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BIOMASS AND LIPID PRODUCTION FROM HETEROTROPHIC AND MIXOTROPHIC FED-BATCH CULTIVATIONS OF MICROALGAE *Chlorella protothecoides* USING GLYCEROL

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BIOMASS AND LIPID PRODUCTION FROM HETEROTROPHIC AND
MIXOTROPHIC FED-BATCH CULTIVATIONS OF MICROALGAE *Chlorella
protothecoides* USING GLYCEROL

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ABSTRACT

Chlorella protothecoides is a microalga that can grow both photo-autotrophically and/or heterotrophically under different culture and environmental conditions. In this study both the heterotrophic growth and mixotrophic growth have been conducted. The heterotrophic experiments were conducted completely in the dark while the mixotrophic experiments had the dark cycles with periodic light exposure. The aim of the study was to independently understand the effect of each mode on biomass and lipid yields.

For the heterotrophic experiments, glycerol was used as an external organic carbon source while yeast extract was used as the nitrogen source. The carbon and nitrogen source were added to a defined culture medium. Three different grades of glycerol were evaluated for their effect on the biomass and lipid yields in the heterotrophic experiments, with the 65% crude glycerol proving best giving an average biomass concentration of 22.13 ± 0.17 g/L and average lipid concentration of 9.75 ± 0.02 g/L at the end of an eight-day fed-batch fermentation. The average biomass concentrations did not increase after the eighth day of fermentation. The pH was maintained at a constant value of 6.8 and temperature at 28^0C. As the experiments were carried out in fed-batch mode, addition of the culture medium was done every 24 hours to maintain the carbon and nitrogen sources at 30g/L and 4g/L respectively till the eighth day. Yeast extract was found to be a good nitrogen source, as it also provides vitamins, amino acids and important growth factors as oppose to some other sources like ammonia

and urea (Shi et al., 2000; Gonzalez-Bashan et al., 2000; Illman et al., 2000; Chen et al., 2006).

The mixotrophic experiments were aimed to expose the algae to alternating light and dark cycles to enhance biomass accumulation during light cycle and lipid accumulation during dark cycles. The light cycle help to assimilate CO_2 and produce energy via photosynthesis, which comprises the catabolic reaction, while the switch to the dark cycle allows anabolic reactions where accumulation of lipids and production of other compounds occur. Here, the algae were exposed to light for 8 hours and dark for 16 hours each day for eight days. The 65% crude glycerol was supplemented as the external carbon source to be utilized by the algae during the dark cycles while yeast extract was used as the nitrogen source. Here the average maximum biomass concentration of 28.95 $\pm$ 0.26 g/L and the average lipid concentration of 13.14 $\pm$ 0.01 g/L were obtained which were found to be higher than the heterotrophic results. With intermittent light exposure, the lipid yields were found to increase from a maximum of 0.44 $\pm$ 0.004 gram lipid/gram biomass for heterotrophic experiments to 0.46 $\pm$ 0.004 gram lipid/gram biomass for mixotrophic experiments. The mixotrophic experiments also provided an increase in the average maximum overall biomass concentration from 22.13 $\pm$ 0.17 g/L in heterotrophic to 28.95 $\pm$ 0.26 g/L in mixotrophic experiments.

DEDICATION

This thesis is dedicated to all my dear and loved ones who have stood by me and helped me learn to be a better person throughout my life. A special thanks to my parents Mrs. and Mr. Sivakaminathan, my sister Pavitra Sivakaminathan and my brother Anirudh Sivakaminathan for their warmth and love. I thank my friend Karthik Gopalakrishnan for all the help and support. I also thank all my other friends here at Clemson and back in India who always believed in me.

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CHAPTER I

I. INTRODUCTION

Over many years now, the exponentially growing demand for energy has led to an unbalanced supply of fuel. Many exhaustible energy sources like coal and petroleum are expected to become mostly depleted or too costly to extract (Benemann et al., 1996). As almost all of the energy needs rely heavily on the use of fossil fuels alone, the urgent necessity is to find an alternate source that is long lasting and at the same time satisfy the enormous energy demand that prevails. Looking at the data from the US Energy Information Administration, most of our current unconventional sources of energy are estimated to last no more than 90 years (Sheehan et al., 1998). Another disadvantage that comes from the use of fossil fuels is that they cause increased greenhouse gas emissions that lead to global warming. Currently, we are increasing the CO_2 emissions at an alarming rate and if not checked will lead to double or triple the current amount, which will lead to an increase in sea level that could threaten the life on earth. About 21% of the total greenhouse gas emissions have been from power stations followed closely by industrial processes. The USA alone is responsible for about 3 times more use of coal and other fossil fuels than other countries. One conclusion is not to burn any more coal. The key is to move beyond coal and fossil fuels for fulfilling energy requirements for a greener future.

Among the renewable sources of energy, there has been tremendous potential found in biofuels. Biofuels are the fuel obtained from biomass or primarily organic substances (Angenent et al., 2004). The biggest advantage is that these use environmentally friendly raw material that are naturally available in nature to produce clean and green energy. Originally, first generation biofuels developed were bioethanol from corn or sugar obtained from food crops. These sources were not considered a favorable option as they were competing with the food industry and led to

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increase in food prices (Boddiger et al., 2007). These biofuels not only displaced the land for food production, but they also produced negative net energy gain when obtained too far from the processing plant, that is, they released more carbon in their production than their food crops could capture during growth (Stein et al., 2007). Another alternative developed was the use of lignocellulosic biomass for second generation biofuel production. This biomass includes non-food crops such as wood, switchgrass and residual wastes that provide a positive net energy gain. However, a considerable amount of the US crop land needs to be displaced for fuel production.

Over recent times, microorganisms have been gaining scientist's attention as an exciting source for fuel production. Among microbes, algae seem to be identified as an attractive option due to many advantages. Such third generation biofuels from algae prove to produce high yields with minimum inputs (Chisti et al., 2007). Microalgae are photosynthetic organisms that grow very easily, occupy much less area for production and can also be grown using land and water that are termed unfit for cultivation of food crops. Compared to the larger land area required by feedstock such as switchgrass, soybean and corn, algae requires only 2% of the total crop land to displace transportation fuels in the US and hence proves to be greatly beneficial (Chisti et al., 2007). Through their natural process of growth, algae sequester large amounts of atmospheric carbon dioxide during the process of photosynthesis for producing cell biomass and hence help lowering the chances of occurrence of global warming (Schenk et al., 2008). The cultivation and harvest time for algae typically lasts only for about 5 to 8 days, which is very fast when compared to crops that require months to reach the same volume. Also in terms of the area cultivated, microalgae are 5-300 times more efficient in output yields (Mittal et al., 2008). Mass and energy balances studies on microalgae-based lipids suggest them as a favorable alternative feedstock for biofuel production due to their production capacity of up to 1,000,000 liter oil per

ha year. This amount of fuel if cultivated with care and commercial plants set up all over the world for the future can displace all the burden of energy demand on fossil fuel (Gong and Jiang et al., 2011). Hence microalgal fuels, with their largely positive net energy gain and carbon neutral nature, are competent enough to be a substitute to conventional fuel.

The species studied here is the green microalga *Chlorella protothecoides* that can be grown both autotrophically (in the presence of light) and heterotrophically (in the absence of light). Though this *Chlorella sp* is photosynthetic, in the absence of light this organism grows on organic carbon substrate (Xu et al., 2006). Among various algae species studied for lipid production, *Chlorella* has been the most understood. All photosynthetic algae have the majority of their fatty acids as saturated and unsaturated C18 s (or 18-carbon fatty acids). This composition being very analogous to that of vegetable oils makes it advantageous and easy to analyze (Benemann and Oswald 1996). Under natural conditions, some fresh water algae like *Chlorella vulgaris* is reported to have lipid accumulation capabilities of up to 30-40% of its dry weight without subjection to any special manipulations (Pratt and Johnson 1963; Nichols et al. 1967; Harris and James 1969; Podojil et al. 1978). The main factor that directly affects the growth of algae and the quality of lipid accumulated is the carbon source that the algae feeds on in heterotrophic growth. The most commonly used organic carbon sources studies widely since the 1960s and 1970s are glucose, acetate and corn powder hydrolysate. However, at that time, the attention was not on lipids but only on the mere growth of algae (Tanner 1969; Komor and Tanner 1971, 1974; Haass and Tanner 1974).

By varying the culture conditions and environmental parameters such as pH, temperature, aeration, agitation, the availability of micro and macro nutrients, the algae can be made to produce a wide range of by-products some of which may find its place in the commercial market.

Some of the most common byproducts produced by algae are proteins, sugars, fats and oils (Miao et al., 2006).

This algal oil when trans-esterified with an alcohol, most commonly methanol, in the presence of a catalyst, acidic or basic, produces FAME (Fatty Acid Methyl Esters) or in other words biodiesel (Fu et al., 2009). Esterification typically requires a fatty acid and an alcohol. Alcoholysis commonly known as transesterification occurs when oil is made to react with an alcohol in order to give esters and glycerol as the products. Usually the equilibrium reaction is made to shift towards the product (ester) side by treating the oil with excess alcohol (Miao et al, 2006). For example, for every mole of oil used, about 6 moles of alcohol is reacted. The first conversion that occurs is the breaking down of the triglycerides (fats) to di and mono glycerides and glycerol (Xu et al, 2006). Two of the catalysts used universally are KOH or NaOH. The methyl ester obtained finally is the biodiesel. Not all fatty acid methyl esters may be passed as biodiesel. There are certain standards that the FAME's have to meet for commercial use as biodiesel.

Originally, the biodiesel industry was restricted in terms of options for the starting material where only vegetable oil, soybean oil, or sunflower oil could be used. The biodiesel produced from these have only a moderate calorific value and the yields obtained are nowhere close to replacing the world's demand or energy (Snare et al., 2008). *Chlorella sp* are an interesting class of microbes that can be manipulated to provide large amounts of lipids to produce commercial quantities of biodiesel.

Biodiesel is very similar in property to conventional diesel. Biodiesel is essentially single alkyl esters of long chain fatty acids. The biodiesel obtained could be of different grades. They can be made available both in the pure form and as blends with conventional diesel (Vasudevan

et al., 2008). A 100% pure biodiesel is called B100 while a 10% biodiesel blend with 90% conventional fuel is called B10 and so on (Fu et al., 2009). The biodiesel obtained from microalgae are also devoid of sulfur and are biodegradable in nature and hence pose no threat to the environment (Chisti et al., 2007).

Objectives

In this study, both the autotrophic and heterotrophic conditions of algae growth have been exploited to understand their effects on biomass and lipid production. The aim of this study is to assess the growth of a microalga *Chlorella protothecoids* in fed-batch mode using different grades of glycerol as substrate, to begin the process for assessing a cost-efficient process for producing high-quality biodiesel.

The three main objectives are:

1. To determine the grade of glycerol that gives the highest biomass concentration in heterotrophic fed-batch cultivation of *Chlorella protothecoides* at the end of eight days.

2. To study and evaluate the lipid yields from each of the three grades of glycerol used in heterotrophic cultivation using fed-batch mode

3. To compare the growth and lipid production of the green microalga *Chlorella protothecoides* cultivated at fixed glycerol and yeast extract concentrations in mixotrophic cultures with periodic light exposures of 8 hours a day in fed-batch mode.

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CHAPTER II

II. LITERATURE REVIEW

2.1. Microalgae

2.1.1. Biology of Microalgae

Algae include a wide range of organisms of differing sizes, shapes, colors and structural make having the common property of photosynthesis involving the evolution of oxygen with carbon dioxide capture and assimilation as a part of their central metabolism. Microalgae are small, green microorganisms that are photo/autotrophic and perform oxygenic photosynthesis very similar to higher plants, but have a much simpler body organization and reproduction capabilities (Mutanda et al., 2007).

Most algae can be classified into two broad categories: Cyanobacteria and Eukaryotic algae (Falkowski et al., 1997). Though both categories fall under the classification of algae, they have fundamental differences that could alter the nature and type of the commercial value-added products that they produce. Cyanobacteria are prokaryotic cells that lack all membrane bound organelles and are more similar to bacteria. Mostly all cyanobacteria could be called as macroalgae and eukaryotic algae are microalgae owing to their sizes. Eukaryotic algae have all organelles and fully functional cells that allow them to grow and reproduce easily (Lee R E., 1980).

Our focus here will be on microalgae due to their superior application in biofuel production. The three main factors based on which scientists have classified microalgae are their color (type of pigment), size (cell structure) and their life cycle (Khan et al., 2009). This classification divides the microalgae into nine categories namely: Chlorophyta, Chlorarachniophyta, Cryptophyta, Dinophyta, Euglenophyta, Glaucophyta, Haptophyta,

Heterokontophyta and Rhodophyta, and two prokaryotic divisions: Cyanophyta and Prochlorophyta (Mutanda et al., 2011).

A microalgal cell contains water at about 60-80% by weight and approximately 98% of the dry weight is comprised of organic molecules and 2% is comprised of inorganic molecules. The organic molecules present predominantly are proteins, carbohydrates and lipids which constitute about 90% while other organics like DNA, RNA and ATP are found in lesser amounts of about 10-12% (Bumbak et al., 2011).

Proteins typically constitute between 11-26 % of the total microalgal weight. Some of the typical proteins found are enzymes, cell wall proteins and intrinsic membrane proteins. These proteins are of different types and display diverse physical, chemical and biological properties. This is one of the main reasons that algae have gained interest as food supplements for humans as well as for animal feed (Bumbak et al., 2011).

Carbon comprises of 50% of the microalgal biomass (Sánchez Mirón et al., 2003). Other carbohydrates are found to comprise 17-24% of the microalgal weight with most of them being complex, large molecules while only few are simple sugars. Their content varies with species and changes with culture conditions. Some examples of sugars found are glucose and sucrose; some cell wall polysaccharides found are cellulose; while energy storage polysaccharides present are starch. Another important component of many microalgal cells is a protein body that is made of Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) called pyrenoid. Pyrenoides are generally surrounded by starch granules (Bumbak et al., 2011). When grown in dark cultures supplemented with an external carbon source like glucose, microalgae tend to accumulate lipids within these starch granules embedded around the pyrenoides. The first formation of oil is witnessed as small droplets around the pyrenoid (Bose S R, 1943).

Lipids vary in their distribution percentage depending on the culture conditions from a minimum of 25% as high as 80% by weight (Metting, 1996; Spolaore et al., 2006). Only a few types of lipids occur in algae. Algal lipids fall under two broad categories: 1.Non polar lipids and 2. Polar/membrane lipids (Bumbak et al., 2011).

Microalgae are considered as cell factories that in the presence of sunlight, convert carbon dioxide to useful biological high-value products like biofuels, proteins, foods and feeds (Walter et al., 2005). From the environmental aspect, these naturally occurring species may be used as biofertilizers as they have nitrogen-fixing abilities in plant soils (Munoz and Guieysse, 2006). These microalgae may also have potential bioremediation applications (Vaishampayan et al., 2001). However, an important application found for microalgae has been its ability to produce oil. Because alternative sources of energy is currently in great need, microalgal lipids may be considered an extremely relevant solution to the growing problem (Gumus M., 2010). The petroleum-based industry has been facing a great threat due to increasing prices and an over burdening demand that may be going beyond the supply capacity (Li Y et al., 2008).

2.1.2 Nutrient requirements for microalgae

Microalgae are aquatic organisms in nature and require water, sunlight and nutrients for constructing their cellular components. Microalgae can be manipulated to grow under three different conditions based on the nutrients provided. The nutrition of algae that utilize only inorganic nutrients like CO_2 as carbon source are called autotrophic, those which use organic nutrients such as glucose as carbon sources are heterotrophic and those which use both inorganic/organic nutrients in synergy as carbon sources are called mixotrophic (Brennan & Owende, 2010; Greenwell et al., 2010).

For phototrophic algae, light energy in precise amounts is considered a prime requirement for microalgae growth in autotrophic condition. Too little light may retard algal growth while too much light may kill algae. Here, microalgae assimilate carbon (typically from dissolved CO_2), oxygen (from H_2O and dissolved O_2) and hydrogen (from H_2O) via photosynthesis to produce algal biomass (Van den et al., 1995). Fig 2.1 shows the schematic of the photosynthesis by algae in a body of water and Fig 2.2 shows the energy and carbon flow through algae.

For heterotrophic growth, microalgae utilize an organic carbon source that is externally supplied to them in optimum amounts. These algae are capable of substituting organic compounds for carbon dioxide as their only source of carbon. Various organic molecules taken up by microalgae are sugars, amino acids and organic acids (Maclntyre and Cullen., 2005).

Nitrogen is the most abundant nutrient required next to carbon, hydrogen and oxygen. There are four major forms of accessible nitrogen namely: N_2 (atmospheric nitrogen gas), NO_3^- (nitrate ion), NH_4^+ (ammonium ion) and organic nitrogen (like an amino acid) (Keller & Zengler., 2004). Not all kinds of algae can use nitrate and ammonium and only certain cyanobacteria could directly fix the atmospheric nitrogen for growth. There are some algae that could substitute the organic nitrogen for inorganic nitrogen (Lee R E., 2008).

The other major nutrients, apart from carbon (C), hydrogen (H), oxygen (O) and nitrogen (N) previously discussed, that are required by microalgae are Magnesium (as dissolved Mg^{2+}), Iron (as dissolved Fe^{2+}/Fe^{3+}), Calcium (as dissolved Ca^{2+}), Potassium (as dissolved K^+) (Barsanti & Gualtieri, 2006), Phosphorous (as dissolved HPO_4^{2-}) (Selekli et al., 2009) and Sulfur (as dissolved SO_4^{2-}) (Martin et al., 200). Most marine algae also require a high concentration of sodium in the form of Na+. There are certain rare species of algae that also need silicon in the

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form of dissolved SiO_2. The minor nutrients required by most microalgae are Copper (Cu), Zinc (Zn), Manganese (Mn), Molybdenum (Mo), Chlorine (Cl) and Bromine (Br) (Molina Grima et al., 1999). They are mostly made available in the dissolved form or chelated form in high enough concentrations required for the algae to assimilate them for their growth (Keller and Zengler., 2004). Most microalgae also require trace quantities of some vitamins like vitamin b_{12}, thiamine (vitamin b_1), biotin (vitamin b_7, riboflavin (vitamin b_2) and pantothenic acid (vitamin b_5).

Grobbelaar et al. (2004) developed an approximate molecular formula of $CO_{0.48}H_{1.83}N_{0.11}P_{0.11}$, which helps to get an estimate of the minimal nutritional requirement for generating microalgal biomass. In addition to this, as the critical elements considered to be most likely for algal growth are carbon, nitrogen and phosphorus, the Redfield Ratio gives the molecular ratio of Carbon : Nitrogen : Phosphorus in microalgae to be 106 C: 16 N : 1 P as a general rule (Hsieh et al., 2009).

2.1.3 Growth Cycle of Microalgae

The idealized growth pattern of a population of microalgae is typically the same as most microorganisms and follows the batch growth curve having the following phases: 1.Lag phase 2.Log/exponential phase 3.Stationary phase and 4.Decline/death phase. Though microalgae prove to be very fast growing species, the duration of each phase is very specific to each kind of algae (Lee R E., 2008).

An inoculum refers to the population of algae that is transferred to a fresh culture medium under sterile conditions. The lag phase occurs immediately after inoculation and marks the period taken by the algae to adapt to the new environment. Numerous factors may affect the duration of the lag phase (Richmond A., 2004). Lack of some important nutrients and growth factors are the most common factors for the algae to remain in the lag phase for an extended

period of time. Sometimes a very small inoculum size or lack of aseptic conditions may delay the length of lag phase (Anderson R A., 2005). Here the algae may not be actually adapting to the environment but just having trouble growing due to poor conditions. It is advisable to use young, active and exponentially growing algae cells well adapted to the required culture conditions before inoculation. In the lag phase the algae do not reproduce and the population remains constant.

The slow growing cells of the lag phase adapt and start growing rapidly and enter the exponential phase or the log phase (Jianqiang Lin et al., 2000). Here, as the name suggests the algal cells multiply at a great pace and increase their cell density. The products found in this growth phase are called primary metabolites (Lin y et al., 2005).

The stationary phase follows at the end of exponential phase that signifies the exhaustion of one or more of the algae's growth requirements. At this stage there is no net growth or rather the growth rate equals the death rate (Wanner et al., 1990). The microalgal population may be treated as neither reproducing not dying. Though the cells may not be growing in the stationary phase, they could produce secondary metabolites (non-growth related products) due to metabolic deregulation (Andersen & Kawachi., 2005).

As the cells grow exponentially in the log phase, the nutrients get utilized and there might not be enough left for cells to grow further. This leads to decrease in the viable cell count. Following the stationary phase, the death phase occurs where cell lysis is caused due to nutrient depletion and /or toxin buildup (Richmond A., 2004).

Accumulation of oils intracellularly by the microorganisms occurs depending on the C/N ratio. Nitrogen depletion is shown to enhance lipid production by triggering formation of triacylglycerol that primarily make up oils (Wynn J et al., 2005). As the organic/inorganic carbon

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source and the nitrogen source play the most crucial role in the lipid accumulation process it is vital to make cautious selection of both of these and monitor their levels during the algae fermentation cycle, they are vital because the C/N ratio plays a major role in the lipid accumulation process (Lin, C et al., 2004). Other conditions like temperature, pH, agitation and aeration have minor effects on lipid accumulation but are essential for the growth and survival of the algae (Giordano et al., 1991). During initial phases of growth, the nutrients are all available in excess and the target is to obtain high levels of biomass and cell density. Slowly, as the cultures are exposed to decreasing nitrogen content, the metabolic shift occurs to accumulate oils. These conditions are best achievable in fed-batch mode of fermentation, where the C/N ratio may be increased once the cells have reached exponential growth (Jones et al., 2007). The growth conditions and microalgal strain also affects the production and composition of lipids. The algae fermentation process must be carefully optimized to obtain both good growth rates of biomass as well as high lipid yields so that the total lipid production is not compromised (Walker et al., 1999).

2.2. Microalgal Applications

There have been lots of research and ideas on using microalgae as a source of fuel since the early days (Sawayama et al., 1995). However, the escalating interests over recent times have been a direct result of the apprehensions in the availability and negative impacts of use of fossil fuels (Gavrilescu and Chisti, 2005). There are a variety of biofuels that microalgae can offer. Depending on the method of treatment and culturing of the algal biomass we can obtain a wide range of bio-energy products. Biohydrogen can be obtained from photo-autotrophically cultured algal biomass (Fedorov et al., 2005; Kapdan and Kargi, 2006), ethanol can be generated by aerobic fermentation of the microalgal biomass (Bush & Hall, 2006) while methane is a direct

product of anaerobic digestion of algal biomass (Spolaore et al., 2006). Biodiesel is produced from the microalgal oils obtained by disrupting the mature algal cells (Banerjee et al., 2002; Gavrilescu and Chisti, 2005).

Microalgal fuels are considered third generation biofuels that do not hamper and come in the way of other bio-diversities or pose a threat to the food industry. They have many benefits over other conventional sources for the following reasons: environment friendly, easily available and have high growth and productivity rates, contain high inherent lipid content and most importantly have a 70% higher energy efficiency. Figure 2.3 shows the various production applications for the lipids/oil obtained from microalgae (Gunstone F D., 2001; Tyson et al., 2004).

High biomass and lipid production under heterotrophic conditions have been achieved with *Chlorella protothecoides* by using different carbon sources (Miao and Wu 2006; Xu et al. 2006). Xu et al. (2006) reported that *C. protothecoides* could accumulate lipid as high as 55% of the cell dry weight after six days of cultivation with feeding of corn powder hydrolysate in fermentors through nitrogen limitation.

2.3 Potential of microalgae derived biodiesel

With the power of the Energy Independence and Security Act of 2007 (EISA 2007), 36 billion gallons of renewable fuel are mandated by 2022 of which 15 billion gallons are corn-based ethanol, 16 billion are "cellulosic biofuels", and at least 1 billion gallons are biodiesel. The bulk amount of biodiesel being produced currently is from plant oils and animal products, but not majorly from microalgae due to the uncertainties in many parameters of the production process (Van Gerpen., 2005). However, this problem is due to be overcome and is likely to soon change owing to the interests expressed by many companies in commercializing microalgal biodiesel

and over the course of time has become a proven fuel technology (Knothe et al., 1997). While the United States biodiesel production relies heavily on soybeans, other sources have been increasing that include canola oil, animal fat, palm oil, corn oil and waste cooking oil (Felizardo et al., 2006; Kulkarni and Dalai, 2006). However, none of these have the capacity of achieving the amounts required to replace petroleum fuel except algae with capabilities of reaching 80% of the weight of their dry biomass (Metting, 1996; Spolaore et al., 2006). The sustainability of the fuel in its nature to co-exist with other industries/technologies is also an important factor. In the United States alone, the conventional sources of oil are all crops that require about 24% of the total available land to meet about 50% of the requirements for transport fuel while microalgae only require about 2-3% of the cropping area for the same purpose (Yusuf Chisti., 2007). Table.1 shows the comparison of different sources of biodiesel with microalgal biodiesel in terms of land area needed and the amount of the transportation fuel needs they can replace (Yusuf Chisti., 2007). The yields reported in Table 2.1 are all experimentally verified biomass productivities in photobioreactors and the actual biodiesel yield per hectare is only about 80% of the yield of the parent crop oil given in the table. Table 2 showcases the oil content in terms of % dry weight of different algae species (Yusuf Chisti., 2007). From Table 2.2 most algae have an average lipid of about 20-50% of their dry weight. Some algae may be manipulated to accumulate lipids up to 80% of their dry weight under specific environmental and growth conditions (Metting, 1996; Spolaore et al., 2006). The oil content of the biomass and the total algae growth contribute simultaneously to the lipid productivity. The lipid productivities are calculated as the mass of lipid produced per liter of microalgal broth per day (Metzger and Largeau., 2005). The microalgae that are capable of giving high lipid productivities are well suited for the biodiesel production process. Most microalgae produce lipids that may be converted to biodiesel easily.

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However, there are certain oils produced by the microalgae that prove unfavorable for biodiesel production (Banetjee et al., 2002).

Microalgae mainly satisfy their carbon requirements primarily from carbon dioxide in the atmosphere (Guschina and Harwood, 2006). Studies have shown that the production of 100 metric tons of algal biomass requires roughly 183 metric tons of carbon dioxide (Yusuf Chisti., 2007). The carbon dioxide exhaust from power plants owing to excessive use of fossil fuels could be redirected for this purpose of algae growth to lower the cost of biodiesel production and reduce carbon dioxide emissions. This will help make microalgal biodiesel production a carbon neutral process (Sawayama et al., 1995; Yun et al., 1997).

2.4. Microalgal Biodiesel

2.4.1. Biodiesel production process

The biodiesel production from microalgal lipids is said to follow the same procedure as biodiesel being produced commercially from commonly used crop oils. Triglycerides are the primary components of the lipids that are involved in biodiesel production (Kulkarni et al., 2006). As the name suggests, three fatty acids make up the triglyceride molecule that are esterified with one molecule of glycerol. The reaction between these triglycerides and alcohol in the presence of a catalyst is called transesterification and the products obtained are fatty acid methyl esters (biodiesel) and glycerol (Barnwal et al., 2005). Many alcohols can be used at this stage but methanol is used widely due to current low cost, ease of separation, and availability. The methanol and oil form two immiscible liquid phases (Yusuf Chisti, 2007). Here the triglycerides are broken down serially to diglycerides, monoglycerides and then glycerol. From Figure 2.4, it is evident that the complete transesterification of triglycerides requires each mole of triglyceride be treated with 3 moles of alcohol to produce 3 moles of biodiesel esters and 1

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mole of glycerol. As it is an equilibrium reaction, to ensure good conversions, an excess of alcohol is always used (Jang et al., 2005). Some industrial processes use up to 6 moles of alcohol so that the reaction is always driven towards the product side (Fukuda et al., 2001). This process is said to give a yield of methyl esters of about 98% on a weight basis.

The catalyst for the transesterification reaction could be acids, bases (Meher et al., 2006) and lipase enzymes (Sharma et al., 2001). Due to a 4,000 times greater efficiency of alkali catalyzed reactions as compared to the acid catalyzed reactions, the more preferred alkali transesterification commonly uses sodium or potassium hydroxide as catalyst at a concentration of 1 % by weight of oil used (Fukuda et al., 2001). Some other alkaloids like methoxides are commonly used as catalyst combined with the alcohol. While some other enzymes like lipases also prove to be beneficial as catalysts, they are expensive and not easily available (Nagle et al., 1990).

Before reacting the alcohol and the oil they are often tested for having as less moisture as possible. The presence of water can trigger formation of soaps by saponification reaction and reduce the yield of biodiesel. Also the oil must be tested to ensure minimum free fatty acid content (Yusuf Chisti, 2007). As the boiling point of methanol is 65°C, the best conditions found for alkali-catalyzed transesterification are at about 60°C under atmospheric pressure. This reaction takes about 90 minutes to achieve completion. Reaction times could typically be reduced by carrying out the reactions at higher temperatures and pressures; however that would substantially increase the cost of the process. Hence, the tradeoff works well with carrying out the reaction for a slightly longer time (Barnwal and Sharma, 2005). The biodiesel product is concentrated with successive water washes that promote the glycerol and methanol being transferred to the aqueous phase (Jang et al., 2005).

2.4.2. Value added co products as a path to viability of the biodiesel production process

2.4.2.1 Glycerol

The main byproduct of the transesterification process is the glycerol (Thompson J C., 2006). From the most prevalent transesterification of vegetable oils or animal fats, an estimate of about 10 lbs of crude glycerol is obtained from every 100 lbs of biodiesel (Aldiguier et al., 2004). While the demand for biodiesel is gradually increasing and the industry expanding remarkably with continuous biodiesel production, the cost of the crude glycerol is decreasing at a similar rate. This is due to the lack of applications or absence of a viable market for the crude glycerol getting accumulated. Figure 2.5 shows the US biodiesel production trend and its impact on the prices of associated crude glycerol from the years of 2004-2006 (Yazdani and Gonzalez., 2007). Statistics have reported that over the past ten years there has been a 10-fold decrease in the cost of crude glycerol due to high availability and low demand (McCoy, 2005). As a matter of fact, there have been reported cases of shut down of glycerol production and refining operations in big industries like Proctor and Gamble Chemicals due to the adverse effects of the glycerol surplus (McCoy, 2006). Taking the statistics from figure as a reference, the revenues that bio-refineries generate would be about three times the gross processing margins if crude glycerol was sold at $0.25/lb - $0.85/ lb/gal giving a glycerin credit of $0.21 (Hazimah et al., 2003). Hence the need of the hour is to find an alternative use for glycerol as a high value-added co-product for reutilization to make the biodiesel production process more economically viable. Capitalizing the current situation of the glycerol availability at low prices while simultaneously helping the biodiesel industry seems a smart and sustainable approach for the future.

2.4.2.2. Spent algal biomass

As discussed in the previous sections, in addition to lipids, the microalgae are also rich in sources like carbohydrates, proteins and other nutrients. Figure 2.6 shows the various uses for microalgal biomass (Sanchez Miron et al., 2003). In many cases the algae is considered a good source of single-cell protein (SCP), which is consumed by humans in small quantities. The omega-3 fatty acids present in the algae, usually sourced from fish stocks, are also fit for human consumption. The spent microalgal biomass coming out of the biodiesel production process, could also serve as animal feed. Another application would be the anaerobic digestion of the spent biomass for methane production (Mata-Alvarez et al., 2000; Raven and Gregersen, 2007). This is highly advantageous as the electricity generated could be used for supplying the power required for the biodiesel production process and other necessities of the biorefinery industry. Though this methane production process may not be as efficient as that from other well established, cheap and easily available substrates, this creates an added advantage of the spent biomass that might not be put to any other use (Mata-Alvarez et al., 2000). While there is biodiesel being produced, we could also simultaneously generate animal feed, protein source, biogas and electricity. The excess power available from these applications could also be sold to compensate for the cost of biodiesel production. Depending on the type of the microalgae used, various other value-added products could also be obtained. There could be possible sugars and amino acids present in the spent algal biomass that could be harvested for various applications.

2.4.3. Research on the acceptability of microalgal biodiesel

All biodiesel being produced in the United States will have to comply in accordance with the standards of ASTM Biodiesel D6751 (Knothe., 2006). Irrespective of the source or feedstock used, these standards have to be strictly met. The level of saturation or unsaturation of the

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oils/lipids and their moisture content are two main factors for the biodiesel to be passed as a transportation fuel. Microalgal lipids have a different chemical and structural composition from other vegetable oils in that they have a large amount of polyunsaturated fatty acids with four or more double bonds (Belarbi et al., 2000). The most commonly observed PUFA in microalgal lipids are eicosapentaenoic acid (EPA, C20:5n-3) having 5 double bonds and docosahexaenoic acid (DHA, C22:6n-3) having 6 double bonds. In general fatty acids as well as FAME (biodiesel) having high levels of unsaturation with 4 or more double bonds are capable of getting oxidized during storage. If oxidized, these fatty acids lose their ability to serve as biodiesel through traditional transesterification processes. Though some vegetable oils, having high amounts of fatty acids like linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3), are also capable of being oxidized upon storage, they have a much higher oxidative stability than EPA and DHA. In certain countries like most European nations, the standards to be satisfied are different depending on if the biodiesel is used as a fuel or for heating purposes only (Knothe., 2006).

The composition of the biodiesel from the microalgae also varies with the type of microalgae under study and the carbon and nitrogen substrate that it grows on. The other prime factor that affects the quality of biodiesel is the growth conditions under which the algae was made to accumulate lipids. Table 2.3 shows the comparison of the microalgal biodiesel characteristics (Han Xu et al., 2006) with those of diesel fuel (Ma and Hanna, 1999; Lang et al., 2001; Al-Widyan and Al-Shyoukh, 2002; Antolin et al., 2002; Vicente et al., 2004), and the ASTM biodiesel standard (Antolin et al., 2002). The microalgae under study here was *Chlorella protothecoides* that was grown on glucose as the carbon substrate in a 5 L stirred tank batch fermenter under heterotrophic conditions (Han Xu et al., 2006). If the unsaturation levels in some

oils are too high and have far too many fatty acids with more than 4 double bonds, they can be reduced by hydrogenation of the oil (Jang et al., 2005; Dijkstra, 2006).

2.5. Metabolism or Nutrition modes of algae

Algae are one of the oldest forms of life on earth that do anything to survive. They are capable of utilizing the available forms of nutrition in the best way possible and hence prove to be diverse species. Microalgae typically showcase 3 main nutritional modes namely Photo-autotrophic, heterotrophic and mixotrophic as tabulated in Table 2.4 (Chun-Yen Chen et al., 2011).

2.5.1. Photo-autotrophic algae

Most naturally occurring microalgae are phototrophic in their nutrition and absorb sunlight to assimilate the atmospheric carbon dioxide as their carbon source. The amount of CO_2 present in air is about 360 ppmv (Chiu et al., 2009). Majority of the microalgae can take up to 150,000 ppmv of CO_2 for utilization (Bilanovic et al., 2009). External sources like power plants or soluble carbonates can be supplied to the algae growth media for algae scale production units but it only increases the cost of production.

2.5.1.1 The photosynthetic reaction in microalgae

The biggest advantage in ordinary algal systems is that the nutrient source is sunlight that is available naturally and free of cost (Janssen et al., 2003). The sole energy source which is light is converted to chemical energy through photosynthetic reactions carried out by the algae. Here, the solar radiation and CO_2 are absorbed by the chloroplasts and converted to adenosine triphosphate (ATP) and O_2 which are used in the respiration process and other cellular levels to support growth of the algae (Falkowski et al., 1997; Zilinskas et al., 1974).

The driving force for this anabolic reaction is the chemical energy in the form of chlorophyll and $NADPH_2$ and other accessory pigments that transfer the absorbed energy to a protein complex for use in biosynthesis and other electron transfers. This electron transfer occurs in accordance with the 'Z scheme' of photosynthesis which allows us to calculate the number of photons used as the energy required to fix one carbon atom as CH_2O (Hill., 1965). There is only a certain range in the electromagnetic spectrum of sunlight that can be used in photosynthesis by the microalgae. This photosynthetic active radiation or PAR ranges from 400-700 nm where the mid wavelength 8 light photons of 550 nm (green light) has the least energy requirement to form CH_2O. This single photon of green light at 550 nm has about 20% more energy than a single photon of red light at 680 nm and 15.5% less energy than a single photon of blue light at 470 nm (Gordon and Polle (2007). From these observations, Matthijs et al. (1995) suggested that both red and blue photons could have equal photosynthetic energy demand on a per quantum basis.

2.5.1.2. Evaluation of effect of different light wavelengths on algae metabolism

There have been studies reported on the effect of exposing microalgae to different wavelength and observing their resulting structural, cellular and chemical compositions (Pirson et al., 1960). These studies were conducted by adjusting the intensities of both blue and red light to produce the same biomass productivity. The chemical composition of the cells exposed to blue light were found to have 15% carbohydrates and 60% proteins while those exposed to red light had 39% carbohydrates and 29% protein respectively. Hence a trend that low intensity blue light along with red light was required to satisfy the photosynthetic demands of the microalgae (Horst., 1982). While the blue radiations help in deriving energy, activation of enzymes and gene regulation, their low exposures also help repair the cell damage caused due to the extended exposure to red light. (Ruyters., 1984). There have also been contradicting reports over recent

times that say that monochromatic exposure to red light helps the healthy growth of microalgae and the partial exposure to blue light does not have any enhanced effect on biomass production of *Chlorella sp* (Matthijs et al., 1995). But these studies do not have any data on the effect of blue LED alone on algal growth and hence we cannot make any conclusions.

Although a wide range of research exists on the growth of microalgae under different light intensities, a thorough understanding of the different light spectra and their effect on the microalgal biomass and fatty acid profiles is needed.

2.5.1.3. Factors limiting growth of phototrophic microalgae

The only limiting factors for algae growth here are light and carbon dioxide. The intensity of sunlight varies throughout the day and through the seasons. This and the availability of carbon affect the algae cell growth and the microalgal oil production. Hence during light limitation conditions, artificial lights may duplicate and augment the optimum conditions of natural growth as closely as possible (Pulz O et al., 1998). The criteria to choose an artificial light source depends on the absorption spectra of the pigments present in the algae. Some green algae have the chlorophyll a and b, and zeaxanthin pigments while some diatoms have chlorophylls a and c, and fucoxanthin pigments (Brennan et al., 2009). Each of these has different absorption maxima and hence would assimilate the different types of light. Most commonly fluorescent lamps are used solely for phototrophic algae cultivations in small-scale and pilot-scale operations. In large industrial scales this could turn out to be far more expensive than natural illumination as artificial light has a considerably higher energy input (Muller et al., 1998). Another disadvantage is that the artificial light derives its power from burning of fossil fuels which digresses from the main focus of using microalgae for oil production, where our interests lie in sustainability and reduced carbon foot prints.

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Most commercial and economically feasible cultivations for photoautotrophic growth occur in open pond systems (Figure 2.7 and 2.8), raceway reactors or photobioreactors (Figure 2.9) (Borowitzka M A., 1999). These phototrophic cultures are mainly for non-energy production where biomass accumulations are fairly low compared to a heterotrophic cultivation (Borowitzka M A., 1997). Table 2.5 compares the features of open pond system and closed photobioreactors (Brennan et al., 2009).

The pattern of light demand shows that for low density initial phases of the cultures the light demand is low but as the cell densities become high towards the end, the light demand becomes high. It becomes critical to ensure that during the initial phases of growth when the light demand is low, the high light incidence may cause photo-inhibition. Thus applying an incremental light incidence rather than constant illumination will help save energy and at the same time prevent photooxidative damage to algae cells due to excessive irradiation.

There are some hybrid designs that try combining the applications of the closed photobioreactors along with the open ponds giving a two-phase algae cultivation system that tries to overcome the limitations of both reactors (Rodolfi L et al., 2008). The first stage consists of the controllable photobioreactor conditions to favor growth and reduce contamination while the second stage aims at exposing cells to nutrient stresses to enhance lipid accumulation (Huntley et al., 2007). Lab scale fermenters help us to understand the growth pattern and the metabolism of the photoautotrophic algae before scaling them up to higher volumes.

2.5.2. Heterotrophic algae

2.5.2.1. Heterotrophy

There are some microalgae that can utilize sources of nutrition other than natural sunlight for their growth. Such a mode of nutrition where the sole carbon and energy source are organic

compounds is called heterotrophy. As the definition suggests the need for a light energy source here is eliminated and so are the associated problems. Low cell densities due to the light limitation and photooxidative damage to the growing algal cells due to excess irradiation are all omitted here. Generally the heterotrophic mode of nutrition is chosen to increase the biomass concentration and the resulting biomass productivities substantially as compared to the slow growing autotrophic cultures. The lipid productivities are also reported to be 20 times higher than those obtained with photoautotrophic cultures. Many photosynthetic microalgae have been reported to grow well in aerobic heterotrophic conditions like: *Chlorella protothecoides, C. vulgaris, , C. sorokiniana, C. regularis, and C. pyrenoidosa, Scenedesmus, Haematococcus, Spirulina, Nitzchia laevis, Chlamidomonas reinhardtii, Scenedesmus obliquus, Synechocystis, Plectonema boryanum, and Nostoc,* with the introduction of organic compounds like glucose, peptone and acetate (Liam et al., 2009; Web Source). However, the sugar based heterotrophic system frequently suffers from problems with contamination.

2.5.2.2. Metabolism of heterotrophic algae

An interesting feature observed in heterotrophic and also mixotrophic cultures is that there is a decrease in the overall chlorophyll content of the algae in course of its growth. There have been studies that have reported that there is up to 94% chlorophyll loss under heterotrophic growth. This could be attributed to the fact that there is reduction in the chlorophyll synthesis as photosynthesis is inhibited and the carbon in its organic form is directly incorporated from the various sugars fed to the algae (Wei Xiong et al., 2010). This causes the microalgal cells to adapt to this kind of carbon assimilation where the synthesis of the unutilized chlorophyll is down

regulated to conserve energy. Hence the metabolic regulation starts the biodegradation of chlorophyll in these heterotrophic cultures (Hortensteiner S et al., 2000; Engel N et al., 1991).

Furthermore, as chlorophyll pose some interference during the transesterification process for biodiesel production, the reduction in chlorophyll content during heterotrophy and mixotrophy only favors the biodiesel production from microalgal feedstock. Besides providing the advantage of eliminating the light requirements, heterotrophy also gives a cultivation process that is much easier to control. The addition of external organic carbon in required amounts also generates a CO_2 rich environment that promotes growth of algae (Wei Xiong et al., 2010). Due to the high cell densities achieved at the end of the heterotrophic process, the biomass harvesting is also cost effective (Chen F et al., 1991). A close study needs to be done with heterotrophy and mixotrophy to obtain an enhanced cultivation technique for microalgal growth.

2.5.2.3. Glycerol fermentation by microorganisms

Glycerol may be assimilated by most microorganisms in the presence of external electron acceptors through their respiration metabolism. However, not many organisms can do so in the absence of electron acceptors or in other words via their fermentative metabolism (Magasanik B et al., 2005; Schuller et al., 2003). Many bacterial species of the Enterobacteriaceae family like Citrobacter freundii and Klebsiella pneumonia have been studied for their ability to metabolize glycerol as a carbon source. The breakdown of glycerol in these organisms is found to follow two pathways, both responsible for breaking down glycerol to a highly reduced 1,3-propanediol (1,3-PDO) product (Bouvet O M et al., 1995). The first pathway is an oxidative pathway where an NAD linked glycerol dehydrogenase (gly DH) dissimilates glycerol to dihydroxyacetone (DHA). This DHA is then phosphorylated by PEP and ATP dependent DHA kinases (DHAK) (Bhooth I et al., 2005). The second parallel pathway is a reductive one where glycerol is

dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde (3-HPA). NADH linked 1,3-PDO dehydrogenase (1,3-PDODH) reduces this 3-HPA to 1,3-PDO while regenerating NAD+ in the process (Bouvet OM et al., 1995). This pathway discussed here is depicted in Figure 2.10 and provides the basis for the fermentative metabolism of glycerol in all microorganisms (Syed Shams Yazdani and Ramon Gonzalez., 2007). Owing to the highly reduced state of the carbon in glycerol, the necessity for an active 1,3-PDO pathway arises. The inclusion of this 1,3-PDO molecule into the cell creates reducing equivalents that are all consumed along the pathway to achieve a redox balance even in the absence of electron acceptors. There are possibilities where glycerol fermentation metabolisms can occur without the 1,3-PDO production. Such a case has been reported for *Escherichia coli* when certain conditions like acidic pH, prevention of fermentative hydrogen gas accumulation and other suitable medium compositions have been maintained (Dharmadi Y et al., 2006).

Glycerol fermenting organisms have also reported to synthesize other products that have applications as fuels and chemicals. *Clostridium pasteurianum* has been found to produce butanol as a major product under specific cultivation conditions of glycerol fermentation (Biebl H., 2001) while *Klebsiella planticola* strain that was isolated from the rumen of red deer was found to produce ethanol and formate as two major products of its glycerol fermentation (Jarvis G N et al., 1997). A certain *Enterobacter aerogens* mutant was found to co-produce ethanol and hydrogen as a result of glycerol fermentation from waste streams (Ito T et al., 2005).

2.5.2.4. Performance of microalgae *Chlorella protothecoides* in heterotrophic mode

There are certain species of microalgae that have a varied cell organization that allows them to alter their metabolism to switch between growth modes by merely manipulating the

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chemical properties of the culture medium (Behrens and Kyle, 1996). *Chlorella protothecoides* is one such microalgae that under different culture conditions grows photoautotrophically, heterotrophically and/or mixotrophically. High biomass concentrations and lipid content in heterotrophic microalgal cells have been observed. By growing microalgae heterotrophically, we not only improve the biomass and lipid yields and efficiency of the process, but also reduce biomass production cost. This type of metabolism can also be used for the production of certain useful metabolites suitable for biofuel production (Wu et al., 1994). By applying this cell metabolism to the principles of fast pyrolysis we can obtain high amounts of microalgal oil. This research on heterotrophic *C. protothecoides* produced a yield of 57.2 % of lipids which was about 3.4 times higher than that from autotrophic cells (16.6%), carried out by fast pyrolysis. The quality of bio-oil produced was also superior to that produced from autotrophic cells and they were found to be comparable to fossil fuels (Xioling et al., 2004). Heterotrophic cultures also make it easier to scale up in industrial settings due to the controllable conditions. In situ sterilization of the closed bioreactors also help control the growth of other microorganisms that is possible in case of open pond phototrophic cultures (Li et al. 2007);

Studies done by *Miao X et al. 2004,* found the chemical compositions of both the autotrophic (AC) and heterotrophic (HC) cultures of *Chlorella protothecoides* to be carbohydrates, proteins and lipids in proportions as shown in Table 2.6. From Table 2.6 it is clear that the lipid content in the heterotrophic cells increased to about 55.2% from the autotrophic 14.57% which is about 4 times higher. As observed under the differential interference microscope depicted in Figure 2.11, the HC cells had a large amount of lipid vesicles that exhibited a higher energy content than protein content as compared to the AC cells, thereby increasing the heating value of HC cells to 1.2 times of AC cells (Table 2.6).

2.5.2.4.1. Effect of carbon substrate on biomass and lipid content of heterotrophic C.protothecoides

The most commonly used and well-studied carbon substrate for microalgae is glucose. Other carbon sources like acetate, glycerol, corn powder hydrolysate and carbonates could be supplied externally. Due to the availability of excess organic carbon, the biomass productivity is enhanced. The cells have been observed to have a very short lag phase with glucose and also an extended log phase (Tamarys et al., 2010). Though the final cell density amplified with a rise in the initial glucose concentration, the specific growth rates were not enhanced at high glucose concentrations. As substrate inhibitions have been observed in other studies, it is important to feed the cultures with the right amount of glucose to prevent low cell densities. An optimum strategy would be to continuously feed the cultures with a low concentration of the substrate. Most studies state that at 5 g/L the glucose was completely consumed at the end of 6 days while at 30 g/L the microalgal growth is found to be enhanced and there is some residual substrate left at the end of the 6-day fermentation cycle (Xiong W et al., 2008). Yen-Hui Chen et al., 2011 reported that for batch cultures of *C. protothecoides*, glucose was fully consumed within four days of fermentation cycle that resulted in retardation of growth due to substrate limitation. The same study done with fed-batch fermentation showed an improved biomass and lipid concentrations with glucose as substrate than the batch cultures. This was mainly due to improved aeration and continuous feeding of the glucose to maintain it at the maximum optimum concentration so that the growing microalgal cells are not starved of the glucose.

Although it is not a commonly preferred carbon source, glycerol has a positive effect on cell growth of *C.protothecoides* in the pure form or in combinations with glucose (John O'Grady et al., 2011). The crux to increasing the acceptability of glycerol as a carbon source for *C.*

protothecoides is by making the inoculum adapt to similar culture conditions in the presence glycerol before its use to inoculate larger cultures (Borowitzka et al., 1988). Yen-Hui Chen et al., 2011 have also reported that the yield of biomass and lipid with pure glycerol is higher than the yields from glucose in both batch and fed batch experiments. Though there are several investigations on the growth of *C. protothecoides* on pure glycerol (Heredia-Arroyo et al. 2010), not much has been established in the fed-batch fermentations to improve yields using crude glycerol and in mixotrophic experiments.

2.5.2.4.2. Effect of nitrogen sources on lipid content of heterotrophic *C.proto*thecoides

A wide range of nitrogen sources have been found acceptable for *C. protothecoides* like yeast extract, ammonia, ammonium nitrate, urea, peptone and other forms of nitrates. Microalgal lipid accumulation is characterized mainly by the nitrogen content and the C/N ratio in the cell cultures. Some minor factors such as low temperature (Renaud S et al., 2002), high salinity (Takagi M et al.,, 2000), high iron levels (Liu Z et al., 2008) and high light intensity (Khotimchenko S V et al., 2005) have also proved to induce lipid accumulation owing to stress conditions. Certain studies on *C.vulgaris* have shown that the biomass content decreases and cellular lipids concentration increases with nitrogen limitation. While the presence of the nitrogen source seems necessary for biomass growth, at the same time the cells start accumulating lipids only when the nitrogen is starved from the cells (Yanna Liang et al., 2009).

Among the effects of four different commonly used nitrogen sources-urea, tryptone, Bacto peptone and yeast extract it was seen that both urea and yeast extract proved to have a positive influence biomass and the total fatty acids (TFA) production as biodiesel feedstock in batch culture of heterotrophic *Chlorella protothecoides* CS-41 grown on glucose. However their concentration proves to be critical to growth (Ratledge C et al., 2004).

Yeast extract seems to be a good source of nitrogen for heterotrophic growth due to its complex nature also providing amino acids, vitamins and essential growth factors that promote algal growth (Shi, X. M et al., 2000; Gonzalez-Bhashen et al., 2000; Illman et al., 2000; Chen G Q et al., 2006). In concurrence with other studies, the *C. protothecoides* cells respond to certain stress factors due to nitrogen deprivation by accumulating high amounts of lipids, fat or starch within their cells. This limitation is found to produce an advantageous change in the chemical composition of the microalgae that causes such a metabolic transformation (Takagi M et al., 200). High amounts of yeast extract helps achieve high biomass over the initial log phase and slowly lipid accumulation is induced by limiting the nitrogen concentrations in the medium (Xiong et al., 2008).

This trend has been consistent in the case of some other species of microalgae like *Chlorella ellipsoidea SK* that accumulated 2.3% of fats in nitrogen-rich medium and 26.8% in nitrogen deprived medium and *Chlorella pyrenoidosa 82* that accumulated 16.7% in cultures having high nitrogen concentrations that increased to 47.1% in those without high nitrogen concentrations (Borowitzka et al., 1988).

2.5.3. Mixotrophic algae

2.5.3.1. Mixotrophy in algae

The nutrition mode of algae where photosynthesis presents to be the main energy source while the presence of organic compounds also are also essential for the growth of the microalgae is called photolothotrophic heterotrophy, more commonly known as, mixotrophy. Another type of mixotrophy is amphitotrophy where the microalgae can live either autotrophically or heterotrophically depending on the relative ratio of concentration of organic compounds to the available light intensity (Chen C-Y et al., 2011). The carbon available here are from two sources-

one from the inorganic carbon dioxide in light and the other from the organic substrate. Hence a simultaneous assimilation of carbon in two forms occurs to rapidly increase the cell densities. The carbon dioxide released as a result of respiration here is then captured and reutilized under light exposure (JoAnn M et al., 2008; Xu et al. 2004). Hence in mixotrophy a dual phase metabolism occurs with autotrophic light energy being converted to chemical energy via photosynthesis and the catabolism of organic compounds giving the energy required for cell synthesis via respiration. In simple words, in mixotrophic growth both photosynthesis and oxidative glucose metabolism co-exist (Yu et al. 2009). Hence, it is evident that the cell biomass and growth are both expected to increase as compared to phototrophic and heterotrophic cultures due to the synergistic advantage of two metabolisms coupled in one (Bockstahler and Coats, 1993a,b). Energy rich compounds such as lipids are synthesized during the photosynthesis cycle with the help of CO_2 (Portis and Parry 2007). Acetyl coenzyme A (Acetyl-CoA) is the fundamental substance that triggers the carbon to branch out on to one of the many metabolic possible pathways such as lipid synthesis and the tricarboxylic acid cycle (TCA cycle). If the conversion of Acetyl-CoA to malonyl-CoA is catalyzed by the acetyl-CoA carboxylase (ACCase), then the algae enter the phase of fatty acid biosynthesis (Cronan and Waldrop, 2002). Many phytoplankton in oligotrophic habitats (reviewed in Jones, 1994, 2000) and many eutrophic estuaries (e.g., Nygaard and Tobiesen, 1993; Jeong et al., 2004) have widely adapted the mixotrophic mode of nutrition for growth and survival.

Some studies suggest that the mixotrophic growth rate can be anticipated to be the sum of the individual photoautotrophic and heterotrophic growths (Martinez and Orus 1991; Marquez et al. 1993). As the two process are assumed to occur independently, the organic carbon sources influence respiration rather than photosynthesis and while biomass concentrations are enhanced,

the photosynthetic efficiency is impaired (Liu et al. 2009). Figure 2.12 compares the pH variation in phototrophic, heterotrophic and mixotrophic cultures of algae (Chen, C.Y et al., 2011).

2.5.3.2. Mixotrophic growth in the presence of glucose

The effects of mixotrophy on 3 microalgae *Nannochloropsis oculata*, *Dunaliella salina* and *Chlorella sorokiniana* grown with glucose as the organic carbon substrate showed that at extremely high glucose concentrations the growth rates of all 3 species were reduced (Minxi Wan et al., 2011). This could be attributed to substrate inhibition (Ip et al. 2004) occurring at such extreme concentrations and this result was found consistent with other species of microalgae (Garcia et al., 2005; Yu et al., 2009). Substrate inhibition could also cause a decrease in the protein and lipid levels and lower the activities of at least 20% of all the enzymes (Reed et al., 2010). In spite of this, the presence of glucose in optimum amounts is vital for high biomass production and protein and lipid accumulations within this biomass. With mixotrophy, the presence of a carbon substrate, like glucose, provides supplementary energy in the form of NADPH and Acetyl-CoA and other material for biosynthesis (Ren et al., 2009).

The consumption pattern of glucose is evidently different in mixotrophy than heterotrophy. Previous studies show that as glucose was the only carbon source in heterotrophic microalgal cultures carries out in absence of light, it was utilized very rapidly and in most cases no residual glucose was left behind at substantially optimum initial concentrations of glucose (Liu et al., 2011). The mixotrophic cultures however showed residual glucose concentrations as the glucose was not completely consumed by the microalgal cells to produce biomass as they also derived carbon from the inorganic carbon dioxide in light. Hence providing the microalgal medium with limited amounts of glucose may provide the best way to obtain high biomass and

improve glucose consumption while also reducing the substrate cost. Figure 2.13 represents metabolic networks for autotrophic, heterotrophic and mixotrophic cultures (Yang et al., 2000).

Consideration of the economics of the process is also important for lipid yields. If glucose was the only main cost affecting factor, we could keep the glucose at lower concentrations to achieve high lipid yields. But very low concentrations of glucose could also affect biomass productivity (Gouveia et al. 2009). Thus there is a need to find a replacement for glucose that can make the process cost effective. Hence the use of glycerol as a potential carbon substrate for mixotrophic growth will prove advantageous and is an area for exploration.

2.5.3.3. Economics of mixotrophy and future prospects

Most heterotrophic and mixotrophic microalgal cultures utilize sugars like glucose or similar organic substrates for growth. The major drawback here is that for commercial production, these sources will contribute to about 60% of the total cost and such an expensive process may not be considered sustainable. Hence use of waste water streams, agricultural wastes or other co-products from similar processes must be diverted to be used for commercial algae production to overcome this problem (Jiang et al., 2009; Xu H et al., 2006; Cheng Y et al., 2009). The presence of an integrated bio refinery where the glycerol, which is the byproduct of the biodiesel pipeline, can be used to feed the microalgae to produce biomass and lipids that can be converted back to biodiesel will make the process a self-sustained one. Also the location of microalgal culture systems in the vicinity of a waste water treatment facility can allow the access to adequate amounts of water streams having the nutrients required for algal growth that can be reused and recycled. Some agricultural wastes like cane molasses which comes as a side product of the sugar industry, comprising 40-50% of the total sugars could bring down the cost to one fifth of those using glucose (Najafpour G D., 2003). In this way both carbon utilization and

waste recovery can be achieved which leads to the production of useful energy and beneficial bio-products.
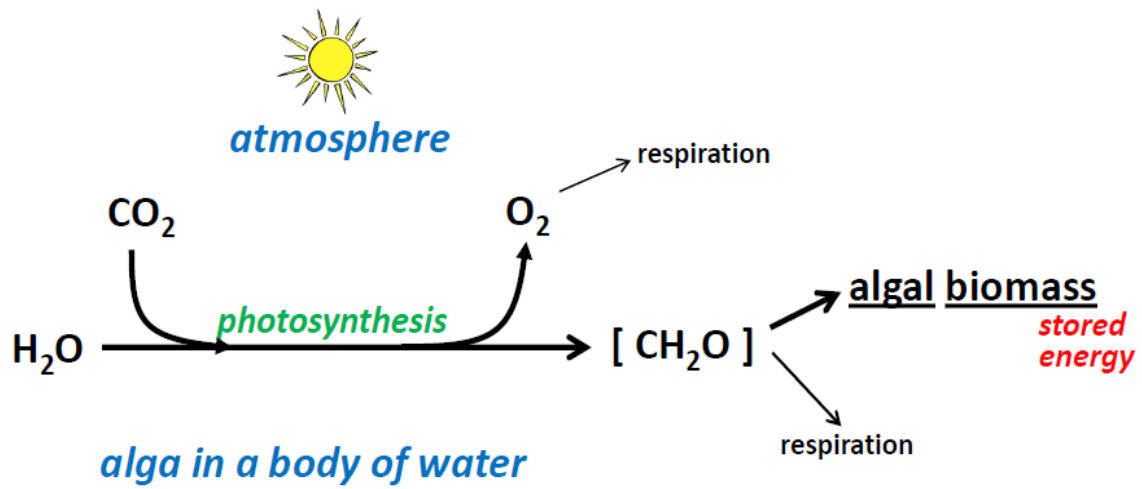
Figure 2.1 Schematic of the photosynthesis by algae in a body of water

Source: Adapted from UTEX Workshop, 2011
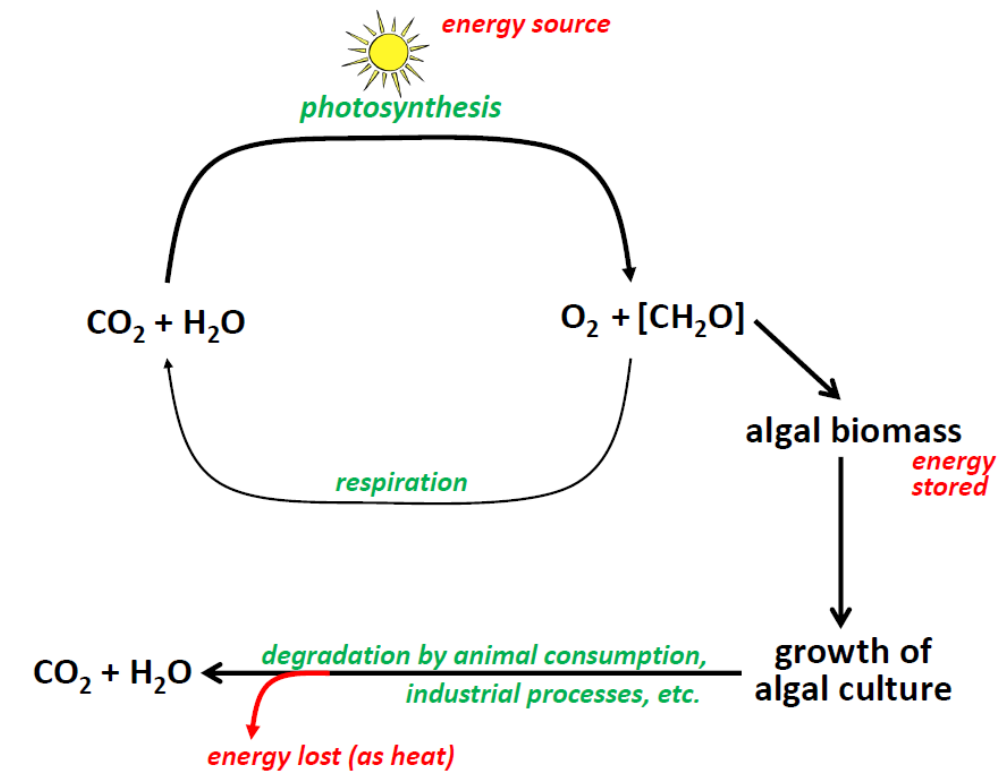
Figure 2.2 Energy and carbon flow through algae.

Source: Adapted from UTEX Workshop, 2011
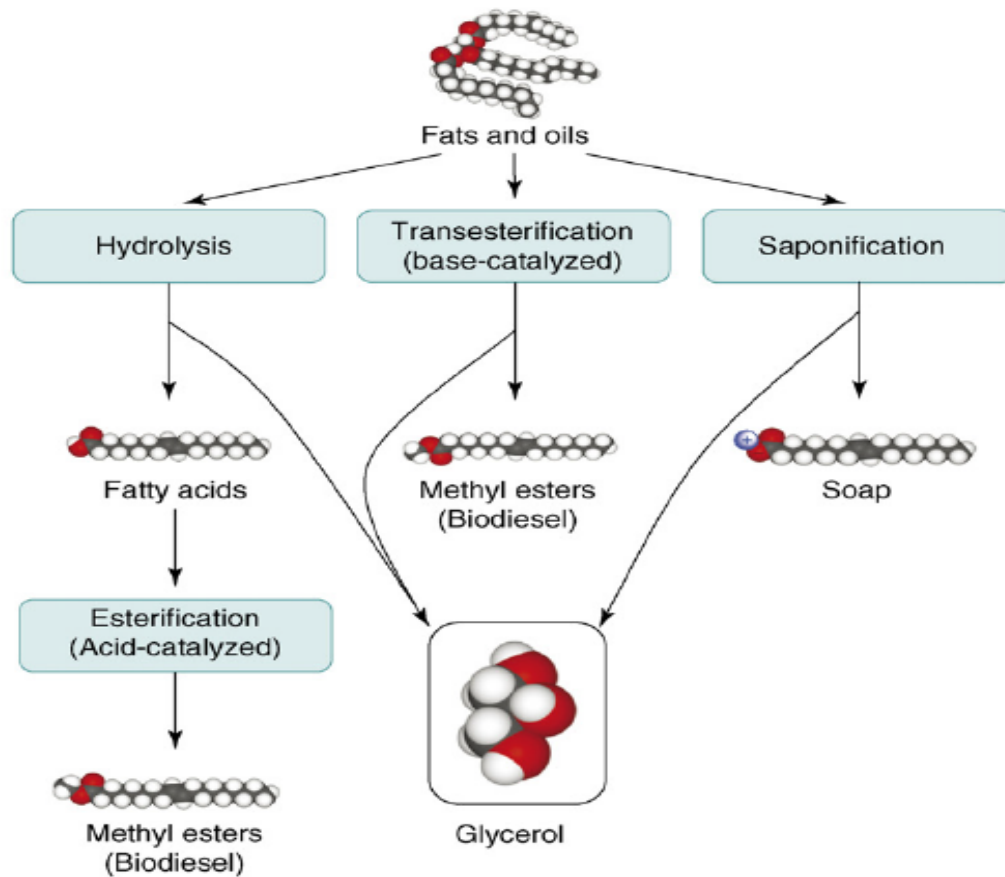
Figure 2.3 Various applications for the conversion of fats and oils.

Source: Gunstone F D, 2001; Tyson et al., 2004.

Table 2.1 Comparison of different sources of biodiesel

Crop	Oil yield (L/ha)	Land area needed (M ha) [a]	Percent of existing US cropping area [a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136,900	2	1.1
Microalgae[c]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the United States.

[b] 70% oil (by wt) in biomass.

[c] 30% oil (by wt) in biomass.

Source: Yusuf Chisti, 2007

Table 2.2 The oil content of some microalgal species

Microalgae	Oil content (% dry weight)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizocgytrium sp.	50-77
Tetraselmis sueica	15-23

Source: Yusuf Chisti, 2007
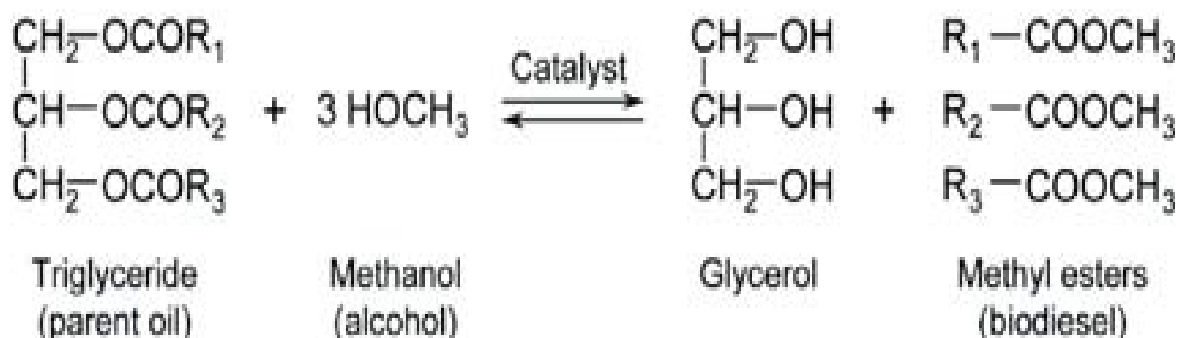
Figure 2.4 Transesterification process of conversion of oil to biodiesel. R1, R2 and R3 are hydrocarbon groups.

Source: Jang et al., 2005
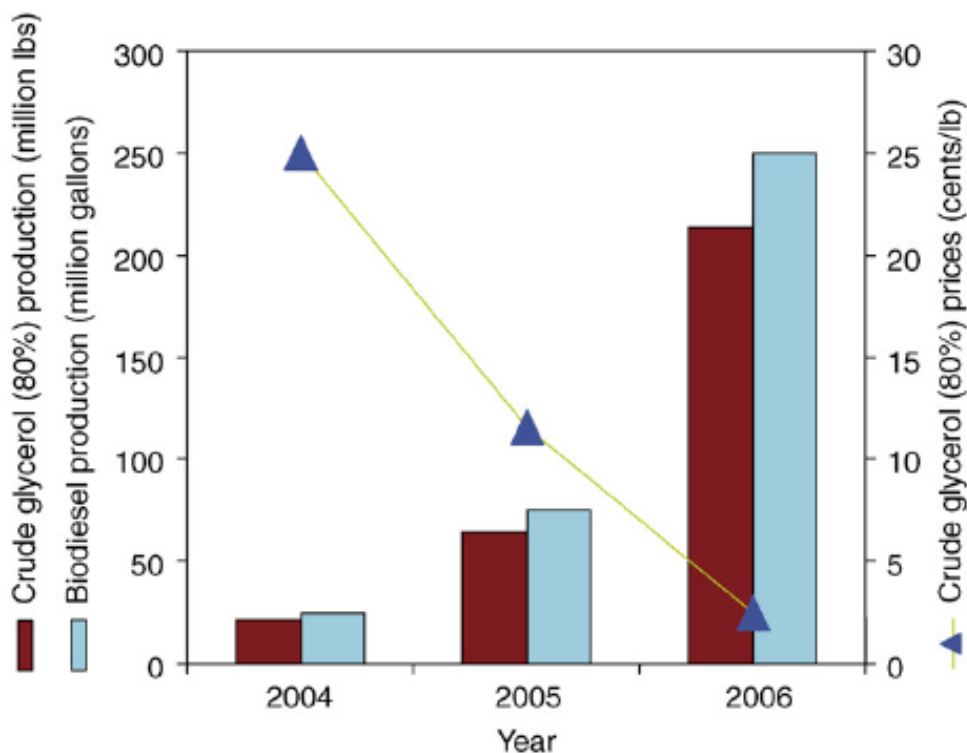
Figure 2.5 United States biodiesel production trend and its impact on the prices of crude glycerol.

Source: Yazdani and Gonzalez, 2007

Microalgal biomass $\xrightarrow{\text{Transesterification}}$ Biodiesel + Spent biomass

$\downarrow$

Proteins + Carbohydrates + Other products + Residue

Animal feed + Methane $\longleftarrow$
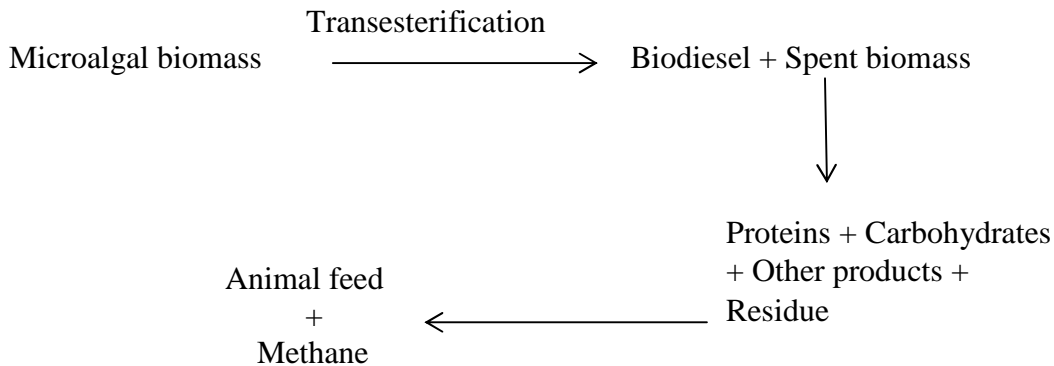
Figure 2.6 Most prominent uses for microalgal biomass.

(Adapted from Sanchez Miron et al., 2003)

Table 2.3 Comparison of the characteristics of microalgae biodiesel, diesel fuel and the ASTM biodiesel standards.

Properties	Biodiesel from microalgal oil[a]	Diesel fuel[b]	ASTM biodiesel standard
Density (kg/L)	0.864	0.838	0.86-0.90
Viscosity (mm^2/sec, cSt at 40°C)	5.2	1.9-4.1	3.5-5.0
Flash point (^{o}C)	115	75	Min 100
Solidifying point (^{o}C)	-12	-50-10	-
Cold filter plugging point (^{o}C)	-11	-3 (max -6.7)	Summer max 0 Winter max < -15
Acid value (mg KOH/g)	0.374	Max 0.5	Max 0.5
Heating value (MJ/kg)	41	40-45	-
H/C ratio	1.81	1.81	-

[a] The data is from Xu H et al., 2006.

[b] The data about diesel fuel was taken from published literature as indicated in the text. (Ma and Hanna, 1999; Lang et al., 2001; Al-Widyan and Al-Shyoukh, 2002; Antolin et al., 2002; Vicente et al., 2004)

Table 2.4 Different microalgae cultivation conditions based on energy source and carbon source

Cultivation condition	Energy source	Carbon source	Microalgae cell density	Reactor scale up	Cost	Issues associated with scale up
Phototrophic	Light	Inorganic	Low	Open pond or photobioreactor	Low	Low cell density High condensation cost
Heterotrophic	Organic	Organic	High	Conventional fermenter	Medium	Contamination High substrate cost
Mixotrophic	Light and organic	Inorganic and organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost

Source: Chun-Yen Chen et al., 2011

Figure 2.7 Open pond for algae cultivation

Source: Chisti, 2007
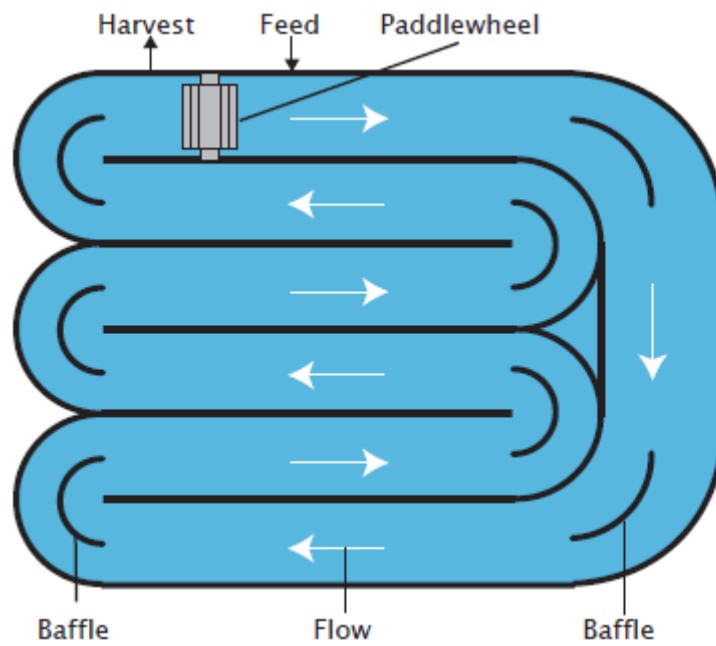
Figure 2.8 Depiction of the operation of an open pond system

Source: Molina Grima et al. 1999
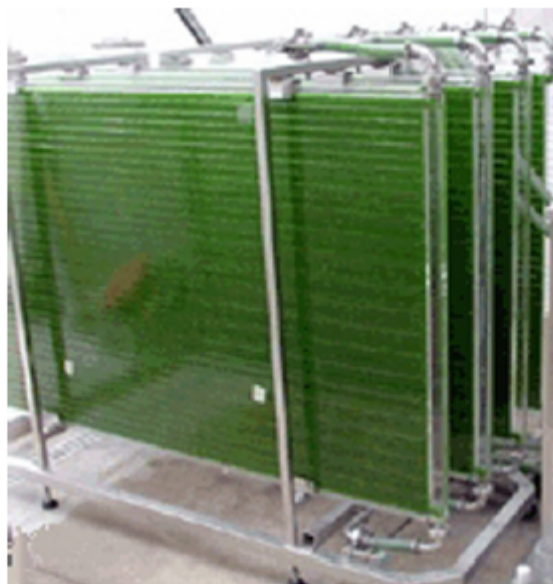
Figure 2.9 Depiction of tubular photobioreactors

Source: Chisti, 2007

Table 2.5 Comparison of the features of an open pond system and closed photobioreactor system

Production system	Advantages	Limitations
Raceway pond	Relatively cheap Easy to clean Low energy inputs Utilizes non-arable land Easy maintenance	Poor biomass productivity Large land area required Limited to fewer strains of algae Poor mixing, light and CO_2 utilization Cultures are easily contaminated
Tubular photobioreactor	Large illumination surface area Suitable for outdoor cultures Relatively cheap Good biomass productivities	Some degree of wall growth Fouling Requires large land space Gradients of pH, dissolved oxygen and CO_2 along the tubes
Flat-plate photobioreactor	High biomass productivities Easy ti sterilize Low oxygen build up Readily tempered Good light path Large illumination surface area Well suited for outdoor cultures	Difficult scale up Difficult temperature control Small degree of hydrodynamic stress Some degree of wall growth
Column photobioreactor	Compact High mass transfer Low energy consumption Good mixing with low shear stress Easy to sterilize Reduced photoinhibition and photo-oxidation	Small illumination area Expensive compared to open ponds Shear stress Sophisticated construction

Source: Brennan et al., 2009
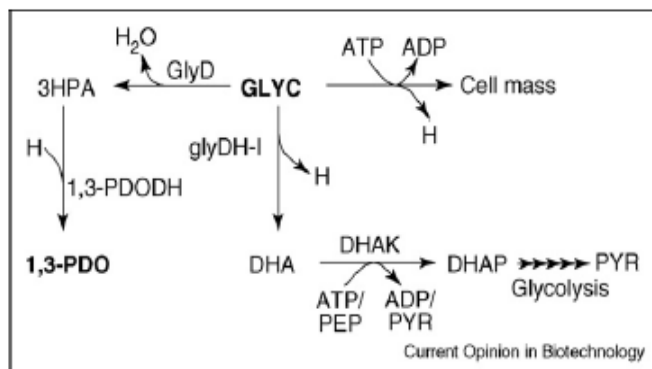
Figure 2.10 Pathway for fermentative metabolism of glycerol by microorganisms.

Abbreviations: DHA, dihydroxyacetone; DHAK, DHA kinase; DHAP, DHA phosphate;

GLYC, glycerol; GlyD, glycerol dehydratase; glyDH-I, glycerol dehydrogenase type I;

PEP, phosphoenolpyruvate; PYR, pyruvate; 1,3-PDO, 1,3-propanediol; 1,3-PDOH,

1,3-PDO dehydrogenase; 3HPA, 3-hydroxypropionaldehyde.

Source: Syed Shams Yazdani and Ramon Gonzalez, 2007

Table 2.6 Comparison of the cell compositions of autotrophic cultures (AC) and heterotrophic

cultures (HC) of *Chlorella protothecoides*

Composition (% dry weight)	AC	HC
Protein	52.64 ± 0.26	10.28 ± 0.10
Lipid	14.57 ± 0.16	55.20 ± 0.28
Carbohydrate	10.62 ± 0.14	15.43 ± 0.17
Ash	6.39 ± 0.05	5.93 ± 0.04
Moisture	5.39 ± 0.04	1.96 ± 0.02
Others	10.42 ± 0.65	11.20 ± 0.61
Heating value (MJ/kg)	23.00 ± 0.08	27.00 ± 0.09

Source: Miao X et al., 2004
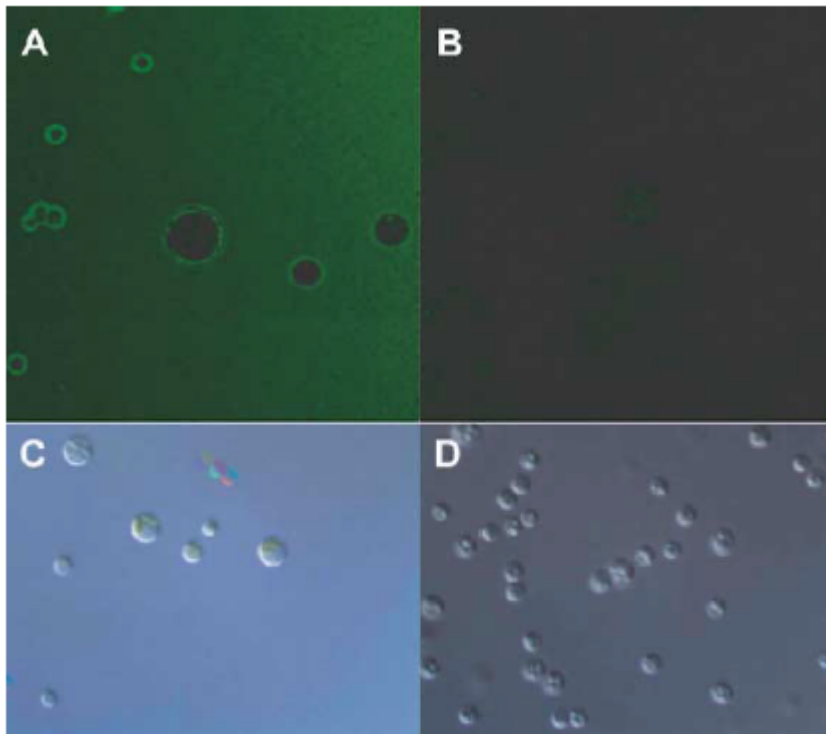
Figure 2.11 Microscope images of autotrophic cultures and heterotrophic cultures of *Chlorella protothecoides*

Source: Miao X et al., 2004

(A,B) depict cells of photoautotrophic and heterotrophic *C. protothecoides* under confocal laser scanning microscope.

(C,D) depict cells of photoautotrophic and heterotrophic *C. protothecoides* under differential interference microscopy

(A) Autofluorescence of photoautotrophic *C. protothecoides* cells with chlorophyll.

(B) Autofluorescence of chlorophyll disappearing cells of heterotrophic *C. protothecoides*.

(C) Almost no lipid vesicles were observed in photoautotrophic *C. protothecoides* cells.

(D) The cells of heterotrophic *C. protothecoides* were full of lipid vesicles

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Photoautotrophic

$$H_2O + HCO_3^- \xrightarrow{hv} C \text{ (biomass)} + \tfrac{1}{2} O_2 + 3OH^- \quad \text{pH increase}$$

Heterotrophic

$$(1+a)CH_2O + O_2 \xrightarrow{hv} C \text{ (biomass)} + aCO_2 + (1+a) H_2O \quad \text{pH decrease}$$

Mixotrophic

$$bHCO_3 + cCH_2O \xrightarrow{hv} (b+(c+a)) C \text{ (biomass)} + 3 OH^- + a CO_2 \quad \substack{\text{pH changes} \\ \text{are not significant}}$$
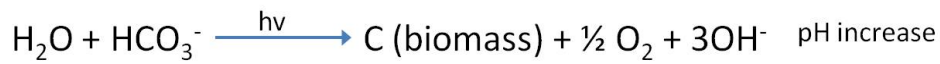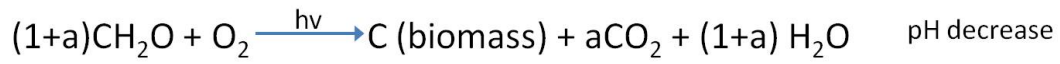
Figure 2.12 Comparison of pH variation in phototrophic, heterotrophic and mixotrophic microalgal cultures.

Source: Chun-Yen Chen et al., 2011

Figure 2.13 Metabolic networks for auto-, hetero- and mixotrophic microbial cultures.

Dotted lines are fluxes for cell mass biosynthesis

Source: Yang et al., 2000

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III. COMPARISON OF BIOMASS AND LIPID YIELDS FROM THE HETEROTROPHIC FED BATCH BIOPRODUCTION OF *Chlorella protothecoides* USING THREE DIFFERENT GRADES OF GLYCEROL

Abstract

Microalgal oil is considered one of the prime sources for satisfying the world wide concerns of fossil fuel depletion and under heterotrophic cultivation microalgae have the ability to produce large amounts of lipids to handle the current energy demand (Li Y et al., 2008). As 60-70 % of cost of the heterotrophic microalgal cultivation process is the substrate, a wise substitute should be chosen to replace the existing cost-intensive sources (Xu J et al., 2006; Gao C et al., 2010). Owing to the large amount of glycerol being generated during biodiesel production without any commercial value, we can study the utilization of glycerol by microalgae to help the process become economically viable (Johnson D T et al., 2007). The aim of this study was to assess the growth of the microalga *Chlorella protothecoids* in fed-batch mode for eight-day fermentation. The eighth day average biomass and lipid concentrations of the *C.protothecoides* grown on 65% crude glycerol were found to be 22.13 ± 0.17 g/L and 9.75 ± 0.02 g/L respectively, that was higher than those grown on 96% pure glycerol producing average biomass and lipid concentration of 20.32 ± 0.12 g/L and 8.10 ± 0.04 g/L, and those grown on 99% pure glycerol producing average biomass and lipid concentrations of 20.06 ± 0.11 g/L and 8.23 ± 0.01 g/L respectively. The lipid yield with the 65% pure glycerol is about 0.44 ± 0.004 gram lipid per gram of dry biomass. Hence the lipid content may be increased to about 44% of the dry weight of algae at the end of the eight day fermentation. These results help establish that the heterotrophic growth was possible for *C.protothecoides* using glycerol and the 65% purity was sufficient and worked best for the microalgal biomass and lipid production. The cost of substrate

and overall cost of the process could be reduced as the crude glycerol from biodiesel production requires no further purification and the impurity in fact enhances the growth of *C. protothecoides*. This work validates that crude glycerol may be utilized as a prospective substrate in place of other expensive sources like glucose to serve as a potential cost cutting step in lipid production.

3.1. Introduction

It has been estimated by The United States Energy Information Administration (EIA) that the non-renewable and exhaustive sources of energy would take only another 90 years to run out of their reserves. The US Department of Energy (DOE) recognized the importance of pursuing research for prospective replacements for fossil fuel as early as 1978, by investing about $25 million in the Aquatic Species Program (ASP) to identify microbial strains that have high lipid yielding capabilities and to develop algae-derived fuel technologies (Sheehan et al. 1998). The finding showed that while the production of fuel from microalgae was feasible, it was expensive. The microalgae under stress conditions of nitrogen deprivation can increase their cellular lipid level to as high as 60–70% of their dry weight when grown in favorable heterotrophic conditions without light.

Biodiesel obtained from microalgal lipids via transesterification with alcohol usually methanol or ethanol, is essentially fatty acid methyl esters (FAME) that showing combustion properties similar to the biodiesel produced from vegetable oil or other plants oils (Vasudevan et al. 2008). Biodiesel can be used in conventional diesel engines of vehicles in its pure form as B100 or in blends of different proportions with diesel from fossil fuel. For example B50 and B20 represent 50% and 20% biodiesel blended with 50% and 80% diesel fuel respectively. Microalgal biodiesel may prove to be an ultimate bioenergy product that will actually bring constructive attributes to the environment with reduced greenhouse gas emissions and carbon sequestration (Fu B S et al., 2009). The main bottleneck for large-scale biodiesel production was the lack of knowledge about availability of alternative starting materials for the biodiesel industry apart from the conventional sources such as soybean oil, canola oil, plant oil or vegetable oil (Hossain et al. 2009). The fuel use may be brought down about 4 times, from 500

billion gallons/year to 50 billion gallons/year, with the high energy efficiencies of biodiesel. Only the microalgal oil seems to have the prospects to completely replace the use of fossil fuels. From the figures estimated by the United States Department Of Energy, only 15,000 square miles (38,849 square kilometers) or about one seventh the amount of land devoted to corn in 2000 would be required for cultivation if algae fuels were to displace the use of all the petroleum in the United States (Xu J et al., 2006) .

Chlorella protothecoides have been found to grow well under heterotrophic conditions to accumulate high amounts of biomass and lipids (Lee Y K., 2001). This microalgae seems to commonly utilize glucose ($C_6H_{12}O_6$), acetate ($C_2H_3O_2^-$), glycerol ($C_3H_5(OH)_3$), and other carbon sources (Syrett P J et al., 1964; Matsuka M et al., 1969,1970; Ferraz et al., 1983; Ceron Garcia et al., 2006). However, there has not been a lot of research on the fed-batch growth of these microalgae using different kinds of glycerol and its effect on the lipid production under different culture conditions. This particular species of microalgae showcases several favorable traits for production of biodiesel. They are a robust species capable of growing on an extensive range of substrates with minimal media and a high resistance to contamination. Typical heterotrophic bioreactors are closed vessels harboring algae in the nutritive medium having the various nutrients distributed in optimal conditions in order to maximize biomass and lipid productivity. The size of these bioreactors varies from small 1 litre reactors to large scale 500,000 litre reactors. The dimensions and shape of these reactors are all dependent on the capital available and application intended. Conversely, the photobioreactors are aimed at maximizing the surface area available for light exposure (Apt and Behrens, 1999). Most importantly they have a capacity to produce lipids up to 55% of their dry weight under heterotrophic conditions (Xu et al., 2006; Garcia M C C et al., 2000). Also, the residual microalgal biomass left behind after lipid

extraction has been found to be non-toxic and can be put to many applications like animal feed, protein feed for humans, for bio-gasification and so on (Day A G et al., 2009).

There has been a series of investigations to see the acceptability of glycerol as a substrate for microalgal growth and to compare them to the growth achieved using glucose. Some preliminary studies suggest that the growth of *C.protothecoides* on glycerol gives greater biomass productivity (Liang y et al., 2010). Just like the growth on glucose, through a unique metabolic adaptation *C. protothecoides* grow readily and with the same ease on glycerol. The nature and composition of glycerol is mainly dependent on two factors: the feedstock of the transesterification process and the biodiesel production process conditions. The purity of the glycerol obtained as a byproduct of biodiesel production also depends on these two factors and can range from a low value to higher values of purity (Liang et al., 2009).

In terms of the industrial advent of this technique, the primary advantage comes in utilizing the waste glycerol stream that builds up with every transesterification process. Currently the commercial value for glycerol is very low due to lack of any significant application that leads to glycerol prices being very low. About one ton of crude glycerol is found to be produced for every 10 tons of biodiesel produced (Huang et al., 2010). If this low-cost substrate that is readily available in excess could be made to synthesize a bioenergy product of high value, we could potentially transcend the economic barriers and create a sustainable and self-sustained process. In this study we are trying to 1. Evaluate the growth of *C. protothecoides* in the presence of three different grades of glycerol having 65% purity, 96% purity and 99.99% purity respectively and 2. Compare the biomass and lipid yields from the three different grades of glycerol to see which one gives the best biomass and lipid concentrations for heterotrophic *C. protothecoides*.

3.2. Materials and methods

3.2.1. Materials

All chemicals were of analytical grade and were obtained commercially from authentic sources.

3.2.1.1. Grades of glycerol

- Crude Glycerol ($\square$ 65% pure) was obtained from the biodiesel plant on Clemson University campus. This glycerol was a by-product of biodiesel produced from the transesterification of vegetable oil with methanol using potassium hydroxide as catalyst.

- Enzymatically derived Glycerol ($\square$ 95% pure) was obtained from treating the above mentioned crude glycerol with an enzyme Novozyme TL-IM to obtain a purer grade.

- Pharmaceutical grade Pure Glycerol ($\square$ 99.99% pure) was obtained commercially from VWR international.

3.2.2. Microorganism and inoculum preparation

*Chlorella protothecoides (*UTEX 256) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX). The basal medium was a modified BG11 culture medium adapted from the research of Chen et al., 2011, with a composition as follows (per liter): 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$, 25 mg $CaCl.2H_2O$, 25 mg NaCl, 3 mg $FeSO_4.7H_2O$, 0.01 mg vitamin B1, and 1 ml A5 solution. The inoculum was prepared by suspending microalgal cells in a 500 mL Erlenmeyer flask having 200mL of the basal medium supplemented with the carbon source (glycerol) at 30 g/L and nitrogen source (yeast extract) at 4 g/L. All media were autoclaved at 121^oC for a 15-20 minute cycle prior to inoculation. The initial pH of the medium was adjusted with 0.5M H_2SO_4 and 0.5M KOH to a value of 6.8 using an Orion Aplus Benchtop pH meter (525A+, Thermo Scientific, USA), prior to autoclaving. The cultures were incubated at 200 rpm in an NBS Classic series C4KC refrigerated shaker incubator

at a growth temperature of 28°C. As the cultures were heterotrophic, all flasks were wrapped with aluminum foil to block out any light. After 3-4 days of incubation, the heterotrophic microalgal cells in the flasks were used for further experiments after observing under a microscope.

3.2.3. Characterizations of different grades of glycerol

The crude glycerol (□ 65% purity) was obtained from the biodiesel plant on Clemson University campus as a by-product of biodiesel produced from the alkali-transesterification of used vegetable oil with methanol using Potassium hydroxide catalyst. The enzymatically derived glycerol (□ 95% pure) was obtained by treating the □ 65% purity crude glycerol with the enzyme Novozyme TL-IM. The characteristics of this □ 65% purity crude glycerol and □ 95% purity enzymatically derived glycerol are both tabulated in Table 3.1 and Table 3.2. The different glycerol and methanol concentrations were determined precisely with the help of a high performance liquid chromatography (HPLC) system described in the analytical techniques section. For evaluating moisture content, all of the samples were dried in an oven at 105°C for 48 hours to obtain constant weight. By subtracting the methanol concentration obtained from HPLC, from this moisture content, the water content was determined. The ash content present was determined by heating the sample to 600°C for two hours. The elemental composition of the crude glycerol and enzymatically derived glycerol were obtained by elemental analysis using the inductively coupled plasma (ICP) method in accordance with the wet ash digestion procedure from the Agricultural Service Laboratory of Clemson University (Clemson, USA).

3.2.4. Fed-batch cultivation of heterotrophic *Chlorella protothecoides*

Heterotrophic fed-batch cultivations were carried out in a 7.5 L working volume bioreactor (BioFlo 110, New Brunswick Scientific, USA) containing 2 L of basal medium

supplemented with 30 g carbon substrate/L (crude glycerol, enzymatically purified glycerol and pure glycerol respectively) and 4 g yeast extract/L. The feed solution was a stock containing 150 g carbon substrate/L (crude glycerol, enzymatically purified glycerol and pure glycerol respectively) and 15 g yeast extract/L to maintain the concentrations of carbon substrate in the culture medium at the desired levels. The pH was maintained at 6.8 by automatic addition of 0.5 M KOH and 0.5 M H_2SO_4 solutions and temperature was maintained at 28 ^{o}C. The dissolved oxygen concentration was aimed to be maintained at 40-50% air saturation by controlling the airflow and agitation speed. The initial values of aeration rate and the agitation speed were set at 1 L/min and 150 rpm, respectively and allowed to vary naturally with the microalgal growth. Sampling was done every 24 hours and samples saved for estimating biomass concentration, substrate utilization and lipid yields. Appendix A illustrates the heterotrophic fed-batch system.

3.2.5. Analytical techniques

All samples were collected every 24 hours throughout the 8 day fed-batch fermentation cycle. Each data reported were taken as an average of 3 readings from the same fermenter. In total, there were eight independent fermenter runs performed for each of the three treatments and the data reported were all average of 8 independent measurements ± standard error.

3.2.5.1. Determination of cell concentration

The *C. protothecoides* cell concentration were determined by recording the absorbance at OD 540 nm using a UV/Visible spectrophotometer and correlated to the cell dry weight to obtain precise values. To obtain the cell dry weight measurements, the culture broth was centrifuged at 3,000 rpm for 15 minutes with 3 cycles of water wash, followed by drying the algae pellet at 105 ^{o}C in an oven for 48 hours or till constant weight was obtained. Weighing of samples was done using an analytical balance (ABS104, Mettler Toledo, Switzerland).

3.2.5.2. Substrate utilization

The substrate concentrations were estimated using a HPLC (Shimadzu Scientific Instruments, Inc., MD, USA), equipped with am Aminex HPX-87H cation exchange column (Bio-Rad, CA, USA) at a temperature of 60^0C with 50 mM H_2SO_4 as the mobile phase. A solvent flow rate of 0.6 ml/min was used and the sample injection volume was 20 µL. The detector used was a pulsed refractive index detector. External standards of known concentrations of pure pharmaceutical grade glycerol were used to obtain the standard curve.

3.2.5.3. Lipid Extraction

The intracellular lipid was extracted from the microalgae using a Polytron homogenizer (PT 1200 model, Kinematica, Switzerland) to mechanically disrupt the algal cell walls and using hexane as a solvent to solubilize the lipids coming out of the ruptured microalgal cells (Cantrell & Walker, 2009; Dong & Walker, 2008a).

The dried algal biomass that was prepared for cell dry weight estimation is extracted with 20 mL hexane in a 50 ml centrifuge, homogenized with a Polytron homogenizer for 5 min, kept at 55^0C for 5 min, and then homogenized again for 5 min. The resulting slurry was then centrifuged at 3,000 rpm for 15 minutes and the supernatant containing lipids and hexane are then transferred to another pre-weighed centrifuge tube. This whole extraction procedure was repeated twice to improve extraction efficiency. The repetition was done to ensure that no lipid is left behind in the biomass. The supernatants containing hexane and lipids were then filtered using 0.6 µm filters and the hexane evaporated using a RapidVap vacuum evaporator system (Labconco, USA). The lipid left behind in the tube was weighed to constant weight with an

accuracy of 0.1 mg on an analytical balance (ABS104, Mettler Toledo, Switzerland). The lipid products were then stored under nitrogen atmospheres to prevent oxidation of microalgal lipids.

3.2.6. Statistical analysis

A Completely Randomized Design (CRD) was used for analysis of the data. The assumptions for carrying out an ANOVA model were satisfied. All experimental data was analyzed using an analysis of variance Fisher's LSD test and the pairwise contrasts were estimated using Statistical Analysis System (SAS v.9.2, SAS Institute, USA). The dependent variables were the Biomass productivity (g/L d) and Lipid content (g/g CDW). The 3 treatments involved were the three grades of glycerol. Prior to randomization, sample sizes were computed using Fishers LSD with an effect size of 8 g/L and a 5% significance level. An experimental design with 8 replicates per treatment was conducted. Experiments were performed in the order as obtained using randomization in SAS v.9.2.

3.3. Results and Discussion

3.3.1. Analysis of the composition of different glycerol grades

The feedstock used for lipid production and the conditions of the biodiesel production process are the two main factors that affect the composition and characteristics of the crude glycerol obtained as a by-product from the transesterification process (Thompon & He., 2006). Figure 3.1 depicts the process flow diagram for the heterotrophic fed batch fermentations of *C.protothecoides*. The crude glycerol obtained here was found to possess a dark brown color with a density of 0.8 g/ml. As summarized in Table 3.1, the chemical composition of the crude glycerol was found to be containing 65% glycerol, 30% methanol, 4% water and other impurities on a dry weight basis. The purity of the glycerol obtained from microalgal fermentative metabolism can lie anywhere in the rage of 42.3% (Liang et al., 2010b) to 85% (Mu et al., 2006),

depending on the different glycerol purification procedures and biodiesel production conditions applied by biodiesel plants. The methanol concentration can be heavily reduced by allowing the biodiesel to bubble for an extended period and then collecting the glycerol that separates out on settling. In this way the methanol concentrations can be reduced by about 80%.

From the ICP elemental analysis shown in Table 3.2, potassium was the main component of both crude and enzymatically derived glycerol. However the amount of potassium in the crude glycerol was as high as 15,532 ppm as opposed to a much lower amount of 524.2 ppm in the enzymatically-derived glycerol. This high potassium concentration was due to the alkali-catalyzed transesterification process that used potassium hydroxide as catalyst. Both the glycerol grades also had a substantial concentration of sodium. As *C. protothecoides* is a microalgal species that has high tolerance to salinity, it is expected to behave as a good candidate for these grades of glycerol. The results from previous studies on the effects of salinity for marine microalgae show that the lipid contents of *Botryococcus braunii* increased from 36% to 51% as the salinity levels increased from 0% to 6%, respectively (Borowitzka et al., 1988). Batch cultures of mixotrophic *C.protothecoides* were also found to give consistent results as the marine algae by producing the highest biomass concentration when at 17.5 g/L of initial salinity as compared to 0 g/L and 35 g/L initial salinity. These experiments also showed that *C. protothecoides* had a strong tolerance to the salinity range as high as sea water up to 35 g/L of NaCl (Tamarys et al., 2010).

Other elements like phosphorus, calcium, and aluminum were also found in detectable amounts. Iron and sulfur were present in trace amounts in both grades of glycerol but the enzymatically derived grade had higher sulfur and iron concentrations. Some other elements like Arsenic, cadmium, molybdenum, selenium, lead and nickel were found to be below the detection

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limits in both grades of glycerol. The concentration of magnesium and boron seemed to have high standard deviations and hence tend to fluctuate.

Many fluorescent studies have been performed previously on microalgae and it has been noted that iron regulated the biomass production in phytoplanktons that grow in both high-nitrogen-low-chlorophyll environments as well as in oligotrophic environment (Behren-feld et al., 2006). The impacts of iron on the biomass and lipid accumulation of marine microalgae have been widely studied. These studies on *Chlorella vulgaris* show that $FeCl_3$ used as an iron source for these microalgae had a profound effect on the final biomass concentration during the exponential phase but did not induce lipid production in the cells. The concentration of this FeCl3 was varied and it was found that concentrations of about 1.2 x 10^{-5} mol per L $FeCl_3$ produced only 56.6% lipids while lower concentrations gave up to 3-7 fold increase in lipid levels (Liu et al., 2008). The presence of certain stress factors other than nitrogen limitation (Illman et al., 200) like high salinity (Rao et al., 2007), reduced phosphorus concentrations (Reitan et al., 1994), silicone deficiency (Lynn et al., 2000) and the presence of certain heavy metals like cadmium (Guschina and Hardwood., 2006) also induced lipid accumulation in most microalgae.

3.3.2. Effect of the three grades of glycerol on the biomass and lipid production of *Chlorella protothecoides* grown in fed batch heterotrophic fermentations

Three heterotrophic fermentations of *C. protothecoides* were carried out in 7.5 L fermenters in the fed batch mode using crude glycerol (□ 65% purity), enzymatically derived glycerol (□ 95% purity) an pure glycerol (□ 99.99% purity) respectively.

3.3.3. Comparison of substrate utilization, biomass and lipid yields from each of the treatments

The biomass and lipid yields from these experiments are tabulated in Table 3.3. The starting concentrations of all the glycerol grades were at 30 g/L. After inoculation, the fed-batch fermentations were carried out under heterotrophic conditions where the variable parameter affecting biomass production was assumed to be the type carbon substrate- crude glycerol, enzymatically derived glycerol and the pure glycerol. Figure 3.2 shows the trend in the biomass concentration and substrate concentration for each of the treatments over the 9-day fermentation period. Figure 3.2 (A) shows that the average biomass concentration on day 1 reached 4.98 ± 0.18 g/L for cultures grown on pure glycerol which was significantly higher than both the average biomass concentrations of 2.54 ± 0.08 g/L, obtained from the cultures grown on crude glycerol (Figure 3.2 C), and 2.87 ± 0.23 g/L obtained from those grown on the enzymatically derived glycerol (Figure 3.2 B). This showed that the cultures grown on pure glycerol seemed to have the smallest lag phase and the cultures adapt readily and start growing most rapidly as compared to both the enzymatically derived glycerol and crude glycerol.

Figure 3.3 (A) gives a day-wise comparison in the biomass concentration of each treatment up to 9 days. Days 2, 3, 4 and 5 depict the exponential phase of growth for *C.protothecoides* where the biomass concentrations of both the crude glycerol and enzymatically derived glycerol are not significantly different and the trend of increase seems to be very similar. However, the pure glycerol seems to be the more favorable substrate in the exponential phase where the average biomass concentration on the 5th day is of 15.83 ± 0.16 g/L for pure glycerol which is significantly higher than those achieved in both the crude and enzymatically derived glycerol with average biomass concentrations of 13.92 ± 0.09 g/L and 14.44 ± 0.34 g/L, respectively. From day 6 onwards the biomass from crude glycerol seems to increase at a higher rate and though at this stage the average biomass concentration from pure glycerol at 17 ± 0.15

g/L is still significantly higher, the average biomass concentration with crude glycerol also reaches 16.35 ± 0.18 g/L.

The prime interest are the day 8 cultures where the biomass concentrations for all three grades of glycerol are different with the crude glycerol giving the highest biomass of 22.13 ± 0.17 g/L as compared to those from enzymatically derived glycerol and pure glycerol at 20.32 ± 0.12 g/L and 20.06 ± 0.11 g/L respectively. Day 9 average biomass concentrations decrease slightly as compared to their respective day 8 concentrations and thereby show the entry of the microalgal cells into a slow decline phase. Hence the fermentations were stopped at 8 days to achieve maximum yields. The cultures could have also been oxygen limited around day 8 that resulted in the biomass increase declining.

From Figure 3.2 we can see that though the rate of increase of biomass was lower for the crude glycerol, there was a high increase in the biomass towards the end of the fermentation. This could be owing to the small quantity of impurities present in the crude glycerol (Chi et al., 2007) that initially slowed down the growth rate of the algae but with time helped prevent substrate inhibition which was a main problem encountered with the pure glycerol cultures after eight days of growth. From Table 3.1 we can see that the methanol concentration for the crude glycerol was about 30% and this could be considered another factor that were enabling the cells to multiply at a faster rate at the end of the exponential phase rather than at the beginning. Table 3.1 also shows high levels of sodium and potassium in both crude glycerol and enzymatically derived glycerol. Literature has also cited many studies that show that *C. protothecoides* is a species well adapted to grow well under saline conditions (Victor M et al., 2003; Tamarys et al., 2010). The slower growth rates for the pure glycerol cultures could be attributed to inhibitory by-products produced during the course of the growth cycle. Though the enzymatically derived

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glycerol had similar properties to the pure glycerol, they had comparatively higher concentrations of sodium, methanol, and other elemental impurities that helped them achieve an average biomass concentration of 20.32 ± 0.12 g/L at the end of 8 days which was higher than the pure glycerol cultures. Hence from Table 3.3 we can say that the biomass productivities for the crude glycerol was the highest at day 8 while those for the pure glycerol cultures were observed to be the lowest. It is important to monitor the airflow and agitation and control the dissolved oxygen levels to maintain the microalgal cultures (Heredia- Arroyo et al., 2010; Xiong et al., 2008).Oxygen limitation could be one of the most important factors affecting cell growth.

From Figure 3.3 (B) it is clear that the glycerol consumption gradually increases in the exponential phase of days 5, 6, 7 and 8 for all three treatments. During days 6, 7 and 8 there was a need to feed more volumes of the stock solution to achieve concentration of roughly 30 grams of glycerol/L with the eighth day having the highest feed volume. Though the amount of stock solution added was high in all 3 cases, the increase in the respective biomass was highest for crude glycerol. This shows that the cells of *C. protothecoides* started increasing their biomass rapidly during the end of the exponential phase in case of the crude glycerol being assimilated as the carbon source. Though glycerol consumption continued on day 9, the fed-batch experiment was stopped on day 8 due to no substantial increase in the average biomass concentration on day 9.

3.3.4. Effect of the three glycerol grades on lipid yield of *C. protothecoides*

Lipid accumulation is very specific to a particular strain of microalgae and varies with the culture conditions. The main factor critical for lipid accumulation in algae is the C/N ratio which is directly influenced by the carbon and nitrogen source used (de morais et al., 2007; Chulanovskaya M V., 1981). The average lipid concentration was found to be the highest for

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crude glycerol on the eighth day at 9.75 ± 0.02 g/L as compared to 8.10 ± 0.04 g/L and 8.23 ± 0.01 g/L for enzymatically derived glycerol and pure glycerol respectively. The average lipid concentrations were found to increase for the crude glycerol over the exponential phase. Table 3.3 shows the average lipid concentrations, lipid productivity and lipid yield values from the three grades of glycerol of the eighth-day fermentation. From figure 3.4 we can see the comparison of the lipid concentration and lipid productivity with the biomass concentration and biomass productivity of the three grades of glycerol. The g/g yield of lipid was the highest for crude glycerol as compared to the lipid yield from the other two grades.

The average biomass cell dry weight was 22.13 ± 0.17 g/L and lipid content was 0.44 ± 0.004 g/g CDW in the crude glycerol, fed-batch fermentation at 8 days (192 hrs) which was significantly higher than the average CDW and lipid content for enzymatically derived glycerol of 20.32 ± 0.12 g/L and 0.40 ± 0.003 g/g CDW, and pure glycerol of 20.06 ± 0.11 g/L and 0.41 ± 0.003 g/g CDW respectively (Figure 3.3, 3.5; Table 3.3). Comparing the crude glycerol with the other two grades, the results related to the average biomass and lipid productivities show that the average maximum biomass and lipid productivity of the crude glycerol was higher than the other two (Table 3.3). The *C. protothecoides* seemed to accumulate an average of about 44% lipids on a dry weight basis when grown on crude glycerol, which is very favorable and cost effective.

3.3.5 Comparison of results with other literature and findings

Chlorella protothecoides is a favorable organism grown heterotrophically and due to their ability to accumulate lipids up to 55% of their dry weight, they have been continuously studied with much research done to improve their biomass and lipid concentrations (Miao and Wu, 2004a). The heterotrophic *C. protothecoides* seem to grow well by utilizing acetate, glucose, glycerol or other organic compounds as carbon source (Endo et al., 1977; Wu et al., 1994).

Factors such as carbon source, nitrogen source, micro nutrients such as magnesium and copper (Baker et al., 1961; Bach et al., 1961; Aslan et al., 2006), pH, temperature, salinity and agitation affect biomass and lipid accumulation (Borowitzka et al., 1986; Bolsunovskii et al., 1996; Alyabyev et al., 2007).

Many studies have established that glycerol is fermentable anaerobically by many microorganisms into value-added products that may serve as chemicals and also fuel. For example *Propionibacteria acidipropionici* and *Propionibacteria freundenreichii* spp have been found to produce propionic acid (Bories A at al., 2004) while *Anaerobiospirillum succiniciproducens* have been found to convert glycerol to succinic acid (Lee P C et al., 2001). Hence the conversion of glycerol to microbial lipid by the microalgae *Chlorella protothecoides* reported in this study is in agreement with these results.

Studies showing the effect of glucose on the heterotrophic batch cultures of *C.protothecoides* have been documented *(*Tamarys et al., 2010) that can be compared to this study. From this study glucose was utilized completely before the end of the growth cycle when fed at 5 g/L and 15 g/L initially, but 30 g/L of initial glucose concentration gave high biomass concentration but large amount of residual glucose was found even after cultures reached the maximum growth peak. These patterns are found consistent with the findings in this study where at 30 g/L initial concentration residual glycerol was also present in considerable amounts after the cells reached the maximum growth rates. Hence a continuous feeding of substrate at lower concentrations could be evaluated for their effects in fed-batch cultivations (Xiong W et al., 2008).

Results from another study of *C. protothecoides* that have reported growth on a crude glycerol obtained from Southeast Biodiesel that used poultry fat for alkali transesterification

gave a biomass and lipid concentrations of 46 g/L and 0.53 g/g CDW over eight days in a fed-batch fermenter (Chen, Y.H. et al., 2011). The results from this study though give a maximum biomass concentration of only 22.13 ± 0.17 g/L, the lipid content obtained was 0.44 ± 0.004 g/g CDW which was a high value but not as high as obtained in the above study. This difference in biomass yields could be due to the characteristics of the crude glycerol used and its purity. Hence, the substrate characterization has a very important role to play in algae cultivation. The dissolved oxygen levels also have a critical role to play in cell growth. From day 5 onwards there seems to be some factors that could have caused oxygen limitation. Improper control in aeration could also be one of the reasons attributing to the relatively low final biomass yields on day 8.

Table 3.4 shows the comparison of results from this study with the heterotrophic cultivations of *Chlorella protothecoides* at various culture conditions. The comparison of various studies show that the biomass concentrations improved drastically with fed batch glycerol cultures as opposed to batch glucose cultures. These results could be attributed to the improved aeration conditions, enhanced substrate feeding technique and improved pH control in case of the fed batch cultures as compared to the batch cultures. The lipid content seemed to be in par with those suggested in these studies. Though some of the studies in fed batch from other experiments seem to yield some higher value of lipid content, this could be due to differences in the composition of the basal used, the algal strain, inoculum type and the growth conditions. Use of different nitrogen sources in combination with glycerol to produce higher biomass and lipid yields could also be a potential study for future work.

3.4. Conclusion

This study indicated that (1) *Chlorella protothecoides* could utilize all three grades of glycerol namely: 65% purity crude glycerol, 96% purity enzymatically derived glycerol and 99.99% pure

glycerol as a carbon substrate for growth; (2) Heterotrophic fed-batch fermentation with the 65% purity crude glycerol gave the highest maximum biomass and lipid concentrations in eight days of fermentation; (3) Though all three glycerol grades proved effective for microalgal cultivation, the use of the 65% purity crude glycerol may prove to be immensely advantageous due to low cost, easy availability and sustainability.

Thus a heterotrophic fed batch fermentation of *Chlorella protothecoides* with crude glycerol as a substrate could help accumulate large amount of lipids that may further be converted to biodiesel by transesterification, thereby regenerating glycerol. The use of expensive substrates like glucose may be completely replaced by the low cost, unrefined crude glycerol. However, the use of mixotrophic cultivation could be investigated to further enhance the lipid productivities and overcome the limitations of heterotrophy.
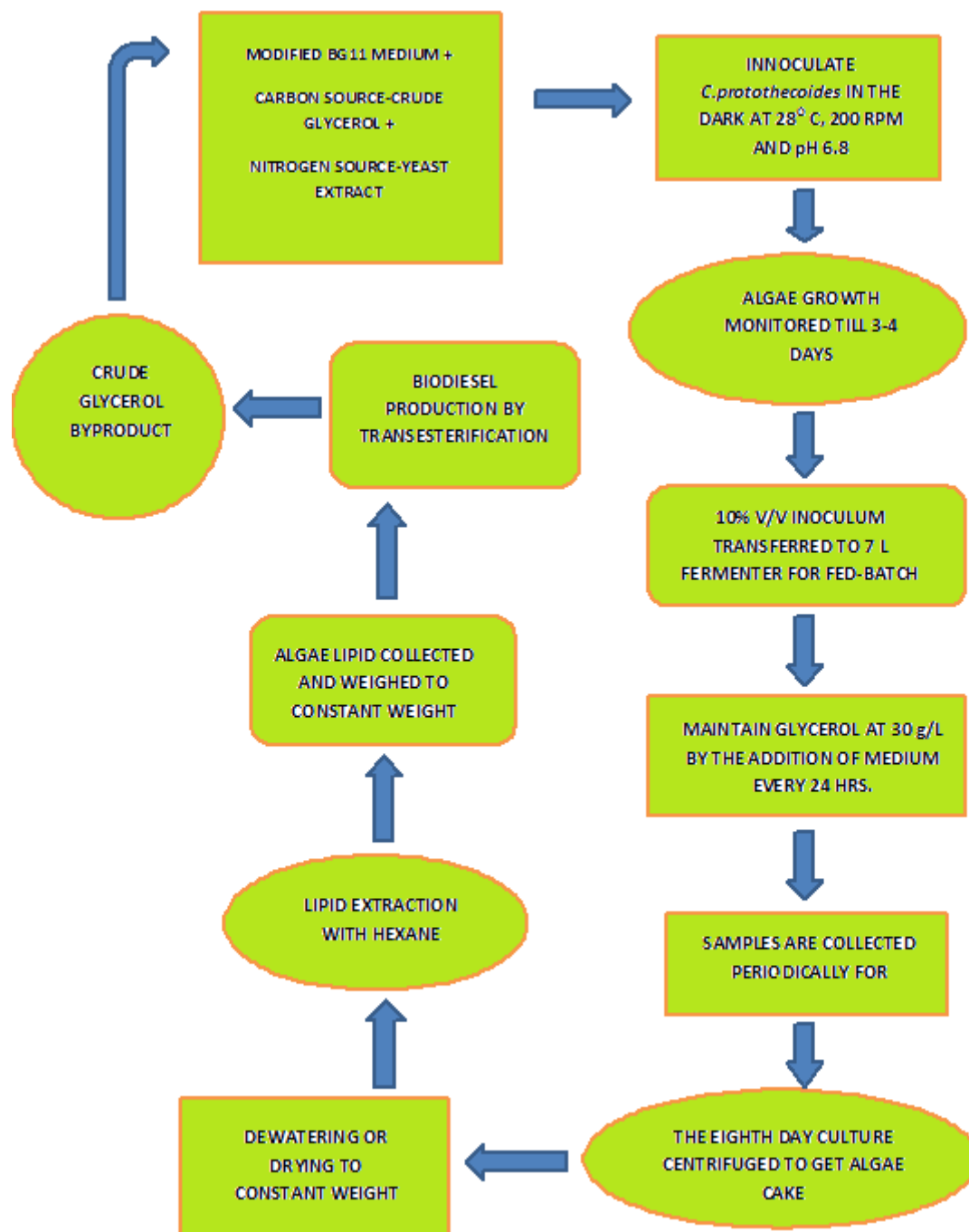
Figure.3.1. Process flow for the heterotrophic fed-batch cultivation of *C.protothecoides*

Table 3.1 Glycerol Compositions

Composition %(w/w)	Crude glycerol %(w/w)	Enzymatically derived glycerol %(w/w)
Glycerol	65 ± 0.07	95 ± 0.12
Methanol	30 ± 0.12	3 ± 0.13
Water	4 ± 0.11	1.5 ± 0.23
Other Impurities	1 ± 0.12	0.5 ± 0.09

Data recorded as an average of 3 independent measurements ± standard error

Table 3.2 ICP elemental analysis of different glycerol grades

Elements	Crude glycerol Parts per million	Enzymatically derived glycerol Parts per million
Aluminum	28.9 ± 2.64	17 ± 4.40
Arsenic	ND	ND
Boron	12.1 ± 0.14	10.8 ± 0.27
Calcium	58 ± 0.98	75.4 ± 7.03
Cadmium	ND	ND
Chromium	1.2 ± 0.21	1.1 ± 0.08
Copper	1.2 ± 0.10	1 ± 0.05
Iron	17.7 ± 2.93	20.4 ± 2.41
Potassium	15532 ± 49.95	524.2 ± 33.26
Magnesium	6.6 ± 0.09	40.2 ± 0.13
Manganese	0.5 ± 0.02	0.5 ± 0.03
Molybdenum	ND	ND
Sodium	121.9 ± 71.27	111.4 ± 38.76
Nickel	ND	ND
Phosphorus	37.6 ± 0.42	38.9 ± 0.34
Lead	ND	ND
Sulfur	23.6 ± 0.25	42.2 ± 0.41
Selenium	ND	ND
Zinc	6.6 ± 0.16	3 ± 0.19

ND represents below detection limit

Data are an average of 3 independent measurements ± standard error

Table 3.3 Results of fed batch fermentations of *C.protothecoides* using 3 grades of glycerol

Different Glycerol grades	Maximum biomass concentration (g/L, CDW)	Biomass productivity (g/L day)	Maximum lipid concentration (g/L)	Lipid Productivity (g/L day)	Maximum lipid yield (g lipid/ g biomass CDW)
Crude glycerol[1]	22.13 ± 0.17^a	2.77 ± 0.02^a	9.75 ± 0.02^a	1.22 ± 0.002^a	0.44 ± 0.004^a
Enzymatically derived glycerol[2]	20.32 ± 0.12^b	2.54 ± 0.02^b	8.10 ± 0.04^b	1.01 ± 0.005^b	0.40 ± 0.003^b
Pure glycerol[3]	20.06 ± 0.11^c	2.45 ± 0.04^c	8.23 ± 0.01^c	1.02 ± 0.002^c	0.41 ± 0.003^c

[1] Calculations for growth on crude glycerol($\square$ 65 % purity) were made on the 8th day

[2] Calculations for enzymatically derived glycerol ($\square$ 95 % purity) were made on the 8th day

[3] Calculations, for pure glycerol ($\square$ 99.99 % purity) were made on the 8th day data points.

Different letters along a column indicate that there is significant difference between the glycerol grades at a significance level of 0.05

Data reported are all averages of 8 independent measurements ± standard error
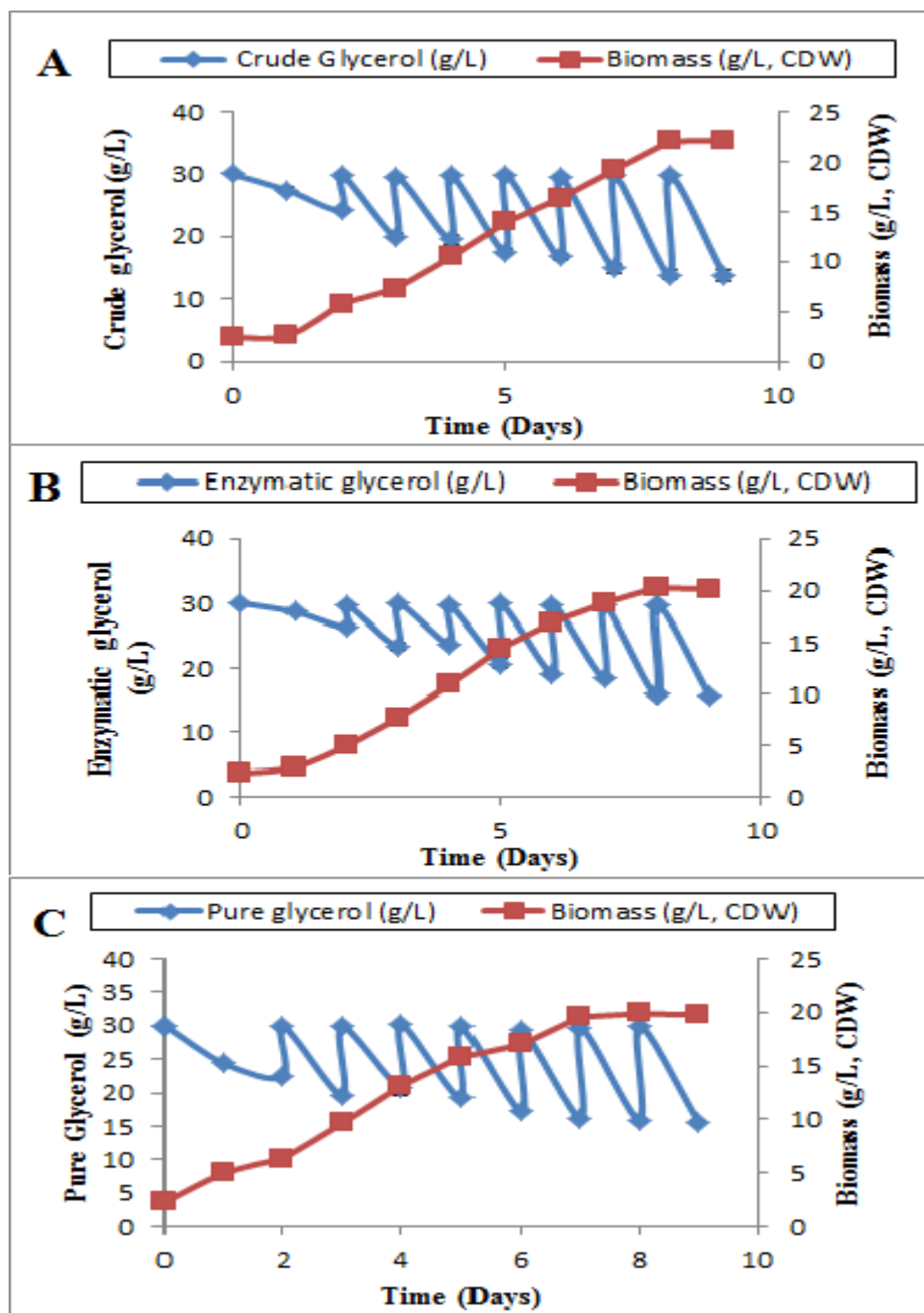
Figure 3.2 Cell biomass growth and substrate utilization of C.protothecoides for (A) crude glycerol,

(B) Enzymatically derived glycerol and (C) pure glycerol.

Each point represents an average of 8 independent measurements ± standard error
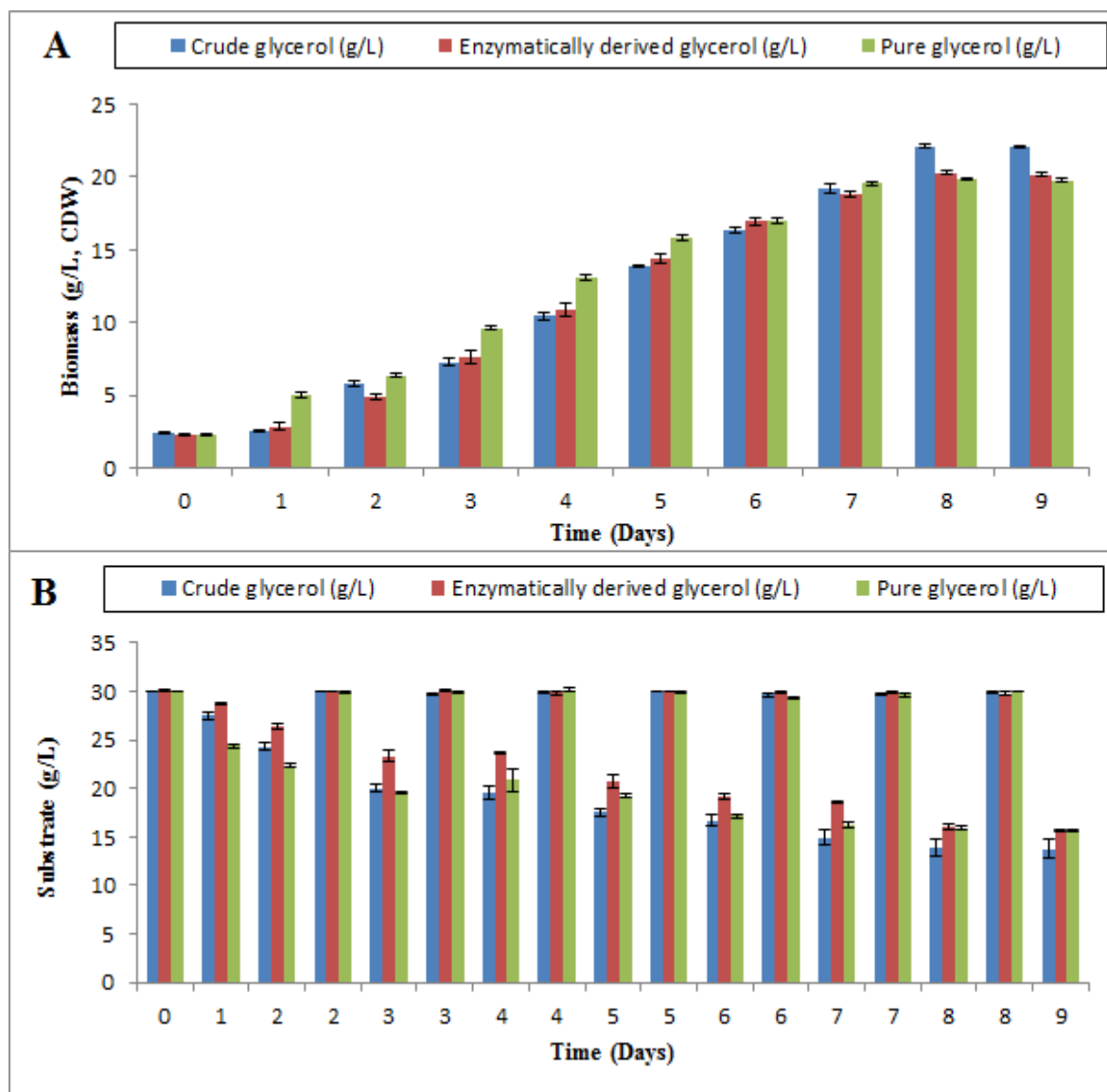
Figure 3.3 Day-wise comparison of biomass yields (A) and substrate utilization (B) for 3 grades of

glycerol for 8 days of heterotrophic fed batch fermentation.

Graph (B) shows the days repeating in the X-axis, where the repeated day indicates the feed

volume added to bring back the substrate concentration back to 30 g/L every 24 hours.

Each point represents an average of 8 independent measurements ± standard error

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Figure 3.4 Comparison of the day 8 average concentrations (A) and productivities (B) of lipid and

biomass for the three grades of glycerol

Each point represents an average of 8 independent measurements ± standard error

Table 3.4. Comparison of results in this work with previous reports in heterotrophic culture of

Chlorella protothecoides

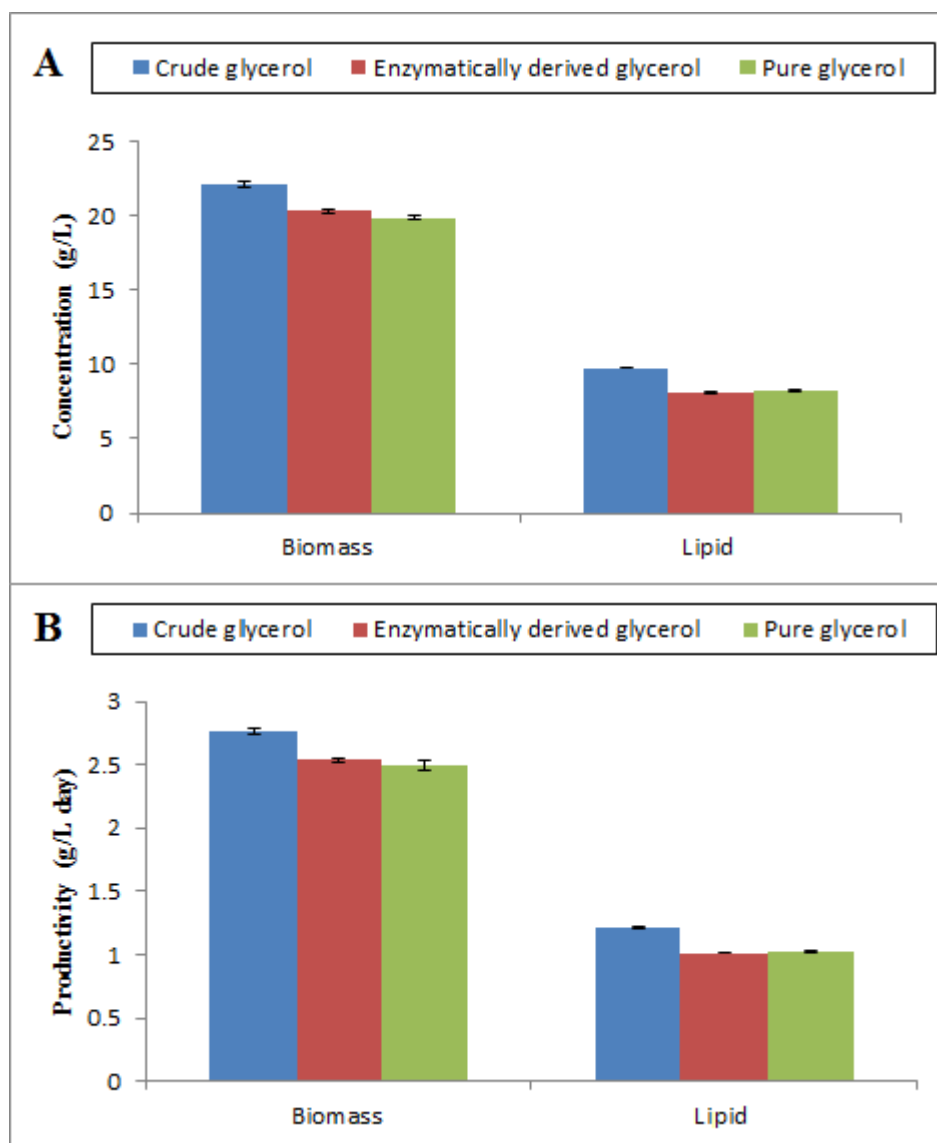| Culture Mode	Carbon substrate and concentration	Nitrogen substrate and concentration	Fermentation time (hrs)	Biomass concentration(g/L)	Maximum lipid concentration (g/L)	Lipid content (% CDW)	Reference
Fed-batch culture in 7.5 L fermenter	crude glycerol at 30 g/L	Yeast extract at 4 g/L	192	22.13	9.75	44.0	This study
Fed-batch culture in 7.5 L fermenter	Pure glycerol 30 g/L	Yeats extract at 4 g/L	192	20.06	8.23	41.0	This study
Batch culture in shake flask	glucose at 40 g/L	Yeast extract at 0.03 mol N /L, 0.5 g/L	240	4.9	-	-	Don Wei et al., 2009
	glucose at 30 g/L	yeast extract at 4.0 g/L	168	17.9	8.3	46	Xiong W et al., 2008
Batch culture in 3.7 L fermentor	glucose at 40 g/L	Urea-N of 0.12 M	142	19.6			Shi X M et al., 2000
Batch culture in 5.0 L fermentor	glucose at ≤10 g/L	Glycine at 0.17 g/L	140	3.2	1.85	57.8	Xiong W et al., 2008
Fed-batch culture in 5.0 L fermentor	Glucose at ≤24 g/L	yeast extract at 4.0 g/L	168	51.2	25.75	50.3	Xiong W et al., 2008
Fed batch culture in 10.0 L fermentor	Glucose at ≤10 g/L	Urea at ≤1.43 g/L	206	50.2	-	-	Wei D et al., 2009
Fed-batch culture in 750 L fermentor 34	glucose at ≤10 g/L	glycine at 0.1 g/L	184	12.8	6.12	48.7	Li X F et al., 2007
Fed-batch culture in 11,000L fermentor	glucose at ≤10 g/L	glycine at 0.1 g/L	200	14.2	6.24	44.3	Li X F et al., 2007

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CHAPTER IV

IV.BIOMASS AND LIPID YIELDS FROM THE MIXOTROPHIC FED-BATCH CULTIVATION of *Chlorella protothecoides* USING BIODIESEL-DERIVED CRUDE GLYCEROL

ABSTRACT

Heterotrophic growth of microalgae has been studied to produce better yields in both biomass and lipids as compared to autotrophic cultures. While the lipid accumulated in phototrophic cultures of *Chlorella protothecoides* is only 18-25%, it can be increased to 55% in the presence of organic carbons (Xu H et al., 2006). Consumption of pure and biodiesel-derived crude glycerol in heterotrophic mode by *C. protothecoides* has also been reported. This study examined the potential of mixotrophic growth with biodiesel-derived crude glycerol. The experiments were carried out by exposing the algae to 8:16 hours light:dark cycle for 8 days. A comparison in biomass and lipid yields were done for cultures grown first in dark and switched to light, and cultures grown first in light and then switched to dark. Under mixotrophic conditions supplied with crude glycerol and also exposed to light at an intensity of 1,000 lux, the fed-batch cultivations of *C. protothecoides* gave an average biomass concentration of 28.95 ± 0.26 g/L for cultures first kept in the dark and then switched to light which was significantly higher than the average biomass concentration of 25.93 ± 0.42 g/L that was obtained for the cultures initially exposed to light and then switched to dark. These results were all found to be higher than the results reported for heterotrophic growth in the same study. The average lipid concentrations were also higher in the mixotrophic experiments as compared to the heterotrophic experiments, where 13.14 ± 0.01 g/L lipids were obtained for cultures first kept in dark then switched to light and 11.69 ± 0.18 g/L of lipids were obtained for cultures initially exposed to light and then switched to the dark respectively. The cultures initially grown heterotrophically and then switched to autotrophic conditions adapted much better to growth as compared to the converse

condition and the average lipid yields of about 0.46 ± 0.004 g lipids/g biomass CDW were obtained. While strict heterotrophy with only external organic substrates may potentially be more cost-prohibitive than photoautotrophy, mixotrophy proves to incorporate the advantages of both types of metabolism thus shortening the growth cycle while increasing favorable biomass and lipid yields.

Keywords: Heterotrophic growth. *Chlorella protothecoides*. Mixotrophic growth. Biodiesel. Glycerol. Lipid yield.

4.1. Introduction

Biodiesel seems to be a very promising advent of biofuel technology that is more sustainable and environmentally friendly as compared to fossil fuel or petroleum. Mixotrophic conditions are expected to not only enhance the biomass production but also improve lipid accumulation by altering the biochemical metabolism in the microalgae. (Xu et al., 2004; Garcia et al., 2005). Certain disadvantages in autotrophic culture like light limitation during high concentrations and limited carbon dioxide availability may be overcome in mixotrophic conditions and achieve high biomass production (Chen 1996; Xu et al. 2004; Liang et al. 2009). All efforts in the microalgal cultivation arena have been to obtain large amount of lipids in the resulting biomass that can be converted to energy efficient biodiesel (Griffiths and Harrison 2009). Minxi Wan et al., 2010 have cited some evidence that the microalgae *C. sorokiniana* CCTCC M209220 has the capability to grow well and achieve large lipid reserves under genetic manipulation via mixotrophic cultivation.

One of the striking features of mixotrophic cultivation is that there are two complementary energy sources available. Both the organic carbon and light source act in tandem and the algae profits from two modes of nutrition- heterotrophic and autotrophic. The CO_2 produced by the algae on consumption of organic carbon sources is reprocessed to be utilized in the photo-autotrophic cycle for growth (Xu et al. 2004). The two processes occur almost as if independent of each other and the final growth rate may be considered as the sum of the individual growth rates in the two modes (Martinez and Orus 1991; Marquez et al. 1993). Mixotrophic cells derive high reserves of biomass and oil by simultaneously undergoing photosynthesis and oxidative metabolism of the carbon substrate, usually glucose (Yu et al. 2009).

Many phytoplankton in oligotrophic habitats (reviewed in Jones, 1994, 2000) and many eutrophic estuaries (e.g., Nygaard and Tobiesen, 1993; Jeong et al., 2004) have widely adapted the mixotrophic mode of nutrition for growth and survival. The microalgal biomass grown in mixotrophic conditions also has high protein contents along with lipid like observed in *C. sorokiniana*. This accumulation of protein and lipid is enhanced by surplus energy in the form of NADPH and Acetyl-CoA and glucose or other carbon substrates (Ren et al., 2009).

Chlorella protothecoides seem to be capable of growing in mixotrophic conditions. When grown in glucose media, colors of the culture broth vary with the autotrophic cultures being green, heterotrophic cultures being yellow mainly due to difference in the color associated with concentration of chlorophyll pigments. Mixotrophic *C. protothecoides* seem to have a specific growth rate of 0.04 h^{-1} as compared to autotrophic cultures that have a much lower specific growth rate of 0.005 h^{-1}. Table 6 shows the comparison of different microalgal species that have the capabilities to grow in various culture conditions.

For mixotrophy, the selection of light intensity or wavelength to be used and the carbon substrate become important factors for consideration. *Nannocloropsis* sp were found to show improved growth giving a maximum specific growth rate of 0.66 ± 0.021 d^{-1} under exposure to blue LED light (Das, P et al., 2011). In an 8-day fermentation with a 12:12 hour dark-photo period, the light intensity of 1,200 lux gave the best performance as compared to the range of 800-1,000 lux (Tamarys et al., 2010). The stages of growth observed were that at low cell densities, exposure to 600, 800 and 1000 lux gave better results in terms of biomass as compared to 1,200 lux, while at high cell densities; exposure to 1,200 gave better results. This is because at a low cell density, 1,200 lux could cause photoinhibition and at high densities, 600 lux can cause light limitation.

.As the studies of heterotrophic growth of *C. protothecoides* have proven to work well using glycerol, similar media may be used to evaluate the mixotrophic growth as well due to the added advantages of glycerol with low cost and availability.

The main aim in this study was to compare the growth and lipid production of the green microalga *Chlorella protothecoides* cultivated at fixed crude glycerol and yeast extract concentrations in mixotrophic cultures with periodic light exposures of 8 hours a day for 8 days in fed-batch fermentations having one treatment being exposed to light cycle first followed by dark cycle, and the other treatment having the dark cycle first followed by the light cycle.

4.2. Materials and methods

4.2.1. Materials

All chemicals were of analytical grade and were obtained commercially from authentic sources.

4.2.1.1. Crude glycerol

Crude Glycerol ($\square$ 65% pure) was obtained from the biodiesel plant on Clemson University campus. This glycerol was a by-product of biodiesel produced from the transesterification of vegetable oil with methanol using potassium hydroxide as catalyst.

4.2.2. Microorganism and inoculum preparation

*Chlorella protothecoides (*UTEX 256) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX). The basal medium was a modified BG11 culture medium adapted from the research of Chen et al., 2011, with a composition as follows (per liter): 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$, 25 mg $CaCl.2H_2O$, 25 mg NaCl, 3 mg $FeSO_4.7H_2O$, 0.01 mg vitamin B1, and 1 ml A5 solution. The inoculum was prepared by suspending microalgal cells in a 500 mL Erlenmeyer flask having 200mL of the basal medium supplemented with the carbon source (glycerol) at 30 g/L and nitrogen source (yeast extract) at 4

g/L. All media were autoclaved at 121^oC for a 15-20 minute cycle prior to inoculation. The initial pH of the medium was adjusted with 0.5M H_2SO_4 and 0.5M KOH to a value of 6.8 using an Orion Aplus Benchtop pH meter (525A+, Thermo Scientific, USA), prior to autoclaving. The cultures were incubated at 200 rpm in an NBS Classic series C4KC refrigerated shaker incubator at a growth temperature of 28^oC.

As the cultures were mixotrophic, 8:16 hour light-dark regime was followed. All flasks were wrapped with aluminum foil to block any light for 16 hours a day during the dark cycle and exposed to blue wavelength LED light lamp at an intensity of 1,000 lux for 8 hours a day during the photoautotrophic cycle. The aluminum foil was removed to expose the flasks to light. The light lamp was placed on the side of the flask and its distance to the flask was adjusted for the desired intensity. Ambient lights in the room were also inclusive of the light available for the microalgal cultures.

Two different set of inoculum were prepared for the two treatments involved. One set of cultures had the phototrophic cycle with light exposure for the first 8 hours in a day which switched to 16 hours of dark, while the other set had the dark cycle of 16 hours first followed by the 8 hours of light exposure. After 3-4 days of incubation, the mixotrophic microalgal cells in the flasks were used for further experiments after observing under a microscope.

4.2.3. Characterization of glycerol

The crude glycerol ($\square$ 65% purity) was obtained from the biodiesel plant on Clemson University campus as a by-product of biodiesel produced from the alkali-transesterification of used vegetable oil with methanol using potassium hydroxide catalyst. The characteristics of the crude glycerol are tabulated in Table 4.1 and Table 4.2. The different glycerol and methanol concentrations were determined precisely with the help of a high-performance liquid

chromatography (HPLC) system described in the analytical techniques section. For evaluating moisture content all the samples were dried in an oven at 105°C for 48 hours or until constant weight was obtained. By subtracting the methanol concentration, obtained from HPLC, from this moisture content, the water content was determined. The ash content present was determined by heating the sample to 600°C for two hours. The elemental composition of the crude glycerol was obtained by elemental analysis using the inductively coupled plasma (ICP) method in accordance with the wet ash digestion procedure from the Agricultural Service Laboratory of Clemson University (Clemson, USA).

4.2.4. Fed-batch cultivation of mixotrophic *Chlorella protothecoides*

Mixotrophic fed-batch cultivations were carried out for both the treatments in a 7.5 L working volume bioreactor (BioFlo 110, New Brunswick Scientific, USA) containing 2 L of basal medium supplemented with 30 g carbon substrate/L (crude glycerol, enzymatically purified glycerol and pure glycerol respectively) and 4 g yeast extract/L. The feed solution was a stock containing 150 g carbon substrate/L (crude glycerol, enzymatically purified glycerol and pure glycerol respectively) and 15 g yeast extract/L to maintain the concentrations of carbon substrate in the culture medium at the desired levels.

The microalgal cultures in the fermenter were illuminated with the help of two LED lamps, placed on either sides of the fermenter, designed to provide light emitted in the blue PAR zone of the spectrum set at a distance to provide an average light intensity of 1,000 lux or 3060 Watts/m^2. The light was measured with the help of a Traceable dual-range light meter purchased from VWR International, placed at the bottom of the fermenter to see the amount of light that was available on the surface for the microalgal culture. The ambient lights present in the laboratory were also inclusive of the light available to the microalgal cultures.

The pH was maintained at 6.8 by automatic addition of 0.5 M KOH and 0.5 M H_2SO_4 solutions and temperature was maintained at 28°C. The dissolved oxygen concentration was set at 40-50% air saturation by controlling the airflow and agitation speed. The initial values of aeration rate and the agitation speed were set at 1 L/min and 150 rpm, respectively and allowed to vary naturally with the microalgal growth. Sampling was done every 24 hours and samples saved for estimating biomass concentration, substrate utilization and lipid yields. Appendix B shows an illustration of the mixotrophic fed-batch system.

4.2.5. Analytical techniques

All samples were collected every 24 hours throughout the 8-day fed-batch cultivation cycle. Each data reported were taken as an average of 3 readings from the same fermenter. In total, there were three independent fermenter runs performed for each treatment and the data reported were all average of 3 independent measurements ± standard error.

4.2.5.1. Determination of cell concentration

The *C. protothecoides* cell concentration were determined by recording the absorbance at OD 540 nm using a UV/Visible spectrophotometer and correlated to the cell dry weight to obtain precise values. To obtain the cell dry weight measurements, the culture broth was centrifuged at 3,000 rpm for 15 minutes with 3 cycles of water wash, followed by drying the algae pellet at 105 ^{o}C in an oven for 48 hours or till constant weight was obtained. Weighing of samples was done using an analytical balance (ABS104, Mettler Toledo, Switzerland).

4.2.5.2. Substrate utilization

The substrate concentrations were estimated using a HPLC (Shimadzu Scientific Instruments, Inc., MD, USA), equipped with an Aminex HPX-87H cation exchange column (Bio-Rad, CA, USA) at a temperature of 60^{0}C with 50 mM H_2SO_4 as the mobile phase. The

solvent flow is maintained at 0.6 ml/min and the sample injection volume is kept at 20 µL. The detector used was a pulsed refractive index detector. External standards of known concentrations of pure pharmaceutical grade glycerol were used to obtain the standard curve.

4.2.5.3. Lipid Extraction

The intracellular lipid was extracted from the microalgae using a Polytron homogenizer (PT 1200 model, Kinematica, Switzerland) to mechanically disrupt the algal cell walls and using hexane as a solvent to solubilize the lipids coming out of the ruptured microalgal cells (Cantrell & Walker, 2009; Dong & Walker, 2008a).

The dried algal biomass that was prepared for cell dry weight estimation was extracted with 20 mL hexane in a 50 ml centrifuge, homogenized with a Polytron homogenizer for 5 min, kept at 55 ^{0}C for 5 min, and then homogenized again for 5 min. The resulting slurry was then centrifuged at 3,000 rpm for 15 minutes and the supernatant containing lipids and hexane are then transferred to another pre-weighed centrifuge tube. This whole extraction procedure was repeated twice to improve extraction efficiency with the three extracts combined for total oil determination. The repetition was done to ensure that no lipid is left behind in the biomass. The supernatants containing hexane and lipids were then filtered using 0.6 µm filters and the hexane evaporated using a RapidVap vacuum evaporator system (Labconco, USA). The lipid remaining in the tube was weighed to constant weight with an accuracy of 0.1 mg on an analytical balance (ABS104, Mettler Toledo, Switzerland). The lipid products were then stored under nitrogen atmospheres to prevent oxidation of microalgal lipids.

4.2.6. Statistical analysis

A repeated measures analysis was used on the data of both the treatments for comparison. The covariance structure of the data was assessed to be unstructured covariance at the beginning

of the statistical analysis. The treatments considered were the L:D and D:L cycles administered with 8:16 hour and 16:8 hour regime respectively. The response measured was the biomass concentration (g/L) and lipid concentration (g/L). Experiments were performed in the random order as obtained using Statistical Analysis System (SAS v.9.2, SAS Institute, USA).

4.3. Results and discussion

4.3.1. Effect of mixotrophic growth mode

The results from this study showed that *Chlorella protothecoides* gave high biomass concentrations and lipid contents under mixotrophic conditions. The color of the mixotrophic algal cultures was similar to the heterotrophic ones in appearance but was darker, having slight green tinges. From Table 4.3 the cultures starting with heterotrophic mode and switched to autotrophic mode (D:L) had a higher average biomass concentration of 28.95 ± 0.26 g/L as compared to the reverse treatment (L:D) reaching an average biomass concentration of only 25.93 ± 0.42 g/L at the end of 8 days. The maximum biomass concentration achieved for treatment D:L was significantly higher than that for L:D. Since autotrophic algae rely on inorganic compounds alone to derive all their energy, this lack of excess inorganic carbon source leads to their low biomass cell density (Hogetsu, D et al., 1977). However as mixotrophic studies incorporate an additional organic carbon source supply, the biomass is expected to increase (Borowitzka et al., 1988). In case of the D:L treatments, the heterotrophic growth occurs rapidly with the low-density cultures utilizing the available glycerol for cell growth. Once the cultures are switched to the autotrophic mode, their cell densities are higher than at the start and hence the cells start shifting their metabolism towards photosynthesis. This shift creates a favorable switch for the microalgae that acts on both accumulating biomass and accumulating lipids during the successive light cycle to dark cycle switch. For the L:D cultures, some amount of photo-

oxidative damage occurs initially as the light irradiated possibly causes some photoinhibition considering the low densities at the start of the culture. Also in this case as the cells are exposed to light initially, the chlorophyll content is comparatively high and their heterotrophic metabolism may not trigger as rapidly as expected. The consumption of the glycerol here occurs in slower rates and the final biomass reached is not as high as the D:L treatments.

From Figure 4.1 (C) and (D), the average maximum lipid concentrations were obtained on day 8 with the treatment L:D having a lower value of 11.69 ± 0.18 g/L as compared the treatment D:L that had 13.14 ± 0.01 g/L. Though the average maximum lipid concentrations achieved between the two treatments were significantly different, the lipid yield in g/g CDW was very similar for both these treatments. With exposure to high intensity of light, higher lipid contents accumulate possibly due to stresses within the organism (Khotimchenko S V et al., 2005). Though the treatment L:D had lower biomass as compared to the treatment D:L, the stress conditions induced in L:D due to light caused the lipid contents to be similar at 0.45 g/g CDW and 0.46 g/g CDW, respectively. Figure 4.2 shows the comparison of biomass and lipid productivities of both treatments L:D and D:L where there is no significant difference between the two.

The nitrogen starvation also is reported to have different effects on lipid and biomass contents. During nitrogen deprivation, the cells have very low amounts of the nitrogen reductase enzyme that is capable of assimilating nitrogen. This slows cell and biomass growth while it promotes lipid accumulation (Darley W M et al., 2004).

Henceforth the mixotrophic cultures of Chlorella protothecoides proved to grow well in a D:L-16:8 hour dark:light regime for 8 days in fed-batch cultivations giving an average maximum biomass concentration of 28.95 ± 0.26 g/L and lipid content of 0.46 ± 0.004 g/g CDW.

4.3.2 Comparison with literature

The mixotrophic cultures of *C. protothecoides* with different concentrations of yeast extract yield high biomass at high nitrogen concentrations while giving higher lipid contents at low nitrogen concentrations (Xiong W et la., 2008). There is also evidence that some *Chlorella* species like *Chlorella ellipsoidea SK* and *Chlorella pyrenoidosa 8* grown on nitrogen-deprived media, but exposed to sufficient light in the presence of organic carbon substrates, tend to accumulate 26.8-47.1 % lipids in their cells (Borowitzka et al., 1988). This study produced 45% lipid using 4 g/L of yeast extract, the nitrogen concentration may still be too high to cause the metabolic change for greater lipid production. Future studies will examine the potential of lowering the organic nitrogen levels for potential improvement. Table 4.1 shows a comparison of different microalgal species that have the capabilities to grow in various nutrient modes.

The light intensity chosen is very critical for mixotrophic growth. From a study done on *Nannochloropsis sp*, the intensity of the spectrums of light showed the best biomass yields in the following order: blue > white > green > red. A comparative study conducted by Wang et al., 2007 on the effect of a wide range of intensities from 300-3000 μmol/m^2/s for different wavelengths of light for *Spirulina platensis* showed that the red light gave high biomass at all intensities while blue light at intensities greater than 300 μmol/m^2/s or 16,200 lux resulted in poor biomass due to photoinhibition. But blue light being most energetic is expected to work best for photosynthesis (Kebede and Ahlgren., 1996).

In a study shown in Figure 4.3, comparing the effect of two light intensities at 400 lux and 1,200 lux on a mixotrophic fresh water microalgae *C. pyrenoidosa* in batch culture, high intensity 1,200 lux produced more differentiated phases during growth. The exponential phase corresponded to mixotrophic growth using the glucose in the medium along with light and went

into the stationary phase when all the carbon was consumed and another autotrophic exponential phase appeared which due to light limitation caused the culture to move into a deceleration phase (Ma E Martinez et al., 1997). The lower intensity light of 400 lux had the mixotrophic exponential phase preceding the stationary phase which was eventually retarded the growth due to light limitation. Table 4.4 shows the various mixotrophic growth rates obtained in this study with glucose. This pattern of growth is in agreement with this study. While the low intensities (400 lux) cause light limitation and high intensities (2,000 lux) cause photo oxidative damage to cells, an optimum moderate intensity used here of 1,000 lux worked well for lipid and biomass accumulation.

Table 4.5 also shows the comparison of biomass and lipid of various species in mixotrophic growth grown on different substrates and culture conditions with the results of this study. The results seemed consistent that the crude glycerol appears to aid lipid accumulation at a greater rate than glucose. The results of this fed-batch study also gave improved yields of both biomass and lipid as compared to the batch cultures. However, a possibility exists that the use of glycerol in combination with some other substrate like glucose enhanced improvement in biomass and lipid accumulation. This opens up some prospect for future studies to increase the lipid yields of mixotrophic *C. protothecoides* to about 55% of their dry weight as suggested in literature.

4.4. Conclusion

This study indicated that (1) *Chlorella protothecoides* could grow efficiently under mixotrophic conditions of alternating light and dark regimes; (2) *C. protothecoides* could also utilize crude glycerol as a carbon substrate and assimilate CO_2 from a light source at an intensity of 1,000 lux

in the mixotrophic mode; (3) fed-batch fermentation could increase the average maximum biomass concentration for mixotrophic growth with a Dark:Light (16:8 hours) regime to 28.95 ± 0.26 g/L as compared to only 25.93 ± 0.42 g/L from the L:D regime ; (3) the average lipid content from both the mixotrophic regimes were found to be similar at 0.45 ± 0.003 g/g CDW and 0.46 ± 0.004 g/g CDW respectively. Considering the high biomass concentrations in mixotrophic growth and the corresponding high lipid content, the use of the 65% crude glycerol from the biodiesel plant seems sufficient for *C. protothecoides* growth and this may lead to a drop in the cost of algae cultivation owing to the economic feasibility and value of the crude glycerol. This will hopefully help in constructing a cost-effective bio-refinery that is self-sustained and energy efficient for the future.

Table 4.1 Composition of crude glycerol

Composition	Crude glycerol
%(w/w)	%(w/w)
Glycerol	65 ± 0.07
Methanol	30 ± 0.12
Water	4 ± 0.11
Other Impurities	1 ± 0.12

Data recorded are an average of 3 independent measurements ± standard error

Table 4.2 ICP elemental analysis of crude glycerol

Elements	Crude glycerol
	Parts per million
Aluminum	28.9 ± 2.64
Arsenic	ND
Boron	12.1 ± 0.14
Calcium	58 ± .98
Cadmium	ND
Chromium	1.2 ± 0.21
Copper	1.2 ± 0.10
Iron	17.7 ± 2.93
Potassium	15532 ± 49.95
Magnesium	6.6 ± 0.09
Manganese	0.5 ± 0.02
Molybdenum	ND
Sodium	121.9 ± 71.27
Nickel	ND
Phosphorus	37.6 ± 0.42
Lead	ND
Sulfur	23.6 ± 0.25
Selenium	ND
Zinc	6.6 ± 0.16

ND represents below detection limit

Data recorded are an average of 3 independent measurements ± standard error

Table 4.3 Comparison of day 8 biomass and lipid contents obtained from mixotrophic fed batch cultivation

Treatment	Maximum Biomass concentration (g/L, CDW)	Biomass productivity (g/L day)	Maximum Lipid concentration (g/L)	Lipid Productivity (g/L day)	Lipid Yield (g/g CDW)
L:D	25.93 ± 0.42^{a}	3.24 ± 0.03^{a}	11.69 ± 0.18^{a}	1.46 ± 0.014^{a}	0.4510 ± 0.003^{a}
D:L	28.95 ± 0.26^{b}	3.62 ± 0.02^{a}	13.14 ± 0.01^{b}	1.64 ± 0.001^{a}	0.4538 ± 0.004^{a}

Treatment L:D signifies the experiments started with autotrophic mode and switched to heterotrophic mode.

Treatment D:L signifies the experiments started with heterotrophic mode and switched to autotrophic mode.

Observations within a column with different letters indicate that the two treatment responses are significantly different at a significance level of 0.05.

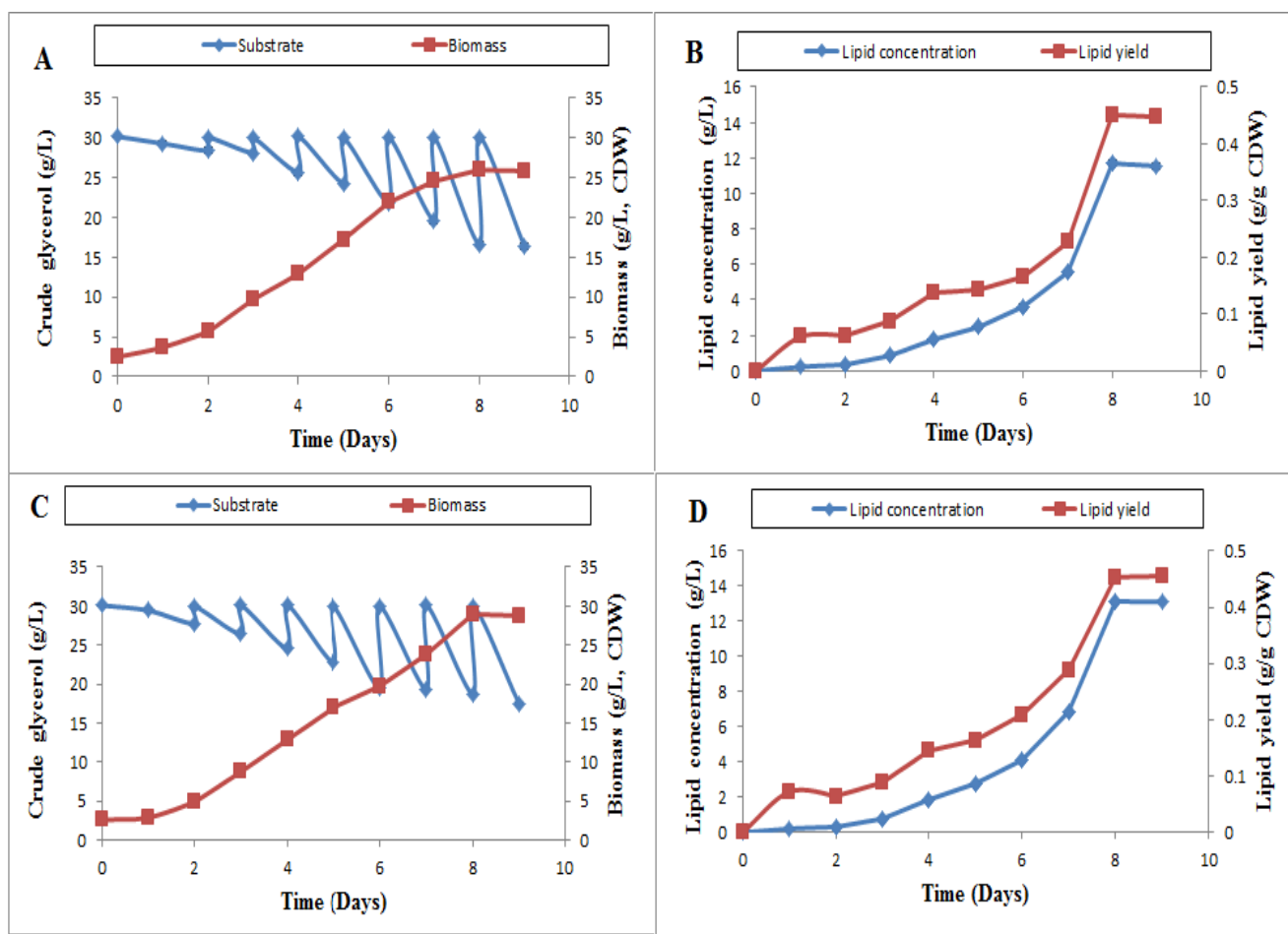All data are an average of 3 independent measurements ± standard error.

Figure 4.1 Biomass, substrate and lipid contents for mixotrophic cultures

(A) and (B) represent treatment L:D

(C) and (D) represent treatment D:L

All readings are an average of 3 independent measurements ± standard error
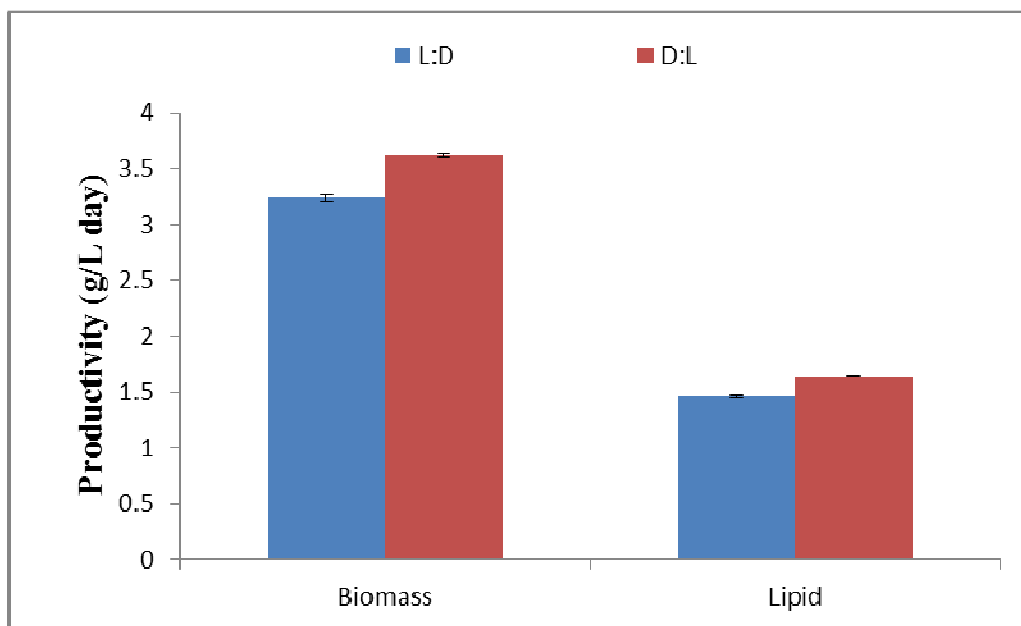
Figure 4.2 Comparison of biomass and lipid productivities for day 8 fed batch mixotrophic fermentation of *Chlorella protothecoides*

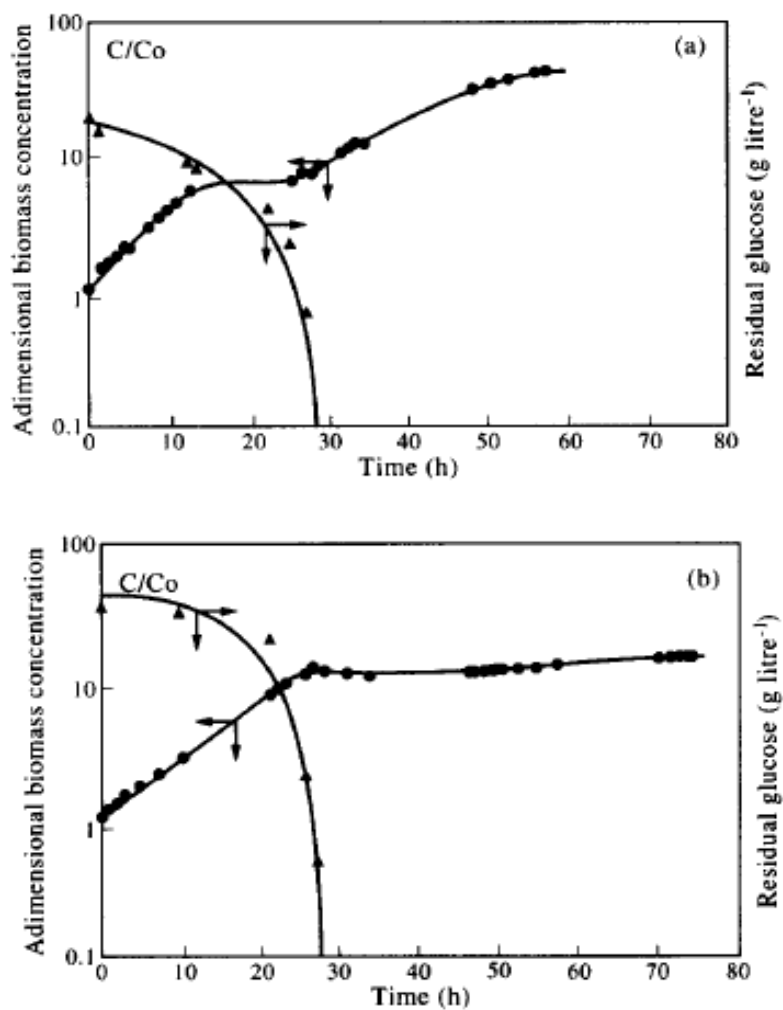All readings are an average of 3 independent measurements ± standard deviation

Figure 4.3 Effect of variation of light intensity on growth and substrate utilization

(a) Io = 2000 lux, So = 0.1 g litre^{-1}. (b) Io = 400 lux, So = 0.5 g litre^{-1}

Table 4.4 Comparison of mixotrophic growth rates of batch *C.pyrenoidosa* grown on glucose using Plant-Gro fluorescent lamps designed to give light in the blue and red regions of the PAR spectrum.

I_o (lux)	S_o (g/L)	μ (hr^{-1})	r^2
2022	0.1	0.1107	0.997
1983	0.5	0.1070	0.995
1907	1.0	0.1094	0.997
1386	0.1	0.0932	0.990
1370	0.5	0.0970	0.998
1255	1.0	0.1000	0.986
814	0.1	0.0880	0.973
809	0.5	0.0874	0.995
802	1.0	0.1027	0.985
372	0.1	0.1047	0.971
395	0.5	0.1058	0.990
369	1.0	0.1195	0.990

Source: Ma. E. Martinez et al.., 1997

Table 4.5 the comparison of different microalgal species in their capabilities to grow in various nutrient modes.

Microalgae	AC	HC	MC	Substrate	Lipid content (%)	Reference
Chlorella protothecoides	x	x	x	Glucose, CO_2/acetate	55.2	Carlsson, A. S et al., 2007
Chlorella vulgaris	x	x	x	Glucose, acetate, lactate, glutamate	11.8-57.9	Borowitzka, M. A et al., 1988
Crypthecodinium cohnii			x	Glucose/acetate	15-70	. Chi, Z. Y et al., 2007
Scenedesmus obliquus	x		x	Glucose/CO_2	14-22	Borowitzka, M. A et al., 1988
Chlamydomonas reinhardtii	x	x	x	Acetate, CO_2	21	Becker, E. W et al., 1994
Schizochytrium sp.	x		x	Glycerol, CO_2	50-77	Chisti, Y., 2007
Spirulina platensis	x		x	Glucose, CO_2	4.2-6.2	Borowitzka, M. A et al., 1988
Botryococcus braunii			x	CO_2	25-75	Chisti, Y., 2007
Dunaliella salina	x	x		CO_2	15-55	Weldy, C.S et al., 2010

AC – autotrophic culture; HC, heterotrophic culture; MC – mixotrophic culture

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CHAPTER V

V.SUMMARY AND RECOMMENDATIONS

This work has confirmed the potential use of crude glycerol by *Chlorella protothecoides* for both heterotrophic and mixotrophic growth. The heterotrophic growth with crude glycerol gives a maximum biomass and lipid concentration of 22.13 g/L and 9.75 g/L respectively. The microalgae *C.protothecoides* was also found to grow well on the pure glycerol giving the maximum biomass and lipid concentrations of 20.06 g/L and 8.23 g/L. The maximum biomass and lipid concentrations were found to be higher for the crude glycerol as compared to the pure glycerol. While the pure glycerol is a useful substrate, the use of crude glycerol may further reduce cost and help in assisting the economic feasibility of the biodiesel production process. Mixotrophic culturing was conducted to see if the beneficial combinations of heterotrophic and autotrophic modes could benefit the algae to improve biomass and lipid yields. Using this crude glycerol, the microalgae grew well in mixotrophic conditions. The average maximum biomass concentration and lipid concentration with crude glycerol improved in mixotrophic conditions to 28.95 ± 0.26 g/L and 13.14 ± 0.01 g/L from 22.13 ± 0.17 g/L and 9.75 ± 0.02 g/L, respectively in heterotrophic conditions. Under both heterotrophic and mixotrophic conditions *C. protothecoides* was able to accumulate lipid content from 44-46 % of their dry weight. This is a big breakthrough in the line of alternative substrate for biodiesel production and these results have a lot of scope for further improvement.

The fed-batch cultivation procedure used here also helped overcome the limitations of improper aeration, lack of pH control and occurrence of substrate limitation that are commonly observed in batch cultures. While the heterotrophic conditions helped evaluate the best grade of glycerol for microalgal lipid production, the mixotrophic conditions helped achieve increased

biomass production that helps increase the overall lipid production. The light intensity, the concentration of carbon substrate and the duration of exposure to light were found as some of the main factors affecting the mixotrophic growth. As different microalgae contain diverse pigments, they absorb light at different wavelengths. Irradiating the algae with the right wavelength at the correct intensity is very important as excess light at low cell densities can retard algal growth due to photooxidative damage and poor exposure to light may reduce biomass concentration due to light limitation. Hence understanding the parameters of mixotrophic growth is very critical. Though heterotrophic growth has been well studied and better understood, mixotrophic cultivation requires more research and understanding especially in the line of microalgal growth for fuel applications.

Though this research provides a good initiative for mixotrophic cultivation using crude glycerol, there is the possibility to improve the lipid and biomass yields substantially from what is obtained here. Some recommendations for future work are: (1) To evaluate the behavior of other microalgae similar to *C.protothecoides,* like say *Chlorella vulgaris,* to see if they have the potential to grow better under the mixotrophic and or heterotrophic conditions when glycerol is used as the carbon substrate; (2) To study the effect of mixed substrates on the growth and lipid production of algae. There have been studies that have suggested that the mixtures of glucose with other carbon substrates seem to improve the usability of certain unconventional carbon substrates like acetate and glycerol by the microalgae (Tamarys et al., 2010). There is some suggested evidence that these mixed carbon substrates might improve the biomass yields and lipid accumulation in the microalgae; (3) To characterize and analyze the microalgal lipids obtained from the above study. There is a need to obtain the characteristics of this microalgal lipid, calculate their efficiency and evaluate their advantages over other oil sources that are

currently used in biodiesel production; (4) To evaluate the effects of different light intensities and duration of light exposure on the biomass and lipid productivities of mixotrophic *C. protothecoides*; (5) To optimize the fed batch feeding technique to improve biomass and lipid yields as suggested by other literature sources; and (6) To study the effect of different nitrogen sources other than yeast extract to improve lipid production of heterotrophic and mixotrophic *C. protothecoides.*

APPENDICES

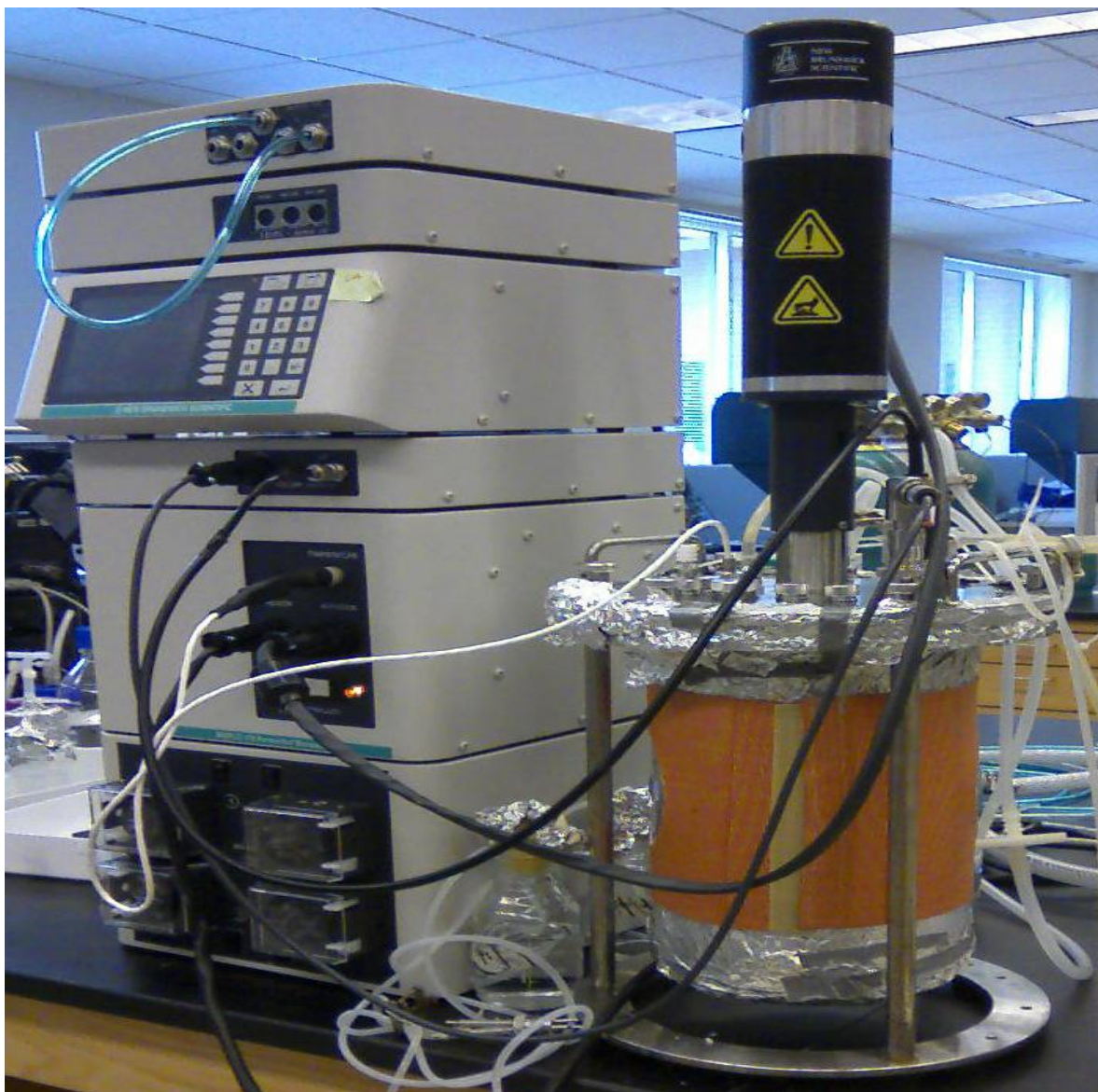**Appendix A – Picture of Heterotrophic fed-batch system**

Figure A1. Heterotrophic fed-batch cultivation system of *C.protothecoides*

Appendix B – Picture of Mixotrophic fed-batch system

Figure B1. Mixotrophic fed-batch cultivation system of *C.protothecoides*