	808000					92000
6			140000		77142.9	137800	
7		1182500		77500			
8	710000		194000		280000		
9				216670			
10	1410000		388000				182500
11		1335000		1280000		71400	
12	1540000						300000
13			460000	1240000	806667		
14						448600	
15					518571		
16	2340000		1355000	2386700	660000		394000
17	2376000						522000
18	2450000		1330000	1846700			626000
19	2963300				1510000	290000	762000
20		2045000	2020000			432500	722500
21				1723300	1972000		
22	2946700	1266667	1520000				
23				1560000	2477500		1260000
24	2972500		1130000			285700	1022000
25	2483300	1880000		1653300			1094000
26	2876700					375600	1316000
27			1133333		2810000		
28	2446700	1846667	1640000	1913300		341670	
29		1580000			3000000		
30				2046650	3113333		
31							
32						418330	

Table A.9 Growth rates versus growth time for Aim 1 experiments

Experiments	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**
Growth time (days)	**Growth Rate (day^{-1})**						
1	3.555		1.012	2.973		1.896	
2	-0.350	2.060					
3			0.417				
4	0.633	0.481		-0.224		0.370	
5	0.748	0.002					0.582
6			0.495		0.341	0.155	
7		0.190		0.146			
8	-0.088		0.163		0.645		
9				0.514			
10	0.343		0.347				0.137
11		0.030		0.888		-0.132	
12	0.044						0.249
13			0.057	-0.016	0.212		
14						0.613	
15					-0.221		
16	0.105		0.360	0.218	0.241		0.068
17	0.015						0.281
18	0.031		-0.009	-0.077			0.182
19	0.190				0.276	-0.087	0.197
20		0.047	0.209			0.400	-0.053
21				-0.019	0.133		
22	-0.002	-0.240	-0.142				
23				-0.041	0.114		0.185
24	0.004		-0.148			-0.104	-0.209
25	-0.180	0.132		0.028			0.068
26	0.147					0.137	0.185
27			0.001		0.031		
28	-0.081	-0.006	0.370	0.045		-0.047	
29		-0.156			0.033		
30				0.032	0.037		
31							
32						0.051	
33				-0.017	0.004		
34						0.126	
Total Growth rate	0.221	0.198	0.207	0.184	0.191	0.138	0.174

Table A.10 Biomass and lipid amounts for Aim 1 experiments

	Dried Biomass (g)	Lipid (mg)	Biomass Productivity	Lipid Productivity	Lipid contents (%)
A1	1.462	12.429	91.674	0.779	0.85
A2	1.070	13.00	67.091	0.815	1.215
A3	0.913	10.044	63.008	0.617	0.98
A4	1.144	11.207	59.292	0.652	1.10
A5	2.672	12.70	142.898	0.679	0.475
A6	1.004	10.90	53.663	0.583	1.086
A7	0.431	5.00	30.133	0.350	1.160

Table A.11 Area obtained from Gas Chromatography analysis to measure fatty acid compositions for Aim 1 experiments

Standard (Marine Oil Mix)	**A1**		**A2**		**A3**		**A4**		**A5**		**A6**		**A7**	
Fatty Acids	**Area**	**Area %**	**Area**	**Area %**	**Area**	**Area %**	**Area**	**Area %**	**Area**	**Area %**	**Area**	**Area %**	**Area**	**Area %**
Methyl Myristate (C14:0)	-	-	12559	0.192	-	-	-	-	17553	0.218	13928	0.261	1428	0.296
Methyl myrostoleate (C14:1)	-	-	-	-	-	-	-	-	-	-	4243	0.080	1734	0.359
-	-	-	23013	0.352	-	-	1450	0.397	56986	0.708	11353	0.213	3243	0.672
-	-	-	-	-	-	-	-	-	-	-	8962	0.168	2336	0.484
-	-	-	-	-	-	-	-	-	-	-	35569	0.667	-	-
Methyl palmitate (C16:0)	96841	30.503	2E+06	25.032	79888	27.711	97136	26.577	2E+06	29.548	1E+06	27.370	147510	30.546
-	-	-	76740	1.173	-	-	3084	0.844	198454	2.467	-	-	-	-
Methyl palmitoleate (C16:1)	3266	1.029	11287	0.172	2737	0.949	3797	1.039	12056	0.150	107525	2.017	13229	2.739
-	3541	1.115	43299	0.662	3682	1.277	2582	0.706	103165	1.282	9091	0.171	7541	1.562
-	-	-	22192	0.339	-	-	-	-	67466	0.839	13615	0.255	-	-
-	-	-	-	-	-	-	-	-	30004	0.373	47225	0.886	-	-
-	-	-	-	-	-	-	-	-	-	-	38303	0.719	-	-
Methyl stearate (C18:0)	7962	2.508	199980	3.055	8623	2.991	12675	3.468	64588	0.803	120136	2.254	13837	2.865
Methyl oleate (C18:1)	10128	3.190	253343	3.871	10953	3.799	15664	4.286	118434	1.472	124519	2.336	7555	1.564
Methyl octadecenoate (C18:1)	-	-	21094	0.322	-	-	-	-	100536	1.250	20167	0.378	3782	0.783
-	-	-	-	-	-	-	-	-	-	-	34048	0.639	-	-
Methyl linoleate (C18:2)	66653	20.994	2E+06	24.612	64508	22.376	100711	27.556	57322	0.712	1E+06	19.474	59988	12.422
-	2891	0.911	46657	0.713	2297	0.797	3047	0.834	-	-	5108	0.096	-	-

-	-	-	-	-	-	-	-	-	-	-	19961	0.374	-	-
Methyl linolenate (C18:3)	-	-	3800	0.058	-	-	-	-	1E+06	12.817	20556	0.386	-	-
-	2283	0.719	-	-	-	-	-	-	-	-	-	-	-	-
-	2445	0.770	-	-	-	-	-	-	-	-	-	-	-	-
Methyl eicosenoate (C20:0)	-	-	2466	0.038	-	-	-	-	6076	0.076	-	-	-	-
Methyl arachidate (C20:1)	-	-	10408	0.159	-	-	-	-	4303	0.053	7155	0.134	-	-
Methyl eicosadienoate (C20:2)	2603	0.820	122001	1.864	3052	1.059	4683	1.281	11026	0.137	134809	2.529	8259	1.710
-	9015	2.840	168816	2.579	3094	1.073	10462	2.863	172459	2.143	99771	1.872	5782	1.197
-	-	-	-	-	5557	1.928	-	-	96014	1.193	-	-	-	-
Methyl arachidonate (C20:4)	75982	23.933	2E+06	26.051	75388	26.150	85547	23.407	2E+06	30.537	1E+06	25.089	140555	29.105
Methyl eicosatrienoate (C20:3)	-	-	6297	0.096	-	-	-	-	10929	0.136	4872	0.091	-	-
Methyl eisocapentaenoate (C20:5)	30103	9.482	566779	8.660	28510	9.889	24646	6.743	1E+06	13.085	615088	11.540	62999	13.045
Methyl behenate (C22:1)	3770	1.187	-	-	-	-	-	-			-	-	-	-
Total Area	317483	-	7E+06	-	288289	-	365484	-	8E+06	-	5E+06	-	482918	-

- No *Methyl erucate (C22:0), Methyl lignocerate (C22:6), Methyl docosahexaenoate (C24:0) and Methyl nervonate (C24:1) were not detected in the P. cruentum cells.*

Table A.12 Saturated, mono and polyunsaturated fatty acids and ARA/EPA ratio from Aim 1 experiments

	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**
Saturated %	33.011	28.318	30.702	30.045	31.292	29.886	33.707
Monounsaturated %	5.406	4.525	4.749	5.325	2.249	4.946	5.446
Polyunsaturated fatty acids (PUFAs) %	55.228	61.341	59.474	58.987	58.795	59.109	56.283
Other Area%	6.355	5.817	5.075	5.643	7.665	6.060	4.564
ARA/EPA	2.524	3.008	2.644	3.471	2.334	2.174	2.231

A.7.2 Aim 2 experimental data

Table A.13 Biomass and lipid amounts for Aim 2 experiments

Experiments	Biomass (g)	Biomass Productivity (g/L)	Lipid (mg)	Lipid Productivity (mg/L)	Lipid Contents %	Growth Time (days)
L1	0.6173	2.058	25.9	86.333	4.196	24
LS1	1.1265	3.755	22.2	74.0	1.971	25
LS2	0.1663	0.554	6.1	20.333	3.668	24
LSW	0.3522	1.174	14.6	48.667	4.145	24
LSW1	0.2282	0.761	1	3.333	0.438	24
LSW2	0.0946	0.315	2.9	9.667	3.066	24
LSW3	0.0489	0.163	1.5	5.0	3.067	22
S1	0.5822	1.941	20.3	67.667	3.487	21
S2	0.0654	0.218	2.1	7.0	3.211	21
S3	0.1027	0.342	1.2	4.0	1.168	21
S4	0.4092	1.364	17.3	57.667	4.228	20
S5	0.0398	0.133	1.6	5.333	4.020	24
S6	0.0771	0.257	0.7	2.333	0.908	24
SW	0.0776	0.259	0.7	2.333	0.902	25
SW1	0.5824	1.941	6.9	23.0	1.185	21
SW2	0.1359	0.453	1.3	4.333	0.957	25
SW3	0.0626	0.209	0.6	2.0	0.958	25
SW4	0.0508	0.169	0.5	1.667	0.984	24
SW5	0.0476	0.159	0.5	1.667	1.050	25
SW6	0.0357	0.119	0.4	1.333	1.120	24
SW7	0.0089	0.030	0.07	0.233	0.787	25
N1	1.3087	4.362	17.6	58.667	1.345	25
N2	1.0089	3.363	22.2	74.0	2.200	20
N3	0.9105	3.035	42.9	143.0	4.712	18
N4	0.4792	1.597	15	50.0	3.130	18

Table A.14 Area obtained from Gas Chromatography analysis to measure fatty acid compositions for Aim 2 experiments

Experiments	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1 [cis-9]	C18:1 [trans]	C18:2	C20:2	C20:4 (ARA)	C20:5 (EPA)	C22:1	ARA/EPA
L1	0.797	-	34.386	1.667	9.364	-	3.611	10.681	1.662	20.526	6.714	9.008	3.057
LS1	1.122	-	35.339	2.443	20.221	-	3.165	6.898	-	21.397	5.609	-	3.815
LS2	0.807	-	41.059	3.842	16.160	-	-	5.997	-	20.030	3.804	-	5.266
LSW	1.145	-	40.636	2.120	19.251	-	-	4.286	-	15.999	7.780	-	2.056
LSW1	1.040	-	38.121	2.293	22.102	-	2.546	4.340	-	16.200	7.170	2.293	2.259
LSW2	0.901	-	35.927	3.387	18.118	-	5.161	3.425	-	17.178	6.880	-	2.497
LSW3	0.850	-	31.144	1.894	17.218	-	2.943	2.299	-	8.551	2.213	-	3.864
N1	0.731	-	31.742	2.096	8.894	3.458	0.869	5.952	0.930	18.959	12.253	0.848	1.547
N2	0.392	-	31.613	2.348	5.817	1.815	0.740	4.082	0.669	21.581	15.155	-	1.424
N3	0.631	-	41.877	1.639	7.681	2.949	2.116	7.902	-	17.504	13.138	-	1.332
N4	-	-	45.978	-	7.755	-	-	8.635	-	20.486	9.197		2.227
S1	0.301	0.151	29.368	1.117	3.266	1.763	0.475	8.084	5.250	15.077	8.275	0.388	1.822
S2	0.280	-	44.739	1.846	3.095	1.318	0.445	11.197	0.392	19.707	9.986	-	1.973
S3	0.457	-	49.187	1.827	3.018	1.404	0.826	10.619	-	15.984	7.650	-	2.089
S4	No Fatty Acids												
S5	0.395	-	46.231	2.906	4.036	0.354	1.264	9.259	-	18.977	12.846		1.477
S6	No Fatty Acids												
SW	No Fatty Acids												
SW1	0.358	0.188	35.254	1.711	4.450	1.253	0.556	7.719	0.484	23.253	14.685	-	1.583
SW2	0.757	-	40.784	1.714	9.898	-	-	5.211	-	15.789	11.115	-	1.421
SW3	0.867	-	36.760	2.165	5.881	1.287	1.142	5.914	-	16.992	12.034	5.206	1.412
SW4	0.476	0.105	35.118	2.228	2.707	0.843	1.338	7.266	0.230	22.744	16.024	-	1.419
SW5	0.812	-	42.672	2.121	5.144	1.094	0.941	6.954	-	16.404	11.178	-	1.468
SW6	0.679	-	26.488	0.825	7.983	0.637	0.735	2.877	10.973	6.702	3.504	0.924	1.912
SW7	2.132	-	45.253	-	31.430	-	-	-	1.882	2.735	-	-	

A 7.3 Biosynthesis pathways to produce EPA and ARA

Long chain polyunsaturated fatty acids such as EPA can be synthesized from C18 fatty acids in microalgal cells using desaturase and elongase enzymes through a series of reactions. Most algae, bacteria, fungi, and mosses possess the desaturase and elongase required for the biosynthesis of EPA. They are the primary producers of these fatty acids in nature. In contrast, animals and other invertebrates lack the requisite Δ12 and ω3 desaturases, and thus cannot synthesize linoleic acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) from oleic acid (OA, 18:1 ω-9). These fatty acids are in the group of the essential fatty acids for human health [9]. The *de novo* synthesis of OA from acetate is the first step followed by conversion of OA to LA and ALA. A number of subsequent stepwise desaturation and elongation steps form the ω-3 PUFA family including EPA (Figure A.1) [10]. *Cis* double bonds can be introduced at specific positions in the fatty acid chain via catalyzing by fatty acid desaturases. Furthermore, the fatty acid elongation performs using malonyl-CoA. It provides two carbon units and commits them to an existing acyl-CoA moiety [11].

The biosynthesis of long chain polyunsaturated fatty acids is initiated by the carboxylation of acetyl-CoA to form acetate or pyruvate using glycolytic enzymes. Acetyl-CoA is converted into malonyl-CoA, which is used to drive a condensation reaction to extend the acyl group to stearic acid (18:0) and desaturate to oleic acid (OA, $18:1^{\Delta 9}$, ω-9) (Figure A.1). Then, oleic acid is desaturated by a Δ12 desaturase and generates linoleic acid (LA, $18:1^{\Delta 9,12}$, ω-6). Subsequently, LA may be desaturated using a Δ15 desaturase to form α-linolenic acid (ALA, $18:1^{\Delta 9,12,15}$, ω-3). The biosynthesis of the three families of fatty acids (ω-3, ω-6 and ω-9) is shown in Figure A.1 and Figure A.2 [10,12]. The three parent fatty acids, OA, LA and ALA, compete with each other

for the Δ6 desaturase. The affinity of the enzyme to the substrate and its quantity determine which metabolic pathway is predominant [13]. In general, the limiting step is the first Δ6 desaturation and the highest affinity for Δ6 desaturase belongs to ALA followed by LA and oleic acid [10]. There are also alternative pathways that starts with Δ9 elongation of LA or ALA to form eicosadienoic acid (EDA, $20:2^{\Delta 11,14}$, ω-6) or eicosatrienoic acid (ETA, $20:3^{\Delta 11,14,17}$, ω-3), respectively, followed by sequential Δ8 and Δ5 desaturations. In the ω-6 and ω-3 pathways, Δ6 desaturation of LA and ALA, further Δ6 elongation and Δ5 desaturation through respective intermediates finally produce ARA or EPA [12]. The key enzyme for EPA biosynthesis is the Δ5 desaturase. Any mutant strain deficient in this enzyme activity cannot synthesize EPA through the desaturation and elongation pathway. The Δ6 and Δ3 pathways can be interconnected by the Δ3 desaturase, which converts Δ6 fatty acids into their Δ3 counterparts [14].

Some microalgae, such as the *Phaeodactylum tricornutum,* have both the omega-3 and omega-6 pathways as active [15]. Nevertheless, sometimes one pathway may surmount over the other one. For example, in the *P. incise* [16], the *eustigmatophytes*, the *Monodus subterraneus* [17], and the *Nannochloropsis sp.* [18,19], the omega-6 pathway is the major pathway. In some microalgae such as *Nannochloropsis sp.* [18], *M. subterraneus* [17], and *P. cruentum* [14], C20 omega-3 desaturation is mediated by Δ17 desaturase, which desaturates ARA to EPA.

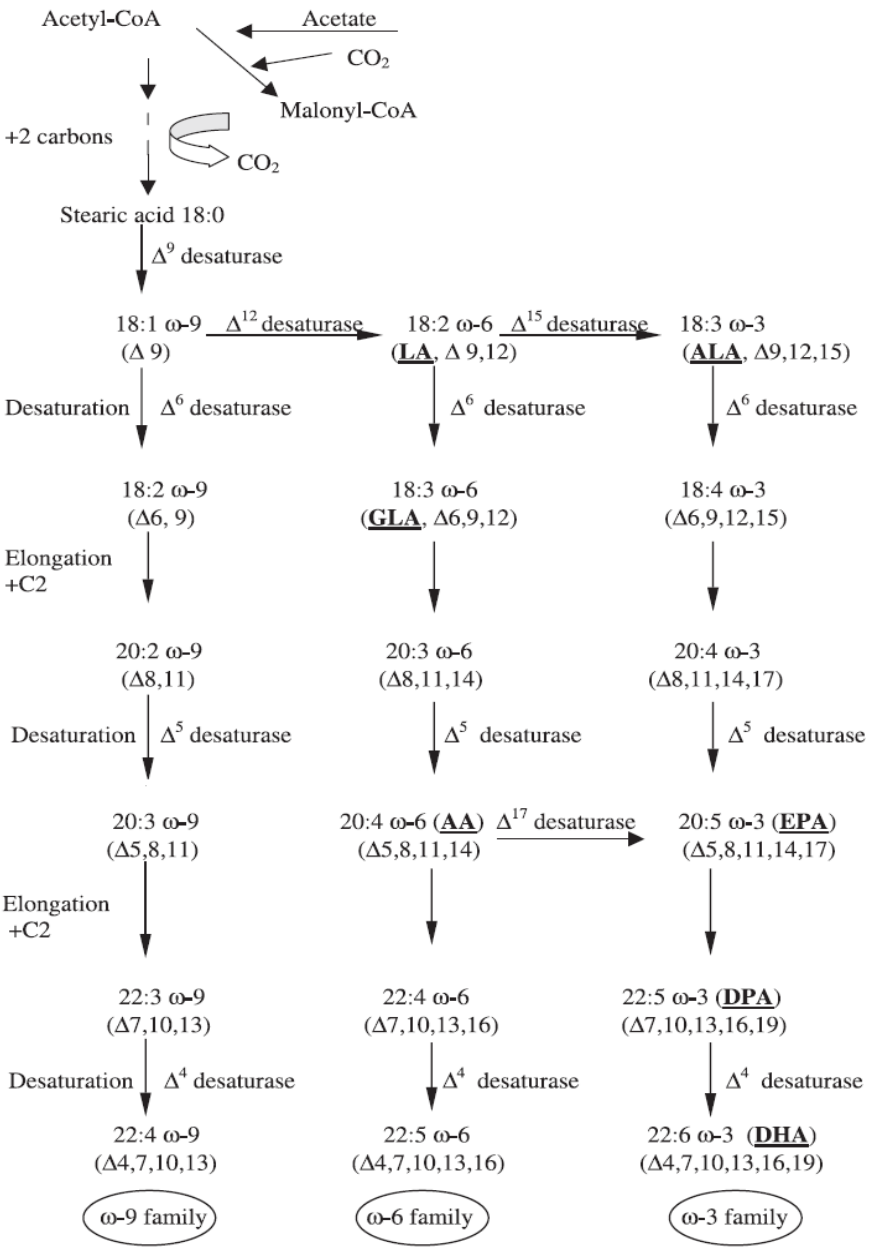

Figure A.1 The biosynthesis of three families of polyunsaturated fatty acids [10].

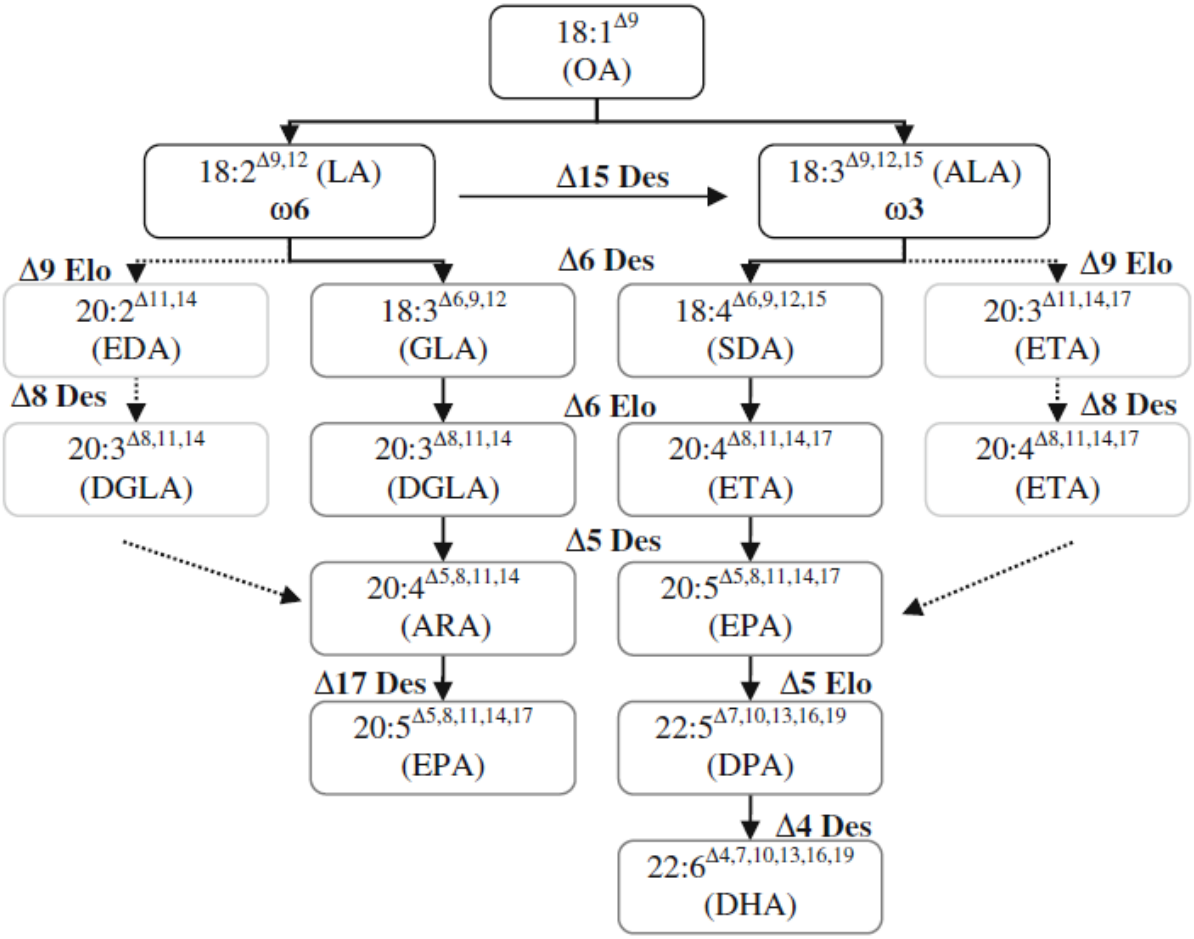

Figure A.2 Pathways for the biosynthesis of long chain PUFAs in microalgae [12].

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APPENDIX B. OXYGEN TRANSFER IN MODIFIED POLYSULFONE HOLLOW FIBER MEMBRANES FOR ARTIFICIAL LUNG APPLICATIONS

B.1 Abstract

Artificial lungs may be employed as a supplementing respiratory support in patients who suffer from chronic or acute respiratory failure. They can remove carbon dioxide as well as doing oxygenation. In this study, polysulfone hollow fiber membranes have been employed to transfer oxygen from liquid phase. The fibers have been modified using peptoid to decrease bio-fouling on the membrane surface. Data from tensile strength and pore size tests showed no significant changes for coated fibers compared to uncoated ones. Results indicate that hollow fiber membranes with peptoid modification have higher oxygen transfer coefficient and less fouling compared to unmodified ones. Over 5 hours, the peptoid surface had a 5.7% drop in flux while an unmodified surface had a 40.6% loss in flux. Overall, these findings reveal that enhanced oxygen transfer achieved by immobilized peptoid fibers to have more efficient oxygenators in respiratory assist devices.

Keywords:

Artificial lung, Bio-fouling, Oxygen transfer, Hollow-fiber membranes, Peptoid, Polysulfone

B.2 Introduction

One of the significant healthcare problems between several hundred thousand adult patients in the US is medical treatment for acute and chronic respiratory failure. The respiratory system (Figure 1) is responsible for supplying the blood with oxygen and releasing unneeded carbon dioxide from the body. The term respiration includes three separate but related functions: Ventilation (breathing); Gas exchange between the air and blood in the lungs (across the walls of tiny air sacs called alveoli) and between the blood and other tissues of the body; Oxygen utilization by the tissues in the energy-liberating reactions of cell respiration. The main tasks of artificial lungs are included to oxygenate a patient's blood and to remove carbon dioxide from it, to realize a long contact time between blood and ventilation gas, a short diffusion length to provide a sufficient gas exchange and an adequate volume flow to ensure that the amount of aerated blood is large enough to supply the patient's body [1].

Hollow fiber membranes (HFMs) have been used as the interface between gas pathways and blood in artificial lung devices. Oxygen transfer and carbon dioxide removal have been investigated during the last 10 years in an attempt to increase survival outcomes for patients suffering from acute lung failure. Development of a modified hollow fiber membrane which facilitates CO_2 removal and O_2 transfer may be required to prepare highly efficient gas transmitting membranes for respiratory assist devices. Several new devices for respiratory assist have been developed by some researchers which increase gas exchange while requiring blood flow rates as small as 300–500 mL/min [2].

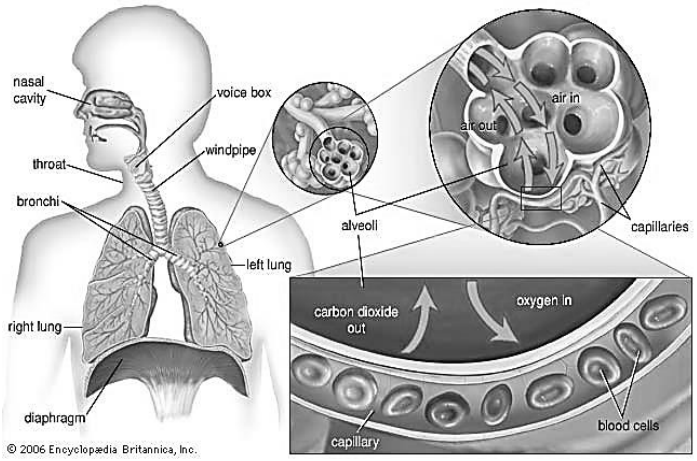

Figure B.1 A diagram of the ventilation system,

including trachea, lungs, bronchi, bronchioles and alveoli [3].

Polysulfone hollow fibers have been widely used as appropriate membranes in biomedical membrane applications due to its high chemical, physical and thermal stability, high porosity, and film forming properties. However, when polysulfone is exposed to biological fluids proteins and other biological materials adsorb to the membrane surface and within its pores, referred to as membrane fouling [4-6]. The fouling of membranes leads to a decrease in flux across the membrane, coagulation, substantial energy consumption, and significant operational cost increases. Therefore another major challenge in a wide range of implanted medical devices is preventing nonspecific protein adsorption on the surface. In the case of implanted medical devices, the adsorption of nonspecific protein can lead to harmful events such as blood clot formation, bacterial infection, compromised implant performance or even implant failure, and increased healthcare costs [7].

There are some artificial lungs in use, but difficult issues remain in the field of membrane development related to gas exchange and fouling. Currently there are external artificial lungs circulating blood outside the body, taking out the carbon dioxide, and inserting oxygenated blood

back into the body. The ExtraCorporeal Membrane Oxygenation (ECMO) machine is an example of this type of machine currently used in hospitals (Figure 2). ECMO takes over the functions for both the lungs and the heart but is only available for short term use by patients with respiratory failure due to infections [8]. Long term exposure to protein in the blood coupled with small surface areas of fibers lead to fouling on the fiber surface in the machine. Gas transfer abilities of the fibers are declined due to fouling on the surface until the machine is no longer effective at exchanging gases with the blood.

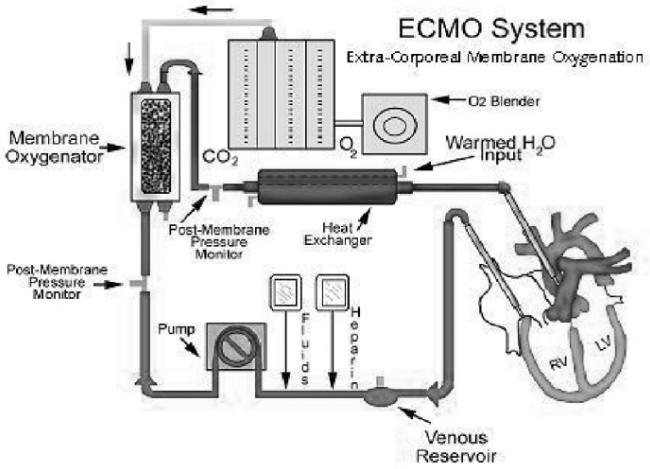

Figure B.2 An example of the ECMO system as an external lung [9]

Some studies developed several novel respiratory assist devices which mechanically improve gas exchange by actively mixing the blood flow over the membrane surface [10]. The relatively small partial pressure difference through the membranes in some current respiratory assist devices limits O_2 transfer and CO_2 removal efficiency. To offset this basic diffusional challenge, Arazawa et al. utilized bioactive hollow fiber membranes to increase CO_2 diffusional gradient. The immobilized enzyme carbonic anhydrase (CA) has been used to covert bicarbonate to CO_2 directly at the fiber surface [10]. They employed a model gas exchange device with

volume of 0.0084 m^2 to test CO_2 removal from the blood. CO_2 removal rate was 108 mL/min/m^2 for the bioactive hollow fibers, with a 36% increase compared to unmodified hollow fibers. The diffusional properties of the bioactive membranes and the effect of enzyme attachment on carbon dioxide removal were investigated by Kaar et al. [11]. Plasma deposition of surface reactive hydroxyls to which CA can be attached, did not affect the gas performance of the hollow fibers in short times and low plasma discharge power. Furthermore, enhanced CO_2 removal rate of 75% was resulted from bicarbonate solutions using the bioactive membranes with no enzyme leaching when employed in a model respiratory assist device [11].

Some studies illustrated mathematical models which can predict the gas exchange performance of artificial lungs. These models would be critical to understand and optimize the gas exchange devices. Potkay (2013) developed a simple mathematical model for gas exchange of artificial lungs. The model was qualified with experimental data from several research groups. This model can be utilized in order to maximize oxygen exchange efficiency. Furthermore, it can be used to set the blood channel dimensions (length and width) required to achieve the desired PO_2, oxygen saturation, or oxygen exchange rate at a given blood flow rate, if the membrane thickness and blood channel height are fixed by the construction technology. However, it has to consider some other factors, such as pressure drop, the size of blood components, shear stress, mechanical durability of the device and others, to provide a full understanding of an artificial lung and provide a powerful tool for comprehending and designing the operation of these devices [2].

Hoganson et al. studied carbon dioxide and oxygen exchange in the device in vitro using three membranes. Plasma impermeable silicon only and silicone-coated microporous polymer were used in this study. The third membrane was a microporous polymer which used as a

control. They illustrated that the silicone and silicone-coated microporous polymer membranes both show promise as gas permeable membranes in a new lung assist device design and there was no difference between gas exchanges of these two membranes. Improving the permeability of the membranes and decreasing the channel diameter in the vascular network while optimizing physiologic blood flow can improve gas exchange in lung assist devices [12].

Sreenivasan et al. fabricated large-area, ultra-thin, polymeric free-standing membranes (FSMs) and composite membranes (CMs) using initiated Chemical Vapor Deposition (iCVD). The FSMs (with 5 μm thick) exhibited a CO_2 permeance of 1.3 times more than the control membrane (8 μm thick spun-cast membrane of silicone). They placed the CMs consisted of a dense iCVD skin layer (0.5-3 μm thick) on top of a polytetrafluoroethylene (PTFE) support membrane (20 μm thick, 100 nm pores). The CO_2 and O_2 permeance values of the CMs were 50-300 times more that the control membrane [13].

An ideal artificial lung should have some characteristic to be most similar to an actual lung and maintain the standard of patient living. Some of these features are included: small enough to fit in a lung cavity, to oxygenate blood and remove carbon dioxide from blood, to operate with no external power supply or pump and little or no biofouling. Although there have been many efforts to develop artificial lungs, but there is still no respiratory device that meet all of these characteristics. This present study sought to further improve oxygen transfer performance, the stability and strength of polysulfone hollow fiber membranes as well as decreasing biofouling on the surface of the fibers without immobilization of peptoid as an antifouling agent. This study demonstrates enhanced oxygen transfer and less biofouling with immobilized peptoid fibers compared to unmodified ones.

B.3 Methods

B.3.1 Polysulfone Hollow Fiber Membrane Fabrication

The polysulfone hollow fiber membranes were made via phase inversion techniques by immersion precipitation. The phase inversion technique is the process in which the polymer solution (polymer plus solvent) is submerged into a precipitation bath containing a non-solvent to the polymer or a mixture of non-solvent and solvent. After immersion, mass transfer occurred between bath and polymer solution and precipitation takes place. The solvent diffuses from the polymer into the precipitation bath, whereas the non-solvent diffuse from bath into the polymer solution. The combination of mass transfer and phase separation influences the membrane structure [14].

The fibers were made using a conventional hollow fiber membrane spinning device (figure 1) consisting of a water bath and spinneret (with ID of 0.8 mm and OD of 1.3 mm). Pressurized nitrogen gas is used to extrude dope and bore solutions into a spinneret (10 psi for bore solution and 90 psi for dope solution). Dope solution contains a volume ratio of polysulfone polymer in N-Methylpyrrilidone (NMP) as a solvent which coagulates to form the wall of the membrane and bore solution constants of the solvent component which created the hollow center of the fibers and disperses through the wall to form pores. The bore solution was the solvent that consists of 15% NMP in water. The solutions were pushed via the spinneret into the water bath and phase inversion occurs causing the liquid Polysulfone to solidify into hollow fiber membranes. The air gap between the water and the spinneret was kept at 1.5 cm. Then, the fibers were pulled under dowels, immersed in the water, and rolled onto a draw wheel at an uptake

speed of 64 m/min (figure 3). Three sets of hollow fibers where made and analyzed with varying percentages (16.5%, 17.8% and 18.5%) of dope fluid content.

The prepared polysulfone hollow fibers were soaked in a 20% (v/v) isopropyl alcohol (IPA) solution at room temperature for 1 hour in an IPA solution to wet membrane pores and keel the porous structure of the polysulfone hollow fibers. Then, membranes are washed with ultra-pure water to remove the alcohol, and stored at 5 ºC in pure water [15].

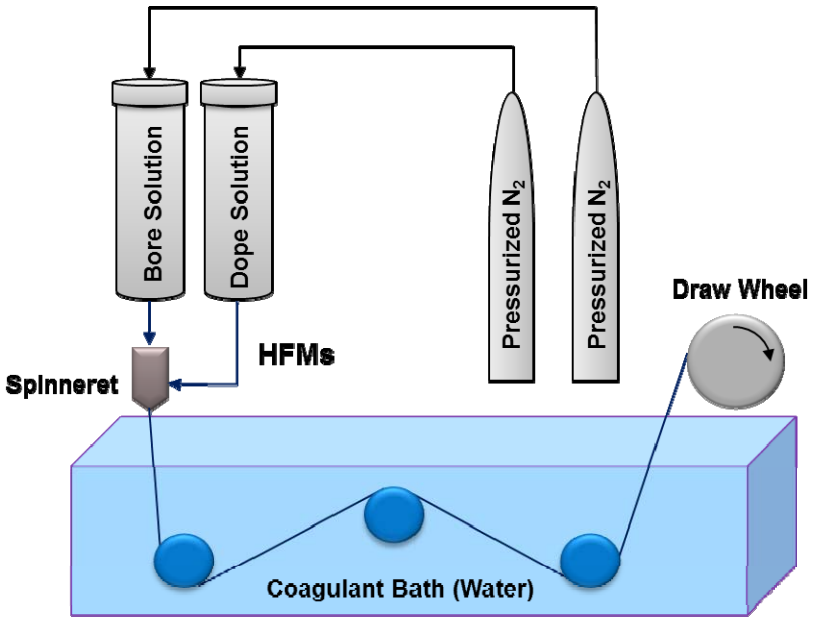

Figure B.3 Hollow fiber membrane spinning apparatus

B.3.2 Modification

Fouling resistance of HFMs can be improved by surface-immobilization of peptoid. Peptoid, or poly-N-substituted glycines, are a class of peptidomimetics whose side chains are appended to the nitrogen atom of the peptide backbone, rather than to the α-carbons (as they are

in amino acids). Peptoid was utilized as an anti-fouling because of its good biocompatibility. Peptoid was immobilized on the surface of polysulfone HFMs using polydopamine as a spacer.

B.3.3 Characterization of the membrane surface

B.3.3.1 Tensile Strength Testing

A biomaterial for a lung must be able to withstand forces while inhaling and exhaling and other various external forces. The tensile strength test was utilized to test different concentrations of unmodified hollow fiber membranes as well as modified fibers. Qualitest ESM301 device was used to measure the tensile strength of different concentrations of polysulfone fibers under peak tension setting.

B.3.3.2 Pore sizes

An evapoporometry technique was used to estimate the average pore size for the HFMs. Evapoporometry is a new technique to measure pore sizes which recently developed by Krantz et al. [16]. This method is based on the Kelvin equation that describes the vapor pressure depression of a volatile wetting liquid which will evaporate from the membrane progressing from largest to smallest pores due to the relationship between the pore radius, r, and the instantaneous vapor pressure, P'. In this technique, the membranes are glued in a specially designed test cell and then they have been pre-saturated with a volatile wetting liquid such as isopropyl alcohol (IPA). The test cell is placed on a microbalance overnight in order to measure the evaporation rate. First, evaporation occurs from the free standing liquid layer on top of the fibers. This evaporation rate should be relatively constant as long as the temperature of the lab remains constant. Once the free standing liquid has completely evaporated, the evaporation from

the pores will begin. It is assumed that the largest pores will evaporate first because liquid draining from certain pores are saturated and the pores smaller than them are supersaturated [17]. The evaporation rate will decrease as the liquid is evaporating from the pores decrease in size. The Kelvin equation is as follows:

$$\ln\frac{P'}{P} = -\frac{2\sigma V}{RTr\cos\theta} \quad (1)$$

where P is the normal vapor pressure, σ the surface tension, P' the instantaneous vapor pressure, V the liquid molar volume, R the gas constant, θ the contact angle, T the absolute temperature and r the principle radii of curvature of the liquid-gas interface [16, 17]. Vapor pressure is related to evaporation rate according to Irving Langmuir's equation:

$$W = (P_v - P_p)\sqrt{\frac{m}{2\pi RT}} \quad (2)$$

where W is the evaporation rate, P_v is the vapor pressure of the liquid, P_p is the partial pressure of the vapor, and m is the mass of each molecule of the vapor when assuming that the partial pressure of the vapor is negligible [17]. The instantaneous evaporation rate can be related to the radius of the pores by the derived equation:

$$r = -\frac{2\sigma V}{RT\cos\theta \ln\left(\frac{W'}{W^o}\right)} \quad (3)$$

where W' is the instantaneous evaporation rate and W^o is the evaporation rate from the free standing liquid [17].

B.3.4 Oxygen transfer in a model oxygenator

Oxygen transfer experiments were performed in a model gas exchange module by potting ten hollow fiber membranes (46 cm) into a 1.5 cm ID nylon tubing. An epoxy resin, Epikure 3030/Epon 828 (Momentive), was used to secure both ends of the fibers to the tubing. The length of uncovered fibers within the module was 30 cm. A recirculating test loop was employed to assess O_2 transfer rates of 17.8% polysulfone hollow fibers and modified fibers with poly-dopamine and peptoid. The experimental setup included gas exchange module, peristaltic pump (Master Flex L/S), fluid container and DO probe. Fluid with 200 ml volume was tested in each experiment with the flow rate of 60 ml/min. The fluid (5 mg/ml bovine serum albumin (BSA) in phosphate buffer saline (PBS)) flowed from a fluid container using a peristaltic pump to the gas exchange module and back to the fluid container. Figure 4 presented a schematic of the oxygen exchange testing module. The inlet oxygen pressure was adjusted to around 15 kpa which is 2.5 SCFH flow rate. The fluid temperature was maintained at 37 °C by a water bath.

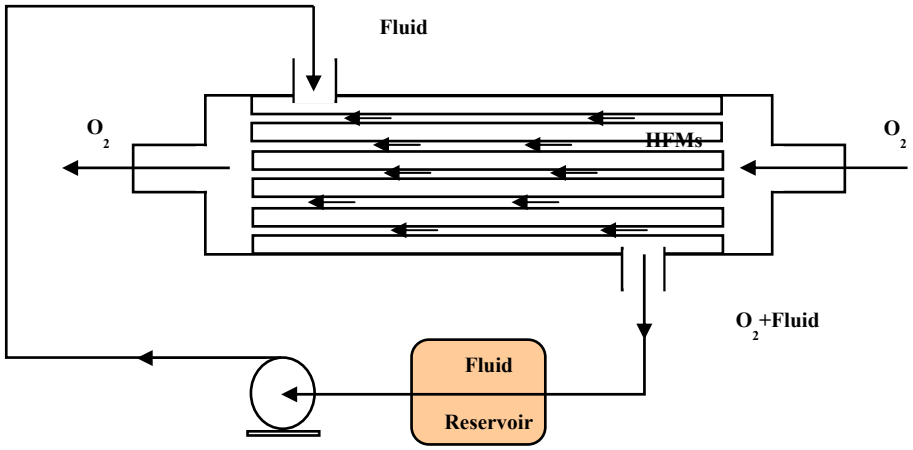

Figure B.4 A schematic view of the oxygen exchange testing module in this study.

B.3.5 Volumetric oxygen transfer coefficient

A dynamic method was employed in this study to measure volumetric oxygen transfer coefficient, k_La. Several assumptions were made in this method including: the main resistance to gas exchange occurs due to diffusion in the liquid side not through the hollow fibers, oxygen concentration remains constant while passing through the fibers, and both liquid and gas are perfectly mixed. Nitrogen was used to de-aerate the liquid phase by purging until only minimum levels of dissolved oxygen remain. Then, oxygen was flowed through the hollow fibers until saturation. Dissolved-oxygen concentration was observed during the operation time using a DO probe [18, 10]. As the oxygen concentration increases, the mass transfer rate is given by the following equation:

$$\frac{dC}{dt} = k_L a(C^* - C) \tag{4}$$

or, in its integral form by

$$Ln\frac{(C-C_0)}{(C^*-C_0)} = -k_L a.t \tag{5}$$

where C is liquid dissolved-oxygen concentration (mg/L); C^* is equilibrium concentration of oxygen between water and gas phase (mg/L); C_0 is liquid oxygen concentration at start of measurement (mg/L); k_La: is volumetric oxygen transfer coefficient (1/s) and t is time (s). k_La can be calculated from slope of $\ln(C-C_0)/(C^*-C_0)$ versus time.

B.4 Results and Disscusion

B.4.1 Tensile strength tests

The tensile strength and elongation tests were performed using an auto tensile tester on hollow fiber membranes with three concentrations of polysulfone in NMP (16.5%, 17.8% and 18.5%) to determine their strength and ductility. The strength and elongation results were shown in Figure 5 and 6, respectively. It can be seen that the maximum strength and elongation were related to the fibers with polymer concentration of 17.8%. Therefore, the experiments were performed with this concentration of polymer as an optimum concentration. Furthermore, average strength for peptoid modified fibers was approximately same as unmodified ones.

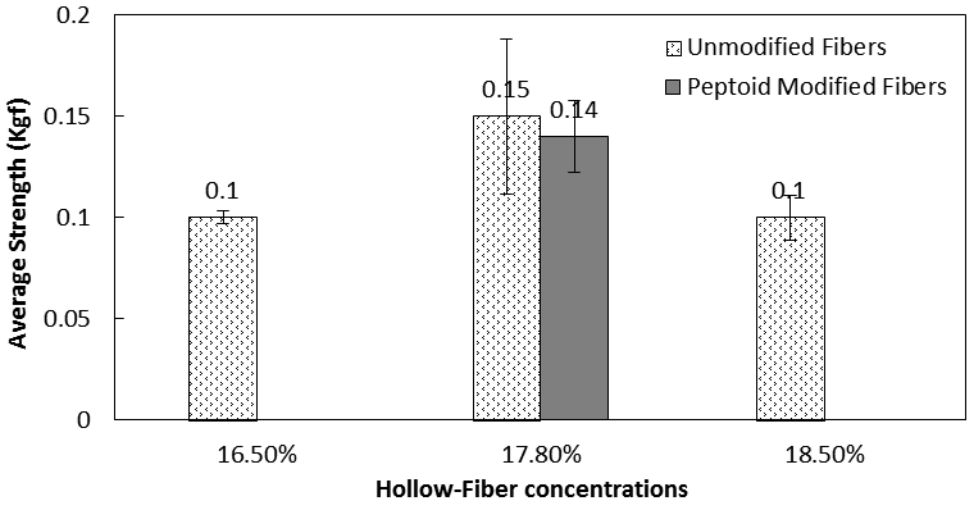

Figure B.5 Average tensile strength for different polymer concentrations of the unmodified hollow fiber membranes (16.5%, 17.8% and 18.5 % polymer) as well as peptoid modified fibers.

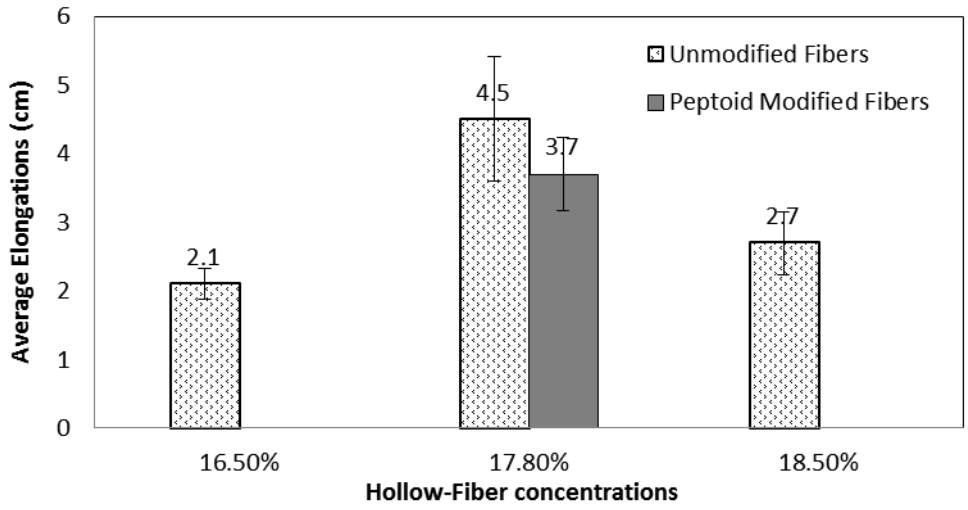

Figure B.6 Average elongation for different polymer concentrations of the unmodified hollow fiber membranes (16.5%, 17.8% and 18.5 % polymer) as well as peptoid modified fibers.

B.4.2 Pores sizes

The pore sizes of the hollow fibers with and without peptoid were measured using evapoporometry technique. The pore size distributions and the average pore sizes were shown in Figures 7 and 8, respectively. The average pore sizes found for unmodified and peptoid modified fibers were 19.7 nm and 16.2 nm, respectively. These data showed that immobilizing peptoid on the surface of polysulfone hollow fiber membrane did not show significant changes in pore sizes.

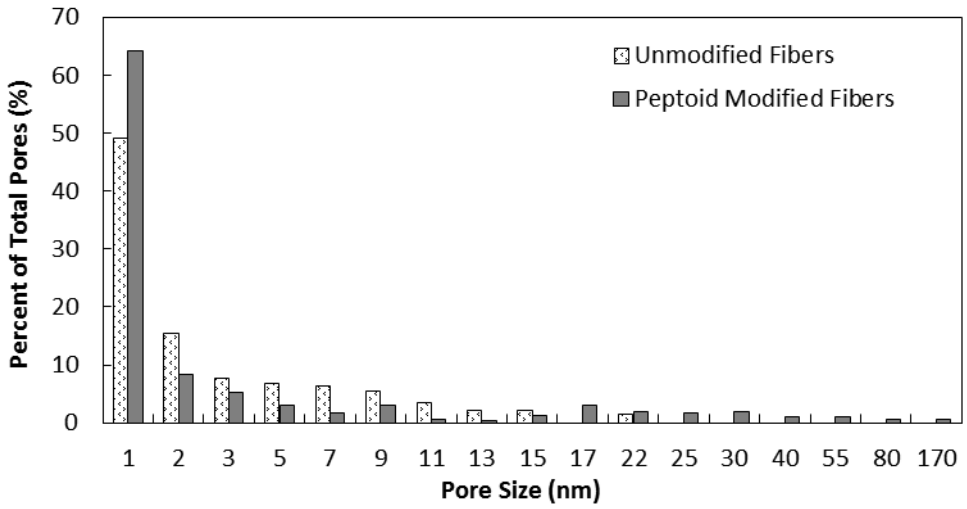

Figure B.7 Comparison of pore size with and without peptoid

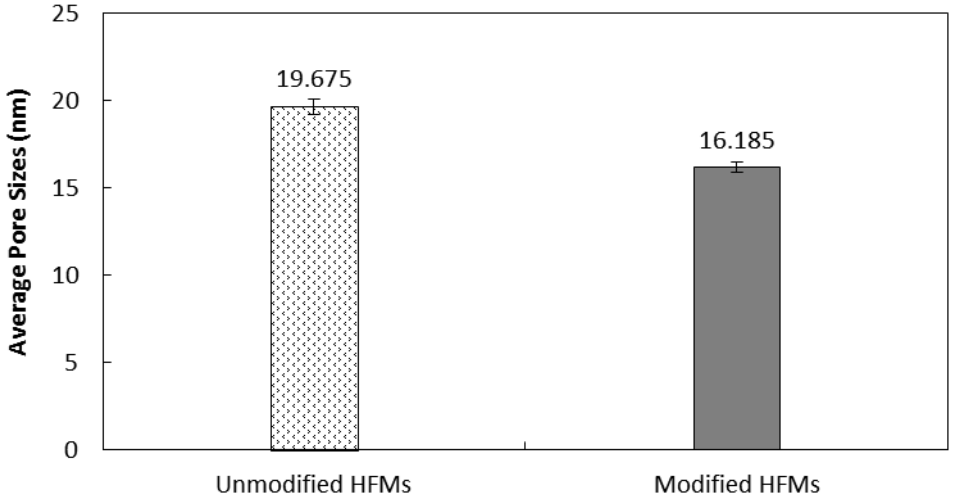

Figure B.8 Pores Sizes of hollow fiber membranes (Modified and Unmodified)

B.4.3 Oxygen transfer coefficient

Oxygen transfer coefficient, k_La, was evaluated for unmodified and peptoid modified hollow fiber membranes in the testing module. Figure 9 showed the oxygen transfer coefficients for unmodified and peptoid modified fibers in a solution of 5% BSA in PBS as liquid phase over

time. PBS was used as blank solution. Oxygen transfer coefficient were slightly increased (1.4%) when peptoid modified fibers was used in the oxygenator compared to unmodified fibers. Moreover, it is obvious from Figure 9 that oxygen transfer coefficients of unmodified fibers diminished over time due to fouling on the surface. Over 5 hours, the peptoid surface had a 5.7% drop in flux while an unmodified surface had a 40.6% loss in flux. In general, results indicate that hollow fiber membranes with peptoid modification have higher oxygen transfer coefficient and less fouling compared to unmodified ones.

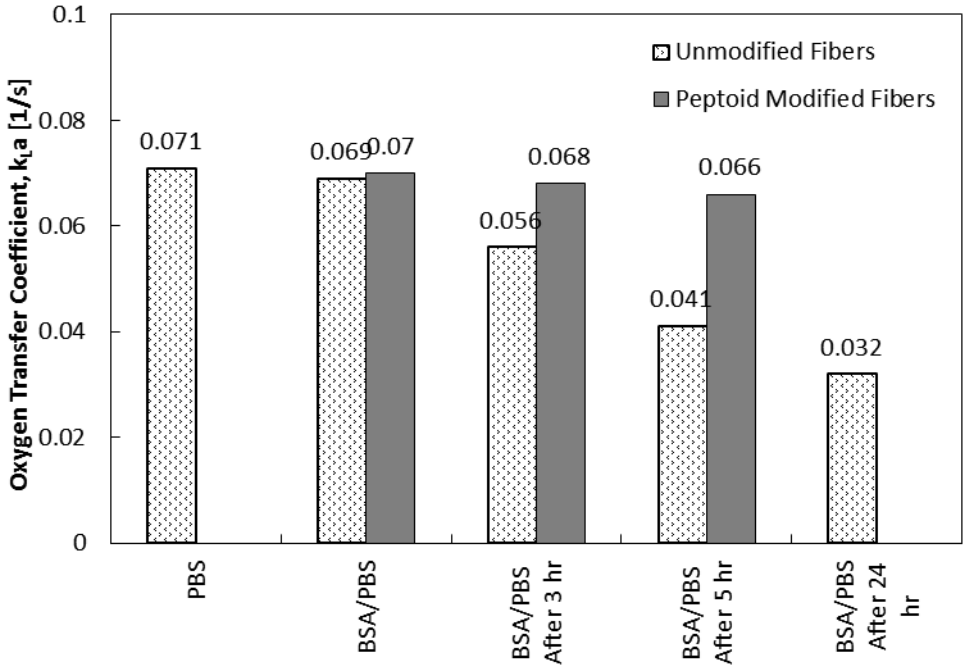

Figure B.9 Oxygen transfer coefficient of polysulfone hollow fiber membranes, unmodified and peptoid modified, in a solution of BSA in PBS over time.

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APPENDIX C. STATISTICAL ANALYSIS ON CHAPTER 2

The data from chapter 2 (Table C.1) representing biomass productivity, lipid productivity and lipid contents were analyzed using a one-way analysis of variance (ANOVA) with 95% confidence. A p-value ≤ 5% was considered significant. Data were presented as mean ± standard deviation.

Table C.1 Treatments and variables

Treatment	T (°C)	Light intensity	CO_2 supp.	Nitrogen level	Biomass Productivity	Lipid productivity	Lipid contents (%)
1	20	140	pure	0.075	91.67	0.78	0.85
2	16	140	Pure	0.075	63	0.61	0.98
3	20	180	Pure	0.075	59.29	0.65	1.1
4	20	140	5%CO_2/air	0.075	67.09	0.81	1.21
5	20	140	Pure	0.3	142.89	0.67	0.47
6	20	140	Pure	0.5	53.66	0.58	1.08
7	20	140	Pure	0.7	30.13	0.34	1.16

Biomass Productivity

The one-way ANOVA showed that there was no significant difference in the yield ($p > 0.05$) between the treatments (temperature, light intensity, nitrate and CO_2 supplementation). Therefore, there was no evidence to suggest that any of these treatments had an impact on biomass productivity.

Table C.2 Biomass Productivity

Biomass productivity (mg L^{-1} day^{-1})			
Temperature± SD	Light intensity± SD	Nitrate± SD	CO_2± SD
77.34±2.27	75.48±22.89	79.59±49.23	79.38±17.38

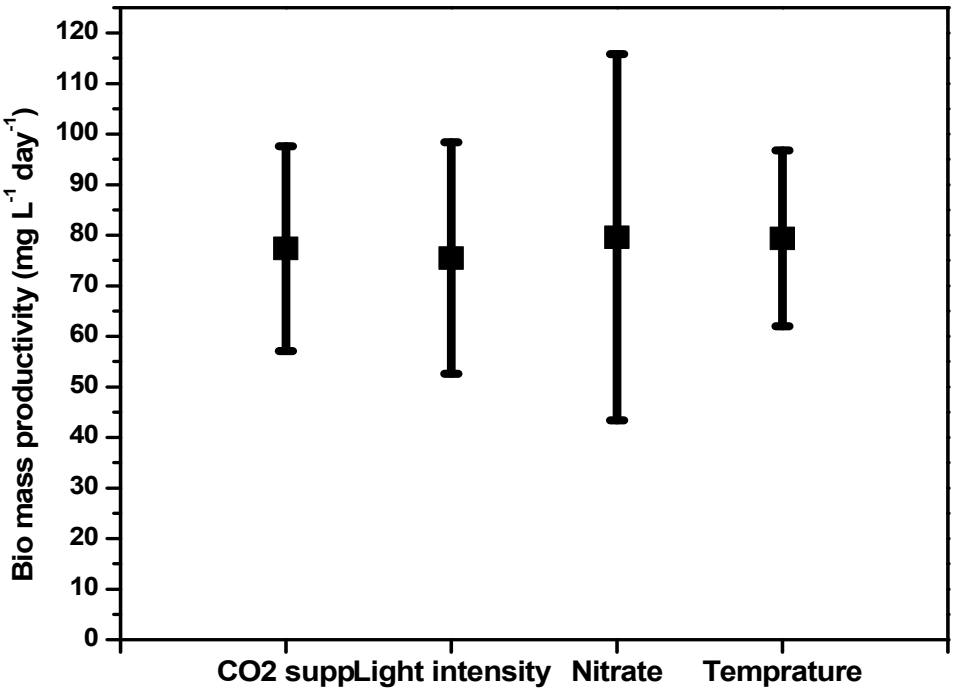

Figure C.1 The effect of CO_2 supplementation, light intensity, Nitrate and Temperature on Biomass productivity

Lipid Productivity

The effects of temperature± SD, light intensity± SD, nitrate± SD as well as CO_2 ± SD supplementation in lipid productivity are presented in table C.3. It was found that the differences in yield were not significant ($p > 0.05$).

Table C.3 Lipid productivity

Lipid productivity (mg L^{-1} day-1)			
Temperature± SD	Light intensity± SD	Nitrate± SD	CO_2± SD
0.69±0.11	0.71±0.089	0.94±0.54	0.79±0.02

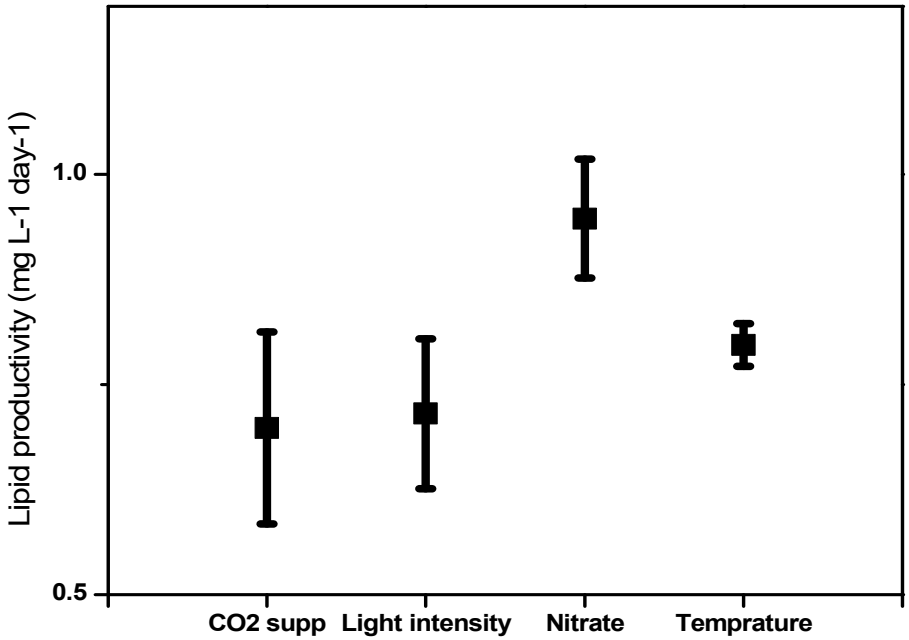

Figure C.2 The effect of CO_2 supplementation, light intensity, Nitrate and Temperature on Lipid productivity

Lipid Contents

The effect of temperature, light intensity, nitrate CO_2 supp average value ± SD are on lipid content presented at table C.4. It was found that the differences in lipid contents were not significant ($p > 0.05$).

Table C.4 Lipid Content

Lipid content (%)			
Temperature± SD	Light intensity± SD	Nitrate± SD	CO_2± SD
0.91±0.09	0.97±0.17	2.05±2.51	1.03±0.25

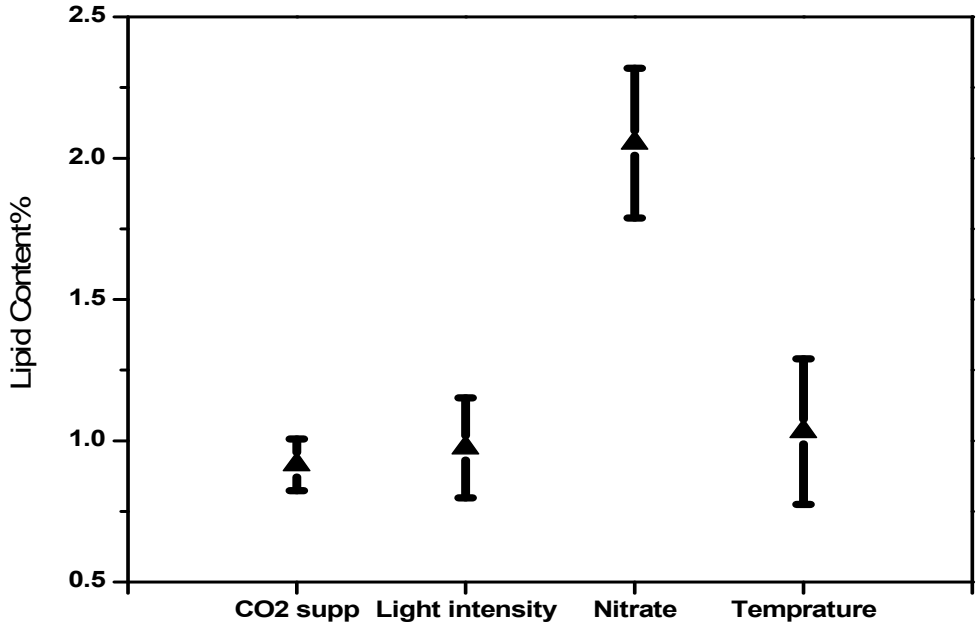

Figure C.3 The effect of CO_2 supplementation, light intensity, Nitrate and Temperature on Lipid contents

The result showed that none of the different parameters (such as, temperature, light intensity, nitrate and CO_2 supplementation) had an impact on biomass productivity, lipid productivity and lipid content.